UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE MEDICINA



TESIS DOCTORAL

Regulation of potassium channel in the pulmonary vasculature. Implication in pulmonary hypertension

Regulación de canales de potasio en la vasculatura pulmonar. Implicación en hipertensión pulmonar

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Gema Mondéjar Parreño

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UNIVERSIDAD COMPLUTENSE DE MADRID

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DEPARTAMENTO DE FARMACOLOGÍA Y TOXICOLOGÍA

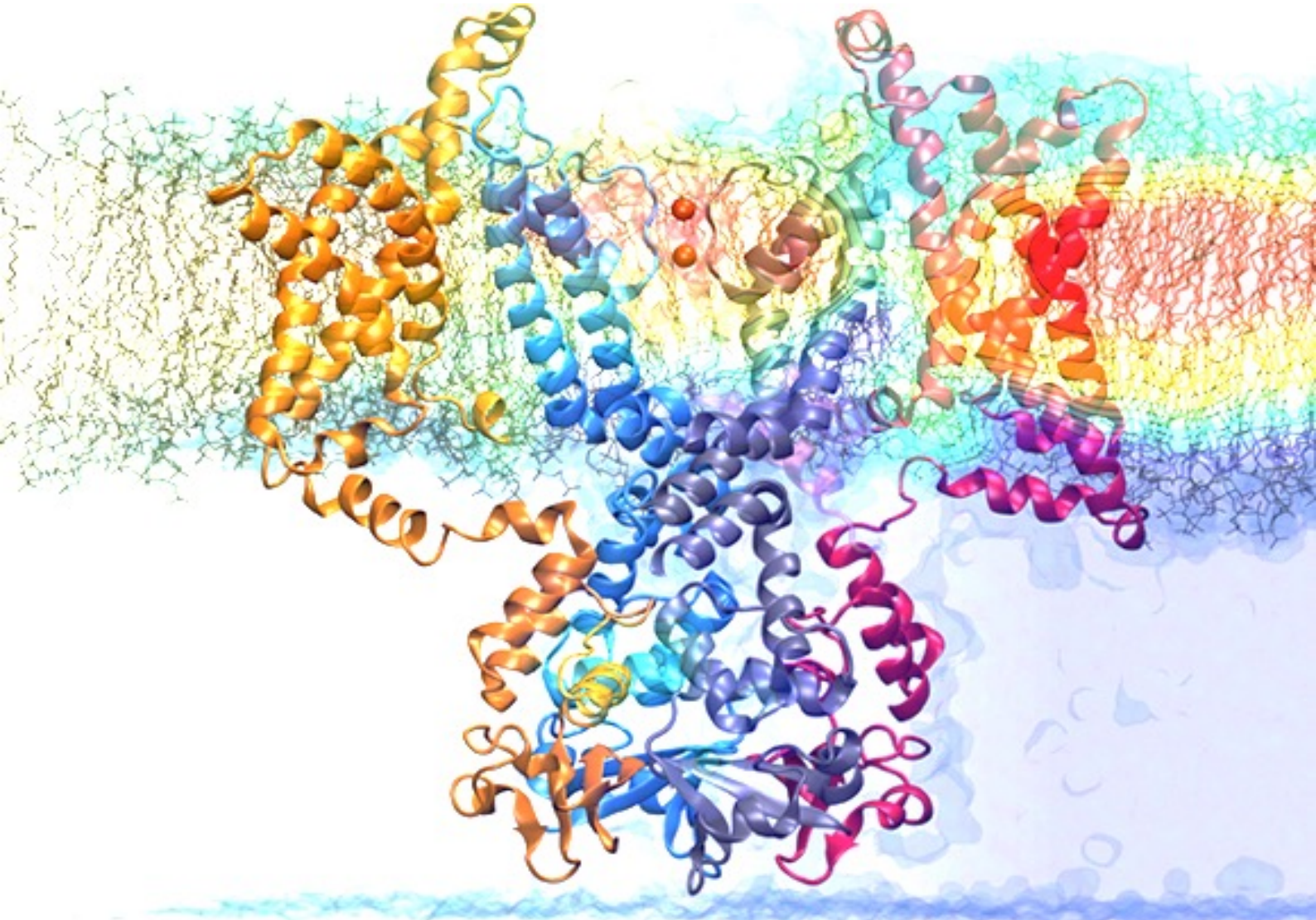
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Departamento de Farmacología y Toxicología

Regulación de canales de potasio en la vasculatura pulmonar. Implicación en hipertension pulmonar.

Memoria para optar al grado de Doctor con mención internacional presentada por

Gema Mondéjar Parreño

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CERTIFICAN:

Que Dña. Gema Mondéjar Parreño, ha realizado bajo su supervisión la tesis doctoral titulada "Regulación de canales de potasio en la vasculatura pulmonar. Implicación en hipertension pulmonar" para optar al grado de Doctor por la Universidad Complutense de Madrid. La presenta tesis doctoral reúne los requisitos de originalidad y contenido exigidos para obtener el grado de doctor en Biomedicina por la Universidad Complutense de Madrid con Mención Internacional.

Y para que así conste, expiden el presente certificado en Madrid, a 22 de Junio de 2020.

Dr. Francisco Pérez Vizcaíno **Dr. Ángel Luis Cogolludo Torralba**

MENCIÓN DE DOCTORADO INTERNACIONAL

La presente tesis doctoral de Dña. Gema Mondéjar Parreño titulada "Regulation of potassium channel in the pulmonary vasculature. Implication in pulmonary hypertension", realizada en el Departamento de Farmacología y Toxicología, de la Facultad de Medicina de la Universidad Complutense de Madrid, cumple los requisitos exigidos por la Universidad Complutense de Madrid para obtener la mención de Doctor Internacional (R.D. 99/2011):

1. Haber realizado una estancia **mínima de 3 meses** fuera de España en un centro de investigación de prestigio, realizando trabajos de investigación, durante el periodo de realización del programa de doctorado:

    - **Institución**: Vascular Biology Research Center, Molecular & Clinical Sciences Research Institute, St George's Hospital University of London.
      Supervisor: Prof. Iain Greenwood. **Duración:** 5 meses (Enero-Junio 2019)
    - **Institución:** Medway Pharmacy School, University of Kent. **Supervisor:** Prof. Ghazwan Butrous**. Duración:** 1.5 meses (Septiembre-Octubre 2017)

2. La tesis doctoral ha sido evaluada por dos expertos doctores pertenecientes a instituciones internacionales:

    - **Thomas Andrew Qvistgaard Jepps**, PhD, Assistant Professor in University of Copenhagen, Department of Biomedical Sciences.
    - **Vincenzo Barrese,** MD PhD, Assistant Professor in University of Naples Federico II, Department of Neuroscience, Reproductive Sciences and Dentistry

3. Un miembro del tribunal evaluador de la tesis doctoral pertenece a un centro de investigación extranjero.

4. La tesis doctoral ha sido redactada en inglés y, al menos, una parte de la defensa será presentada en una de las lenguas habituales para la comunicación científica en su campo de conocimiento, distinta a cualquiera de las lenguas oficiales en España.

El trabajo descrito en la presente Tesis Doctoral ha sido llevado a cabo en el Laboratorio de farmacología y fisiopatología vascular en el Departamento de Farmacología y Toxicología de la Facultad de Medicina y ha sido financiado por los siguientes proyectos de investigación:

- MicroRNAs implicados en disfunción vascular pulmonar: implicaciones fisiopatológicas y terapéuticas (2015-2017). Plan Nacional de I+D (CICYT) SAF2014- 55399R. PI: Francisco Pérez Vizcaíno & Ángel Cogolludo Torralba
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"Nuestra sociedad, en la que reina un urgente deseo de riquezas y lujos, no entiende el valor de la Ciencia. No se da cuenta de que la Ciencia es una de las partes más preciosas de su patrimonio moral. Ni ha asumido de modo suficiente, el hecho de que la Ciencia está en la base de todo el progreso que aligera la carga de la vida y disminuye su sufrimiento."

Maria Salomea Skłodowska-Curie [1867-1934]

A mi madre

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Abbreviations

AC, adenylate cyclase

ALK1, activin receptor-like kinase 1

AP-1, activator protein 1

AQP1, aquaporine 1

ATP, adenosine triphosphate

ATIIR, angiotensin II receptor

BK_{Ca}, big conductance Ca^{2+}-activated K^+ channel

BMPR2, bone morphogenetic protein receptor II

BSP, beraprost sodium

$[Ca^{2+}]_{cyt}$, cytosolic calcium concentration

Ca^{2+}-CaM, calcium-calmodulin complex

cAMP, cyclic adenosine monophosphate

Ca_v, voltage-dependet-L-type calcium channel

CAV-1, caveolin-1

cGMP, cyclic guanosine monophosphate

CGRP, calcitonin gene-related peptide

CHIP, c-terminus of HSP70-interacting protein

CHO, chinese hamster ovary cells

COS, simian kidney *cells* transformed by SV40

CREB, cAMP response element-binding protein

CS, Cantu Syndrome

DAG, diacylglycerol

DNA, deoxyribonucleic acid

DPO-1, diphenylphosphine oxide

ECE-1, endothelin-converting enzyme-1

Em, membrane potencial

EMA, European Medicines Agency

E_K, equilibrium membrane potencial

eNOS, endothelial nitric oxide synthase

ENG, endoglin

EP4, prostaglandin E_2 receptor 4

ER, endoplasmic reticulum

ETC, electron transport chain

ET-1, endothelin-1

FDA, Food and Drug Administration

GoF, gain of function

HEK-293, human embryonic kidney 293 cells

HIF1, hypoxia-inducible factor 1

HIV, human immunodeficiency virus

H_2O_2, hydrogen peroxide

HPV, hypoxic pulmonary vasoconstriction

HSP, Heat Shock Proteins

5HT, serotonin

IK_{Ca}, intermediate Ca^{2+}-activated K^+ channel

I_{KN}, non-inactivating K^+ current

IL6, interleukin-6

IP, prostacyclin receptor

IP3, inositol-1,4,5-triphosphatase

K_{ATP}, ATP-sensitive K^+ channel

K_{Ca}, calcium-activated K^+ channel

KCO, K_{ATP} channel opener

K_{IR}, inward rectifier K^+ channel

K_{Ca}, voltage-dependent K^+ channel

LoF, loss of function

MLC-K, myoxin light-chain kinase

MLCP, phosphorylates myosin light-chain

mPAP, mean pulmonary artery pressure

NADPH, nicotinamide adenine dinucleotide phosphate oxidase

Nef, negative regulatory factor protein

NFAT, transcription nuclear factor of activated T cells

NF-κB, nuclear factor κ-light-chain-enhancer of B cells

NO, nitric oxide

Nox, NADPH oxidase

ONO-RS-082, phospholipase A2 inhibitor

PA, pulmonary artery

PAEC, pulmonary artery endothelial cells

PAH, pulmonary arterial hypertension

PASMC, pulmonary artery smooth muscle cells

PAWP, pulmonary arterial wedge pressure

PDE-5, phosphodiesterase-5

PDGF, platelet-derived growth factor

PGD_2, prostaglandin D_2

PGE_2, prostaglandin E_2

PGF_{2a}, prostaglandin $F_{2\alpha}$

PGI_2, prostacyclin

PIP_2, phosphatidylinositol 4,5-bisphosphate

PH, pulmonary hypertension

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PPAR, peroxisome proliferator-activated receptor

PVR, pulmonary vascular resistance

RAAS, renin-angiotensin-aldosterone signalling

RNA, ribonucleic acid

ROCK, Rho-associated protein kinase

ROS, reactive oxygen species

RV, right ventricular

sGC, soluble guanylate cyclase

SK_{Ca}, small conductance Ca^{2+}-activated K^+ channel

SMase, sphingomyelinase

SNP, single nucleotide polymorphisms

SQSTM1, sequestosome1

STAT3, activator of transcription 3

SUR, sulfonylurea receptor

VGCC, L-type voltage-gated Ca^{2+} channels

VSMC, vascular smooth muscle cells

TASK, two pore acid-sensitive K^+ channel

Tat, transactivating regulatory protein

TBX4, T-box4 transcription factor

TGF, transforming growth factor

TK, tyrosine kinase

TM, transmembrane

TNF, tumor necrosis factor

TP, thromboxane receptor

TXA_2, thromboxane A2

UTR, untranslated region

VDR, vitamin D receptor

VitD, vitamin D

VPU, viral protein U

WHO, world health organization

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1. Abstract

The large K^+ channel functional diversity in the pulmonary vasculature results from the multitude of genes expressed encoding K^+ channels, alternative RNA splicing, the post-transcriptional modifications, the diversity of homomeric or heteromeric assemblies of the pore-forming α-subunits and the existence of accessory β-subunits which can modulate the channel functional properties. Furthermore, K^+ channels can be regulated at multiple levels by different factors controlling channel activity, trafficking, recycling and degradation. The activity of K^+ channels is the main determinant of membrane potential (Em) in pulmonary artery smooth muscle cells (PASMC) and pulmonary artery endothelial cells (PAEC), providing an important regulatory mechanism to dilate or contract pulmonary arteries (PA). The activity of K^+ channels is implicated in the regulation of the population and phenotype of PASMC in the pulmonary vasculature, since they are involved in cell apoptosis, survival and proliferation. Notably, K^+ channels play a major role in the development of pulmonary hypertension (PH). Impaired K^+ channel activity in PH results from: 1) Loss of function mutations, 2) Downregulation of its expression which involves transcription factors and microRNAs; and 3) Decreased channel current resulting from an increase in vasoactive factors (e.g. hypoxia, 5-HT, endothelin-1 or thromboxane) which inhibit channel current, by drugs blocking the channel, or by a reduction in factors which positively regulate their activity (e.g. NO and prostacyclin).

The main objective of this Doctoral Thesis was to increase the knowledge of physiological, pathophysiological and pharmacological aspects of the regulation of K^+ channels in the pulmonary vasculature that may have an impact on our understanding and treatment of PH.

The NO/cGMP pathway represents a major physiological signalling controlling vascular tone in PA and drugs activating this pathway are used to treat PAH. The contribution of Kv channels in the electrophysiological and vasodilating effects evoked by NO donors and the sGC stimulator (riociguat) was analysed in PA. The results showed NO donors and riociguat enhance Kv7 currents leading to PASMC hyperpolarization. This mechanism contributes to NO/cGMP-induced PA vasodilation. Our study identified Kv7 channels as a novel mechanism of vasodilator drugs used in the treatment of PAH.

In recent years, a key role of Kv7 channels and KCNE ancillary subunits in the control of vascular tone has been demonstrated. Moreover, reduced Kv7 channel activity has been implicated in different cardiovascular pathologies such as diabetes, hypertension, erectile dysfunction, atrial fibrillation or long QT syndrome. However, the physiologic regulation of Kv7 channel activity in the pulmonary vasculature is still poorly understood. The objective was to study the role of Kv7 channels/KCNE subunits in the pulmonary vasculature and their possible alteration in PAH. We found that, although the total K^+ current is reduced, Kv7 current is preserved in PASMC from PAH-animals (Su/Hpx) resulting in a higher contribution to the net K^+ current. Likewise, enhanced vascular responses to Kv7 channel modulators were found in PAH rats. Accordingly, KCNE4 subunit was highly upregulated in lungs from PAH animals and patients. Additionally, Kv7 channel activity was enhanced in the presence of Kv1.5 and TASK-1 channel inhibitors and this was associated with an increased KCNE4 membrane abundance. Compared with systemic arteries, PA showed a poor response to Kv7 channel modulators, which was associated with reduced expression and membrane abundance of Kv7.4 and KCNE4. Our data indicate that Kv7 channel function is preserved and KCNE4 is upregulated in PAH. Therefore, compared to other downregulated channels, the contribution of Kv7 channels is increased in PAH resulting in an enhanced sensitivity to Kv7 channel modulators. This study provides insight into the potential usefulness of targeting Kv7 channels in PAH.

MicroRNAs are non-coding RNAs that regulate gene expression by binding to the 3'-UTR region of specific mRNAs. The expression of miR-1 is increased in plasma from idiopathic PAH (iPAH) patients) compared to healthy controls. miR-1 was proposed as a possible biomarker; however, its pathophysiological role remained unknown. We aimed to analyse the effects of miR-1 on Kv channel function in PA. We found an increased miR-1 expression levels in lungs from PAH animal model (Su/Hpx). PASMC from PAH rats show hypertrophy, a decreased expression and activity of Kv1.5 channels and a membrane more depolarized. miR-1 directly targets Kv1.5 channels analysed by luciferase activity assay. The transfection of miR-1 in PA reduces Kv1.5 activity and induces membrane depolarization.

Antagomir-1 prevents Kv1.5 channel downregulation and the depolarization induced by Su/Hpx exposition. These data indicate that miR-1 reduces the activity and expression of Kv1.5 channels, depolarizes the membrane and induce hypertrophy in PASMC suggesting a pathophysiological role in PAH.

Human immunodeficiency virus (HIV) infection is an established risk factor for PAH; however, the pathogenesis of HIV-related PAH remains unclear. Since K^+ channel dysfunction is a common marker in most forms of PAH, our aim was to analyse whether the expression of HIV proteins is associated with impairment of K^+ channel function in the pulmonary vascular bed. HIV transgenic mice express 7 of the 9 HIV viral proteins. PASMC from HIV-mice had reduced K^+ currents and were more depolarized than those from *wt* mice. TASK-current was nearly abolished in PASMC from HIV-mice. HIV-mice had reduced lung expression of Kv7.1 and Kv7.4 channels and decreased responses to the Kv7.1 channel activator L-364,373. Although we found pulmonary vascular remodelling and endothelial dysfunction in HIV-mice, this was not accompanied by changes in hemodynamic parameters. In conclusion, the expression of HIV proteins *in vivo* impairs pH-sensitive and Kv7 currents. This negative impact of HIV proteins in K^+ channels was not sufficient to induce PAH, but may play a permissive or accessory role in the pathophysiology of HIV-associated PAH.

The data from this Doctoral Thesis provide new evidence of the pathophysiological role of K^+ channels in PH, new mechanisms of action of drugs used in the treatment of PH and suggest a new family of K^+ channels that could constitute a new therapeutic target in PH.

2. Resumen

La gran diversidad funcional de canales de K^+ en la vasculatura pulmonar es el resultado de la multitud de genes que codifican estos canales, el splicing alternativo del ARNm, las modificaciones postranscripcionales, la presencia de ensamblajes homoméricos o heteroméricos de las subunidades α que constituyen el poro del canal y la existencia de subunidades β accesorias que modulan las propiedades funcionales del mismo. Los canales de K^+ también pueden ser regulados a múltiples niveles por diferentes factores que controlan su actividad, tráfico, reciclaje o degradación. La conductancia de estos canales es el principal determinante del potencial de membrana (Em) en las células del músculo liso y en las células endoteliales de arterias pulmonares (CMLAP y CEAP, respectivamente), proporcionando un importante mecanismo regulador para dilatar o contraer las arterias pulmonares (AP). Otra de sus funciones es la regulación de la población y el fenotipo de las CMLAP en la vasculatura pulmonar, ya que participan en supervivencia, lviabilidad, apoptosis y proliferación celular. La alteración de los canales de K^+ juegan un papel fisiopatológico clave en el desarrollo de la hipertensión pulmonar (HP). El deterioro de la actividad de los canales de K^+ en la HP se debe a: 1) mutaciones de perdida de función, 2) la disminución de su expresión mediada a través de factores de transcripción y microARNs; y 3) la disminución de la corriente de K^+ como resultado de un aumento de los factores vasoactivos que inhiben la actividad de estos canales (por ejemplo, la hipoxia, 5-HT, endotelina-1 o tromboxano), por fármacos que bloquean el canal o por una reducción de los factores que regulan positivamente su actividad (por ejemplo el NO y la prostaciclina). El objetivo principal de esta Tesis Doctoral fue aumentar el conocimiento sobre la regulación farmacológica, fisiológica y fisiopatológica de los canales de K^+ en la vasculatura pulmonar y su posible implicación en el contexto de la HP.

La vía del NO/cGMP representa una importante vía fisiológica que controla el tono de las AP. Los fármacos que activan esta vía a diferentes niveles se utilizan en el tratamiento de la hypertension arterial pulmonar (HAP). Uno de los objetivos de esta Tesis doctoral fue analizar la contribución de los canales Kv1.5 y Kv7 en AP en los efectos electrofisiológicos y vasodilatadores inducidos por los donantes de NO y el riociguat, un estimulador de guanilato ciclasa soluble (GCs).

Los resultados mostraron que los donantes de NO y el riociguat activan canales Kv7, lo que conduce a la hiperpolarización del Em de CMLAP. Este mecanismo contribuye a la vasodilatación de AP inducida por la vía del NO/cGMP. Por tanto, nuestro estudio ha permitido identificar los canales Kv7 como un mecanismo de acción novedoso de fármacos vasodilatadores utilizados en el tratamiento de la HAP.

En los últimos años, se ha demostrado el papel clave de los canales de Kv7 y las subunidades auxiliares de KCNE en el control del tono vascular. Además, se ha observado una reducción de la actividad de estos canales en diferentes patologías cardiovasculares como la diabetes, la hipertensión, la disfunción eréctil, la fibrilación auricular o el síndrome del QT largo. Sin embargo, la regulación fisiológica de la actividad de canales Kv7 en la vasculatura pulmonar ha sido poco estudiada. En esta Tesis Doctoral, hemos estudiado el papel de los canales de Kv7 y subunidades KCNE reguladoras en la vasculatura pulmonar y su posible alteración en la HAP. Encontramos que, a diferencia de otros canales de K^+ que están alterados, la corriente Kv7 se conserva en CMLAP de animales con HAP (Su/Hpx), lo que resulta en una mayor contribución relativa a la corriente de K^+ total. Asimismo, encontramos respuestas vasculares mejoradas a moduladores de canales Kv7 en AP de animales con HAP en comparación con controles. También encontramos que la expresión de canales Kv7 y de las subunidades auxiliares KCNE está afectada en HAP. Específicamente, la expresión de la subunidad KCNE4 está aumentada tanto en pulmones de animales como de pacientes con HAP. Además, la actividad de canales Kv7 se potencia en presencia de inhibidores selectivos de los canales Kv1.5 y TASK1, debido al aumento de la abundancia en la membrana de la subunidad KCNE4. Nuestros datos sugieren que hay una mayor contribución de los canales Kv7 en el control de Em y del tono vascular pulmonar en HAP debido a que su actividad está preservada en comparación con otros canales K^+. En conjunto, nuestros datos indican que las AP muestran una mayor sensibilidad a los activadores de los canales Kv7 en la HAP, por lo que estos canales podrían representar un nuevo objetivo farmacológico en esta enfermedad.

Los microARN son ARN no codificantes que regulan la expresión de los genes uniéndose a la región 3'-UTR de determinados ARNm. La expresión de miR-1 está aumentada en el plasma de pacientes con HAP idiopática (HAPi) en comparación con controles sanos. Por ello, miR-1 ha sido propuesto como un posible biomarcador en la HAP, pero su función fisiopatológica era desconocida. Nos propusimos analizar los efectos del miR-1 sobre la función de los canales Kv1.5 en AP. Encontramos que los niveles de miR-1 están aumentados en pulmones de ratas con HAP (Su/Hpx). Además, las CMLAP de ratas Su/Hpx presentaban una disminución de la expresión y actividad de los canales Kv1.5 lo que se traducía en una despolarización del Em y un aumento del tamaño celular o hipertrofia. Mediante el ensayo de actividad de la luciferasa confirmamos que el extremo 3'-UTR del gen *KCNA5*, que codifica los canales Kv1.5, es una diana directa de miR-1. Asímismo, observamos que la transfección de miR-1 en AP reducía la expresión y actividad de canales Kv1.5 e inducía la despolarización de la membrana. El tratamiento con antagomiR-1 previno la regulación negativa de los canales Kv1.5 y la despolarización inducida por la exposición a Su/Hpx. Estos datos indican que miR-1 reduce la expresión y la actividad de los canales Kv1.5, despolariza la membrana e induce hipertrofia de las CMLAP sugiriendo un posible papel fisiopatológico en el desarrollo de HAP.

La infección por el virus de la inmunodeficiencia humana (VIH) es un factor de riesgo establecido para el desarrollo de HAP; sin embargo, la patogénesis de la HAP asociada con el VIH sigue sin conocerse con exactitud. Dado que la disfunción de canales de K^+ es un marcador común en la mayoría de las formas de HAP, nuestro objetivo fue analizar si la expresión de las proteínas del VIH está asociada con el deterioro de la función de canales de K^+ en el lecho vascular pulmonar. Se utilizaron ratones transgénicos VIH-1 que expresan 7 de las 9 proteínas virales. Las CMLAP de los ratones con VIH presentan una reducción en la corriente de K^+ y un Em más despolarizado que las CMLAP controles. La corriente sensible a pH, que implica principamente la activación de canales TASK, estaba prácticamente abolida en las CMLAP de los ratones con VIH. Asimismo, los ratones con VIH presentaban una reducción de la expresión pulmonar de los canales Kv7.1 y Kv7.4, lo cual condujo a una respuesta disminuida al activador del canal Kv7.1, L-364,373. A pesar de que los ratones VIH presentaban una modesta remodelación vascular pulmonar y disfunción endotelial de AP, esto no fue acompañado por cambios en los parámetros hemodinámicos. En conclusión, la

expresión de las proteínas del VIH *in vivo* afecta a las corrientes sensibles al pH y a las corrientes Kv7. Este impacto negativo de las proteínas del VIH en los canales K^+ no fue suficiente para inducir HAP, aunque podría desempeñar un papel permisivo o accesorio en la fisiopatología de HAP asociada al VIH.

En definitiva, esta Tesis Doctoral aporta nuevas evidencias sobre la relevancia de los canales de K^+ en la HP. Identifica la apertura de canales de K^+ como un mecanismo de acción novedoso a través del cual actúan fármacos usados en el tratamiento de la HP, muestra como la expresión de canales de K^+ se ve alterada por factores fisiopatológicos de la enfermedad y sugiere una nueva familia de canales de K^+ como posible diana terapéutica en la HP.

3. Introduction

3.1 K^+ channels in the pulmonary vasculature

K^+ channels are transmembrane proteins that form a pore connecting the cytoplasm to the extracellular environment and setting an outward K^+ current flow. They are the dominant ion conductance of the vascular smooth muscle cell (VSMC) membrane which primarily determine and regulate the membrane potential (Em) [115]. K^+ channels are present in all tissues and each cell expresses a different set of K^+ channels. This structural diversity is further generated from alternative RNA splicing, post-transcriptional modifications, the diversity of homomeric or heteromeric assemblies of the pore-forming α-subunits and the existence of accessory β-subunits modulating the localization and functional properties of the channel [7, 51]. Furthermore, K^+ channels can be regulated at multiple levels by different factors controlling their activity, biosynthesis, trafficking, insertion into the cell membrane, recycling and degradation. The diversity of K^+ channels, their differential tissue expression and partially functional redundancy underlies a physiological fine-tuning within each cell type. A wide variety of K^+ channels are functionally expressed in pulmonary artery smooth muscle cells (PASMC) and pulmonary artery endothelial cells (PAEC). K^+ channel conductance plays a fundamental role in the pulmonary arterial function at three different levels: 1) Regulating the Em of PASMC which will lead in contraction or vasodilation of pulmonary arteries (PA); 2) Regulating the population of PASMC through controlling cell apoptosis, survival and proliferation [37]; and 3) Maintaining the resting Em and the Ca^{2+} entry in PAEC, and thus regulating the release of endothelial vasodilator factors. Therefore, an adequate vascular K^+ channel function ensures an optimal blood flow distribution within the lungs, safeguarding the heart from excessive vascular afterload. Conversely, alterations in the K^+ channel density and activity at the cell surface strongly impact in the pulmonary circulation.

3.1.1 α-subunits

K^+ channels are categorised into three major groups based on the number of transmembrane domains (TM) for each pore-forming α-subunit (2, 4 or 6), which correlates with different electrophysiological and pharmacological properties [246]: 2TM, 4TM and 6TM (Figure 1). The first group comprises inward rectifier K^+ channels (K_{IR}) and ATP-sensitive

K^+ channels (K_{ATP}). Four α subunits form the transmembrane channel for K^+ ion conduction. The function of K_{IR} channels can be regulated by small substances and by phosphorylation. The small substances include ions such as H^+, Mg^{2+}, and Na^+; polyamines and membrane-bound phospholipids [99]. There are 7 K_{IR} channel subfamilies and 15 different isoforms that can be classified into four functional groups: 1) classical K_{IR} channels (K_{IR}2.1-2.4) which are constitutively active, 2) G protein-gated K_{IR} channels (K_{IR}3.1-3.4), 3) K_{ATP} (K_{IR}6.1-6.2) which are tightly linked to cellular metabolism and 4) K^+ transport K_{IR} channels (K_{IR}1.1, K_{IR}4.1-4.2, K_{IR}5.1, and K_{IR}7.1) [99]. The most relevant K_{ir} channel expressed in the vasculature belongs to the K_{IR}2 and K_{IR}6 subfamilies. K_{ir}2.1 channel expression predominates in VSMC and endothelial cells (EC) [64, 5]. K_{ATP} channels are assembled as octameric complexes of K_{IR} channel (K_{IR}6.1-6.2) subunits associated with regulatory sulfonylurea receptor-1 (SUR1) or sulfonylurea receptor-2 subunits (SUR2A and SUR2B) [76]. The K_{ATP} channels are opened in response to lower ATP levels and close in response to higher ATP levels [76]. The composition of K_{ATP} channels is tissue specific, for example, K_{ATP} channels comprises SUR1 and K_{IR}6.2 subunits in the pancreatic β cell; in the cardiac and skeletal myocytes, are composed by SUR2A and K_{IR}6.2; in VSMC predominates SUR2B and K_{IR}6.1 expression while, SUR2B and K_{IR}6.2 subunits in non-VSMC [280]. The 4TM channels family is comprised by two α subunits and are often referred as two-pore domain K^+ channels (K_{2P}). They are mostly voltage-independent, contributing to setting the resting Em. Their activity is modulated by physical and chemical stimuli such as mechanical stretch, pH and volatile anaesthetics. There are 6 subfamilies of K_{2P} channels and 15 different isoforms denoted as 1) TREK [TREK1(K_{2P}2.1), TREK2(K_{2P}10.1) and TRAAK (K_{2P}4.1)], 2) TALK [TALK1(K_{2P}16.1) TALK2(K_{2P}17.1), and TASK2(K_{2P}5.1)], 3) TASK [TASK1(K_{2P}3.1), TASK3(K_{2P}9.1) and TASK5(K_{2P}15.1)], 4) TWIK [TWIK1 (K_{2P}1.1), TWIK2 (K_{2P}6.1) and KCNK7 (K_{2P}7.1)], 5) THIK [THIK1(K_{2P}13.1) and THIK2(K_{2P}12.1)] and 6) TRESK channels [K_{2P}18.1] [162]. TWIK1, TREK1 and TASK1 channels expression predominates in vascular vessels [264]. Finally, the 6TM channels are classified into voltage-dependent K^+ channels (Kv) and calcium-activated K^+ channels (K_{Ca}). There are 12 subfamilies and 42 different isoforms of Kv channels: [Kv1.1-1.10, Kv2.1-2.2, Kv3.1-3.4, Kv4.1-4.3, Kv5.1, Kv6.1-6.4, Kv7.1-7.5 also known as KCNQ, Kv8.1-8.2, Kv9.1-9.3, Kv10.1-10.2 also known as *ether-à-go-go* (EAG), Kv11.1-11.3 also known as eag-*related gene* (ERG) and Kv12.1-12.3 also known as eag-*like (*ELK)] [265]. Resistance arteries and arterioles express a diverse array of K_V channels; however, Kv1, Kv2.1, Kv3.1, Kv4, Kv7 and Kv9 channels are

particularly important [246]. Kv9 do not form by themselves active channels but can heterodimerize with other Kv channels. The K_{Ca} channels subfamilies are subdivided into BK ($K_{Ca}1.1$), IK ($K_{Ca}3.1$), and SK ($K_{Ca}2.1$–2.3) channels according to their conductance (big, intermediate and small, respectively) [28, 133]. BK channels have a Ca^{2+} binding region (bowl), which causes its opening to increase when cytosolic calcium rises and an additional TM domain (7TM) also known as S0 segment, which is required for β-subunit modulation [254]. VSMC express particularly BKCa channel; while, EC express IK ($K_{Ca}3.1$), SK ($K_{Ca}2.3$) and to a lesser extent BK_{Ca} [128].

Extensive studies have identified the essential role of specific subgroups of K^+ channels as key regulators of PASMC excitability. Among them, it is worth highlighting the importance of Kv1.2/Kv1.5/Kv2.1 and Kv7.4/Kv7.5 channels (Kv channels), TASK1 channels (K_{2P}), Kir6.1/SUR2 (K_{ATP}) and $K_{Ca}1.1$ (BK_{Ca} channels) since they have been shown to play an essential role in the context of pulmonary vascular physiology and pathology [216, 170, 174, 163, 80, 223, 28]. Annex I includes the biophysical and pharmacological properties of the K^+ channels studied in this Doctoral Thesis.

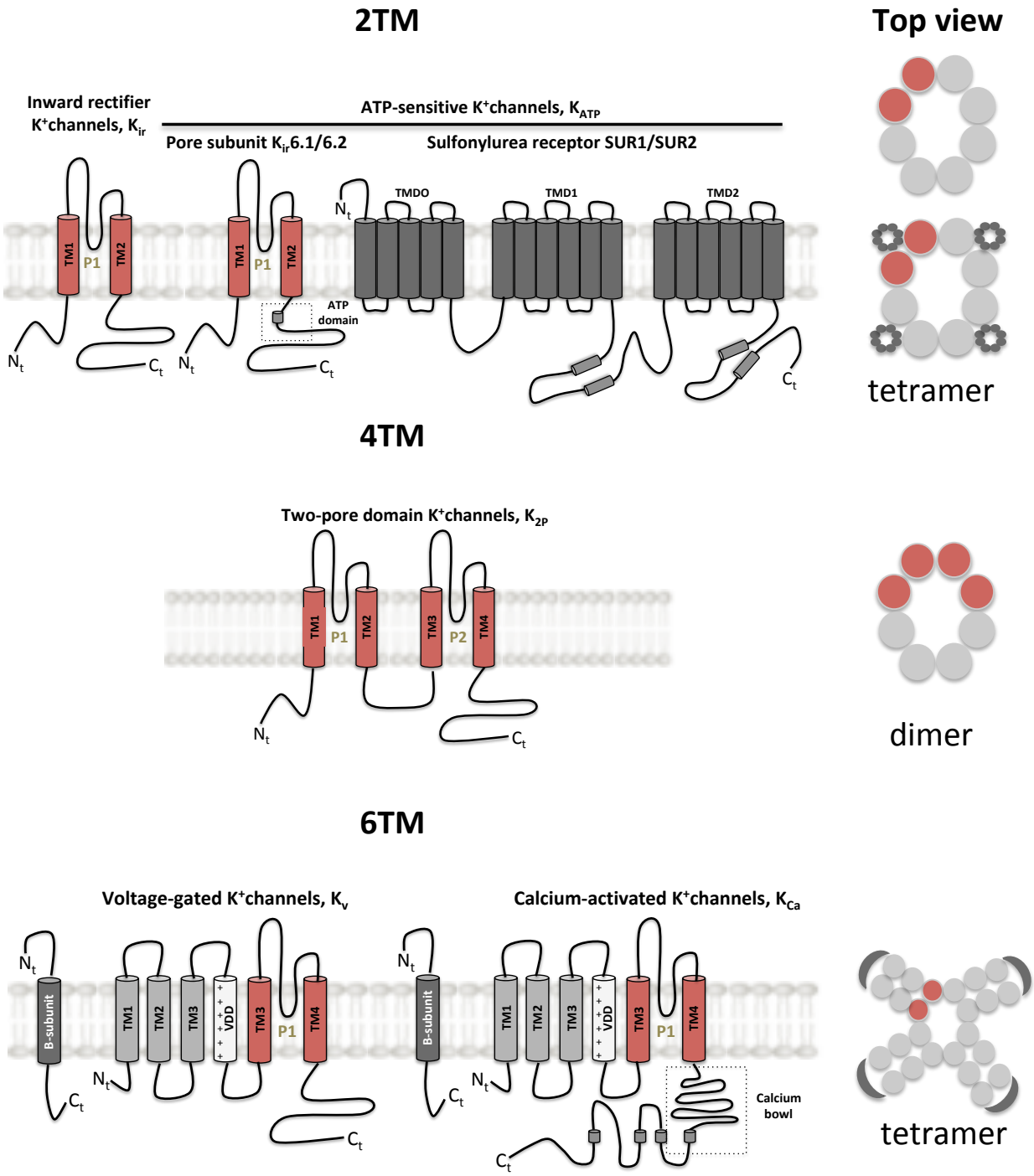


Figure 1. Classification and structure of potassium channels expressed in the pulmonary vasculature. TM: transmembrane; VDD: Voltage-detection domain; β-subunit: single transmembrane domain protein attached to voltage-dependent α-subunit K^+ channels.

3.1.2 β-subunits in 6TM channels

6TM K^+ channels, i.e. Kv and BK_{Ca} channels can be regulated by single transmembrane domain proteins composed of 103 to 177 residues known as accessory β ancillary subunit. The β-subunit is attached to α-subunit and can regulate the ion channel trafficking, its biophysical and pharmacological properties [2]. Three main regulatory Kvβ subunits, Kvβ1-3, are associated to Kv1 α-subunits subfamily. All Kvβ1-3 genes have been detected in PASMC [74]. Four β-subunits interact with four α subunits to form a functional channel [199]. As cytoplasmic regulatory domains, Kvβ1.1 and Kvβ1.2 are regulated by the cellular redox state [207]. The Kvβ2.1 subunit has several potential phosphorylation sites that could regulate assembly or interaction with the α-subunit. The coexpression Kvβ2.1/Kv1.5 in HEK293 cells resulted in a early activation of Kv1.5 channel and an almost 2-fold increase in the degree of slow inactivation [247]. Kv7 channels can be regulated by KCNE(1-5) ancillary subunits which present a single transmembrane domain. The best known is the KCNE1 ancillary subunits modulation of Kv7.1 in the cardiomyocytes. KCNE1 also increased Kv7.5 currents by precluding their activation. KCNE4 enhanced Kv7.4 current amplitude, whereas KCNE3 abolished Kv7.4 and Kv7.5 currents [75]. Jepps TA *et al.,* demonstrated that KCNE4 knockdown leads to reduced Kv7.4 membrane abundance in mesenteric artery myocytes [120]. However, the role of Kv7 channels and KCNE subunits is not well established in the pulmonary vasculature.

BK channel activity is regulated by the tissue-specific expression of auxiliary β-subunits. Two distinct classes of BK channel auxiliary subunits have been identified: four β subunits (β1–β4) and four γ subunits (γ1–γ4) [146]. Four β-subunits have been determined in VSMC, but BK_{Ca}β1 appears to be the main isoform altering channel gating kinetics and increasing the Ca^{2+} sensitivity of the α-subunit [246]. The four BK_{Ca}β subunits (β1–β4) appear to be expressed at mRNA levels in PASMC which are targets for various physiological factors released from the endothelium [74, 137]. A recent study showed that, the BK_{Ca}β1 does not affect pulmonary vascular tone under basal conditions, but increase contraction under hypoxic conditions (either acute or chronic) [15, 13, 6]. In PAEC, the expresion of β_1- and β_4-subunits, but not that of β_2- and β_3-subunits, have been identified [250]. The auxiliary γ-subunits habe been identified recently; therefore, information about their regulation is very scarce [146].

3.2 Role of K^+ channels in pulmonary artery smooth muscle contraction

The ability of VSMC to contract and relax determines the control of pressure and the distribution of blood flow in small arteries and arterioles to different organs and tissues [34]. PASMC have a high K^+ channel activity, which provides them protection against membrane depolarization contributing to the maintenance of a lower blood pressure than in systemic vessels. At physiological K^+ ion concentrations in PASMC (3–5 mM K^+ extracellular and 140 mM K^+ intracellular), the electrochemical gradient or the driving force for K^+ movement is outward toward the theoretical equilibrium Em for K^+ ions (E_k=−90 mV) [70, 186, 137]. This means that opening K^+ channels will lead to diffusion of K^+ ions out of the cell, the loss of positive charges in the cytosol and the subsequent E_m hyperpolarization. *In vivo,* PASMC have a negative resting Em from −85 to −60 mV close to E_k. *Ex-vivo*, the resting *Em* of PASMC varies from −65 to −40 mV [137, 70, 41, 28, 74, 9]. Hyperpolarization reduces the open probability of voltage-gated Ca^{2+} channels (VGCC) located at membrane level, decreasing Ca^{2+} entry and causing vasodilation. Conversely, K^+ channel inhibition causes a decrease in the hyperpolarizing K^+ current and membrane depolarization [37, 186, 235]. Membrane depolarization opens VGCC, which increases cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$ [59]. The Ca^{2+}-Calmodulin complex ($(Ca^{2+})_4$-CaM)-mediated activation of myosin light chain kinase (MLCK) and phosphorylation of 20 kDa myosin regulatory light chains (LC20) at serine-19 (MLC-pLC20-S19) leading to enhanced actomyosin-pS-19 ATPase activity, cross-bridge cycling and contraction (Figure 2) [258]. There are two main negative-feedback mechanisms involving Kv and BK_{Ca} channels which repolarize the cell membrane and therefore permit a precise control of arterial diameter [8, 177, 206, 268, 278]. The activation of Kv channels is a direct consequence of the membrane depolarization [8, 177, 206, 268, 278]. In contrast, the activation of BK_{Ca} channels has been attributed the rise in $[Ca^{2+}]_{cyt}$ due to the release of Ca^{2+} from the sarcoplasmic reticulum (SR) and Ca^{2+} influx via VGCCs [31, 268]. MLC-pLC20-S19 is dephosphorylated by myosin light chain phosphatase (MLCP) to induce relaxation. The balance of MLCK and MLCP activity determines LC20 phosphorylation level and force generation in the steady-state [59]. The stimulation of PKC activity by Ca^{2+} owing to influx or release from SR stores leads to MLCP inhibition [236, 100] causing a leftward shift in the relation between LC20 phosphorylation or force generation versus $[Ca^{2+}]_{cyt}$ [59].

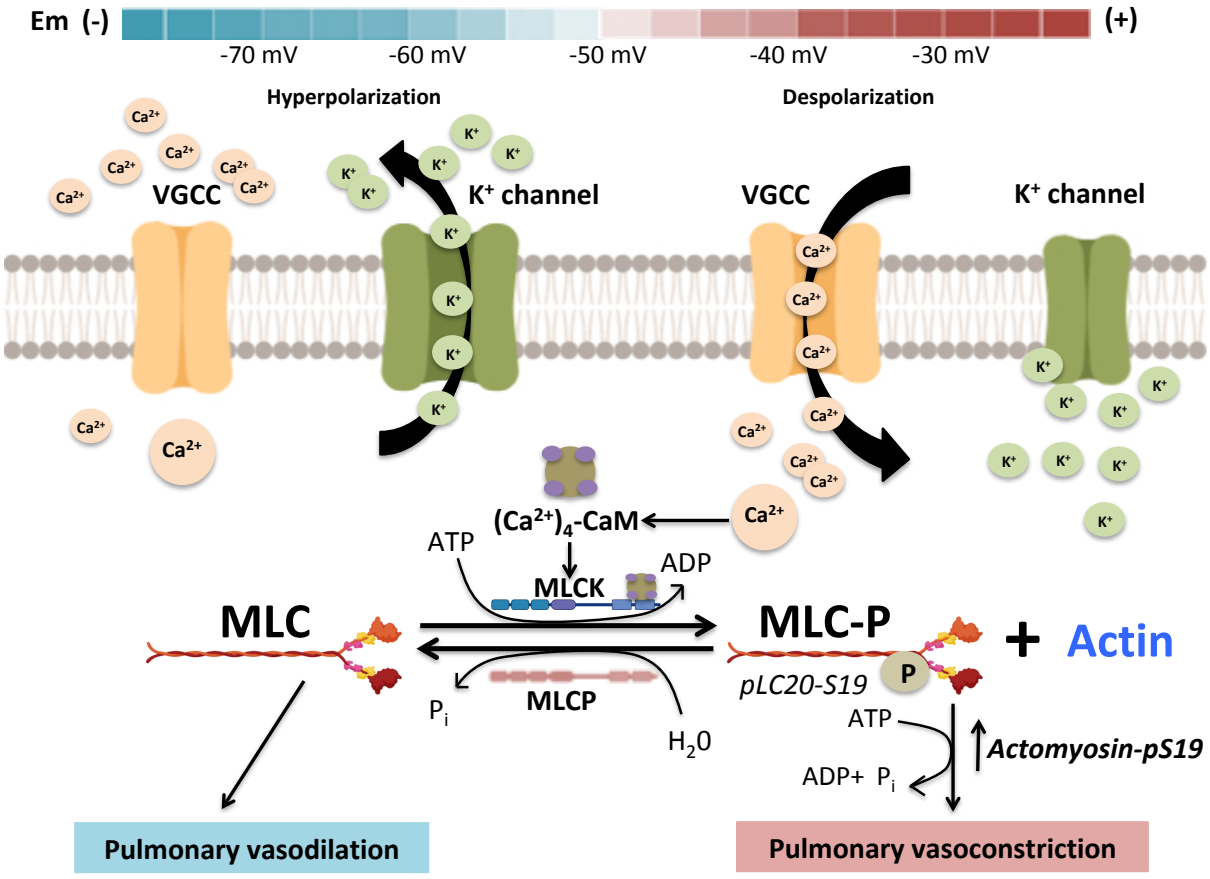


Figure 2. Role of K+ channel in the regulation of vascular tone. VGCC: L-type voltage-gated Ca^{2+} channels Ca-CaM: Ca^{2+}-calmodulin complex; MLCK: myosin light chain kinase; MLCP: myosin light chain phosphatase; MLC: myosin light chain.

3.3 Role of K^+ channels in pulmonary artery endothelial cells

Pulmonary arterial vasoconstriction and vasodilatation are importantly regulated by factors released from PAEC. Although their role is less studied than in PASMC, a large variety of K^+ channels are expressed in PAECs playing an important role in vascular tone control. Different studies have confirmed the functional expression of K^+ channels in fresh rat PAEC. The K^+ channels function in these cells is to regulate resting Em (in non-excitable cells is around −55 to −30 mV). In PAECs, which do not express VGCC, hyperpolarization induces Ca^{2+} entry inside the cell (by increasing the electrical driving force), activates the production of NO and subsequent endothelium-dependent vasodilation in the pulmonary vasculature. So far, the expression of $K_{IR}2.1/2.2$ [190, 262], K_{ATP} [44], Kv1.5 [105], $K_{Ca}2.3/3.1$ [131, 251] and TASK-1/2 channels [79] has been reported in PAEC. Furthermore, other studies have proved some these channels are sensitive to shear stress as K_{ATP} channels [44] or can be activated by PGI_2 analogues such as K_{Ca} channels [148].

3.4 Role of K^+ channels in cell survival, apoptosis and proliferation.

K^+ channels regulate several key cellular processes such as migration, differentiation, apoptosis and proliferation [37]. An appropriate balance between proliferation and apoptosis is required for regular PASMC homeostasis; the imbalance of these two mechanisms contributes to the PA vascular remodelling. K^+ channels have been implicated in both early and late stages of apoptosis. The decrease in cell volume is one of the earliest and necessary morphological changes observed in cells undergoing apoptosis. Apoptotic volume decrease is accomplished in similar manner as regulatory volume decrease associated to changes in hypo- and hypertonic conditions [43]. Active K^+ channels in the plasma membrane leads to K^+ and Cl^- outflow through K^+ and Cl^- channels, this generates an osmotic gradient leading to an outward water transport through aquaporines which results in a decrease in cell volume [160]. Several K^+ channels are involved in regulation of cell volume, including Kv channels (Kv1.3 and Kv1.5), K_{Ca} channels (SK_{Ca}, IK_{Ca} and BK_{Ca}), and K_{2P} (TREK1, TRAAK and TASK2) [37]. Notably, the overexpression of human KCNA5 gene (encoding Kv1.5 channel) increases Kv current and enhances apoptosis [32]. In addition, using a mitochondrial protonophore, was shown to cause apoptosis by increasing IK_{Ca} and BK_{Ca} channels in rat and human PASMC and the block of these channels with iberiotoxin or TEA inhibited protonophore induced-apoptosis [130]. In later stages of apoptosis, the augmented $[K^+]_{int}$ suppresses caspase and nuclease activity and, conversely, the decreased $[K^+]_{int}$ caused by K^+ channel activation promotes apoptosis [37].

K^+ channels also participate cell cycle controlling $[Ca^{2+}]_{int}$, an important second messenger for cell proliferation. Since only certain types of Ca^{2+} signals can lead to the activation of growth-promoting genes, the ability of K^+ channels to modulate the amplitude and duration of Ca^{2+} signalling can influence cell growth. The sustained increase in Ca^{2+} influx through VGCC triggers CRE- and c-Jun-mediated gene transcription, activates transcription factors (CREB, NF-AT and NF-kB) that are involved in cell proliferation, protein synthesis and inflammation [65]. $[Ca^{2+}]_{int}$ is also required for cell-cycle progression such as the G_0 to G_1 transition, DNA synthesis and mitosis [37]. The inhibition of Kv channels is associated with cell proliferation in PASMC, consistent with the idea that the depolarization-induced increase in $[Ca^{2+}]_{int}$ allows for cell cycle progression.

Proliferating PASMCs from PAH-patients demonstrate many cellular abnormalities linked to K^+ channels, including downregulated expression of various K^+ channels, decreased K^+ current, more depolarized Em, higher $[Ca^{2+}]_{int}$ and inhibited apoptosis [207]. In addition, several studies have shown that restoring K^+ channel activity inhibits proliferation and enhanced apoptosis in PAH-PASCM [10, 165, 166, 181, 267, 281].

However, according to the K^+ channel isoform, expression levels, heteromers, splice variants, regulatory subunits, the cell type and the stage of differentiation the effects of K^+ channels activity are diverse. For instance, K^+ channels activity may exhibit proliferative or anti-proliferative roles [215]. Moreover changes in the phenotypes, signalling pathways and pattern of expression of K^+ channels may be observed in proliferative cells, which increases the complexity of K^+ channels regulation on cell viability.

3.5 Pulmonary hypertension

The normal mean pulmonary artery pressure (mPAP) at rest is 14±3 mmHg, i.e. five fold lower that the mean systemic blood pressure, with an upper limit of approximately 20 mmHg [129]. Pulmonary hypertension (PH) is a disorder defined by an elevated mPAP as assessed by right heart catheterization [103]. PH symptoms are non-specific and intimately related to progressive right ventricular (RV) dysfunction [78]. Initial symptoms are generally induced by exertion such as fatigue, weakness, shortness of breath, dry cough, nausea, vomiting, angina and syncope. Symptoms at rest, as abdominal distension and ankle oedema, generally appear in advanced cases. Based on pathophysiological findings, clinical presentation, haemodynamic characteristics and therapeutic considerations, PH is categorized into five groups by World Health Organization (WHO): pulmonary arterial hypertension (PAH), PH due to left-sided heart disease, PH due to lung disease or hypoxia, chronic thromboembolic PH and multifactorial PH [78]. PH is becoming a substantial global health issue. Recent estimates suggest a PH prevalence of about 1% of the global population, which increases up to 10% in individuals aged more than 65 years [104]. However, the diverse forms of PH differ considerably in incidence and prevalence. Worldwide, left-sided heart (group 2) and lung diseases (group 3) are the most frequent causes of PH [78].

Approximately 80% of affected patients live in developing countries, where PH is widely associated with congenital heart disease, various infectious disorders (schistosomiasis or HIV) and rheumatic heart disease. These last forms of PH occur predominantly in those younger than 65 years. Most researchers, clinicians, and the pharmaceutical industry have focused on certain forms of PH, mainly PAH which is considered a multifactorial and rare condition. The PAH group describes a subpopulation of PH patients that is hemodynamically distinguished by precapillary PH with mPAP ≥ 20 mm Hg, normal pulmonary arterial wedge pressure (PAWP ≤ 15 mmHg) and with elevated pulmonary vascular resistance (PVR >3 Wood units) [78, 103, 153]. PAH can be further categorized into idiopathic (iPAH), heritable (hPAH), induced by drugs and toxins, and associated PAH (aPAH). aPAH occurs in the setting of a variety of conditions such as connective tissue disease, congenital heart disease, portal hypertension, schistosomiasis or HIV infection [17].

In the developed word, the PAH reported incidence is 1.1–7.6 per million adults per year and the prevalence is 6.6–26.0 per million adults [69, 111, 200]. PAH affects predominantly younger individuals, mostly females; remarkably for hPAH, which affects twice as many females as males younger 50 years [217]. However females have better survival than males [253]. The pathophysiology of PAH is complex, it involves endothelial dysfunction with impaired production of vasodilators as NO or PGI_2 and the overexpression of vasoconstrictors such as ET-1. These molecular alterations affect vascular tone and promote pathological vascular remodeling, which leads to pulmonary arterial vasoconstriction, medial hypertrophy, intimal proliferation, fibrosis, inflammation, *in-situ* thrombosis and, occasionally, complex plexiform intimal lesions produced by the transition from endothelial to mesenchymal cells [213]. It is translated in a decrease arterial lumen and increased PVR [212, 221]. The ensuing rise in PVR leads to increase right ventricular afterload and dilatation. Without effective therapeutic interventions, PAH patients eventually die from right heart failure [245].

HIV-associated PAH (HIV-PAH) is a well-recognized cardiovascular complication of HIV infection that is associated with an adverse prognosis irrespective of the stage of disease and with significant mortality [118]. Despite the success of the highly active antiretroviral treatment (HAART), the risk of developing cardiovascular disease is amplified in HIV-patients

due to HAART-associated dyslipidaemias and insulin resistance as well as the direct toxic effects of HIV-related proteins which results in chronic endothelial dysfunction, impaired fibrinolysis and a chronic inflammatory state [14]. PAH occurs in approximately 0.5% of HIV-infected persons, in comparison with the incidence of iPAH in the general population (1-2 per million); HIV-infected patients have a 2500-fold increased risk of developing PAH [232]. Recent echocardiographic studies of HIV-infected outpatients found that between 15% and 35% had elevated systolic PAP, indicating that PAH may be even more common than previously thought [180]. Despite the improvements in HIV-associated morbidity and mortality, the prevalence of PAH has not changed significantly in the post-HAART era [211]. The clinical presentation of HIV-PAH can be associated with nonspecific and insidious symptoms, which can often be mistakenly attributed to HIV itself. The most common presenting symptom noted in HIV-PAH patients is progressive dyspnea [118]. Even with diagnosis and treatment, prognosis remains poor for HIV-PAH. The exact pathogenic mechanism that links HIV to PAH is not known. Histologically, the features of concentric intimal fibrosis, medial hypertrophy and plexiform lesions are seen in up to 78% of patients with HIV-PAH, and this closely resembles changes seen in other PAH forms [204]. Several HIV viral proteins are implicated in the pathogenesis although the exact mechanism remains unknown (See Chapter IV). The possible HIV-PAH pathogenic mechanism are related to the disruption of TGF-β and BMPR2 pathways, induction of ROS production and increase inflammation factors such as IL-6 [118]. In the current guidelines for treatment of PH, therapeutic options for HIV-PAH are less well established, as conflicting data on the role of HAART in the management of patients with HIV-PAH. HIV-PAH treatment is similar to that for all PAH conditions and includes lifestyle changes, general treatments and disease-specific treatments [47, 61].

The classic strategies of PH treatment have harnessed the known dilatation pathways to restore the balance between the vasodilator and vasoconstrictor mediators released by the endothelium. The FDA/EMA approved drugs for PH can be classified into 6 groups (Figure 3): endothelin receptor antagonists (bosentan, ambrisentan and macitentan), Ca^{2+} channel blockers (nifedipine, diltiazem and amlodipine), inhaled NO, PGI_2 analogues (epoprostenol, teprostinil, iloprost, beraprost and selexipag), sGC activators (riociguat) and PDE5 inhibitors (sildenafil and tadalafil) [248].

Vasodilator drugs are beneficial in most patients but depending on the mechanism that produces mPAP increase, they do not always produce effective vasodilation. On the other hand, their poor pulmonary selectivity leads to the appearance of systemic side effects. Despite effective current therapies, no cure exists for PAH and lung transplantation remains the last treatment option for eligible PAH patients [140]. Several additional pathways have been suggested to be involved in the pathogenesis of PAH, and may represent innovative therapies [172]. These include, antiproliferative therapies such as tyrosine kinase inhibitors (imatinib, PDGF receptor inhibitor) witch have been shown to have benefits in clinical trials but with important side effects [102] , ROCK inhibitors (fasudil and hydroxyfasudil) that also play key roles in PASMC contraction [40], peroxisome proliferator–activated receptor (PPAR) agonists [90], several antioxidants [239], inhibitors of other kinases and drugs interfering the BMPR2 or the TGFβ signalling pathways. There is increasing evidence to suggest an important role for inflammation in the onset and progression of PH, then immunosuppressant approaches should allow the development of innovative treatments [54].

Impairment of K^+ channel activity is considered a hallmark of PAH [10, 28, 88, 135, 167, 173, 255, 271, 273, 274]. The K^+ channel disturbance in PAH may occur at 3 different levels due to: 1) the presence of polymorphisms and mutations in the K^+ channels-encoding genes, 2) downregulation of the expression of K^+ channels and 3) due to pathophysiological factors that alter the activity or location of K^+ channel.

3.5.1 K^+ channel polymorphisms and mutations in PAH.

Genetics could partially elucidate the abnormal phenotypes of pulmonary vascular cells in PAH. The most commonly associated gene, BMPR2, which encodes bone morphogenic protein receptor type 2 (a member of the TGFβ super family of receptors) is mutated in 56 ~70% of patients with hPAH [113, 154]. Several mutations have been identified in the TGFβ super-family genes including the activin receptor-like kinase 1 (ALK1), endoglin (ENG) [92], Caveolin-1 (CAV1) [12], T-box4 transcription factor (TBX4) and many cytoplasmic proteins encoded by *SMAD* genes [122]. More recently three new predisposing genes were identified: ATPase 13A3 (ATP13A3), Aquaporine 1 (AQP1) and SRY-Box (SOX17); even though, constitute a susceptibility factors for the development of PAH are neither necessary

nor sufficient [83]. Other large group of identified mutations or polymorphisms in genes encoding K^+ channels have demonstrated their relevancy in the development of PAH. Different K^+ channel-Single Nucleotide Polymorphisms (SNP) in KCNA5 gen and gene mutations in KCNK3, KCNJ8 and ABCC8/9 have been related to hPAH, as detailed as bellow (Figure 3).

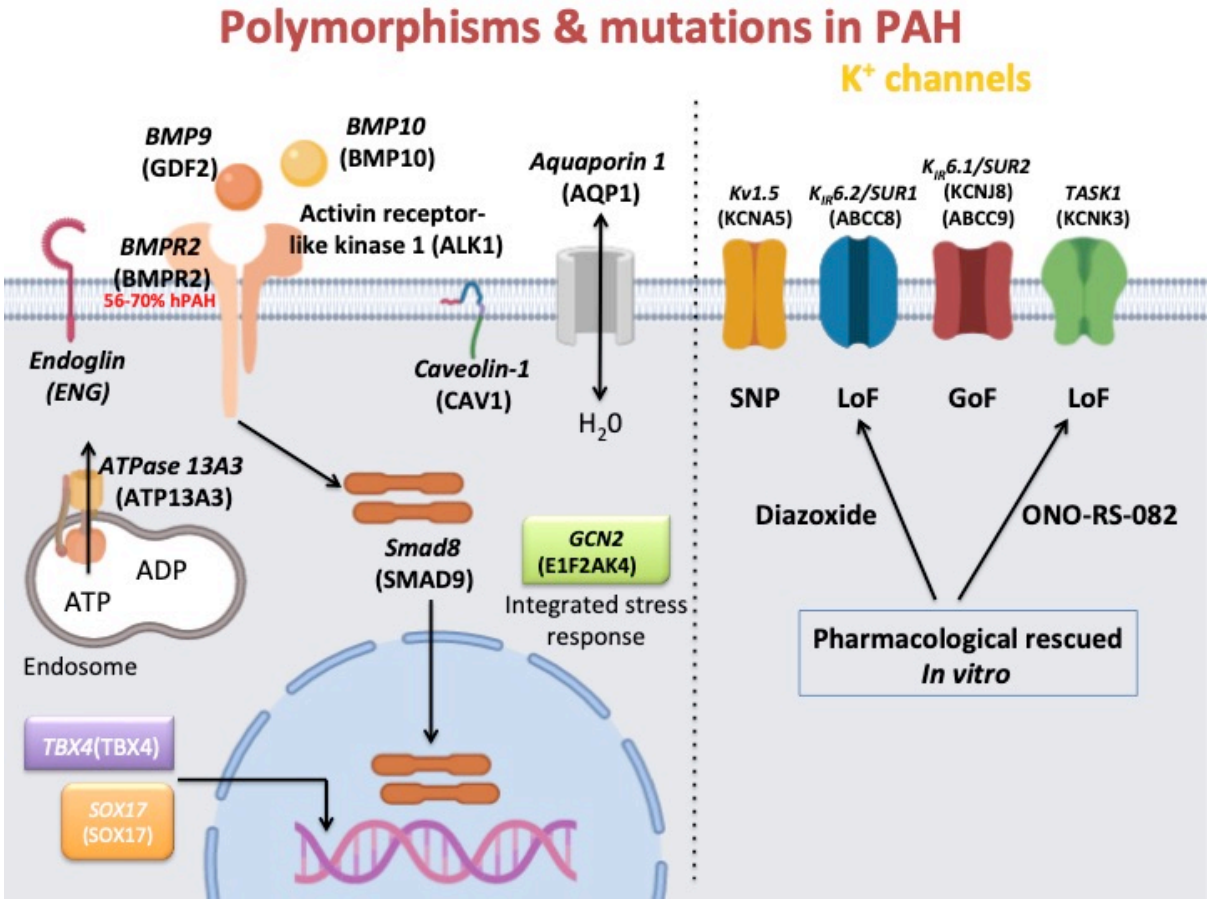


Figure 3. Polymorphisms and mutations PAH-associated.

3.5.1.1 Single Nucleotide Polymorphisms in KCNA5 gene

Kv1.5 channels encoded by *KCNA5* gene are functional Kv channels highly expressed in PASMC, being the ones that most contribute to the total Kv current (Figure 5). The reduced Kv1.5 channel expression and function is a common denominator in PASMC from PAH patients [11, 126] and PH animal models [167, 207, 255] (Figure 5); suggesting their important role in the pathogenesis of various forms of PH [28]. Overexpression of Kv1.5 channel in human PASMC and other cell lines (HEK-293, CHO and COS cells) causes membrane hyperpolarization and enhances apoptosis [32]. Conversely, downregulation of

Kv1.5 channels causes membrane depolarization, an increase of $[Ca^{2+}]_{cyt}$, contraction, migration, and proliferation of PASMC [10, 214]. In 2007, Remillard CV *et al*. identified 17 SNPs in KCNA5 gene; 7 of which had not been previously reported. There were 12 SNPs in the 5'UTR region, 2 of which were proposed to alter transcription factor binding sites in the promoter, 2 non-synonymous SNPs in the coding region, 2 SNPs in the 3'-UTR and 1 SNP in the 3'-flanking region [216]. Additionally, two SNPs (T-937a and G2870a) were suggested to correlate with the nitric oxide-mediated decrease in mPAP. Allele frequency of two other SNPs (C828t and C136t) in patients with a history of fenfluramine and phentermine use was significantly different from patients who have never taken anorexigens. The two non-synonymous SNPs (G773a and G861c) were located in the heteromerization or polymerization domain where Kvα channel and β-subunits interacts with each other and could affect assembly of functional tetrameric Kv channels [270]. The other two SNPs (A2578t and T2806t/g) located in the 3-UTR may alter the regulatory effects of protein kinases on channel activity or may be involved in post-translational regulation of Kv1.5 channel expression. However, in the absence of any independent replication studies or comprehensive examination of functional consequences, the causality of this gene mutation is disputed [238].

3.5.1.2 Loss of Function (LoF) mutations in KCNK3 gen

To date, 15 different *KCNK3* mutations have been described in PAH patients. In 2013, Ma L *et al*; described the first channelopathy known to cause PAH. Six different heterozygous mutations were identified in *KCNK3* gene in sporadic and familial PAH patients [24, 155]. *KCNK3* gene encodes for a K_{2P} channel also referred to as TASK-1 *(TWIK-Related Acid-Sensitive K^+ Channel 1)* that determines a non-inactivating K^+ conductance (I_{KN}) and largely contributes to resting Em of PASMC. These channels are enhanced by halothane or alkaline pH and inhibited by Zn^{2+} or anandamide [137]. The knockdown of *KCNK3* in human PASMC induced a significant depolarization of resting Em (from −40 mV to −25 mV), confirming the TASK-1 channels implication in the resting Em [193]. *Ma* and colleagues demonstrated that the identified *KCNK3* mutations result in non-functional channels, indicating a loss of function (LoF) mutation. However they also revealed that the function of some of these mutated channels can be at least partly restored with the use of a phospholipase A2

inhibitor (ONO-RS-082) [155]. In 2017, two additional *KCNK3* mutations (p.Gly106Arg and p.Leu214Arg) have been identified in a Spanish cohort of PAH patients. Interestingly, this report described the first case of PAH occurring in a patient with homozygous *KCNK3* mutations associated with an aggressive form of PAH diagnosed at birth [185]. These two mutations have a LoF compared to *wt* TASK-1 channels. These mutated TASK-1 channels are located at the plasma membrane to the same degree as *wt* TASK-1 channels. Functional current through these mutated channels could not be restored using TASK-1 activators, pH 8.4, ONO-RS-082 or the sGC activator, riociguat [63]. Two additional *KCNK3* mutations were identified in an American cohort of PAH patients the same year [20].

3.5.1.3 Gain-of-function (GoF) mutations in ABCC9 and KCNJ8 genes

K_{ATP} channels are assembled as octameric complexes of pore-forming $K_{IR}6.1$ or $K_{IR}6.2$ subunits associated with regulatory SUR1 or SUR2 subunits. SUR1 is encoded by *ABCC8* gene, located on human chromosome 11, immediately preceding the gene encoding the $K_{IR}6.2$ subunit (*KCNJ11*), whilst the paralogous *ABCC9* (SUR2) and *KCNJ8* ($K_{IR}6.1$) gene pair are immediately adjacent to each other on chromosome 12 [189]. Autosomal dominant mutations in *ABCC9* and *KCNJ8* genes cause the complex heritable disorder, Cantu Syndrome (CS), characterized by vasodilation, decreased systemic blood pressure, pronounced cardiac hypertrophy and PH [33, 91]. Both mutations converge in a common pathophysiology; the GoF of SUR2/$K_{IR}6.1$-dependent K_{ATP} channels. The $K_{IR}6.1$/SUR2 complex is expressed in the pulmonary vasculature [50, 62], consequently, exaggerated pulmonary vasodilatation with reduced mPAP should be anticipated in CS patients. Paradoxically, CS patients present high mPAP and an aggressive form of PH [125, 196]. The adverse effects of K_{ATP} channel openers (KCOs) such as diazoxide and minoxidil overlap interestingly with the clinical features observed in CS, including PH [187, 243]. The KCOs will trigger compensatory feedback in response to their potent systemic blood pressure lowering effect [117]. It is hypothesized that the compensatory effects include elevated sympathetic activity and the up-regulation of renin-angiotensin-aldosterone signalling (RAAS) due to decreased renal perfusion. Activation of RAAS leads to elevated salt and water retention and blood plasma volume expansion (hypervolemia), which helps to normalize systemic blood pressure [163]. The elevated RAAS activity can contribute to the

development of PH due to volume-overload of the pulmonary circulation [179, 178]. The causality of this gene mutation is not universally recognized [238].

3.5.1.4 Loss of Function (LoF) mutations in ABCC8 gen

A recent study has identified *de novo* and inherited heterozygous LoF mutations in a novel candidate gene, *ABCC8*, potentially associated with idiopathic, familial and congenital heart disease-associated in both paediatric and adult PAH [23]. The penetrance of these mutations is incomplete and it can also depend on additional genetic or environmental factors. As mentioned above, *ABCC8* gene encodes SUR1, a regulatory subunit of K_{ATP} channels that associates with $K_{IR}6.2$ subunit. The eleven identified missense *ABCC8* variants are all rare, located at residues highly conserved across species and reside in intracellular or transmembrane domains of SUR1, including nucleotide binding fold regions. *ABCC8* was shown to be moderately expressed in human lungs, however the molecular characterization of K_{ATP} channels in PASMC and PAEC demonstrates a predominance of *ABCC9* gene (SUR2) expression [62], not *ABCC8* (SUR1) which is instead highly expressed in the pancreas and neurons. In fact, the $K_{IR}6.2$/SUR1 association in lung is peculiar because these channels are critical regulators of pancreatic β-cell excitability and *ABCC8* LoF mutations are a recognized cause of congenital hyperinsulinism [67]. However, PAH patients with *ABCC8*-variant do not exhibit hyperinsulinism. The mechanism of SUR1-LoF likely varies based on mutation location, may affect the SUR1 trafficking to the plasma membrane, impair magnesium-nucleotide activation and impacts in the viability for pharmacological rescue [188]. Functional analyses demonstrated reduced K_{ATP} channel activity in all *ABCC8* mutants tested. The channel activity can be pharmacological rescued *in vitro* by the K_{ATP} channel activator diazoxide [23]. Moreover, SUR1 expression can also mediate apoptosis induced by the SUR-ligands and this response does not require K_{ATP} channel activity. Hence, PAH-associated *ABCC8* mutations may impair apoptosis through a K_{ATP}-independent effect of SUR1, promoting vascular remodelling in PAH [4, 89].

3.5.2 Downregulation of K^+ channel activity and expression in PAH

3.5.2.1 Human PAH

Due to the complexity of obtaining human samples from patients with PH, few studies have been carried out to study the alteration of K^+ channels in the pulmonary vasculature in patients with PH. In 1998, *Yuan XJ* and colleagues demonstrated for the first time an attenuated expression of Kv1.5 mRNA in PH-PASMC. The decreased number of the Kv1.5 channels and an increased fraction of Kv1.5/Kv1.1 heteromultimers led to reduced Kv current amplitude and accelerated Kv inactivation in PAH-PASMC [271]. The resultant membrane depolarization and increase in $[Ca^{2+}]_{cyt}$ was involved in the enhanced vasoconstriction and PASMC proliferation. These observations indicated that the K_V channels are dysfunctional PH-PASMC [271, 273]. Other study has found dowregulation of Kv1.1 and Kv4.3 channels mRNA in PASMC from PAH patients [137]. Most recently, it has been proved that KCNK3 expression and activity are strongly reduced in PASMC and PAEC in PAH. KCNK3 inhibition promoted proliferation, vasoconstriction and inflammation demonstrating that loss of KCNK3 is a key event in PAH pathogenesis [10].

3.5.2.2 Reduced K^+ channel in experimental animal models of PAH

Most of our knowledge of the responsible molecular mechanisms for regulating K^+ channel in the pulmonary vasculature under physiological and pathological conditions is derived from experimental PAH models which attempt to mimic the human PAH pathophysiology. Impairment K^+ channel activity of has been reported in many preclinical rodents animal models of PAH (Table 1). This table summarizes the available data on K^+ channel dysregulation and the underlying mechanisms, in animal models of PAH.

Experimental PH model	Induction	Pathophysiological features	Effects on K^+ channels	Mechanism
Monocrotaline (rat & mice)	A single dose of MCT (40–100 mg/kg, subcutaneously), an 11-membered macrocyclic pyrrolizidine alkaloid derived from the seeds of the *Crotalaria spectabilis* plant [81].	• Vascular remodeling • Proliferation PASMC • Endothelial dysfunction • Inflammatory cytokines • Right ventricle failure	Downregulation Kv1.5 mRNA & protein in PASMCs	Decreased Kv1.5 channel expression might be caused by the ET-1 and ROS pathways [275]
			Downregulation Kv1.7 mRNA & protein in PASMCs	Decreased Kv1.7 channel expression caused by the ET-1 and ROS pathways [143]
			Downregulation TASK1 mRNA & protein in PASMC and PAEC	Unknown [10]
			Upregulation $K_{Ca}3.1$ protein in PASMC	Up-regulation by ET-1 [142]
			Up-regulation $K_{IR}6.1$ mRNA & protein in PASMCs.	Compensatory mechanism throught ET-1 [143]
Chronic hypoxia (rat & mice)	Exposing animals to 10% oxygen for 2–4 weeks [156].	• Vascular remodeling • Proliferation PASMC • Endothelial dysfunction • Obstructive pulmonary arterial intimal lesions. • Right ventricle failure • Reversible when return to normal oxygen.	Downregulation BK_{Ca} mRNA & protein in PASMCs	Unknown [26]
			Downregulation Kv1.1, Kv1.5, Kv1.6, Kv2.1, and Kv4.3 mRNA & protein in PASMC [256]	Involve modulation of a transcription factor: hypoxia-inducible factor-1 (HIF-1) [225] or endothelin-1 [263]
			Downregulated Kv7.4 mRNA in PASMC	Unknown [174] [223]
			Downregulated TASK1 mRNA & protein in PASMC	The changes in acid-base status might affect TASK channel activity[60].
			Downregulated KCa3.1 and K(Ca)2.3 protein expression was upregulated in PAEC	Unknown [132]
			Downregulation K_{ATP} channel (Activity) in PASMCs	Unknown [233], [266]
Sugen 5416-Hypoxia (rat & mice)	After a single dosis of Sugen5416 (20 mg/kg subcutaneous) of the vascular endothelial growth factor receptor type 2 inhibitor, rats are exposed to hypoxia to 10% oxygen for three weeks. After this time, rats return to normoxia for an additional 2–5 weeks [3].	• Vascular remodeling • Proliferation PASMC • Endothelial dysfunction • Inflammatory cytokines • Plexiform lesions.	Downregulation Kv1.5 mRNA & protein in PASMC	KCNA5 is a direct target of miR-1, microRNA upregulated in Sugen 5416/hypoxia lung [167]
			Downregulated TASK1 mRNA & protein in PASMC [39]	Unknown
BMPR2 $^{+/-}$ (rat & mice)	*BMPR2* mutation monoallelic deletion of 71 bp or 140bp in exon 1 in rats [94] or doxycycline-induced Rosa26-$Bmpr2^{R899X}$ mice [121]	• 20% develop PAH • Susceptible to hypoxia • Perivascular fibrillar collagen • Inflammatory cytokines • Proliferation PASMC • Apoptosis PAEC	Downregulation TASK1 activity in PASMC and PAEC	Changes in *KCNK3* localization [94]
			Downregulation Kv1.5 mRNA & protein in PASMC	Mediated by the overexpression of the Bcl-2 antiapoptotic factor [157, 269, 276]
			Upregulation SUR1 mRNA & protein in PASMCs	Protective response for the decrease of SUR2 [23] [46].
SERT$^+$ (female mice)	Over-expressing 5-HT transporter in transgenic mice[158]	• Elevated right ventricular pressures, • Right ventricular hypertrophy • Vascular remodeling • Increase vascular reactivitY • Less sensitive to acute hypoxia	Downregulation Kv1.5 and Kv2.1 mRNA & activity in PASMC	Direct inhibition of the current, internalization of the channel and downstream effects of 5-HTT activation may involve NADPH oxidase activity, which alter Kv channel expression and/or activity [58, 85]
			Upregulatied Kv7 activity in PA	Unknown [174]
HIV- asssociated (mice & rats)	Expressing seven of the nine HIV-1 viral proteins in transgenic mice [169].	• Non-PAH • Vascular remodeling • Endothelial dysfunction	Downregulated TASK activity in PASMC	Viral protein U (Vpu) interacts with TASK-1 channels perturbing their function [108]
			Downregulated Kv7.1 mRNA & activity	Unknown [108]
			Downregulated Kv7.4 (mRNA)	Unknown [108]
	Expressing seven of the nine HIV-1 viral proteins in transgenic Fischer 344 (F344) male rats (4-9 months) [151, 208]	• Elevated mPAP and RVSP • RV hypertrophy, PA remodeling • Exacerbated hipoxia-induced PAH • Increased PVR	Downregulated Kv1.5 mRNA	Unknown

Fawn hooded rats (FHRs)	A strain of rat with a hereditary bleeding tendency due to a genetic defect in platelet aggregation and develop PH spontaneously at 4 week old [219]	• Increase mPAP • Increase total pulmonary resistance • Right ventricular enlargement • Vascular remodeling • Not severe hypoxia • heritable basis • Reversed by continuous exposure to a mildly oxygen-enriched environment	Downregulated Kv1.5 and Kv3.1b channels (protein and function)	Mitochondrial abnormality actives HIF-1 which t inhibited expression of oxygen-sensitive, voltage-gated K^+ channels [27].
***Kcnk3*-Mutated Rat & TASK-1$^{-/-}$ mice**	Rat: Using CRISPR/Cas9 technology in male wistar rats was generated a 94 bp out of frame deletion in exon 1 of *Kcnk3* gene: heterozygous (Kcnk3$^{\Delta 94ex1/+}$) and homozygous rats (Kcnk3$^{\Delta 94ex1}$/$^{\Delta 94ex1}$) [136]	• Age-dependent PH associated with low serum-albumin concentration. • More sensitive to MCT or chronic hypoxia exposure. • Elevation of the RVSP and ldistal neomuscularization and perivascular extracellular matrix activation. • Alteration of right ventricular cardiomyocyte excitability • Overexpression of ERK1/2 and AKT, HIF1-α survivin and VWF (Von Willebrand factor). • Reduced endothelial-NOS expression, desensitization of EDHF and severe loss of sildenafil-induced relaxation.	Downregulatation of TASK1 channels (mRNA, protein and function)	
	Mice: TASK-1−/− (*KCNK3*−/−) and wild-type male and female mice C57BL/6 at 20–24 weeks of age and 1 year of age [124]	• Female and male TASK-1-/- mice do not develop hemodynamic or vascular evidence for PH.	Information not avalaible	

Table 1. Impairment of K^+ channel expression/activity in animal models of PH.

In this Doctoral Thesis, two PH rodent models have been used: Sugen5416-Hypoxia (Su/Hpx) rat model and HIV-1 transgenic mice, which are related and attempt to reproduce the pathophysiology of two different forms of human PAH (PH class I), iPAH and HIV-associated PAH, respectively. Both animal models were induced in male rodents, since, despite the fact that PAH affects mostly females, males have a worse prognosis and survival [253]. To study the regulation of different K^+ channels and ancillary subunits implicated in pulmonary vascular control by different pathophysiological factors related to PAH, we used Su/Hpx rats, the rodent model which best recapitulates human PAH, since it mimics plexiform vascular lesions and K^+ channel remodelling characteristic of human PAH. To study the impact that HIV-1 proteins can have on the pulmonary vasculature, we used a non-infectious transgenic mouse model that expresses 7 of the 9 HIV-1 proteins. The two missing proteins (*gag and pol*) are responsible for the infectious virus component. Therefore, this animal model represents HIV patients treated with antiretroviral therapy. The detailed description of these two PAH animal models can be found in *Table 1* and in the section of *Materials & Methods* available in each article that composes this Doctoral Thesis.

3.5.2.3 Pathophysiological factors impairing K^+ channel expression

3.5.2.3.1 HIF1 α

In the lung, acute reductions in oxygen lead to hypoxic pulmonary vasoconstriction (HPV, see *section 3.5.3.2*), whereas prolonged exposures to hypoxia result in sustained vasoconstriction, vascular remodelling and the development of PH [1]. Adaptation to sustained hypoxia involves the synchronized transcriptional regulation of a variety of genes and is largely coordinated by the hypoxia-inducible factor 1 (HIF-1), an oxygen-sensitive transcription factor [209]. HIF-1 is a heterodimer formed from HIF-1α and HIF-1β subunits. At normal oxygen concentrations, HIF-1α is inactivated or marked for degradation by hydroxylation of its prolyl hydroxylase domain (PHD). During hypoxia, PHD activity decreases resulting in HIF-1α protein stabilization and translocation into the nucleus, where it binds HIF-1β and recruits coactivator proteins to form a HIF-1-transcriptional complex modulating the transcription of a multitude of target genes [101]. HIF-1α is upregulated in PAH and has been shown to play a key role in this disease [227]. Chronic hypoxia reduces Kv current density and depolarized PASMC by downregulating the expression of several O_2-sensitive K^+ channels. In PASMCs from *Hif1*α $^{(+/-)}$ mice, hypoxia-induced Kv current decrease and depolarization was significantly blunted [228]. Following *in vitro* or *in vivo* exposure to chronic hypoxia (≥24 h), gene and protein expression of Kv1.2, Kv1.5, Kv2.1, and Kv9.3 channels are reduced [66]. In addition, endothelin-1 (ET-1) expression was enhanced three-fold in the lungs of *HIF2*$^{-/+}$ mice, suggesting that HIF-2α may upregulate ET-1. A previous study showed that ET-1 causes pulmonary vasoconstriction and stimulates PASMC proliferation by, at least in part, inhibiting Kv channel function and expression [228]. In addition, Kv channels impairment contribute to pulmonary remodeling by reducing apoptosis [227].

3.5.2.3.2 microRNAs

MicroRNAs are noncoding RNAs of approximately 22 nucleotides that behave as key regulators of gene expression. They contain complementary sequences to the 3'-UTR region of specific mRNAs and bind to them, leading to a reduced mRNA translation or to mRNA degradation. Some microRNAs are released with exosomes, circulate in plasma, and may function in cell-to-cell communication. MicroRNAs play a fundamental role in physiological

processes. In addition, up- or downregulation of miRNAs are involved in multiple diseases (Annex II) [168]. The expression profile of circulating microRNAs is different in animal models and PAH patients compared to healthy volunteers. Several microRNAs that regulate K^+ channels expression have been found altered in PAH models or patients. Recently it has been demonstrated that the expression of miR-1 is increased in lungs from PAH rat model (Chapter III, Annex III and IV). PASMC from PAH-animals are more depolarized and show decreased expression and activity of Kv1.5 channel [167]. Lv *et al.* showed that this channel can also be inhibited by increased expression of miR-206 in PASMC from PAH following intrauterine growth retardation rats [152]. Other study identified miR-29b highly expressed in iPAH-PASMC compared to control PASMC (>2.5-fold difference). The overexpression of miR-29b in control PASMC decreased BK_{Ca} channel currents and downregulated BK_{Ca} channel β1-subunit expression [13]. Li SS and colleagues demonstrated that hypoxia produces a significant increase of miR-190 in PA which results in Kv7.5 channel impairment [147]. Moreover, microRNA-138 promotes proliferation and suppresses mitochondrial depolarization in human PASMCs through targeting TASK-1 channel [150]. Due to their ability to, totally or partially, reverse the effects of these microRNAs in PASMC, antagomiRs may represent an innovative and promising therapeutic strategy for targeting the K^+ channel impairment in PH.

3.5.2.3.3 Vitamin D deficiency

Vitamin D receptor (VDR) regulates the expression of several genes involved in signalling pathways affected in PAH, among them KCNK3 gene that encoded TASK1-channels [226]. Vitamin D (VitD) deficiency has been related to increased cardiovascular mortality in large epidemiological studies [218, 222]. VitD is reduced in PAH-patients according to epidemiological studies [38]. It has been shown that PASMC isolated from vitamin D-deficient rats had reduced acid-sensitive K^+ currents (TASK-like). Moreover, vitamin D-free diet in PAH-rats exacerbated the increase in mPAP induced by chronic hypoxia, worsened endothelial function, increased the hyperreactivity to 5HT, enhanced arterial muscularization, decreased TASK-1 currents and further depolarized the PASMC membrane compared to PAH-rats with standard diet. These data strongly suggest Vitamin D deficiency downregulates TASK-1 channels and aggravates PAH [39].

3.5.2.4 Pathophysiological factors impairing K^+ channels activity

3.5.2.4.1 Vasoactive factors

Pulmonary vascular tone is controlled by the interaction of a multitude of different circulating or locally released factors with vasoconstrictor or vasodilating properties. The endothelium plays a critical role in the regulation of the local vasoactive factors. It synthesizes and releases potent vasodilators such as nitric oxide (NO) or prostacyclin (PGI_2) and vasoconstrictors, as endothelin-1 (ET-1) and thromboxane A_2 (TXA_2). Other key factors controlling pulmonary vascular tone include oxygen and serotonin (5-HT). Endothelial dysfunction in pulmonary arteries, a typical feature of PA from PAH patients and animal models, is characterized by an altered production of various endothelial-derived relaxing factors, such as NO and PGI_2, which are decreased, and contracting factors, such as ET-1, serotonin, and TXA_2, which are increased [36]. Acute modulation of the activity of K^+ channels has been extensively shown to contribute to the vascular effects of these vasoactive factors (Figure 4). Thus, vasodilators activate several K^+ channels and, conversely, vasoconstrictors block them [109, 116, 170, 192]. Accordingly, the decrease in NO, PGI_2 and oxygen and the increase in ET-1, TXA_2 and 5-HT observed in PAH impact on a reduced activity of K^+ currents and membrane depolarization.

3.5.2.4.1.1 The NO pathway

Endothelial NO synthase (eNOS) promotes the formation of NO in the vascular endothelium from L-arginine and oxygen in a calcium-dependent process. Once synthesized NO diffuses into PASMCs where it acts stimulating the soluble guanylate cyclase (sGC) resulting in the accumulation of its product, cyclic GMP (cGMP). This second messenger activates PKG which, in turn, targets a number of downstream mechanisms to induce vasodilation [25]. The intracellular concentration of cGMP is mostly regulated by phosphodiesterases, which rapidly degrade cGMP *in vivo*. Among them, phosphodiesterase-5 (PDE-5) is highly expressed in PA and represents a predominant isoform responsible for cGMP hydrolysis [30]. The activation of K^+ channels is considered an important mechanism mediating NO/cGMP-induced vasodilation by promoting membrane hyperpolarization and, subsequently, closure of L-type VGCC channels [55, 57, 220]. NO-induced relaxation of PA has been related to both cGMP-dependent and independent activation of BK_{Ca} channels [25] and Kv channels [272,

57] (Figure 4). Intriguingly, controversial effects of NO donors have been shown on Kv1.5 currents expressed in heterologous systems [216, 192]. However, the identity of other Kv channels involved in NO-induced relaxation of PA was unknown (Chapter I).

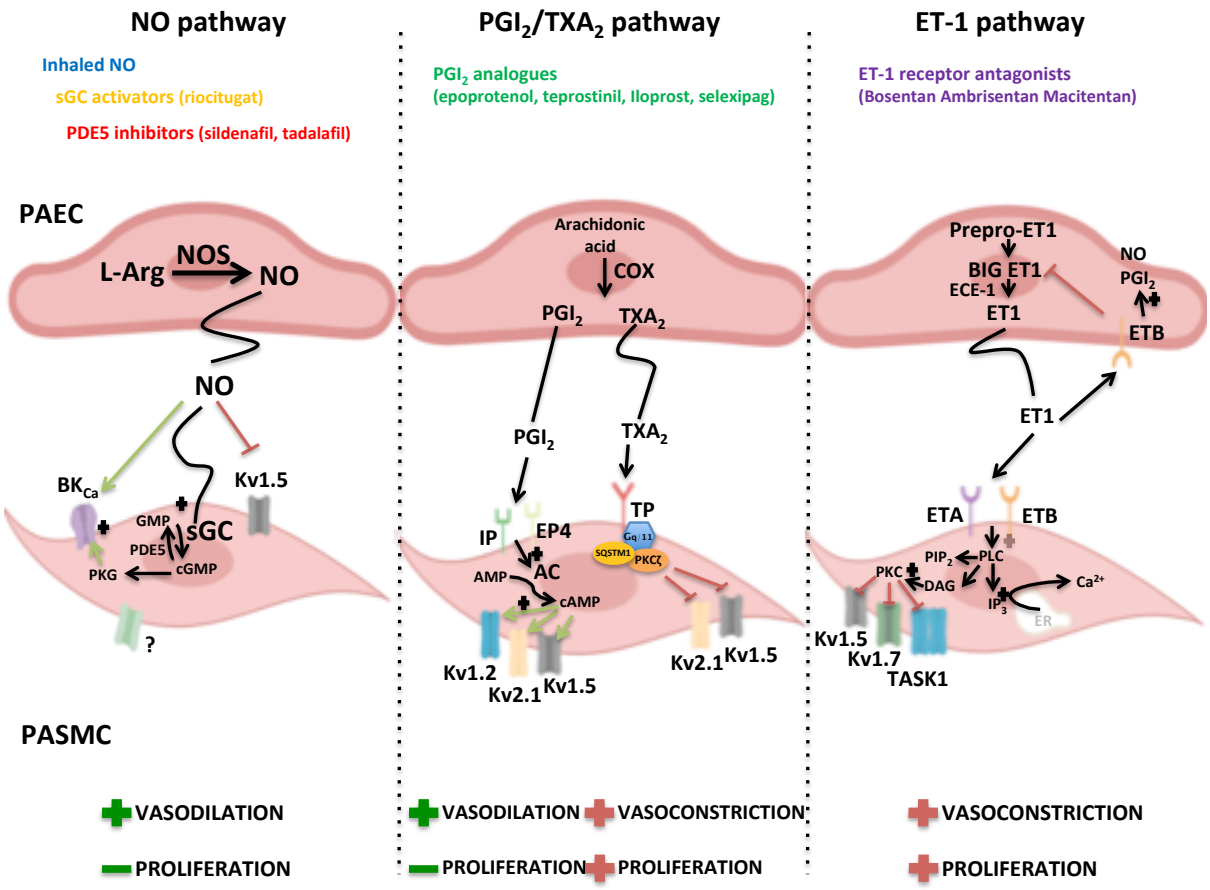


Figure 4. Mechanism of regulation of K^+ channels through vasoactive factors derived from the endothelium and places of action of the drugs used in the treatment of pulmonary hypertension.

3.5.2.4.1.2 The eicosanoid pathway

The prostanoid family comprises two groups: prostaglandins (PGD_2, PGE_2, PGI_2 and PGF_{2a}) and thromboxane (TXA_2) [139]. Prostanoid target receptors can be classified into two categories: relaxant receptors (IP, DP_1, EP_2 and EP_4) and contractile receptors (EP_1, EP_3, FP and TP). PGI_2 is synthesised predominantly by PAEC and it is a natural ligand for IP receptor that is expressed in PASMCs and whose primary effects are the induction of dilation and the inhibition of proliferation through the activation of adenylate cyclase (AC) and cyclic AMP

synthesis [182]. Li *et al*; showed that the PGI_2 analogues iloprost and treprostinil activate TASK-1 and BK_{Ca} channels in human PASMC, contributing to their vasodilating effects [148]. Additionally, it has been demonstrated that O_2-sensitive Kv channels (Kv1.2, Kv1.5, and Kv2.1) in PASMC can be up-regulated by the PGI_2 analogue, beraprost sodium (BPS), via the IP and EP4 receptor-related pathway [242]. TXA_2 elicits contrary effects, as a potent pulmonary vasoconstrictor and activator of platelet aggregation [49]. TXA_2 contracts vascular smooth muscle by binding to specific G protein–coupled TP receptors, which leads to an increase in $[Ca^{2+}]_{cyt}$ and sensitization of the contractile proteins to Ca^{2+} [202]. Previously was reported that TXA_2 inhibited Kv channels in rat PASMC though PKCzeta (PKCζ) activation, playing a critical role in TXA_2-induced pulmonary vasoconstriction [54]. The formation of a signalling complex involving sequestosome1/p62 complex (SQSTM1), as a scaffolding protein of PKCζ, and Kvβ subunits (PKCζ-SQSTM1-$K_v\beta$) [114] was suggested to account for TXA_2-induced inhibition of Kv channels and PA vasoconstriction [176]. Moreover, we also found TP receptor activation increases ROS via activation of NADPH oxidase [42]. NADPH oxidase-derived superoxide and the subsequent production of hydrogen peroxide (H_2O_2) are involved in the Kv channel inhibition and the contractile response induced by TP receptor activation in rat PA [52]. Other studies also affirm that K^+ conductance through K_{2P} channels (TWIK-2) [195] and BK_{Ca} channels produce outward hyperpolarizing currents that normally attenuate the hypercontractile to a TXA_2 mimetic [164].

3.5.2.4.1.3 The endotelin pathway

ET-1 is a potent vasoconstrictor peptide that also stimulates the proliferation of PASMC, acts as a co-mitogen and a proinflammatory mediator [112]. The product of ET-1 transcription is prepro-ET-1, which is cleaved by a neutral endopeptidase to form the active precursor pro-ET-1 or big ET-1. Big ET-1 is converted to the mature peptide by the metalloproteinase endothelin-converting enzyme-1 (ECE-1) [107]. ET-1 signals through two G protein-coupled receptors, endothelin receptor A (ET_A) and endothelin receptor B (ET_B). Activation of ET_A receptors causes sustained vasoconstriction and proliferation of PASMCs; whereas ET_B receptors mediate pulmonary ET-1 clearance and induce the production of NO and PGI_2 by PAEC [19, 71]. ET-1-stimulated effects can be classified into short-term and long-term responses, which are regulated trough different signalling pathways. In short-

term responses, ET-1-induced phospholipase C (PLC) activation leads to the generation of inositol-1,4,5-triphosphatase (IP3) resulting in Ca^{2+} influx from endoplasmic reticulum (ER) and diacylglycerol (DAG) resulting in PKC activation. ET-1 stimulates the PKC_{cyt} translocation to the plasma membrane and subsequently PKC phosphorylates specific cellular proteins, resulting in multiple biological effects [249]. In long-term responses, ET-1 plays a significant role in the chronic modulation of cellular proliferation and differentiation [231]. It has been well documented that ET-1 inhibits native Kv currents in rat and human PASMC [229]. In *KCNA5*-transfected HEK-293 cells, acute application of ET-1 caused a 32% reduction of the Kv1.5 current [216]. *Lee* and colleagues also demonstrated that a decrease in Kv1.7 channel expression might be caused by the ET-1 in PASMC [143]. Moreover, TASK1 was documented as being inhibited in human PASMCs by ET-1 through an ETA–PLC–PIP2–DAG–PKC-dependent signalling cascade [241].

3.5.2.4.1.4 Serotonin

5-HT is an effective pulmonary vasoconstrictor and induces vascular smooth muscle hyperplasia [141, 159]. The 5-HT is a key determinant of vessel remodelling, high 5-HT level had been reported in platelets and plasma from PAH-patients [98]. PAEC can produce high levels of these neurotransmitter under pathological conditions and promote proliferation and contraction in PASMC [68]. Previous studies showed that the activation of $5\text{-}HT_{2A}$ receptors inhibits K_V current in PASMC leading to membrane depolarization via PLC-PKC-TK. In addition, $5\text{-}HT_{2A}$ receptors and caveolin-1 co-immunoprecipitated with Kv1.5 channels. Noteworthy, Kv1.5 channels were shown to be internalized upon acute stimulation with 5-HT [58]. Thus, 5-HT signalling appears to modulate Kv current in PASMCs through direct alterations in Kv1.5 trafficking and surface expression.

3.5.2.4.1.5 Calcitonin gene-related peptide

The sensory neurotransmitter calcitonin gene-related peptide (CGRP) is released by sensory nerves. CGRP was shown to hyperpolarize both PASMCs and PAECs independently and complementary via activation of K_{ATP} channels through PKA, leading to PA dilation in a concentration-dependent manner [191]. CGRP-availability is diminished in pulmonary vascular disease, implying a compromised functional role for this vasodilator [97, 237, 244].

3.5.2.4.1.6 Vasoactive intestinal polypeptide

Vasoactive intestinal polypeptide (VIP) is an endogenous neuropeptide normally present in lungs and other organs, that acts as a potent systemic and pulmonary vasodilator [277]. Clinical studies have shown that VIP administration results in substantial improvement of hemodynamic and prognosis of the disease without evident side effects [205]. The activation of different K^+ channels of PASMC has been proposed as potential mechanisms contributing to VIP-induced PA vasodilation. Zhang S *et al*., demonstrated that one of the primary targets of VIP in PA is the G-protein-coupled VIP receptors, which leads to activation of the adenylyl cyclase-PKA pathway and the downstream target is the K_{ATP} channels [277]. Other study pointed out that PAEC contains functional VIP receptors whose activation results in the increase of K_{IR} channels through a G protein [198].

3.5.2.4.2 Acute hypoxia

Hypoxic pulmonary vasoconstriction (HPV) reflects an intrinsic property of PASMC, which allows shifting blood flow from hypoxic to normoxic lung areas, thereby coupling ventilation and perfusion [257]. Mitochondrial electron transport chain (ETC) and NADPH oxidases (Nox) have been proposed as possible oxygen sensors playing key roles in coupling the sensors to the HPV contractile machinery. However, whether Reactive Oxygen Species (ROS) increase or decrease in PASMC during hypoxia and the source of ROS is a matter of intense debate. Several types of K^+ channels highly sensitive to redox changes have been proposed to contribute to HPV and chronic hypoxia-induced PH [259]. Ceramide is a sphingolipid-derived second messenger molecule synthesized from membrane sphingomyelin by acid (aSMase) or neutral sphingomyelinases (nSMase) [56]. Previous studies have reported that nSMase and ceramide trigger the activation of NADPH oxidase via phosphorylation and translocation of p47phox [53]. Moreover, inhibition of nSMase or neutralization of ceramide prevented the increase in NADPH-derived ROS, Kv channel inhibition and vasoconstriction induced by hypoxia [77]. We demonstrated that in freshly isolated rat PASMC, tert-butyl hydroperoxide (H_2O_2 analogue) caused a marked inhibition of the native Kv current and this effect was prevented by catalase [52]. While acute hypoxia acts, at least partly, *via* inhibition of Kv channel activity, during chronic hypoxia K^+ channel density and Kv channel protein expression (Kv1.5 and Kv2.1) is decreased, although a Kv current is still detectable

[252]. Other studies have demonstrated that hypoxia-induced inhibition of TASK-1 contributed to PASMC depolarization and HPV [193, 82]. Since TASK-1 cannot sense O_2 itself, Nox4 has been suggested to be the O_2-sensing partner modulating the O_2 sensitivity of TASK-1 [144]. Other study shows TASK-1, TASK-3, and TASK-1/3 heteromer expressed in HeLa cells were activated by H_2O_2. This effect was independent of the oxidation of –SH groups, suggesting that H_2O_2 acts directly on the channel protein [197].

3.5.2.4.3 Inflamatory factors

Inflammation is recognised to play a significant role in the pathogenesis of PAH; however, relatively few studies have investigated the functional effects of pro-inflammatory cytokines on K^+ current in PASMC [93, 212]. Inflammatory cells are present within and around the remodelled PA and PAH-patients have elevated levels of inflammatory cytokines, including tumor necrosis factor-α (TNFα) or interleukin-6 (IL-6). Human PASMCs exposed to TNFα or IL-6 adopt a PAH phenotype with decreased K^+ current even though the specific molecular mechanisms remain unclear [240].

3.5.2.4.4 Drugs

The 6th World Symposia on PH (Nice 2018) proposed a new classification of drug-induced PAH in two categories “definite association” and “possible association” [230]. Many of these drugs are able to directly or indirectly disrupt the activity of K^+ channels. Anorectic drugs were the first class of drugs identified as definite cause of PAH. The appetite-suppressant or anorectic drugs, including aminorex, fenfluramine and its d-isomer, dexfenfluramine, are commonly used for the treatment of obesity. Since 1967, their consumption has been associated with diverse epidemics of PH [261]. The anorectics drugs decrease appetite through their effects on catecholamine or 5-HT levels in the brain; but also release 5HT from platelets and inhibit its reuptake in plasma [21]. Different studies have indicated that anorectic agents can inhibit Kv current in rat PASMC, cause membrane depolarization, open voltage-sensitive Ca^{2+} channels, increase in $[Ca^{2+}]_{cyt}$ and stimulate pulmonary vasoconstriction, suggesting this mechanism as responsible for initiating pulmonary hypertension in susceptible individuals [260]. Single channel experiments demonstrated that open probability and open duration of hKv1.5 expressed in mammalian cell line were decreased by fluoxetine and sibutramine [201]. Recent data have pointed to dasatinib as a

drug with a definite association to PAH. This inhibitor of the Src family tyrosine kinase (SrcTK) is used for the treatment of chronic myelogenous leukaemia and has been was associated with cases of severe PAH, in part reversible after its withdrawal [84, 171, 194]. SrcTK may control TASK1 channel function in hPASMCs in response to changes in oxygen tension and the clinical use of a SrcTK inhibitor has resulted in partly reversible PH. SrcTK is co-localised with the TASK-1 channel and the Inhibition of SrcTK by moderate hypoxia or the SrcTK inhibitors such as Dasatinib decreases TASK-1 current density and results in considerable depolarization through decrease the tyrosine phosphorylation state of the TASK-1 channel [184]. Another group of recently classified drugs with straight association to PAH are the methamphetamines. Different studies have demonstrated that methamphetamine use has cardiovascular complications involving the modulation of the activity of various K^+ channels [127, 149, 183, 210]. Though it is not clearly demonstrated, it would be expected that methamphetamines might also be able to modulate directly or indirectly the activity of different K^+ channels expressed in pulmonary vasculature contributing to the development of PAH. Even though a definitive association has not been demonstrated, a possible risk factor for PAH is the pergolide use, a drug with clinical benefit in Parkinson disease by stimulating dopamine D1 and D2 receptors. Pergolide is an inhibitor of voltage-gated K^+ channels, including Kv1.5, and causes pulmonary vasoconstriction [106, 119].

3.5.2.4.5 Surface expression, recycling and trafficking pathways

Surface expression and trafficking are regulated by different physiological stimuli such as changes in gene expression, interactions with accessory subunits, by phosphorylation, stimulation of protein internalization/endosomal recycling pathway and by cellular components that regulate their trafficking to the cell surface. However, the research on channel trafficking is an area to be exploited. Kv1.5 continuously recycles between the cytosol and plasma membrane. A previous study demonstrated that in PA that 5-HT signals through an interaction between Kv1.5, 5-HT_{2A} and caveolin-1 and Kv1.5 internalization, leading to a decrease in surface Kv1.5 protein and pulmonary vasoconstriction [58]. The regulation of Kv1.5 channel trafficking has been associated with changes in the intravascular pressure and Em in rat mesenteric arteries VSMC. Thus, intravascular pressure was shown

to stimulate VSMC membrane depolarization, leading to, voltage-dependent-L-type calcium channel ($Ca_V1.2$) channel activation [123]. The increase in $[Ca^{2+}]_{cyt}$ inhibits Kv1.5 channel degradation, allowing Kv1.5 to recycle to the plasma membrane. A reduction in intravascular pressure leads to membrane hyperpolarization which promotes the degradation of Kv1.5 protein through a process that involves both proteasomes and lysosomes [123]. Although several groups have investigated K^+ channel trafficking, little is known about the molecules and machinery involved in these processes. Surface expression requires movement from the endoplasmic reticulum through the Golgi apparatus to the plasma membrane. Motifs in the C termini and pore domains of Kv channels have been implicated in their differential surface expression, presumably through effects on forward trafficking [145, 161, 279]. A study showed that Kv1.5 surface expression is modulated by retrograde trafficking of newly endocytosed channels by the dynein motor [48]. Direct interference with dynein function, the disruption of the microtubule cytoskeleton or direct interference with endocytosis itself caused a significant increase in Kv1.5 surface expression and Kv1.5 currents. The SH3-binding domain in the Kv1.5 channels N-terminus is required to interact with the dynein motor complex in both heterologous cells and rat cardiac myocytes, supporting the role of this complex in Kv1.5 channel trafficking [48].

Other study showed that bone morphogenetic protein receptor type II *(BMPR2)* mutations predisposed rats to PA vasoconstriction through functional TASK1 current inhibition and subsequent PASMC depolarization. Thus, in BMPR2 deficient rats KCNK3 expression was not affected at the mRNA level, however TASK1 channels were not present at the cell surface of PASMCs and were abnormally retained in the cytosol and sarcoplasmic reticulum compartments [73]. K^+ channel trafficking can also be regulated by altered protein folding via changes in the interaction with molecular chaperones such as Heat Shock Proteins (HSPs) [72]. Aberrant interactions between HSP90 and its client proteins could contribute to pulmonary vascular diseases [29]. Barrese V *et al.* demonstrated that Ang II decreases the expression and function of Kv7.4 channels in VSMC by reducing its interaction with HSP90 and altering protein stability, which lead to the recruitment of the ubiquitin-ligase CHIP (C-terminus of HSP70-interacting protein) and the subsequent protein degradation via the proteasome. Curiously, HSP90 is preferentially accumulated in the mitochondria in PH-PASMC, to ensure cell survival by preserving mitochondrial DNA integrity and bioenergetics

functions [16]. The cytosolic HSP90 inhibition could be altering the stability and induce the degradation of different K^+ channel proteins in PH-PASMC [29]. Thus, increasing HSP90 activity might represent a new strategy to stabilize K^+ channels function.

3.5.3 Ion channel remodelling: its role in the transition from the contractile to the proliferative phenotype

The PASMC transformation is the starting point for the vascular remodelling after which thickening of the vessel intima-media wall occurs from proliferation, anti-apoptosis and migration of dedifferentiated PASMC [95]. The excessive proliferation of PASMC can be partially explained by the imbalance between vasoconstrictor and vasodilator factors as well as angio-proliferative factors produced by ECs [110]. In recent years, it has been understood that increased or decreased expression of various ion channels is intimately involved in the transformation of PASMC [96, 138].

The ion channel remodelling begins with the decreased Kv1.5 and TASK-1 channel function (Figure 5A and 5B) and expression (Figure 5C) in PASMC, which is a hallmark in both animal models and PAH patients. The marked downregulation in Kv1.5 and TASK1 currents, two of the most relevant K^+ currents in PASMC, results in membrane depolarization of PAH-PASMC compared to healthy-PASMC (Figure 5D). This pathophysiological feature of PAH involving membrane depolarization due to dysfunctional Kv1.5 and TASK1 channels in PASMC can be pharmacologically mimicked using selective K channel inhibitors. Thus, Figure 5E shows a continuous Em recording and how the addition of a Kv1.5 channel selective blocker (DPO-1) together with a TASK1 channel selective blocker (ML365) turn the Em more depolarized reaching values (≈ -40/-30 mV), similar to those recorded in PASMC from the PAH model (Figure 5D).

Another change that occurs in PAH-PASMC, is the replacement of $KCa_{1.1}$ (BK_{Ca}) by $KCa_{3.1}$ (IK_{Ca}) channels with intermediate K^+ conductance (Figure 6). In PASMC the Ca^{2+}-influx from outside of the cell is mainly governed by VGCC, which are involved in the excitation-contraction process and whose activity strongly depend on the Em. In PAH-PASMC, the expression of VGCC is decreased and replaced by transient receptor potential channels (TRP), which are activated and opened even in the vicinity of resting Em, with almost no effect from the Em [95]. The Em depolarization and persistently elevated $[Ca^{2+}]_{cyt}$ are major pathological factors in the stimulation of cell proliferation.

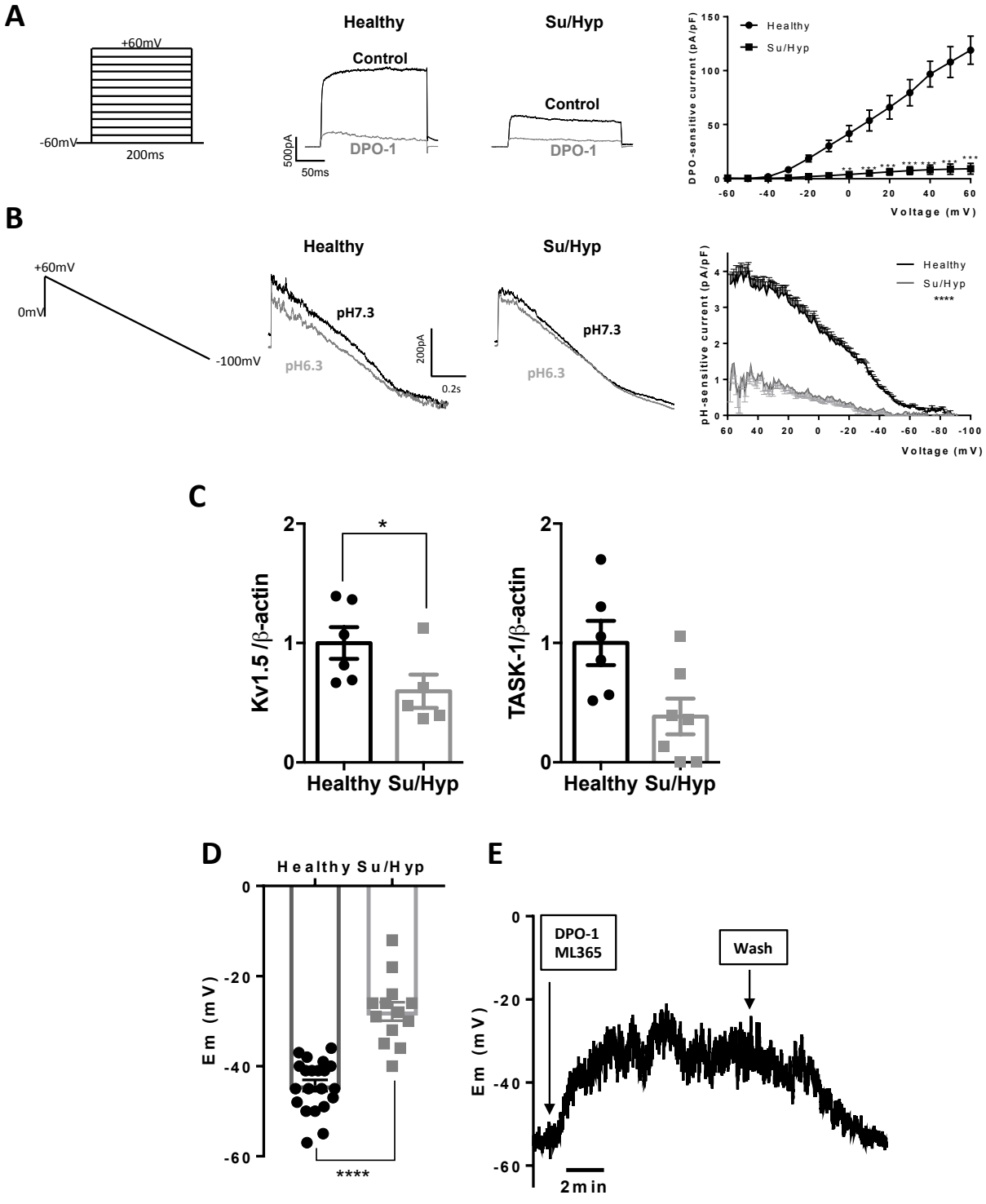
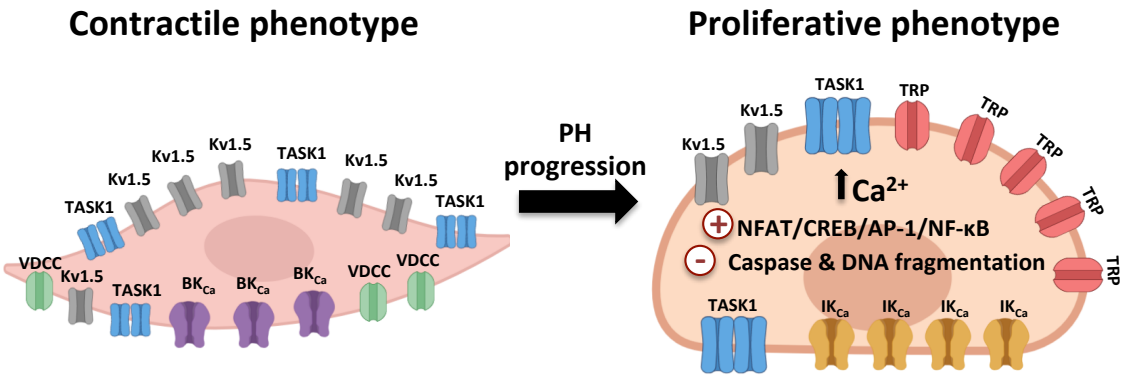


Figure 5. Decreased activity and expression of Kv1.5 and TASK1 channels in PAH. A) Characterization of Kv1.5 currents: electrophysiological protocol applied, original recordings at +60 mV in the absence and in the presence of 1 µM DPO-1 in healthy and Su/Hyp-PASMC and current-voltage relationship of the DPO-1-sensitive current calculated from the subtraction of the basal current and the current in the presence of the blocker. B) Characterization of TASK1 currents: electrophysiological protocol applied, original recordings of 1s ramp from +20 to -60 mV perfusing at pH7.3 or pH 6.3 in healthy and Su/Hyp-PASMC and current-voltage relationship of the pH-sensitive current measured at all potentials. C) Kv1.5 and TASK1 channel protein expression in lung from from healthy and Hyp/Su5416 rats. D) Resting Em measured under current clamp mode in PASMCs from healthy and Hyp/Su5416 rats. E) Representative trace of the effects of DPO-1 (1uM) and ML365 (1uM) on Em. These data were obtained from previous studies [122, 28]. Results are the mean ± SEM. * P < 0.05, ** P < 0.01 and *** P < 0.001 vs. Healthy.

This state is advantageous for the activation of intracellular calcium concentration-dependent transcription factors such as NFAT/CREB/AP-1/NF-κB promoting proliferation and migration [18]. PAH-PASMCs are thought to have increased resistance to apoptosis. This resistance to apoptosis is reported to be produced by downregulation of Kv channels [87]. The K^+-flow out of the cell through K^+ channel activation is important not only with respect to the decrease in cell volume, but is also thought to play a major role in inhibition of caspase activity and in DNA fragmentation [37]. It has been suggested that mitochondrial Kv1.5, K_{ATP} and BK_{ca} channels are involved in the apoptotic resistance of PAH-PASMC [216]. In summary, the ion channel remodelling produced by different pathophysiological factors in PH causes the contractile phenotype of PASMC to be dedifferentiated into a proliferative, anti-apoptotic and migratory phenotype (Figure 6)



K^+ channel	Kv1.5 TASK1 $K_{Ca}1.1$ (BK_{Ca})	↓ Kv1.5 ↓ TASK1 $K_{Ca}3.1$ (IK_{Ca})
$Em_{ex\text{-}vivo}$	-60/-50 mV	-40/-30 mV
Ca^{2+} influx	VDCC	TRP
Stimulus Ca^{2+} influx	Em-dependent	Em-independent Current increased by driving force
Size	Elongated	Increased volume, rounded
Features	Differentated PASMC Antiproliferative Controlled apoptosis	Inmature PASMC Proliferative Anti-apoptosis

Figure 6. Ion channel remodelling of PASMC in pulmonary hypertension. Characteristics of contractile phenotype versus proliferative phenotype.

4. Rationale & Hypothesis

K^+ channel conductance plays a fundamental role in the control of the pulmonary artery vascular tone at three different levels: 1) Regulating the membrane potential of PASMC and thereby controlling PA tone. 2) Regulating the population of PASMC by controlling cell apoptosis, survival and proliferation. 3) Maintaining resting Em of PAEC, and thus regulating Ca^{2+} entry and the production and release of vasoactive factors. Therefore, the impairment of K^+ channels contributes to the development of pulmonary vascular diseases such as pulmonary hypertension. A better understanding of the physiological and pathophysiological molecular mechanisms of K^+ channel regulation in the pulmonary vasculature may contribute to anticipate or ameliorate the development and progression of pulmonary hypertension, as well as to elucidate potential new therapeutic targets to help fight this currently incurable disease.

We hypothesize that drugs and pathophysiological factors with relevance in the context of pulmonary hypertension may influence pulmonary vascular tone by targeting particular K^+ channels.

5. Objectives

General objective

To increase our knowledge about the pharmacological, physiological and pathophysiological regulation of K^+ channels in the pulmonary vasculature and their possible involvement in the context of pulmonary hypertension.

Specific objectives

I. To analyse the contribution of Kv channels in the electrophysiological and vasodilating effects evoked by nitric oxide donors and the soluble guanylyl cyclase stimulator, riociguat, in pulmonary arteries.

II. To shed light on the role of Kv7 channels and KCNE ancillary subunits in the pulmonary vasculature and their possible alteration in PAH.

III. To analyse the pathophysiological effect of microRNA-1 on the expression and activity of Kv1.5 channels in pulmonary arteries.

IV. To analyse whether the expression of HIV-1 proteins is associated with impairment of K^+channel function in the pulmonary vascular bed.

6. Results

Chapter I: Activation of K_v7 channels as a novel mechanism for NO/cGMP-induced pulmonary vasodilation

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RESEARCH PAPER

Activation of K_v7 channels as a novel mechanism for NO/cGMP-induced pulmonary vasodilation

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Background and Purpose: The NO/cGMP pathway represents a major physiological signalling controlling tone in pulmonary arteries (PA), and drugs activating this pathway are used to treat pulmonary arterial hypertension. K_v channels expressed in PA smooth muscle cells (PASMCs) are key determinants of vascular tone. We aimed to analyse the contribution of $K_v1.5$ and K_v7 channels in the electrophysiological and vasodilating effects evoked by NO donors and the GC stimulator riociguat in PA.

Experimental Approach: K_v currents were recorded in isolated rat PASMCs using the patch-clamp technique. Vascular reactivity was assessed in a wire myograph.

Key Results: The NO donors diethylamine NONOate diethylammonium (DEA-NO) and sodium nitroprusside hyperpolarized the membrane potential and induced a bimodal effect on K_v currents (augmenting the current between −40 and −10 mV and decreasing it at more depolarized potentials). The hyperpolarization and the enhancement of the current were suppressed by K_v7 channel inhibitors and by the GC inhibitor ODQ but preserved when $K_v1.5$ channels were inhibited. Additionally, DEA-NO enhanced $K_v7.5$ currents in COS7 cells expressing the KCNQ5 gene. Riociguat increased K_v currents at all potentials ≥−40 mV and induced membrane hyperpolarization. Both effects were prevented by K_v7 inhibition. Likewise, PA relaxation induced by NO donors and riociguat was attenuated by K_v7 inhibitors.

Conclusions and Implications: NO donors and riociguat enhance K_v7 currents, leading to PASMC hyperpolarization. This mechanism contributes to NO/cGMP-induced PA vasodilation. Our study identifies K_v7 channels as a novel mechanism of action of vasodilator drugs used in the treatment of pulmonary arterial hypertension.

*A.C. and F.P.-V. share the senior authorship.

Abbreviations: DEA-NO, diethylamine NONOate diethylammonium; K_v, voltage-gated potassium channels; PA, pulmonary arteries; PASMC, PA smooth muscle cell; Phe, phenylephrine; sGC, soluble GC; SNP, sodium nitroprusside

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1 | INTRODUCTION

NO is a key endogenous vasodilator playing a pivotal role in maintaining pulmonary vascular tone (Barnes & Liu, 1995; Coggins & Bloch, 2007). The classical NO signalling pathway involves the activation of **soluble GC (sGC)** and the subsequent generation of **cGMP** (Barnes & Liu, 1995; Kraehling & Sessa, 2017), while alternative mechanisms (such as S-nitrosylation) may also take place (Coggins & Bloch, 2007; Hess, Matsumoto, Kim, Marshall, & Stamler, 2005). This second messenger activates **PKG** which, in turn, targets a number of downstream mechanisms to induce vasodilation (Cogolludo et al., 2001; Sausbier et al., 2000).

Activation of K^+ channels is considered an important mechanism mediating NO/cGMP-induced vasodilation by promoting membrane hyperpolarization and, subsequently, closure of L-type calcium channels (Cogolludo, Moreno, & Villamor, 2007; Cogolludo et al., 2001; Jackson, 2018; Sausbier et al., 2000). Thus, NO-induced relaxation of systemic and pulmonary arteries (PA) has been related to both cGMP-dependent and -independent activation of large-conductance calcium-activated potassium (**BKCa**) channels (Bolotina, Najibi, Palacino, Pagano, & Cohen, 1994; Robertson, Schubert, Hescheler, & Nelson, 1993) and voltage-gated K^+ (**K_v**) channels (Cogolludo et al., 2001; Plane, Sampson, Smith, & Garland, 2001; Yuan, Tod, Rubin, & Blaustein, 1996). More than 20 years ago, Yuan et al. (1996) showed that activation of K_v channels contributed to NO-induced hyperpolarization of PA smooth muscle cells (PASMCs) and PA relaxation. Later, activation of these channels was also reported to contribute to NO-induced apoptosis of PASMCs (Krick et al., 2002). Among the different members of the Kv channel family expressed in PASMCs, including **$K_v1.5$**, **$K_v2.1$**, and **$K_v1.2$** channels, the former are considered major contributors to the total K_v current, especially in resistance PASMCs (Archer et al., 1998; Moral-Sanz et al., 2011; Smirnov, Beck, Tammaro, Ishii, & Aaronson, 2002). Intriguingly, NO donors have been shown to either activate (Remillard et al., 2007) or inhibit (Núñez et al., 2006) $K_v1.5$ channels when expressed in heterologous systems. Thus, the identity of the K_v channel activated by NO in PASMCs remains unclear.

During the last decade, K_v channels encoded by *KCNQ1–5* genes (**$K_v7.1$**–**$K_v7.5$**) have been reported to regulate resting membrane potential and contractility in various blood vessels (Barrese, Stott, & Greenwood, 2018; Chadha, Zunke, Davis, et al., 2012; Mackie & Byron, 2008; Yeung et al., 2007). Likewise, a number of studies have shown that activation of K_v7 channels contributes to the relaxation induced by agents stimulating the production of **cAMP** (Chadha, Zunke, Zhu, et al., 2012; Khanamiri et al., 2013; Mani et al., 2016; Morales-Cano et al., 2015, 2016). However, the possible contribution of these channels in the relaxation induced by the NO/cGMP pathway remains poorly studied.

What is already known

- K_v channels and the NO/cGMP pathway are key determinants of pulmonary vascular tone.

What this study adds

- K_v7 channel activation contributes to the electrophysiological and relaxant effects of NO donors and riociguat.

What is the clinical significance

- K_v7 channels represent a new target for drugs activating the NO/cGMP pathway in pulmonary vasculature.

Impairment of the NO/cGMP pathway has been implicated in the pathogenesis of cardiovascular diseases, including pulmonary hypertension (PH; Klinger & Kadowitz, 2017; Schermuly, Ghofrani, Wilkins, & Grimminger, 2011). Moreover, pharmacological stimulation of this pathway at different levels using inhaled NO, **PDE5** inhibitors (eg, **sildenafil**), or sGC stimulators (eg, **riociguat**) represents a key strategy in the treatment of pulmonary vascular diseases such as PH (Galiè et al., 2015; Kraehling & Sessa, 2017; Lau, Giannoulatou, Celermajer, & Humbert, 2017; Stasch, Pacher, & Evgenov, 2011). Thus, the elucidation of novel mechanisms underlying the effects of these drugs would have great therapeutic relevance.

In the present study, we aimed to shed light on the functional modulation of K_v currents by the NO/cGMP pathway in freshly isolated PASMCs. We examined the dependence of $K_v1.5$ and K_v7 channels on NO/cGMP-mediated responses in PA. The findings of our study provide evidence that K_v7 channels contribute to the pulmonary vasodilation induced by NO donors and riociguat.

2 | METHODS

2.1 | Animal preparation

All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committee of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, ref. number PROEX-301/

16). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010). Male Wistar rats (250–300 g) were obtained from Envigo (RRID: RGD_5508396, Barcelona, Spain). All animals were kept with free access to standard rat chow and water in an enriched environment and maintained at 24°C under a 12-hr light/12-hr dark cycle. The rats were killed using the CO_2 method, which provided a rapid, painless, stress-free death. The CO_2 flow rate was displaced from 10% to 30% of the cage volume per minute following 2013 AVMA guidelines (Leary & Golab, 2013). CO_2 flow was maintained for at least 1 min after respiratory arrest. Death was verified by observation for vital signs.

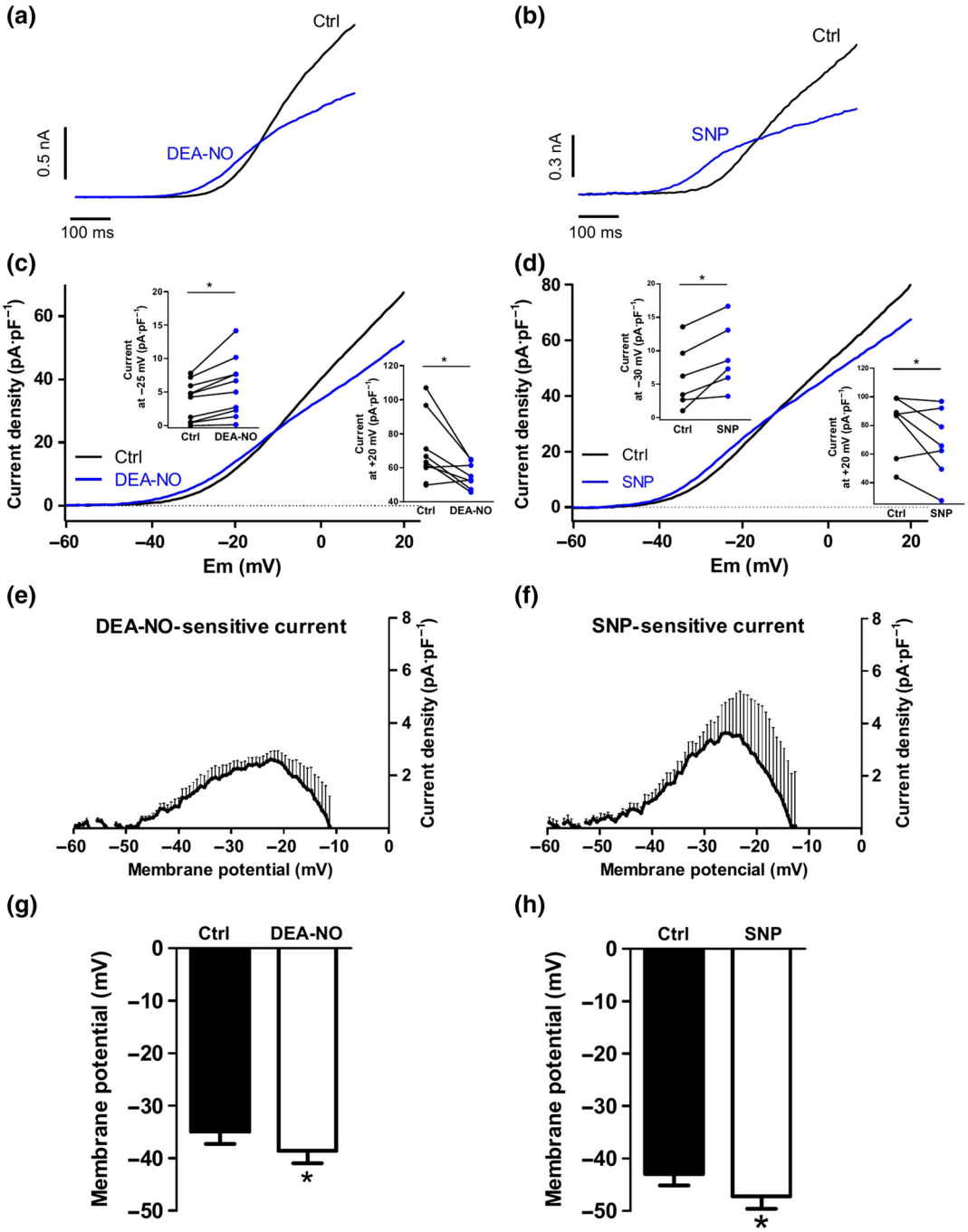


FIGURE 1 Bimodal modulation of K_v currents by NO donors in PASMCs. (a and b) Representative traces and (c and d) mean values of K_v currents recorded after the application of depolarizing ramps from −60 to +20 mV in the absence (black lines) or the presence (blue lines) of DEA-NO (10 μM, *n* = 10 from seven animals) or SNP (10 μM, *n* = 7 from six animals), respectively. Inset shows the current density at −30 and +20 mV before and after the addition of DEA-NO or SNP. (e and f) Average values of DEA-NO- and SNP-sensitive currents obtained by subtracting the current in the presence and in the absence of the drug. (g and h) Mean values of the membrane potential in PASMCs in the absence (Ctrl) and in the presence of DEA-NO (*n* = 9 from seven animals) and SNP (*n* = 7 from six animals). *$P < 0.05$ versus Ctrl (Student's paired *t* test). Results are means ± *SEM*

2.2 | Tissue and cell isolation

Rat resistance intrapulmonary arteries (250–500 μm) were isolated from the lungs and cut into rings. Rings were randomly assigned to different experimental groups. For isolation of PASMCs, PA were placed into a nominally Ca^{2+}-free physiological salt solution of the following composition (in mM): NaCl 130, KCl 5, $MgCl_2$ 1.2, glucose 10, and HEPES 10 (pH 7.3 with NaOH) containing (in mg·ml^{-1}) papain 1, DTT 0.8, and albumin 0.7 for 8–12 min as previously described (Cogolludo et al., 2006). PASMCs were dissociated using a wide bore, smooth-tipped pipette, stored in Ca^{2+}-free physiological salt solution (4°C) and used within 8 hr of isolation.

2.3 | Cell culture

COS7 (ATCC Cat# CRL-1651, RRID:CVCL_0224) cells were grown in DMEM containing 10% FBS and supplemented with 100-U·ml^{-1} penicillin/streptomycin. Human wild-type KCNQ5 cDNA was transfected in COS7 cells at nearly 80% confluence. COS7 were transfected with 1-μg pEYFP-N1-KCNQ5 plasmid using the FUGENE6 transfection method (Promega). The ratio μg DNA:μl Fugene was 1:3. Twenty-four hours after transfection, cells were washed in PBS and used.

2.4 | Electrophysiological studies

Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA, USA) using the whole-cell configuration of the patch-clamp technique. Freshly isolated PASMCs were superfused with an external Ca^{2+}-free HEPES solution (see above) and a Ca^{2+}-free pipette (internal) solution containing (in mM) KCl 130, $MgCl_2$ 1.2, Na_2ATP 5, HEPES 10, and EGTA 10 (pH adjusted to 7.3 with KOH). K_v currents were evoked following the application of depolarizing ramps from −60 to +20 mV. In some experiments, long (4 s) depolarizing steps from −60 to +20 mV were applied. K_v currents were initially monitored for 3–5 min, and only cells seen to have stable currents were then used for experiments. Under these conditions, currents remained stable and were usually reproducible for at least 20 min. The electrophysiological effects induced by the NO donors diethylamine NONOate diethylammonium (DEA-NO) and sodium nitroprusside (**SNP**) and the sGC stimulator riociguat were tested until they reached steady state. In some experiments, cells were exposed for 10 min to selective inhibitors of K_v1.5 (DPO-1, 1 μM), K_v7 (**XE991**, 10 μM or **linopirdine**, 10 μM), or BKCa channels (**iberiotoxin**, 0.1 μM), or sGC (**ODQ**, 10 μM) and then challenged with NO donors or riociguat in the continuous presence of these inhibitors. Currents were normalized for cell capacitance

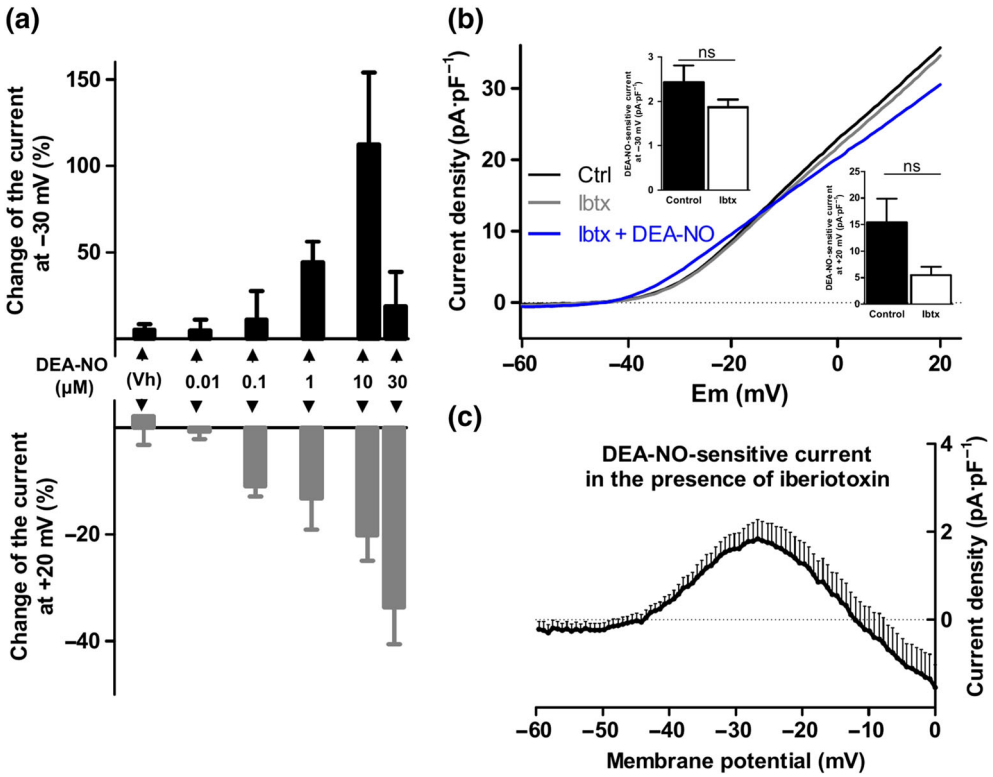


FIGURE 2 Concentration-dependence and lack of BKCa channels involvement, in the electrophysiological effects of NO donors. (a) Changes in the current at −30 mV (black, top) and at +20 mV (grey, bottom) induced by vehicle (DMSO, 0.1%) or different concentrations of DEA-NO (0.01–30 μM, *n* = 7–10 for each concentration from three to eight animals). (b) Mean values of K_v currents in PASMCs recorded after the application of depolarizing ramps from −60 to +20 mV in the absence and in the presence of the BKCa channel inhibitor iberiotoxin (Ibtx, 0.1 μM, *n* = 9 from five animals) before (grey lines) and after (blue lines) the addition of DEA-NO (10 μM). Inset shows the DEA-NO-sensitive current at −30 and +20 mV under control conditions or in the presence of Ibtx. (c) DEA-NO-sensitive currents in the presence of Ibtx, obtained by subtracting the current in the presence and in the absence of the NO donor. Results are means ± *SEM*

and expressed in pA·pF^{-1}. Membrane potential was recorded under the current-clamp mode.

K_v7.5 currents expressed in COS7 were recorded using the perforated-patch configuration of the patch-clamp technique (amphotericin B, 125 μg·ml^{-1} in the internal solution) to prevent rundown using an Axopatch 200B amplifier (Axon Instruments) as previously described (Moreno et al., 2017; Oliveras et al., 2014). The intracellular pipette filling solution contained (in mM) K-aspartate 80, KCl 50, phosphocreatine 3, KH_2 PO_4 10, MgATP 3, HEPES-K 10, and EGTA 5 and was adjusted to pH 7.25 with KOH. The bath solution contained (in mM) NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES-Na 10, and glucose 10 and was adjusted to pH 7.40 with NaOH. All experiments were performed at room temperature (22–24°C).

2.5 | Recording of arterial reactivity

For contractile tension recording, PA rings were mounted in a wire myograph with Krebs buffer solution maintained at 37°C and bubbled with 21% O_2, 74% N_2, and 5% CO_2. Vessels were stretched to give an equivalent transmural pressure of 30 mmHg. Preparations were firstly

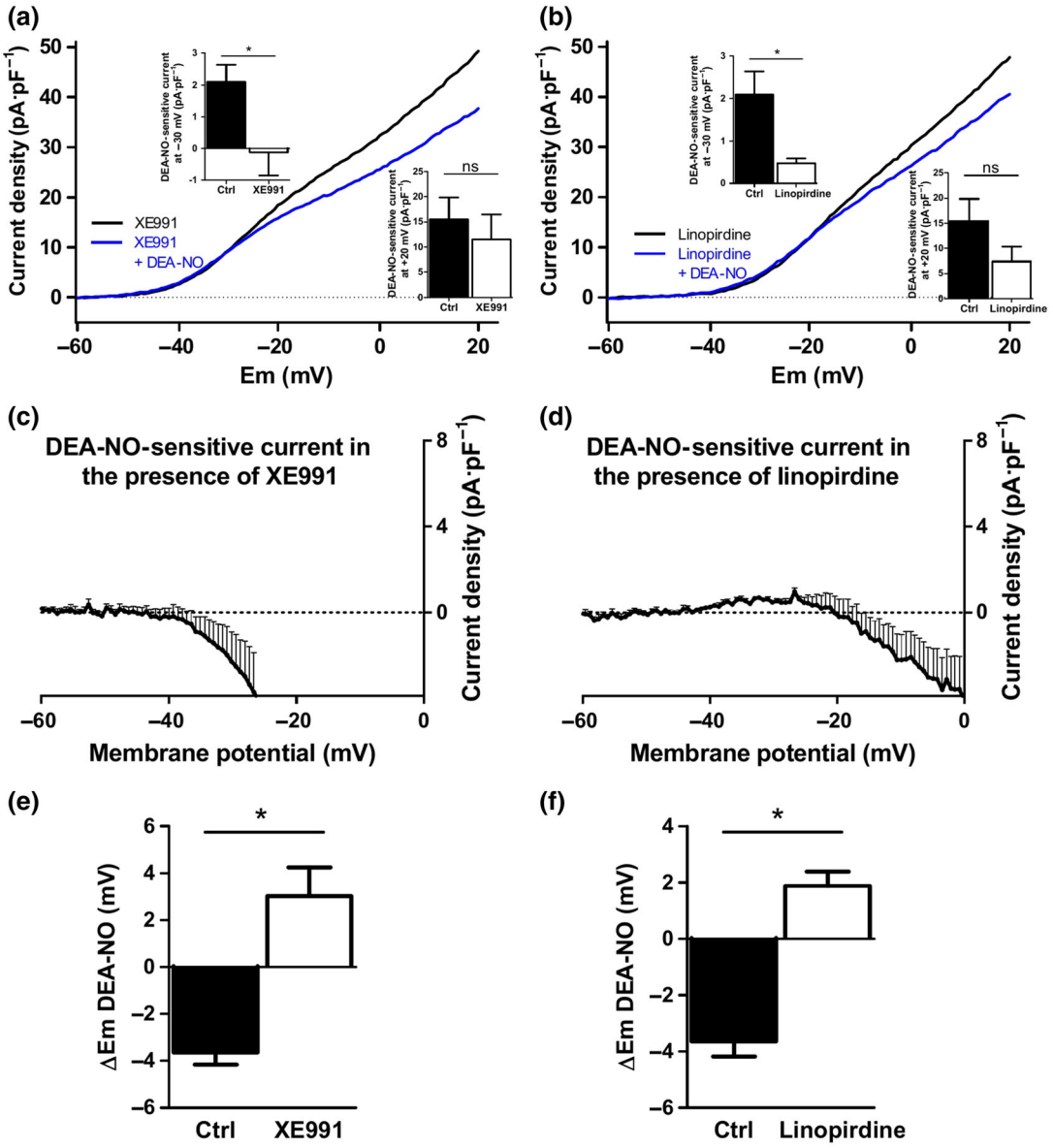


FIGURE 3 The activation of K_v current and hyperpolarization induced by DEA-NO is prevented by K_v7 channel inhibitors. (a and b) Mean values of K_v currents in PASMCs recorded after the application of depolarizing ramps from −60 to +20 mV in the presence of the K_v7 channel inhibitors (a) XE991 (10 μM, *n* = 6 from five animals) or (b) linopirdine (10 μM, *n* = 6 from five animals) before (black lines) and after (blue lines) the addition of DEA-NO (10 μM). Inset shows the DEA-sensitive current at −30 and +20 mV under control conditions (*n* = 10 from seven animals) and in the presence of XE991 or linopirdine (*n* = 6 from five animals). (c and d) Average values of DEA-NO-sensitive currents under both conditions obtained by subtracting the current in the presence and in the absence of DEA-NO. (e and f) Mean values of the changes in the membrane potential induced by DEA-NO in the absence (Ctrl) and in the presence of the K_v7 channel inhibitors (XE991, *n* = 6 from five animals, or linopirdine, *n* = 5). *P < 0.05 versus Ctrl (Student's unpaired *t* test). Results are means ± *SEM*

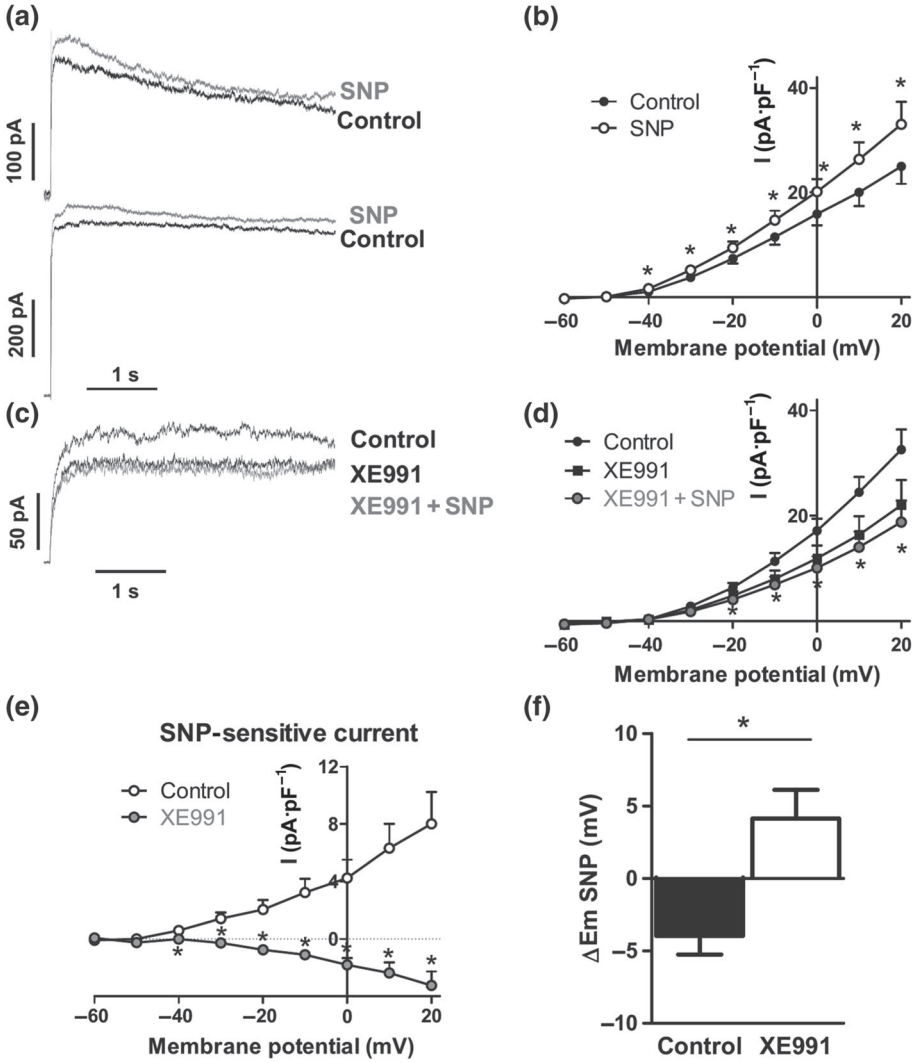


FIGURE 4 SNP-induced activation of K_v current and hyperpolarization is prevented by K_v7 channel inhibition. (a) Representative experiments in PASMCs showing inactivating (top) or non-inactivating (bottom) K_v currents obtained after 4-s depolarization pulses from −60 to +20 mV in the absence and in the presence of SNP (10 μM, *n* = 11 from eight animals). (b) Current–voltage relationships in the absence and in the presence of SNP. (c) Representative current traces and (d) average values of the K_v currents under control conditions or following the addition of XE991 (10 μM) and XE991 + SNP (10 μM, *n* = 7 from five animals). (e) Average values of SNP-sensitive currents in the absence (control) or the presence of XE991 obtained by subtracting the current in the presence and in the absence of SNP. (f) Mean values of the changes in the membrane potential induced by SNP in the absence (Ctrl, *n* = 11 from eight animals) and in the presence of XE991 (*n* = 6 from five animals). **P* < 0.05 versus Ctrl (Student's paired or unpaired *t* test). Results are means ± *SEM*

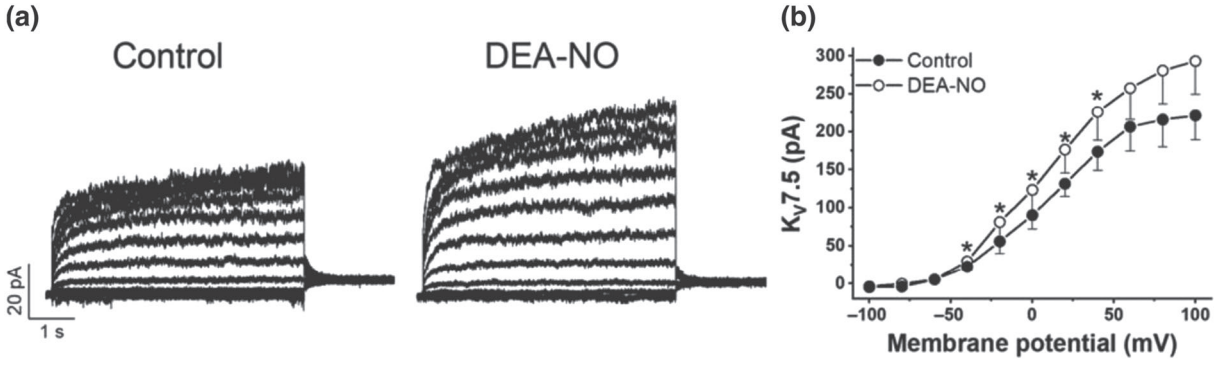


FIGURE 5 DEA-NO enhances K^+ currents in COS7 cells expressing $K_v7.5$ channels. (a) Representative current traces and (b) average values of the $K_v7.5$ currents in COS7 in the presence of DEA-NO (10 μM, *n* = 5). Currents were evoked by series of 5.5-s pulses from −100 to +100 mV in 20-mV steps from a holding potential of −80 mV. **P* < 0.05 versus Ctrl (Student's paired *t* test). Results are means ± *SEM*

stimulated by raising the K^+ concentration of the buffer (to 80 mM) in exchange for Na^+. Vessels were washed three times and allowed to recover before a new stimulation. The relaxant effects induced by the NO donors DEA-NO and SNP and riociguat were examined in PA stimulated with **phenylephrine** (Phe, 1 μM). Relaxation was expressed as a percentage of the reduction in Phe-induced contraction. Some experiments were performed in the presence of XE991, iberiotoxin, DPO-1, or ODQ.

2.6 | Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018). The data analyst was blinded, whereas blinding for the operator was not possible since some drugs used (i.e., DEA-NO) had to be prepared immediately before their use due to their fast decomposition. All data were analysed using GraphPad Prism 5.01 (RRID:SCR_002798). Data are expressed as mean ± SEM; *n* indicates the number of independent experiments performed at different preparations (PA or PASMC derived from different PA) from at least five animals. Statistical analysis was performed using Student's *t* test for paired or unpaired observations or one-way ANOVA followed by a Bonferroni test for multiple comparisons. Current–voltage relations were analysed by two-way repeated-measures ANOVA with the Bonferroni post hoc test. Differences were considered statistically significant when *P* was less than 0.05.

2.7 | Materials

Drugs and reagents were obtained from Sigma-Aldrich Quimica (Madrid, Spain), except riociguat (MedChem Express Europe, Sollentuna, Sweden). Drugs were dissolved in DMSO, and final vehicle concentrations were always ≤0.1%.

2.8 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Fabbro et al., 2017; Alexander, Striessnig et al., 2017).

3 | RESULTS

3.1 | Bimodal effects of NO donors on total K_v current

Figure 1 shows original traces (panels a and b) and mean data (panels c and d) of the K^+ currents recorded following the application of a depolarizing ramp from −60 to +20 mV. The NO donors DEA-NO (10 μM) and SNP (10 μM) induced a bimodal effect on K^+ currents, augmenting the current between ≈−40 and −10 mV and decreasing it at more depolarized potentials. The onset of the responses to NO donors was fast (≈30 s for the inhibitory effects and ≈60–90 s for

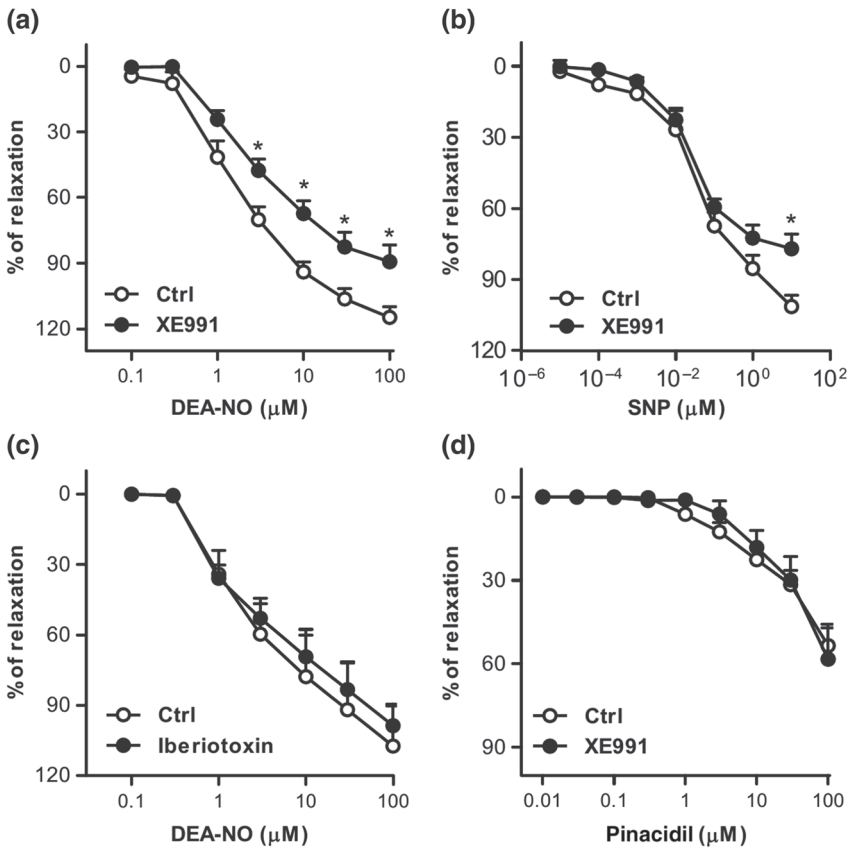


FIGURE 6 Pulmonary vasodilation induced by NO donors is attenuated by K_v7 channel inhibition. (a and b) Averaged data showing the concentration-dependent relaxant responses induced by DEA-NO (0.1–30 μM, *n* = 8–11) or SNP (0.00001–10 μM, *n* = 5–6) in the absence or the presence of XE991 (10 μM). (c) Averaged data showing the concentration-dependent relaxant responses induced by DEA-NO in the absence or the presence of iberiotoxin (0.1 μM, *n* = 6). (d) Averaged data showing the concentration-dependent relaxant responses induced by pinacidil in the absence or the presence of XE991 (10 μM, *n* = 6). **P* < 0.05 versus Ctrl. Results are means ± *SEM*

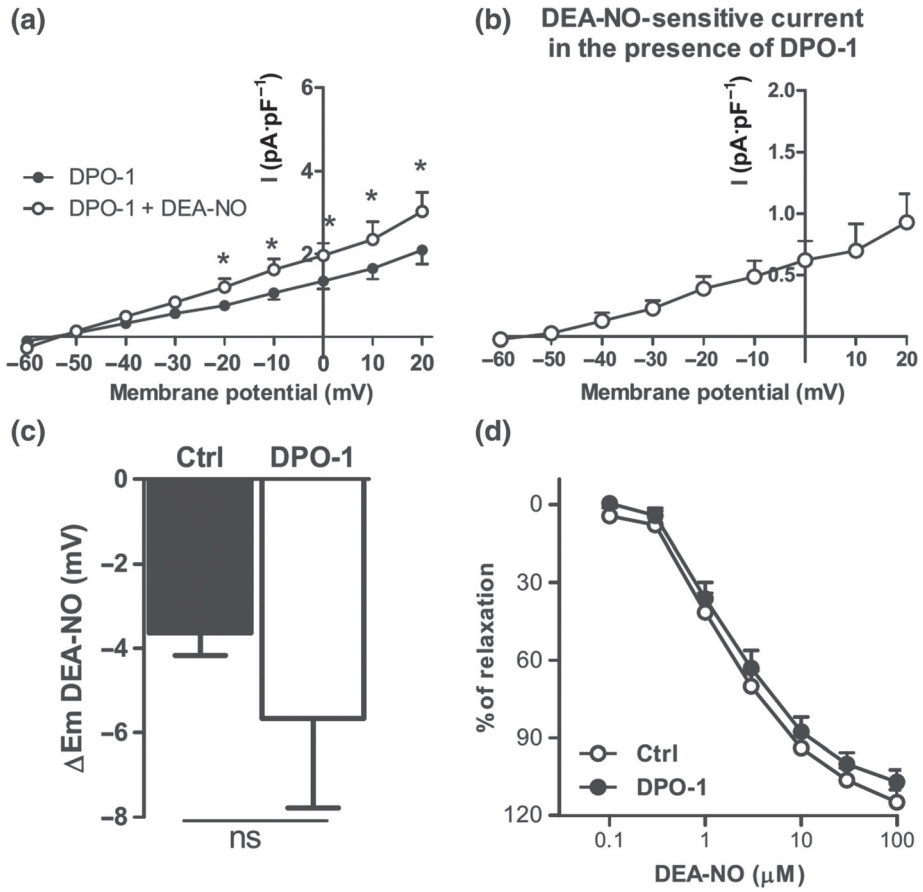


FIGURE 7 DEA-NO-induced activation of K_v current, hyperpolarization, and relaxation is not affected by K_v1.5 channel inhibition. (a) Average values of the K_v currents obtained after 4-s depolarization pulses from −60 to +20 mV in PASMCs perfused with the K_v1.5 channel inhibitor DPO-1 (1 μM, *n* = 10 from seven animals) in the absence and in the presence of DEA-NO (10 μM). (b) Average values of DEA-NO-sensitive currents in the presence of DPO-1 obtained by subtracting the current in the presence and in the absence of DEA-NO. (c) Mean values of the changes in the membrane potential induced by DEA-NO in the absence and in the presence of DPO-1 (*n* = 8–9 from five to seven animals). (d) Averaged data of the concentration-dependent relaxant responses induced by DEA-NO (0.1–30 μM) in the absence (*n* = 11) or the presence of DPO-1 (*n* = 6). **P* < 0.05 versus DPO-1 (Student's paired *t* test). Results are means ± *SEM*

the enhancing effect), and stable responses were reached within 4–5 min. The increase of the current at −30 mV and the decrease of the current at +20 mV induced by both NO donors are depicted in inset panels. Figure 1e,f illustrates the current sensitive to DEA-NO and SNP, respectively, obtained by subtracting the current in the absence and in the presence of the drug. Both NO donors enhanced the K_v current at similar membrane potentials, within a physiological range. Likewise, this was associated with PASMC hyperpolarization (Figure 1g,h). We also studied the concentration dependence of DEA-NO-induced effects. Both the increase (Figure 2a, top) and the decrease (Figure 2a, bottom) of the current were evident at concentrations ≥0.1 μM and reached a maximal response at 10 and 30 μM, respectively.

3.2 | K_v7 channel inhibitors prevent the increase in Kv current induced by NO donors

In the next set of experiments, we tested the electrophysiological effects of DEA-NO in the presence of potassium channel inhibitors. In the presence of the BKCa channel inhibitor iberiotoxin, the bimodal effects of DEA-NO were still observed (Figure 2b,c), and the changes at −30 and +20 mV were similar to those recorded under control conditions (inset). On the other hand, in the presence of the K_v7 channel inhibitors XE991 (10 μmol·L^{-1}; Figure 3a,c) or linopirdine (10 μmol·L^{-1}; Figure 3b,d), only the inhibitory, but not the enhancing, component was evident. Thus, DEA-NO-sensitive current at −30 mV was markedly reduced in the presence of these inhibitors (top insets), while at +20 mV, no statistical differences were found (insets bottom). Under these conditions, DEA-NO behaved as a K_v channel inhibitor at potentials positive to ≈−25 mV (Figure 3c,d). XE991 and linopirdine also prevented the hyperpolarization induced by DEA-NO (Figure 3e,f).

In another set of experiments, long (4 s) depolarizing pulses were applied to increase the contribution of the slowly activating channels (such as K_v7) to the total current. Using this protocol, we found that some cells showed a clear time inactivation, while others showed apparently no inactivation (Figure 4a, top and bottom). Addition of SNP led to a similar increase in the current in both cases. Thus, rather than inducing a bimodal effect, SNP exclusively enhanced the K_v

current at all potentials tested ≥−40 mV (Figure 4b), resulting in a positive SNP-sensitive current at all these potentials (Figure 4e). When PASMCs were perfused with XE991, a modest reduction on the K_v currents was observed (Figure 4c,d). Under these conditions, the addition of SNP in the continuous presence of XE991 did not produce any effect. Thus, the SNP-sensitive current was blunted in the

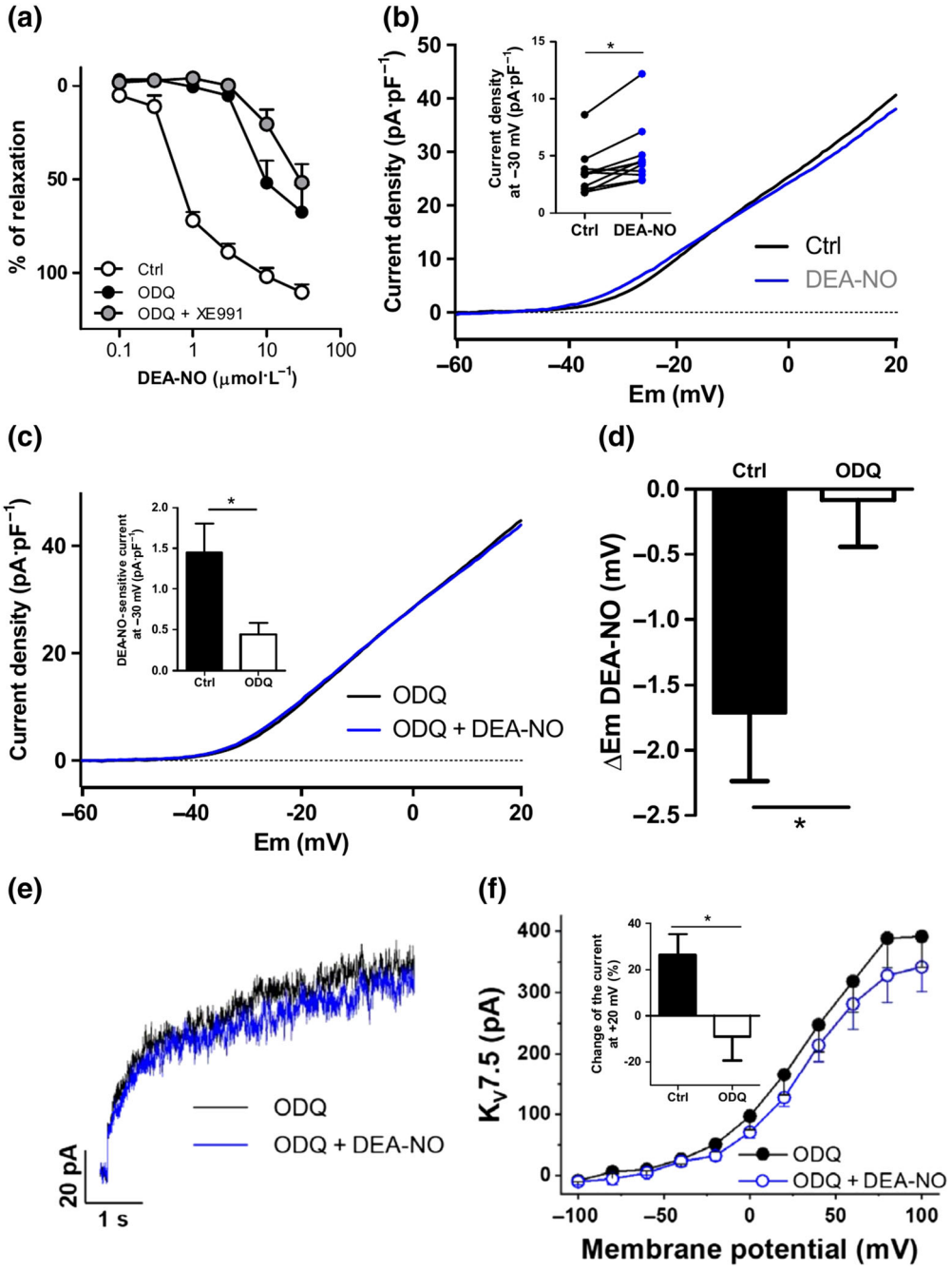


FIGURE 8 Role of sGC in DEA-NO-induced effects. (a) Averaged data of the concentration-dependent relaxant responses induced by DEA-NO (0.1–30 μM) in the absence (Ctrl, *n* = 15 from 12 animals) or the presence of the sGC inhibitor ODQ (10 μM, *n* = 9 from eight animals) or ODQ + XE991 (10 μM, *n* = 7 from five animals). (b) Mean values of the K_v currents recorded after the application of depolarizing ramps from −60 to +20 mV in the absence or the presence of DEA-NO (1 μM, *n* = 11 from six animals). Inset shows the current density at −30 mV before and after the addition of DEA-NO. (c) Mean values of K_v currents recorded after the application of depolarizing ramps from −60 to +20 mV in PASMCs perfused with ODQ in the absence or the presence of DEA-NO (1 μM, *n* = 11 from five animals). Inset shows the changes in current density at −30 mV induced by DEA-NO in the absence and in the presence of ODQ. (d) Mean values of the changes in the membrane potential induced by DEA-NO in the absence and in the presence of ODQ (*n* = 7–12 from five animals). (e) Representative current traces at +20 mV and (f) average values of K_v7.5 currents in COS7 perfused with ODQ (10 μM) before and after the addition of DEA-NO (10 μM, *n* = 6). Inset shows the changes in the current at +20 mV (expressed as a percentage) induced by DEA-NO in the absence and in the presence of ODQ. **P* < 0.05 versus Ctrl (Student's paired or unpaired *t* test). Results are means ± *SEM*

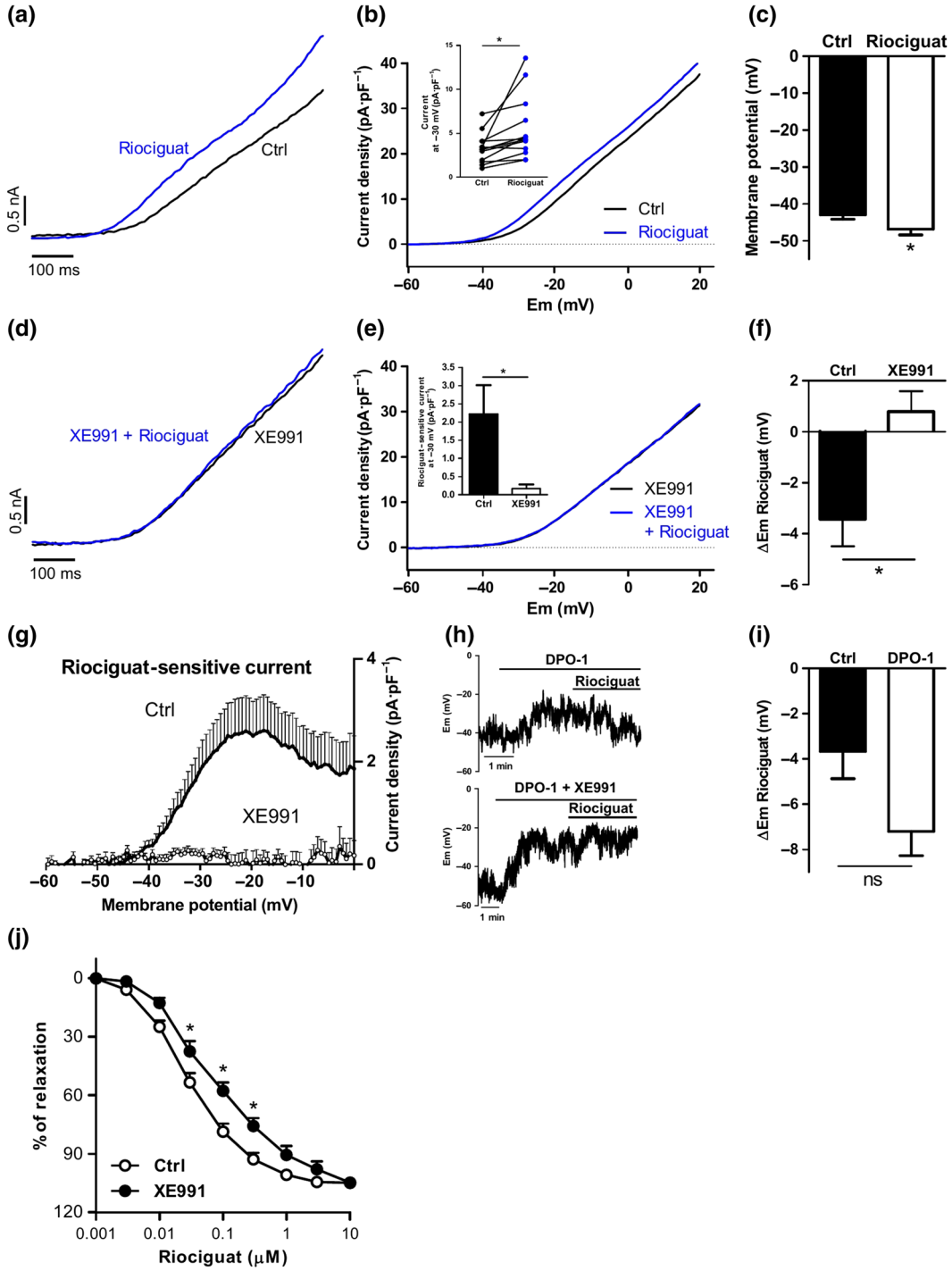


FIGURE 9 Role of K_V7 channels in riociguat-induced electrophysiological and vascular responses in PA. (a) Representative traces and (b) mean values of K_V currents recorded after the application of depolarizing ramps from −60 to +20 mV in the absence or the presence of riociguat (0.3 μM, *n* = 15 from eight animals). Inset shows the current density at −30 mV before and after the addition of riociguat. (c) Mean values of the membrane potential in PASMC before and after the addition of riociguat (*n* = 14 from eight animals). (d) Representative traces and (e) mean values of K_V currents recorded after the application of depolarizing ramps from −60 to +20 mV in PASMCs perfused with XE991 before and after the addition of riociguat (0.3 μM, *n* = 7 from five animals). Inset shows the changes in the current at −30 mV induced by DEA-NO riociguat. $^*P < 0.05$ versus Ctrl (Student's unpaired *t* test). (f) Mean values of the changes in the membrane potential induced by riociguat in the absence and in the presence of XE991 (*n* = 5–14 from five to eight animals). (g) Averaged values of riociguat-sensitive currents obtained by subtracting the current in the presence and in the absence of the drug. (h) Representative traces of the effects of riociguat on the membrane potential in the presence of DPO-1 (1 μM) or DPO-1 + XE991 (10 μM). (i) Mean values of the changes in the membrane potential induced by riociguat in the absence and in the presence of DPO-1 (*n* = 5–14 from five to eight animals). (j) Averaged data of the concentration-dependent relaxation induced by riociguat (0.001–10 μM) in PA in the absence (*n* = 6) or the presence of XE991 (10 μM, *n* = 6). $^*P < 0.05$ versus Ctrl (Student's paired or unpaired *t* test). Results are means ± *SEM*

presence of the K_v7 channel blocker (Figure 4e). In addition, XE991 prevented the hyperpolarizing effect (Figure 4f) induced by SNP.

3.3 | DEA-NO activates $K_v7.5$ channels

In order to confirm the regulation of NO on K_v7 channels observed in isolated PASMC, we tested the electrophysiological effects of DEA-NO in COS7 cells expressing $K_v7.5$ channels. As previously reported, $K_v7.5$ currents were evident at membrane potentials more positive than −50 mV (Figure 5a,b). The application of DEA-NO increased $K_v7.5$ current amplitudes at potentials positive to −40 mV reaching statistical significance at potentials ranging from −40 to +40 mV.

3.4 | Activation of K_v7 channels contributes to the pulmonary vasodilation induced by NO donors

To ascertain if the electrophysiological effects of NO donors contribute to their relaxing effect, we studied the vasodilation induced by DEA-NO and SNP in isolated PA. Phe-stimulated PA were relaxed in a concentration-dependent manner by DEA-NO (Figure 6a) and SNP (Figure 6b). XE991 (Figure 6a,b), but not iberiotoxin (Figure 6c), attenuated this relaxant response. Moreover, XE991 did not affect the relaxation induced by the selective KATP channel opener **pinacidil** (Figure 6d). These data indicated that K_v7 channels contributed to NO-induced pulmonary vasodilation.

3.5 | The effects of DEA-NO are not affected by $K_v1.5$ channel inhibition

So far, our data suggested that activation of $K_v1.5$ channels did not contribute to the activation of K_v current and the hyperpolarization induced by NO donors. To confirm this, we analysed the effects of DEA-NO in the presence of the $K_v1.5$ channel blocker DPO-1. Current amplitude in the presence of DPO-1 was reduced in relation to control conditions (cf. Figure 7a vs. Figure 4b). We observed that the enhancing effect of this NO donor on K_v currents was preserved in the presence of DPO-1 (Figure 7a). Moreover, under these conditions, DEA-NO caused an increase in the outward current amplitude at all potentials, rather inhibiting the current at positive potentials (Figure 7b). In line with these data, the hyperpolarizing (Figure 7c) and the vasodilating (Figure 7d) effects of DEA-NO were unaffected by the $K_v1.5$ channel inhibitor.

3.6 | Role of the sGC

We studied the involvement of sGC in the relaxation induced by NO donors. We found that the sGC inhibitor ODQ, at 10 μM, induced a marked inhibition of DEA-NO-induced vasodilation (Figure 8a). Interestingly, in the presence of the ODQ, the K_v7 channel inhibitor XE991 did not significantly affect DEA-NO-induced relaxation. Moreover, ODQ suppressed the relaxations elicited by concentrations of DEA-NO $\leq$3 μM, indicating that at concentrations up to 3 μM, the relaxation to DEA-NO was due to an increase in cGMP levels. Thus, we tested the electrophysiological effects of 1-μM DEA-NO in the presence of ODQ. Notably, ODQ markedly reduced the increase in K_v current (Figures 8c) and the hyperpolarization (Figure 8d) induced by the NO donor in PASMC. Likewise, ODQ prevented the enhancement of $K_v7.5$ currents expressed in COS7 cells which was induced by DEA-NO (Figure 8e,f).

3.7 | Activation of K_v7 channels contributes to the relaxation induced by riociguat

We then tested the electrophysiological effects of the sGC stimulator riociguat. This drug induced a substantial increase in K^+ currents at all potentials $\geq$−40 mV within the first 1–2 min and reached a stable effect at 5–7 min (Figure 9a,b). In addition, riociguat induced hyperpolarization of the PASMC (Figure 9c). Both effects were inhibited in the presence of XE991 (Figure 9d–f). Thus, riociguat-sensitive K^+ current was essentially abolished in the presence of the K_v7 channel inhibitor (Figure 9g). Notably, the hyperpolarizing effect induced by riociguat persisted in the presence of the $K_v1.5$ channel inhibitor DPO-1 (Figure 9h,i) but was blunted under the combination of DPO-1 plus XE991 (Figure 9h). Remarkably, the pulmonary vasodilation induced by riociguat was attenuated by XE991 (Figure 9j), indicating that activation of K_v7 channels contributes to its main pharmacological effect.

4 | DISCUSSION

Herein, we demonstrate that NO donors induce a bimodal effect on K_v currents in PASMC, with an enhancement at potentials ranging between −40 and −10 mV and a reduction at more depolarized potentials. The increased K_v currents and the hyperpolarization of PASMC induced by NO donors are triggered by the activation of K_v7, but not $K_v1.5$, channels. We also identify $K_v7.5$ channels as targets of NO donors. The increase of K_v7 channel activity involves the activation of sGC. Accordingly, the sGC stimulator riociguat increases K_v7 currents leading to membrane hyperpolarization. Remarkably, K_v7 channel activation contributes to the pulmonary vasodilating effect induced by drugs stimulating the NO/cGMP pathway.

More than two decades years ago, Yuan et al. (1996) showed that NO activated K_v channels in cultured PASMC and proposed that this effect contributed to NO-induced hyperpolarization of PASMC and PA relaxation. Intriguingly, controversial effects of NO donors on $K_v1.5$ channels, which represent the major component (around 80%) of the total K_v current present in PASMC (Archer et al., 1998; Moral-Sanz et al., 2011), have been reported. Thus, NO donors such as SNAP or SNP have been shown to activate (Remillard et al., 2007) and inhibit (Núñez et al., 2006) $K_v1.5$ currents in KCNA5-transfected HEK 293 and *Ltk*$^-$ cells, respectively. In the present study, we aimed to gain more insight into the modulation of K_v channels by NO in pulmonary arteries. Since culture conditions may lead to a dramatic down-regulation of several potassium channels including $K_v1.5$ channels (Manoury, Etheridge, Reid, & Gurney, 2009), we used freshly isolated PASMCs which represent a more reliable model for

characterizing the impact of ion channels in the regulation of vascular reactivity. We found that the NO donors DEA-NO and SNP hyperpolarized PASMC and exerted a bimodal effect on the total K_v current recorded following the application of a depolarizing ramp. Thus, these drugs increased the current activity at negative potentials (from −40 to −10 mV) and decreased the current at more positive potentials in a concentration-dependent manner. The enhancement of the current at −30 mV induced by DEA-NO reached a maximal value at 10 μM and decreased at a higher concentration, probably counterbalanced by the inhibitory component. Upon the application of long depolarizing pulses (to enhance the contribution of the slowly activating K_v7 channels), we only observed the activating component, with no evidence of the inhibitory component. Both the increase in K_v current and the hyperpolarization of PASMC induced by DEA-NO still occurred in the presence of the selective $K_v1.5$ channel inhibitor DPO-1. These data strongly suggested that $K_v1.5$ channels present in freshly isolated PASMC were not activated by NO donors but rather might contribute to the inhibitory component of NO donors in line with previous observations in *Ltk*$^-$ cells (Núñez et al., 2006).

Since the net effect of NO donors was PASMC hyperpolarization, according with an enhancement of the K_v currents at physiological membrane potentials, we focused in characterizing this activating component of the K_v current. Although activation of BKCa channels has been shown to contribute to NO-induced relaxation (Bolotina et al., 1994; Robertson et al., 1993), we found that the selective inhibitor iberiotoxin did not affect the enhanced K_v current or the relaxation induced by DEA-NO. K_v7 channels have recently emerged as key players regulating systemic (Chadha, Zunke, Zhu, et al., 2012; Khanamiri et al., 2013; Mani et al., 2016; Morales-Cano et al., 2015, 2016) and pulmonary (Joshi, Sedivy, Hodyc, Herget, & Gurney, 2009; Ng et al., 2011; Sedivy et al., 2015) vascular tone. Accordingly, we observed that the K_v7 inhibitor XE991 consistently depolarized the membrane potential but to a lower extent (3 mV) than previously reported (6 mV, Eid & Gurney, 2018, and 15 mV, Joshi et al., 2009). One possible explanation for these discrepancies is that, in comparison with ours, these studies were performed in the presence of other K^+ channel inhibitors (**glibenclamide** and glibenclamide plus **TEA**, respectively) which could potentiate XE991-induced depolarization. Whereas many studies have highlighted their role in cAMP-induced relaxation, information on the modulation of K_v7 channels by the NO/cGMP vasodilation pathway is very scarce (Jepps, Olesen, Greenwood, & Dalsgaard, 2016; Stott, Barrese, Jepps, Leighton, & Greenwood, 2015). Thus, we tested this possibility and found that NO donors failed to increase K_v currents in the presence of XE991 or linopirdine indicating that K_v7 channels mediated the enhanced NO-sensitive current in PASMC. Likewise, these K_v7 channel inhibitors prevented the hyperpolarizing effects induced by DEA-NO or SNP. Our study is, to the best of our knowledge, the first to show the activation of K_v7 channels by NO in isolated vascular myocytes. Importantly, these electrophysiological effects had functional significance, since K_v7 channel inhibitors also attenuated their relaxant responses. These data indicated that pulmonary vasodilation induced by NO donors is driven, at least partly, through the activation of vascular K_v7 channels.

While the main mechanism of NO-induced pulmonary vasodilation involves the activation of sGC and the subsequent increase in cGMP levels (Barnes & Liu, 1995; Cogolludo et al., 2001; Sausbier et al., 2000), alternative mechanisms (i.e., S-nitrosylation) have been reported (Hess et al., 2005). In order to gain more insight into the mechanism of NO-mediated K_v7 channel activation, we performed experiments with the sGC inhibitor ODQ. This compound prevented DEA-NO-induced increase in $K_v7.5$ currents, enhancement of K_v currents and hyperpolarization in PASMC, and relaxation, consistent with an involvement of the canonical NO/cGMP pathway in the activation of K_v7 channel by NO. This idea was also supported by the lack of inhibitory effects of XE991 on DEA-NO-induced relaxation in the presence of ODQ. To further confirm this, we analysed the electrophysiological effects of the sGC stimulator riociguat. In contrast to the NO donors, riociguat did not inhibit but only enhanced K_v currents at all membrane potentials, and this effect was associated with PASMC hyperpolarization. Both the increase in K_v current and the hyperpolarization induced by riociguat were suppressed by the K_v7 channel inhibitor XE991, while the inhibition of $K_v1.5$ channels failed to prevent riociguat-induced hyperpolarization. Conversely, the reduction of K_v currents induced by the NO donors was unaffected by K_v7 channel inhibition, according with a plausible involvement of $K_v1.5$ channels as discussed above. Altogether, our data support the notion that the bimodal effects of NO donors on total K_v current may reflect an opposite modulation of different K_v channels by NO. Thus, while NO activates K_v7 channels by a cGMP-dependent mechanism, it inhibits other K_v channels (most likely $K_v1.5$ channels) through a cGMP-independent mechanism, which may involve S-nitrosylation as reported (Núñez et al., 2006; Zhao, Wang, Rubin, & Yuan, 1997).

Interestingly, K_v7 channels have been reported to be negatively regulated by NO in neurons (Ooi, Gigout, Pettinger, & Gamper, 2013). The higher concentrations reported to induce this effect, the mechanism involved (S-nitrosylation) and the possible K_v7 channel subtypes targeted may explain the differences with respect to our study. Among the different K_v7 channels expressed in vascular smooth muscle (Morales-Cano et al., 2015, 2016; Yeung et al., 2007) including the pulmonary circulation (Joshi et al., 2009; Morales-Cano et al., 2014), **Kv7.4** and $K_v7.5$ channels are considered to play a predominant role in regulating vascular tone (Barrese et al., 2018). A previous study (Stott et al., 2015) showed that $K_v7.4$ currents are increased following the application of cGMP in HEK-293 cells. Our study extends this observation identifying $K_v7.5$ channels as another NO target. Thus, it is possible that both $K_v7.4$ and $K_v7.5$ channels may contribute to the enhanced K_v7 current induced by the NO/cGMP pathway in PASMC. The mechanisms leading to $K_v7.5$ channel activation observed herein remain unknown, but a direct phosphorylation could play a role since the analysis of the peptide sequence of these channels reveals the presence of several sites for phosphorylation by PKG. Alternatively, activation of **PKA** by cGMP has been shown to contribute to the relaxation induced by high concentrations of NO (Sausbier et al., 2000). Thus, it is plausible that cGMP may activate K_v7 channels through a PKA-dependent

activation of K_v7 channels (Chambard & Ashmore, 2005; Mani et al., 2016). Interestingly, we and other authors have shown that $K_v7.5$ may form functional heterotetrameric channels with $K_v7.1$ (Oliveras et al., 2014) and $K_v7.4$ (Brueggemann et al., 2014) in vascular tissues. Thus, it is conceivable that activation of $K_v7.1/K_v7.5$ or $K_v7.4/K_v7.5$ heterotetramers could contribute to cGMP-mediated responses as previously proposed (Stott et al., 2015).

The results from the present study are of great interest from a physiological, pathological, and pharmacological point of view. The NO/cGMP vasodilator pathway is a key player determining pulmonary vascular tone (Barnes & Liu, 1995). In fact, impairment of this classic pathway at any level is known to importantly contribute to the pathophysiology of PH (Klinger & Kadowitz, 2017; Schermuly et al., 2011). Detrimental effects of vascular K_v7 loss have been reported in experimental models of essential hypertension (Barrese et al., 2018; Carr et al., 2016; Jepps et al., 2011) and diabetes (Morales-Cano et al., 2015, 2016). Likewise, several studies suggest that K_v7 channels may also be impaired in murine models of PH (Morales-Cano et al., 2014; Morecroft, Murray, Nilsen, Gurney, & MacLean, 2009; Sedivy et al., 2015). It is therefore likely that the impairment of K_v7 channels in the pulmonary circulation could have detrimental consequences associated with an altered response to the NO/cGMP pathway. Interestingly, KCNQ5, which encodes for $K_v7.5$ channels, has been shown to be significantly down-regulated in a model of congenital diaphragmatic hernia (Zimmer, Takahashi, Hofmann, & Puri, 2017), a cause of PH in which the therapeutic response to inhaled NO is very poor (Neonatal Inhaled Nitric Oxide Study Group, 1997; Putnam et al., 2016). Finally, our study is of great pharmacological significance since it identifies a novel mechanism by which riociguat, a drug approved for the treatment of pulmonary arterial hypertension and chronic thromboembolic PH, may exert, at least partly, its therapeutic effect. Likewise, this mechanism is expected to contribute to the pulmonary vascular effects of other drugs activating this pathway such as PDE5 inhibitors (sildenafil or **tadalafil**).

In summary, our study is the first to show that the stimulation of the NO/cGMP pathway enhances K_v7 currents and induces hyperpolarization in PASMC. Importantly, activation of these channels partly mediates the relaxation induced by NO donors and riociguat. Our study identifies a novel mechanism that may contribute to the therapeutic effect of drugs activating the NO/cGMP pathway indicated for the treatment of PH.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

A.C. and F.P.-V. conceived and designed the research. G.M.-P., J.M.-S., B.B., T.G., A.D.C., and M.C. performed the experiments. G.M.-P., J.M.-S., B.B., T.G, A.D.C., D.M.-C., M.C., S.E.-R., and A.C. analysed the data. A.C., F.P.-V., G.M.-P., T.G, L.M., and C.V. interpreted the results of the experiments. A.C., G.M.-P, A.D.C., T.G., and B.B. prepared the figures. A.C. drafted the manuscript. A.C., G.M.-P., T.G., L.M., C.V., and F.P.-V. edited and revised the manuscript. All authors approved the final version of the manuscript.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

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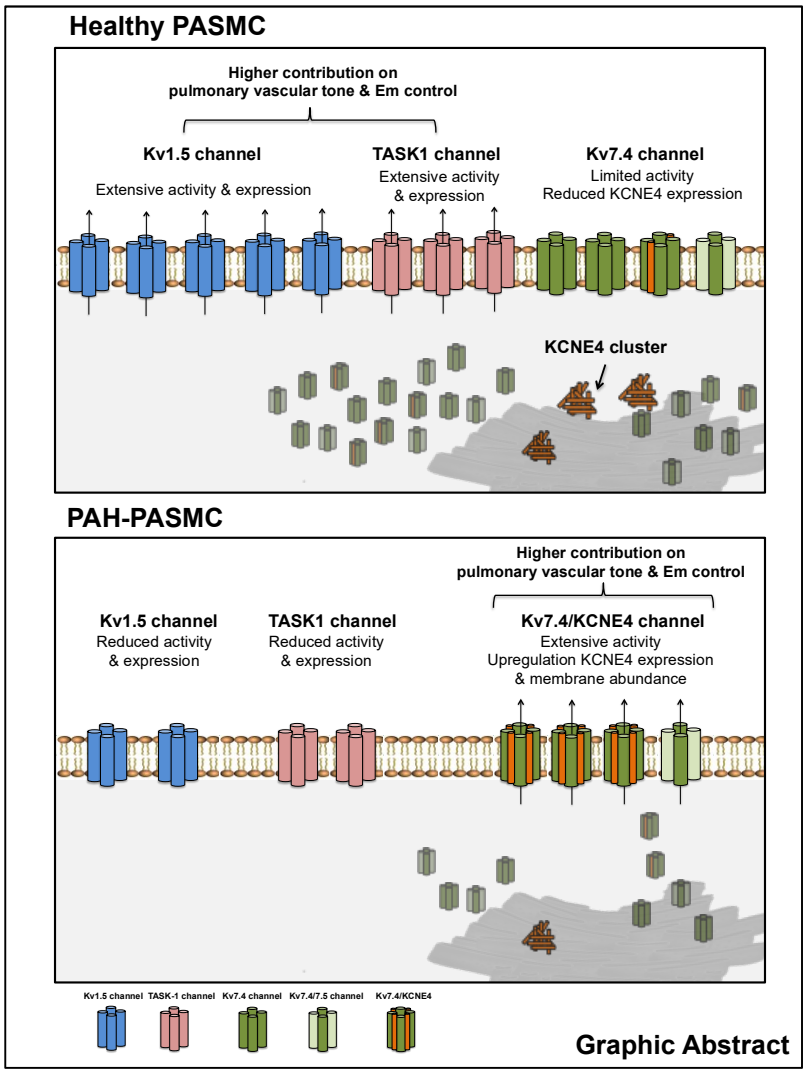
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Chapter II: An uncovered contribution of Kv7 channels to pulmonary vascular tone in pulmonary arterial hypertension

Original Article Submitted

An uncovered contribution of Kv7 channels to pulmonary vascular tone in pulmonary arterial hypertension



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Short title:

Kv7 channel in the pulmonary vasculature

Keywords: K_v7 channels, KCNE subunits, pulmonary vasculature, vascular tone, ion channel remodelling, pulmonary arterial hypertension

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Novelty and Significance

What Is New?

- Compared to systemic vessels, pulmonary arteries display low Kv7 channel expression and activity.
- However, Kv7 channel function turns much more relevant in PAH.
- The ion channel remodelling in PAH involves a greater contribution of Kv7 channels

What Is Relevant?

- Since pulmonary hypertension is associated with a net loss of K^+ channel activity, targeting the preserved Kv7 channels may be an interesting strategy in PAH.
- The development of available Kv7 channels enhancers in PAH would provide vasodilator effects with selectivity for diseased versus healthy pulmonary vessels.

Summary.

- We have found Kv7 channels assume a remarkably higher functional role in the regulation of pulmonary arterial tone during the development of PAH.

Abstract. K^+ channels play a fundamental role regulating membrane potential (Em) of pulmonary artery (PA) smooth muscle cells (PASMCs) and their impairment is a common feature in pulmonary arterial hypertension (PAH). Kv7 channels (*KCNQ1-5*) and their KCNE regulatory subunits are known to regulate vascular tone, but whether Kv7 channel function is impaired in PAH and how this can affect the rationale for targeting Kv7 channels in PAH remains unknown. Herein we have studied the role of Kv7/KCNE subunits in rat PA and their possible alteration in PAH. Using the patch-clamp technique we found that, although the total K^+ current is reduced, Kv7 current is preserved in PASMC from PAH-animals (Su/Hpx) resulting in a higher contribution to the net K^+ current. Likewise, enhanced vascular responses to Kv7 channel modulators were found in PAH rats. Accordingly, KCNE4 subunit was highly upregulated in lungs from PAH animals and patients. Additionally, Kv7 channel activity was enhanced in the presence of Kv1.5 and TASK-1 channel inhibitors and this was associated with an increased KCNE4 membrane abundance. Compared with systemic arteries, PA showed a poor response to Kv7 channel modulators which was associated with reduced expression and membrane abundance of Kv7.4 and KCNE4. Our data indicate that Kv7 channel function is preserved and KCNE4 is upregulated in PAH. Therefore, compared to other downregulated channels, the contribution of Kv7 channels is increased in PAH resulting in an enhanced sensitivity to Kv7 channel modulators. This study provides insight into the potential usefulness of targeting Kv7 channels in PAH.

Introduction. The four classes of K^+ channels (voltage-gated, calcium-activated, inward-rectifier and two-pore-domain K^+ channels) comprises 16 families and 77 genes and may bind with several accessory β-subunits[1]. These channels are present in almost every tissue, and most cells

express different K^+ channels. This large diversity of K^+ channels, their differential tissue expression and partially functional redundancy underlies a physiological fine-tuning within each cell type. In the cardiovascular system a wide variety of K^+ channels are functionally expressed and their conductance plays a fundamental role in the control of membrane potential (Em) of vascular smooth muscle cells (VSMC) and vascular tone. Redundant channel function ensures that blockade or downregulation of a given channel can be partly compensated for by another(s) channel(s), safeguarding the tissues from reduced perfusion and/or the heart from excessive vascular resistance.

Pulmonary arterial hypertension (PAH) is a rare and multifactorial disease defined as an increase in mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg at rest [2]. The pathophysiology of PAH is characterized by vascular remodelling in pulmonary arteries (PA) attributable to persistent vasoconstriction, proliferation, inflammation and *in situ* thrombosis [3]. It is translated in a decreased arterial lumen and increased pulmonary vascular resistance [4]. Downregulation of K^+ channels in PA smooth muscle cells (PASMC) is considered an early contributor to the pathophysiology of the disease, that results in a more depolarized Em, increased intracellular calcium concentration ($[Ca^{2+}]_i$), vasoconstriction, hypertrophy and proliferation [5–9]. In particular, downregulation of Kv1.5 channels and TASK-1 channels is a common denominator of human and experimental PAH [10–15,5,16–18]. In addition mutations in genes encoding for TASK-1 and Kv1.5 channels, *KCNK3* and *KCNA5* respectively, have been identified in PAH patients [14,19–21]. Altogether these studies strongly suggest that dysregulation of K^+ channels plays an important pathophysiological role in PAH.

During the last years many studies have highlighted a critical role of Kv7 channels (encoded by *KCNQ* genes) in the regulation of vascular tone [22–26]. Kv7 channel activation in VSCM causes K^+ outward efflux and the subsequent hyperpolarization dampens cell excitability. Moreover, Kv7 channels can be regulated by different vasodilator and vasoconstrictor agents [27–30]. Transcripts for *KCNQ* genes have been detected in different arteries, including mesenteric, renal, pulmonary, aortic, cerebral, coronary and penile arteries [31]. Noteworthy, the expression of Kv7.1, Kv7.4, and Kv7.5 channels predominates in VSMC whereas Kv7.2 and Kv7.3 expression is practically negligible [32]. In VSMC, Kv7.4 and Kv7.5 α-subunits formed as homo or heteromers seem to play an important role as regulators of contractility [33,31,34]. Likewise, VSMC express β-subunits KCNE1-5 encoded by *KCNE* genes [31,34] which can associate with the Kv7 channels and regulate their expression and function [34]. The physiological relevance of Kv7 channels in the cardiovascular system is supported by the fact that their dysfunction is associated with several cardiovascular disorders including diabetes, hypertension, erectile dysfunction, atrial fibrillation and long QT syndrome [29,35–37,31]. In addition, direct pharmacological modulation of Kv7 channels has been shown to influence blood vessel contractility and diameter [15,29,32,36,38], and Kv7 channel activators have been proposed as potentially useful drugs to treat cardiovascular diseases [39,22,24]. The functional expression of Kv7 channels in the pulmonary circulation of rats [40,41] and mice [42,30] has also been reported and, their activation has recently been involved in the pulmonary vasodilator effect induced by drugs increasing cGMP levels [30]. Interestingly, Sedivy *et al.,* reported that short term exposure to hypoxia reduced mRNA expression of Kv7.4 and suggested a loss of Kv7 channel function in the early onset of pulmonary hypertension [41]. Moreover, Li SS *et al.,* showed a reduced mRNA level of Kv7.5 channels in PA from rats exposed to hypoxia for 9 days and proposed an increase in microRNA-190, a microRNA upregulated during chronic hypoxia,

as a potential mechanism involved [43]. Curiously, against the potential decrease in Kv7 channel expression and activity, Kv7 channel activators have been shown to attenuate the development of chronic hypoxia-induced pulmonary hypertension in mice and to reverse established pulmonary hypertension in $SERT^+$ mice [42]. Therefore, it remains unclear whether Kv7 channel function is impaired in PAH and how this can affect the rationale for targeting Kv7 channels in PAH. Thus, in the present study we aimed to study the activity and expression of Kv7 channels and their KCNE ancillary subunits in PAH, by using an experimental model that recapitulates major features of the disease (Sugen5416 plus chronic hypoxia rat model) and lung samples from human patients.

Methods

Ethical Approval. All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-301/16). Animals were taken care of and used according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes and experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals [44].

Table 1. *Details of* Taqman *primers employed for the RT-PCR* (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA). *Rn: rat*

Gen	***Taqman Assay***
KCNQ1 | *potassium voltage-gated channel subfamily Q member 1*	Rn00583376_m1
KCNQ4 | *potassium voltage-gated channel subfamily Q member 4*	Rn01518851_m1
KCNQ5 | *potassium voltage-gated channel subfamily Q member 5*	Rn01512013_m1
KCNA5 | *potassium voltage-gated channel subfamily A member 5*	Rn00564245_s1
KCNE3 | *potassium voltage-gated channel subfamily E regulatory subunit 3*	Rn02377069_s1
KCNE4 | *potassium voltage-gated channel subfamily E regulatory subunit 4*	Rn01769979_s1
KCNK3 | *potassium two pore domain channel subfamily K member 3*	Rn04223042_m1
18S|small subunit 18S ribosomal RNA	Hs03003631_g1
B2M | *beta-2-microglobulin*	Rn00560865_m1

All investigators understand the ethical principles under which the journal operates and that their work complies with the journal's animal ethics checklist. This manuscript adheres to the AHA Journals' implementation of the Transparency and Openness Promotion (TOP) Guidelines.

Animal model of PAH. Pathogen-free male Wistar rats were obtained from Envigo (Barcelona, Spain). All animals were kept with free access to standard rat chow and water in an enriched environment throughout the whole experiment period and maintained at 24 ºC under a 12h light/12h dark cycle. PAH was induced in some animals by the combined exposition to the VEGFR type 2 inhibitor SU5416 plus hypoxia (Su/Hpx). Thus, rats of 220 g were injected subcutaneously with a single dose of SU5416 (20 mg/Kg) or vehicle [45,46]. Then, SU5416-treated animals were introduced in glass cages and ventilated with 10% O_2 (hypoxia) for 2 weeks. CO_2 and water vapour produced by the animals were captured with soda lime and silica gel, respectively. Control rats (Ctrl) were kept in the same room. Oxygen was monitored using an oxygen sensor (DrDAQ Oxygen Sensor, Pico Technology, UK) in the chamber outflow. The chambers were opened for 20-30 minutes daily for regular animal care. Animals were euthanized by decapitation, following 2013 AVMA guidelines [47] and according to the European Directive 2010/63/EU.

Human samples. The expression of Kv7 channels was analysed in lung samples from case-control study. The cases included 9 idiopathic PAH patients, whose lung samples had been deposited at the Biobank of Ciberes (Barcelona, Spain) (*http://biobancopulmonar.ciberes.org*). According to International guidelines when tissues were collected, PAH was defined as a mean PAP of more than 25 mmHg, with pulmonary capillary wedge pressure (PCWP) less than 15 mmHg and pulmonary vascular resistance [PVR] >3 Wood units. Controls were obtained from the same biobank which included 10 lung samples from donor subjects, which were discarded for transplantation. Patient informed consent was obtained. The protocol was approved by the research ethics committee (Hospital Gregorio Marañón) and Ciberes Biobank ethics committee.

Electrophysiology. PASMC were isolated by enzymatic digestion as previously described [48]. Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA, USA) using the whole-cell configuration of the patch-clamp technique. Cells were superfused with an external Ca^{2+}-free Hepes solution and a Ca^{2+}-free pipette (internal) solution containing (mM): KCl 130, $MgCl_2$ 1.2, Na_2ATP 5, HEPES 10, EGTA 10 (pH adjusted to 7.3 with KOH). Potassium currents were evoked following the application of 4s depolarizing pulses from −60 mV to +20 mV in 10 mV increments in order to enhance the contribution of slow activating Kv channel as described [49]. To characterise the contribution of Kv7 channels to the total K^+ current, cells were exposed to the selective Kv7 inhibitor XE991 (10^{-5}M) or the Kv7.2-7.5 channel opener retigabine (10^{-5}M). Some experiments were performed in the presence of Kv1.5 and TASK-1 channel inhibitors, DPO-1 (10^{-6}M) and ML365 (10^{-6}M), respectively Currents were normalized for cell capacitance and expressed in pA/pF. Membrane potential was recorded under the current-clamp mode. All experiments were performed at room temperature (22-24ºC).

Vascular reactivity study. For contractile tension recording, 2nd to 3rd order PA (250-500 µm) and 2nd order mesenteric arteries (MA) were mounted in a wire myograph with physiological salt solution maintained at 37°C and bubbled with 95% O_2 and 5% CO_2. Unless otherwise stated, vessels were stretched to give an equivalent transmural pressure of 30 mmHg or 100 mmHg for PA and MA, respectively. Preparations were

first stimulated by raising the K^+ concentration of the buffer (to 80 mM) in exchange for Na^+. The contractile response induced by XE991 (10^{-8}-10^{-5}M) was examined in PA in the absence of any pretone. In some experiments the effect of XE991 (10^{-6}M) was tested in the absence or in the presence of DPO-1 (10^{-6}M) and ML356 (10^{-6}M). The vascular responses of the Kv7 enhancer retigabine (10^{-8}-$3x10^{-5}$M) were examined in 5-HT (10^{-5}M) precontracted PA in the absence (vehicle) or in the presence of DPO-1 (10^{-6}M) plus ML356 (10^{-6}M). In another set of experiments the vasodilator responses induced by selective Kv7 enhancers (retigabine, S1, ML213 and ML277) were comparatively examined in PA and MA precontracted with methoxamine (10^{-5}M) plus U46619 ($3x10^{-7}$M).

RNA extraction and Quantitative RT-PCR. Total RNA was extracted from PA or MA using RNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. RNA concentration and quality was checked using NanoDropTM 1000 Spectrophotometers (Thermo Scientific). For mRNA determination, 1 µg of RNA was reverse transcribed into cDNA using iScriptTM cDNA Synthesis Kit (Biorad) following manufacturer's instructions. Gene expression was determined by quantitative real-time PCR (qRT-PCR) with a Taqman system (Applied Biosystems) in the Genomic Unit of the Complutense University (Madrid) using specific primers specified in *Table 1 (see above).*

The delta-delta Ct method was used to quantify relative changes. mRNA expression was normalized by the expression of 18S and β2-microglobulin.

Table 2. *Details of antibodies employed for the molecular biology techniques.*

Primary antibody	*Specie*	*Supplier*	*Reference*	*Reactivity*	*Dilution*
Anti-Kv7.1	Rabbit	Alomone	APC-022	H, R	1:200
Anti-Kv7.4	Mouse	Santa Cruz Bio.	Sc-271320	H	1:200
	Rabbit	Santa Cruz Bio.	Sc-50417	R	1:100
Anti-Kv7.4	Rabbit	Abcam	Ab65797	R	1:200
Anti-Kv7.5	Mouse	Santa Cruz Bio.	Sc-293305	H	1:200
Anti-Kv7.5	Rabbit	Alomone	APC-155	R	1:200
Anti-KCNE3	Mouse	Atlas Antibodies	HPA014849	H,R	1:100
Anti-KCNE4	Rabbit	Atlas Antibodies	HPA011420	H,R	1:100
Anti-β-actin	Mouse	Sigma-Aldrich	A1978	R	1:10000
Anti-vinculin	Mouse	Santa Cruz	Sc-25336	H	1:500

Western Blotting analysis. Lung tissues were homogenized with a lysis buffer containing Trizma Pre-set cystals pH 7.5, DL-dithiothreitol (DTT) 1M, NP40 1% and supplemented with protease (Protease inhibitor cocktail tablets, Roche Diagnosis GmbH) and phosphatase inhibitor (PhosSTOP, Roche Diagnostics GmbH) cocktail in a Tissuelyser device (Qiagen, Hilden, Germany). Homogenates were run on a sodium dodecyl sulphate-polyacrylamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, incubated with primary rabbit antibodies against Kv7 channels and KCNE3 and E4 subunits (details are showed in *Table 2)* overnight and then with the secondary peroxidase conjugated antibodies. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Blots were imaged using an Odissey Fc System (Li-COR, Biosciences) and were quantified by densitometry using Quantity One software. Samples were normalized through expression of smooth muscle β-actin and vinculin for rat and human, respectively.

Inmunocytochemistry. Freshly dissociated rat MA smooth muscle cells (MASMC) or PASMC were fixed in 3% paraformaldehyde (Sigma) in PBS for 30 min at room temperature as previously described [23]. Cells were then incubated for 10 min at room temperature with the membrane marker wheat germ agglutining (WGA) (1:200, Life technologies, W11261). Cells were treated with 0.1 M glycine for 5 min and incubated 45 min with blocking solution (PBS-0.1% Triton X-100-10% bovine serum). Following the incubation overnight at 4°C with the primary antibody (*Table 2*), cells were then washed for 20 min with PBS, incubated for 1 h at room temperature with secondary conjugated antibody (Alexa Fluor-568, 1:100, Invitrogen, Ref. 1826664) and counterstained with Hoechst 33258 (0.2 µg/ml, 10 min at RT; Sigma-Aldrich) to visualize cells nuclei. Coverslips were analysed using a Zeiss LSM 510 Meta argon/krypton laser scanning confocal microscope (Image Resource Unit St George's University of London) and confocal laser scanning fluorescence microscope SP2 ACS APO 60x (Unidad de Microscopía Confocal Hospital Universitario Gregorio Marañón). Plasma membrane abundance of Kv7.4 and KCNE4 subunits was quantified using Image J software by performing a line scan analysis of fluorescence, as previously described [23]. Briefly, plasma membrane was identified as the regions where WGA fluorescence intensity was above a threshold, set at 5 Arbitrary Units, and average fluorescence intensities of Kv7.4 and KCNE4 signals in the corresponding regions were measured. Co-localization of Kv7.4 channels and KCNE4 with the membrane marker WGA was also assessed.

Proximity ligation assay (PLA). PLA technology was used to determine the co-localization of Kv7.4 and Kv7.5 channels in freshly dissociated rat MASMC and PASMC using the Duolink *in situ* (PLA) detection kit 563 (Olink, Uppsala, Sweden) in accordance with the manufacturer's instructions and as previously described [23]. Briefly, cells were fixed for 20 min in PBS containing 3% paraformaldehyde, before being permeabilized in PBS containing 0.1% Triton X-100 for 5 min. To reduce non-specific binding, cells were then blocked for 1 h at 37°C in Duolink blocking solution, and incubated overnight at 4°C with pairs of primary antibodies (all at dilution 1:200) in Duolink antibody diluent solution. Control experiments omitted the primary antibody. The primary antibodies employed in the present study were goat anti-Kv7.4 (sc-20882; Santa Cruz Biotechnology) and rabbit anti-Kv7.5 (ABN1372, MERCK). Cells were subsequently labelled with Duolink PLA anti-rabbit and anti-goat PLUS, as appropriate, for 1 h at 37°C. The secondary antibodies of PLA PLUS and MINUS probes are attached to synthetic oligonucleotides

and, if the target proteins are localized sufficiently close together (<40 nm), the oligonucleotides bound to the antibodies will hybridize and form a circular DNA strand. The hybridized oligonucleotides are then ligated for 30 min at 37°C prior to rolling circle amplification for 100 min at 37°C. Fluorescently-labelled oligonucleotides hybridize to the rolling circle amplification product. Green punctae, indicating co-location of two proteins were visualized using an LSM 510 confocal microscope (Carl Zeiss). Analysis of mid-cell *xy* sections was performed using Image-J software.

Statistical analysis. Data are expressed as mean ± SEM. Statistical comparisons were performed using unpaired or paired *t* tests or two-way ANOVA followed by Bonferroni test. $P<0.05$ was considered statistically significant.

Drugs. 5HT, U46619, methoxamine DPO-1 and XE991 were purchased from Sigma-Aldrich Quimica (Madrid, Spain), S1 and retigabine were from NeuroSearch A/S (Denmark). SUGEN5416, ML213, ML277 and ML356 were from TOCRIS (U.K).

Results

Total K^+ current is decreased while Kv7 current is preserved in Su/Hpx-PASMC . Whole cell patch clamp recordings from normal PASMC produced large rapidly activating voltage-dependent K^+ currents (Figure 1). The total K^+ current was dramatically reduced in PASMC from Su/Hpx rats (Figure 1A and 1B). This was associated with a more depolarized membrane potential in Su/Hpx-PASMC compared to control-PASMC (Figure 1C). To characterize the contribution of the Kv7 channels to the total K^+ current, we used a specific Kv7 channel inhibitor, XE991 (10^{-5}M) (Figure 1D). XE991 inhibited the K^+ current at test potentials more positive than -30mV in both control and Su/Hpx PASMC. In contrast to total current, the XE991-sensitive current amplitude, calculated by subtracting the current in the absence and in the presence of XE991, was no different in PASMC from control or Su/Hpx treated animals (Figure 1E). Thus, the percentage of the current sensitive to XE991 was significantly higher in Su/Hpx-PASMC compared to control-PASMC (Figure 1F) indicating a greater contribution of Kv7 channels to the total current in experimental PAH.

Enhanced response to Kv7 channel modulators in PA from PAH animals. The cumulative addition of XE991 caused a minor effect in the basal tone of control-PA, whereas a clear vasoconstrictor effect was observed in PA from Su/Hpx rats (Figures 2A and B). In line with these results, we observed that the Kv7 channel opener retigabine had a negligible vasodilator effect in control-PA, but caused a marked vasodilator response in PA from Su/Hpx rats (Figure 2C and 2D). These results, together with the electrophysiological data, suggested a greater functional role of Kv7 channels in PA from PAH as compared to control animals.

Altered lung expression of Kv7 and KCNE ancillary subunits in PAH. We analysed the protein expression of Kv7.1, Kv7.4 and Kv7.5 channels by Western blot in lungs from Su/Hpx rats and human PAH patients compared to controls. We found no changes in the expression of Kv7.1 or Kv7.5 subunits but there was a clear downregulation of Kv7.4 channel expression in Su/Hpx-lungs compared to controls (Figure 3A). Nevertheless, no differences in any of the subunits were found in human samples (Figure 3B). Since these results could not help to explain the increased contribution of Kv7 channels in Su/Hpx-PA, we also analysed the levels of expression of the regulatory KCNE subunits. Interestingly, the expression of KCNE4, but not that of KCNE3, was upregulated in both Su/Hyp (Figure 3C) and PAH patients (Figure 3D) lung samples compared to controls.

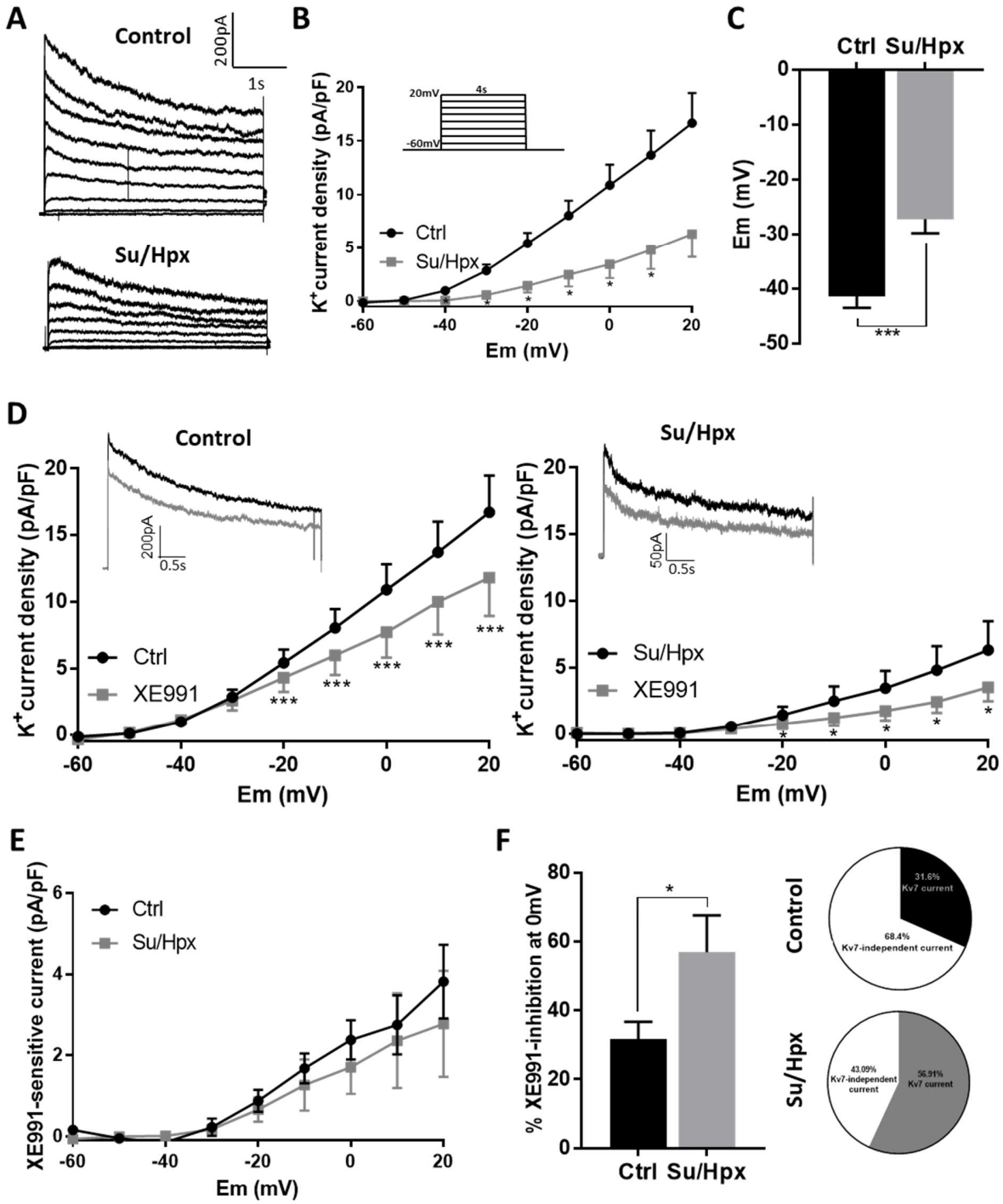


Figure 1. Kv7 current is preserved and provides a higher contribution to total K^+ current in Su/Hpx-PASMC compared to control-PASMC. A) Representative K^+ current traces and B) K^+ current-voltage relationships measured at the end of the depolarising pulses. C) Mean values of the resting membrane potential (E_m). D) K^+ current-voltage relationships measured before and after the addition of XE991 (10^{-5}M). Insets show representative current traces measured at +20 mV in the absence and presence of XE991 in control (left) and Su/Hpx-PASMC (right). E) XE991-sensitive current obtained by subtracting the K^+ current in the presence of XE991 from the current in the absence of the drug. F) XE991-induced inhibition of K^+ currents measured at 0 mV expressed as a percentage. *P < 0.05, and ***P<0.001 vs control. Data was analysed by paired t-test in panel D and by unpaired t-test in panels B, C and E. n≥5 cells from n=3-5 different animals.

Enhanced response to Kv7 channel modulators under conditions mimicking the ion channel remodelling that occurs in PAH. Downregulation of TASK-1 and Kv1.5 channels is a hallmark of PAH [10–15,5,16–18]. We performed additional experiments with Kv7 modulators in the presence of TASK-1 and Kv1.5 channel inhibitors, ML-365 and DPO-1, respectively to mimic the characteristic ion channel remodelling of PAH. The effects of retigabine (10^{-5}M) on K^+ currents were tested in the absence (vehicle) or in presence of ML365 (10^{-6}M) plus DPO-1 (10^{-6}M) in PASMC from control rats (Figure 4A). Unexpectedly, we observed a slight decrease in the K^+ current following the addition of the Kv7 activator, which turned into an increase in the current in the presence of ML365+DPO-1 (Figure 4A, right panel). Thus, the retigabine-sensitive current was negative under control conditions and positive in the presence of the TASK-1 and Kv1.5 channel inhibitors (Figure 4B).

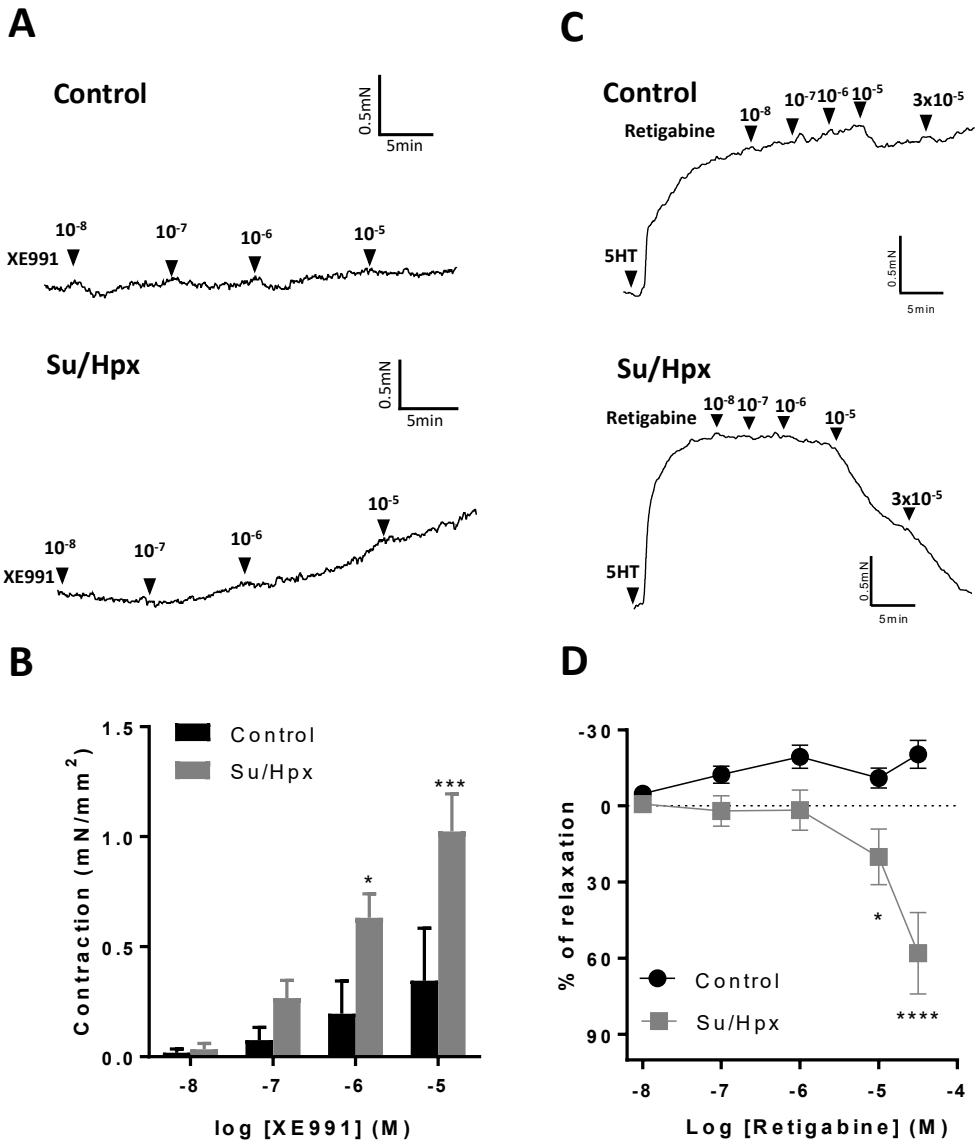


Figure 2. Enhanced response to Kv7 channel modulators in Su/Hpx compared to control PA. A) Representative recordings and (B) averaged data of the concentration-response curves to XE991 (10^{-8} -10^{-5}M) in Su/Hpx and control PA. C) Representative recordings and D) averaged data of the concentration response curves to retigabine (10^{-8} -$3x10^{-5}$M) in 5HT-precontracted PA from Su/Hpx and control rats. *P < 0.05 and ***P < 0.001 vs control, respectively. n≥5 PA from n=3 different animals.

Moreover, retigabine had no apparent effect on Em under baseline conditions (Figure 4C and D). However, in the presence of ML365+DPO-1 (Figure 4C and E), which led to a marked membrane depolarization, a clear repolarising effect was observed following the addition of retigabine. Thereafter, we analysed the vascular responses induced by XE991 and retigabine in the absence and in the presence of ML365+DPO-1. Remarkably, higher contraction to XE991 (Figure 5A and 5B) and relaxation to retigabine (Figure 5C and 5D) was observed in PA from control rats incubated with ML365+DPO-1 compared to vehicle. Therefore, the results in this *in vitro* model of ion channel remodelling resemble those observed in the PAH experimental model and strongly suggest an increased contribution of Kv7 channels when TASK-1 and Kv1.5 channels are inhibited. To further analyse the potential mechanisms responsible for the increased Kv7 activity we studied the subcellular location of Kv7.4 channel and the KCNE4 subunit in isolated PASMC in the absence and the presence of ML365+DPO-1 by immunofluorescence (Figure 5E). Kv7.4 channels staining was detected in PASMCs both in the absence or in the presence of ML365+DPO-1, showing some co-localization with the membrane marker WGA (Figure 5E), and rendering similar membrane abundance under both conditions (Figure 5G). Line scan fluorescence intensity analysis in control PASMCs revealed that the expression of Kv7.4 (red) was mainly observed between the peaks of highest intensity of the membrane marker WGA (green), suggesting that its expression was mostly cytosolic (Fig 5F-1). Such subcellular distribution was unaffected in cells treated with ML365+DPO-1 (Fig 5F-3). Immunofluorescence experiments also revealed KCNE4 expression in control PASMCs (Fig 5E), showing a mainly cytosolic distribution (Fig 5G). However, a higher location of KCNE4 in the plasma membrane was observed when cells were treated with ML365+DPO-1 (Figure 5E and 5F), leading to a net increase in the KCNE4 membrane abundance (Figure 5H).

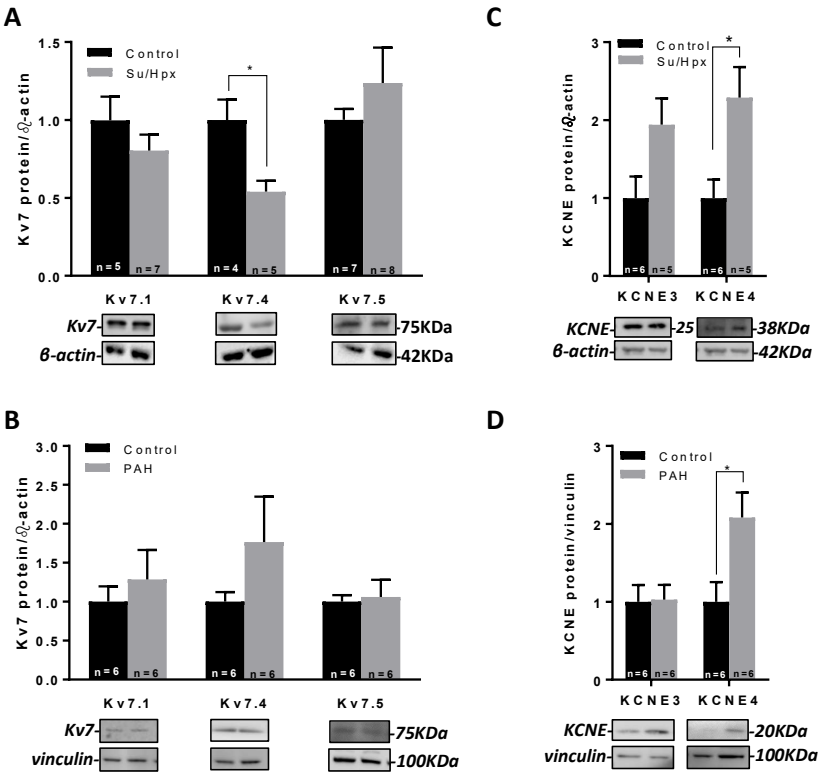


Figure 3. Altered lung expression of Kv7 channels and KCNE ancillary subunits in experimental and human PAH. Kv7 channel protein expression analysed by Western blot in lung samples from A) Su/Hpx rats and B) human PAH patients compared to controls. KCNE subunits protein expression analysed by Western blot in lung samples from C) Su/Hpx rats and D) human PAH patients compared to controls. *P < 0.05 vs control.

Kv7 channel activators produce a negligible relaxation in PA compared to MA. A surprising finding in our study was the negligible effect of XE991 and retigabine in control-PA (Figure 2). In order to confirm this unexpected result, we tested the effects of different selective Kv7enhancers in PA and, for comparison reasons, in MA. A similar pattern of attenuated pulmonary vasodilation was observed with all tested Kv7 enhancers (S1, retigabine, ML213 and ML277).

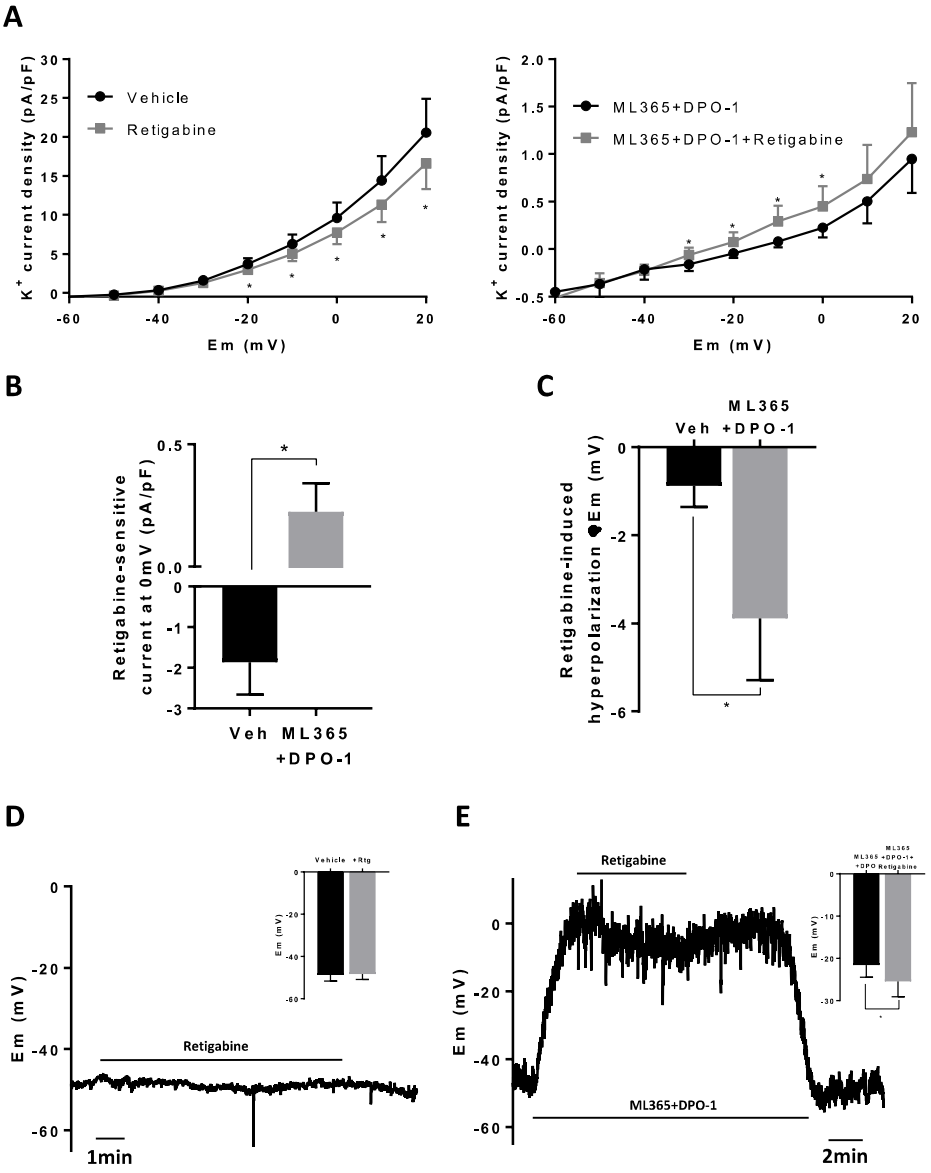


Figure 4. Retigabine increase K^+ currents and induces membrane hyperpolarization in PASMC upon inhibition of Kv1.5 and TASK-1 channels. A) K^+ current-voltage relationships measured before and after the addition of retigabine (10^{-5} M) in the absence and in the presence of ML365+DPO-1 (both at 10^{-6} M). B) Retigabine-sensitive current measured at 0 mV. C) Retigabine-induced hyperpolarization in absence and in presence of ML365+DPO-1. Representative recordings the effects of retigabine on Em in absence (D) or presence (E) of ML365+DPO-1.* P<0.05 vs vehicle. Panels A, D and E were analysed by paired t-test and B and C by unpaired t-test. n=9 cells from n=4-5 different animals.

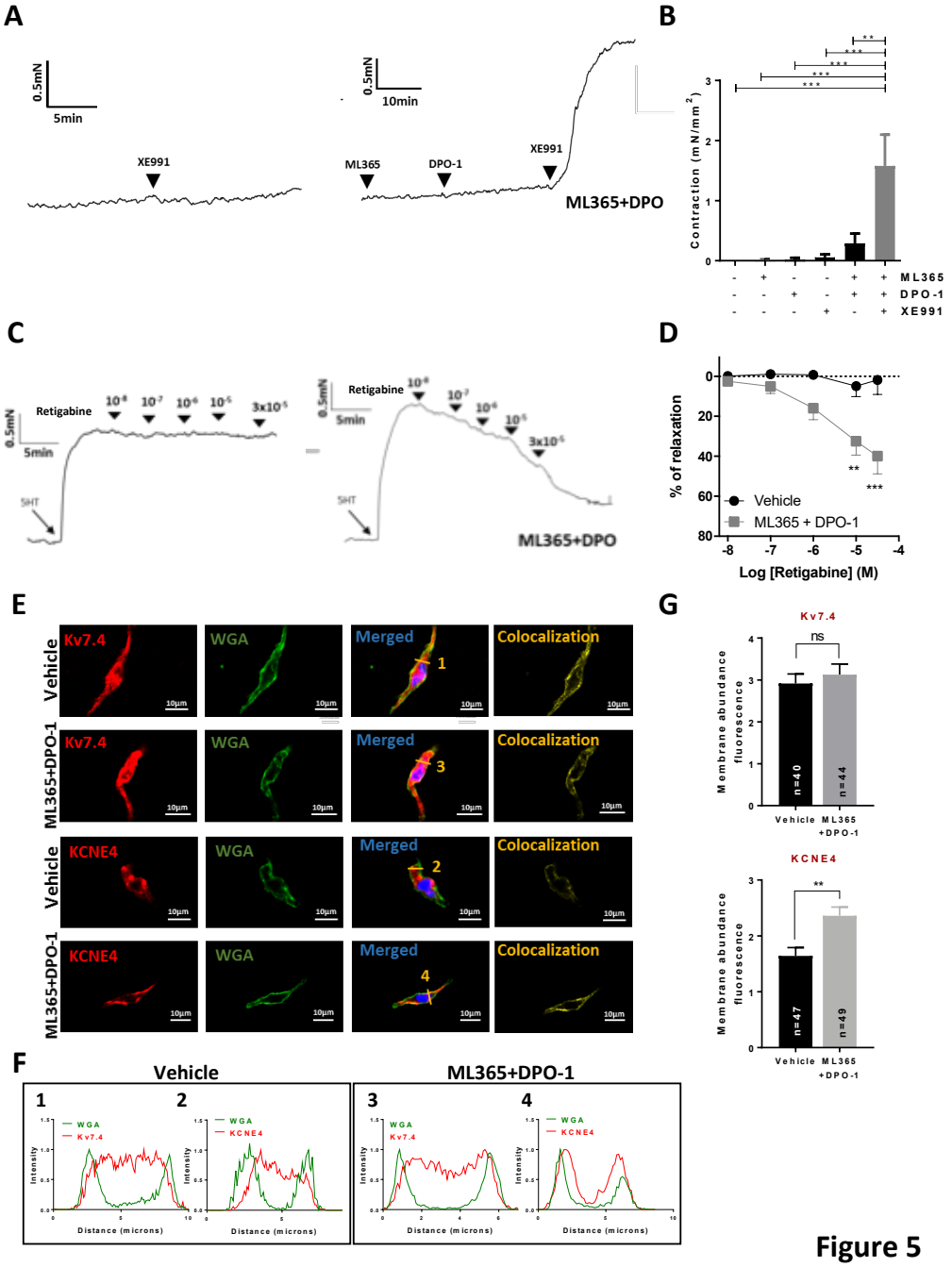


Figure 5

Figure 5. Enhanced PA reactivity to Kv7 channel modulators in the presence of Kv1.5 plus TASK-1 inhibitors. Representative recordings (A) and averaged data (B) of contractions induced by 10^{-5}M XE991 in PA in the absence and in the presence of ML365+DPO-1 (both at 10^{-6}M). Representative recordings (C) and averaged data (D) of the relaxation induced by increasing concentrations of retigabine (10^{-8} -3x10^{-5}M) in precontracted-PA in the absence (vehicle) and in the presence of ML365+DPO-1. E) Kv7.4 channel and KCNE4 subunit protein expression (red) analysed using immunocytochemistry in isolated PASMCs. Wheat Germ Agglutinin (WGA) was used as a membrane marker (green) and DAPI for nuclear staining (blue). F) Representative graph of Kv7.4 and KCNE4 channel (red) protein expression in the membrane determined by colocalization with WGA (green) in PASMC. G) Kv7.4 channel and H) KCNE4 subunit membrane abundance determined by colocalization with WGA (yellow). *P< 0.05, **P < 0.01 and ***P < 0.001 vs vehicle. n≥5 PA from 4-5 animals for functional experiments and n≥36 cell analysed from 3 different animals for immunocytochemistry.

However, all these Kv7 channels enhancers elicited a robust relaxation in MA (Figure 6A and 6B). In addition, the negligible pulmonary relaxant response of retigabine in PA was also observed under different O_2 concentrations and after applying different transmural pressures, even reaching values similar to those applied to the MA (Supplemental Figure 1).

PA display lower expression of Kv7.4 and KCNE4 subunits compared to systemic arteries. To elucidate the differences observed in the effects of Kv7 channel modulators between pulmonary and systemic vessels, we analysed the mRNA expression of the different Kv7 channels (*KCNQ1, KCNQ4* and *KCNQ5*) and KCNE ancillary subunits by RT-PCR in PA compared to MA (Figure 6C). Our data revealed that PA have much lower expression of *KCNQ4* and *KCNE4* than systemic arteries. These data could explain the difference in Kv7 channel activity between these two types of arteries. We also compared the expression of other K^+ channels in the pulmonary vasculature versus MA and found a similar expression level of *KCNA5* but a much higher expression of *KCNK3* in PA than in MA (Supplemental Figure 2).

Differential cellular location of Kv7 channels in PASMC compared to MASMC. We next assessed the possible differences in subcellular-location of Kv7 channels in isolated PASMC and MASMC by immunofluorescence. As stated above, we found that the expression of the Kv7.4 subunit was essentially cytosolic in PASMC, while in MASMC these channels were expressed at high level in the plasma membrane where they are functional (Figure 6D). Line scan analysis showed that MASMC, in contrast to PASMC, had the highest levels of Kv7.4 channel expression matching with the plasma membrane marker WGA (Figure 6E). We also observed that Kv7.5 channels and KCNE3 (Supplemental Figure 3) showed poor membrane localization in PASMC, in contrast to what has been reported in systemic vascular myocytes or heterologous systems [36,50,51]. This may also contribute to the differential functional role of Kv7 channels in PA and systemic arteries. Finally, since functional Kv7.4/Kv7.5 heterotetramers have been suggested to play an essential role in the relaxation of systemic arteries [4,33,50,52] we assessed their existence in PASMC using a proximity ligation assay (PLA). Figure 6F shows that Kv7.4/Kv7.5 heterotetramers are present in PASMC and their abundance is comparable to that found in MASMC (Figure 6F).

Discussion

Our primary finding of the present study is that pulmonary vascular Kv7 channels have a higher functional role during the development of PAH. This is associated with an upregulation of subunit KCNE4 and is observed under *in vitro* conditions that mimic the characteristic ion channel remodelling of PAH. Unexpectedly we found that, compared to systemic arteries, control PA displayed modest Kv7 current, low Kv7 channel expression and negligible response to Kv7 channel modulators. Therefore, the Kv7 channels present in the pulmonary circulation have a relatively poor role compared to those present in systemic arteries, but can become functionally highly relevant during the development of PAH.

A number of K^+ channels are be downregulated in experimental or human PAH, including Kv1.5, Kv2.1 and TASK-1 among others [53,10,12,14,5,16]. In the Su/Hpx rats, the rodent model which best recapitulates PAH, we have previously reported a reduced activity of Kv1.5 [13] and TASK-1 channels [54] in PASMC. Accordingly, herein we found a profound reduction of total K^+ channel activity and a marked depolarization in PASMC from Su/Hpx rats. In contrast, Kv7 channel activity (defined as the XE991-sensitive current) was essentially preserved in PAH rats.

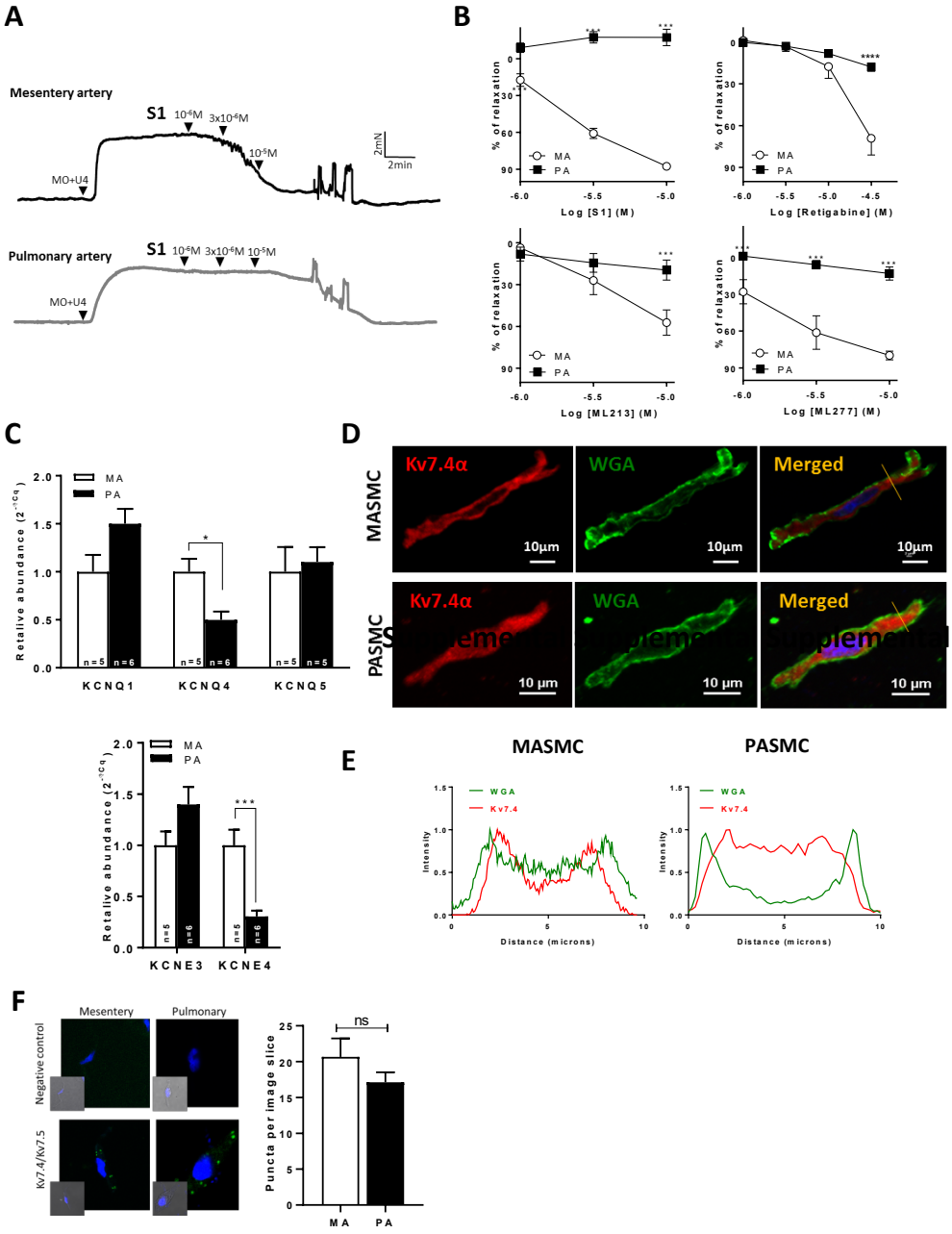
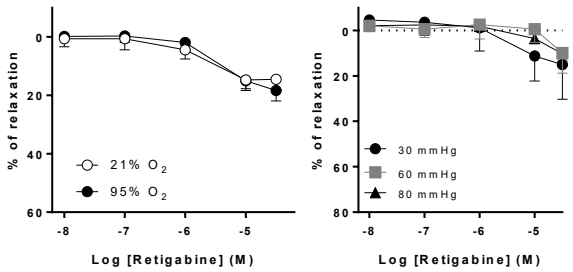


Figure 6. Differences in Kv7 channel activity and expression in PA vs MA. A) Representative recordings of the vasodilator responses induced by increasing concentrations of the Kv7 channel enhancer (S1) in PA and MA precontracted with methoxamine (10^{-5}M) plus U46619 ($3x10^{-7}$M). B) Concentration-response curves to S1, retigabine (Kv7.2-7.5-enhancer), ML277 (Kv7.1-enhancer) and ML213 (Kv7.4-enhancer) in MA and PA. n≥5 from n=5 different animals. C) *KCNQ* and *KCNE* subunits mRNA expression analysed by RT-PCR in PA compared to MA. n≥5 from 4-5 different animals. D) Kv7.4 channel protein expression (Red) analysed using immunocytochemistry in isolated PASMC compared to MASMCs. Wheat Germ Agglutinin (WGA), a membrane marker (Green) and DAPI for nuclear staining (Blue). E) Representative graph of Kv7.4 channel (Red) protein expression in the membrane determined by colocation with WGA (Green) in PASMC compared to MASMC. F) *In situ* PLA detection of Kv7.4 and Kv7.5 protein interactions in PASMC compared to MASMC. Representative fluorescence and transmitted light confocal mid-cell xy-sections probed with Kv7.4 and Kv7.5 channel primary antibody combination. Green punctae indicate target proteins are in close proximity (<40 nm). Nuclei are shown in blue as stained by DAPI. Quantification of the mean±SEM number of PLA signals per mid-cell *xy*-section for Kv7.4/7.5 combination. n=3 different animals for immunocytochemistry. Results are the mean ± SEM. **P < 0.01 and ***P < 0.001 vs MA, respectively.

Moreover XE991 inhibited the K^+ current to a greater extent in PAH, indicating that Kv7 channels make a much higher contribution to the net K^+ current in PAH than in healthy PASMC. In line with the electrophysiological data, Kv7 channel activity assessed as retigabine-induced vasodilation and XE991-induced vasoconstriction was greatly augmented in PA from Su/Hpx rats. Thus, the vascular effects of the Kv7 channel modulators turned from negligible in control-PA to very robust in Su/Hpx-PA, again indicating a greater functional contribution of Kv7 channels in the setting of PAH.

Previous studies suggest that drugs activating Kv7 channels may have beneficial effects in PH experimental models [41,42]. Thus, Morecroft *et al.,* reported that flupirtine, a Kv7 channel activator, significantly attenuated the development of PH in chronic hypoxic mice and reversed established PH in mice over-expressing the 5-HT transporter (SERT) [42].

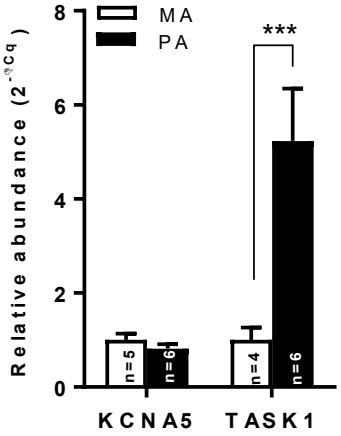
In addition, Sedivy V et *al*., found that flupirtine caused pulmonary relaxant effects in rats exposed to short-term (3-5 days) hypoxia but not in those exposed to normoxia [41]. Of note, the enhanced response to flupirtine in this hypoxic model was unrelated to an increased expression of the most relevant vascular Kv7 channels encoded by *KCNQ1, KCNQ4* and *KCNQ5*. In fact, paradoxically, a robust reduction of *KCNQ4* mRNA expression in lung homogenates from rats exposed to hypoxic environment was observed. Our data in the present study are consistent with these observations and suggest that the enhanced response to Kv7 modulators observed in PH models is not due to an augmented expression of the Kv7 α-subunits.



Supplemental Figure 1. Concentration-response curve to retigabine in U46619-precontracted PA. Responses were evaluated under different oxygen conditions (21% or 95%) (A) or after applying different transmural pressures (30, 60 or 80 mmHg) (B). Results are the mean ± SEM. n≥5 PA from n=4-5 different animals.

Kv7 channel activity is regulated by auxiliary subunits (KCNE1-5) and many arteries express KCNE3 and KCNE4 predominantly. Co-expression of KCNE4 increases Kv7.4 currents in heterologous expression systems [55–57]. Of note, Jepps *et al.,* found that KCNE4 co-localizes with Kv7.4 channels and Kv7.4/Kv7.5 heteromeric channels in freshly isolated MASMC and that knocking down this regulatory subunit reduced Kv7 currents, depolarized MASMC and markedly attenuated the vascular responses to Kv7 channel modulators [56]. Interestingly, we found an upregulation of KCNE4 in lungs from PAH rats, which could contribute to the preservation of the Kv7 current in a condition where Kv7.4 expression is reduced. Remarkably, our study shows for the first time that Kv7 subunits are also expressed in human lungs and, consistent with our data in the animal model, we found an upregulation of KCNE4 in samples from PAH patients.

As stated above, the development of PAH is associated with downregulation of Kv1.5 and TASK-1 channels [10–15,5,16–18]. To mimic these conditions we performed *in vitro* experiments in which the Kv7 channel function was assessed in the presence of Kv1.5 and TASK-1 channel inhibitors.



Supplemental Figure 2. *KCNA5* and *KCNK3* mRNA expression analysed by RT-PCR in PA compared to MA. Results are the mean ± SEM. n≥5 PA or MA from n=4-5 different animals. ***P < 0.001 vs MA.

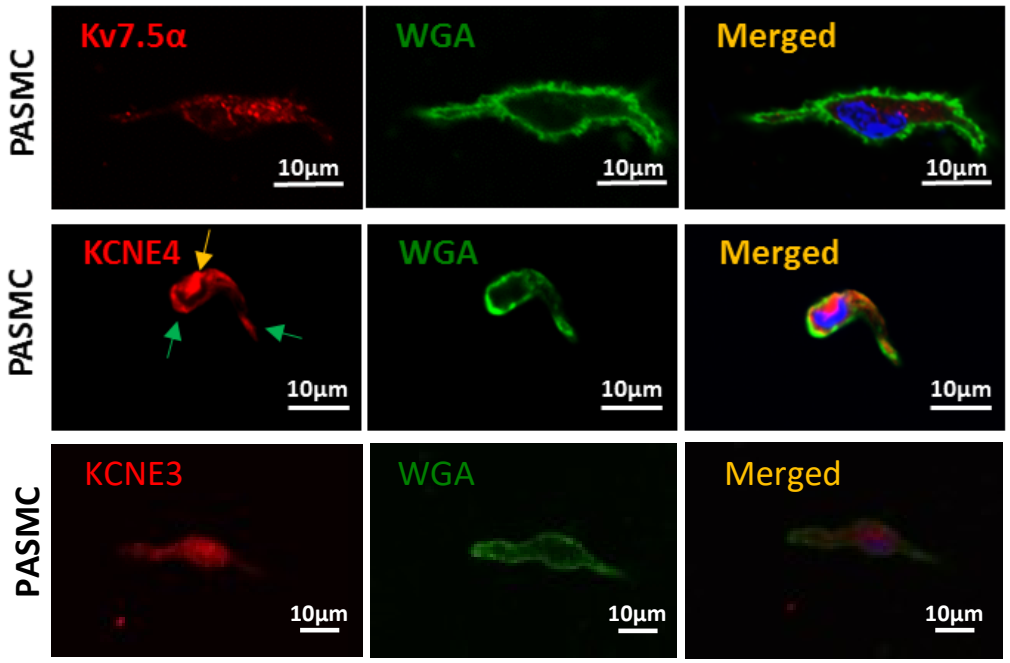
Following acute exposure to these inhibitors the ability of retigabine to increase K^+ current amplitude and hyperpolarise the membrane potential of PASMC became evident. In addition, this was associated with a profound enhancement of retigabine-induced PA vasodilation and XE991-induced pulmonary vasoconstriction. Altogether these data suggest that Kv7 channel activity is unmasked under conditions mimicking the ion channel remodelling characteristic of PH. The simplest explanation for this would be that: 1) this ion channel remodelling would bring the membrane potential up to the voltage threshold for activation of Kv7 channels and/or 2) the redundant nature of K^+ channels would result in the Kv7 channels assuming a major role when other members (i.e. Kv1.5 and TASK-1) are not active. Alternatively, other potential mechanisms might contribute. Since the enhanced Kv7 channel activity occurred after an acute exposure to the K^+ channel inhibitors it is very unlikely that the underlying mechanism involve changes in protein expression. We therefore studied if the acute inhibition of Kv1.5 and TASK-1 channels could regulate the trafficking of the channels. No apparent changes in location or membrane abundance of Kv7.4 were observed, while KCNE4 trafficked from the cytosol to the plasma membrane, resulting in higher membrane abundance. Thus, the higher confinement of this enhancer subunit in the membrane would lead to a greater Kv7 channel activity compensating for the loss of Kv1.5 and TASK-1 channel function in PASMC. Our findings indicate that the activation of Kv7 channels under conditions mimicking the ion channel remodelling occurring in PAH would be crucial to control membrane potential and limit an exaggerated depolarization in PASMC. Moreover, the upregulation of KCNE4 found in our *in vivo* and *in vitro* models of PAH, as well as in patient samples, is very likely to contribute to the enhanced Kv7 channel activity during the onset of the disease.

Currently, Kv7 channels are considered key regulators of membrane potential and vascular tone in many vessels including coronary [36,38], mesenteric [50,56], renal [28,29,50] and cerebral arteries [26] derived from several species. Accordingly, MA were efficiently relaxed by a range of Kv7 channel openers in the present study. Surprisingly, we found that under control conditions PA were essentially insensitive to Kv7 channel modulators. This finding, confirmed in two independent labs, was consistent with differential expression profile of Kv7 channels in MA and PA. Thus, in comparison with MA, PA had similar expression of *KCNQ1*, *KCNQ5* and *KCNE3* but markedly reduced expression of *KCNQ4* and *KCNE4*, which are considered major contributors to the Kv7 current in systemic vessels [56,26]. Additionally, Kv7.4 and KCNE4 were abundantly expressed at the membrane level (where the channel is functional) in MASMC [56], while in PASMC Kv7.4 channels expression was essentially cytosolic and KCNE4 appeared to be located in cytoplasmic

accumulations around the nucleus (probably close to the endoplasmic reticulum) and in plaques near the membrane. By contrast, Kv7.4/7.5 heterotetramers, which have been postulated as a predominant functional channel subtype in vascular smooth muscle cells [11,26,33,52] were similarly detected in MASMC and PASMC.

A few studies have previously assessed the role of Kv7 channels in the pulmonary circulation using rat [30,40,58] or mice models [42,49]. Joshi et al., first reported that Kv7 channels blockers induced a marked vasoconstriction in rat PA but showed negligible effects in MA [40]. Thereafter, these authors reported a substantial pulmonary vasodilator effect by Kv7 channel openers and suggested a greater functional role of Kv7 channels in the pulmonary as compared to the systemic circulation. This was associated with higher *KCNQ4* expression in PA than in MA.

In an attempt to explain these discrepancies between ours and previous studies, we examined the pulmonary vasodilator responses to retigabine under different experimental conditions. The negligible response to retigabine was found to be independent of the vasoconstrictor stimulus, the transmural pressure (even when applying tension values similar to those used for MA) or oxygen levels. Alternatively, it is likely that differences in rat strains (Sprague-Dawley in the previous studies versus Wistar in this work) may account for the variable responses to the Kv7 channel modulators observed in the pulmonary arteries. In fact a lack of pulmonary vasodilation by the Kv7 channel opener flupirtine in control Wistar rats has been reported [41].



Supplemental Figure 3. Kv7.5 and KCNE3 (Red) subunit protein expression analysed using immunocytochemistry, Wheat Germ Agglutinin (WGA), a membrane marker (Green) and DAPI for nuclear staining (Blue) in isolated PASMCs.

In addition these authors reported that the Kv7 channel blocker linopirdine did not increase pulmonary arterial pressure in this rat strain unless lungs were previously primed with a stimulus to increase vasoreactivity (i.e pre-exposition to angiotensin II-hypoxia cycles). Likewise linopirdine or XE991 were unable to contract Wistar rat PA unless previously primed with U46619 [58] or K^+ channels inhibitors (herein). In summary, it is therefore likely that Wistar rats display reduced Kv7 channel function in the pulmonary circulation compared to Sprague-Dawley rats, which could contribute to their higher susceptibility to develop pulmonary hypertension [59–61].

Perspectives

Our study shows that Kv7 channels acquire a more relevant role controlling pulmonary vascular tone in the onset of pulmonary hypertension. As a result, targeting Kv7 channel may represent an attractive strategy in the treatment of PAH. In agreement with this idea preliminary studies in animal models suggest that Kv7 channel openers are able to prevent and even reverse pulmonary hypertension [41,42]. While two of the commercially available pan-Kv7 channel openers (i.e. retigabine and flupirtine) have been withdrawn from the market in recent years a number of new molecules with higher selectivity for particular Kv7 channel subunits are under development [62,63]. Remarkably, activation of Kv7 channels has been demonstrated to contribute to the vasodilation induced by drugs increasing cGMP [30] and cAMP levels [53] which represent current strategies for the treatment of PAH. It is therefore likely that the activation of Kv7 channels may contribute to the therapeutic effect of drugs increasing cGMP and cAMP in pulmonary hypertension patients. Our present study supports this notion and provides molecular insight into the mechanisms underlying the potential usefulness of therapies targeting Kv7 channels.

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Conflict of interest

The authors declare no conflicts of interest.

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Chapter III: miR-1 is increased in pulmonary hypertension and downregulates Kv1.5 channels in rat pulmonary arteries.

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miR-1 is increased in pulmonary hypertension and downregulates Kv1.5 channels in rat pulmonary arteries

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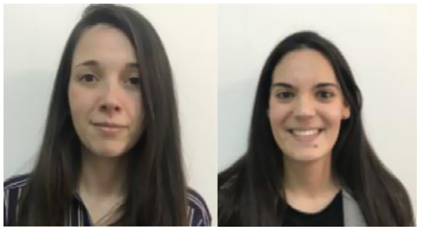
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Key points

- The expression of miR-1 is increased in lungs from the Hyp/Su5416 PAH rat model.
- Pulmonary artery smooth muscle cells from this animal model are more depolarized and show decreased expression and activity of voltage-dependent potassium channel (Kv)1.5.
- miR-1 directly targets Kv1.5 channels, reduces Kv1.5 activity and induces membrane depolarization.
- Antagomir-1 prevents Kv1.5 channel downregulation and the depolarization induced by hypoxia/Su5416 exposition.

Abstract Impairment of the voltage-dependent potassium channel (Kv) plays a central role in the development of cardiovascular diseases, including pulmonary arterial hypertension (PAH). MicroRNAs are non-coding RNAs that regulate gene expression by binding to the 3′-untranslated region region of specific mRNAs. The present study aimed to analyse the effects of miR-1 on Kv channel function in pulmonary arteries (PA). Kv channel activity was studied in PA from healthy animals transfected with miR-1 or scrambled-miR. Kv currents were studied using the whole-cell configuration of the patch clamp technique. The characterization of the Kv1.5 currents was performed with the selective inhibitor DPO-1. miR-1 expression was increased and Kv1.5 channels were decreased in lungs from a rat model of PAH induced by hypoxia and Su5416. miR-1 transfection increased cell capacitance, reduced Kv1.5 currents and induced membrane depolarization in isolated pulmonary artery smooth muscle cells. A luciferase reporter assay indicated that KCNA5, which encodes Kv1.5 channels, is a direct target gene of miR-1. Incubation of PA with Su5416 and hypoxia (3% O_2) increased miR-1 and induced a decline in Kv1.5 currents,

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which was prevented by antagomiR-1. In conclusion, these data indicate that miR-1 induces pulmonary artery smooth muscle cell hypertrophy and reduces the activity and expression of Kv channels, suggesting a pathophysiological role in PAH.

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Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene function by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs. The miRNA–3′UTR interaction leads to cleavage of target mRNA or to translational repression, resulting in a decrease in the targeted protein. Mature miRNAs play a key role in global cellular processes, such as differentiation, proliferation, apoptosis and organogenesis, as a result of their capacity to target multiple mRNAs (Bartel, 2004). Dysregulation of miRNAs has been involved in a number of clinically important diseases, ranging from myocardial infarction to cancers, highlighting their potential usefulness as diagnostic and prognostic tools (Sayed & Abdellatif, 2011; Meloche *et al.* 2014). Moreover, modulating the levels of under- and overexpressed miRNAs has recently emerged as a promising therapeutic strategy. miRNA-based therapeutic strategies (van Rooij & Kauppinen, 2014) can be formulated by either antagonizing (with antagomirs) or restoring (with mimetics) the functions of miRNAs. Mimetics are synthetic RNA duplexes designed to mimic the endogenous functions of the miRNA of interest, with modifications for stability and cellular uptake, whereas antagomirs are modified anti-sense oligonucleotides harbouring the full or partial complementary reverse sequence of a mature miRNA.

Pulmonary hypertension (PH) is defined as a mean pulmonary arterial pressure of at least 25 mmHg at rest. Class I PH (i.e pulmonary arterial arterial hypertension; PAH) originates from alterations in the small precapillary pulmonary arteries (PA) (Galie *et al.* 2016). It is a disorder with significant burden in terms of both severity and prevalence, affecting young and middle aged individuals, preferentially women (Galie *et al.* 2016). The disease progresses with increasing pulmonary vascular resistance, right heart failure and ultimately death. It is characterized by sustained pulmonary vasoconstriction, thrombosis *in situ* and excessive pulmonary vascular remodelling, involving an increase in medial thickness as a result of hypertrophy and proliferation of pulmonary artery smooth muscle cells (PASMCs) (Meyrick & Reid, 1980; Tajsic & Morrell, 2011). These factors contribute to a reduced lumen diameter and increased peripheral resistance.

Ionic remodelling, comprising the downregulation of voltage-gated K^+ channels (Kv) channels (notably the α subunit Kv1.5), is also considered to be an early contributor to the pathophysiology of the disease (Yuan *et al.* 1998; Wang *et al.* 2005; Barnes *et al.* 2017) via a more depolarized membrane potential in PASMCs, leading to increased intracellular calcium, vasoconstriction, hypertrophy and proliferation (Shimoda *et al.* 2001; Cogolludo *et al.* 2007; Burg *et al.* 2008).

We hypothesized that specific miRNAs are involved in key pathophysiological features of pulmonary vascular diseases, such as ionic remodelling. miR-1 has been previously reported as a biomarker of PH (Sarrion *et al.* 2015; Sysol *et al.* 2018). Therefore, the present study aimed to analyse the effects of miR-1 on pulmonary Kv channel currents.

Methods

Ethical approval

All experimental procedures utilizing animals were carried out in accordance with the Care and Use of Laboratory Animals and were approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-251/15). The present study conforms with the principles and regulations described by Grundy (2015). All of the investigators understand the ethical principles under which the journal operates and state that their work complies with the journal's animal ethics checklist.

Animals and models of PAH

Pathogen-free male Wistar rats were obtained from Envigo (Barcelona, Spain). All animals were kept with free access to standard rat chow and water in an enriched environment throughout the whole experiment period and maintained under a 12:12 h light/dark cycle at 24°C. Rats were killed using CO_2.

For the rat model of Su5416 plus hypoxia-induced PAH (Hyp/Su5416), rats weighing 220 g were injected S.C. with a single dose of the vascular endothelial

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growth factor (VEGF) receptor type 2 inhibitor Su5416 (20 mg kg^{-1}) or vehicle (Taraseviciene-Stewart *et al.* 2001; Gomez-Arroyo *et al.* 2012). Then, Su5416-treated animals were introduced into glass cages and ventilated with 10% O_2 (hypoxia) for 3 weeks. CO_2 and water vapour produced by the animals were captured with soda lime and silica gel, respectively. Control rats (normoxia) were kept in the same room. Oxygen was monitored using an oxygen sensor (DrDAQ Oxygen Sensor; Pico Technology, St Neots, UK) in the room and in the chamber outflow. The chambers were opened for 20–30 min daily for regular animal care.

Haemodynamic measurements

At the end of the experimental protocol, rats were anaesthetized I.P. with 80 mg kg^{-1} ketamine plus 8 mg kg^{-1} xylazine and ventilated with room air (tidal volume 9 mL kg^{-1}, 60 breaths min^{-1}, positive end-expiratory pressure 2 cmH_2O). Right ventricular systolic pressure and diastolic pressure, as well as systolic, diastolic and mean pulmonary arterial pressures, were then measured in open-chest rats with a pressure transducer via a catheter advanced through the right ventricle into the PA (Morales-Cano *et al.* 2014). After haemodynamic measurements, rats were killed by exsanguination under anaesthesia ketamine/xylazine (80 mg/8 mg kg^{-1}, I.P.).

In silico analysis for miR target prediction

Target gene prediction analysis was completed using the microRNA.org target prediction resource, utilizing miRanda sites and miRSVR scoring. The target sites predicted using miRanda are scored for likelihood of mRNA downregulation using a regression model trained on sequence and contextual features of the predicted miRNA:mRNA duplex.

Incubation and transfection of PA

Rat PA were isolated from the lungs and cut into rings. In preliminary experiments, we assessed the transfection efficiency of this protocol using a fluorescent miRNA (miRIDIAN mimic transfection control-Dy547, CP-004500-01-05; Dharmacon, Lafayette, CO, USA). To increase transfection efficiency into the smooth muscle cells, PAs were first partially digested with a Ca^{2+}-free solution containing (in mg mL^{-1}): 1.125 collagenase, 0.1 elastase and 1 albumin for 4 min at 4°C followed by 1 min at 37°C. To introduce miR-1 (hsa-miR-1-3p mirVanaTM, miRNA mimic, MC10617; Applied Biosystems, Foster City, CA, USA), scrambled miR (miRNA mimic negative control, 4464058) and the antagomiR-1 (hsa-miR-1-3p mirVanaTM miRNA inhibitor, MH10617; Applied Biosystems) into isolated PA, a combination of reverse permeabilization (Moreno *et al.* 2014) and Lipofectamine RNAiMAX (Life Technologies, Grand Island, NY, USA) was used. miRNA duplexes with a final concentration of 100 nM and Lipofectamine RNAiMAX were mixed with Opti-MEM (Life Technologies). Briefly, PA were exposed to three successive solutions (4°C) containing (in mM): (i) miRNA duplexes, 10 EGTA, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$ and 20 Hepes (pH 6.8; 30 min); (ii) miRNA duplexes, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$ and 20 Hepes (pH 6.8; 180 min); and (iii) miRNA duplexes, 120 KCl, 5 Na_2ATP, 10 $MgCl_2$ and 20 Hepes (pH 6.8; 30 min). Subsequently, PA were bathed in a fourth solution containing (in mM) 120 NaCl, 5 KCl, 5 Na_2ATP, 10 $MgCl_2$, 5.6 glucose and 10 Hepes (pH 7.1, 4°C), in which $[Ca^{2+}]$ was gradually increased from 0.001 to 0.01, 0.1 and 1 mM every 15 min. Vessels were then placed in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (1 g L^{-1}), non-essential amino acid solution (1×), penicillin (100 U mL^{-1}), streptomycin (0.1 mg mL^{-1}), amphotericin B (0.25 μg mL^{-1}) and maintained for 48 h in a normoxic incubator (21% O_2 and 5% CO_2) or hypoxic incubator (3% O_2 and 5% CO_2 in the presence of 10 μmol L^{-1} Su5416). Under these conditions, we achieved a higher efficiency of transfection as determined in pilot experiments with the fluorescent miRNA. It should be noted that Kv currents are downregulated in cells isolated from PA incubated for 48 h in culture by ∼50% compared to the freshly isolated cells, whereas the permeabilization /transfection produced no effect on these current (not shown). In some experiments, miR-1 was measured as described below in PA placed in DMEM supplemented with glucose (4.5 g L^{-1}), non-essential amino acid solution (1×), penicillin (100 U mL^{-1}), streptomycin (0.1 mg mL^{-1}), amphotericin B (0.25 μg mL^{-1}) and maintained for 48 h in a normoxic incubator (21% O_2 and 5% CO_2) or hypoxic incubator (3% O_2 and 5% CO_2 in the presence of 10 μmol L^{-1} Su5416).

Electrophysiology

PASMCs were isolated by enzymatic digestion as described previously (Cogolludo *et al.* 2006). Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA, USA) using the whole-cell configuration of the patch clamp technique. Cells were superfused with an external Ca^{2+}-free Hepes solution (see above) and a Ca^{2+}-free pipette (internal) solution containing (mol L^{-1}): 130 KCl, 1.2 $MgCl_2$, 5 Na_2ATP, 10 Hepes, 10 EGTA (pH adjusted to 7.3 with KOH). Kv currents were evoked after the application of 200 ms depolarizing pulses from −60 mV to +60 mV in 10 mV increments. To characterize the contribution of Kv1.5 channels to the total Kv current, cells were

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exposed to the selective inhibitor DPO-1 (1 μmol L^{-1}) (Lagrutta *et al.* 2006; Du *et al.* 2010). Cell capacitance was calculated from the integral of the capacitive transient current elicited by 10 mV hyperpolarizing pulses from a holding potential of −70 mV. Currents were normalized for cell capacitance and expressed as pA pF^{-1}. Membrane potential was recorded under the current clamp mode. All experiments were performed at room temperature (22–24°C).

miRNA extraction and quantitative RT-PCR

miRNA was extracted from lung tissue using miRNeasy Mini Kit (Qiagen, Hilden, Germany) and a NucleoSpin miRNA-RNA purification kit (Macherey-Nagel GmbH & Co. KG, Germany) was used to isolate miRNA from PA, in accordance with the manufacturer's instructions. RNA concentration and quality was checked using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). For miRNA analysis, complementary DNA analysis was synthesized from total RNA using specific stem-loop reverse-transcription primers (Taqman MicroRNA Reverse Transcription Kit; Applied Biosystems). Gene expression was determined by quantitative RT-PCR with a Taqman system (Applied Biosystems) in the Genomic Unit of the Complutense University (Madrid) using specific primers (U6 Ref. 001973 and miR-1 Ref. 002222). The $\Delta\Delta C_t$ method was used to quantify relative changes. miRNA expression was normalized by the expression of U6.

Luciferase gene expression reporter assay

Luciferase activity assays were performed in the African green monkey kidney-derived cell line (COS-7) transfected with the 3′-UTR region of *Kcna5* (NM_002234) cloned into pMirTarget vector (OriGene Technologies, Rockville, MD, USA) containing the sequence of a red fluorescent protein (RFP) and a firefly luciferase as a reporter. COS-7 cells placed into a 96-well plate were cotransfected with cloned vector (50 μg) and miR-1 or scrambled miR (both at 10 nM), using Lipofectamine 2000 transfection reagent (Invitrogen, Thermo Fisher Scientific), in accordance with the manufacturer's instructions. Forty-eight hours after transfection, luciferase activity was measured using the Firefly Luciferase Assay Kit 2.0 (Biotium, Fremont, CA, USA), in a luminometer (Fluoroskan Ascent, Thermo Scientific) for 5 min. Luminescence was normalized to the red fluorescence of RFP.

Western blotting analysis

Homogenates were subjected to SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, incubated with primary rabbit polyclonal antibodies against Kv1.5 (dilution 1:200; APC-004; Alomone Labs, Jerusalem, Israel) overnight and then with the secondary peroxidase conjugated antibodies. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Blots were imaged using an Odissey Fc System (Li-Cor, Lincoln, NE, USA) and were quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA, USA). Samples were re-probed for expression of smooth muscle α-actin (dilution 1:10,000; A2547; Sigma-Aldrich, Madrid, Spain).

Immunocytochemistry

Enzymatically isolated PASMCs from PAs were fixed in polylysine coated glass coverslips, blocked and permeabilized as described previously (Morales-Cano *et al.* 2016). The coverslips were exposed to primary rabbit anti-Kv1.5 (dilution 1:100; APC-004; Alomone Labs) antibodies overnight and exposed afterwards to secondary antibody Cy3 conjugated donkey anti-rabbit (dilution 1:200; Jackson ImmunoResearch, Ely, UK) and stained with 4′,6-diamidino-2-phenylindole (DAPI). Image analysis was performed using a Confocal laser scanning fluorescence microscope (SP2; Leica Microsystems, Wetzlar, Germany) with oil immersion lens ACS APO 40× (Unidad de Microscopía Confocal Hospital General Universitario Gregorio Marañón, Madrid, Spain).

Drugs

All drugs were obtained from Sigma-Aldrich, except Su5416 (Tocris Bioscience, Bristol, UK).

Statistical analysis

Data are expressed as the mean ± SEM. Statistical comparisons were performed using one- or two-tailed unpaired *t* tests or two-way ANOVA followed by a Bonferroni test as appropriate. $P < 0.05$ was considered statistically significant.

Results

PASMCs from the Hyp/Su5416 model have increased cell capacitance and depolarized membrane potential

We used an established model of PAH triggered by exposition to Su5416 plus hypoxia (10% O_2, Hyp/Su5416). As expected, haemodynamic assessment revealed a robust elevation of mean pulmonary arterial pressure in Hyp/Su5416 rats (Fig. 1*A*). In addition, PASMCs from Hyp/Su5416 rats had a significantly increased cell capacitance (Fig. 1*B*), as an index of PASMC hypertrophy.

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Moreover PASMCs from these PAH animals had a more depolarized membrane potential (Fig. 1*C*).

Ionic remodelling in an animal model of PAH. To investigate the possible ionic remodelling, we analysed the total Kv currents in freshly isolated PASMCs from control or Hyp/Su5416 rats. Figure 2*A* shows representative original traces of the total Kv currents recorded in freshly isolated PA myocytes under both conditions. Kv currents measured at the end of the 200 ms depolarizing pulses were significantly decreased in PASMCs from Hyp/Su5416 compared to those from control rats (Fig. 2*B*). We analysed whether the Kv1.5 current, which represents a main component of the total Kv current in the pulmonary vasculature (Moral-Sanz *et al.* 2011), was affected in Hyp/Su5416 rats. We found that the selective Kv1.5 channel inhibitor DPO-1 (1 μmol L^{-1}) induced a marked reduction of the Kv currents in control and Hyp/Su5416 rat PASMC (Fig. 2*C*). Thus, DPO-1 sensitive currents, which reflect the Kv1.5 channel component, were markedly smaller in Hyp/Su5416 rats (Fig. 2*D*). In agreement with these data, the expression of Kv1.5 channels was reduced in lung homogenates from Hyp/Su5416 rats compared to those exposed to normoxia (Fig. 2*E*).

Expression of Kv1.5 channels is downregulated by miR-1. We hypothesized that the expression of Kv1.5 could be regulated by miRNAS. Thus, we performed an *in silico* analysis using the microRNA.org database and identified that *KCNA5*, which encodes the Kv1.5 channel protein, is a predicted mRNA target of miR-1 (Fig. 3*A*). Remarkably, lungs from Hyp/Su5416 rats showed a marked upregulation of miR-1 (∼4-fold increase) (Fig. 3*B*). To confirm that miR-1 binds to the 3′-UTR of the *KCNA5* mRNA, we constructed a vector containing the 3′-UTR of *KCNA5* mRNA and the firefly luciferase. COS-7 cells were cotransfected with this vector and miR-1 or a scrambled miR. The luciferase activity of COS-7 cells was significantly reduced after 48 h of co-transfection with miR-1 (Fig. 3*C*), confirming that *KCNA5* is a direct target of miR-1. To assess the potential pathophysiological impact of the regulation of *KCNA5* by miR-1 levels, we transfected PA with miR-1 or a scramble sequence. Transfection of miR-1 in PAs produced a decrease in the immunostaining of Kv1.5 channels (Fig. 3*D*).

miR-1 reduces Kv1.5 currents and induces membrane depolarization in PASMCs. Interestingly, the transfection of miR-1 in PAs mimicked the electrophysiological findings observed in the PAH model. Thus, after miR-1 transfection, PASMC showed an increase in cell capacitance (Fig. 4*A*), strongly reduced Kv currents (Fig. 4*B* and 4*C*) and a more depolarized membrane potential (Fig. 4*D*) compared to scrambled miR transfected cells. Moreover, we also found that the selective Kv1.5 channel inhibitor DPO-1 (1 μmol L^{-1}) induced a smaller reduction of the Kv currents in PASMCs transfected with miR-1 compared to the scrambled miR (Fig. 5*A*). Therefore, in the presence of DPO-1, the current was similar in cells transfected with the control or with the active miR. Figure 5*B* shows that DPO-1 sensitive current (i.e. the Kv1.5 channel component) was smaller in miR-1 transfected cells. These data indicate a lower contribution of Kv1.5 channels to whole Kv currents in the presence of miR-1. DPO-1 induced a strong depolarization in both scrambled miR and miR-1 treated cells so that the differences in membrane potential were abolished after Kv1.5 inhibition (Fig. 5C).

AntagomiR-1 prevents hypoxia-induced ionic remodelling. Finally, we evaluated the role of

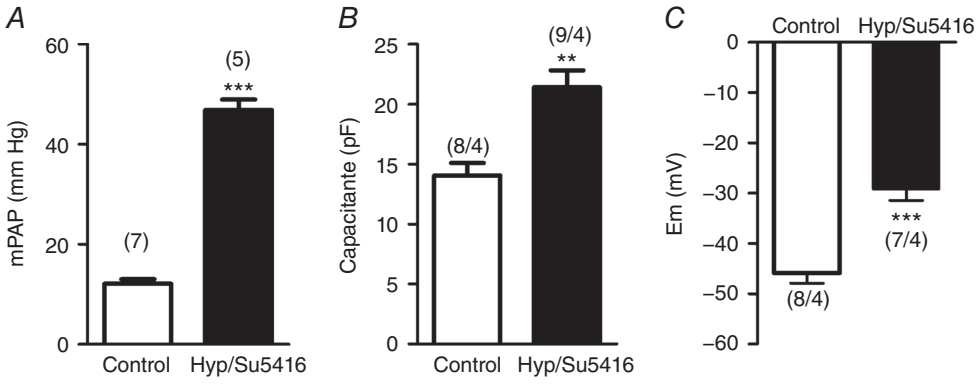


Figure 1. Hypertrophy and membrane depolarization in PASMCs from the Hyp/Su5416 model
A, mean pulmonary artery pressure (mPAP). *B*, average cell capacitance of PASMCs from normoxic and Hyp/Su5416 rats. *C*, resting membrane potential in PASMC measured under current clamp mode. Results are the mean ± SEM. The number of animals in each group is indicated in parenthesis. $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs.* normoxia, respectively.

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antagomiR-1 in PA exposed to normoxia and hypoxia (3% O_2) plus Su5416 (10 μmol L^{-1}) for 48 h *in vitro*. We also used scrambled miR as a negative control for antagomir-1. Under normoxic conditions, antagomiR-1 transfection did not produce changes in cell capacitance (scrambled: 16.44 ± 0.77 pF and antagomiR-1: 16.80 ± 1.01 pF, $n = 7$ and 9, respectively; $P > 0.05$), in total Kv currents or in membrane potential in PASMCs compared to scrambled miR (Fig. 6*A*). We analysed the expression of miR-1 in PA from rats incubated 48 h in Hyp/Su5416 respect to normoxic conditions. The results showed a significant increase of miR-1 in PA incubated with Hyp/Su5416 (Fig. 6*B*). PASMCs isolated from PA transfected with the inactive miR and exposed to Hyp/Su5416 showed an increase in cell capacitance (23.11 ± 2.21 pF, $n = 7$; $P < 0.05$) and a marked reduction in total Kv currents compared to normoxia (Fig. 6*C vs.* Fig. 6*A*). AntagomiR-1 transfection prevented the increase in cell capacitance (16.31 ± 2.17 pF, $n = 6$; $P > 0.05$), produced a significant increase in Kv currents and hyperpolarized

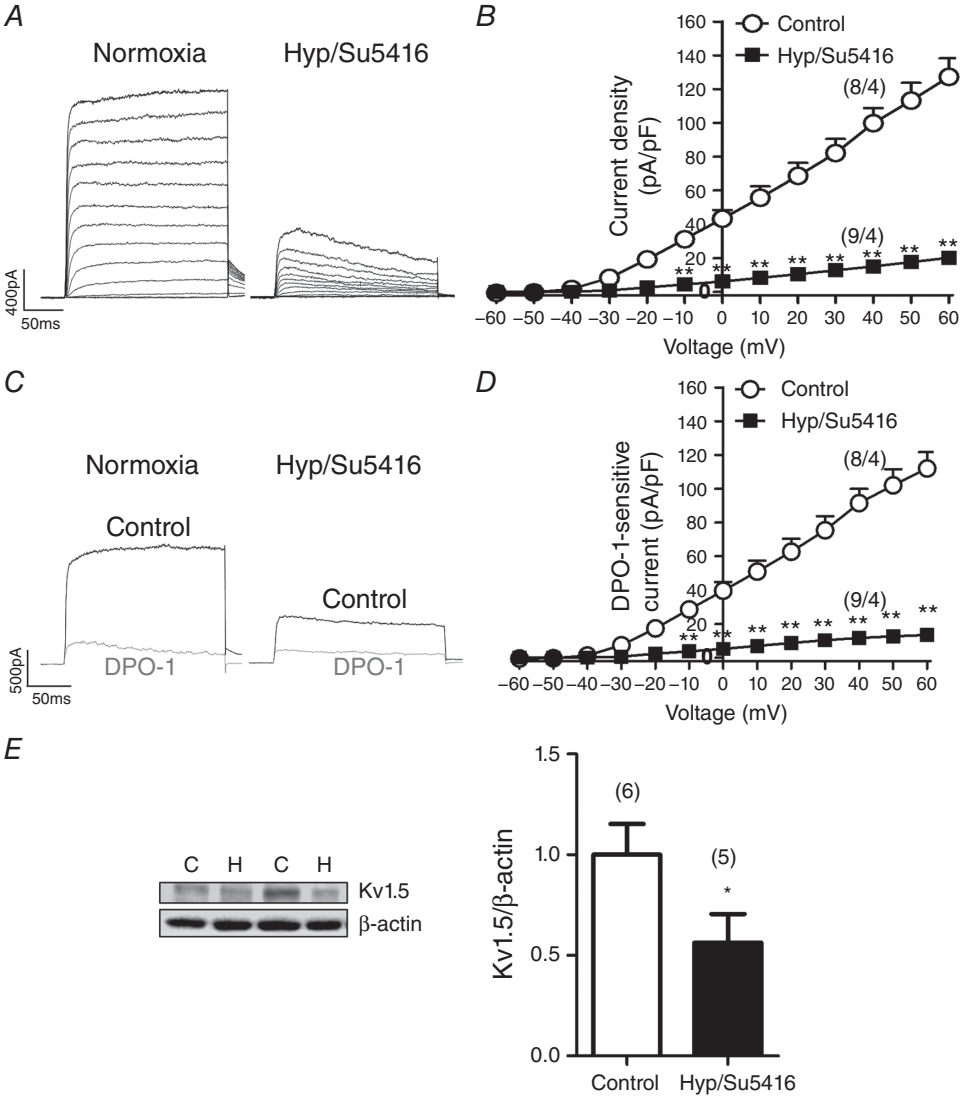


Figure 2. Ionic remodelling in Hyp/Su5416 rats
A, representative current traces for 200 ms depolarization pulses from –60 mV to +60 mV in 10 mV increments from a holding potential of -60 mV in PASMC. *B*, average current–voltage relationships of K^+ currents, normalized by cell capacitance, measured at the end of the pulse from experiments as those shown in (*A*). *C*, representative current traces measured before (black) and after (grey) the addition of the Kv1.5 channel blocker DPO-1 (1 μmol L^{-1}). *D*, mean DPO-1-sensitive currents obtained by subtracting the current in the absence and in the presence of DPO-1. *E*, Kv1.5 protein expression in lung homogenates from rats exposed to normoxia (N) or Hyp/Su5416 (H) analysed by western blotting and normalized by β-actin expression. The parentheses indicate the number of cells or arteries and the number of animals from which these cells or arteries were obtained, respectively. Results are the mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ *vs*. normoxia, respectively.

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PASMCs isolated from PA exposed to Hyp/Su5416 (Fig. 6*C*). DPO-1 induced a stronger blockade in PASMC from PA transfected with antagomiR-1 compared to scrambled miR under hypoxia plus Su5416. Therefore, DPO-1-sensitive currents were increased by antagomiR-1 (Fig. 6*D*), suggesting that antagomiR-1 partially prevents the hypoxia plus Su5416-induced decline in Kv1.5 currents. This was associated with a significantly higher Kv1.5 channel expression in Hyp/Su5416-exposed PA transfected with antagomiR-1 compared to those transfected with scrambled miR (Fig. 6*E*).

Discussion

In the present study, we show that miR-1 is upregulated and Kv1.5 channels are downregulated in lungs from rats with PAH induced by Su5416 plus hypoxia. Transfection with miR-1 of PA induced a strong decline in whole Kv currents, which was accompanied by membrane depolarization. We confirmed *KCNA5* mRNA, which encodes Kv1.5, as a direct target of miR-1. Finally, antagomiR-1 prevented the downregulation of Kv currents and Kv1.5 channel expression, as well as the depolarization induced by Hyp/Su5416 in PA.

Among the miRNAs found in the literature that were increased in PAH but had not been previously functionally characterized with respect to PA function, miR-1 was the one with the highest upregulation (8-fold increase in microarray experiments and 12-fold increase in the validating PCR analysis) in plasma from 12 idiopathic PAH patients (Sarrion *et al.* 2015). miR-1 is also upregulated in peripheral blood mononuclear cells in essential hypertensive patients (Kontaraki *et al.* 2014) and in pre-eclampsia (Hromadnikova *et al.* 2015). By contrast, the expression of miR-1 showed a strong downregulation in the buffy coat of a cohort of 31 subjects with pulmonary hypertension, including all classes of pulmonary hypertension, such as chronic obstructive pulmonary disease, interstitial lung disease, obstructive lung disease, scleroderma, etc. (Wei *et al.* 2013). Very recently, it was also reported that miR-1 was downregulated in cultured PASMCs from four patients with idiopathic PAH (Sysol *et al.* 2018). To further understand these conflicting results and because the expression of miR-1 has not been analysed in lungs with PAH, we analysed miR-1 levels in a widely used animal model of PAH, consisting of a combination of hypoxia plus the VEGF antagonist Su5416. The Hyp/Su5416 model, as opposed to hypoxia alone, is considered to best conform to class 1 pulmonary hypertension (i.e. PAH) because it develops complex vascular lesions, including plexiform-like lesions, and it is irreversible

A

Gene Symbol	Gene description	Complementary sequence	mirSVR score
KCNA5	Potassium voltage-gated channel, shaker-related subfamily, member 5	3' uauguaugaagaaaUGUAAGGu 5' hsa-miR-1 ||||||| 5' uuuuaaaaaguaccACAUUCCa 3' KCNA5	-1.07

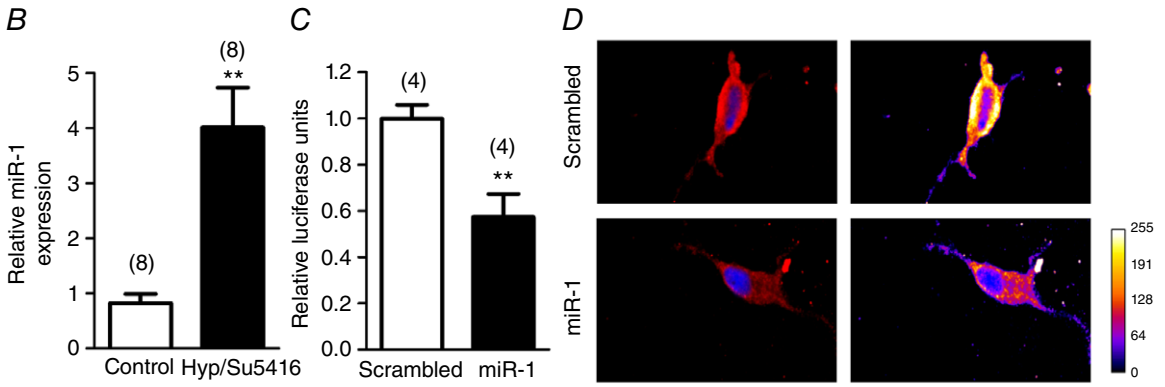


Figure 3. Changes in miR-1 expression in the PAH model
A, predicted sequence alignment between miR-1 and *KCNA5* 3′-UTR mRNA in *Homo sapiens* from microRNA.org. *B*, expression of miR-1 was analysed in lung homogenates from rats with PAH induced by Hyp/Su5416 and controls. *C*, luciferase activity in Cos7 cells co-transfected with miR-1 and the pMirTarget vector containing the 3′-UTR region of *KCNA5* and the luciferase gene. Results are the mean ± SEM. The parentheses indicate the number of experiments. $^{**}P < 0.01$ *vs*. scrambled miR. *D*, representative confocal images (left) and pseudocolour scale images (right) of PASMCs incubated with rabbit anti-Kv1.5 (red) and DAPI nuclear staining (blue) following transfection with scrambled miR or miR-1. Images are representative of cells from three different rats. [Colour figure can be viewed at wileyonlinelibrary.com]

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after returning to normoxia (Gomez-Arroyo *et al.* 2012; Ryan *et al.* 2013), rather than pulmonary hypertension as a result of respiratory diseases and hypoxia (i.e. class 3). We found a very strong upregulation of miR-1 in the lungs of Hyp/Su5416 treated rats. By contrast, the lungs from mice exposed to hypoxia (class 3 PH), as well as cultured human PASMC exposed to hypoxia *in vitro*, had reduced miR-1 levels (Sysol *et al.* 2018). Our *in vitro* studies show that cultured PA under hypoxia and Su5416 had increased miR-1 levels. Altogether, the data from human samples and animal models show that changes in miR-1 are not apparently uniform in pulmonary hypertension, indicating that plasma miR-1 levels do not appear to be a reliable biomarker of the disease. These variable levels of miR-1 may contribute to explaining the heterogeneity in the different forms of the pathology.

Kv channels play a fundamental role in controlling PASMC membrane potential and pulmonary arterial tone. Kv channel activity is decreased by several vasoactive factors involved in PAH, such as endothelin-1, 5-HT, thromboxane A_2 and hypoxia (Shimoda *et al.* 1998; Cogolludo *et al.* 2006; Cogolludo *et al.* 2009). Moreover, decreased PASMC expression of Kv1.5 and Kv2.1 in humans with PAH is considered to play an important pathophysiological role (Yuan *et al.* 1998; Michelakis *et al.* 2001; Wang *et al.* 2005). Thus, diminished transcription of Kv α-subunits may reduce the number of functional Kv channels in the membrane resulting in the decreased Kv current and leading to pulmonary artery vasoconstriction, hypertrophy and proliferation. Interestingly, we found that PASMC size, as reflected in the measurement of cell capacitance, was increased in PH rats, consistent with PASMC hypertrophy. A similar increase in cell capacitance has been reported previously (Shimoda *et al.* 2001) in PASMC derived from the chronic hypoxia PH mice model. Moreover, to our knowledge, the present study is the first to show a downregulation of Kv currents in the Hyp/Su5416 model of PAH. This effect is much more evident in this model than in the monocrotaline or in the hypoxic rat model reported previously (Wang *et al.* 2005; Morales-Cano *et al.* 2014). Indeed, the current sensitive to the Kv1.5 channel blocker DPO-1 (i.e. the component attributable to Kv1.5 channels) was almost

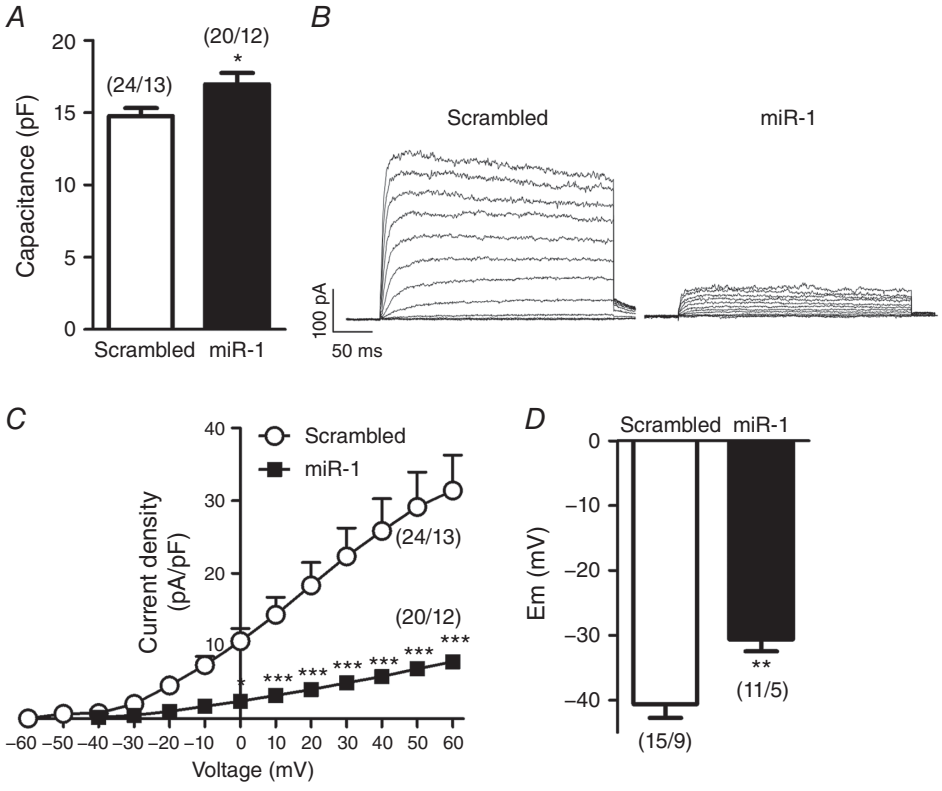


Figure 4. miR-1 reduces Kv currents and induces membrane depolarization in PASMCs
A, average cell capacitance of PASMCs transfected with scrambled miR or miR-1. *B*, representative Kv current traces and (*C*) current–voltage relationships of Kv currents measured at the end of the pulse in PASMCs transfected with scrambled miR or miR-1. *D*, resting membrane potential values in PASMCs transfected with scrambled miR or miR-1. Results are the mean ± SEM. The parentheses indicate the number of cells and the number of animals from which these cells were obtained, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. scrambled miR, respectively.

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completely abolished. In agreement with this finding, we found a lower expression of the Kv1.5 channels in lungs from Hyp/Su5416 rats. A potential limitation of the present study is that the inhibitory effect of DPO-1 on Kv1.5 channels can be influenced by multiprotein channel composition, such as the presence of the ancillary Kvbeta1.3 subunits (Du *et al.* 2010). Accordingly, we cannot rule out that changes in the expression of these regulatory subunits could also contribute to the different sensitivity to the drug. Unfortunately, this also appears to occur with other Kv1.5 channel inhibitors (Gonzalez *et al.* 2002; Decher *et al.* 2005).

The *KCNA5* mRNA, which encodes for the Kv1.5 channel protein, was predicted by microRNA.org to be a target of miR-1. We subsequently validated this interaction of miR-1 with the human *KCNA5* 3′ -UTR by a luciferase reporter assay. The data indicate that the levels of Kv1.5 protein may be regulated by miR-1. Therefore, to analyse the functional consequences of the miR-1–*KCNA5* interaction, we investigated the Kv currents in rat PA transfected with miR-1. The expression of miR-1 in the PA myocytes produced an increase in cell capacitance and a dramatic decline of the whole Kv current and specifically of the DPO-1-sensitive current. miR-1 also significantly depolarized the membrane potential. Therefore, miR-1 transfection mimicked the changes on Kv currents in the Hyp/Su5416 animal model of PAH and in human PAH. Thus, we hypothesized that miR-1 might play a role in the downregulated Kv currents in PAH. For this purpose, we analysed the effects of the antagomiR-1 on the PA exposed to hypoxia (3% O_2) plus Su5416 for 48 h, an *in vitro* correlate to the *in vivo* model. The *in vitro* exposure to Hyp/Su5416 produced a marked downregulation of the Kv current and increased miR-1, also mimicking the *in vivo* model. Interestingly, antagomiR-1 prevented PASMC hypertrophy and increased Kv currents and Kv1.5 channel expression in Hyp/Su5416-treated PA. Altogether, these data indicate that miR-1 downregulates Kv1.5 channels and suggest that that elevated miR-1 may be implicated in the inhibition of Kv channel expression in the Hyp/Su5416 animal model of PAH.

Kv downregulation for pulmonary hypertension associated with chronic hypoxia has been previously reported to be the result of hypoxia-inducible factor-1α (HIF-1α)-dependent induction of endothelin-1 (ET-1) (Whitman *et al.* 2008). The results of the present study may represent an alternative mechanism. Bioinformatic analysis and reporter assays revealed miR-1 binding sites in the 3′-UTR region of both the HIF-1α and the ET-1 mRNAs (Lu *et al.* 2014). miR-1 has also demonstrated to downregulate both proteins in colorectal cancer cells (Xu *et al.* 2017) and hepatocarcinoma cells (Li *et al.* 2012). Because HIF-1α and ET-1 play a fundamental role in pulmonary hypertension, it would be important to understand their relationship with miR-1 in the context of PAH. We speculate that the reduced miR-1, as found in some series of patients with pulmonary hypertension and cell culture exposed to hypoxia (discussed above), might allow a higher overexpression of HIF-1α and ET-1, finally resulting in reduced Kv1.5. However, although HIF-1α overproduction in endothelial cells or in the whole lung appears to play a clear pathophysiological role, changes

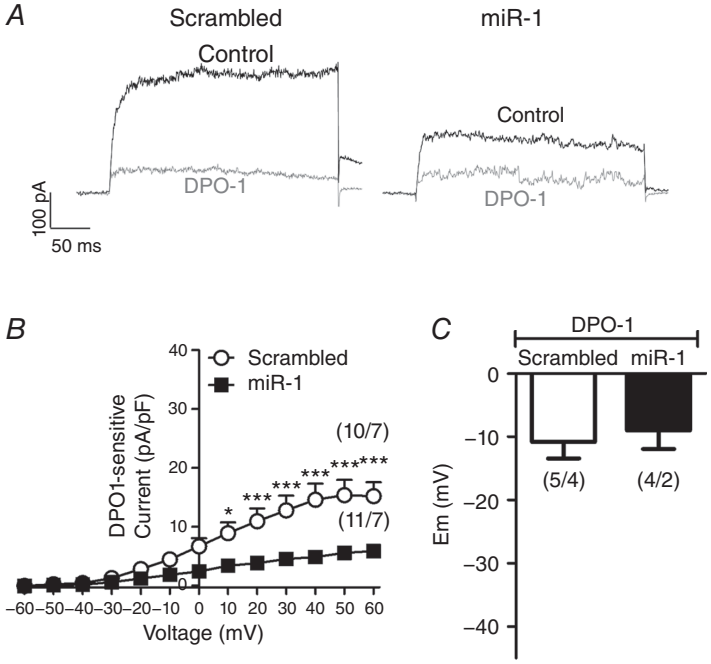


Figure 5. miR-1 reduces Kv1.5 currents in PASMCs
A, representative Kv currents traces recorded in the absence (control, black) and in the presence of the Kv1.5 channel blocker DPO-1 (1 μmol L^{-1}, grey). *B*, DPO-1 sensitive current in PASMCs transfected with scrambled miR and miR-1. *C*, resting membrane potential values in PASMCs transfected with scrambled or miR-1 in the presence of the Kv1.5 channel blocker DPO-1 (1 μmol L^{-1}). Results are the mean ± SEM. The parentheses indicate the number of cells and the number of animals from which these cells were obtained, respectively. $^{*}P < 0.05$ and $^{***}P < 0.001$ *vs*. scrambled miR, respectively.

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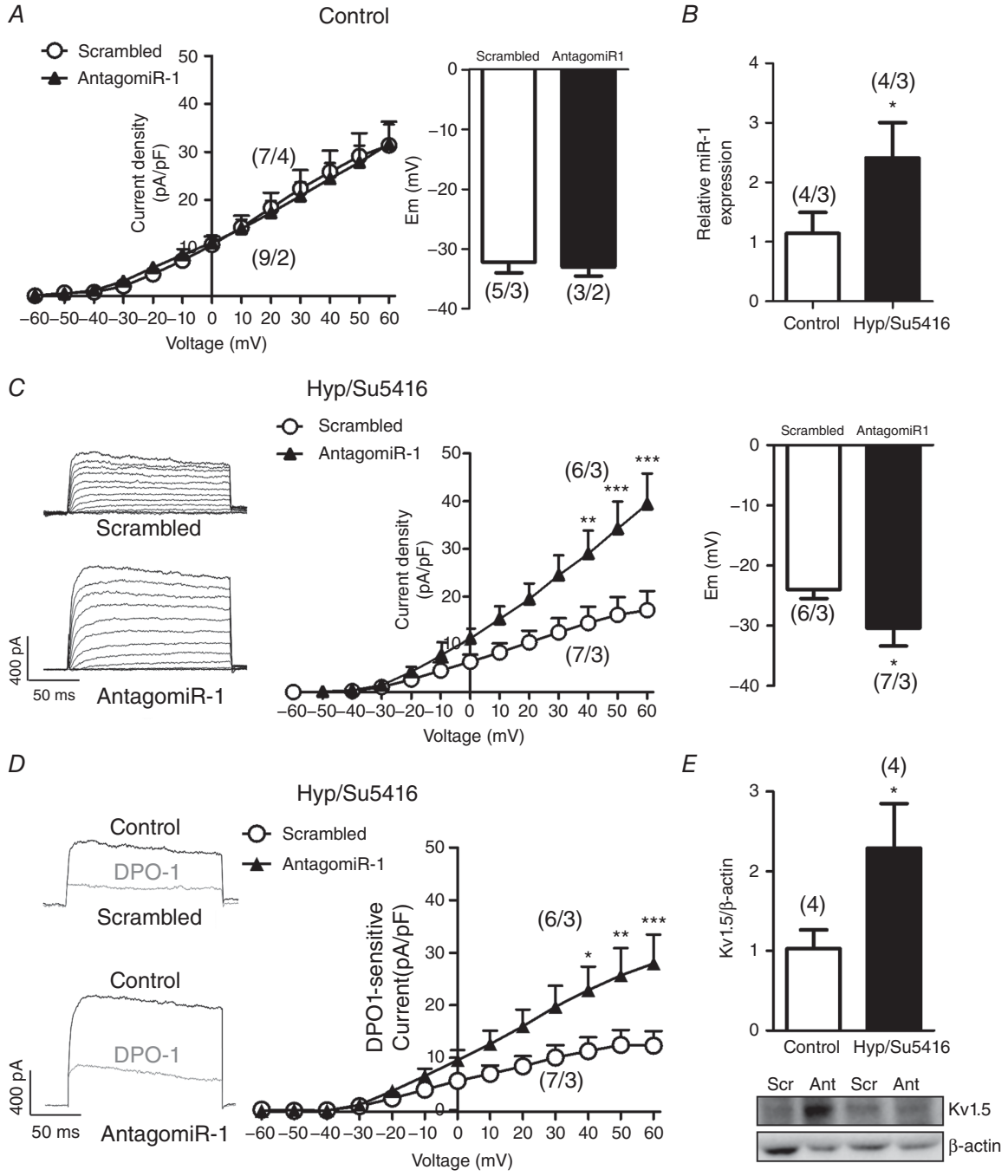


Figure 6. Effects of antagomiR-1 on hypoxia-induced ionic remodelling
A, current–voltage relationships of Kv currents measured at the end of the pulse and membrane potential values in PASMCs from PAs transfected with scrambled miR or antagomiR-1. *B*, expression of miR-1 was analysed in PA from rats incubated 48 h in Hyp/Su5416 or normoxia. *C*, representative current traces for 200 ms depolarization pulses from −60 mV to +60 mV in 10 mV increments from a holding potential of −60 mV, current–voltage relationships of Kv currents measured at the end of the pulse and membrane potential in PASMCs isolated from PAs transfected with scrambled miR or antagomiR-1 maintained in hypoxia (48 h, 3% O_2). *D*, representative Kv current traces measured before (black) and after (grey) the addition of the Kv1.5 channel blocker DPO-1 (1 μmol L^{-1}) and the DPO-1-sensitive currents obtained by subtracting the current in the absence and in the presence of the drug in myocytes from PA transfected with scrambled miR or antagomiR1 maintained in hypoxia. *E*, Kv1.5 protein expression in Hyp/Su5416-exposed PA transfected with scrambled (Scr) or antagomir-1 (Ant) analysed by western blotting and normalized by β-actin expression. Results are the mean ± SEM. The parentheses indicate the number of cells or arteries and the number of animals from which these cells or arteries were obtained, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ *vs*. control, respectively.

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in HIF-1α in smooth muscle cells are controversial. Reduced Kv1.5 protein expression has been reported to be concurrent with diminished HIF-1α in the smooth muscle layer of PA from idiopathic PAH (Barnes *et al.* 2017). Regarding the context of the present study, Su5416 is known to reduce HIF1α expression in several cell types, including PASMCs (Dean *et al.* 2017). Taken together, Kv downregulation in PASMC may result from at least two mechanisms: (i) HIF-1α- and ET-1-dependent and (ii) miR-1-dependent pathways. We speculate that the former might be more important in the context of hypoxia and respiratory diseases (class 3 PH) and the later in PAH (i.e. class 1 PH).

In conclusion, these data indicate that miR-1 reduces the activity and expression of Kv channels. *KCNA5* is a direct target of miR-1 and its downregulation accounts for decreased Kv currents and membrane depolarization in miR-1 transfected cells. The diminished Kv currents in Hyp/Su5416 treated PA was prevented by antagomiR-1. All of these data suggest that miR-1 upregulation may play a pathophysiological role, at least in some forms of PAH.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

GMP, MC, BB, DMC and SE performed and analysed the experiments. GMP and MC drafted the manuscript. AC and FPV conceived the study and designed the experiments. FPV wrote the manuscript with significant conceptual contributions from GMP, MC, LM and AC. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Chapter IV: HIV transgene expression impairs K^+ channel function in the pulmonary vasculature

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RESEARCH ARTICLE

HIV transgene expression impairs K^+ channel function in the pulmonary vasculature

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Mondejar-Parreño G, Morales-Cano D, Barreira B, Callejo M, Ruiz-Cabello J, Moreno L, Esquivel-Ruiz S, Mathie A, Butrous G, Perez-Vizcaino F, Cogolludo A. HIV transgene expression impairs K^+ channel function in the pulmonary vasculature. *Am J Physiol Lung Cell Mol Physiol* 315: L711–L723, 2018. First published August 23, 2018; doi:10.1152/ajplung.00045.2018.—Human immunodeficiency virus (HIV) infection is an established risk factor for pulmonary arterial hypertension (PAH); however, the pathogenesis of HIV-related PAH remains unclear. Since K^+ channel dysfunction is a common marker in most forms of PAH, our aim was to analyze whether the expression of HIV proteins is associated with impairment of K^+ channel function in the pulmonary vascular bed. HIV transgenic mice (Tg26) expressing seven of the nine HIV viral proteins and wild-type (WT) mice were used. Hemodynamic assessment was performed by echocardiography and catheterization. Vascular reactivity was studied in endothelium-intact pulmonary arteries. K^+ currents were recorded in freshly isolated pulmonary artery smooth muscle cells (PASMC) using the patch-clamp technique. Gene expression was assessed using quantitative RT-PCR. PASMC from Tg26 mice had reduced K^+ currents and were more depolarized than those from WT. Whereas voltage-gated K^+ channel 1.5 (Kv1.5) currents were preserved, pH-sensitive noninactivating background currents (I_{KN}) were nearly abolished in PASMC from Tg26 mice. Tg26 mice had reduced lung expression of Kv7.1 and Kv7.4 channels and decreased responses to the Kv7.1 channel activator L-364,373 assessed by vascular reactivity and patch-clamp experimental approaches. Although we found pulmonary vascular remodeling and endothelial dysfunction in Tg26 mice, this was not accompanied by changes in hemodynamic parameters. In conclusion, the expression of HIV proteins in vivo impairs pH-sensitive I_{KN} and Kv7 currents. This negative impact of HIV proteins in K^+ channels was not sufficient to induce PAH, at least in mice, but may play a permissive or accessory role in the pathophysiology of HIV-associated PAH.

HIV; Kv7; potassium channels; pulmonary hypertension; TASK channels

INTRODUCTION

Human immunodeficiency virus (HIV) infection and its associated pathologies constitute a global health concern. It is estimated that ~37 million people are living with HIV globally (45). Despite the success of highly active antiretroviral therapy, chronic inflammation persists and is independently associated with cardiovascular complications (44). Among the cardiovascular complications, HIV-associated pulmonary arterial hypertension (PAH) is especially severe and is associated with significant morbidity and mortality (4). Moreover, HIV patients have a 2,500-fold increased risk of developing PAH and have a poorer prognosis than PAH in the general population (9). Although only a small proportion (0.46%) of patients with HIV will develop HIV-related PAH (42), there may be as many as 200,000 HIV-infected patients affected by PAH worldwide.

PAH is a complex disorder characterized by excessive vasoconstriction, inflammation, and vascular remodeling that lead to reduced lumen of pulmonary arteries (PA) and increased pulmonary vascular resistance (34). The pathogenesis of PAH is rather multifactorial, but impairment of K^+ channels is considered an early and common feature in most, if not all, forms of the disease (3, 34).

A variety of K^+ channels have been shown to play an essential role in controlling pulmonary vascular tone, and their impairment results in a more depolarized membrane potential in PA smooth muscle cells (PASMC), leading to increased intracellular calcium, vasoconstriction, and proliferation (3, 8). Dysfunction of K^+ channels, particularly voltage-gated K^+ channel 1.5 (Kv1.5, encoded by *KCNA5*; 2, 3, 30, 46) and a member of the two-pore domain K^+ channel, TWIK-related acid-sensitive K^+ channel 1 (TASK-1, encoded by *KCNK3*; 1, 26, 37), has been found in experimental and clinical PAH and is considered a contributing factor in the development of the disease (3, 37). During the last decade, Kv7 (Kv7.1–Kv7.5) channels encoded by *KCNQ1–5* have emerged as key candidates to control vascular tone in several blood vessels, including the pulmonary circulation (6, 22, 24, 31). Interestingly, reduced *KCNQ4* expression has been reported in early phases of hypoxia-induced pulmonary hypertension (PH; 41), but this

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has not been consistently confirmed in other forms of the disease.

Although the mechanisms underpinning the pathophysiology of HIV-associated PAH still remain largely unknown, several HIV viral proteins have been proposed to contribute (4, 21). Thus, negative factor (Nef), transactivator protein (Tat), and envelope glycoprotein gp120 (gp120) induce endothelial dysfunction (12, 23, 38) and have been proposed to initiate pulmonary vascular remodeling (4). Tat represses the transcription of bone morphogenic protein receptor 2 (BMPR2; 5), whose deficiency plays a key important role in the onset and progression of PAH (34). Viral protein U (Vpu) interacts with TASK-1 channels and disrupts their function (18), and expression of Nef has been associated with the development of complex pulmonary vascular lesions in simians (28). We hypothesized that the expression of HIV proteins is associated with impairment of K^+ channel function in the pulmonary circulation as occurs in other forms of PAH. To address this issue, we undertook experiments using the Tg26 transgenic noninfectious mouse model, which expresses seven of the nine proteins of the HIV.

MATERIALS AND METHODS

Animals. All experimental procedures utilizing animals were performed according to Spanish Royal Decrees 1201/2005 and 53/2013 on the care and use of laboratory animals and approved by the institutional ethical committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animal Welfare (Comunidad de Madrid, ref. no. PROEXO-301/16). Age-matched (10-wk-old) male FVB/NJ [wild type (WT)] and HIV-1 transgenic mice on the FVB/NJ background [FVB/N-Tg(HIV)26Aln/PkltJ; Tg26] from the Jackson Laboratory were provided by Charles River Laboratories. This HIV-1 Tg26 mouse model expresses a transgene containing a portion of the HIV including envelope glycoprotein (Env) and Tat, Nef, regulator of expression of viral proteins (Rev), viral infectivity factor (Vif), viral protein R (Vpr), and Vpu accessory genes but lacking part of the gag-pol region, rendering the virus noninfectious (11). Animals were kept under standard conditions of temperature 22 ± 1°C (mean ± SE) and 12:12-h dark-light cycle with free access to food and water.

Genotyping. Genomic DNA was isolated from tail biopsies of WT and Tg26 mice with a lysis buffer of the following composition (in mmol/l) for 16 h at 55°C: 10 Tris·Cl (pH 8), 10 EDTA, 100 NaCl, and 0.5% SDS containing 0.1 mg/ml proteinase K. Then, genomic DNA was extracted with phenol-chloroform-isoamyl alcohol and purified. One hundred nanograms of genomic DNA were used as template for standard PCR using the primers listed in Table 1 according to the genotyping protocol from the Jackson Laboratory.

Tissue and cell isolation. Resistance PA were carefully dissected free of surrounding tissue and cut into rings (1.8–2-mm length). For cell isolation, PA rings were cut into small segments and placed into a nominally Ca^{2+}-free physiological salt solution (PSS) of the following composition (in mmol/l) for 7–10 min: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 glucose, and 10 HEPES (pH 7.3 with NaOH) containing (in mg/ml) 1 papain, 0.8 dithiothreitol, and 0.7 albumin. Thereafter, artery segments were incubated for an additional 3–5 min in Ca^{2+}-free PSS containing (in mg/ml) 1 collagenase F, 0.3 collagenase H, and 0.7 albumin. PASMC were dissociated using a wide-bore, smooth-tipped pipette. Cells were stored in Ca^{2+}-free PSS (4°C) and used within 8 h of isolation.

Recording of arterial reactivity. For contractile tension recording, PA rings were mounted in a wire myograph with Krebs buffer solution maintained at 37°C and bubbled with 95% O_2-5% CO_2. Vessels were stretched to give an equivalent transmural pressure of 20 mmHg. Preparations were first stimulated by raising the K^+ concentration of the buffer (to 80×10^{-3} M) in exchange for Na^+. Vessels were washed three times and allowed to recover before a new stimulation. The relaxant effects induced by acetylcholine (ACh, 10^{-9}–10^{-5} M), sodium nitroprusside (SNP, 10^{-11}–10^{-5} M), or Kv7 channel activators were examined in arteries stimulated with serotonin (5-HT, 10^{-5} M). Contraction induced by Kv1.5 or Kv7 channel inhibitors was assayed in the absence of agonist-induced preconstriction ("pretone").

Electrophysiological studies. Membrane currents were recorded with an Axopatch 200B and a Digidata 1322A (Axon Instruments, Burlingame, CA) using the whole cell configuration of the patch-clamp technique. Total Kv currents were recorded following the application of 200-ms voltage steps ranging from −60 to +20 mV (7, 32). In some experiments, long (4-s) depolarizing steps were applied to minimize the contribution of time-dependent delayed-rectifier potassium currents (such as Kv1) and maximize the contribution of Kv7 currents as reported (31). Current-voltage relationships were constructed by measuring the currents at the end of the pulse. To record TASK-like currents, defined as the noninactivating background K^+ current (I_{KN}), sensitive to pH, PASMC were clamped at 0 mV for 5 min to inactivate Kv channels, and subsequently, a 1-s ramp from +60 to −100 mV was applied (16, 36) at pH 6.3 and 7.3. To minimize activation of large-conductance, Ca^{2+}-activated K^+ channels, cells were superfused with an external Ca^{2+}-free PSS (see above) and a Ca^{2+}-free pipette (internal) solution containing (mmol/l): 110 KCl, 1.2 $MgCl_2$, 5 Na_2ATP, 10 HEPES, and 10 EGTA (pH adjusted to 7.3 with KOH). Currents were normalized for cell capacitance and expressed in pA/pF as previously described (7, 32). All experiments were performed at room temperature.

PASMC culture. Primary PASMC were obtained from intralobar PA explants and grown in Smooth Muscle Cell Growth Medium 2 supplemented with Smooth Muscle Cell Growth Supplement (C-22062; PromoCell, Heidelberg, Germany). The smooth muscle phe-

Table 1. *List of primers used*

Primer Name	Assay ID	Forward (5′–3′)	Reverse (5′–3′)
HIV-1 transgene		TCCAGTTTGGAAAGGACCAG	TTGCCACACAATCATCACCT
Positive control		CTCCCAACCCCAGAGGTAGT	AGACCCCAGATCCAGAAAGG
KCNA5	Mm00524346_s1		
KCNK3	Mm04213388_s1		
KCNQ1	Mm00434640_s1		
KCNQ4	Mm01185500_m1		
KCNQ5	Mm00524346_s1		
Actb	Mm02619580_g1		
NOS3		GGTATTTGATGCTCGGGACT	TGTGGTTACAGATGTAGGTGAACA
BMPR2		GAGCCCTCCCTTGACCTG	GTATCGACCCCGTCCAATC

Actb, β-actin; BMPR2, bone morphogenetic protein receptor 2; HIV-1, human immunodeficiency virus type 1; KCNA, voltage-gated K^+ channel subfamily A; KCNK, K^+ channel subfamily K; KCNQ, voltage-gated K^+ channel subfamily KQT; NOS3, endothelial nitric oxide synthase.

notype was confirmed by positive immunofluorescent staining using an anti-α-actin antibody (clone 1A4; Sigma-Aldrich). PASMC between *passages 1* and *2* were used for quantitative RT-PCR studies.

Quantitative RT-PCR analysis. Total RNA was isolated and purified either from whole lung homogenates or from PASMC using the miRNeasy extraction kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). Total RNA was reverse transcribed into cDNA using iScript cDNA Synthesis Kit (Bio-Rad) following the manufacturer's instructions. Real-time PCR was performed using a TaqMan system (Roche-Applied Biosystems, Mannheim, Germany) in the Genomic Unit of Universidad Complutense de Madrid. Custom sense and antisense primers for *NOS3* and *BMPR2* with TaqMan probe no. 56 (cat. no. 04688538001; Roche) and no. 67 (cat. no. 0468866001; Roche), respectively, and commercial primers from Applied Biosystems for *KCNA5*, *KCNK3*, *KCNQ1*, *KCNQ4*, *KCNQ5*, and *ACTB* expression were used (Table 1). The comparative threshold cycle method was used to quantify mRNA. Target gene expression was normalized to the expression of *ACTB*.

Echocardiography. A two-dimensional motion-mode transthoracic echocardiography was performed at a frame rate above 230 frames/s using a Vevo 2100 system and a 30-MHz linear probe (VisualSonics, Toronto, ON, Canada). Echocardiography examination was blindly performed by an expert operator. Color and pulse wave Doppler were acquired with a pulse repetition frequency of 40 kHz to study PA flows. Pulse wave Doppler was displayed just at the beginning of the PA. The pulmonary acceleration time (PAT), ejection time (ET), and the ratio of PAT to ET (PAT/ET) were measured to estimate right ventricular systolic pressure (RVSP; 43). Right ventricular (RV) systolic function was estimated using the tricuspid annular plane systolic excursion (TAPSE) obtained measuring the maximum lateral tricuspid annulus motion in a four-chamber apical view. Mice were lightly anesthetized with 0.5–1.5% isoflurane in oxygen to maintain heart rate during the experiments. Mice were positioned in supine position and kept in normothermia during the experiments with a heating platform and a warmed ultrasound gel.

Hemodynamic measurements. Mice were anesthetized intraperitoneally with a mixture of 80 mg/kg ketamine (Merial, Lyon, France) plus 8 mg/kg xylazine (KVP Pharma und Veterinär Produkte, Kiel, Germany). Before initiation of surgical procedure, general anesthesia was confirmed by assessing the absence of response to any stimulus. Then, animals were placed in a supine position on a thermostatically controlled electric heating blanket (Homeothermic Blanket Control Unit; Harvard Apparatus, March-Hugstetten, Germany) to maintain body temperature at 38°C. The tracheostomy was performed by a ventral neck incision followed by insertion of a 1.3-mm-outer diameter tracheostomy cannula in the trachea. Animals were ventilated with room air (tidal volume of 9 ml/kg, 100 breaths/min, and a positive end-expiratory pressure of 2 cmH_2O) with a rodent ventilator (MiniVent type 845; Harvard Apparatus). After sternotomy, RVSP and systolic, diastolic, and mean PA pressures (sPAP, dPAP, and mPAP) were measured in open-chest mice as previously reported in rats (30). Measurements were recorded with a pressure transducer via a 0.7-mm-internal diameter catheter (24 GA, BD Insyte; Becton Dickinson) introduced in the RV and then advanced to the main PA.

Assessment of RV hypertrophy. At the end of the recordings, hearts were excised, and the RV and the left ventricle plus septum (LV + S) were carefully dissected and weighed. The Fulton index [RV/(LV + S)] and the ratio of RV weight to body weight were calculated to assess RV hypertrophy (30).

Lung histology. The right lung was inflated in situ with formol saline through the right bronchus and embedded in paraffin. Hematoxylin-eosin staining and elastic van Gieson (eVG) staining were performed in lung sections according to common histopathological procedures and examined by light microscopy. Small arteries (25–100-μm outer diameter) were analyzed in a blinded fashion and categorized as muscular, partially muscular, or nonmuscular as previously described (30). Medial wall thickness was defined as the area between the external elastic lamina and the internal elastic lamina marked by eVG staining. Around 500 representative vessels within a range of diameters from 20 to 70 μm were analyzed per sample.

Reagents. Drugs and reagents were obtained from Sigma-Aldrich Química, except retigabine (Axon, Groningen, The Netherlands) and L-364,373 (Tocris, Bristol, United Kingdom). Drugs were dissolved in DMSO, and final vehicle concentrations were ≤0.1%.

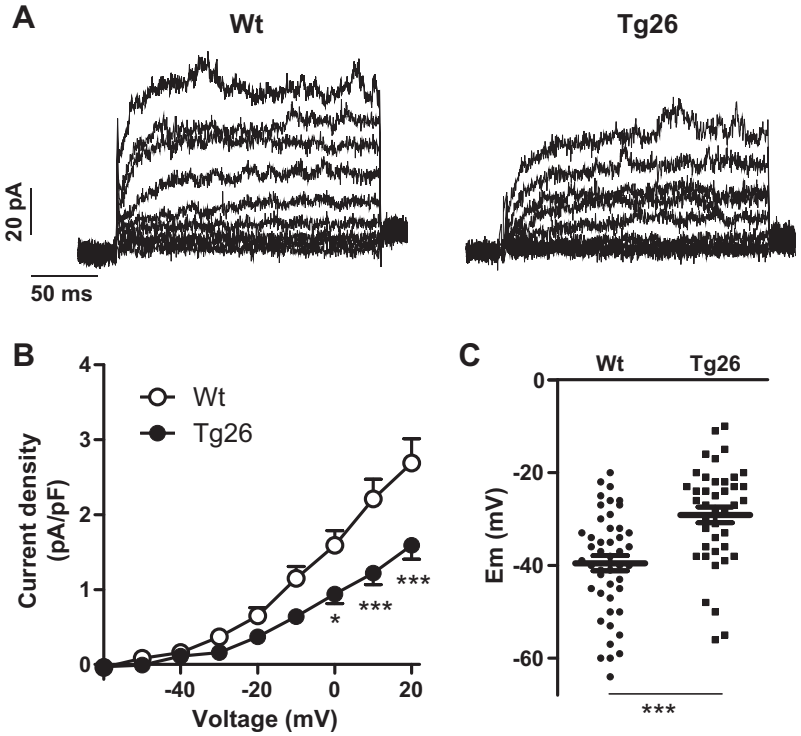


Fig. 1. K^+ channel activity is reduced in pulmonary artery smooth muscle cells (PASMC) from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A*: representative current traces for 200-ms depolarization pulses from −60 to +20 mV in 10-mV increments from a holding potential of −60 mV in PASMC from wild-type (WT) or Tg26 pulmonary arteries. *B*: current-voltage relationships of K^+ currents measured at the end of the pulse in myocytes from WT (n = 32 from 11 different animals) or Tg26 (n = 26 from 9 different animals). *C*: resting membrane potential (E_m) values in PASMC from WT (n = 46 from 13 different animals) or Tg26 (n = 40 from 12 different animals). *$P < 0.05$ and ***$P < 0.001$ vs. WT; nested ANOVA.

Statistical analysis. Data are expressed as means ± SE; *n* indicates the number of experiments from different animals, unless otherwise stated. Statistical analysis was performed using a Student's *t*-test for paired or unpaired observations or one-way ANOVA followed by a Newman-Keuls test for multiple comparisons. When >1 sample came from the same animal, the nested ANOVA was applied. Differences were considered statistically significant when P was <0.05.

RESULTS

K^+ channel dysfunction in PASMC from HIV-1 mice. PASMC isolated from Tg26 mice had similar membrane capacitance (20.3 ± 0.7 pF) compared with those isolated from WT mice (19.7 ± 0.6 pF; $P > 0.05$). Figure 1*A* shows original traces of the total Kv currents recorded in myocytes from both strains. Kv currents measured at the end of the 200-ms depolarizing pulses were significantly smaller in PASMC from Tg26 mice than in those from WT mice at potentials more positive than −10 mV (Fig. 1*B*). Moreover, resting membrane potential was more depolarized in PASMC from Tg26 mice than in those from WT (Fig. 1*C*).

Kv1.5 channels are not affected in PA from Tg26 mice. We studied whether Kv1.5 currents, which represent a main component of the total Kv current in the pulmonary vasculature (32), were affected in Tg26 mice. We found that the selective

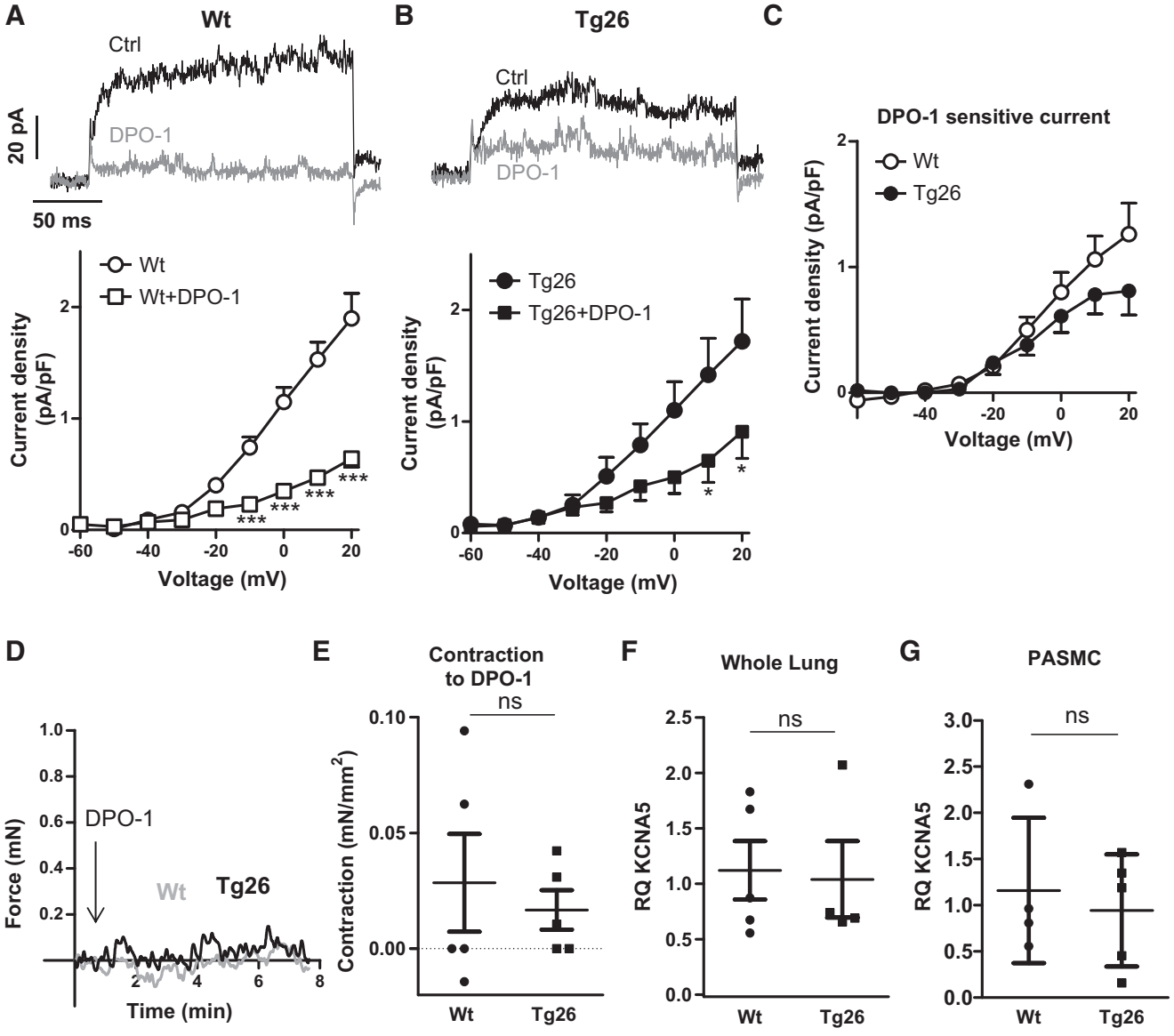


Fig. 2. Voltage-gated K^+ channel 1.5 (Kv1.5) activity is not altered in pulmonary artery smooth muscle cells (PASMC) from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A* and *B*, *top*: representative current traces obtained when applying a depolarizing pulse to +20 mV before (black) and after (gray) the addition of the Kv1.5 channel blocker diphenylphosphine oxide-1 (DPO-1, 10^{-6} M). Ctrl, control. *Bottom*: current-voltage relationships of K^+ currents measured at the end of the pulse before and after the addition of DPO-1 in myocytes from wild type (WT; $n = 7$ from 6 different animals) or Tg26 ($n = 7$ from 5 different animals), respectively. $^*P < 0.05$ and $^{***}P < 0.001$ vs. in the absence of DPO-1; nested ANOVA. *C*: mean data of the DPO-1-sensitive currents obtained by subtracting the current in the absence and in the presence of the drug. *D* and *E*: representative traces and averaged values of the contractile response induced by DPO-1 in pulmonary arteries from WT and Tg26 mice. *F* and *G*: *KCNA5* mRNA expression by RT-PCR in WT and Tg26 whole lung or PASMC homogenates, respectively. Results are means ± SE of 4–5 samples, normalized by the expression of *ACTB*; ns, not significant.

Kv1.5 channel inhibitor diphenylphosphine oxide-1 (DPO-1, 10^{-6} M) induced a marked reduction of the Kv currents in both WT-derived (Fig. 2*A*) and Tg26-derived (Fig. 2*B*) PASMC. Thus, DPO-1-sensitive currents, which reflect the Kv1.5 channel component, were not significantly different in both cell types (Fig. 2*C*). In addition, DPO-1 had negligible effects on vascular tone in arteries isolated from both strains (Fig. 2, *D* and *E*). In line with these data, the gene encoding Kv1.5 (*KCNA5*) was similarly expressed in lungs (Fig. 2*F*) or PASMC (Fig. 2*G*) isolated from both strains. These data suggest that K^+ channels other than Kv1.5 were impaired in PA from Tg26 mice.

pH-sensitive I_{KN} currents are impaired in PA from HIV-1 Tg26 mice. Figure 3*A* shows original recordings of I_{KN} at different pH during the application of voltage ramps from 60 to −100 mV from a holding potential of 0 mV. In WT PASMC, I_{KN} was inhibited when switching the pH of the external solution from 7.3 to 6.3. The current-voltage relation of the pH-sensitive current yielded a non-voltage-gated current as expected for TASK-like currents (Fig. 3*B*). On the other hand,

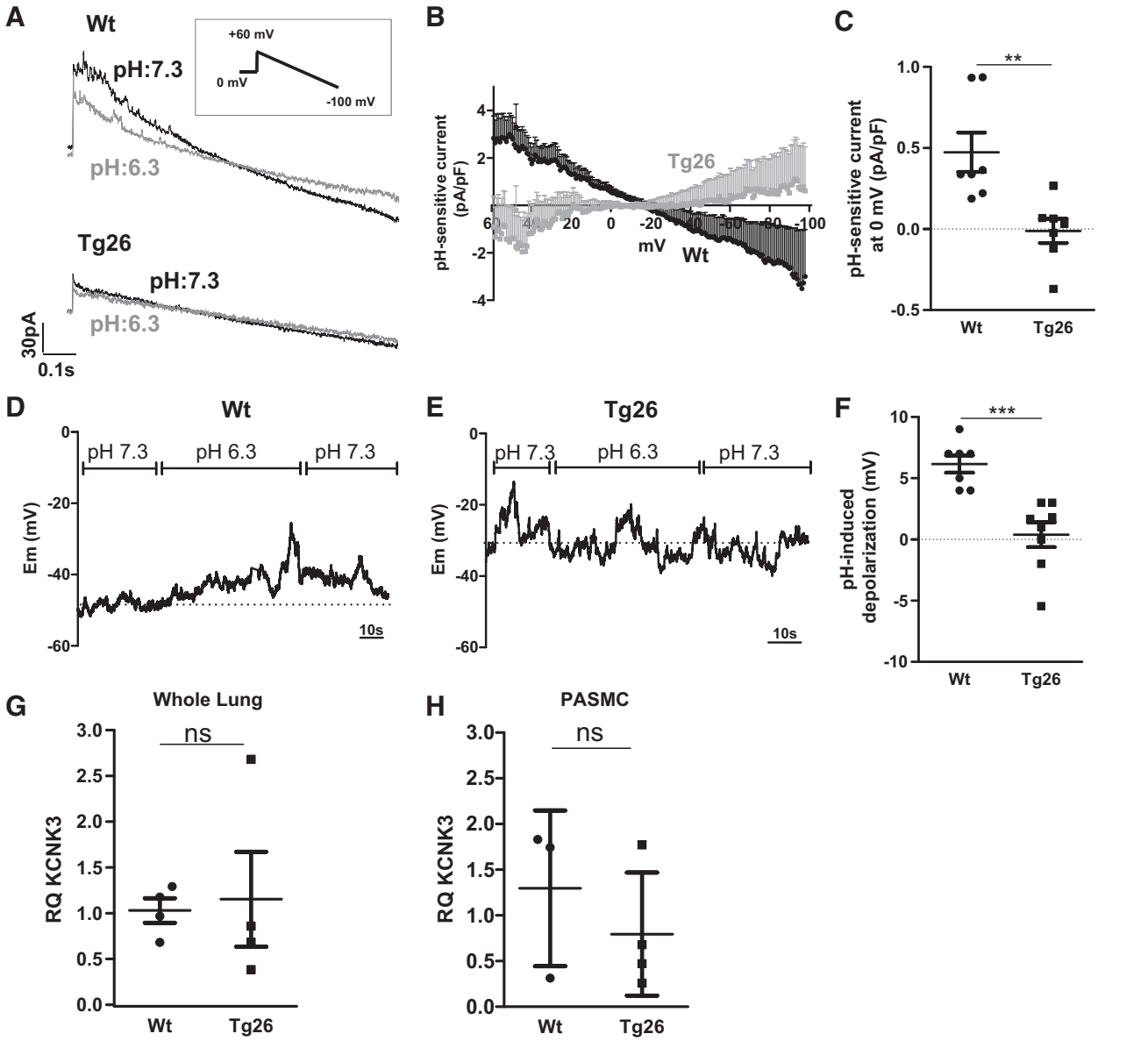


Fig. 3. Loss of TWIK-related acid-sensitive K^+ channel (TASK) currents in pulmonary artery smooth muscle cells (PASMC) from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A*: ramp protocol and original noninactivating background K^+ current (I_{KN}) recorded in PASMC from wild-type (WT) or Tg26 mice. Representative traces recorded at pH 7.3 (black) and after changing to pH 6.3 (gray) are shown. *B*: current-voltage relationships of the pH-sensitive current obtained by measuring the difference in K^+ current at external pH values of 7.3 and 6.3 in PASMC from WT ($n = 7$ from 6 different animals) or Tg26 ($n = 7$ from 5 different animals). *C*: pH-sensitive current at 0 mV. $^{**}P < 0.01$ vs. WT. *D* and *E*: original recordings of the effects of switching the pH of the external solution from 7.3 to 6.3 on membrane potential (E_m). *F*: acid-induced membrane depolarization in PASMC from WT ($n = 7$ from 6 different animals) or Tg26 ($n = 8$ from 5 different animals). $^{***}P < 0.001$ vs. WT. *G* and *H*: KCNK, K^+ channel subfamily K member 3 (*KCNK3*) mRNA expression by RT-PCR in lungs or PASMC, respectively, from WT or Tg26 mice. RQ, relative quantification. Results are means ± SE of 3–4 samples, normalized by the expression of *ACTB*.

currents from Tg26 mice were not inhibited by extracellular acidification (Fig. 3, *A* and *B*). In addition, acidification led to membrane depolarization in WT (Fig. 3*D*), but not in Tg26 (Fig. 3*E*), PASMC. Thus, both pH-sensitive K^+ current (Fig. 3*C*) and pH-induced depolarization (Fig. 3*F*) were markedly attenuated in cells from Tg26 mice. These differences were unrelated to changes in the expression of *KCNK3* in lungs or PASMC (Fig. 3, *G* and *H*). Consistent with previous reports (35), we were unable to analyze TASK-1, the protein encoded by *KCNK3*, in lung samples

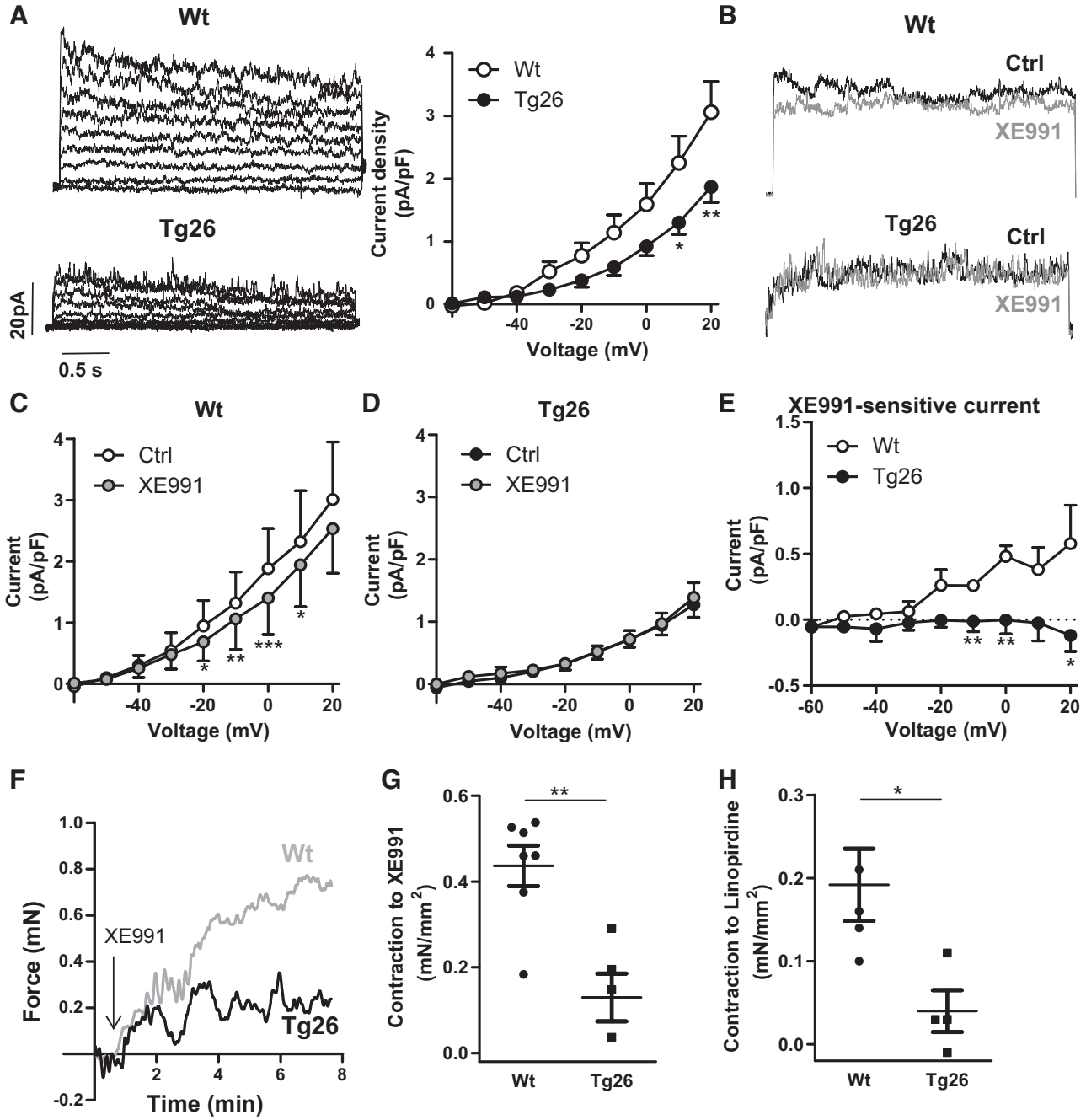


Fig. 4. Voltage-gated K^+ channel 7 (Kv7) currents are reduced in pulmonary artery smooth muscle cells (PASMC) from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A*: representative current traces and current-voltage relationships of K^+ currents measured at the end of the 4-s depolarization pulses from −60 to +20 mV in 10-mV increments from a holding potential of −60 mV in PASMC from WT (n = 31 from 14 different animals) or Tg26 (n = 30 from 8 different animals). *B–D*: representative current traces at +20 mV (*B*) and current-voltage relationships (*C* and *D*) of K^+ currents measured at the end of the pulse before and after the addition of the Kv7 channel blocker 10,10-bis(4-pyridinyl-methyl)-9(10H)-anthracenone (XE991, 3×10^{-7} M) in myocytes from WT (n = 7 from 5 different animals) or Tg26 (n = 6, from 5 different animals), respectively. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. in the absence of XE991. Ctrl, control. *E*: mean data of the XE991-sensitive currents obtained by subtracting the current in the absence and in the presence of the drug. $^*P < 0.05$ and $^{**}P < 0.01$ vs. WT. *F–H*: original recordings (*F*) and mean data (*G* and *H*) of the vasoconstriction induced by XE991 (n = 5–7) and linopirdine (n = 4–5), respectively. $^*P < 0.05$ and $^{**}P < 0.01$ vs. WT.

because commercially available antibodies did not identify the protein in the Western blots.

Kv7 channels are impaired in PA from HIV-1 Tg26 mice. The noninactivating voltage-gated Kv7 currents were studied by applying long (4-s) depolarizing pulses as previously reported (31). The K^+ current measured at the end of these pulses was diminished in PASMC from Tg26 mice (Fig. 4, *A* and *B*). To further characterize the role of Kv7 channels, cells were exposed to the selective Kv7 channel inhibitor 10,10-bis(4-pyridinyl-methyl)-9(10H)-anthracenone (XE991). At 3×10^{-7} M, this drug reduced the current in WT-derived (Fig. 4, *A* and *C*), but not in Tg26-derived (Fig. 4, *A* and *D*), PASMC. Thus, the current sensitive to XE991 was abrogated in PASMC from Tg26 mice (Fig. 4*E*). In PA isolated from WT mice, XE991 (3×10^{-6} M) elicited a robust contraction that was significantly smaller in PA from Tg26 mice (Fig. 4, *F* and *G*). Similar results were obtained with linopirdine (10^{-5} M), another Kv7 channel inhibitor (Fig. 4*H*). Quantitative analysis

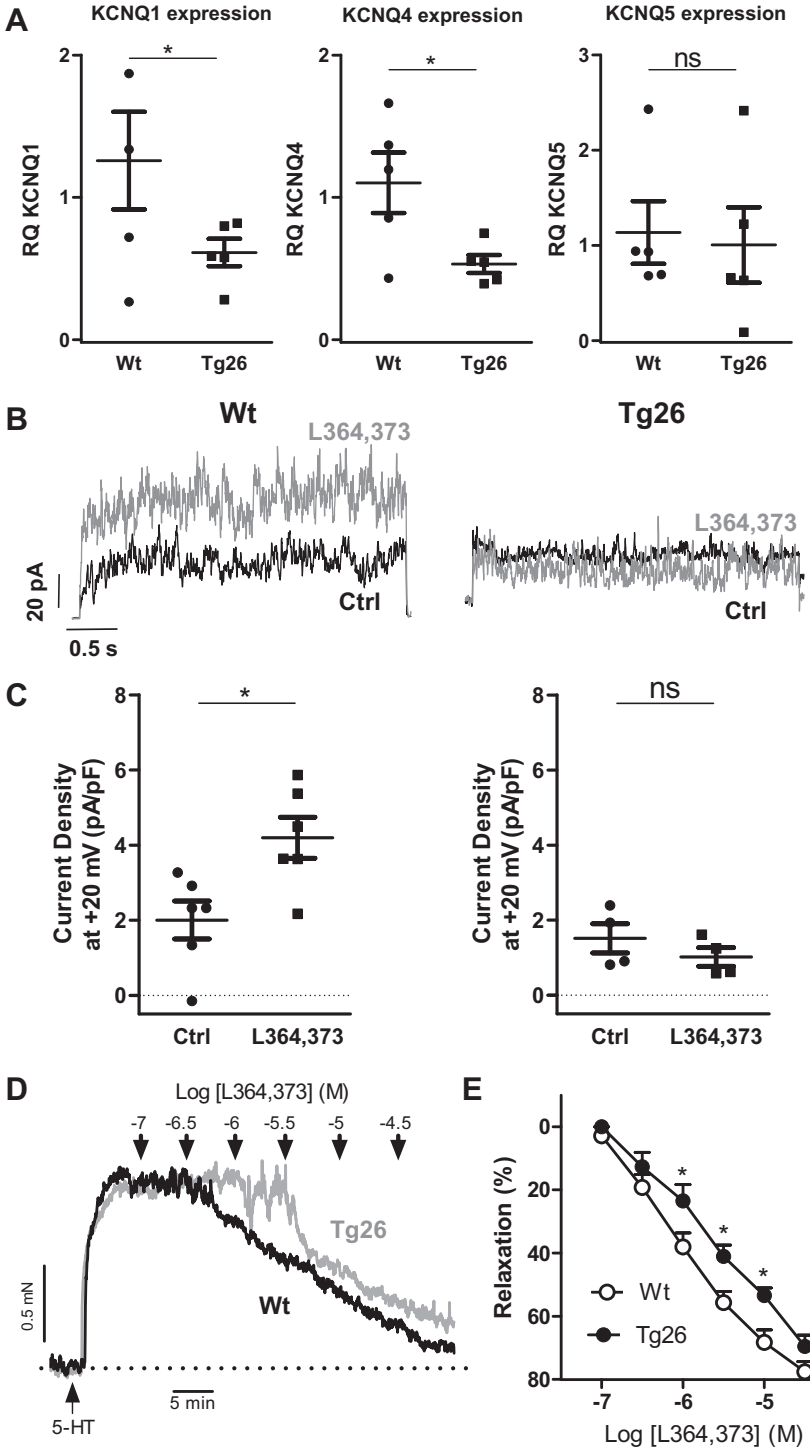


Fig. 5. Attenuated expression of and activity of voltage-gated K^+ channel 7.1 (Kv7.1) in pulmonary arteries from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A*: voltage-gated K^+ channel subfamily KQT member 1 (*KCNQ1*), *KCNQ4*, and *KCNQ5* mRNA expression by RT-PCR in wild-type (WT) and Tg26 lungs. Results are means ± SE of 4–5 samples, normalized by the expression of *ACTB*. $*P < 0.05$ vs. WT; ns, not significant. RQ, relative quantification. *B* and *C*: representative current traces at +20 mV and current density before and after the addition of the Kv7.1 channel activator L-364,373 (10^{-5} M) in pulmonary artery smooth muscle cells from WT ($n = 6$ from 3 different animals) and Tg26 mice ($n = 4$ from 3 different animals), respectively. Ctrl, control. *D* and *E*: original recordings and mean data of the relaxation induced by L-364,373 in serotonin-stimulated pulmonary arteries from WT ($n = 8$) or Tg26 ($n = 7$). Results are means ± SE. $*P < 0.05$ vs. WT.

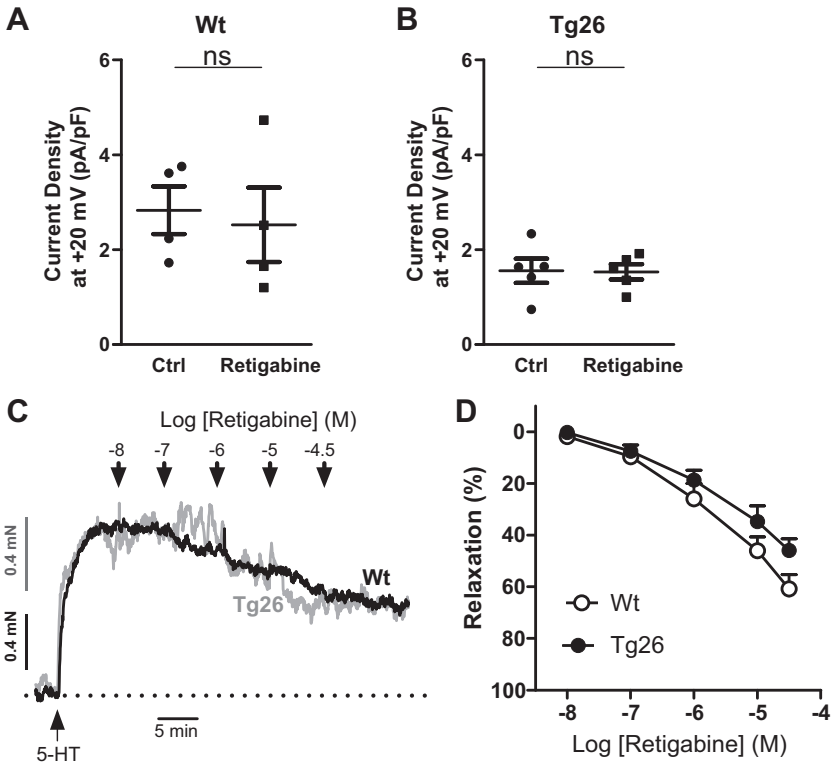


Fig. 6. Electrophysiological and relaxant effects induced by retigabine. *A* and *B*: current density at +20 mV before and after the addition of the voltage-gated K^+ channel 7.2–7.4 (Kv7.2–Kv7.4) activator retigabine (10^{-5} M), in pulmonary artery smooth muscle cells from wild-type (WT; $n = 4$ from 3 different animals) and human immunodeficiency virus type 1 transgenic (Tg26) mice ($n = 4$ from 3 different animals), respectively. Ctrl, control; ns, not significant. *C* and *D*: original recordings and mean data of the relaxation induced by retigabine in serotonin-stimulated pulmonary arteries from WT ($n = 9$) or Tg26 ($n = 8$). Results are means ± SE.

of mRNA expression of specific genes encoding the most predominant Kv7 channels (Kv7.1, Kv7.4, and Kv7.5) in the vascular bed revealed a reduced expression of *KCNQ1* and *KCNQ4*, but not *KCNQ5* (Fig. 5*A*), in lungs from Tg26 compared with WT mice. Thus, we assessed the effects of L-364,373, which selectively activates Kv7.1 channels, and retigabine, which activates Kv7.2 through Kv7.5 channels. The addition of L-364,373 led to an enhancement of the current in PASMC from WT mice, but not in those from Tg26 mice (Fig. 5, *B* and *C*). On the other hand, retigabine had no effect on the current in either cell type (Fig. 6, *A* and *B*). We also analyzed the relaxant responses induced by both drugs. The relaxation induced by L-364,373 was significantly reduced in PA from Tg26 mice (Fig. 5, *D* and *E*). However, no differences were found in the relaxant responses induced by retigabine between WT and Tg26 PA (Fig. 6, *C* and *D*).

Assessment of pulmonary hemodynamics and remodeling. Table 2 summarizes the hemodynamic parameters obtained by Doppler echocardiography in WT and HIV-1 Tg26 mice. PAT, ET, PAT/ET, and TAPSE values were similar in both strains. Figure 7*A* shows original recordings of PA pressure by PA catheterization. In line with the echocardiography findings, no differences were found in pulmonary hemodynamics (mPAP, sPAP, and dPAP, Fig. 7*B*), RVSP (Fig. 7*C*), or heart rate (Fig. 7*D*) assessed by catheterization. Assessment of cardiac remodeling by the Fulton index [RV/(S + LV), Fig. 7*E*] or RV weight-to-body weight ratio (Fig. 7*F*) revealed no significant RV hypertrophy in Tg26 compared with WT.

Table 2. *Noninvasive estimation of hemodynamic parameters in WT and Tg26 mice by Doppler echocardiography*

	WT	Tg26
PAT, ms	16.53 ± 1.03	16.53 ± 0.21
ET, ms	57.41 ± 2.04	61.76 ± 1.99
PAT/ET, %	28.98 ± 2.03	26.88 ± 0.78
TAPSE, cm	10.5 ± 0.5	10.5 ± 1

Values are means ± SE. ET, ejection time; PAT, pulmonary acceleration time; TAPSE, tricuspid annular plane systolic excursion; Tg26, human immunodeficiency virus type 1 transgenic; WT, wild type.

PA from HIV-1 Tg26 exhibit endothelial dysfunction. Contractile responses induced by KCl and 5-HT were similar in PA isolated from both Tg26 and WT mice (Fig. 8, *A–C*). We then analyzed the relaxation induced by the nitric oxide-cGMP pathway in 5-HT-contracted arteries. The relaxation induced by ACh (endothelial dependent, Fig. 8*D*), but not that of SNP (endothelial independent, Fig. 8*E*), was attenuated in PA from Tg26 mice. This endothelial dysfunction was not associated with changes in the expression of genes encoding endothelial nitric oxide synthase (*NOS3*) or BMPR2 (*BMPR2*; Fig. 8, *G* and *H*). We also assessed the endothelial-mediated relaxation in mesenteric arteries. Although there was a trend for reduced relaxation in mesenteric arteries from Tg26, differences did not reach statistical significance (Fig. 8*F*).

Figure 9*A* shows representative images of hematoxylin-eosin-stained lung sections from WT and HIV-1 Tg26 mice. PA were classified into nonmuscular, partially muscular, and muscular arteries (Fig. 9*B*). A modest vascular remodeling consisting of an increased percentage of partially muscular and muscular PA and decreased percentage of nonmuscular PA was observed in Tg26 mice. Moreover, a significant increase in medial wall thickness, assessed by the eVG staining, was found in Tg26 compared with WT mice (Fig. 9, *C* and *D*).

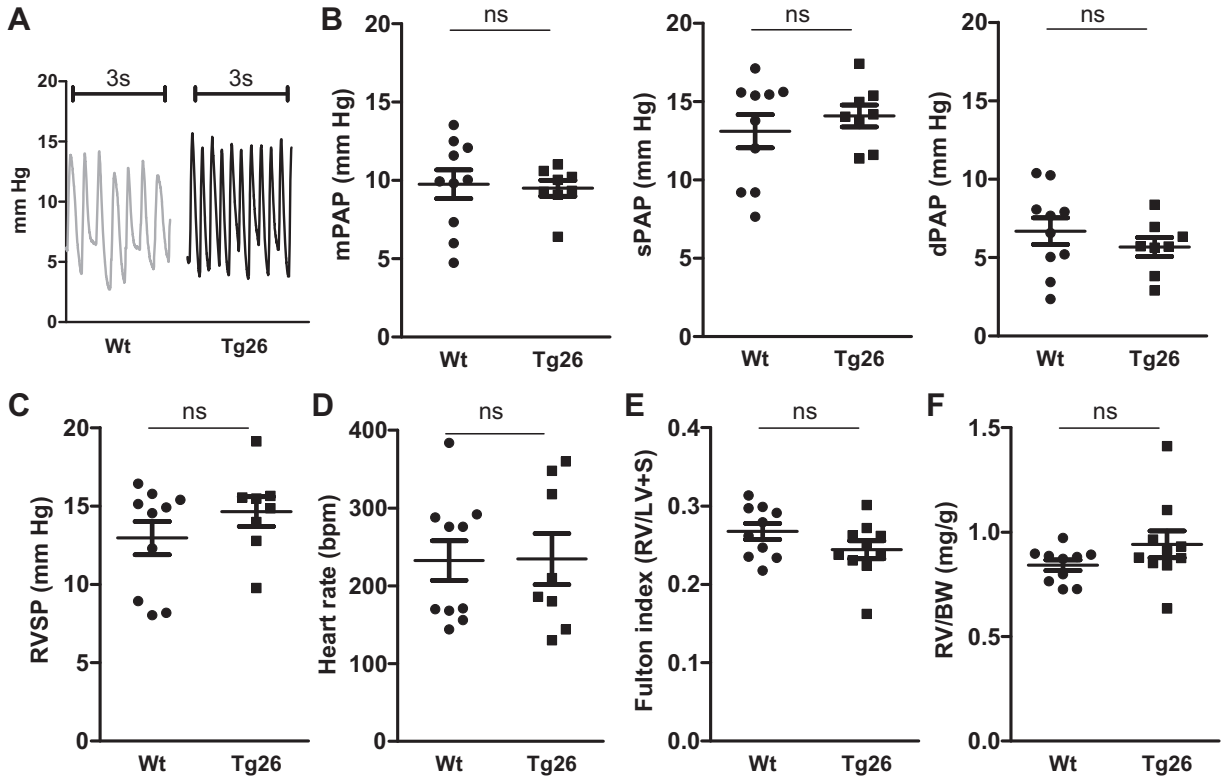


Fig. 7. Human immunodeficiency virus type 1 transgenic (Tg26) mice do not exhibit pulmonary hypertension. *A*: original recordings of pulmonary arterial pressure in wild-type (WT; gray) and Tg26 (black) mice. *B*: mean, systolic, and diastolic pulmonary arterial pressure (mPAP, sPAP, and dPAP, respectively) in WT and Tg26 mice. Results are means ± SE (n = 8–10). *C* and *D*: right ventricular systolic pressure (RVSP) and heart rate, respectively, in WT and Tg26 mice. Results are means ± SE. *E* and *F*: right ventricular (RV) weight relative to left ventricle + septum (LV + S) or to body weight (BW), respectively. Results are means ± SE (n = 10); bpm, beats/min; ns, not significant.

DISCUSSION

In the present study, we report that HIV transgene expression leads to attenuation of endothelial-dependent relaxation and impairment of K^+ channel activity in the pulmonary vasculature. In particular, a marked reduction of pH-sensitive I_{KN} and Kv7 currents was found in PASMC from mice expressing HIV proteins. Our study identifies novel pathogenic factors that could play a role in the development of PAH associated with HIV. This is the first study reporting K^+ channel dysfunction by HIV in the pulmonary circulation.

With the advent of combination antiretroviral therapy, HIV-associated comorbidities such as cardiovascular diseases have become a leading cause of death in HIV-infected patients. Among them, PAH is a life-threatening complication of HIV infection (4). The mechanisms involved in the pathogenesis of HIV-PAH are not completely understood, but HIV proteins such as gp120, Tat, and Nef are considered candidate contributors (4, 21). Thus, to gain insight into the mechanisms involved in PAH associated with HIV, we took advantage of transgenic mice expressing seven of the nine HIV proteins.

Our noninvasive (by echocardiography) and invasive (by PA catheterization) hemodynamic assessments revealed that PA pressures were not elevated in Tg26 mice. Accordingly, we found no changes in RV weight. Previous studies in HIV transgenic rats have shown elevated PA pressure and RV hypertrophy (25, 29), whereas another study, by Porter et al. (40), showed that Fischer 344 rats expressing HIV proteins were normotensive, even though these animals had exacerbated PAH in response to hypoxic exposure. Compared with these studies, animals used in our study were much younger (10 wk vs. 4–9 mo), so we cannot rule out that these animals may develop PAH at older ages. However, most likely the lack of PAH in HIV Tg26 mice can be attributed to the notable differences in the development of this disease between rats and mice (15). These include, among others, the development of less PA thickening and PH in mice than rats after chronic hypoxia or the induction of reversible (in mice) versus nonreversible and fatal (in rats) PH induced by exposure to SUGEN5416/hypoxia (15). These data strongly suggest that the expression of HIV-1 may predispose to, but appears insufficient to cause, PAH induction in the mouse model. It is noteworthy that the majority of patients with HIV infection are also not affected of PAH. Albeit HIV-1 Tg26 mice had PA pressures similar to WT mice, we observed changes characteristic of PAH such as pulmonary vascular remodeling, endothelial dysfunction, and K^+ channel impairment. Likewise, severe PA muscularization can occur in mice without the development of PAH or RV hypertrophy (10). We also found that PA from HIV-1 Tg26 mice had endothelial dysfunction as demonstrated by attenuated relaxation in response to ACh with unaffected relaxation in response to SNP. Whereas similar results have been observed in large systemic arteries from HIV-1 transgenic mice (17), in our study the endothelial-dependent vasodilation was not impaired in mesenteric arteries. Several mechanisms

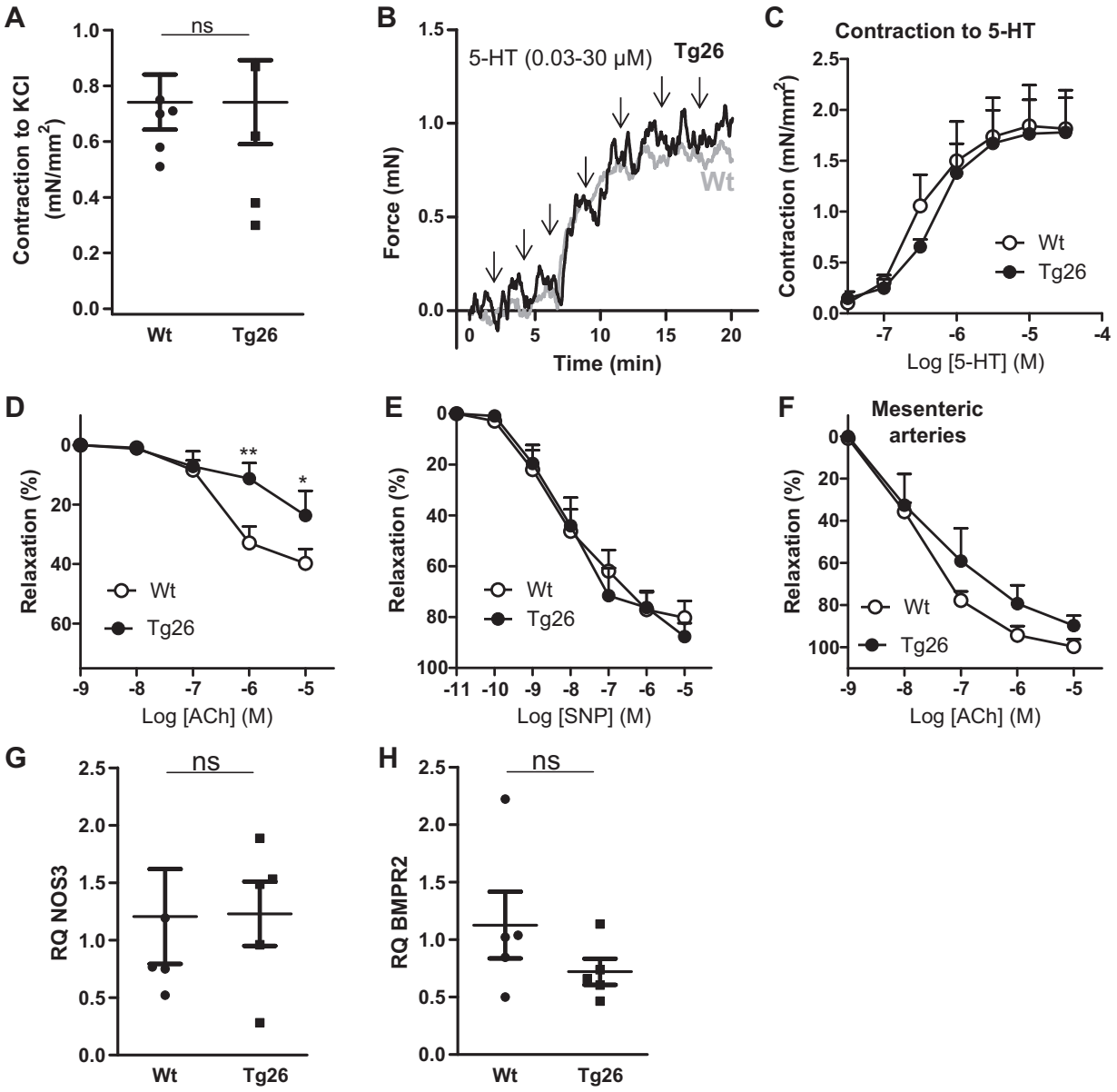


Fig. 8. Endothelial-dependent relaxation is impaired in pulmonary arteries (PA) from human immunodeficiency virus type 1 transgenic (Tg26) mice. *A*: vasoconstrictor responses induced by KCl. *B* and *C*: original recordings (*B*) and mean data (*C*) of the vasoconstriction-induced serotonin. *D* and *E*: relaxation induced by acetylcholine (ACh) or sodium nitroprusside (SNP), respectively, in serotonin-stimulated PA. *F*: relaxation induced by ACh in serotonin-stimulated mesenteric arteries. *G* and *H*: endothelial nitric oxide synthase (*NOS3*) and bone morphogenic protein receptor 2 (*BMPR2*) mRNA expression by RT-PCR in wild-type (WT) and Tg26 lungs. ns, Not significant; RQ, relative quantification. Results are means ± SE. $*P < 0.05$ and $**P < 0.01$ vs. WT (n = 5–6 mice per group).

have been proposed to account for the endothelial dysfunction in systemic arteries from HIV-associated PAH including downregulated *BMPR2* or *NOS3* gene expression (5, 12, 23, 38). However, we found unaltered *BMPR2* or *NOS3* expression in lungs from Tg26 mice. Alternative mechanisms proposed for HIV-1-induced endothelial dysfunction, which we did not explore further, include increased production of reactive oxygen species and accelerated NO degradation (19, 20) or increased levels of asymmetric dimethylarginine, which competitively inhibits endothelial nitric oxide synthase activity (39).

K^+ channels play a central role in governing membrane potential in PASMC, and their impairment is associated with depolarization, increased vasoconstriction, and proliferation (3, 8, 37). Our experiments demonstrate that total K^+ currents are attenuated in PASMC from HIV-1 Tg26 mice and this is associated with a more depolarized resting membrane potential. Among the different K^+ channels expressed in PASMC, special attention has been given to Kv1.5 and TASK-1 channels because of their role in controlling pulmonary vascular tone and in the pathogenesis of PAH. Reduced expression and function of Kv1.5 channels are common characteristics in many forms of human and experimental PAH (2, 3, 30, 46). Similarly, Lund et al. (25) found that expression of *KCNA5*, the gene encoding Kv1.5 channels, is reduced in lungs from HIV-1

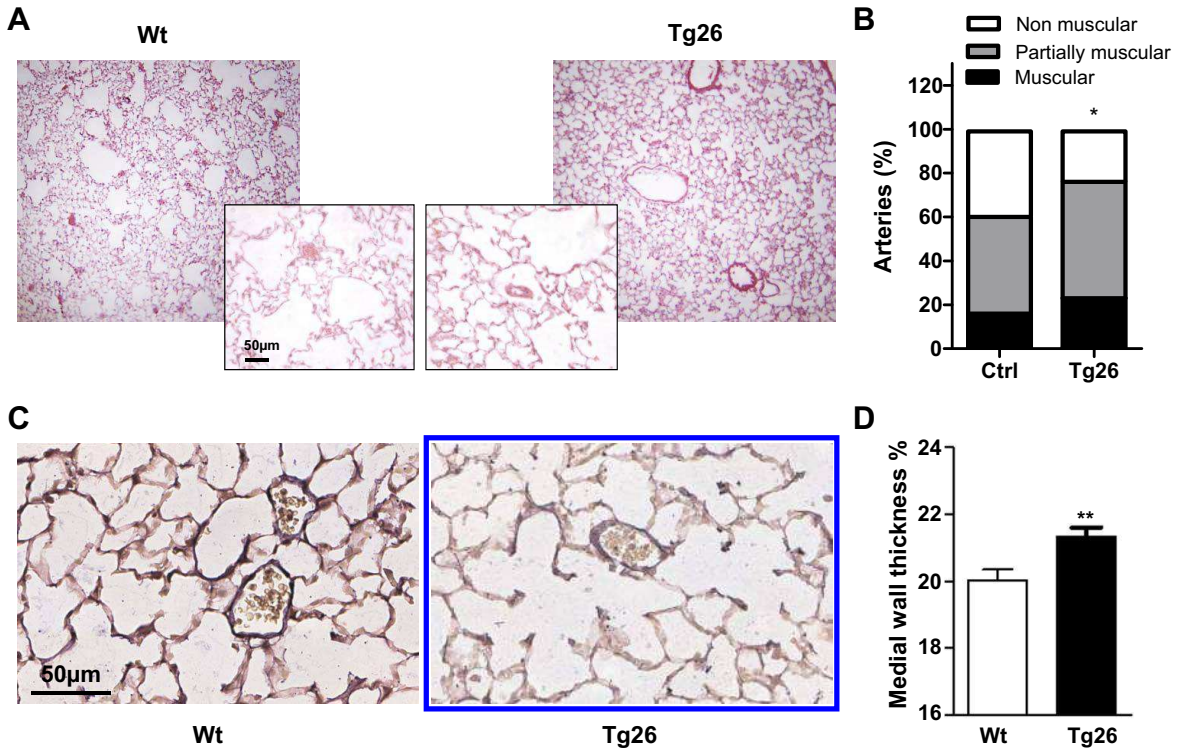


Fig. 9. Human immunodeficiency virus type 1 transgenic (Tg26) mice exhibit a modest pulmonary vascular remodeling. *A*: representative hematoxylin-eosin staining of paraffin-embedded lung sections from the wild-type (WT) and Tg26 mice. *B*: percentage of muscular, partially muscular, and nonmuscular pulmonary arteries in WT and Tg26 mice. *C*: representative photomicrographs of elastic van Gieson staining. *D*: percent wall thickness of pulmonary vessels in WT and Tg26 mice ($n = 4$ per group). A total of 495–500 vessels were measured per group. Scale bars = 50 μm. Ctrl, control. *$P < 0.05$ and **$P < 0.01$ vs. WT.

transgenic rats that exhibit PH. Herein, we comparatively analyzed the expression and activity (using the selective inhibitor DPO-1) of Kv1.5 channels in both WT and HIV-1 Tg26 mice. We found that DPO-1 exerted similar effects on total Kv current and contraction in both strains strongly suggesting a similar contribution of Kv1.5 channels. Intriguingly, DPO-1-sensitive current in PASMC from Tg26 mice reached an apparent plateau at positive potentials, which could be due to the partial contribution of residual large-conductance, Ca^{2+}-activated K^+ channel current. Likewise, no differences were found in *KCNA5* expression in lungs or PASMC from WT versus Tg26 mice. The reason for the discrepancies between our and Lund et al.'s study is unknown but may rely on the different animal species used. Another possible explanation is that downregulation of *KCNA5* is secondary to the development of PH, as occurred in the Lund et al. study.

There is compelling evidence that impairment of TASK-1 channels (encoded by *KCNK3*), members of the two-pore domain background K^+ channel family, plays a role in PAH (3, 37). Thus, the association between loss of function of *KCNK3* and PAH has been identified in hereditary and other forms of the disease (1, 26, 37). However, its role in PAH associated with HIV-1 remains unknown. A distinguishing feature of TASK channels is their pH dependence; thus we aimed to compare the activity of TASK channels in WT and Tg26 mice by examining the acid sensitivity of the background current I_{KN}. In PASMC from WT we found that acidification of extracellular pH inhibited the I_{KN} current leading to membrane depolarization, suggesting a functional role of TASK-like channels. These results are in line with previous data in rat (14), rabbit (16), and human (36) PASMC, albeit this pH-sensitive current was not observed in a previous study in mouse PASMC (27). In contrast to what we observed in WT cells, PASMC from Tg26 mice were essentially insensitive to pH. The mechanism underlying the reduced pH-sensitive current in HIV-1-expressing mice remains unknown, but it is tempting to speculate a role of the HIV-1 accessory protein Vpu. It is worth highlighting that Vpu shares structural homology with the NH_2-terminal region of the TASK channel family members, including TASK-1, and that Vpu and TASK-1 oligomerize in vitro and in lymphoid tissues from AIDS patients (18). Moreover, the coexpression of Vpu and TASK-1 in heterologous systems leads to the suppression of the TASK current. Conversely, overexpression of TASK-1 suppresses HIV-1 replication (13). However, we could not confirm the protein expression of Vpu in the lung (with a commercially available antibody, ab81532).

Findings from the last years suggest that Kv7 channels are key regulators of vascular tone, and reduced expression and activity of Kv7 channels have been reported in several cardiovascular diseases including essential hypertension (6, 22) and diabetes (31). Kv7 channels also make an important contribution to the regulation of pulmonary vascular tone (24). Moreover, the vascular responses to Kv7 channel modulators are depressed in two murine models of PH (33) suggesting a Kv7 channel impairment. In fact, reduced *KCNQ4* expression has been noticed in PA from rats exposed to hypoxia for 3 days, which corresponds to the onset of PH development (41).

Herein, we dissected the Kv7 noninactivating voltage-gated K^+ currents by applying long depolarizing pulses and by the use of Kv7.1–Kv7.5 channel blocker XE991. The amplitude of this Kv7 current was markedly reduced in PASMC from Tg26 mice. Accordingly, XE991 as well as another Kv7 channel blocker, linopirdine, elicited greater vasoconstrictor responses in PA from WT than from Tg26. To ascertain whether these differences were related to loss of channel expression in Tg26 lungs, we analyzed the gene expression of the most relevant Kv7 channels in the vasculature. Our data confirmed a reduced expression of *KCNQ1* and *KCNQ4*, but not *KCNQ5*, in lungs from HIV-1 Tg26. We also tested the effects of the selective Kv7.1 activator L-364,373 and retigabine (which activates Kv7.2 through Kv7.5). We observed that the electrophysiological and relaxant effects of L-364,373 were reduced in PASMC from HIV-1 Tg26, whereas retigabine did not enhance the currents and had comparable relaxation in PA from both strains. Altogether, our data indicate that Kv7 channel (especially Kv7.1) activity and expression are impaired in mice expressing HIV-1 proteins.

In conclusion, we demonstrate that the expression of HIV proteins in vivo impairs TASK-like and Kv7 channel activities but preserves Kv1.5 channel currents. Decreased Kv7 currents can be explained by downregulated gene expression of the channel and was functionally correlated with reduced vasodilation in response to a Kv7.1 channel activator. This negative impact of HIV proteins in pulmonary K^+ channels was not sufficient to induce PAH, at least in mice, but may play a permissive or accessory role in the pathophysiology of HIV-associated PAH.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

A.M., G.B., F.P.-V., and A.C. conceived and designed research; G.M.-P., D.M.-C., B.B., M.C., and S.E.-R. performed experiments; G.M.-P., D.M.-C., B.B., M.C., and A.C. analyzed data; G.M.-P., D.M.-C., J.R.-C., L.M., A.M., F.P.-V., and A.C. interpreted results of experiments; G.M.-P., D.M.-C., B.B., and A.C. prepared figures; A.C. drafted manuscript; G.M.-P., D.M.-C., L.M., A.M., G.B., F.P.-V., and A.C. edited and revised manuscript; G.M.-P., D.M.-C., B.B., M.C., J.R.-C., L.M., S.E.-R., A.M., G.B., F.P.-V., and A.C. approved final version of manuscript.

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7. Global overview

The Em of PASMC is regulated by the activity of different Kv, K_{Ca}, K_{IR} and K_{ATP} channels providing an important mechanism to relax or contract PA [29]. K^+ channels are also expressed in PAEC where their function is focused on maintaining resting Em, enable Ca^{2+} entry and regulate the production of different vasoactive factors. Furthermore, the activity of K^+ channels is a key regulator of proliferation, apoptosis, migration and survival of PASMC [37] [95]. The regulation of K^+ channels in the pulmonary vasculature is quite complex. They can be modulated by many physiologic and pathological stimuli. In the last decade, several studies have highlighted the role of inflammatory cells in the development of PH [212, 93, 35]. Reduced activity of several K^+ channel has demonstrated to play a crucial role in PAH. This reduction may result from 1) LoF mutations, 2) reduced activity of the channels by the effects of vasoactive factors, hypoxia or drugs 3) reduced protein expression and/or a reduced trafficking of the channels to the membrane.

In this Doctoral Thesis two rodent PH models were used: Sugen5416-Hypoxia rat model and HIV-1 transgenic mice, which are related and attempt to reproduce the pathophysiology of two different forms of human PAH (PH class I), iPAH and HIV-associated PAH, respectively. Both animal models were induced into male rodents, since, despite the fact that PAH affects mostly females, males have a worse prognosis and survival. To study the regulation of different K^+ channels and ancillary subunits implicated in pulmonary vascular control by different pathophysiological factors related to PAH, we used Su/Hpx rats, the rodent model which best recapitulates human PAH, since it mimics plexiform vascular lesions and K^+ channel remodelling characteristic of human PAH. The results obtained using this animal model could better benefit iPAH; but the findings could be extended to other forms of PAH; since, ion channel remodelling is a hallmark of PH. To study the impact that HIV-1 proteins can have on the pulmonary vasculature, we used a non-infectious transgenic mouse model that expresses 7 of the 9 HIV-1 proteins. The two missing proteins (*gag* and *pol*) are responsible for the infectious virus component. Therefore, this animal model could represent HIV patients treated with antiretroviral therapy. HIV-mouse do not exhibit an increase in pulmonary pressure; however, they developed certain pathophysiological characteristics related to PH such as impairment of K^+ channels, endothelial dysfunction and vascular remodelling, which in the long term could contribute to the development of PAH.

The results of this study can benefit millions of HIV-infected people differentiating cardiovascular complications due to direct contact with HIV-proteins from the cardiovascular complications related to HAART-treatment.

Different published study support that K^+ channels regulation in PH is mainly subjected to transcriptional changes and intracellular protein trafficking regulation leading to reduced membrane expression, for this reason the clinical benefit of selective K^+ channel openers appears questionable. An alternative strategy may be to prevent K^+ channel downregulation in PAH. For instance, dichloroacetate (a metabolic modulator that increases mitochondrial oxidative phosphorylation) treatment almost completely reversed the downregulation of Kv1.5 channel observed in isolated PASMCs from PAH rats, improving Kv1.5 current. Similar results were observed in PAH animals model using cyclosporine-A (an inhibitor of calcineurin–NFATc binding), fluoxetine (a selective serotonin reuptake inhibitor), Plumbagin (a STAT3 inhibitor) and a selective survivin inhibitor [28]. Alternatively, different antagomiRs have demonstrated to reverse the effects, at least partially, of altered microRNAs that downregulate K^+ channels in PAH-PASMC such as the decrease in Kv1.5 channel expression by microRNA-1, representing an innovative therapeutic strategy. Moreover, it has been demonstrated that intranasal or intratracheal instillation and inhaled administration of such antagomiRs is effective in mice models and may represent a more selective way to target the lung in respiratory diseases [168].

One of the most remarkable results of this Doctoral Thesis is the activation of Kv7 channels contributes to the physiological relaxation of PA induced by the NO/cGMP pathway [170]. Although the activation of Kv7.5, and possibly Kv7.4 channels, occurs through the activation of sGC and the subsequent increase of cGMP, it is not known accurately if cGMP acts directly on the channel or there is an intermediary factor such as a trafficking protein or a ancillary β-subunit. Moreover, there is considerable evidence of implicating NO/cGMP signalling dysfunction the PAH pathophysiology process, and therefore, drugs restoring this pathway are used to treat PAH [86, 45]. Another important finding of this Thesis is that pulmonary vascular Kv7 channels have a higher functional role during the development of PAH associated with an upregulation of subunit KCNE4.

The mechanisms that dictate the K^+ channels membrane abundance in PASMC is yet to be fully elucidated but it has already been described different cellular processes that modulate

K^+ channel transcription, retrograde and anterograde trafficking and protein degradation. Recently, an increasing number of ion channel-transporter "chansporter" complexes has been discovered [2]. The chansporter complex is advantageous over the isolated ion channel as the overcoming of spatial barriers, the acceleration of traffic to the membrane, the interaction between ion channels and other substrates that they can transport with themselves resulting in faster signal integration. The association between K^+ channel α- and β-subunits in PASMC as SUR2-K_{IR}6.1, SUR1-K_{IR}6.2 and KCNE4-Kv7.4 can result in complexes that may have other activities in addition to carrying ionic currents through the membrane. However, it is necessary to explore their biological roles and the pathophysiological consequences of their disruption of this association in the pulmonary vasculature.

The findings of this Doctoral Thesis regarding Kv7 channels of this Doctoral Thesis support that targeting Kv7 channel may represent an attractive strategy in the treatment of PAH. According to these findings, NO-cGMP-relaxation of pulmonary arteries through Kv7 channels may be maintained in PAH, and Kv7 channels may contribute largely to the therapeutic effect of drugs increasing cGMP and cAMP in PH patients. However, it needs to be confirmed by further studies. Moreover, in agreement with this idea preliminary studies in animal models suggest that Kv7 channel openers are able to prevent and even reverse pulmonary hypertension [175, 223]. While two of the commercially available pan-Kv7 channel openers (i.e. retigabine and flupirtine) have been withdrawn from the market in recent years a number of new molecules with higher selectivity for particular Kv7 channel subunits are under development [22, 224]. The specific expression of certain Kv7 channel in different types of cells is of great pharmacological interest, since their selective modulation could provide beneficial therapeutic effects while avoiding the adverse effects associated with other isoforms (Annex V).

Other K^+ channels openers (e.g., minoxidil, lemakalim, nicorandil, diazoxide or pinacidil) have demonstrated to be able to relax PA [134]; nevertheless, their powerful systemic vasodilatory effects may exacerbate PH in long-term administration [163]. Hence, given the functional roles and ubiquitous expression of Kv7 channels in many tissues, drugs modulating Kv7 channels could cause off-target effects including alterations of the QT interval at the heart level, systemic hypotension, urinary retention or neuronal effects.

In fact, an important feature of an ideal vasodilator drug for the treatment of PH would be the selectivity on the pulmonary vasculature to avoid the associated systemic effects. However, this could be largely avoided by localized drug delivery to the lungs by inhalation.

Altogether, these data suggest that Kv7 channels represent new promising therapeutic targets in the field of PH even when there is still much to be learned about their physiological role, regulation and possible impairment by pathological processes.

8. Conclusions

I. The activation of Kv7 currents is a novel mechanism involved in the pulmonary vasodilator effects of the NO/cGMP pathway and therefore is a therapeutic target of drugs indicated for the treatment of PH, such as NO and riociguat.

II. Kv7 channel function is preserved and KCNE4 is up-regulated in PAH. Therefore, the relative contribution of these channels to regulate vascular tone in PAH is increased compared to other downregulated K^+ channels, resulting in an enhanced sensitivity to Kv7 channel modulators. This study provides an insight into the potential usefulness of targeting Kv7 channels in PAH.

III. miR-1 reduces the activity and expression of Kv1.5 channels and depolarizes the PASMC and its inhibition by antagomir-1 prevents the diminished Kv currents in an in vitro model of PAH. These data suggest that miR-1 upregulation may play a pathophysiological role, at least in some forms of PAH.

IV. The expression of HIV proteins in mice impairs TASK-like and Kv7 channel activities but preserves Kv1.5 channel currents. The negative impact of HIV proteins in pulmonary K^+ channels was not sufficient to induce PAH, at least in mice, but may play a permissive or accesory role in the pathophysiology of HIV-associated PAH.

This Doctoral Thesis provides evidence of new mechanisms of action of drugs used in the treatment of PAH via activation of K^+ channels, the pathophysiological role of K^+ channels in PAH and suggest a new family of K^+ channels that could be a new therapeutic target in PA

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9. Future directions

One of the most relevant finding of this Doctoral Thesis is that Kv7 channel function is preserved providing a higher contribution to the basal K^+ current in PASMC unlike other K^+ channel such as Kv1.5 and TASK1 channel whose activity is seriously diminished in PAH. Moreover, Kv7 channel activation contributes to NO/cGMP-induced PA vasodilation, a major physiological signalling controlling tone in PA. These findings point to Kv7 channels as a potential novel pharmacological target for the treatment of PAH. Future research in line with this Thesis should focus in answering different issues for a proper understanding of the role of Kv7 channels in the pathophysiology of PAH as:

- Is NO-induced PA vasodilation through Kv7 channels maintained or potentiated in PAH? This issue could be solved analysing the effects of NO and riociguat in the absence and presence of Kv7 channel inhibitors in PA or PASMC isolated from PAH-animals compared to controls using vascular reactivity and patch clamp techniques, respectively.
- Does cGMP directly activate Kv7.4 and/or Kv7.5 channels or is necessary an intermediary factor such as the KCNE4 subunit? Silencing the KCNE4 subunit and analysing the Kv7 channel activity induced by the activation of the NO/cGMP pathway could be of help to solve this question.
- Does the cellular location of Kv7.4 channels and KCNE4 subunits in PAH-PASMC correspond to what happens when Kv1.5 and TASK1 channels are pharmacologically inhibited? This question could be solved analysing Kv7.4 channels and KCNE4 subunits cell location in isolated PASMC from PAH-animals compared to controls using immunocytochemistry technique.
- There is a demanding need to develop modulators of specific Kv7 subunits to allow for tissue-specific treatments. Probably, the next-generation of Kv7 channels therapeutics could target KCNE subunits as well as specific isoforms of Kv7 channels linked to KCNE subunits. Consequently, analysing the effect of specific Kv7.4 or Kv7.4/KCNE4 channel activators in a PAH animal model through inhaled therapy would complete this line of research.

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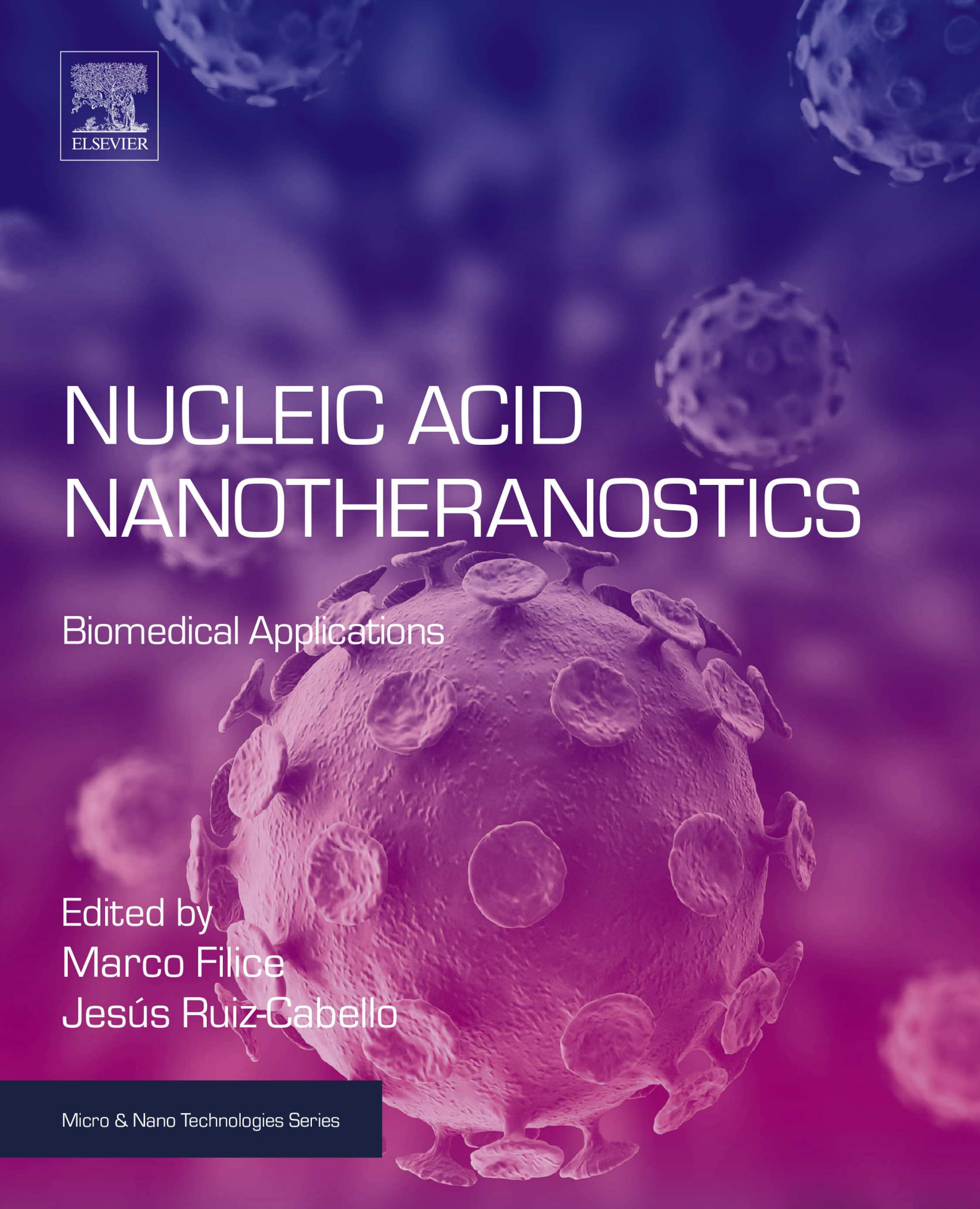
Annex I: K^+ channels biophysical & pharmacological properties

Biophysical and pharmacological properties of the K^+ channels studied in this Doctoral Thesis.

Protein	Gene & Location	Structure	β-subunits	Biophysical properties	Pharmacological properties		References (PMID)
					Activators/agonists (IC50)	Inhibitors/antagonists (IC50)	
Kv1.5 potassium voltage-gated channel subfamily A member 5	*KCNA5* 12p13.32	6TM, 1PORE,Kv	β1.2, β1.2 and β2.1: Shift the steady-state activation and inactivation curves to more negative potentials β1.3 induces a fast and incomplete inactivation of the current β3 rapid inactivation	$V_{0.5}$=-14 τ_a= <5 τ_d=23 G=8 P_o=0.8		Diphenyl phosphine oxide (0.31µmol/L) kaliotoxin (0.65 nmol/L) ShK toxin (11 pmol/L) Psora-4 (3 nmol/L) PAP-1 (2 nmol/L) TEA (330 mmol/L) 4-AP (0.27 mmol/L) correolide (1.1 µmol/L) Psora-4 (7.7 nmol/L) PAP-1 (45 nmol/L)	20500153 31459020 18485768 22426702 27796577 22426705 30851197 21159668
Kv7.1 potassium voltage-gated channel subfamily Q member 1	*KCNQ1* 11p15.5-p15.4	6TM, 1PORE,Kv	KCNE1 (++) KCNE2 (+*) KCNE3 (+*) KCNE4 (--) KCNE5 (++)	$V_{0.5}$=-20 τ_a=90 τ_d=456 G=1-4 P_o=0.1	ML277 (0.26µmol/L) ML213 (>10 µmol/L) R-L3 (L-364)(<1 µmol/L)	TEA (5 mmol/L) Linopirdine (40 µmol/L) XE991 (0.8 µmol/L) Chromanol 293B (0.5–63 µmol/L) HMR-1556 (120 nmol/L) L735821 (173 nmol/L) Retigabine (~100 µmol/L)	27826710 28992433 10025409 9425900 10713961 9834139 9834138 16251430 15175377 12702742 16287990 10025409 10816588 10713961 16914890 9834139
Kv7.4 potassium voltage-gated channel subfamily Q member 4	*KCNQ4* 1p34.2	6TM, 1PORE,Kv	KCNE1 (+) KCNE2 (+) KCNE3 (-) KCNE4 (++) KCNE5 (+)	$V_{0.5}$=-20 τ_a=160 τ_d=103 G=2 P_o=0.07	ML277 (>30 µmol/L) ML213 (0.5–0.8 µmol/L) Retigabine/Flupirtine (5.3 µmol/L)	TEA (3 mmol/L) Linopirdine (14 µmol/L) XE991 (5.5 µmol/L)	
Kv7.5 potassium voltage-gated channel subfamily Q member 5	*KCNQ5* 6q13	6TM, 1PORE,Kv	KCNE4 (+)	$V_{0.5}$=-40 τ_a=150 τ_d=125 G=2 P_o=0.1	Retigabine/Flupirtine (6.4 µmol/L) ML213 (700 nmol/L)	XE991 (65 µmol/L) Linopirdine (14 µmol/L)	
TASK1 potassium two pore domain channel subfamily K member 3	*KCNK3 2p23.3*	4TM, 2 PORE,K_{2P}		Voltage-independent Open/close rapidly. Mean open times (–120 mV)=0.8 ms G_{inward}=14 $G_{outward}$=10 P_o= 0.04	Halothane (0.3-0.4 mmol/L) ONO-RS-082 (7 µmol/L) pH 8.3	Anandamide (10 µmol/L) Zn^{+2} (100 µmol/L) ML365 (4 nmol/L) A293 (0.2 µmol/L) Lidocaine (222 µmol/L) pH 6.3	30851197 17652773 27826710 11053038

β-subunits: -, reduced current; + and ++ enhanced current; *, loss of time-dependence; NP, not performed; Note: variations may occur in different expression system.
Biophysical properties: $V_{0.5}$ voltage dependence activation (mV); τ_a rate of activation +40mV (ms); τ_d rate of deactivation at -60 mV; G single-channel conductance (pS); P_o,Open probability at 0mV for Kv and -120mV for TASK1 channels.

Annex II: microRNAs in Respiratory Diseases


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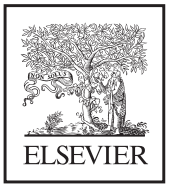
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CHAPTER

4

MicroRNAs in Respiratory Diseases

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1 WHAT ARE MicroRNAs?

Gene expression is regulated by transcriptional and posttranscriptional pathways, which are crucial for optimizing gene output and for coordinating cellular programs.[1] Whereas the roles of proteins as gene regulatory factors are well established, the functions of regulatory RNA molecules in development are just beginning to emerge. microRNAs (miRNAs) are a class of small regulatory RNA encoded in the genomes of plants and in invertebrate and vertebrate animals that have recently generated considerable excitement. miRNAs were first identified as being involved in *Caenorhabditis elegans* larval development, and today are recognized as important regulators of most evolutionary conserved processes in plants and animals. *lin-4, the first microRNA discovered,* is essential for the normal temporal control of diverse postembryonic developmental events in *C. elegans*. *lin-4* acts by negatively regulating the level of LIN-14 protein, creating a temporal decrease in LIN-14 protein starting in the first larval stage (L1).[2] Years later, it was discovered that let-7, another gene in the *C. elegans* heterochronic pathway, encoded a second 22 nucleotides (nt) regulatory RNA. The let-7 RNA acts to promote the transition from late-larval to adult cell fates. Furthermore, homologs of the let-7 gene were identified in the human and fly genomes, and let-7 RNA itself was detected in human, Drosophila, and eleven other bilateral animals.[3,4]

At present, the human genome contains over 1500 annotated miRNA genes and each one is thought to target on average more than 400 individual mRNAs.[5,6] Expression profiles of miRNAs are critically important in defining functional states of specific tissues, and their dysregulation in a number of diseases suggest their dynamic roles in maintaining homeostasis. MicroRNAs negatively regulate gene function by binding to complementary sequences of the 3′-untranslated region (3′UTR) of specific messenger RNA (mRNA). The miRNA-3′UTR interaction leads to cleavage of target mRNA or to translational repression, resulting in a decrease in the targeted protein.[3] Mature miRNAs are

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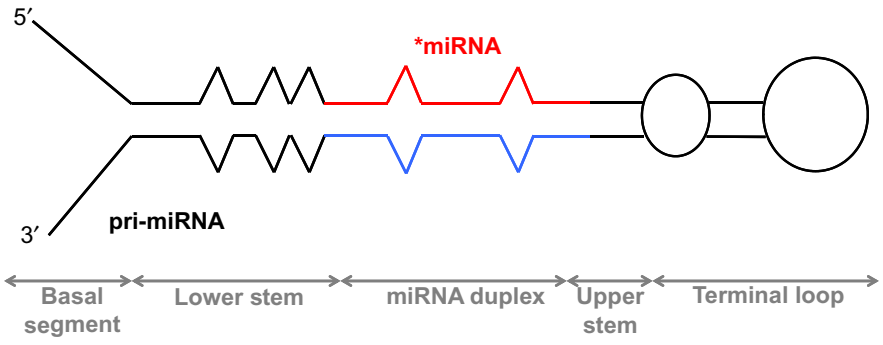


FIG. 1 Proposed stem-loops structure of the precursor or pri-miRNA is shown with the mature miRNA (guide strand, on top) and the complementary sequence (complementary strand, at the bottom).

~21 nucleotides long and excised from the precursors of 60 to 80 nucleotides double-stranded RNA fold-backs, dsRNA hairpins, or pri-miRNAs (Fig. 1).

An interesting, common feature of miRNAs is that *in silico* secondary structure prediction indicates that the ~70-nt putative precursor forms a stem-loop structure with some bulges[4,7] (Fig. 1). MiRNAs are uncapped, unpolyadenylated molecules that are processed from primary transcripts in sequential steps (see below). Each type of miRNA carries a highly conserved sequence of 7–8 nts, usually called "seed," which participates in the process of target recognition.[8,9]

2 MicroRNA NOMENCLATURE

The rules to designate miRNA sequences are[10,11]:

(1) Use the prefix "mir" followed by a dash and a number. The number designates new given sequences as they are classified as miRNAs (e.g., mir-248).

(2) Uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-1" refers to the mature (functional) form.

(3) The species of origin is designated with a three-letter prefix (e.g., hsa, *Homo sapiens*; rno, *Rattus norvegicus*; mmu, *Mus musculus*).

(4) miRNAs with nearly identical sequences are annotated with an additional lowercase letter. In the example below, conserved nucleotides within the two miRNA sequences can be appreciated.

hsa-mir548a

UGCAGGGAGGUAUUAAGUUGGUGCAAAAGUAUUGUGAUUUUUGCCAU
UAAAAGUAACGACAAAACUGGCAAUUACUUUUGCACCAAACCUGGUAUU

hsa-mir548b

CAGACUAUAUAUUUAGGUUGGCGUAAAAGUAAUUGUGGUUUUGGCCUU
UAUUUUCAAUGGCAAGAACCUCAGUUGCUUUUGUGCCAACCUAAUACUU

(5) Pre-miRNAs leading to 100% identical mature miRNAs but that are located at different sites in the genome are indicated with an additional dash-number suffix, for example:

hsa-mir-548a-1	Chromosome 6	Start position 185720150	Strand +
hsa-mir-548a-2	Chromosome 6	Start position 135560298	Strand +
hsa-mir-548a-3	Chromosome 8	Start position 105496597	Strand -

(6) When two mature miRNAs originate from opposite arms of the same pre-miRNA, they are denoted with the -3p or -5p suffix, for example:

hsa-mir-548b

CAGACUAUAUAUUUAGGUUGGCGCAAAAGUAAUUGUGGUUUUGGCC
UUUAUUUUCAAUGGCAAGAACCUCAGUUGCUUUUGUCGCCAACCUAA

hsa-miR-548-5p
AAAAGUAAUUGUGGUUUUGGCC

hsa-miR-548-3p
CAAGAACCUCAGUUGCUUUUGU

3 BIOGENESIS AND CELLULAR FUNCTIONS

This section will cover the canonical miRNA biogenesis pathway that is responsible for the maturation of most miRNA families. For simplicity we will focus on the mammalian enzyme components.[12] miRNA genes are located throughout the genome. Many miRNA genes are noncoding genes whose sole transcriptional product is the miRNA.[13] In other cases, the miRNA is located within an intron or untranslated region (UTR) of a protein-coding gene. A hallmark characteristic of miRNA genes is the stem-loop precursor RNA structure, with one strand of the stem as the source of the mature miRNA[14] (Fig. 2).

In the canonical pathway, transcription of the miRNA host gene by RNA polymerase II in the nucleus leads to the long pri-miRNA, or

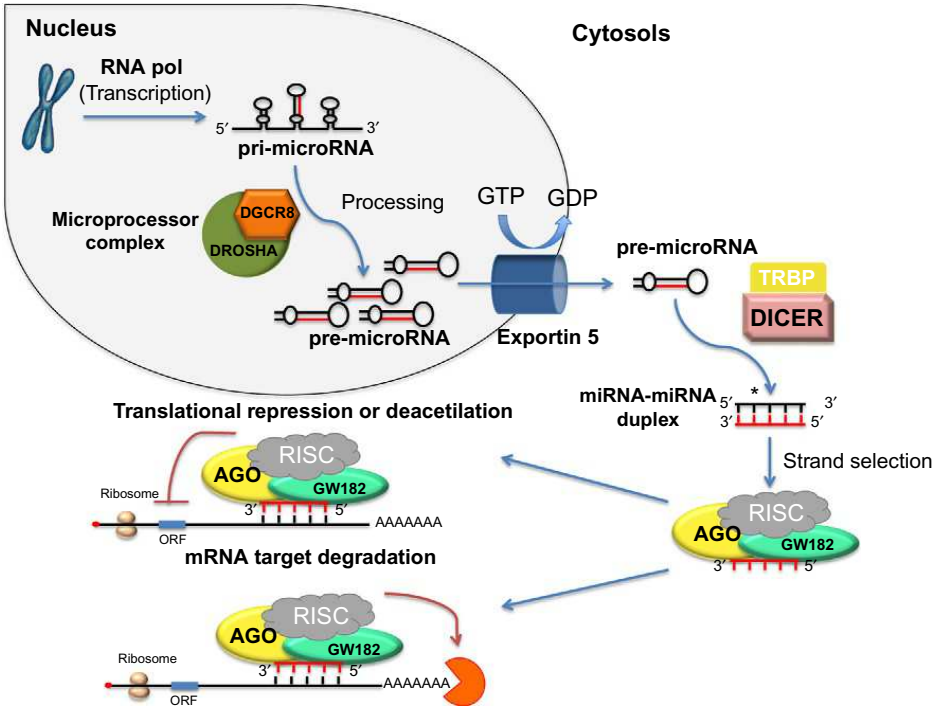


FIG. 2 Canonical pathway of miRNA biogenesis and functions of miRNAs. The biogenesis of most miRNAs is governed by the activity of RNA polymerase II (pri-miRNA) and by the subsequent processing mediated by the Drosha-DGCR8 microprocessor complex to generate the pre-miRNA. The export of the pre-miRNA to the cytoplasm occurs by means of the exportin-5-GTP-dependent process. Once in the cytoplasm, the pre-miRNA is processed by Dicer and generates the mature (double-stranded) miRNA. The mature miRNA is incorporated into the RISC-AGO-GW182 complex, where the strand (the leader 5′-3′, or the complementary 3′-5′) that will exert the regulation of the target mRNA is selected. The miRNA:miRNA* duplex gives rise to two types of sequences: the 5p sequence and the complementary 3p sequence (designated with an asterisk). The miRNA-loaded RISC is capable of binding to target mRNAs and results in the degradation of mRNA or blocking translation.

primary miRNA transcript.[15] Pri-miRNAs are single transcripts, ~100–120 nts long (in some cases up to 1000 nts), with a modified nt (7-methylguanosine triphosphate) at the 5′-end, a long adenine sequence at the 3′-end, and a 1–6 hairpin loop structure in the middle (each hairpin loop adopts a double-stranded structure and it a miRNA precursor).[10] The pri-miRNA requires two endonuclease processing steps before it becomes a mature, active miRNA. In the first one, the double-stranded RNA structure of the hairpin loop in a pri-miRNA is recognized by a nuclear protein known as DiGeorge syndrome critical region 8 (DGCR8). DGCR8 binds with Drosha (an RNase III enzyme) to form the "Microprocessor" complex. This microprocessor complex frees hairpins from pri-miRNAs by cleaving RNA. The resulting product (≈70–100 nts with a hairpin structure) has a 2-nt overhang at its 3′-end (pre-miRNA).[15,16] Pre-miRNAs bind to the nuclear export factor exportin-5 (a RanGTP-dependent double-stranded ribonucleic acid binding protein [dsRNA-binding protein]), and then are shuttled to the cytoplasm along with guanosine 5′-triphosphate (GTP) hydrolysis.[17] In the cytoplasm, the Dicer enzyme (a specific double-stranded RNA endonuclease) and the TAR RNA binding protein (TRBP) interact with pre-miRNA and cleave both extremes (the "head" of the hairpin and both endings), yielding an imperfect miRNA duplex of about 22 nts in length.[18] The miRNA:miRNA duplex gives rise to two types of sequences: the 5p sequence and the complementary 3p sequence. Although 3p miRNAs were initially considered as a waste product, they have been recently shown to play significant roles in cellular function and to be associated with human diseases.[19]

The miRNA duplex is loaded in a multiprotein complex called the RNA-induced silencing complex (RISC).[18] Each strand of the miRNA duplex has a different fate: (1) the guide strand (in most cases the 5p strand) is selected by the RNase Argonaute and integrated into RISC; and (2) the passenger strand (in most cases the 3p strand) is degraded by RISC. In particular, nucleotides 2–7 of the miRNA, termed the "seed" region, are important for target association. The 3′ end of the miRNA can also contribute to target recognition, and centrally matched targets have been identified. Once it finds the target mRNA the protein Argonaute in the RISC complex cleaves and degrades the mRNA.[20] This process is called RNA interference and is considered one of the most potent posttranscriptional mechanisms regulating gene expression. However, for some miRNAs, both strands may be loaded into RISC at similar frequencies. In this case, the strand from the 5′ end of the stem-loop is termed "5p" and the 3′ strand the "3p." In fact, while RISC loading may strongly favor incorporation of one strand, recent next generation sequencing (NGS) data have demonstrated a small fraction of star strand loaded for essentially all miRNA families.[21] Next, the AGO-miRNA complex assembles into a larger structure known as the RNA-induced silencing complex (RISC), located in cytoplasmic P-bodies and stress granules, which targets the miRNA to the 3′ UTR of an mRNA.[22,23] Argonaute recruits the GW182 complex that facilitates removal of the poly(A) tail and eventual degradation of the mRNA target.[24] miRNAs interact with their targets in different ways:

- Direct miRNA-mRNA interaction:
  - **(i).** Translational repression or deacetylation. Partial miRNA-mRNA pairing usually results in reduced protein expression through a variety of mechanisms that involve mRNA degradation and translational repression.[25]
  - **(ii).** mRNA target degradation. Complete miRNA-mRNA pairing leads to target degradation by RISC.
- Indirect miRNA-mRNA interaction:
  - **(i).** Modulation of the "global" methylation pattern.
  - **(ii).** Targeting transcription factors.

The expression of miRNA is highly regulated. Some of the different mechanisms for the

regulation of miRNA expression are the following: transcription factors, inflammatory stimulus, posttranscriptional modification, processing disturbances, epigenetic modification, and DNA alterations. A single miRNA may target multiple sites within a single 3′ UTR and often regulates multiple genes within the same signaling network. Moreover, the high evolutionary conservation of miRNAs across eukaryotes and the redundancy of miRNA genes, as exhibited by families of miRNAs possessing the same targeting sequence, suggests their vital role in controlling biological processes.[26] It is predicted that evolutionary constraints maintain miRNA-mRNA pairing in greater than 60% of human genes, while more than 84% of protein output is miRNA-controlled.[27] Since their discovery almost 20 years ago, the expression of different microRNAs has been associated with different cellular functions, such as fat metabolism, insulin secretion, epigenetics regulation, development, cell proliferation, apoptosis, and cell differentiation (Fig. 3).

4 MicroRNAs AS BIOMARKERS

There is a need to find biomarkers that reveal the different aspects of complexity and heterogeneity characteristic of respiratory diseases and help in the diagnosis and prognosis of the disease. Although, in recent years, a large number of studies have been carried out with the aim of identifying new biomarkers, very few have reached clinical practice, mainly due to the lack of validation. Mature miRNAs are known to be involved in global cellular processes, such as differentiation, proliferation, apoptosis, and organogenesis. Excessive or defective expression of specific miRNAs has been correlated with a number of clinically important diseases, highlighting their potential usefulness as diagnostic and prognostic tools. Due to their

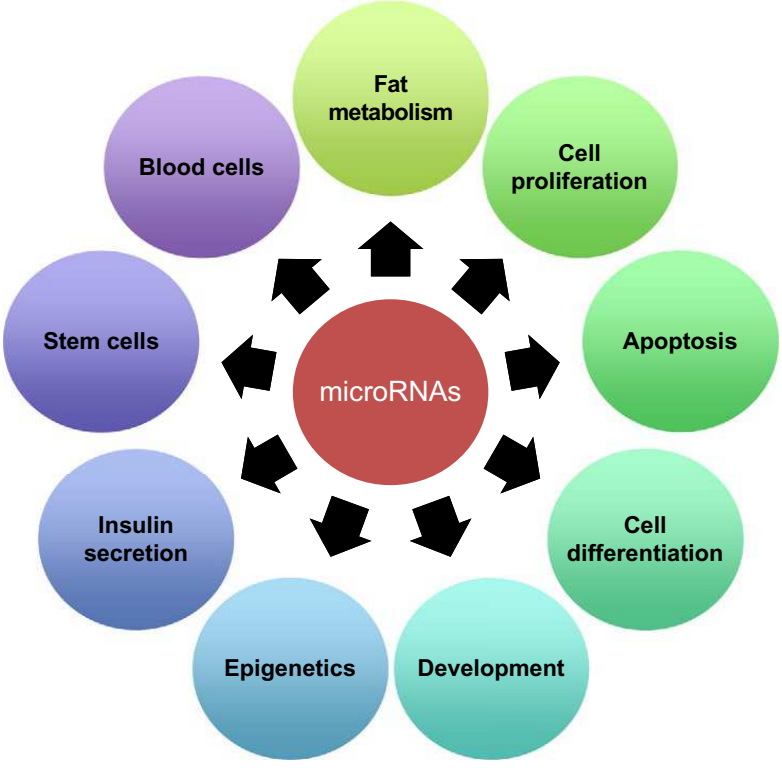


FIG. 3 microRNAs regulate a number of cellular functions by inhibiting the translation or degradation of the target microRNA implicated in different pathways.

inherent ability to repress multiple gene targets simultaneously, miRNAs have been postulated as ideal candidates to orchestrate an integrated control of these diseases.

Although first reported decades ago, the possibility that extracellular RNA could survive in the blood was not widely accepted because plasma contains potent ribonucleases that should, in theory, destroy any free RNA.[28] However, the existence of circulating RNA is a remarkable finding because RNA is more labile than DNA and ribonuclease is known to be present in blood. Recently, the presence of circulating extracellular RNA in serum and plasma has been documented and it has also been shown that this RNA is somehow protected from degradation by plasma ribonucleases.[29] At present, the exact mechanisms that protect circulating RNA are still unclear. The circulating miRNA may be complexed to lipids, exosomes,[30] lipoproteins, or phospholipids bound with DNA in nucleosomes[31]; or protected within apoptotic bodies[32] or other vesicular structures. Circulating miRNA can be captured by distant cells in which they execute regulatory functions.

In the last decade, different studies have shown that miRNAs could stably subsist extracellularly and be detected in body fluids, such as blood, sputum, saliva, and breast milk, suggesting their potential as disease biomarkers. The differential cellular expression patterns of miRNAs observed in a number of chronic lung diseases suggests that profiling extracellular miRNAs could provide a less invasive diagnostic and prognostic approach. One example is a study analyzing circulating miRNAs as potential biomarkers for idiopathic pulmonary fibrosis (IPF), in which the expression profile in the serum collected from patients with IPF and healthy controls were compared. The levels of miR-21 and miR-155 were significantly elevated in IPF patient serum with respect to the control.[33] The upregulation of serum miR-21 was confirmed as a predictor of poor prognosis by two other groups.[34] Another study identified that let-7d was significantly downregulated in serum from IPF patients compared with healthy controls[35] and this was also associated with acute exacerbation of IPF and a rapid decline in lung function.[36]

4.1 MicroRNA Detection

In order to establish microRNAs as potential biomarkers, there must be specific, diagnostically sensitive, and reproducible detection methods. One of the most used techniques at present is the microRNA detection by quantitative reverse transcription-PCR (RT-PCR) in homogenate of different tissues, plasma, or serum.[37] Optimal RNA isolation and purification is necessary to achieve consistent and reproducible miRNAs level detection. However, most studies can only isolate RNA from 50 to 500 μL of plasma/serum or tissue, and this probably poses difficulties in extending the method for RNA isolation from smaller volumes.[38] Recently, serum-based miRNA signature (miR-test) has been developed, which is a serum-based test that measures a signature of 13 miRNAs,[39] and a plasma-based miRNA test (MSC) that categorizes patients into low, intermediate, or high risk of disease based on predefined positivity for 24 miRNA expression ratios.[40]

A breakthrough in the detection of microRNAs has also been achieved through the use of an ultrasensitive optical biosensor based on the electrostatic absorption of the microRNA on the surface of gold nanoparticles with positive charge.[41]

5 MicroRNAs AS THERAPEUTIC TOOLS: MicroRNA MIMICS AND ANTAGOMIRS

As indicated above, circulating or tissue microRNAs are considered as potential biomarkers of respiratory diseases. In addition, it is becoming increasingly apparent that the aberrant expression of miRNAs is causally related to a variety of lung diseases. The upregulation

of miRNAs can lead to the downregulation of a set of targeted mRNAs, whereas downregulation of miRNAs can result in upregulation of target mRNAs because of the loss of tonic inhibitory control of the miRNA on its target mRNA. Ultimately, it is the pattern of miRNA-induced gene expression that contributes to the resultant disease phenotype.[42] The apparent roles of miRNAs in respiratory disease pathogenesis, as well as in promoting lung health, have led to an increasing interest in miRNA regulation as a novel potential therapeutic strategy designed to target miRNA function. Moreover, miRNA expression is generally tissue specific so their modulation could provide therapeutic benefit with limited adverse effects on healthy cells.[43]

The potential relevance of miRNAs in the development of diseases and the ability to manipulate them in vivo provides a unique opportunity to exploit miRNAs therapeutically. However, miRNA-based therapeutics pose a different set of challenges from those associated with classic drugs (Table 1).

Antisense technology and gene therapy approaches can be adapted to manipulate miRNA levels in vivo. Depending on the context of miRNA function in disease, two approaches are currently available and employed to modulate selectively miRNA activity (Fig. 4). The first one is to restore the function of a miRNA using miRNA mimics to elevate the levels of miRNAs with beneficial functions (Fig. 4A). The second one is to inhibit the function of a miRNA using modified antisense oligonucleotides targeting the mature miRNA sequence, antagomiRs, to reduce the levels of pathogenic or aberrantly expressed miRNAs (Fig. 4B).

It is important to mention that miRNAs act as inhibitors of gene expression. Thus, the consequence of adding a specific miRNA mimic to a system is the decrease in mRNA expression controlled by the miRNA. On one hand, the addition of a specific miRNAs inhibitor would result in relieving the inhibition of the genes normally targeted by the miRNA.[42] Thus, the primary effect of a miRNA inhibitor is activation of gene expression, whereas that of a miRNA mimic is suppression of gene expression.

TABLE 1 Advantages and Potential Challenges of miRNA-Based Therapeutics Compared With Conventional Drug Approaches

Advantages	Potential Challenges
• miRNAs regulate multiple components of the same pathway/cellular process • Long-lived, sustained effects • The target miRNA is defined • Effective in vivo regulation of the miRNA	• Delivery of the miRNA modulator. • Off-target effects of unintended or unidentified targets of a miRNA • A single miRNA can have both beneficial and pathogenic effects • Toxicity of the miRNA modulator

5.1 Restoring miRNA Function: miRNA Mimics

Under conditions in which a reduction in miRNA level is associated with disease development, an increase in the concentration of that specific miRNA could be a therapeutically beneficial approach. One strategy is to use synthetic RNA duplexes that harbor chemical modifications to improve stability and cellular uptake, such as the addition of a cholesterol[44] or the conjugation to *N*-acetyl-D-galactosamine.[45] In such double-stranded miRNA mimics, the strand identical to the miRNA of interest is the guide (antisense) strand, while the opposite (passenger or sense) strand is less stable and can be linked to a molecule, such as cholesterol, to enhance cellular uptake.[46]

In addition, the passenger strand may contain chemical modifications to prevent RISC loading. Care must be taken in the design of such chemical entities because the passenger strand may act as a new miRNA, which can lead to erroneous results and/or misinterpretation of the data.

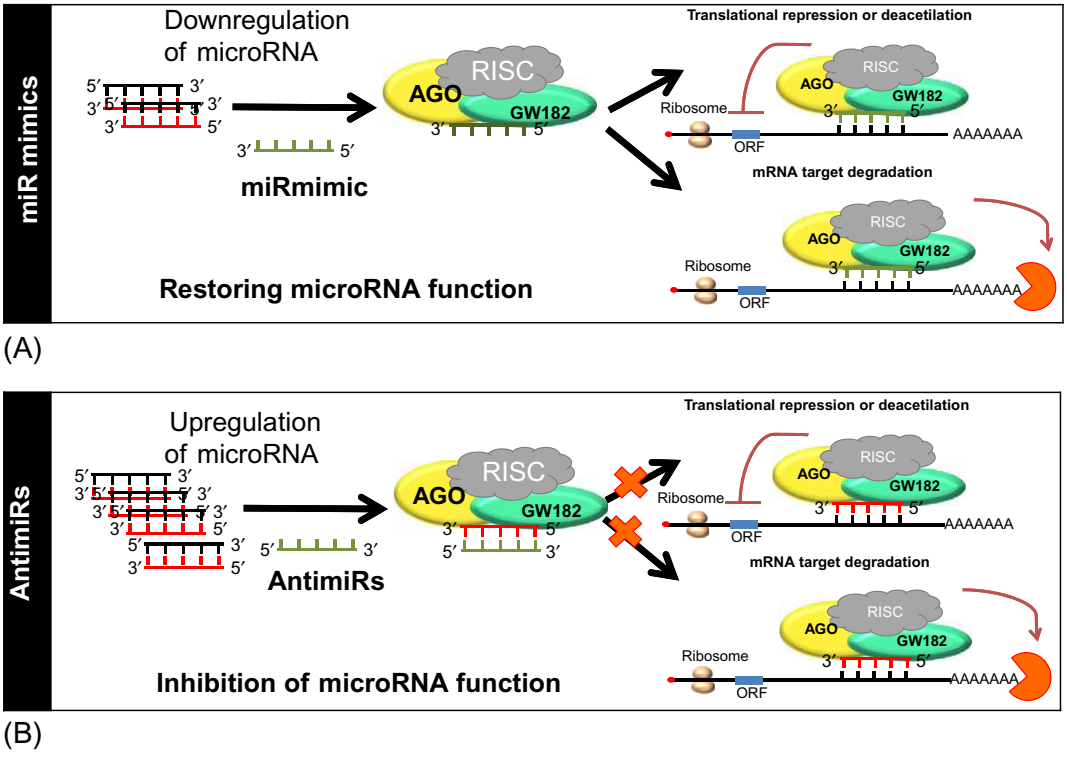


FIG. 4 MicroRNAs as therapeutic tools: microRNA mimics and antagomirs. (A) miRNA mimics are short double-stranded oligonucleotides or single-stranded with necessary modifications in order to be recognized by RISC. As may be observed, miRNA mimic is identical to the mature miRNA sequence (guide strand). Subsequent to the efficient loading of the guide strand in the RISC complex, the endogenous miRNA can replace the levels of the miRNA of interest and block gene expression. (B) AntimiRs or antimiRs are single-stranded, antisense oligonucleotides designed to inhibit miRNA function. The perfect sequence complementarity allows the antisense oligonucleotide to bind to the miRNA loaded on the RISC-AGO-GW182 complex and interfere with miRNA function.

Thus, bioinformatic and chemical modification approaches are required to ensure that only one strand is used.[47] Because the miRISC needs to recognize the guide strand as a miRNA, the chemical modifications that can be used are limited. To prevent degradation by RNases and increase targeting, chemical modifications to the RNA can be introduced, including locked nucleic acids (LNA),[48] 2′-fluoro (2′-F) modification,[49] and the addition of 2′-*O*-methyl groups.[50] Examples of potential mimic designs are shown in Fig. 5. Unfortunately, miRNA mimic therapy may have a number of limitations. Thus, double-stranded miRNA mimics can potentially induce a nonspecific interferon response through Toll-like receptors.[51] Another issue with miRNA replacement therapies is the challenge of restoring the level of a downregulated miRNA, while preventing the introduction of supraphysiological levels of the miRNA.[43] Additionally, systemic delivery of miRNA mimics can also result in uptake by nontarget tissues that normally do not express the miRNA of interest, resulting in off-target effects.

5.2 Inhibition of miRNA Function: AntagomiRs

Efficient silencing in vivo of dysregulated miRNAs requires chemical modification of the antagomiRs oligonucleotides to improve their binding affinity, biostability, and pharmacokinetic properties. Additionally, because the expression level of a given miRNA varies greatly depending

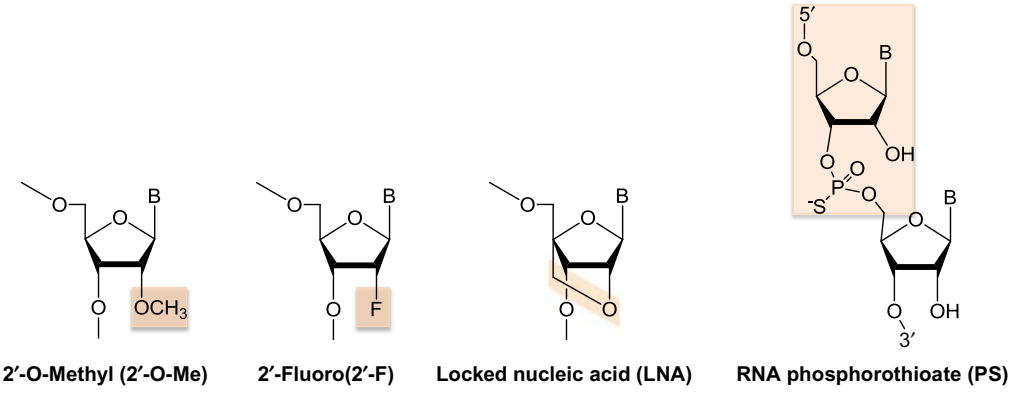


FIG. 5 Chemical modifications introduced into the microRNA to prevent degradation by RNases and increase targeting. Example of modifications for miRNA mimic designs are 2′-*O*-Methyl, 2-Fluoro, and Locked nucleic acid. An example of an antagomiR is the modification of the phosphate backbone with PS linkages.

on the cell or tissue type, as well as disease stage, extensive preclinical studies in animal models are needed to determine the optimal level of miRNA inhibition. A reduction of an upregulated miRNA and with a causal role in a pathological condition would be therapeutically desirable. Inhibition of miRNA activity can be achieved through the use of antagomiRs, chemically modified single-stranded reverse complement oligonucleotides. The synthetic reverse complement oligonucleotide approach can theoretically act at multiple levels to affect miRNA levels[42]:

I. Binding to the mature miRNA within the RISC and acting as a competitive inhibitor.
II. Binding to the pre-miRNA and preventing its processing or entry into the RISC.
III. Interfering with the processing or export of the pre- or pri-miRNA from the nucleus.

In summary, the result of the antagomiR application is a reduction in the concentration of a specific miRNA-programmed RISC.

5.3 Delivery of miRNA Modulators

RNA activity and safety in humans is restricted by stability, cellular uptake, target specificity, and immune activation. Therefore, advances in miRNA therapeutics are limited by the availability of specialized delivery technologies. Thus, targeted delivery of miRNA mimics or antagomiRs to the appropriate cell or tissue type is going to be important to prevent unwanted off-target effects using this therapeutic approach. The main challenge for the development of miRNA-based therapeutics is to achieve an efficient and safe delivery of miRNA mimics and antagomiRs. Two strategies have been used to deliver miRNA replacement therapies in vivo:

I. Formulated, synthetic, or double-stranded miRNA mimics.
II. Viral constructs over-expressing the lost or downregulated miRNA.[52]

Intravenously and intratumorally injected miRNA mimics conjugated with nanoparticles, polyethyleneimine, or atelocollagen have been used to restore the functions of various tumor-suppressive miRNAs in mouse cancer models.[53] Furthermore, intranasally administered lentivirus-based expression constructs were utilized to deliver tumor-suppressive miRNAs in vivo.[54]

In addition, two main strategies have been utilized to enhance in vivo delivery of antagomiR oligonucleotides:

I. Cholesterol conjugation.
II. Modification of the phosphate backbone with PS linkages (Fig. 5).

It has been shown that administration of such antagomiRs in mice, either by intravenous, intraperitoneal, or subcutaneous injections resulted in antagomiR uptake in the tissue of interest, leading to inhibition of miRNA function and derepression of direct target mRNAs[55,56] Intranasal or intratracheal instillation or inhaled administration may represent a more selective way to target the lung in respiratory diseases.[57–59] At present, different clinical trials are being carried out with both miRNA mimics and antagomiRs to treat several conditions, but clinical trials for testing their potential in respiratory diseases are still missing.

6 MicroRNAs IN RESPIRATORY DISEASES (UNREGULATED MicroRNAs AND MAIN TARGETS)

Most data from miRNA research in the lung suggest that their primary function is to fine-tune gene expression and maintain signaling pathway homeostasis. The dysregulation of multiple miRNAs and pathways may play a significant role in respiratory disease phenotypes and progression. In addition, the potential therapeutic usefulness of miRNAs mimics or antagomiRs in respiratory diseases are also being studied. Although many advances have been achieved in this field during the last decade, better tools are still needed to fully understand the functional implications of miRNAs in lung health and disease. Importantly, specific miRNAs play a key role in maintaining lung homeostasis, the alteration of which underlies a range of pulmonary disorders. Thus, in this section, we will discuss the link between dysregulation of miRNAs and the pathogenesis of different respiratory diseases. We also highlight the miRNAs with the best characterized potentials as diagnostic, prognostic, or therapeutic tools.

We will focus on several relevant respiratory diseases because of their high prevalence or their severity in terms of morbidity and mortality, such as Pulmonary Hypertension (PH), Chronic obstructive pulmonary disease (COPD), asthma, lung cancer, Idiopathic pulmonary fibrosis (IPF), Bronchopulmonary dysplasia (BPD), Obstructive Sleep Apnea (OSA), Cystic Fibrosis (CF), and Acute Respiratory Distress Syndrome (ARDS).

6.1 Pulmonary Hypertension

The clinical problem. Pulmonary hypertension (PH) refers to a heterogeneous group of diseases, clinically defined by mean pulmonary arterial pressure (PAP) of at least 25 mmHg at rest.[60] It is a disorder with a significant burden in terms of both severity and prevalence. For instance, some forms of PH, namely pulmonary arterial hypertension (PAH) and chronic thromboembolic PH (CTEPH), are rare diseases but entail extremely poor prognosis and there is no known cure, except for transplantation. The mean survival of patients with PAH (<4 years) is lower than that of a patient with metastatic breast cancer (Stage III) while the cost of therapy for a patient with advanced PAH is much higher.[61] PAH affects young and middle-aged persons, preferentially women, exerting substantial impact on their quality of life, daily life activities, and employment.[62] On the other hand, PH may also develop in prevalent chronic respiratory diseases, particularly in COPD and interstitial lung diseases, as well as in acute respiratory failure. In some of these conditions PH may be highly prevalent, affecting more than 50% of patients with advanced disease, and its presence is associated with poorer outcomes—survival, exacerbations—and increased use of health resources.[63]

Pathophysiology. PAH is a severe and progressive vascular disease largely restricted to small pulmonary arteries (PA) that results in increased pulmonary vascular resistance (PVR), right heart failure, and ultimately death. Although the molecular origins are still poorly defined, sustained pulmonary vasoconstriction,

excessive pulmonary vascular remodeling, and thrombosis in situ are considered the three major causes of elevated PVR in patients with PH.[64] PH is often considered a proliferative disorder and some similarities with cancer have been highlighted. Factors leading to cell proliferation include inflammatory abnormalities, bone morphogenetic protein receptor mutations, overexpression of growth factors, and metabolic changes. The excessive vasoconstriction and proliferation occur as a result of a pathologic imbalance of complex molecular pathways in different vascular cell types, including pulmonary arterial endothelial cells (PAECs) and smooth muscle cells (PASMCs).

The expression profile of circulating microRNAs is different in animal models and PAH patients compared with healthy volunteers. Herein, we summarize some of the main microRNAs dysregulated in PH.

6.1.1 *Upregulated MicroRNAs*

miR-1. The expression of miR-1 is increased in lungs from the Hyp/Su5416 PAH rat model. PASMCs from this animal model are more depolarized and show decreased expression and activity of the voltage-dependent potassium channel Kv1.5. miR-1 directly targets Kv1.5 channels, reduces Kv1.5 activity and induces membrane depolarization. Antagomir-1 prevents the effects induced by exposure to hypoxia/Su5416.[65] miRNA-1-2, extracted from serum, is increased in idiopathic pulmonary arterial hypertension (iPAH) compared with healthy controls.[66] In addition, miR-1 is upregulated in peripheral blood mononuclear cells in patients with essential hypertension.[67] However, other reports have found decreased miR-1 in PA as mentioned below.

miR-9. miR-9 has been shown to be upregulated by hypoxia in rat PASMC.[68] Overexpression of miR-9 in normoxia promoted a proliferative phenotype with increased expression of contractile genes in vascular smooth muscle cells, while its inhibition attenuated the proliferation of PASMCs induced by hypoxia. The increase of miR-9 during hypoxia appears to be HIF-1α-dependent as several putative HIF-1α binding motifs are found in miR-9.[68]

miR-17-92. It has been demonstrated that members of the miR-17-92 cluster are significantly altered in experimental PH.[69,70] This was associated with an inhibition of the miR-17 target PDZ and LIM domain 5 (PDLIM5) and subsequent induction of the TGF-β3/SMAD3 pathway[70] and reduced p21 expression and increased proliferation of smooth muscle cells.[69] Moreover, in vivo administration of miR-17 antagomiR induced protective effects in hypoxia-induced PH in mice and in monocrotaline-induced PH in rats.[69] Similar protection was reported in MC-specific miR-17∼92 knockout mice.[70]

miR-21. miR-21 is upregulated in pulmonary tissue from several rodent models of PH and in humans with PH.[71] This is associated with a reduced expression of bone morphogenetic protein receptor type 2 (BMPRII), which plays a protective role in PH.[72] Moreover, administration of an antagomir-21 reduced right ventricular systolic pressure, and decreased pulmonary arterial muscularization in the hypoxic PH mouse model.[69] However, the involvement of miR-21 in PH appears to be complex because miR-21 deletion is not associated with protection, but even with exaggerated manifestation of PH, in the chronic hypoxia plus SU5416 mice model of PH.[71] In contrast, other reports have found decreased miR-1 in AP as mentioned below.

miR-27a/b . The exposure to hypoxia (10% O_2) for 3-weeks increased levels of miR-27a and ET-1 and reduced peroxisome proliferator-activated receptor (PPAR)-γ levels in the lungs of C57BL/6 mice and in human pulmonary arterial endothelial cells (hPAEC).[73] In addition, miR-27b expression is upregulated in MCT-induced PAH and inversely correlated with the levels of (PPAR)-γ, and miR-27b inhibition attenuated MCT-induced endothelial dysfunction and remodeling and prevented PAH associated right ventricular hypertrophy and systolic pressure in rats.[74]

miR-130/301. The expression of miR-130a was found to be upregulated in the circulation of PH subjects.[75] The miR-130/301 family is a master regulator of cellular proliferation in PH via regulation of subordinate miRNA pathways. Pulmonary vessels and plasma from mammalian models and human PH subjects exhibited upregulation of miR-130/301 expression. In endothelial cells, miR-130/301 modulated apelin-miR-424/503-FGF2 signaling, while in smooth muscle cells, miR-130/301 modulated STAT3-miR-204 signaling to promote PH-associated phenotypes.[76]

miR-143/145. One of the most studied miRNA expressed by vascular smooth muscle cells (VSMC) is the miR-143/145 cluster, which has a critical role in VSMC differentiation.[77] The miR-143/145 cluster is related to the development of plexiform lesions (PL) and concentric lesions (CL) in lungs with PAH. The expression of miR-143/145 and its target proteins (e.g., myocardin, smooth muscle myosin heavy chain) were significantly higher in CL than in PL. These data show that morphologic changes of plexiform vasculopathy in the end-stage PAH lung are reflected by alterations at the miRNA-143/145 expression level.[78]

miR-190. Hypoxia produces a significant increase of miR-190 in the pulmonary artery (PA). The use of a synthetic miR-190 can improve the vasoconstriction responses to phenylephrine (PE) and KCl. The member of the voltage dependent K^+ subfamily, Kcnq5, is a target for miR-190. Meanwhile, miR-190 antisense oligos can partially reverse the effects of miR-190 in PASMCs.[79]

miR-210. miR-210 was shown to be a predominant miRNA induced by hypoxia in human PASMC.[80] Furthermore, inhibiting miR-210 expression increased mitogen-activated protein kinase phosphatase 1 (MKP-1), a protein involved in inflammation and proliferation,[81] in hPASMCs and decreased cell proliferation under hypoxia.[82] In addition, White et al. reported that miR-210 targets ISCU1/2, causing deficiencies in iron-sulfur (Fe-S) clusters in vivo and promoting PH.[83]

miR-361. Hypoxia has been shown to elevate miR-361-5p levels in pulmonary artery smooth muscle cells (PASMC)[84] in an HIF-1α-dependent manner. In addition the inhibition of this miRNA suppressed PASMC survival and migration by targeting ABCA1 and inhibiting the JAK2/STAT3 pathway.[84]

miR-424. The levels of circulating miR-424 (322) are higher in PH patients when compared with healthy subjects. In the monocrotaline rat model of HP, an association was found between the circulating levels of miR-424(322) and the stage of right ventricular hypertrophy of the right ventricle, as well as an inverse correlation between the levels of miR-424(322) and SMURF1 in the hypertrophied right ventricle.[85]

6.1.2 *Downregulated MicroRNAs*

miR-1. miR-1 has been shown to be reduced in hypoxic pulmonary hypertension.[86] miR-1 downregulation led to the upregulation of its target sphingosine Kinase 1 (SphK1), which plays a role in pulmonary vascular remodeling. In addition the in vivo administration of miR-1 prevented the development of this pathological condition in mice.[87] Likewise, the expression of miR-1 was downregulated in the buffy coat of a cohort of pulmonary hypertension patients.[75]

miR-21. Human lung tissue and serum from patients with idiopathic PAH have downregulated miR-21 levels. Interestingly, a reduction of miR-21 was found in the rat model of PAH induced by monocrotaline, but not in the chronic hypoxic rat model.[88] These authors also reported reduced levels of miR-22, miR-30, Let-7a, and Let-7f in both animal models of pulmonary hypertension.[88]

miR-100. Mammalian Target of Rapamycin (mTOR) plays a key role in the proliferation and survival of pulmonary artery smooth muscle cells (PASMCs) in PH patients and animal PH models.[89] Of note, an upregulation of mTOR in the rat model of hypoxic PH has been associated with the downregulation of miR-100.[90]

miR-124. PAH is associated with cellular and mitochondrial metabolic dysfunction, which appears to underlie the pathology of this disease.[91] Overexpression of miR-124 or siRNA silencing of PTPB1 restored normal proliferation and glycolysis in PAH blood outgrowth endothelial cells, which recapitulate the metabolic abnormalities observed in pulmonary artery endothelial cells from patients with PAH.[92] Likewise, downregulation of miR-124 dysregulates glycolytic genes in the rat SUGEN-hypoxia model of severe PAH. Additionally, miR-124 overexpression reversed the glycolytic phenotype and rescued mitochondrial reprogramming, and decreased cell proliferation in PH fibroblasts.[93]

miR-140. miR-140 expression is known to be reduced in both PAH patients and in the monocrotaline-induced PAH rat model.[94]

miR-150. miR-150 levels were significantly reduced in circulating microvesicles from patients with PAH and the lungs of the monocrotaline rat. Remarkably, reduced circulating miR-150 levels are associated with poor survival in PAH.[95]

miR-193. Sharma et al. have recently reported that miR-193 is downregulated in the lung tissue and serum from both patients with pulmonary arterial hypertension and animals with PH. Notably, miR193 overexpression in the lungs rescued preexisting PH and resulted in downregulation of lipoxygenases and insulin-like growth factor-1 receptor.[96]

miR-204. Courboulin et al.[97]reported that miR-204 expression is downregulated in PASMCs in both human and rodent PAH and the administration of synthetic miR-204 significantly reduced the severity of PAH.[97] Interestingly, the master miR-130/301 has been involved in the miR-204 downregulation observed in PH-associated phenotypes though the activation of STAT3.[76] Direct modulation of Forkhead box M1 (FOXM1) has been involved in the pathophysiological role of miR-204 in PAH.[98] FOXM1 is a transcription factor known to positively regulate the expression of genes essential for DNA repair, cell cycle progression, and cell survival, all of which are important features of PAH.[99]

miR-206. miR-206 has been suggested to be a regulator of proliferation, apoptosis, and differentiation of PASMCs, and could potentially be used as a novel pharmacological strategy in PAH.[100] Importantly, reduced miR-206 serum levels have been reported in pulmonary hypertension patients due to left heart diseases, and this is associated with increased pulmonary artery systolic pressure.[101]

miR-328. miRNA-328 was found to be downregulated in pulmonary arteries exposed to hypoxia. Moreover, transgenic mice overexpressing miRNA-328 had decreased right ventricular systolic pressure and pulmonary arteries wall thickness under both normoxia and hypoxia.[102]

miR-424. Kim et al. found that miR-424 is downregulated in PAH and induced antiproliferative effects in pulmonary artery endothelial cells.[103] In addition, the administration of miR-424 reduced pulmonary pressure in experimental models of PH.

miR-451 and miR-1246. The downregulated miRNAs (miR-451, miR-1246) in the circulation of PH human subjects are directly proportional to the degree of PH. These miRNAs may be considered as potential biomarkers for early detection of PH.[75]

miR-503. miR-503 has been reported to be downregulated in PAH[103] and may play a pathophysiological role in the disease by exerting antiproliferative effects via targeting FGF2 and FGFR1. Moreover, these authors showed that in vivo administration of a miR-503 mimic ameliorated the severity of pulmonary hypertension in three models of the disease.

6.2 Chronic Obstructive Pulmonary Disease

The clinical problem. Chronic obstructive pulmonary disease (COPD) is one of the primary causes of death globally, affecting about

64 million people. COPD is predicted to become the third most common cause of death by 2030.[104] COPD is a common, preventable, and treatable disease, characterized by chronic inflammation of the airways and lungs, persistent airflow limitation, and a progressive decline in lung function. It is correlated with significant morbidity and increased mortality, even in patients with minor air flow obstruction. Despite this, the disease is often undiagnosed and untreated until it has progressed to a point at which lung function and quality of life are severely compromised.[105] The principal method for COPD diagnosis is spirometry testing, such as postbronchodilator spirometry. Postbronchodilator forced expiratory volume in 1 s (FEV1)/forced vital capacity <0.7 or less than the lower limit of normal confirms the presence of airflow limitation, the severity of which can be measured by FEV1% predicted.[106] Cigarette smoke is the principal risk factor for developing COPD, and disease occurrence correlates with the frequency and duration of smoking. One out of four smokers 45 years or older present respiratory symptoms, with not fully reversible airflow limitations compatible with COPD and are mostly undiagnosed.[107]

Pathophysiology. The pathogenesis of COPD is based on the exacerbated innate and adaptive inflammatory immune response to the inhalation of toxic particles and gases. Many other genetic causes, such as alpha1 antitrypsin deficiency (AATD),[108] and environmental factors, such as atmospheric pollution, contribute to the pathology of COPD. The immune inflammatory changes associated with COPD are linked to a tissue-repair and remodeling process that increases mucus production and causes emphysematous destruction of the gas-exchanging surface of the lung. Inflammation, fibrosis of the bronchial wall and excess mucus, produced by hypertrophy of submucosal glands, obstruct air passage and an imbalance of the ventilation/perfusion ratio. The destruction of alveolar tissue decreases the surface of gas exchange and the loss of elastic fibers leads to the collapse of the airways. This loss of pulmonary elasticity accompanied by air spaces distal to the terminal bronchi and the destruction of alveolar walls and capillary beds leads to the formation of emphysema. The disorganization of elastin is produced by protease enzymes secreted by polymorphonuclear leukocytes, macrophages, and CD8 + T lymphocytes.

To evaluate the involvement of miRNAs in COPD, many recent studies have identified panels of dysregulated miRNAs in patients or in rodents exposed to tobacco smoke.[109] Most of the differentially expressed miRNAs in bronchial epithelial cells from current smokers, compared with those who never smoked, were downregulated in smokers.[110] Decreases in global microRNA abundance were also observed in populations of alveolar macrophages from cigarette smokers.[111]

6.2.1 *Upregulated MicroRNAs*

miR-15b. An increase in the expression of miR-15b was found in COPD samples with obstruction compared with smokers without obstruction and this increase in expression was located in areas of emphysema and fibrosis.[112] Expression of SMAD7, which was validated as a target for *miR-15b and* inhibits the TGF-β signaling pathway, was decreased in bronchial epithelial cells in COPD.[112]

miR-101. Hassan et al. demonstrated that miR-101 is highly expressed in lung samples from patients with severe chronic obstructive pulmonary disease (COPD) when compared with control patients.[113] The miR-101 expression directly targeted the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) 3′UTR and suppressed the expression of the CFTR protein, which is a chloride channel that plays a critical role in the lung by maintaining fluid homeostasis.[113]

miR-135b. A study in mice exposed to smoke found that the abundance of miR-135b increased in the respiratory tract cigarette smoke in an interleukin 1 receptor (IL-1R1)-dependent

manner.[114] Induced expression of miR-135b acts to signal the presence of danger and initiates the inflammation. miR-135b can bind to its own regulator (IL-1R1) in a negative feedback loop, as well as its downstream effector Caspase-1, in an attempt to stop further expansion of the inflammatory process.[114]

miR-199a. It has been reported that the expression of the hypoxia-inducible factor-1α (HIF-1α) is reduced in lungs from patients with COPD[115] and the increase of miR-199a-5p expression is strongly associated with more severe disease and with lower levels of Hif-1α.[116] In contrast, another study found that miR-199a-5p is reduced in these patients as mentioned below.[117]

miR-223. Reduced activity of histone deacetylase 2 (HDAC2) has been described in patients with COPD, but the mechanisms resulting in decreased expression of this important epigenetic modifier remain unknown.[118] miR-223 was upregulated in human pulmonary artery endothelial cells (HPAEC) from the lungs of smokers and this increase was correlated with decreased Histone deacetylase 2 (HDAC2) expression, which led to elevated expression of a pro-inflammatory chemokine, CX3CL1.[119]

6.2.2 *Downregulated MicroRNAs*

miR-30. In lung tissue from COPD patients, it was downregulated.[110] A previous study reported that it specifically inhibits the expression of hypoxia inducible factor HIF2α.[120]

miR-34a. *miR-34c* expression is downregulated in COPD patients compared with controls, and also in moderate emphysema patients, compared with mild emphysema.[121] The expression of miR-34c was correlated with SERPINE1 levels, a gene that encodes a protease inhibitor that is sensitive to TGF-β and it is involved in the pathogenesis of COPD.[122]

miR-128. In the analysis of whole-genome miRNA and mRNA expression in bronchial airway epithelium from current and never smokers, it was found that mir-218 expression is reduced in primary bronchial epithelium exposed to cigarette smoke condensate (CSC),[109] and alteration of mir-218 levels in these cells diminishes the induction of the predicted mir-218 target MAFG (v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog G)[123] in response to CSC.

miR-146b. In one study it was observed that miR-146a levels were reduced after cytokine treatment in fibroblasts of patients with COPD compared with non-COPD smokers.[124] miR-146a caused degradation of COX-2 mRNA, which is expected to reduce PGE_2.

miR-199a. miR-199a-5p was found to be reduced in patients with COPD compared with unaffected smokers and may be involved in modulating the adaptive immune balance in favor of a Th1 and Th17 response.[117]

miR-218. miR-218-5p was found to be significantly downregulated in lung tissue of smoke-exposed mice and humans.[125] The same group also demonstrated that cell-free bronchoalveolar lavage (BAL) supernatant of mice exposed to air or cigarette smoke (CS) for 4 or 24 weeks had a decrease in miR-218 levels and this can correlate inflammation in BAL.[126] Moreover, miR-218 acts by repressing TNFR1-mediated activation of NF-κB, which is involved in MUC5AC hyperproduction and inflammation in smoking-induced bronchiolitis of COPD.[127]

miR-503. In COPD, lung fibroblast repair functions are altered in multiple ways, including extracellular mediator release.[128] MiR-503 expression is decreased in COPD lung fibroblasts and reduced miR-503 augments VEGF release from lung fibroblasts in patients with COPD. Because VEGF contributes to disturbed vasculature in COPD, altered miR-503 production might play a role in modulating fibroblast-mediated vascular homeostasis in COPD.[129]

6.3 Asthma

The clinical problem. Asthma is a chronic inflammatory pulmonary disorder with episodic airway narrowing and remodeling and

hyperresponsiveness of the airways, induced by many types of stimuli. Environmental (pollutants and allergens) and genetic factors play a role in the pathogenesis of the disease.[130] The manifestations consist of repeated breathlessness attacks, wheezing, coughing, and chest tightness, often occurring after exposure to allergens, pollutants, infections, or drugs.[131] Spirometry is used to confirm the diagnosis. Asthma can be controlled by a combination of corticosteroids (antiinflammatory drugs) and beta-2 adrenergic agonists. Unfortunately, 10% of patients do not respond to these therapies.[132] Alternative treatments include antimuscarinic drugs, theophylline, inhibitors of leukotriene receptors (zafirlukast, or montelukast) or antibodies against IgE (omalizumab). Globally, about 300 million people are affected by asthma, and the incidence of the disease is increasing, affecting 1 in 10 children and 1 in 12 adults.[130] Individuals suffering from this condition widely range from teenage children to elderly people. According to the World Health Organization, asthma is annually responsible for over 180,000 deaths.[133]

Pathophysiology. During disease progression, different changes in the airways occur, such as bronchial smooth muscle hypertrophy, epithelial shedding, fibroblast activation, extracellular matrix deposition, and neovascularization.[134] Different asthma phenotypes have been established due to the existing interindividual heterogeneity, and in the present it comprises five different subtypes characterized by the age of onset, the presence of inflammation of type 2 T cells (Th2), the implication of eosinophilia, the level of obesity, and whether the attack is induced by exercise.[135] Chronic inflammation of the respiratory tract, bronchi, and bronchioles results in a greater contraction of smooth muscle cells. Typical changes that occur in the airways include an increase in eosinophils and thickening of the reticular lamina. The smooth muscle cells of the respiratory tract may increase in size along with an increase in the number of mucous glands. Other types of inflammatory cells are also involved, including T lymphocytes, macrophages, and neutrophils. There may also be participation by other components of the immune system, including cytokines, chemokines, histamine, and leukotrienes. The main pathophysiological characteristics of asthma are commonly associated with an abnormal response of the Th2 lymphocytes, with upregulation of proinflammatory cytokines, such as the interleukins IL-4, IL-5, and IL-13, and increased levels of circulating IgE.[136] Airway epithelial cells produce mucus, which is a major contributor to airway obstruction in fatal asthma,[137] and even in patients with stable mild and moderate asthma.[138]

miRNAs have been suggested to play roles in allergic airway inflammation,[139] even the exposure to air pollutants has the potential to dysregulate miRNA.[140] However, Williams et al.[141] did not find significant expression in miRNA profiles in airway biopsies in 227 participants with mild asthma compared with healthy volunteers. After that, Jardim et al. demonstrated that bronchial epithelial cells from mild asthmatic and healthy subjects harbor differing miRNA profiles.[142] This study only focused on one cell type, bronchial epithelial cells, while Williams and colleagues used airway biopsies from a mixed cell population. Later, in 2012, Yamamoto et al.[143] clarified that miRNA profile changes can be detected in the peripheral blood of asthmatic subjects undergoing allergen inhalation and that changes in miR-192 in peripheral blood was altered after exposure to inhaled allergen in human asthmatics. Thus, they suggested that miR-192 may be involved in the mechanisms of asthma.

Exposure to allergens is one of the important factors to induce and enhance asthma pathogenesis. Several studies have previously reported the role of miRNA in asthma using the allergen inhalation in animal models that mimic acute and chronic human asthma.[139,144]

6.3.1 Upregulated MicroRNAs

miR-9. Corticosteroid-resistant asthma occurs predominantly in patients with severe and neutrophilic asthma.[145] miR-9 has been reported to regulate corticosteroid-resistant airway hyperresponsiveness. miR-9 expression is increased in pulmonary macrophages and lung samples in the IFN/LPS-induced mouse model of steroid-resistant asthma.[146] In fact, levels of IFN and LPS are often increased in these patients.[147] Flavia et al. also showed that miR-9 is upregulated in PMNs and monocytes and it has a key role in the inflammatory response.[148]

Let7f, miR-487b and miR-181c. This group of miRs is upregulated in bronchial epithelial cells from asthmatic donors isolated via bronchoscopy. In this study,[142] Jardim et al. identified a total of 165 miRNAs by a miRNA microarray analysis in these cells. Furthermore, they found 24 miRNA whose expression was greater than or equal to twofold between these two groups. In fact, Let7f, miR-487b, and miR-181c were the three miRNAs with higher expression.[142] Inhibition of let-7 family members reduces the allergic phenotype and IL-13 expression.[149]

miR-21. Several studies reported an increase of miR-21, and it is considered as one of the most important mediators of the disease. Two independent models of allergen-induced allergic airway inflammation observed an overexpression of miR-21. In fact, miR-21 was the most highly induced in IL-13 transgenic mice.[139] It is well known that lung overexpression of IL-13, a key T helper type 2 (Th2) cell derived effector cytokine in asthma pathogenesis, induces allergic airway inflammation.[150] In this study, Lu et al. noted that miR-21 overexpression likely contributes to the action of IL-13 in the lung, and possibly asthma pathogenesis. In the same way, Hwa Young Lee et al. found an upregulation of miR-21 in a mouse model of acute bronchial asthma, and antagomiR-21 suppressed the development of allergic airway inflammation, inhibiting Th2 activation.[151] miR-21-deficient animals exhibited a reduction in eosinophilic lung infiltration, increase of IL-12/IFN, and a decreased level of Th2 cytokine IL-4.[139] Moreover, in humans with bronchial asthma, miR-21 expression increases in bronchial epithelial cells.[152] Even in children with an allergic disease, Sawant et al. found elevated levels of miR-21.[153]

mir-145. Collison et al. demonstrated an induction of miR-145 in the airway wall from an animal model of allergic airways inflammation that mimics the hallmark features of allergic asthma. Inhibition of miR-145 inhibited eosinophilic inflammation, Th2 cytokine production, airway hyperresponsiveness and mucus hypersecretion.[154] They suggested that miR-145 played a proinflammatory role for the onset of allergic airways disease, and the effects of miR-145 antagonism were comparable to steroid treatment.

miR-126. Compared with naïve mice, there was a notable upregulation of miR-126 in the airway wall tissue from animals with chronic asthma. Anti-miR-126 significantly reduced recruitment of intraepithelial eosinophils into the airways, but had no effect on the chronic inflammatory response or on airway remodeling.[155] Moreover, Mattes et al. analyzed the potential role of miR-126 in allergic asthma. This miR is rapidly upregulated in the airways after allergen exposure. Selective blockade of miR-126 suppressed the asthmatic phenotype, resulting in diminished inflammation via Th2 responses, airway hyperresponsiveness, and eosinophil recruitment. So, probably, targeting miR-126 in the airways may lead to antiinflammatory treatments for allergic asthma.[156]

miR-155. miR-155 has emerged as a critical regulator of immune cell development, function, and disease. Interestingly, miR-155 is found at low levels in most of immune cell types until it is activated by antigens.[157] In 2014, Malmhäll and colleagues explored miR-155 in the regulation of allergic airway inflammation

in a mouse model. MiR-155 knockout mice presented a diminished inflammation and mucus hypersecretion, supported by a reduction in Th2 cell number and cytokine levels.[158] Others studies found the same effect of miR-155-mediated pathways regulating Th2 cells[159] and altering the normal immune physiology.[160]

6.3.2 *Downregulated MicroRNAs*

miR-34/449. The miR-34/449 family includes four members: miR-34c-5p, miR-34b-5p, miR-449a, and miR-449b-5p. Repression of this miR-34/449 family has been found in two asthma cohorts.[161] The miR-34/449 family members have also been implicated in airway epithelial cell differentiation and proliferation.[162] All four members of the miR-34/449 family reported to be decreased in asthma were significantly repressed by IL-13, in a time- and dose-dependent manner. So, these data further provide new insights into mechanisms of epithelial miRNA regulation by IL-13 as a mediator that can induce many of the miRNA changes seen in asthma.[161]

miR-203. miR-203 is downregulated in human bronchial epithelial cells isolated from patients with asthma. Moreover, the top-ranked predicted targets of this strongly downregulated miRNA in asthmatic cells was the aquaporin gene AQP4, which is considered a novel asthma-associated gene. AQP4 transcript levels were higher in bronchial epithelial cells from asthmatic patients, and it is strongly implicated in tissue swelling, a pathological characteristic observed in asthmatic lungs.[142,163]

miR-200 family (200b and 141). Levänen et al. demonstrated the presence of miRNAs in exosomes from BALF of both healthy subjects and asthmatic patients.[164] The miR-200 family has been shown to regulate epithelial to mesenchymal transition, which has been implicated in airway remodeling in asthmatic patients.[165]

miR-192. Yamamoto et al. showed that miR-192 in peripheral blood was altered after exposure to inhaled allergen in human asthmatics and suggested that miR-192 levels may be implicated in pathophysiology of asthma.[143,166] Moreover, miR-192 suppressed T follicular helper cell differentiation in childhood asthma.[167]

miR-133a. miR-133a is implicated in the development of bronchial smooth muscle hyperresponsiveness, a characteristic feature of allergic bronchial asthma. The level of miR-133a was significantly decreased in bronchial smooth muscles of asthmatic animals. miR-33a downregulation causes an upregulation of RhoA, resulting in an augmented bronchoconstriction. IL-13 also caused an upregulation of RhoA mRNA and a downregulation of miR-133a in human bronchial smooth muscle cells.[168] Another study has also focused on the role of miR-133a and its negative regulation of the RhoA translation, suggesting that the miR-133a/RhoA pathway may be a putative therapeutic target for asthma.[169]

6.4 Lung Cancer

The clinical problem. Lung cancer is the most common malignancy worldwide with 1.8 million new cases and approximately 1.6 million deaths in 2012.[170] Smoking is the leading cause of lung cancer, accounting for 85% of cases. Additional risk factors for lung cancer include second-hand smoke, asbestos, radon, and other environmental factors. Lung cancer is histologically divided into two main types: small cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC). SCLC comprises about 15% of lung cancer while NSCLC comprises approximately 85%.[171]

Pathophysiology. SCLC is divided into the two subtypes of oat cell carcinoma and combined-SCLC. Combined SCLC is defined as SCLC with non-small-cell components, such as squamous cell or adenocarcinoma. SCLC carcinogenesis can occur by various pathways that disrupt normal DNA repair mechanisms. Common mutations in SCLC include loss of RB1

tumor suppressor gene and TP53(17p13) mutations, which decrease the pro-apoptotic activity of cancer cells. Almost all SCLC tumors have a deletion in a section of the short arm of chromosome 3p, which contains the tumor suppressor gene FHIT. SCLC is defined by light microscopy as a tumor with small cells, round to fusiform, scarce cytoplasm, finely granular nuclear chromatin, and absent or poorly visible nucleoli. The necrosis can be extensive and the cells manifest a high mitotic rate. SCLC originates from epithelial cells, and up to 90% express thyroid transcription factor-1 (TTF1).[171] NSCLC is characterized by an abnormal expression of Notch signaling pathway members. The Notch signaling pathway serves important roles in cell fate determination, proliferation, differentiation, and apoptosis.[172] Several studies have demonstrated that the plasma or serum miRs has a potential clinical value in the early detection of lung cancer and would play a critical role in the preliminary screening of lung cancer in the general population.[39,173]

6.4.1 *Upregulated MicroRNAs*

miR-155. miR-155 is one of the best studied miRs in lung cancer. Wang et al. performed a metaanalysis that included 19 studies. They observed that miR-155 was significantly associated with worse nonsmall-cell lung cancer survival. This metaanalysis provides evidence that miR-155 could be a predictor factor for nonsmall-cell lung cancer prognosis.[174] miR-155 levels are elevated in NSCLC and are proportional to the progression of the disease.[175] In NSCLC, miR-155 exerts an oncogenic role.[176,177] It is reported that restoration of miR-155 represses the growth of early-stage NSCLC malignant cells.[175,178,179] Moreover, in the A549 cancer cell line, miR-155 also modulates cellular apoptosis and DNA damage.[180]

miR-145. Several studies reported upregulation of miR-145 in different types of lung cancer. For example, in early-stage NSCLC miR-145 could be potential biomarker. Geng et al. demonstrated in plasma that miR-145 showed the best predictive value in 25 early-stage NSCLC patients and smokers.[181] Previously, Tang et al. revealed that miR-145 could serve as a noninvasive screening tool in early detection of lung cancer.[182]

miR-9. miR-9 is overexpressed in lung cancer and plays different roles in different cancer types. Xu et al., found that miR-9 expression is increased in human NSCLC tissues.[183] Upregulation of miR-9 expression was correlated with adverse clinical features and unfavorable survival. In nonsmall-cell lung cancer, miR-9 enhanced cell invasion and adhesion via TGF-β1.[184] The miR-9 inhibitor greatly reduced TGF-β1-induced NSCLC cell invasion.[184] These studies indicated that miR-9 is a poor prognosis biomarker in patients with nonsmall-cell lung cancer.

miR-21. Recently, Yang et al. demonstrated that serum miR-21 was upregulated in nonsmall-cell lung cancer patients compared with cancer-free controls.[185,186] miR-21 expression was also greater in lung cancer associated with COPD.[187] This study suggests that upregulation of miR-21 expression and its methylation may partly underlie the increased susceptibility of COPD patients to develop lung tumors. Moreover, it is reported that miR-21 promoted tumor angiogenesis in the LIN28B mouse model.[188] LIN28B is an RNA-binding protein whose expression is associated with tumor initiation, progression, resistance, and poor outcome in several solid cancers, including lung cancer.[189,190]

miR-221. miR-221 plays an important role in the development of tumors. Bioinformatic analyses showed that miR-221 was significantly increased in lung cancer patients.[191] In NSCLC, miR-221 promotes cell proliferation, cell migration, and invasion by directly targeting TIMP2, while downregulation of miR-221 exhibited the opposite role.[192] Moreover, miR-221 levels were significantly upregulated in exosomes extracted from lung adenocarcinoma plasma samples.[193]

6.4.2 Downregulated MicroRNAs

Experimental data has provided evidence that exposure to various environmental or lifestyle factors, such as environmental cigarette smoke, result in extensive alterations to miRNA expression in the lung. Most miRNAs are downregulated in tumors when compared with normal tissues because of the association between microRNA levels and cellular differentiation.

miR-34. The miR-34 family includes three tumor-suppressive miRs: miR-34a, miR-34b, and miR-34c. miR-34 downregulation is a frequent observation in lung cancer.[194] miR-34 has been demonstrated as a potential regulator of the metastatic process in several cancer types. In nonsmall-cell lung cancer patients, miR-34 suffered a methylation that could be used as a powerful marker for the prognosis of this type of lung cancer.[195,196] In the same way, others studies have also showed that miR-34 methylation predicts poor disease-free survival in the early stage of NSCLC and lung adenocarcinoma.[197] miR-34 prevents cancer initiation and progression in a mouse model of lung adenocarcinoma.[198] Transcription of miR-34 is directly induced by the tumor suppressor p53 in response to DNA damage and inhibits cell proliferation.[199]

miR-200. It is reported that tobacco smoke carcinogens can generate epigenetic silencing, promoting the downregulation of miR-200.[200] The miR-200 family and their potential targets are functionally involved in canonical pathways of immune response, metastatic signaling, cell-cell communication, proliferation, and DNA repair.[165] The downregulation of miR-200, identified in metastatic NSCLC cells, provides new insights into miR-200 regulation in lung cancer metastasis and consequent clinical outcome, and may provide a potential basis for innovative therapeutic approaches for the treatment of this deadly disease.[201]

miR-15a/16 cluster. The miR-15/16 family is consistently downregulated and has a tumor suppressor function, so restoring miR-16 expression represents a novel therapeutic approach in cancer.[202] In NSCLC, miR-16 and miR-15a are implicated in cell cycle control and likely contribute to the tumorigenesis.[203,204] miR-15/16 family overexpression significantly inhibited cell proliferation and induced apoptosis via p27, Bax, pro-caspase 3 and cleaved caspase 3. On the other hand, miR-16 suppression resulted in completely opposite effects.[204,205] Downregulation of miR-16 promotes growth and motility by targeting heparin binding growth factor (HDGF) in nonsmall-cell lung cancer cells. It causes autophagy and apoptosis in paclitaxel-resistant lung cancer cells.[206] MiR-16 targets Bcl-2 in paclitaxel-resistant lung cancer cells and overexpression of miR-16 along with miR-17 causes unprecedented sensitivity by simultaneously modulating autophagy and apoptosis.

miR-187. miR-187 showed a significant downregulation in lung tumors. Xuelian et al. suggested that miR-187 could be a potential biomarker for predicting survival in lung adenocarcinoma, so that patients could be divided into high and low risk based on their miR-187 levels.[207,208] miR-187 was significantly downregulated in primary tumor tissues and very low levels in NSCLC cell lines. In this context, ectopic expression of miR-187 in NSCLC cell lines suppressed cell growth through inhibition of BCL6. Sun et al. also demonstrated that miR-187 induced apoptosis via upregulation of the activities of caspase-3 and caspase-7.[209]

miR-124. miR-124 was downregulated in both clinical NSCLC samples and in tumor cell lines. Ectopic expression of miR-124 inhibited the proliferation and enhanced apoptosis of nonsmall-cell lung cancer.[210,211] Wang et al. also indicated that the miR-124/STAT3 pathway acts as a tumor suppressor in NSCLC.[211] Moreover, Jin et al. showed that overexpression of miR-124 suppressed NSCLC growth by inhibiting the Akt pathway, and systemic delivery of miR-124 dramatically suppressed tumorigenesis, by increasing apoptosis and inhibiting cell proliferation.[212]

miR-203. miR-203 was downregulated during tumorigenesis and inhibited the

proliferation and invasion of lung cancer cells.[213] miR-203 expression was decreased in nonsmall-cell lung cancer. Furthermore, overexpressing of miR-203 with a miR-203 mimic promotes a decrease in cell proliferation and migration.[214] Wei et al. suggested that miR-203//TGF-β/SMAD2 expression plays an important role as a tumor suppressor gene in patients with NSCLC.[214] In patients, Tang et al. concluded that the survival of the low-miR-203-expression group was markedly shorter than that of the high-expression group.[215]

6.5 Idiopathic Pulmonary Fibrosis

The clinical problem. Idiopathic pulmonary fibrosis (IPF) is a chronic diffuse interstitial lung disease of unknown cause characterized pathologically by inflammation and fibrosis of the lung parenchyma, commonly diagnosed in older adults (>50), and usually fatal.[216] Although estimates of frequency are limited and variable, the prevalence has been estimated to range from 3 to 5 per 100,000, although this is based on case series and reports. The disease significantly affects health-related quality of life and is also associated with a large number of comorbidities, which further add to the burden of disease associated with the condition.[217] Although the cause of IPF is not clear, several risk factors are known, including cigarette smoke, agriculture, pollutants, gastroesophageal reflux, and viral infections.[218] A small percentage of cases of IPF are grouped within families, suggesting that there are genetic risk factors. The average life expectancy following diagnosis is approximately 3 years, as this is the worst of all idiopathic interstitial pneumonias. Currently, therapy is aimed at reducing the decline in lung function associated with IPF but cannot stop the course of the disease.

Pathophysiology. The pathogenesis of the disease is characterized by the remodeling of the tissue of the respiratory tract through an excess of extracellular matrix protein deposition (ECM) and the presence of fibroblastic foci, which are observable by histology.[219] The remodeling begins with the formation of repeated lesions in the pulmonary epithelium, followed by the destruction of the basement membrane and the activation of myofibroblasts involved in ECM secretion and tissue repair. Myofibroblasts can arise from both resident and circulating fibroblasts, as well as possibly by transdifferentiation of epithelial cells through the epithelial to mesenchymal transition (EMT).[220] Also, it has been shown that the signaling pathway of TGF-β is involved in the basis of many of these changes.[221] Perhaps the most common experimental model of IPF is bleomycin induction of pulmonary fibrosis in mice. Following intraperitoneal or intratracheal instillation, bleomycin stimulates inflammatory and fibrotic responses within 2 weeks.[222] The extremely rapid onset of symptoms and partial reversibility of fibrosis in this model limits the direct applicability of findings to the clinic.

6.5.1 Upregulated MicroRNAs

miR-9. Increased expression of miR-9-5p abrogates TGF-β1-dependent myofibroblast phenotypic transformation. In the mouse model of bleomycin-induced lung fibrosis, miR-9 reduces fibrogenesis and inhibition of miR-9 and prevents its anti-fibrotic effect both in vitro and in vivo. In lung specimens from patients with IPF, high levels of miR-9 are found.[223]

miR-21. The upregulation of miR-21 is found in lungs from IPF patients and bleomycin-treated mice.[224] Increasing miR-21 levels promotes the pro-fibrogenic activity of TGF-β1 in fibroblasts through regulation of the expression of an inhibitory Smad, Smad7. In addition, it contributes to epithelial-mesenchymal transition in pulmonary epithelial cells.[225]

miR-145. The expression of smooth muscle α-actin (SMA-α) by fibroblasts defines the phenotypic transition to myofibroblasts and plays an important role in the development of pulmonary fibrosis.[226] miR-145 expression is upregulated in TGF-β1-reated lung fibroblasts. miR-145

expression is also increased in the lungs of patients with idiopathic pulmonary fibrosis as compared with normal human lungs. Overexpression of miR-145 in lung fibroblasts increased SMA-α expression, enhanced contractility, and promoted formation of focal and fibrillar adhesions.

miR-155. In mice treated with bleomycin, miR-155 is overexpressed and the keratinocyte growth factor (KGF) is a direct target of miR-155. Following miR-155 transfection, KGF was released at lower levels by lung fibroblasts in response to pro-inflammatory cytokine stimulation.[227] Elevated levels of miR-155 contribute to the proinflammatory expression of IL-8 in lung epithelial cells by lowering SHIP1 expression, thereby activating the PI3K/Akt signaling pathway, suggesting a multifunctional role of miR-155 in lung inflammation.[228]

6.5.2 *Downregulated MicroRNAs*

Let-7d. The data collected on let-7d-5p demonstrate that its expression is downregulated in the skin of IPF patients.[57] The activity of this miR negatively correlated with markers of fibrosis and fibroblast phenotypes.[229]

miR-17∼92. Compared with control samples, miR-17∼92 expression was reduced in lung biopsies and lung fibroblasts from patients with IPF and in the lungs of bleomycin-treated mice, whereas DNA methyltransferase 1 (DNMT-1) expression and methylation of the miR-17∼92 promoter was increased.[230]

miR-26a. The expression of miR-26a is downregulated in lung specimens from IPF patients and in bleomycin-treated murine lungs compared with controls.[231] miR-26a overexpression partly reduces the exaggerated deposition of collagen[232] and, in human alveolar type 2 cells, reduces the epithelial-mesenchymal transition.[233]

miR-29. A screen for miRNAs dysregulated in bleomycin-treated mice identified miR-29 as significantly reduced compared with PBS-injected controls.[234] Levels of miR-29 were inversely correlated with the expression of collagen and directly regulated several pro-fibrotic genes, including integrins and extracellular matrix remodelers. A recent study suggests that decreased miR-29 could be caused by impaired signaling through protein phosphatase 2A (PP2A) and histone deacetylase 4 (HDAC4) following fibroblast interactions with collagen 1.[235] Moreover, miR-29 expression was suppressed by TGF-β via its downstream effector, SMAD3, which likely occurs through PI3K/Akt pathway activation.[236,237] Transposon-mediated gene transfer of miR-29 or direct injection of miR-29 mimics into bleomycin-treated mice successfully decreased TGF-β signaling and collagen expression, while improving phenotypes of fibrosis.[238]

miR-153. miR-153 expression was dysregulated in the lungs of mice with experimental pulmonary fibrosis and TGF-β1 decreased miR-153 expression in pulmonary fibroblasts. Moreover, increased miR-153 levels attenuated, whereas the knock down of miR-153 promoted, the pro-fibrogenic activity of TGF-β1 and miR-153 reduced the contractile and migratory activities of fibroblasts.[239]

miR-200. The family members of miR-200 are significantly downregulated in the lungs of patients with IPF and in mice with bleomycin-induced lung fibrosis. This family inhibits the TGF-β1-induced epithelial-mesenchymal transition of alveolar epithelial cells and can reverse the fibrogenic activity of pulmonary fibroblasts. The introduction of miR-200c diminishes experimental pulmonary fibrosis in mice, suggesting that restoring miR-200 expression in the lungs may represent a novel therapeutic approach for the treatment of pulmonary fibrotic diseases.[240]

miR-326. Transforming growth factor (TGF)-β has a well-established role in lung fibrosis.[241] miR-326 regulates TGF-β1 expression. Moreover miR-326 levels are inversely correlated with TGF-β1 protein levels in multiple human cell lines. Thus, the increase in TGF-β1 expression during the progression of bleomycin-induced lung fibrosis in mice was associated with the loss

of miR-326. Restoration of miR-326 levels by intranasal delivery of miR-326 mimics was sufficient to inhibit TGF-β1 expression and attenuate the fibrotic response. Moreover, human IPF lung specimens had markedly diminished miR-326 expression as compared with nonfibrotic lungs. Additional targets of miR-326 controlling TGF-β signaling and fibrosis-related pathways were identified, and miR-326 was found to downregulate profibrotic genes, such as Ets1, Smad3, and matrix metalloproteinase 9, whereas it upregulates antifibrotic genes, such as Smad7.[58]

6.6 Bronchopulmonary Dysplasia

The clinical problem. Bronchopulmonary dysplasia (BPD) is a chronic lung disease that primarily affects preterm infants. At present, BPD primarily occurs in extremely premature infants (between 23 and 28 weeks of gestation) born during the late canalicular/early saccular stage of lung development. This disease is associated with a chronic state of inflammation, which disturbs the growth and alveolarization of lungs and leads to abnormal angiogenesis. Compromised angiogenesis can lead to pulmonary hypertension (BPD-PH), which is a severe complication of BPD.[242] There are many risk factors, such as low gestational age, pulmonary immaturity, prenatal (chorioamnionitis) and postnatal infections, persistent ductus arteriosus (PDA), lack of prenatal steroid therapy, and surfactant insufficiency. Prolonged ventilation is often required, which often worsens this condition. Currently, genetic factors are also taken into consideration. So far, there are no effective treatments for BPD.

Pathophysiology. The pathogenic mechanisms that underlie BPD and BPD associated with PH are not well understood, but it has been recognized in the disruption of fetal lung development and perinatal infection/inflammation. Mechanical ventilation and hyperoxia cause pulmonary inflammation that can have a deleterious impact on both the airways and alveoli, as well as the pulmonary vasculature.[243] The evolution of pathology in BPD is characterized by the appearance of epithelial severe lesions (hyperplasia, squamous metaplasia), smooth muscle hyperplasia of the airways, extensive fibroproliferation, prominent vascular hypertensive lesions, and a decrease of the internal surface area and alveoli.[244]

In recent years, several studies have been carried out on microRNA expression profiles in bronchopulmonary dysplasia (BPD) in mammalian lung tissues. Several metaanalyses compared the expression profiles of miRNA between lung tissues of BPD and normal lung tissues. Several microRNA have been described that play a role in branching morphogenesis, a key step in early lung development. Results of the different metaanalyses revealed that different upregulated miRNAs (miR-21, miR-34a, miR-431, miR-449a-5p and Let-7f) were differentially expressed in BPD lung tissues compared with normal groups. In addition, eight miRNAs (miR-146b, miR-29a, miR-503, miR-411, miR-214, miR-130b, miR-382, and miR-181a-1) and one downregulated miRNA (miRNA-335) were found to show differential expression not only in the process of normal lung development, but also during the progress of BPD.[245]

6.6.1 Upregulated MicroRNAs

miR-21. Bhaskaran et al. have reported an upregulation of miR-21 in neonatal rat lungs in response to hyperoxia exposure.[246] In addition, a specific target of miR-21 is 15-hydroxiprostaglandin dehydrogenase (15-PGDH, HPGD), which participates in the initial inactivation of prostaglandins (PG), which are potent mediators of several biological processes, including inflammation.[245]

miR-34a. Lung miRNA-34a levels are significantly increased in lungs of neonatal mice exposed to hyperoxia and it is also increased in type 2 alveolar epithelial cells in neonates

with respiratory distress syndrome and BPD.[246,247] Moreover, the deletion or inhibition of miR-34a improves the pulmonary phenotype and BPD-associated pulmonary hypertension in BPD mouse models, which, conversely, is worsened by miR-34a overexpression.[247] Syed et al. reported that the administration of angiopoietin-1, which is one of the downstream targets of miR34a, is able to ameliorate the BPD.[247]

miR-183. Elevated CO_2 concentrations (hypercapnia) occur in patients with severe lung diseases, such as BPD.[248] High CO_2 levels lead to increased levels of miR-183, which in turn decrease the expression of IDH2 (isocitrate dehydrogenase 2), thus impairing mitochondrial function and cell proliferation.[249] Thus, the overexpression of IDH2 or inhibition of miR-183 ameliorates the effects of high CO_2 on cell proliferation and enables cells to grow in a galactose medium.[249]

6.6.2 *Downregulated MicroRNAs*

miR-17. Plasma miR-17 levels are significantly lower in the first week of life in human infants with severe BPD compared with mild or moderate BPD.[250] miR-17 levels inversely correlated with DNA methyltransferase (DNMT) expression and promoter methylation. These data support a plausible role for epigenetically altered miR-17 in the pathogenesis of severe BPD.[250] Otherwise, Rogers et al. reported that altered miR-17∼92 cluster expression contributes to the molecular pathogenesis of bronchopulmonary dysplasia.[251]

miR-150. This microRNA was found to be downregulated in an experimental model of BPD induced by hyperoxia.[246] The glycoprotein nonmetastatic melanoma protein b (Gpnmb) was identified and validated as a direct target of miR-150. These data suggested that decreased miR-150 levels would lead to increased Gpnmb expression, which was indeed observed in experimental BPD. Although the expression of miR-150 was altered in experimental BPD, a miR-150(−/−) knockout mouse did not exhibit appreciable protection in a BPD animal model.[252]

miR-196a. Go et al. demonstrated that miR-196a is degraded in hyperoxia, resulting in limited heme oxygenase-1 (HO-1) induction in neonatal mice lungs.[253] miR-196a binds to the 3′-untranslated region of the transcriptional repressor BTB and CNC homology 1 (Bach1) and regulates its expression, and subsequently leads to higher levels of lung HO-1 mRNA compared with levels in adults.[253]

miR-206. BPD mice had decreased expression of miR-206 compared with the control mice and the decreased expression of miR-206 was also reflected by low plasma concentrations in BPD patients.[254] The downregulated miR-206 leads to an upregulation in patients of Fibronectin 1 (FN1), which is a direct target of this miRNA.[254] Moreover, miR-206 inhibits FN1 expression and proliferation and promotes apoptosis of rat type II alveolar epithelial cells, suggesting that miR-206 may be used as a potential molecular target for BPD therapy in the future.[255]

miR-342-3p. miR-342-3p is downregulated in neonatal rat lungs in response to hyperoxia exposure.[246] Raffay et al. using a neonatal mouse model of BPD, showed that hyperoxia increases the activity and expression of S-nitrosoglutathione (GSNO) reductase, endogenous bronchoconstriction mediator, via downregulation of miR-342-3p. In addition, this adverse effect can be overcome by supplementing its substrate, GSNO, or by inhibiting the enzyme itself.[256]

miR-489. Infants with bronchopulmonary dysplasia (BPD) reduced the miR-489 lung and increased the Igf1 and Tnc direct targets compared with premature or normal term infants.[59] miR-489 is necessary to complete alveolar septation and using LNA-miR-489 attenuated hyperoxia-induced inhibition of septation and improved compliance and pulmonary resistance.[59]

6.7 Obstructive Sleep Apnea

The clinical problem. Obstructive sleep apnea (OSA) is a common illness, characterized by episodic upper airway narrowing during sleep. Sleep-disordered breathing is associated with major morbidity and mortality. However, its prevalence has mainly been studied in populations at risk for cardiovascular diseases, diabetes, and metabolic syndrome.[257] The condition is characterized by repeated intermissions in breathing during sleep, which leads to the fragmentation of sleep and decreases in oxyhemoglobin saturation. The physiologic spectrum of sleep-disordered breathing ranges from partial airway collapse and increased upper-airway resistance, manifested as loud snoring and episodes of hypopnea, to complete airway collapse and episodes of apnea lasting 60 seconds or more.[258] Polysomnography is the current standard for the evaluation of sleep-disordered breathing. It provides detailed data on respiratory effort, airflow, oxygenation, sleep state, and other variables, but it is costly and requires subjects to sleep overnight in a laboratory.

Pathophysiology. OSA pathogenesis is multifactorial, mainly due to a pharyngeal anatomic abnormality that may be corrected by surgical procedures. Nonanatomic pathogenic factors are upper airway dilator muscle dysfunction, heightened chemosensitivity, low arousal threshold and insulin sensitivity, and dysregulation of the glucose homeostasis.[259] The airway narrowing during sleep leads to intermittent hypoxia (IH) with recurrent arousals for at least 10 s. The IH is associated with hypertension, increased sympathetic nerve activation, metabolic dysregulation, vascular endothelial dysfunction, oxidative stress, and possibly systemic inflammation. IH recurrent intrathoracic pressure swings and sleep fragmentation are thought to be key pathological processes in the development of cardiovascular disease in OSA.[260] OSA is a cause of systemic hypertension and is associated with an increased incidence of stroke, heart failure, atrial fibrillation, and coronary heart disease.

6.7.1 Upregulated MicroRNAs

miR-130. miR-130a may be involved in the progression of OSA-associated pulmonary hypertension by targeting the growth-arrest-specific homeobox (GAX) gene.[261] An increase in serum miR-130 levels was found in patients with OSA associated with pulmonary hypertension. This increase in miR-130 was related to a decrease in the GAX gene and this is associated with the increase of ET-1 and VEGF expressions and a decrease in NO and SOD expressions in the PH group associated with OSA.[261]

miR-193. Endothelial dysfunction is the main pathogenic mechanism of cardiovascular complications induced by obstructive sleep apnea/hypopnea syndrome (OSAHS).[262] Chronic intermittent hypoxia (CIH) is the primary factor of OSAHS-associated endothelial dysfunction. Downregulation of miR-193 inhibits Chronic Intermittent Hypoxia (CIH) induced endothelial injury and apoptosis- or autophagy-related protein expression.[263]

6.7.2 Downregulated MicroRNAs

miR-185. In an OSA canine model developed by the group of Ding et al., it was found that the fibrotic tissue and the infiltration of inflammatory cells were located predominantly around the bronchi and vascular tissue in the lung.[264] OSA activated the expression of TGF-β, which subsequently suppressed miR-185 and promoted collagen type I (CoL A1), GAP-43, tyrosine hydroxylase and NGF expression. This signaling cascade leads to pulmonary remodeling.[264]

miR-664a. Early prediction of atherosclerosis is important in the treatment of patients with obstructive sleep apnea (OSA).[265] MiR-664a-3p has been shown to decrease in serum from patients with OSA. In addition, the expression of miR-664a-3p can be correlated with the thickness of the carotid intima-media.[266]

miR-630. The expression of exosomal miRNA-630 was reduced in children with endothelial dysfunction and normalized after therapy along with restoration of endothelial function. miR-630 regulates targets in endothelial cells that include the Nrf2 and AMP kinase.[267]

miR-365. Intermittent hypoxia acts as a potent proinflammatory stimulus, resulting in IL-6 induction and M1 macrophage polarization.[268] Mature microRNA-365 has been demonstrated to regulate IL-6 expression, and miR-365 expression was decreased in the setting of intermittent hypoxia.[268]

miR-107, miR-574-5p and miR-199-3p. The downregulation of hsa-miR-107, hsa-miR-574-5p, and hsa-miR-199-3p was found in serum samples from OSA patients with respect to healthy controls. These miRs might participate in the control of metabolic signaling pathways in OSA.[269]

6.8 Cystic Fibrosis

The clinical problem. Cystic fibrosis (CF) is a rare disease. Although this disease affects all racial and ethnic groups, Caucasians are the most affected. One in every 2000–3000 European newborns presents CF.[270] CF is caused by mutations in the CF transmembrane conductance regulator gene (*CFTR*). The most common mutation is deletion of a phenylalanine residue at position 508 (ΔF_{508}del), which occurs on approximately 70% of patients with CF. The mutation causes misfolding, endoplasmic reticulum retention, and degradation of the CFTR protein.[271] *CFTR* encodes a chloride ion channel that, when defective, damages chloride, sodium, and bicarbonate transport and leads to airway dehydration and accumulation of thick mucus secretions, promoting the development of persistent infections. Due to dense accumulation of mucus, pathogens cannot be eliminated efficiently, which leads to chronic inflammation at the sites of infection.

Pathophysiology. Evidence suggests that pulmonary inflammation of the airways in CF is associated with increased production of proinflammatory cytokines produced by the infiltration of macrophages and neutrophils. Several studies have found high concentrations of proinflammatory cytokines, such as interleukin-1 (IL-1), IL-6, IL-8, and tumor necrosis factor alpha (TNFα), in sputum and bronchoalveolar fluid (BALF) of patients with CF.[272] Their synthesis is promoted by the transcription factor nuclear factor-κB (NF-κB), which plays an important role in intracellular signaling for the production of proinflammatory cytokines.[273] IL-10, IL-1 receptor antagonist protein (IRAP), and soluble TNFα receptor (TNFsR) are antiinflammatory cytokines that are relatively downregulated in CF airway cells. Autophagy removes pathogens and aggregates of dysfunctional proteins within macrophages. However, this process is impaired in CF because its macrophages show limited autophagy activity.[274] Lung damage progressively worsens with age and is the main cause of mortality. Treatments that restore CFTR channel function are not currently beneficial for all patients with CF, suggesting the existence of modifier genes that control the respiratory phenotype independent of the CFTR locus.

6.8.1 Upregulated MicroRNAs

mirc1/mir17∼92. Clustering mirc1/mir17∼92 is a potential negative regulator of autophagy. The absence or reduced expression of the group increases the expression of autophagy proteins, suggesting the canonical inverse relationship between mirc1/mir17∼92 and the expression of the autophagy gene. In vivo, downregulation of mir17 and mir20a partially restored autophagy expression.[275]

miR-9. Anoctamine 1 (ANO1) was identified as the main Ca^{2+} channel activated to compensate for the CFTR deficiency[276] in airway epithelial cells. Recently it has been found that the

upregulation of miR-9 correlates with downregulation of ANO1 in CF cells and the decrease in ANO1 in CF patients contributes to the severity of the disease.[277]

miR-101. Air pollutants, such as cigarette smoke and cadmium, negatively regulate the expression of CFTR by affecting several steps in the biogenesis of CFTR protein.[278] Cigarette smoke and cadmium upregulate the expression of miR-101 that is predicted to target CFTR in human bronchial epithelial cells. Mice exposed to cigarette smoke for 4 weeks demonstrated an upregulation of miR-101 and suppression of CFTR protein in their lungs.[113]

miR-145. Evaluation of microRNA prediction algorithms and subsequent in vitro luciferase-based reporter assays show that miR-145 and miR-494 directly target the 3′ UTR of *CFTR* and reduce its expression.[279] Similar studies by additional groups identified increased expression levels of these miRNAs in the airway epithelial cells of CF patients compared with healthy controls.[280]

miR-155. There is overexpression of miR-155 in CF lung epithelial cells in culture. The increase of miR-155 was associated with the positive regulation of IL-8 activity by direct repression of SHIP1 (an inositol phosphate phosphatase), which led to enhanced PI3K/Akt signaling, an increased inflammatory response, and worsening of the disease.[228]

miR-494. The expression of miR-494 directly targets the 3′ UTR of *CFTR* and reduces its expression.[279] The level of expression of miR-494 is increased in the airway epithelial cells of CF patients with respect to healthy controls.[281] Moreover, miR-494 expression was found to be directly correlated with *CFTR* levels in vivo.[280]

miR-509. The genetic variants in CFTR 3′ UTR, can be typical targets of posttranscriptional gene regulation of microRNAs.[282] A study showed that patients with CF with the F508del genotype had different clinical expression depending on the presence or not of a mutation in CFTR 3′ compared with controls. The 1043A > C mutation is found in a binding region of miR-433 and miR-509-3p, increases the affinity for miR-509-3p, and decreases slightly for miR-433. Both miRNAs cause reduced expression of the CFTR protein in vitro. Therefore, the 1043A > C mutation may act as a mild CFTR mutation that improves affinity for miRNA-inhibitors as a new pathogenic mechanism in CF.[283]

6.8.2 *Downregulated MicroRNAs*

miR-16. It was shown that miR-16 is capable of rescuing F508del CFTR traffic defects, probably through the suppression of the chaperone protein 90 (HSP90). Then, a miR-16 mimic can efficiently rescue F508del-CFTR protein function in airway cell lines and primary cultures of differentiated human bronchial epithelia from F508del homozygotes.[284]

miR-17. Interleukin (IL)-8 levels are higher than normal in cystic fibrosis (CF) airways, causing neutrophil infiltration and nonresolving inflammation.[285] miR-17 regulates IL-8 and its expression was decreased in adult cystic fibrosis bronchial brushings, βENaC-transgenic mice and bronchial epithelial cells chronically stimulated with *Pseudomonas*-conditioned medium. These results implicate defective CFTR, inflammation, neutrophilia, and mucus overproduction in regulation of miR-17.[286]

miR-126. The expression of miR-126 is significantly decreased in endobronchial brushings from CF patients and its downregulation increased the expression of Myb1 (TOM1), which is involved in intracellular protein trafficking, a negative regulator of innate immune responses in the CF lung.[287] The expression of TOM1 resulted in a significant increase in NF-κB regulated IL-8 secretion.

miR-138. CFTR expression is regulated by miRNA-138 through its interactions with the transcriptional regulatory protein SIN3 homolog A (SIN3A), a transcriptional regulatory protein involved in CTCF-mediated gene

regulation. SIN3A controls a subset of genes in the biogenesis of wild type and mutant CFTR. Manipulating miR-138 levels is possible to improve F508del CFTR biosynthesis and expression in airway epithelial cells by targeting.[288]

miR-1343. The cytokine transforming growth factor β (TGF-β) has a pivotal role in promoting lung fibrosis and is implicated in respiratory disease severity.[289] miR-1343 attenuates pathways of fibrosis by targeting the TGF-β receptors[290] and TGF-β is often overexpressed in the CF lung and is a known modifier of lung disease severity, suggesting a role for miR-1343 in this pathology.

6.9 Acute Respiratory Distress Syndrome

The clinical problem. Acute Respiratory Distress Syndrome (ARDS) or Acute Lung Injury (ALI) is a type of acute, diffuse, inflammatory lung injury, leading to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue. ARDS represents a major cause of acute respiratory failure in Intensive Care Units. Risk factors for this condition include infection, trauma, or other systemic conditions. The social and economic impact of ARDS is documented by an incidence of 48–90 cases per 100,000 inhabitants per year, the high associated mortality rate (around 50%), as well as the important sequelae in surviving patients that often require prolonged rehabilitation treatment. ARDS management remains largely supportive, with mechanical ventilation forming the cornerstone of therapy and the lack of approved pharmacological therapies reflects major deficiencies in our understanding of the pathogenesis of ARDS. Despite little social concern, the death toll from ARDS is greater than that of other socially recognized conditions (e.g., similar to myocardial infarction and much higher than breast cancer or HIV).

Pathophysiology. ARDS is a disorder of acute inflammation that causes disruption of the lung endothelial and epithelial barriers. It is characterized by pulmonary edema and alveolar collapse, resulting in severe arterial hypoxemia. Cellular characteristics include loss of alveolar-capillary membrane integrity, excessive transepithelial neutrophil migration and release of pro-inflammatory and cytotoxic mediators. A complex interplay of regulatory cytokines counteracts the inflammatory mediators; similarly, matrix deposition is balanced by the actions of the metalloproteases. The inflammatory process is driven in part by cytokines, including TNF-α and IL-1β, IL-6, and IL-8. In addition to the altered barrier function of the microcirculation, dysfunction in the contractility of pulmonary vessels is a prominent feature of ARDS. Mild PH is frequently seen in patients with ARDS and loss of normal control over pulmonary vasomotor tone is an important mechanism underlying refractory hypoxemia. Moreover, the magnitude of the increase in PAP has been shown to correlate with the severity of lung injury in patients with ARDS and has an adverse prognostic significance independent of the level of hypoxemia.[291] Thus, alterations of vascular smooth muscle tone lead to hypoxic pulmonary vasoconstriction (HPV) failure, resulting in ventilation-perfusion mismatch, PH, and arterial hypoxemia. As occurs in PH, endothelial dysfunction is an important contributor to the pathogenesis of ARDS, but unlike PH, Kv channel activity seems to be preserved.[292]

6.9.1 Upregulated MicroRNAs

miR-21. Lee et al. demonstrated that miR-21 is positively regulated in an ALI model induced by oleic acid.[293] Another group reported that miR-21 is highly upregulated in another animal model induced by high-tidal-volume ventilation.[294] The alveolar-arterial oxygen difference and protein levels in bronchoalveolar lavage were lower and lung compliance was preserved in mice treated with anti-miR-21 as compared with mice treated with pre-miR-21 or negative miRNA.[294] In addition, a miR-21 inhibitor mimicked the effects of lipoxin A4 (LXA4),

which has antiinflammatory and antiedema properties.[295–297] Moreover, overexpression of miR-21 abolished the protective effects of LXA4 in A549 cells.[298]

miR-23a. One study showed that miR-23a levels are increased in the serum of rats with ALI induced by LPS, suggesting that miR-23a-5p might be employed as a potential biomarker for ARDS in early stages.[299]

miR-34a. miR-34a levels are significantly increased in lungs of neonatal mice exposed to hyperoxia and the administration of angiopoietin-1, which is one of the downstream targets of miR34a, is able to ameliorate the ALI.[247] However, the excessive autophagic activity of alveolar type II epithelial (AT-II) cells is one of the main causes of ALI and Song et al. reported that miR-34a might suppress the excessive autophagic activity in AT-II cells via targeting FoxO3 to reduce the damage of LPS-induced ALI.[300]

miR-127. Upregulation of miR-127 expression has been observed in a rat model of ARDS[301] and has been suggested to contribute to the development of ventilator-induced lung injury (VILI) though an increase in the inflammatory response involving NF-κB and P38 MAPK signaling pathways.[302,303] On the other hand, miR-127 has been shown to inhibit pulmonary inflammation by targeting IgG Fcγ receptor.[304]

miR-146. miR-146a regulates Toll-like receptors (TLRs) and cytokines through downregulation of target proteins, such as IL-1 receptor activated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), which are mediators for NF-κB activation.[305] TLR4 signaling has a very important role in the upregulation of inflammation and the release of inflammatory cytokines in animal models and cellular models.[306] miR-146a mimic treatment suppressed LPS-mediated pro-inflammatory cytokines induction in normal alveolar rat macrophage cells and also suppressed the expression of inducible nitric oxide synthase (iNOS), favoring M2 macrophage phenotype and ameliorating the development of ALI in mice lungs exposed to acid and LPS-treated macrophages.[307] Therefore, miR-146a could be a therapeutic target in ALI.

miR-155. miR-155 is a proinflammatory factor after LPS stimulation, and alveolar macrophages-derived miR-155 has an important role in LPS-induced ALI.[308] Likewise, miRNA155 is significantly increased in patients with severe sepsis and sepsis-induced ALI as compared with control subjects, thus it has been proposed as biomarkers predictive of mortality and treatment outcome of sepsis-induced ALI.[309]

miR-185. Lung epithelial cell death is a prominent feature involved in the development of ALI.[310] Hyperoxia strongly induced miR-185 and its precursor in human and mouse lung epithelial cells in a time-dependent manner.[311] The hyperoxia-induced reactive oxygen species suppress the function of histone deacetylase 4 (HDAC4), which results in an elevated miR-185 transcription, and this microRNA contributes to the death of epithelial cells associated with oxidative stress through improved DNA damage.[311]

miR-200. Chen et al. reported that miR-200 and other miRNAs are transported from primary human bronchial epithelial cells (HBECs), bronchoalveolar lavage fluid, mesenchymal stem cells, and dendritic cells and that it could have an important role in lung diseases.[312] Also, it has been reported that miR-200 in parallel with miR-429, regulates alveolar macrophage inflammatory cytokine production and is involved in LPS-induced acute lung injury.[313] However, another group affirmed that miR-200b/c exerts a protective effect by targeting ZEB1/2, which may be associated with the inhibition of p38 MAPK and TGF-β/smad3 signaling pathways during the development of pulmonary fibrosis induced by LPS.[314]

miR-203. microRNA-203 expression in the lung tissues of LPS-challenged mice was found to be significantly upregulated.[315] Of note, miR-203 overexpression in A549 cells significantly promoted cell apoptosis by inducing S-phase cell-cycle arrest and also inhibiting the

protein expression of PI3K catalytic subunit alpha (PIK3CA), thereby suppressing the PI3K/Akt pathway.[315]

miR-211. Interestingly, downregulation of miR-211 has been proposed to protect lung tissue against sepsis-induced ARDS by upregulation of SIRT1[316].

miR-223. The upregulation of miR-223 limits the number of Ly6G+ neutrophils and inhibits the activity of the NLRP3 inflammasome to alleviate ALI induced by mitochondrial damage-associated molecular patterns.[317] In line with these beneficial effects, Neudecker et al. showed that coculture of alveolar epithelial cells with polymorphonuclear neutrophils (PMNs) induced a selective increase in lung epithelial cell expression of miR-223,[318] which dampens acute lung injury through repression of PARP-1.

miR-429. Xiao et al. demonstrated that p38 MAPK, which is a critical regulator in lung inflammation, can be inactivated by DUSP1, a direct target of miR-429.[313] In vivo, LPS induced the expression of miR-429 in the lung and this increases the production of pro-inflammatory cytokines.[313]

miR-1246. The expression of miR-1246, a direct regulator of Angiotensin-converting enzyme 2 (ACE2), was increased in LPS-exposed pulmonary microvascular endothelial cells (PMVECs) and ALI in mouse models.[319] Then, silencing of miR-1246 prevented LPS-induced inhibition of ACE2, which was accompanied by reduced apoptosis and production of IL-1β and TNF-α, attenuating lung inflammation, neutrophil infiltration, and vascular permeability.[319]

6.9.2 *Downregulated MicroRNAs*

miR-16. miR-16 is significantly downregulated in LPS-induced ALI in mice while its upregulation inhibits the expression level of IL-6 and TNFα.[320] Tamarapu Parthasarathy et al. demonstrated that miR-16 was downregulated in mice exposed to hyperoxia[321] aggravating pulmonary edema, which was correlated with reduction of epithelial sodium channel (ENaC) expression and increase in serotonin transporter (SERT) expression. Moreover, miR-16 inhibits hyperoxia-induced cell apoptosis in human alveolar epithelial cells.[322]

miR-17. Studies have shown that FoxA1 expression is upregulated during ALI and may play an important role in this pathological condition by promoting the apoptosis of alveolar type II epithelial cells.[323] Interestingly, Xu et al. showed that downregulation of miR-17 is involved in the overexpression of FOxA1 in this model.[324]

miR-125b. A potential role of miR-125b in the development of ARDS has been proposed by Guo et al.[325] Upregulation of miR-125b produced beneficial effects in LPS-induced ALI. Additionally, serum miR-125b levels were inversely correlated with the disease severity in patients with ARDS. These data suggest a potential therapeutic application for miR-125b in ARDS.[325]

miR-126. The miR-126-5p levels in pulmonary tissues were markedly decreased in the LPS-induced ALI model in mice. Interestingly these were associated with a marked augmentation of vascular endothelial growth factor-A, a direct miR-126-5p target. Moreover, overexpression of this miRNA attenuated LPS-induced effects in alveolar type II cells.[326]

miR-150. Deletion of miR-150 has been reported to induce a persistent increase in vascular permeability though angiopoietin 2 (Ang2) and, thereby, to contribute to ARDS. Likewise, administration of a miR-150 mimic decreased Ang2 levels and sepsis-induced mortality.[327]

miR-181. Cai et al.[320] found a downregulation of miR-181 in a mouse model of lipopolysaccharide (LPS)-induced lung injury. Intriguingly, downregulation of miR-181a can significantly reduce LPS-induced acute lung injury in mice and A549 cells by targeting Bcl-2.[303,328]

miR-199a. miR-199a was found to be downregulated in lipopolysaccharide (LPS)-induced mouse acute.[320] Interestingly, downregulation

of miR-199a has been proposed for protecting tissue against sepsis-induced ARDS by upregulation of SIRT1.[329]

miR-374a. Administration of hsa-miR-374a has been proposed to be therapeutically useful to reduce inflammatory lung injury[330] by downregulating the expression of the pre-B-cell colony-enhancing factor (PBEF)/NAMPT, which contributes to the pathophysiology of ARDS.[330]

7 CONCLUSIONS AND PERSPECTIVES

In recent years, the role of miRNAs in the pathogenesis and therapy of different chronic respiratory diseases has become increasingly evident. The dysregulation of the expression of miRNAs can be observed in the different lung tissues (bronchus, endothelium, parenchyma, smooth muscle cells…) and in the different types of inflammatory cells that intervene at the pulmonary level. MicroRNAs in serum or plasma are distal mechanisms of cell-to-cell communication and may serve as a clinical diagnostic or as prognostic biomarkers of disease. In this chapter, we have extensively, but not exhaustively, compiled the available evidence so far regarding the changes in microRNAs and their potential pathological role (Fig. 6). In addition, many of these changes correlate with the risk or severity of the disease, though the specific mechanisms by which this dysregulation occurs remain largely unknown. Several miRNAs appear to be involved in multiple distinct respiratory diseases, suggesting the importance of these miRNAs, and the pathways that they regulate, in maintaining global lung homeostasis.

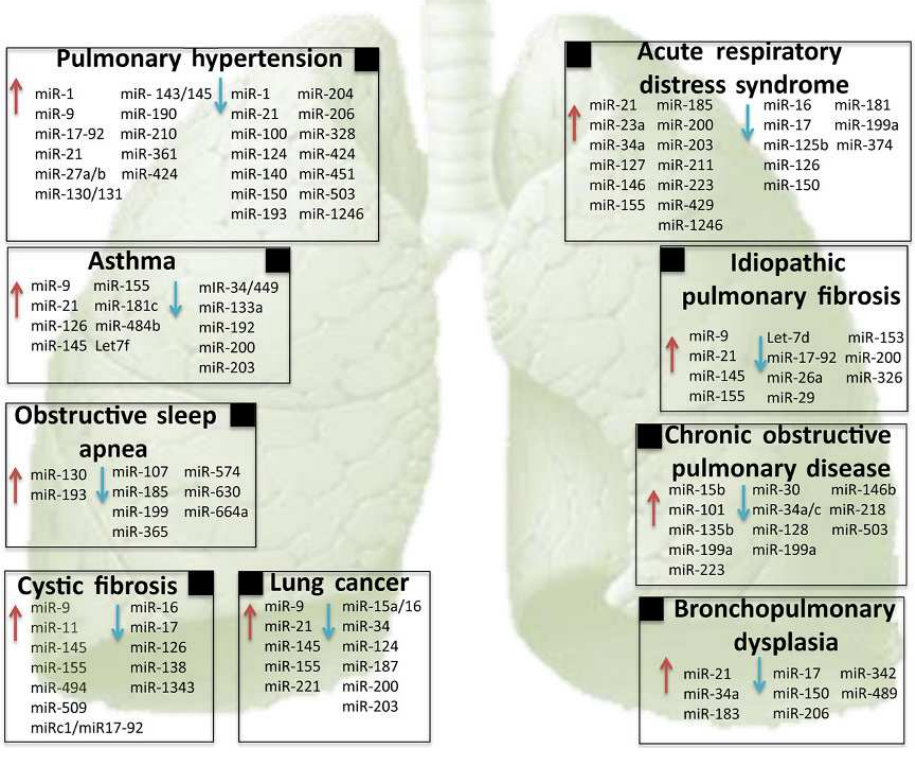


FIG. 6 Summary of the best-studied altered microRNAs in respiratory diseases.

Therefore, these commonly altered miRNAs are not selective diagnostic biomarkers but may rather be indicators of poor prognosis.

Different studies propose miRNAs or their antagonists as possible therapeutic options for the coming years. However, it is still necessary to advance in the optimization of delivery in order to guarantee the safety and efficacy of miRNA-based treatments. Before miRNAs can become feasible clinical tools, several issues related to the biology of miRNAs must be resolved. The first point concerns the reproducibility of the studies, as different groups use similar experimental conditions and obtain contradictory results. Though this may be due to variability among patients, the lack of standard methods for quantifying miRNA expression remains a problem in the field. Therefore, it is necessary to promote the development of more sophisticated tools and standardized methods to quantify microRNAs.

Another important question to be resolved in most studies is whether the dysregulation of the microRNAs is causative of the disease or whether it is just an epiphenomenon. In the latter case, treatments targeting the miRNA will probably not be effective, but its use as a biomarker could still be a good option. Finally, the large number of target genes regulated by the same miRNA and the variety of cell types involved in each disease make it difficult to identify their precise role in the pathogenetic mechanisms of the disease. Currently, different investigations are being carried out to incorporate microRNAs into different systems, such as exosomes or nanoparticles, to improve their delivery to the target tissues.

The growing availability of RNA-sequencing methods (as opposed to microarray- and in silico-based tools), together with single cell technologies, should provide more accurate data on miRNA profiles dysregulated in disease and the role of each miRNA in a given cell type. With the development of new technologies and tools to analyze and manipulate the miRNA function, knowledge about the control of chronic respiratory diseases through miRNAs will progress, with the ultimate objective of creating improved disease management strategies and better outcomes for the patient.

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NUCLEIC ACID NANOTHERANOSTICS

Edited by Marco Filice and Jesús Ruiz-Cabello

Nucleic Acid Nanotheranostics: Biomedical Applications offers a comprehensive overview of improvements and new trends in the fabrication of nanostructures as theranostic multifunctional carriers in gene therapy. With a strong focus on medical applications, comprising diagnosis, therapy, imaging, and combinations thereof, the book also examines gene therapy in the cells or tissues of an individual patient to treat a wide variety of diseases, such as oncology, as well as cardiovascular and respiratory diseases. Sections also cover the biomedical and diagnostic applications of nucleic acids, biologic and synthetic advanced nanostructures for specific nucleic acid delivery, and important considerations in nanomedicine.

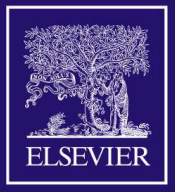
Key Features

- Provides a unique source of knowledge (theoretical as well as practical) on nanotheranostic materials for gene therapy at all levels and related scientific areas.
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This book is a valuable guide for materials scientists, biochemists, biomedical engineers, pharmaceutical chemists, physicians, academic researchers, and industry professionals who work with nanomaterials for healthcare applications.

Dr. Marco Filice is an associate researcher at the Dept. of Chemistry in Pharmaceutical Sciences, Faculty of Pharmacy, Complutense University of Madrid (UCM, Spain); head of the Nanobiotechnology for Life Sciences Group (UCM); and researcher at the Spanish Biomedical Network of Research in Respiratory Diseases (CIBERES), part of the National Institute of Health Carlos III. His research work is mainly focused on the multidisciplinary-based design and creation of nanosized-material-biomacromolecule artificial hybrids showing highly improved or de novo biological properties. Prepared artificial nanobiohybrids have shown a wide application scope, mainly in advanced synthesis and catalysis, as well as in the development of novel ultrasensitive molecular diagnostic and nanomedicines, especially the theranosis of cardiovascular, pulmonary, cancer, and immune-based diseases.

Prof. Jesús Ruiz-Cabello is a full professor at the Faculty of Pharmacy, Complutense University of Madrid (UCM, Spain); Ikerbasque Research Professor and Head of the Molecular and Functional Biomarkers group at the CIC BiomaGUNE in San Sebastian/Donostia; and Scientific Assistant Director of the Spanish Biomedical Network of Research in Respiratory Diseases (CIBERES), part of the National Institute of Health Carlos III. His fundamental research is in the field of pulmonary and cardiovascular molecular and functional imaging. This research is directed toward biomarker (multimodality imaging, metabolic, and different omics) searches. He is the coauthor of over 180 articles and book chapters and coinventor of six patents, four of them European, and he is the cofounding partner of a technology-based company, Numio Technologies.



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Annex III: miR-1 induces endothelial dysfunction in rat pulmonary arteries

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ORIGINAL ARTICLE

miR-1 induces endothelial dysfunction in rat pulmonary arteries

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Abstract
Endothelial dysfunction plays a central role in the pathophysiology of pulmonary arterial hypertension (PAH). MicroRNAs (miRNAs) are small single-strand and non-coding RNAs that negatively regulate gene function by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs. microRNA-1 (miR-1) is upregulated in plasma from idiopathic PAH patients and in lungs from an experimental model of PAH. However, the role of miRNA-1 on endothelial dysfunction is unknown. The aim of this study was to analyze the effects of miR-1 on endothelial function in rat pulmonary arteries (PA). Endothelial function was studied in PA from PAH or healthy animals and mounted in a wire myograph. Some PA from control animals were transfected with miR-1 or scramble miR. Superoxide anion production by miR-1 was quantified by dihydroethidium (DHE) fluorescence in rat PA smooth muscle cells (PASMC). Bioinformatic analysis identified superoxide dismutase-1 (SOD1), connexin-43 (Cx43), caveolin 2 (CAV2) and Krüppel-like factor 4 (KLF4) as potential targets of miR-1. The expression of SOD1, Cx43, CAV2, and KLF4 was determined by qRT-PCR and western blot in PASMC. PA incubated with miR-1 presented decreased endothelium-dependent relaxation to acetylcholine. We also found an increase in the production of O_2^- and decreased expression of SOD1, Cx43, CAV2, and KLF4 in PASMC induced by miR-1, which may contribute to endothelial dysfunction. In conclusion, these data indicate that miR-1 induces endothelial dysfunction, suggesting a pathophysiological role in PAH.

Keywords Posttranscriptional regulation · miRNA-1 · Endothelial dysfunction · Superoxide dismutase · Pulmonary arterial hypertension

Introduction

Pulmonary arterial hypertension (PAH) is a rare and progressive disease characterized by an elevation of the mean pulmonary arterial pressure. It is a disorder with a significant burden in terms of both severity and prevalence. PAH affects young and middle-aged persons, preferentially women [9]. The disease progression involves an increase in pulmonary vascular resistance (PVR) leading to right heart failure and eventually death. PAH is characterized by an imbalance in endothelial-derived vasoactive factors, inflammation, and structural remodelling of pulmonary arteries (PA) [28].

The endothelium is a single layer of cells that lines the lumen of blood vessels and plays an important physiological role in vascular homeostasis [13]. Endothelial dysfunction is defined as a chronically impaired production of vasodilators such as nitric oxide (NO) leading to a proliferative, pro-oxidant, pro-inflammatory, and pro-thrombotic status. Various pathological conditions including diabetes, hyperlipidemia, erectile dysfunction [33], hypertension [5], and PAH [12] may impact on endothelial function by disrupting the molecular mechanisms regulating NO bioavailability, which is an independent predictor of poor prognosis of these diseases. Thus, decreased NO production and/or increased NO degradation by reactive oxygen species (ROS), mainly superoxide anions, are considered early markers of endothelial

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dysfunction [17, 42]. Several studies have highlighted the close relationship between mitochondrial ROS production, endothelial dysfunction, and excess cardiovascular risk. Superoxide dismutases (SOD) are a family of enzymes that catalyze the conversion of superoxide anion to hydrogen peroxide (H_2O_2). In particular, SOD1 plays a key role in scavenging superoxide anions in the pulmonary vasculature [8].

MicroRNAs (miRNAs) are single-strand and non-coding RNAs of 18–24 nucleotides that negatively regulate gene function by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs. The interaction between the miRNA and the 3′UTR sequence of the specific mRNA leads to translational repression and/or degradation of the mRNA, resulting in a decrease of the target protein. Mature miRNAs play a key role in different cellular processes, such as proliferation, apoptosis, differentiation, and organogenesis [2]. Aberrant regulation of miRNA expression has been implicated in a large number of clinically important cardiovascular diseases. In the last decade, multiple studies have proposed the analysis of miRNA as a potential diagnostic and prognostic tool [19, 32]. Therapeutic strategies based on the upregulation or downregulation of different miRNAs have recently emerged [29].

Upregulated miRNAs can be antagonized using antagomirs, while downregulated miRNAs can be restored with miRNA mimetics. miRNA mimetics are synthetic RNA duplexes mimicking the endogenous miRNA, while antagomirs are modified antisense oligonucleotides that harbor the complete or partial complementary sequence of a mature miRNA.

Recent studies suggest that different miRNAs are dysregulated in patients with PAH and in PAH experimental models, particularly in pulmonary artery smooth muscle cells (PASMCs) and in pulmonary artery endothelial cells (PAECs) [43]. Among the miRNAs that are increased in PAH, miR-1 was the one with the highest upregulation (8-fold increase in microarray experiments and a 12-fold increase in the PCR analysis) in plasma from 12 idiopathic PAH patients [31]. miR-1 is also upregulated in peripheral blood mononuclear cells in essential hypertensive patients [16] and in preeclampsia [11]. Moreover, we have recently demonstrated that lungs from rats with PAH induced by Su5416, a vascular endothelial growth factor type 2 receptor (VEGFR) inhibitor, plus 3 weeks under hypoxia (10%, Hyp/Su5416) showed a marked upregulation of miR-1 (≈4-fold increase) [20]. We also found an increased expression of miR-1 in pulmonary arteries from healthy animals incubated for 48 h under a hypoxic environment (3% O_2) in the presence of Su5416, mimicking the condition of human and animal models of PAH. Our study also showed that miR-1 downregulates the voltage-dependent potassium channel Kv1.5 in PASMCs, which plays an important role in the control of pulmonary vascular tone and whose activity is impaired in patients and in different animal models of PAH.

Herein, we hypothesized that miR-1 could also be involved in an additional key pathophysiological feature of pulmonary vascular diseases, such as endothelial dysfunction. Therefore, the aim of the present study was to analyze the effects of miR-1 on pulmonary endothelial function.

Material and methods

Ethical approval

All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-251/15). All investigators understand the ethical principles.

Animals and in vitro models of PAH

Male Wistar rats of 220–250 g ($n = 22$) were obtained from Envigo (Barcelona, Spain). All animals were kept with free access to standard rat chow and water in an enriched environment throughout the whole experiment period and maintained at 24 °C under a 12 h light/12 h dark cycle. Rats were euthanized using CO_2.

For the in vivo model of Su5416 plus hypoxia-induced PAH (Hyp/Su5416), rats were injected subcutaneously with a single dose of the VEGFR type 2 inhibitor Su5416 (20 mg/Kg) or vehicle [10, 37]. Then, SU5416-treated animals were introduced in glass cages and ventilated with 10% O_2 (hypoxia, $n = 5$) for 3 weeks. CO_2 and water vapor produced by the animals were captured with soda lime and silica gel, respectively. Control rats (normoxia, $n = 3$) were kept in the same room. Oxygen was monitored using an oxygen sensor (DrDAQ Oxygen Sensor, Pico Technology, UK) in the room and in the chamber outflow. The chambers were opened for 20–30 min daily for regular animal care.

For in vitro models of PAH, isolated pulmonary arteries (PA) from male Wistar rats were isolated from the left lung. Vessels were then placed in Dulbecco's Modified Eagle Medium (DMEM) supplemented with glucose (4.5 g/L), non-essential amino acid solution (1×), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml), and maintained for 48 h in a normoxic incubator (21% O_2 and 5% CO_2) or a hypoxic incubator (3% O_2 and 5% CO_2 in the presence of 10 μM Su5416) before assessing vascular reactivity.

In silico analysis for miR target prediction

Target endothelial gene prediction analysis was completed using the MicroRNA.org target prediction resource, utilizing miRanda sites and miRSVR scoring. The target sites predicted using miRanda are scored for the likelihood of mRNA downregulation using a regression model trained on the sequence and contextual features of the predicted miRNA: mRNA duplex.

Cell culture

PASMCs were isolated from explants of PA from 5 different control rats. Briefly, cells were isolated from endothelium-denuded vessels digested in a Ca^{2+}-free solution containing (in mg/ml) collagenase 1.125, elastase 0.1, and albumin 1 for 4 min at 4 °C followed by 1 min at 37 °C. Following digestion, tissues were washed in the Ca^{2+}-free HEPES solution containing (in mM) NaCl 130, KCl 5, HEPES 10, $MgCl_2$ 1.2, and glucose 10 (pH adjusted to 7.3 with NaOH) and disaggregated using a wide bore, smooth-tipped pipette, to make a cell suspension. Cell suspension was plated into 35-mm Petri dishes and incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C in DMEM containing 20% heat-inactivated FBS, pyruvate (1.1 mg/ml), 1% non-essential amino acids, streptomycin (100 mg/ml), penicillin (100 unit/ml), and amphotericin B (250 ng/ml) for 1 week. Cells were subcultured in 75-cm^2 sterile flasks in DMEM supplemented with 10% FBS and used within passages 2–3.

Transfection of miR into PASMCs

PASMCs were transfected with miR-1 (hsa-miR-1-3p mirVanaTM miRNA mimic, MC10617) or a scramble miR (miRNA mimic negative control, 4464058) from Applied Biosystems using Lipofectamine™ RNAiMAX (Life Technologies) following manufacturer's instructions. miRNAs with a final concentration of 10 nM and Lipofectamine™ RNAiMAX were mixed with Opti-MEM (Life Technology) supplemented with 2% FBS and 1% antibiotic/antimycotic solution. The mixture of miRNA/LipofectamineTM RNAiMAX was kept at room temperature for 5 min to form the transfection complexes and then added to the cells. After incubation for 24 h at 37 °C in a humidified incubator (21% O_2/5% CO_2), transfection complexes were replaced with complete growth media (DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic solution) and kept for 24 h.

Transfection of PA

PA from 5 different rats were isolated from the left lung and cut into rings. For the study of endothelial function, to introduce miR-1 and scramble miR (final concentration 100 nM) into isolated PA, reverse permeabilization was used as previously reported [23]. Briefly, PA were exposed to three successive solutions (4 °C) containing (in mM) (i) miRNA duplexes, 10 EGTA, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$, 20 HEPES (pH 6.8; 30 min); (ii) miRNA duplexes, 120 KCl, 5 Na_2ATP, 2 $MgCl_2$, and 20 HEPES (pH 6.8; 180 min); and (iii) miRNA duplexes, 120 KCl, 5 Na_2ATP, 10 $MgCl_2$, and 20 HEPES (pH 6.8; 30 min). Subsequently, PA were bathed in a fourth solution containing (in mM) 120 NaCl, 5 KCl, 5 Na_2ATP, 10 $MgCl_2$, 5.6 glucose, and 10 HEPES (pH 7.1, 4 °C), in which [Ca^{2+}] was gradually increased from 0.001 to 0.01, 0.1, and 1 mM every 15 min. Vessels were then placed in DMEM culture medium supplemented with glucose (1 g/L), non-essential amino acid solution (1×), penicillin (100 U/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 μg/ml), and maintained for 48 h in a normoxic incubator (21% O_2 and 5% CO_2) before assessing vascular reactivity. Previously, the transfection efficiency was assessed of this protocol using a fluorescent microRNA (Dharmacon miRIDIAN mimic transfection control-Dy547, CP-004500-01-05). We found that endothelial function was preserved, and transfection was achieved successfully for endothelial cells.

Recording of arterial reactivity

For contractile tension recording, PA rings (1.7–2 mm long, ~ 0.8 mm internal diameter) were mounted in a wire myograph, as previously reported [20]. Vessels were stretched to give an equivalent transmural pressure of 30 mmHg. Preparations were first stimulated by raising the K^+ concentration of the buffer (to 80 mM) in exchange for Na^+. Vessels were washed three times and allowed to recover before a new stimulation. Then, endothelial function was assessed by cumulative addition of acetylcholine (ACh, 1 nM−10 μM) in PA rings as described [18].

Lung histology

The left lung was inflated in situ with 4% formol saline through the left bronchus and embedded in paraffin. Lung sections were stained with hematoxylin and eosin and examined by light microscopy, and elastin was visualized by its green auto-fluorescence [22].

Detection of O_2^- production

O_2^- release in rat PASMCs was quantified by dihydroethidium (DHE) fluorescence. Briefly, confluent PASMCs in 96-well plates were transfected with scramble or miR-1 (10 nM), as described above. Forty hours post-transfection, cells were incubated as described above with

HEPES buffered solution supplemented with 1.5 mM $CaCl_2$ at 37 °C for 30 min in the absence or presence of SOD mimetic, MnTMPyP (100 μM). Then, DHE (30 μM) or the nuclear stain 4,6-diamidin-2-phenylindol dichlorohydrate (DAPI, 0.3 μM) was added. The fluorescent intensity was followed for 1 h at an excitation and emission wavelengths of 530 nm and 620 nm, respectively, for DHE and of 355 nm and 460 nm, respectively, for DAPI, using a plate spectrofluorimeter (Fluoroskan Ascent, Thermo Scientific). DHE fluorescence was normalized by the fluorescence of DAPI.

RNA extraction and quantitative RT-PCR

Total RNA and microRNA were extracted from PASMCs using miRNeasy Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. RNA concentration and quality were checked using NanoDropTM 1000 Spectrophotometers (Thermo Scientific, Massachusetts, USA). For mRNA determination, 1 μg of RNA was reverse transcribed into cDNA using iScriptTM cDNA Synthesis Kit (Biorad, California, USA) following the manufacturer's instructions. For miRNA amplification, complementary DNA analysis was synthesized from total RNA using specific stem-loop reverse transcription primers (Taqman MicroRNA Reverse Transcription Kit, Applied Biosystems, Table 1). Gene expression was determined by quantitative real-time PCR (qRT-PCR) with a Taqman system (Applied Biosystems, Thermo Fisher Scientific, Massachusetts, USA) in the Genomic Unit of the Complutense University (Madrid) using specific primers (Table 1). The delta-delta Ct method was used to quantify relative changes. mRNA expression was normalized by the expression of β-actin with a Taqman probe number #69 (Roche, Cat: 04688686001), and U6 for miRNA analysis.

Western blot analysis

Homogenates were run on a sodium dodecyl sulfate-polyacrylamide electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes, incubated with primary rabbit polyclonal antibody against SOD-1 (1:10000, #SOD-101, Stressgen, San Diego, CA) overnight and then with the secondary peroxidase conjugated antibodies. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). Blots were imaged using an Odissey Fc System (Li-COR, Biosciences) and were quantified by densitometry using Quantity One software. Samples were re-probed for the expression of smooth muscle α-actin (1:10000, A2547, Sigma-Aldrich, Spain).

Drugs

All drugs were from Sigma-Aldrich (Spain) except Su5416 (Tocris, UK).

Statistical analysis

Data are expressed as means ± s.e.m. Statistical comparisons were performed using two-tailed unpaired *t* tests or two-way ANOVA followed by Bonferroni test as appropriate. $P < 0.05$ was considered statistically significant.

Results

Endothelial dysfunction in in vitro and in vivo models of pulmonary hypertension

Figure 1a shows hematoxylin and eosin–stained lung sections from control or Hyp/Su5416 animals. The increase of medial wall thickness can be observed by hematoxylin and eosin staining (Fig. 1a, above), and elastin was visualized by its

Table 1 Details of primers employed for the PCR

Gen		Taqman assay
Gap junction protein, alpha 1 (Gja1)		Rn01433957_m1
Superoxide dismutase 1 (Sod1)		Rn00566938_m1
Caveolin 2 (Cav2)		Rn00590969_m1
Krüppel-like factor 4 (Klf4)		Rn00821506_g1
Gen	Primer forward 5′–3′	Primer reverse 5′–3′
Actin, beta (Actb)	5′-GCCCTAGACTTCGAGCAAGA-3′	5′-TCAGGCAGCTCATAGCTCTTC-3′
miRNA	Taqman assay	Sequence
U6	001973	GTGCTCGCTTCGGCAGCACATATACTAAAATTGGA ACGATACAGAGAAGATTAGCATGGCCCCTGCGCAA GGATGACACGCAAATTCGTGAAGCGTTCCATATTTT
miR-1	002222	UGGAAUGUAAAGAAGUAUGUAU

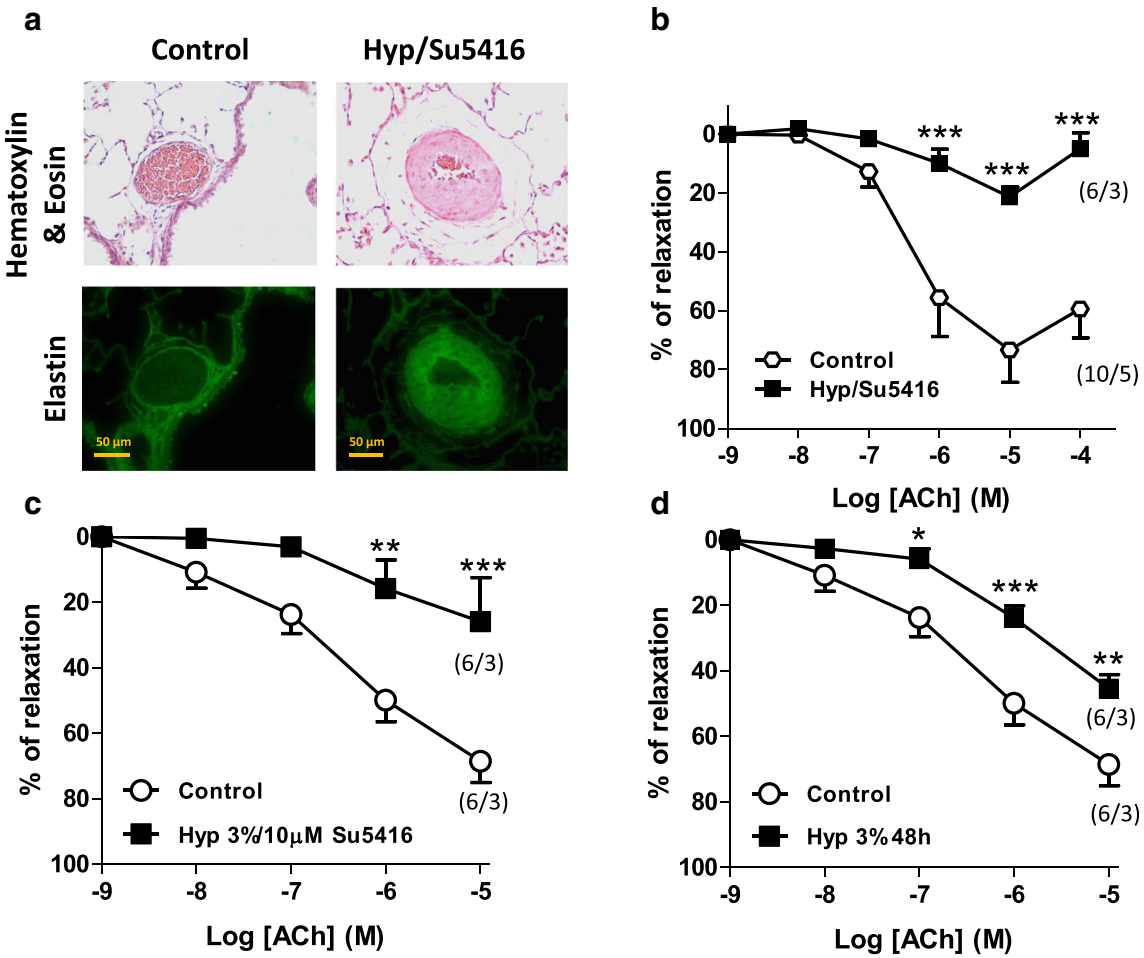


Fig. 1 Vascular remodeling and endothelial dysfunction in vivo and in vitro models of pulmonary hypertension associated to upregulated miR-1. **a** Representative images (× 40) of vascular remodelling of pulmonary arteries in Hyp/Su5416 animals compared with control. Concentration-response curves to cumulative addition of ACh (1 nM–100 µM) as an indicator of endothelial function in rat PAs constricted by phenylephrine (1 µM) from **b** in vivo model of Hyp/Su5416 and in rat PAs constricted by 5-HT (10 µM) from **c** in vitro model Hyp 3% for 48 h/10 µM Su5416 and **d** in vitro model of Hyp 3% for 48 h. Results are means ± S.E.M. The number of pulmonary arteries/animals in each group is indicated in parenthesis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control

green auto-fluorescence (Fig. 1a, bottom). A marked vascular remodelling with an increase of medial wall thickness was present in PA from the in vivo Hyp/Su5416 animal model compared with control PA. We analyzed the endothelium-dependent relaxant response to ACh in isolated PA pre-contracted with serotonin (5-HT, 10 µM). The relaxation induced by ACh was attenuated in PAs from Hyp/Su5416 indicating that PA from Hyp/Su5416 rats overexpressing miR-1 exhibit endothelial dysfunction (Fig. 1b, $P < 0.001$). In addition, the relaxant responses to ACh were attenuated in PA incubated for 48 h at 3% O_2 in the presence (Fig. 1c) or absence (Fig. 1d) of Su5416 ($P < 0.001$) compared with PA incubated under normoxic conditions (5% CO_2 in air at 37 °C).

miR-1 induces endothelial dysfunction in rat pulmonary arteries

The endothelium-dependent relaxation induced by ACh was also studied in PA transfected with miR-1 compared with scramble miR. PA transfected with miR-1 showed a decreased relaxant response to ACh (Fig. 2a, $P < 0.01$), indicating that miR-1 induces endothelial dysfunction. Forty-eight hours post-transfection with the miR-1 mimetic, the miR-1 levels were markedly increased compared with scramble miR transfected PASMC (Fig. 2b, $P < 0.001$). We also evaluated the production of ROS in PASMCs by DHE fluorescence. The DHE fluorescent signal was strongly reduced in the presence of the SOD mimetic MnTMPyP, suggesting that it was essentially due to O_2^- production. miR-1 increased DHE

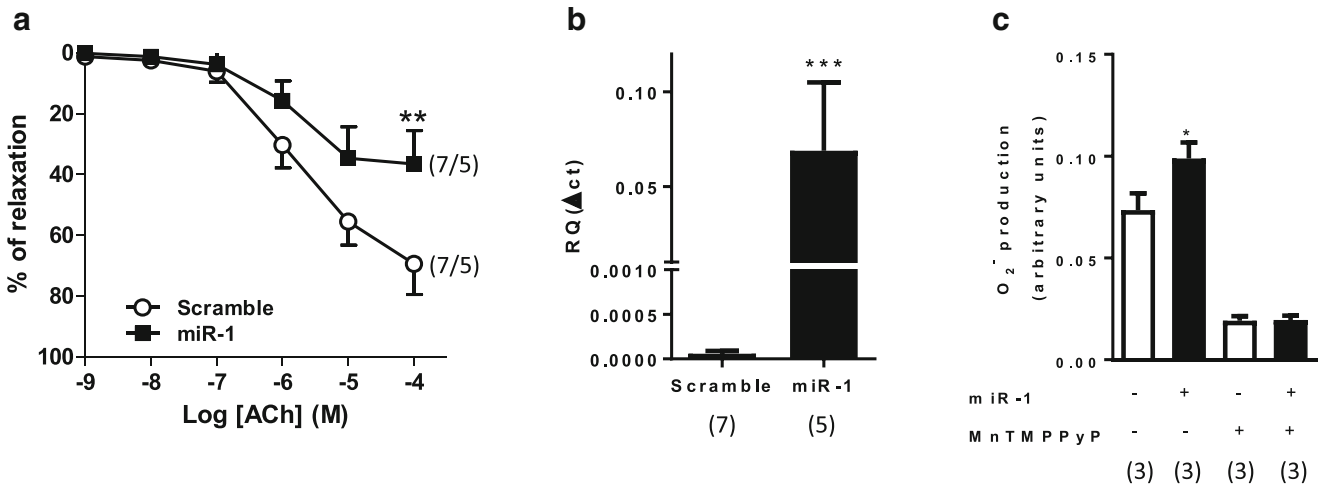


Fig. 2 miR-1 induces endothelial dysfunction in rat pulmonary arteries. Effects of miR-1 on **a** endothelium-dependent relaxation to ACh (1 nM–100 μM) in rat PA rings precontracted with 5-HT (10 μM) transfected with miR-1 or scramble miR (negative control) for 48 h. **b** miR-1 expression analyzed by RT-PCR in PASMC transfected with scramble miR or miR-1. **c** O_2^- production in transfected rat PASMCs. O_2^- release was quantified by DHE (30 μM) fluorescence. Average values of DHE fluorescence were normalized to the DAPI (30 μM) fluorescence in rat PASMCs incubated in the presence or absence of the SOD mimetic MnTMPyP (100 μM). Results are means ± S.E.M. The parentheses indicate the number of arteries or PASMC cell cultures, and the number of animals from which these arteries were obtained, respectively. *$P < 0.05$, **$P < 0.01$

fluorescence compared with scramble miR (Fig. 2c, $P < 0.05$), and this increase was abolished in the presence of MnTMPyP.

In silico *analysis* for the prediction of miR-1 endothelial targets

In order to assess the potential pathophysiological impact of the upregulation of miR-1 levels, we performed an in silico analysis for the prediction of miR-1 targets using the microRNA.org database. Among those mRNAs whose downregulation could be involved in endothelial dysfunction, we selected the *Sod1*, *Gja1*, *Cav2*, and *Klf4* mRNAs (Table 2) which encode for superoxide dismutase 1, Gap junction protein α1 or connexin 43 (Cx43), caveolin-2, and Krüppel-like factor 4. The table contains the gene symbol, gene description, complementary sequence, and mirSVR score for each gene. A more negative mirSVR score indicates a more favorable union site.

Table 2 Selected mRNA targets of miR-1 in *Homo sapiens* that may be involved endothelial dysfunction (*SOD1, GJA1, CAV2,* and *KLF4*). Data predicted from www.microRNA.org

Gene symbol	Gene description	Complementary sequence	mirSVR score
SOD1	Superoxide dismutase 1, soluble	3′ uauguaugaagaaaUGUAAGGu 5′ hsa-miR-1 ||||||| 5′ ----------ACAUUCCc 3′ SOD1	−0.81
GJA1	Gap junction protein α1 (connexin 43)	3′ uauguauGAAGA-AAUGUAAGGu 5′ hsa-miR-1 || || |||||| 5′ uaaguccCUGCUAAAACAUUCCa 3′ GJA1 3′ uauGUA-UGAAGAAAU-GUAAGGu 5′ hsa-miR-1 ||| ||||:|| | |||||| 5′ uacCAUCACUUUUUCAUCAUUCCu 3′ GJA1 3′ uaUGUAUGAAGAAA-UGUAAGGu 5′ hsa-miR-1 || |:|| ||| ||||||| 5′ uuACUAAUUUGUUUGACAUUCCa 3′ GJA1	
CAV2	Caveolin 2	3′ uauguaUGAAG--AAAUGUAAGGu 5′ hsa-miR-1 || || || ||||||| 5′ aucagaACCUCAAUUGACAUUCCu 3′ CAV2	−0.54
KLF4	Krüppel-like factor 4 (gut)	3′ uaugUAUGAAGAAAUGUAAGGu 5′ hsa-miR-1 || |||| | |||||| 5′ cuggAU-CUUC-UAUCAUUCCa 3′ KLF4	−0.17

miR-1 directly targets superoxide dismutase 1 (SOD1)

After the evaluation of ROS production, we analyzed if miR-1 altered the expression of *Sod1*, an enzyme responsible for the metabolism of superoxide ions. Our data showed that there were no changes in the expression of *Sod1* mRNA in PASMCs transfected with miR-1 compared with scramble miR, analyzed by qRT-PCR (Fig. 3a). However, we found a decrease in protein SOD1 levels in PASMCs transfected with miR-1 compared with scramble miR as analyzed by western blot and normalized by α-actin expression (Fig. 3b, $P < 0.05$).

Changes in miR-1-targeted endothelial genes

As additional potential mechanisms for impaired endothelial function, we analyzed the mRNA expression of several target genes of miR-1 (Fig. 4). Impairment of endothelial-dependent relaxation in PA transfected with miR-1 was associated with a reduction in the expression of the genes encoding for connexin43 (Fig. 4a, $P < 0.001$), caveolin-2 (Fig. 4b, $P < 0.01$), and Krüppel-like factor 4 (Fig. 4c, $P < 0.05$) in rat PASMCs transfected with miR-1 compared with scramble miR, analyzed by qRT-PCR and normalized by β-actin expression.

Discussion

Herein, we show that endothelial dysfunction is a common pathophysiological feature for several in vitro and in vivo models of pulmonary hypertension. miR-1, which is upregulated in the lungs from rats with PAH induced by Su5416 plus hypoxia, induced endothelial dysfunction and increased the production of ROS in rat pulmonary arteries. Several miR-1 targets, which could be involved in impaired endothelial function, i.e., *Sod*1, *Cav-2*, *Gja1*, and *Klf4* were downregulated.

miR-1 has been found upregulated in the plasma from 12 idiopathic PAH patients [31] and in lungs from the Hyp/Su5416 model, which best represents class 1 pulmonary hypertension (i.e., PAH) [10, 30]. Despite this fact, other studies affirm that the levels of miR-1 are downregulated in the buffy coat of a cohort of 31 subjects with pulmonary hypertension including all classes of pulmonary hypertension (COPD, interstitial lung disease, obstructive lung disease, scleroderma, etc.) [41] and in lungs from mice exposed to hypoxia (class 3 PH) as well as cultured human PASMC exposed to hypoxia in vitro [35]. The heterogeneity of miR-1 expression levels is greatly contributed by the difference in the cell tissues analyzed and the different conditions applied in each study. Overall, the data indicate that miR-1 does not appear to be a reliable biomarker of the disease. It may be due to the fact that a single miRNA can regulate hundreds of genes or proteins and conversely multiple miRNAs can regulate one protein [7]. These variable levels of miR-1 also may contribute to explain the heterogeneity in the different forms of the pathology.

Endothelial dysfunction is an early key player in the pathophysiology of multiple cardiovascular diseases, including pulmonary hypertension [12]. A reduced endothelial-derived nitric oxide is a widely used biomarker of endothelial dysfunction [14]. Nitric oxide bioavailability can be classically evaluated by the relaxation induced by ACh, which stimulates the release of NO from the endothelium. Both the in vivo model (present results and [3]) as well as in vitro model of Hyp/Su5416 showed NO-dependent endothelial dysfunction. We found that miR-1 reduced the ACh-induced relaxation in rat PA, reproducing the characteristics of PAH in both humans and animal models. The fact that incubation of isolated pulmonary arteries in vitro with Su5416 plus hypoxia [20] also increases miR-1 suggests that the effect of miR-1 is local. Regarding the cellular source of miR-1, our experiments do not address specifically the cell type involved. However, the effects of miR-1 impact on both endothelial and smooth

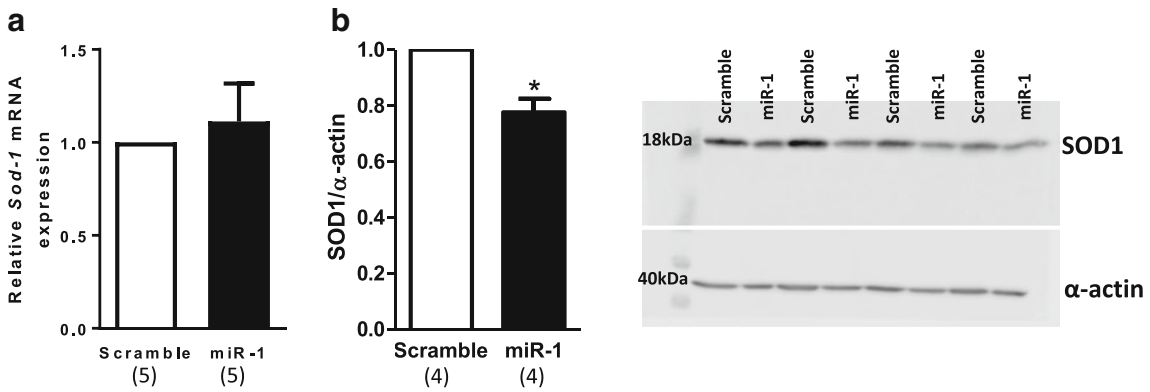


Fig. 3 miR-1 targets superoxide dismutase 1 (SOD1). a mRNA expression by qRT-PCR *of Sod1* in rat PASMCs transfected with miR-1 or scramble miR (negative control). **b** SOD1 protein expression in rat PASMCs transfected with miR-1 or scramble miR analyzed by western blot and normalized by α-actin expression. Results are means ± S.E.M. The parentheses indicate the number of cell cultures used for analysis. *$P < 0.05$

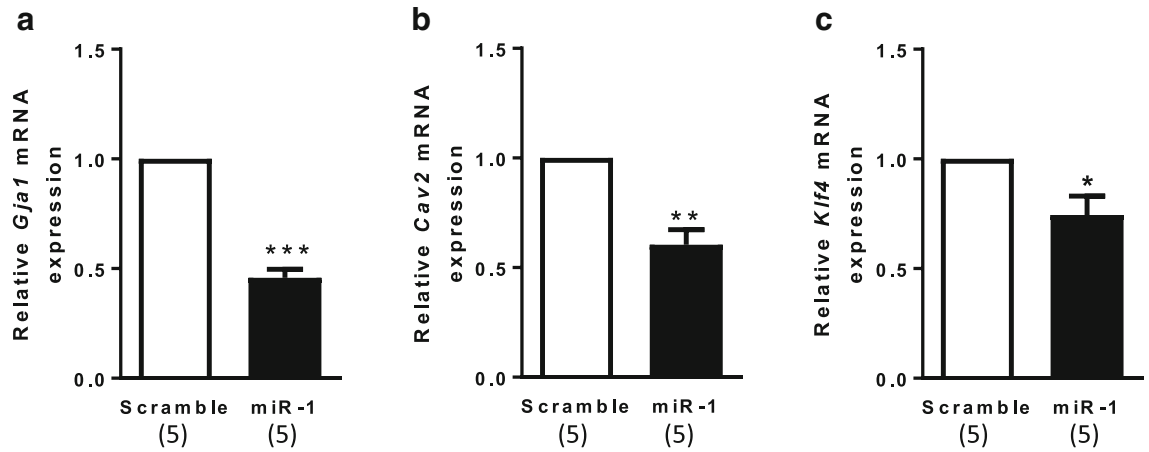


Fig. 4 Changes in miR-1-targeted genes that encode connexin 43 (Gja1), caveolin-2 (Cav2), and Krüppel-like factor 4 (Klf4). mRNA expression by qRT-PCR of **a** *Gja1*, **b** *Cav2*, and **c** *Klf4* in rat PASMCs transfected with miR-1 or scramble miR (negative control). Results are means ± S.E.M. The parentheses indicate the number of cell cultures used for the analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs control

muscle cells. Our functional studies clearly indicate that endothelial-dependent vasodilation is affected by miR-1.

NO inactivation by ROS, mainly superoxide, is a widely recognized mechanism for endothelial dysfunction [36]. We found increased production of superoxide by miR-1, suggesting that endothelial-derived NO is inactivated by superoxide. In fact, transgenic mouse overexpressing miR-1 show increased ROS levels which are likely to be due to a reduced superoxide metabolism by a miR-1 induced post-transcriptional repression of *Sod1* [40]. In line with this, SOD1-deficient mice exhibit PAH [27]. We found that *Sod1* mRNA was unchanged but SOD1 protein was reduced in PASMC transfected with miR-1. This suggests that the miR-1 interaction with 3′-UTR mRNA of *Sod1* results in reduced transduction but not mRNA

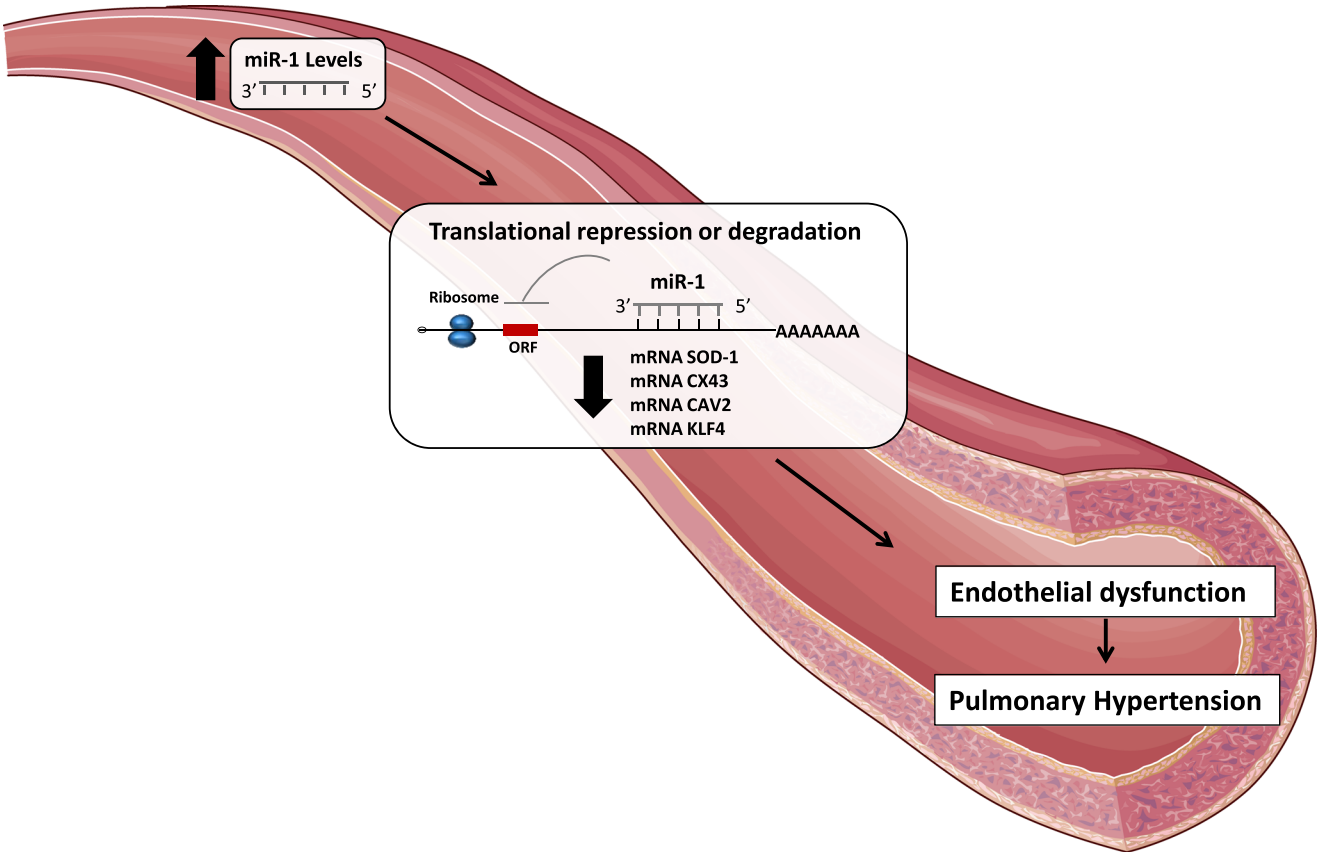


Fig. 5 miR-1 upregulation in pulmonary arteries causes translational repression or degradation of SOD1, Cx43, CAV2, and KLF4 mRNA leading to endothelial dysfunction

degradation. The fact that the 3′-UTR sequence of SOD1 is a direct target of miR-1 has been already demonstrated by luciferase reporter assays in HEK293 cells [40].

Connexin43 is a gap junction protein necessary for the endothelial cell to cell communication which is another target of miR-1 [15]. It exhibits reduced expression in PAH patients resulting in altered gap junctional communication, barrier function, and angiogenesis [38]. We also confirmed that miR-1 reduced *Gja1*, the gene encoding Connexin43 expression at the mRNA level. Other miR-1 targets which may be interesting in the context of pulmonary vascular dysfunction include caveolin-2 and Klf4. We found that the expression of both genes was reduced by miR-1 in PA. Caveolin-2 is a membrane protein which co-expresses and heterooligomerizes with caveolin-1. Decreased caveolin-2 expression is found in the Hyp/Su5416 model of PAH [1]. Klf4 is a transcription factor expressed in the vascular endothelium, where it promotes anti-inflammatory and anticoagulant states, and increases endothelial nitric oxide synthase expression. Its expression was reduced in lungs from patients with PAH, and its loss exacerbated pulmonary hypertension in mice [34]. Therefore, we identified multiple miR-1 targets potentially involved in endothelial dysfunction. Another study in the context of erectile dysfunction supports the fact that the upregulation of miR-1 in the corpus cavernosum of rats produces endothelial dysfunction by downregulating the eNOS/NO/PKG and the PGE1/PKA pathways [25]. In contrast, other studies have shown that decreased miR-1 levels cause endothelial dysfunction by increased endothelin-1 (ET-1) mRNA in the case of diabetes [6] or by increased endothelial permeability in an atherosclerosis mice model [39].

In recent years, a large number of studies have proposed the potential use of miRNA mimics or antagonists (antagomirs) as therapeutic strategies in multiple conditions, including respiratory diseases [21]. Importantly, mimics and antagomirs can be formulated for inhaled administration, which may represent a more selective way to target the lung in respiratory diseases [4, 24, 26]. The identification of miR-1 as a potential factor contributing to key pathophysiological mechanisms of PAH such as endothelial dysfunction (present study) and ionic remodelling [20] suggests that antagonizing miR-1 (e.g., with antagomirs) may be a useful strategy for the treatment of PAH.

In conclusion, our data indicate that miR-1 induces endothelial dysfunction, increased production of ROS and downregulated *Sod1*, *Cav2*, and *Gja1* and *Klf4* expression. As depicted in Fig. 5, all these data suggest that miR-1 upregulation may play a pathophysiological role, at least in some forms of PAH.

Author contributions GMP, MC, BB, DMC, and SE performed and analyzed the experiments. GMP and MC drafted the manuscript. AC and FPV conceived the study and designed the experiments. FPV wrote the manuscript with significant conceptual contributions from GMP, MC, LM, MF, and AC.

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Compliance with ethical standards

All experimental procedures utilizing animals were carried out according to the Care and Use of Laboratory Animals and approved by the institutional Ethical Committees of the Universidad Complutense de Madrid (Madrid, Spain) and the regional Committee for Laboratory Animals Welfare (Comunidad de Madrid, Ref. number PROEX-251/15). All investigators understand the ethical principles.

Conflict of interest The authors declare that they have no conflict of interest.

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Annex IV: Kv1.5 channel down-regulation in pulmonary hypertension is nothing short of MiR-1-aculous!

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Pulmonary Vascular Function in Health and Disease



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The Journal of Physiology

PERSPECTIVES

K_V1.5 channel down-regulation in pulmonary hypertension is nothing short of MiR-1-aculous!

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Pulmonary hypertension (PH), defined as a pulmonary artery (PA) pressure of ≥25 mmHg, can arise idiopathically, as a consequence of inheritance, or secondary to other conditions. The most severe form of PH, pulmonary arterial hypertension (PAH; Group 1 PH), is characterized by an increased pulmonary vascular resistance associated with PA vasoconstriction and severe pulmonary vascular remodelling; this leads to right heart failure, with a grave prognosis. Although an integrated understanding of PAH pathogenesis remains elusive, abundant evidence indicates that down-regulation of several types of K^+ channels in PA smooth muscle cells (PASMC), causing membrane depolarization and a consequent increase in intracellular [Ca^{2+}], may play an important causative role. In particular, the expression of K_V1.5 (*KCNA5*) the most important voltage-gated K^+ current (VGKC) in PASMC, is diminished in various animal models and clinical manifestations of PH, including PAH.

The mechanisms responsible for K_V1.5 down-regulation in PAH are unknown. However, in the current issue of *The Journal of Physiology*, a paper from Francisco Perez-Vizcaino and Angel Cogolludo's laboratory at the Universidad Complutense de Madrid (Mondejar-Parreño *et al.* 2019) presents novel evidence that the decreased expression of PASMC K_V1.5 is caused by increased levels of microRNA-1 (miR-1) in a model of PH which recapitulates many of the features of human PAH.

MicroRNAs (miRs) are small (~22 nucleotides) single-stranded non-coding RNAs that inhibit gene expression post-transcriptionally. They bind to microRNA responsive elements (MREs) in the 3'UTR of their target mRNAs, causing them to be degraded and/or inhibiting their translation into protein. MicroRNAs can be transcribed like any gene: they may contain promoters and have tightly regulated transcription. They can also arise from processing of intronic or exonic sequences of coding and non-coding genes. The base pairing between microRNAs and their targets is imperfect; thus one single microRNA can target hundreds of different mRNAs, thereby regulating an entire biological pathway. MicroRNAs are involved in virtually all biological processes from development to ageing, inflammation and cardiac development. MicroRNAs can also be applied exogenously as pre-microRNAs or inhibited using anti-microRNAs (antagomiRs).

The possibility that miR-1 might be affecting K_V1.5 expression emerged when Mondejar-Parreño *et al.* carried out an *in silico* analysis identifying *KCNA5* as a potential miR-1 target. They then showed that the expression in COS-7 cells of a luciferase reporter vector incorporating the 3'UTR of *KCNA5* was diminished by co-transfection with miR-1, but not with (negative) scrambled miR. It is worth pointing out, however, that they used the 3'UTR from human *KCNA5* in this construct (and also employed human mimic miR-1 in the over-expression experiments described below), whereas they should have used a rat miR-1 mimic and demonstrated direct targeting of the rat *Kcna5* 3'UTR. Whilst the sequences of human and rat miR-1 differ by only one nucleotide, which is outside of the 'seed-region' (the primary determinant of target specificity), changes outside of this region may affect targeting (Broughton *et al.* 2016). Although it is very likely that the human mimic miR-1 was binding to the rat *Kcna5* 3'UTR given the evidence of functional effect presented in the rest of the manuscript, ideally they would have pinpointed the predicted binding site of miR-1 by showing that site-directed mutagenesis of this site prevents miR-1 from down-regulating the reporter; this was not done. Thus, the exact mechanism by which the miR-1 mimic they used was acting on *Kcna5* was not established.

They compared the VGKC and K_V1.5 expression in PASMC from control rats and those in which PH was induced by combined treatment with chronic hypoxia and the VEGF antagonist Su5461. The VGKC, which was largely suppressed by the K_V1.5-selective antagonist DPO-1, was profoundly reduced in the PH animals compared to controls. Moreover, protein expression of K_V1.5 was diminished in lung homogenates from the PH rats compared to controls, whereas miR-1 expression was increased fourfold. In order to determine whether these events were causally linked, they used an innovative method of transfecting miR-1 into isolated PA, which involved briefly digesting the tissues with collagenase and elastase prior to incubating them with miR-1 (and/or scrambled miR or antagomiR-1) in the presence of Lipofectamine, at the same time applying a reverse permeabilization protocol in which extracellular Ca^{2+} was removed and then gradually replaced.

Using this approach, they found that, compared to PASMC treated with scrambled miR, cells from arteries transfected with miR-1 showed decreased K_V1.5. protein levels, were depolarized, and exhibited a much smaller VGKC. Moreover, transfection with antagomiR-1 greatly increased the amplitude of the VGKC and the protein expression of K_V1.5 in PA which had been exposed to hypoxia and Su5416 for 48 h, whilst it did not affect either the K^+ current or channel expression in control normoxic PA.

In summary, the results demonstrate that pulmonary miR-1 expression is upregulated in an important animal model of PAH, and that this causes attenuation of K_V1.5 expression in PASMC. However, both increases (Sarrion *et al.* 2015) and decreases (Wei *et al.* 2013) in miR-1 expression have been reported in patients with PH, and could reflect heterogeneous consequences associated with different types of the disease. Indeed, a pathway involving HIF1α and ET-1, both of which contribute importantly to PH pathogenesis, has been shown to

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suppress $K_V1.5$ channel expression in the chronic hypoxia rat model of PH (Whitman *et al.* 2008), and since there is evidence that miR-1 can reduce levels of HIF1α and ET-1 in some types of cells (Xu *et al.* 2017), it is possible that a *decrease* in miR-1 levels could also down-regulate $K_V1.5$ channels in some types of PH, as the authors discuss. Therefore, a better understanding of the precise role(s) of miR-1 in the regulation of these channels during PH will require additional studies using cells from patients with different types of PH. Hopefully this pioneering study will engender an increased effort among workers in this field to further elucidate the involvement of miR-1 and other microRNAs in the pathogenesis of this perplexing disease.

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Additional information

Competing interests

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Annex V: Kv7 channels in lung diseases

frontiers in Physiology

REVIEW
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Kv7 Channels in Lung Diseases

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Lung diseases constitute a global health concern causing disability. According to WHO in 2016, respiratory diseases accounted for 24% of world population mortality, the second cause of death after cardiovascular diseases. The Kv7 channels family is a group of voltage-dependent K^+ channels (Kv) encoded by *KCNQ* genes that are involved in various physiological functions in numerous cell types, especially, cardiac myocytes, smooth muscle cells, neurons, and epithelial cells. Kv7 channel α-subunits are regulated by KCNE1–5 ancillary β-subunits, which modulate several characteristics of Kv7 channels such as biophysical properties, cell-location, channel trafficking, and pharmacological sensitivity. Kv7 channels are mainly expressed in two large groups of lung tissues: pulmonary arteries (PAs) and bronchial tubes. In PA, Kv7 channels are expressed in pulmonary artery smooth muscle cells (PASMCs); while in the airway (trachea, bronchus, and bronchioles), Kv7 channels are expressed in airway smooth muscle cells (ASMCs), airway epithelial cells (AEPs), and vagal airway C-fibers (VACFs). The functional role of Kv7 channels may vary depending on the cell type. Several studies have demonstrated that the impairment of Kv7 channel has a strong impact on pulmonary physiology contributing to the pathophysiology of different respiratory diseases such as cystic fibrosis, asthma, chronic obstructive pulmonary disease, chronic coughing, lung cancer, and pulmonary hypertension. Kv7 channels are now recognized as playing relevant physiological roles in many tissues, which have encouraged the search for Kv7 channel modulators with potential therapeutic use in many diseases including those affecting the lung. Modulation of Kv7 channels has been proposed to provide beneficial effects in a number of lung conditions. Therefore, Kv7 channel openers/enhancers or drugs acting partly through these channels have been proposed as bronchodilators, expectorants, antitussives, chemotherapeutics and pulmonary vasodilators.

Keywords: Kv7 channels, KCNE, respiratory diseases, asthma, chronic obstructive pulmonary disease, pulmonary hypertension

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LUNG DISEASES: ONE WORLD'S HEALTH CONCERN

Lung diseases constitute a global health concern causing severe physical limitations and premature death, and entails enormous costs associated with treatment, hospital-based and primary care (Tobin, 2005). Mortality from overall lung diseases is very striking according to data collected by the WHO in 2016, in which respiratory diseases account for 24% of world population mortality, the second cause of death after cardiovascular diseases (WHO Global Health Estimates, 2020). At the top of mortality rate within lung diseases, the 9% of the population dies of tuberculosis. Second and third positions include chronic obstructive pulmonary disease (COPD) and lower respiratory infections with 5.4 and 5.2%, respectively. These are followed by lung cancer (affecting trachea, bronchus, and lungs) with 3% and, lastly, asthma and other respiratory

diseases with a 0.7 and 0.6%, respectively (**Figure 1**). Most major respiratory diseases are avoidable and can be mitigated by reducing exposure to air pollution, toxic smoke of biomass fuel, tobacco smoke, and different allergens (Laumbach and Kipen, 2012). Additional factors, such as aging, genetic predisposition, nutritional status, obesity, physical inactivity, raised blood pressure, human immunodeficiency virus 1 infection (HIV-1), or other infections may influence the risk of developing lung diseases. Lung diseases can be classified into three main groups depending on the lung tissue involved. First, airways diseases including asthma, COPD, emphysema, cystic fibrosis, or bronchitis in which the bronchial tubes are narrowed or blocked and the transport of oxygen and other gases are affected (Athanazio, 2012). Second, lung tissue diseases which affect the structure of alveoli or the interstitium (Corvol et al., 2009), such as pneumonia, tuberculosis, pulmonary edema, acute respiratory distress syndrome, sarcoidosis, and pulmonary fibrosis, where the lungs are unable to expand fully (Dellaripa, 2018). The third group includes diseases affecting the pulmonary vessels, such as pulmonary hypertension characterized by vasoconstriction, vascular remodeling, inflammation, and thrombosis (Cummings and Bhalla, 2015). This last group of diseases may end up affecting heart function.

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has recently rapidly evolved into a global pandemic. To date, risk factors associated with admission to ICU and mortality include cardiovascular comorbidities while respiratory diseases paradoxically do not appear to increase the prevalence or severity of the viral infection (Halpin et al., 2020; Li et al., 2020).

Kv7 CHANNEL STRUCTURE AND REGULATION

The Kv7 channel family, is a group of voltage-dependent K^+ channels subfamily Q, which are involved in various physiological activities of numerous cell types, especially, cardiac myocytes, smooth muscle cells, neurons, and epithelial cells (Stott et al., 2014). The different Kv7 channels isoforms are encoded by *KCNQ1–5* genes located at chromosomal loci *11p15*, *20q13*, *8q24*, *1p34*, and *6q13*, respectively (Barrese et al., 2018). *KCNQ* genes encode for five Kv7 proteins, Kv7.1–Kv7.5 channels, comprising approximately 650–940 aminoacids. Each pore-forming α-subunit of Kv7 channels has six helices transmembrane domains (TM). The TM5 and TM6 are part of the pore while the TM4 constitutes the voltage-detection domain. Four α-subunits form the transmembrane pore assembling into a homo- or hetero-tetrameric channel (Taylor and Sanders, 2017). Kv7 channels can be regulated by potassium voltage-gated channel subfamily E regulatory subunits (KCNEs) ancillary subunits (KCNE1–5, also called minK-related peptides), which present a single transmembrane domain with 103–177 residues, encoded by the five *KCNE1–5* genes located at chromosomal loci *21q11*, *21q11*, *11q13*, *2q36*, and *Xq23*, respectively (Lundquist et al., 2006). These KCNE subunits modulate several features of Kv7 channels such as biophysical properties, cell localization, channel trafficking, and sensitivity to different drugs (Barrese et al., 2018).

Mutations in all five *KCNQ* and *KCNE* genes have been shown to underlie excitability hereditary disorders (Jentsch, 2000; Maljevic et al., 2010; Lehman et al., 2017; Barrese et al., 2018). Particularly, mutations affecting *KCNQ1* gene cause disorders related to cardiac electrical activity such as long-QT syndrome, short-QT syndrome, and atrial fibrillation leading to severe arrhythmias and sudden death (Li et al., 1998; Chen et al., 2003). *KCNQ2* and *KCNQ3* gene mutations have been identified in different epilepsy syndromes (Neubauer et al., 2008) and, moreover, *KCNQ3* gain-of-function variant have been related to autism and developmental disability (Sands et al., 2019). Kv7.4 channels expressed in the cochlea are essential for normal hearing, and *KCNQ4* mutations are linked to hearing loss (Kubisch et al., 1999; Jung et al., 2019). Lastly, mutations in the Kv7.5 channel encoded by *KCNQ5* gene cause epilepsy and intellectual disability (Lehman et al., 2017). Different mutations have also been found in all five *KCNE* genes, mainly related to cardiac arrhythmias (Splawski et al., 2000; Lundby et al., 2008; Ohno et al., 2009; Abbott, 2016).

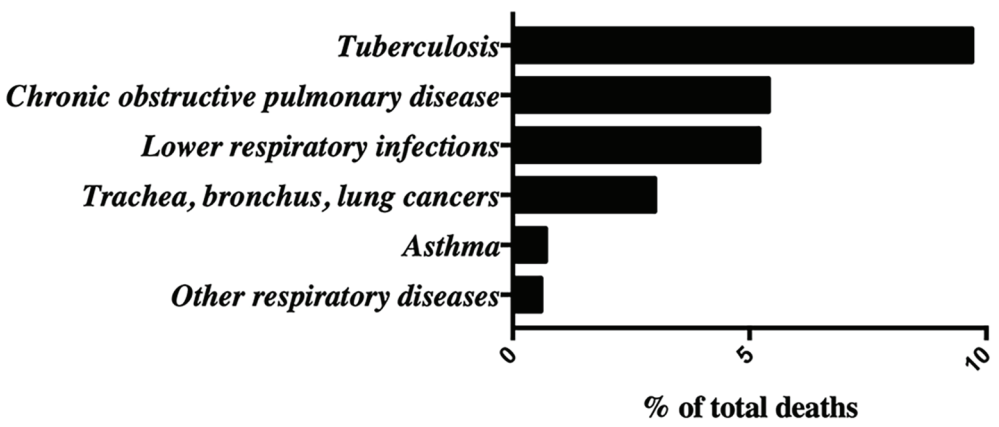


FIGURE 1 | Global lung diseases epidemiological data adapted from the *World Health Organization (WHO) Website*: Mortality rates in 2016 measured as % of total deaths.

Different combinations of Kv7 channels and KCNE subunits can be found depending on the cell type. Thus, there is a Kv7 channel isoform-specific expression playing different roles depending on tissue types (see section Kv7 Channel Expression in the Lung). Many KCNE subunits have been shown to modulate Kv7 channels α-subunits expressed in heterologous systems, providing a valuable experimental platform for elucidating structure-function relationships and mechanistic differences. Thus, all five KCNE proteins have been demonstrated to modulate Kv7.1 channels expressed in heterologous systems with diverse effects (Van Horn et al., 2011; Sun and MacKinnon, 2020). For instance, KCNE1 causes the Kv7.1 channel to activate at more positive voltages, slows its activation and deactivation, increases its conductance, and suppresses its inactivation (Sun and MacKinnon, 2020). While, KCNE2 and KCNE4 have inhibitory effects on Kv7.1 activity (Grunnet et al., 2002; Vanoye et al., 2009; Hu et al., 2019), KCNE3 stabilizes Kv7.1 channels voltage sensors (S4 segment) in an activated state turning the channel voltage-independent (Barro-Soria et al., 2015). Such a mechanism seems to require the participation of the signaling lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) in non-excitable cells such as lung epithelial cells (Zhou et al., 2019; Sun and MacKinnon, 2020). Sun and MacKinnon have recently identified binding sites of PIP_2 on Kv7.1 channels present in the S0, the S2-S3 loop, and the S4-S5 linker, and proposed a model in which the conformational change following PIP_2 interaction would results in dilation of the pore's gate (Sun and MacKinnon, 2020). In addition, KCNE subunits regulate traffic and cell surface expression of Kv7.1 channels (Roura-Ferrer et al., 2010; Hu et al., 2019). The information about the regulation of Kv7.2 and Kv7.3 channels by KCNE subunits is more limited. In *Xenopus* oocytes, KCNE1 was found to slow Kv7.2/7.3 heteromer currents and decreased their magnitude (Yang et al., 1998). Likewise, KCNE2 was shown to accelerate the deactivation kinetics of Kv7.2 and Kv7.2/7.3 channels transiently expressed in COS cells (Tinel et al., 2000). Whereas, KCNE1, KCNE2, and especially KCNE4 and KCNE5 increased Kv7.4 currents, co-expression with KCNE3 led to a strong attenuation (Strutz-Seebohm et al., 2006; de Jong and Jepps, 2018). Moreover, KCNE4 was shown to co-localize with Kv7.4 channels, regulating its activity and membrane abundance in mesenteric arterial myocytes (Jepps et al., 2015). Finally, Kv7.5 channels were shown to be affected by KCNE1 and KCNE3, but not by other KCNE subunits in *Xenopus* oocytes and HEK-293 cells (Roura-Ferrer et al., 2009, 2012). Thus, while KCNE1 was shown to slow activation and increase Kv7.5 currents, KCNE3 drastically inhibited these currents (Roura-Ferrer et al., 2009, 2012).

The activity of Kv7 channels is modulated by G protein-coupled membrane receptors (GPCRs). Thus, activation of different GPCR have been shown to inhibit M-type (Kv7) currents, with a dominant participation of the G_q and G_{11} α-subunits (Caulfield et al., 1994; Haley et al., 1998; Delmas and Brown, 2005). For instance, in neurons, Kv7 activity is decreased by M_1 and M_3 muscarinic and H_1 histaminic receptors coupled to $G_{q/11}$ proteins through different signal transduction pathways, including depletion of PIP_2, activation of protein kinase C (PKC), or increased intracellular calcium (Delmas and Brown, 2005; Higashida et al., 2005; Taylor and Sanders, 2017; Barrese et al., 2018). In human airway smooth muscle cells, histamine was found to inhibit Kv7.5 channels though a PKC-dependent phosphorylation of Ser441 (Haick et al., 2017). On the other hand, it is well recognized that stimulation of β-adrenergic receptor increases the activity of different Kv7 channels in different tissues such as cardiac (Kv7.1), neuronal (Kv7.2/Kv7.3), and vascular (Kv7.4/Kv7.5 and Kv7.5) channels (Jentsch, 2000; Marx et al., 2002; Chadha et al., 2014; Mani et al., 2016; Barrese et al., 2018). Likewise, in human airway smooth muscle cells, Kv7.5 channels are robustly enhanced by activation of β-adrenoceptors *via* a mechanism involving cyclic adenosine monophosphate/protein kinase A (cAMP/PKA)-dependent phosphorylation (Brueggemann et al., 2018). This effect might be shared with other GPCRs but does not seem to account for Kv7.4 activation. The mechanism may involve increased membrane expression of Kv7 channels mediated by colchicine-sensitive microtubule disruption (Lindman et al., 2018). Kv7 channels are also regulated by GPCR-independent mediators activating soluble guanylate cyclase (sGC) such as nitric oxide (NO) (Mondejar-Parreño et al., 2019) and natriuretic peptide (Stott et al., 2015). The implications in respiratory diseases and drug therapy are discussed below.

In addition to regulation by KCNE β-subunits and GPCRs, Kv7 channels can be regulated by scaffolding proteins, sodium-coupled myo-inositol transporter (SMIT), lipids, microRNAs, and/or further signaling mechanisms as extensively reviewed elsewhere (Delmas and Brown, 2005; Zaydman and Cui, 2014; Taylor and Sanders, 2017; Barrese et al., 2018; Byron and Brueggemann, 2018).

Kv7 CHANNEL EXPRESSION IN THE LUNG

Kv7 channels are expressed mainly in pulmonary arteries (PAs) and in the bronchial tubes (**Figure 2**). In PA, Kv7 channels are expressed in pulmonary artery smooth muscle cells (PASMCs); while in the bronchial tubes (trachea, bronchus and bronchioles), Kv7 channels are expressed in airway smooth muscle cells (ASMCs), airway epithelial cells (AEPs) and vagal airway C-fibers (VACFs). The functional role of Kv7 channels may vary depending on the cell type. While most of the available studies have provided relevant information on the particular expression of α-subunits in these cell types, evidence for the expression of heteromeric Kv7 complexes or KCNE subunits is still very scarce.

Pulmonary Artery Smooth Muscle Cells

The expression of Kv7 channels in mice and rat PA has been demonstrated in several studies. Specifically, their expression is located in PASMC. In these cells, the expression of Kv7.1, Kv7.4, and Kv7.5 channels predominates; but Kv7.4 channels expression appears to be the most relevant. The expression of Kv7.2 and Kv7.3 channels in PASMCs is still controversial (Joshi et al., 2009; Morecroft et al., 2009; Chadha et al., 2012; Li et al., 2014a; Sedivy et al., 2015; Eid and Gurney, 2018; Mondejar-Parreño et al., 2018). The existence of Kv7.1/7.5 or Kv7.4/7.5 heteromeric

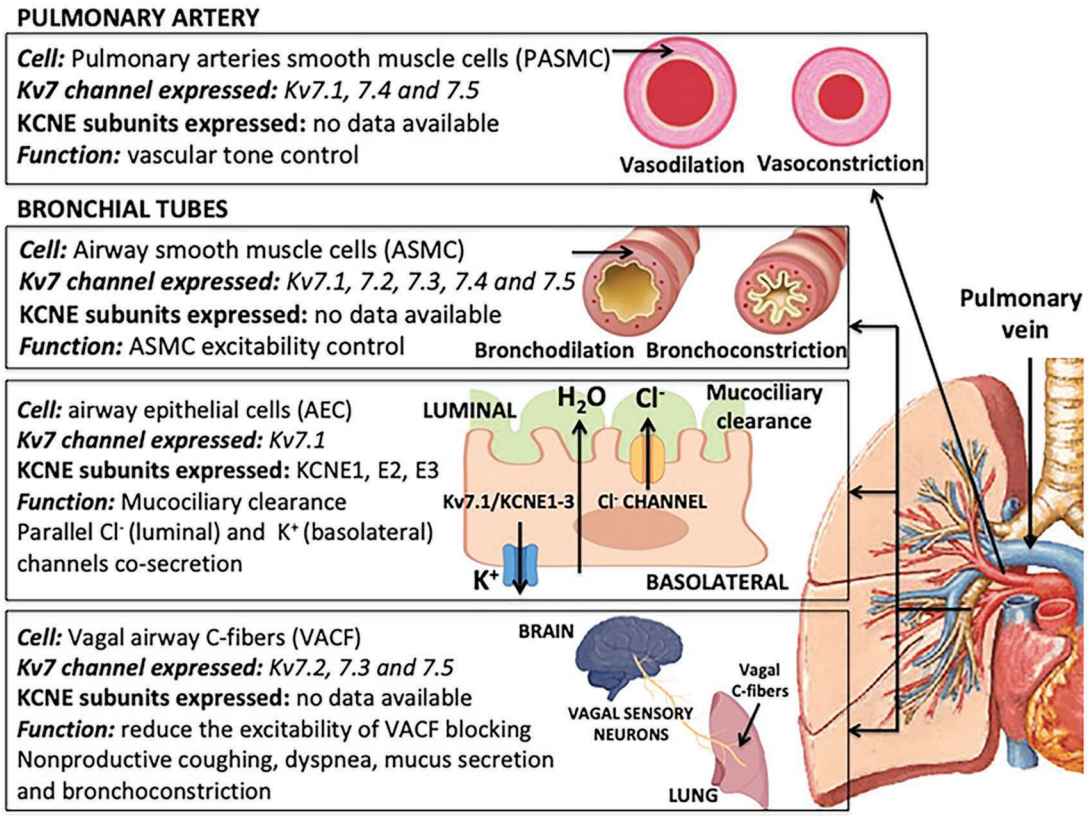


FIGURE 2 | Expression and functions of isoform-specific Kv7 channel and potassium voltage-gated channel subfamily E regulatory subunits (KCNE) ancillary subunit in lung cells.

complexes in vascular smooth muscle has been postulated on several studies (Chadha et al., 2014; Oliveras et al., 2014; Barrese et al., 2018). In particular, Kv7.4/7.5 heteromers have been proposed as the dominant functional vascular Kv7 channels, supported by electrophysiological data showing that Kv7 currents in freshly isolated vascular smooth muscle cells have phenotypes intermediate to the respective homomeric Kv7.4 and Kv7.5 channels (Brueggemann et al., 2014a; Chadha et al., 2014; Fosmo and Skraastad, 2017; Barrese et al., 2018). We have recently confirmed that Kv7.4/7.5 heteromeric complexes are also present in PASMC (authors' unpublished observations). Although, in other types of arteries, the expression of Kv7 channels has been located also in endothelial cells (Goodwill et al., 2016), whether this is also the case for pulmonary artery endothelial cells (PAECs) remains unknown. The main function of these channels is to regulate pulmonary vascular tone, due to their contribution to maintain the membrane potential (Em) of PASMC (Joshi et al., 2009; Sedivy et al., 2015; Mondejar-Parreño et al., 2018, 2019).

Airway Smooth Muscle Cells

There is some interspecies variability in the expression of Kv7 channel isoforms in the airways. Thus, ASMCs from guinea pigs were shown to express Kv7 channels with a relative abundance of *KCNQ2* > *KCNQ5* > *KCNQ4* > *KCNQ3* and, to a lesser extent, *KCNQ1* (Brueggemann et al., 2011). Comparatively, *KCNQ* mRNA expression in ASMC revealed a high expression of *KCNQ4* followed by a fainter expression of *KCNQ5* and *KCNQ1* transcripts (Brueggemann et al., 2014b). Studies in human tracheal muscle revealed abundant expression of *KCNQ1* relative to guinea pig, a modest expression of *KCNQ4* and *KCNQ5* and no detectable expression of *KCNQ2* or *KCNQ3* (Brueggemann et al., 2011). A further study showed that cultured human ASMCs express all *KCNQ* genes with a predominant relative abundance of *KCNQ5* mRNA followed by *KCNQ3* or *KCNQ4*, while *KCNQ2* and *KCNQ1* mRNA are expressed to a lesser extent (Brueggemann et al., 2018). These differences in the expression profile of Kv7 channels in human tissue could be due to phenotypic changes associated with cell culture. Measurements of Kv7 currents and functional studies assessing airway smooth muscle contractility in the bronchioles from different species have pointed to Kv7 channels as important players in maintaining, resting Em, and controlling ASMC excitability (Evseev et al., 2013; Brueggemann et al., 2014b, 2018).

Airway Epithelial Cells

Airway epithelial cells (AECs) predominates the expression of Kv7.1 channels, also named KvLQT1, along with the expression of KCNE1 and KCNE3 ancillary subunits (Mall et al., 2000;

Boucherot et al., 2001; Trinh et al., 2007; Preston et al., 2010; Frizzell and Hanrahan, 2012; Kroncke et al., 2016; Hynes and Harvey, 2019). Nevertheless, KCNE2 has also been found to be expressed in *Calu-3* cells, a model for human submucosal serous cells of the lung, where it has been suggested to regulate *KCNQ1* (Cowley and Linsdell, 2002a). Chloride (Cl^-) secretion in AEC is essential to mucociliary clearance and requires the co-activation of Cl^- channels in luminal membrane and, in parallel, of K^+ channels in the basolateral membrane which involves Kv7.1 channels (Frizzell and Hanrahan, 2012).

Vagal Airway C-Fibers

Using single-cell mRNA, Sun et al. have recently demonstrated that *KCNQ2*, *KCNQ3*, and *KCNQ5* are consistently expressed in mouse airway sensory afferent nerves and remarkably, the expression of *KCNQ3* mRNA was the most prevalent (Sun et al., 2019). Most vagal sensory nerves in the respiratory tract are nociceptive C-fibers. Increased VACF activity may contribute to the nonproductive coughing, dyspnea, parasympathetic reflex mucus secretion, and bronchoconstriction. Thus, it has been proposed that activation of Kv7 channels may reduce the vagal C-fibers excitability innervating the airways, and being useful to treat chronic cough.

Kv7 CHANNELS IN LUNG DISEASES

This section covers the potential pathophysiological role of Kv7 channels in different respiratory diseases such as cystic fibrosis, asthma, COPD, chronic coughing, lung cancer, and pulmonary hypertension.

Cystic Fibrosis

The airway surface liquid layer represents a key primary defense mechanism of mucociliary clearance, which crucially depends on the transport of chloride into the lumen of the airways, and the subsequent movement of water. Cl^- ion transport across the apical membrane is mainly driven by a cAMP-regulated Cl^- channel named cystic fibrosis transmembrane conductance regulator (CFTR) and by the calcium-activated Cl^- channels (CaCC) (Frizzell and Hanrahan, 2012; Bartoszewski et al., 2017). However, a possible overlap between these two pathways has also been proposed (Billet and Hanrahan, 2013). Cystic fibrosis is an autosomal recessive disease due to mutations in the gene encoding CFTR and characterized by alterations in the composition and volume of secreted luminal fluids. The CFTR is a member of the ATP-binding cassette (ABC) transporter superfamily known to function as an ion cannel that conducts chloride ions across epithelial cell membranes and is responsible for salt secretion in response to cAMP/PKA stimulation. Thus, mutations of the CFTR gene lead to defective cAMP-dependent Cl^- secretion and enhanced Na^+ absorption throughout the apical membrane of airway epithelia.

CFTR has been shown to influence both function and properties of other membrane channels, such as epithelial Na^+ channels, inward rectifying renal outer medullary K^+ channel (ROMK), outwardly rectifying Cl^- channels, and aquaporin 3 (Schwiebert et al., 1999). Cl^- secretion in AEC requires the parallel co-activation of Cl^- channels and K^+ channels, in the luminal surface and the basolateral membrane, respectively. Whereas Cl^- channels are required for Cl^- efflux to the luminal side of the AEC, K^+ channels are needed for K^+ recycling *via* the basolateral membrane (Boucherot et al., 2001; Bartoszewski et al., 2017). Kv7.1 channels have been shown to contribute to the basolateral cAMP-activated K^+ conductance in human airway epithelium (Mall et al., 1996, 2000; Bardou et al., 2009). This study also showed that the Kv7.1 channel inhibitor chromanol 293B inhibited Cl^- secretion suggesting an important role of these K^+ channels in maintaining cAMP-dependent Cl^- secretion in human airways. Since CFTR is a modulator of other ion channels, several studies have analyzed whether it controls the activity of Kv7.1 channels. Studies analyzing this issue have led to contradictory results. Thus, while initial studies suggested that CFTR could directly modulate Kv7.1 conductance (Cunningham et al., 1992; Loussouarn et al., 1996; Mall et al., 2000), this was not the case in other studies (Boucherot et al., 2001). Interestingly, using CFTR KO mice, Belfodil et al. (2003) found that cAMP-sensitive Kv7.1 currents were regulated by CFTR in renal distal but not in proximal tubule cells, suggesting that the possible modulation of Kv7.1 is unlikely to involve a direct interaction.

As stated above, human airway epithelia also express CaCC that are activated by extracellular nucleotides [adenosine triphosphate (ATP) and/or uracil triphosphate (UTP)] and other Ca^{2+}-increasing agonists. CaCC was shown to be preserved or enhanced in human and murine cystic fibrosis airways as a mechanism to compensate for the lack of CFTR (Clarke et al., 1994; Grubb et al., 1994). Since, CaCC-mediated Cl^- secretion appears to be preserved in cystic fibrosis its pharmacological stimulation has been suggested as a therapeutic tool for bypassing dysfunctional CFTR. Mall et al. demonstrated that activation of the cAMP pathway increased CaCC-mediated secretion in cystic fibrosis and, conversely, chromanol 293B, an inhibitor of cAMP-activated Kv7.1 channels was able to fully abolish nucleotide-activated Cl^- secretion (Mall et al., 2003). Hence, the authors proposed that activation of Kv7.1 channels, together with hSK4 K^+ channels, may improve Cl^- secretion *via* CaCC in human cystic fibrosis airway tissues (Mall et al., 2003). Altogether, these studies indicate the Kv7.1 channel activation may help to improve mucociliary clearance in cystic fibrosis airways.

Elevated levels of reactive oxygen species (ROS) lead to oxidative stress and DNA damage in several tissues providing a common pathological mechanism in all inflammatory lung diseases. Hydrogen peroxide (H_2O_2) has been proven to stimulate several components of the Cl^- secretion in the airway epithelia, including the cAMP-dependent activation of CFTR and the basolateral K^+ channels (Cowley and Linsdell, 2002b; Ivonnet et al., 2015). The basolateral K^+ conductance was abrogated by clofilium, a Kv7.1 channel inhibitor, suggesting a role for these channels in the H_2O_2-stimulated response (Cowley and Linsdell, 2002b; Ivonnet et al., 2015). In line with this, H_2O_2 has been reported to potently enhance Kv7 currents (Gamper et al., 2006; Kim et al., 2016). Activation of transmembrane adenylyl cyclase by H_2O_2 has been proposed as a triggering

mechanism for the cAMP-activated basolateral Kv7.1 current (Ivonnet et al., 2015). The stimulated anion secretion by ROS could act as a compensatory protective mechanism to keep the airways clear from damaging radicals and to hold acceptable airway surface liquid levels.

While the Kv7.1 channel is the member of the *KCNQ* family with the strongest evidence of its functional expression and physiological role in lung epithelial cells, others (*KCNQ3* and *KCNQ5*) have been detected in the apical or lateral membranes (Moser et al., 2008). In addition, mRNA transcripts for several KCNE accessory subunits (*KCNE2* and *KCNE3*) have been found in human airway Calu-3 cells (Cowley and Linsdell, 2002a). Kv7.1 channels expressed in *Xenopus* oocytes were shown to be activated by an increase in intracellular cAMP concentration, independently of co-expression with KCNE1 or KCNE3 ancillary subunits (Boucherot et al., 2001). On the other hand, Potet et al. demonstrated that recombinant Kv7.1/KCNE1 complexes expressed in *Cos-7* cells were totally insensitive to cAMP (Potet et al., 2001). The co-assembly of Kv7.1 with KCNE3 subunits markedly affects Kv7.1 channel properties (abolishing its voltage dependence) and has been proposed to form the cAMP-activated basolateral K^+ channels contributing to Cl^- secretion in colonic epithelial cells (Schroeder et al., 2000). Interestingly, Preston et al. evidenced that cAMP-stimulated Cl^- secretion across tracheal epithelia was severely reduced in *KCNE3*$^{(-/-)}$ mice (Preston et al., 2010). While the involvement of KCNE3 in airway secretion requires confirmation in future studies, the structural basis of the interaction of Kv7.1 with KCNE3 leading to a constitutive activation of Kv7.1 channel activity has been recently established (Kroncke et al., 2016; Sun and MacKinnon, 2020).

Asthma

Asthma is characterized by hyper-contractility of bronchial smooth muscle attributed to an increased concentration of different bronchoconstrictor agonists, or enhanced sensitivity to them, leading to aberrant narrowing of the airways (Penn, 2008). Acetylcholine, leukotriene D4, endothelin, and histamine represent the most relevant locally released bronchoconstrictor agonists that activate GPCR on ASMC, leading to increased cytosolic Ca^{2+} concentration and contraction (Jude et al., 2008; Penn, 2008). This together with the strong inflammation leads to an acute hypersensitivity to different air stimulants that produce ASMC bronchospasm and that, in the long term, promote smooth muscle proliferation and hypertrophy (Stott et al., 2014). Kv7 channels are considered negative regulators of ASMC contraction due to their major contribution to maintain negative Em negative, preventing L-type-Ca^{2+} channel activation and the subsequent bronchoconstriction (Brueggemann et al., 2011). Several members of the Kv7 family are expressed in ASMCs (Brueggemann et al., 2011; Bartoszewski et al., 2017). The relative abundance expression of the Kv7 members appears to differ depending on the location (trachea vs. bronchioles) and depending on the animal species as mentioned above (Brueggemann et al., 2011, 2014b; Evseev et al., 2013). Several studies have evidenced that bronchoconstrictor agonists negatively regulate the activity of Kv7 channels. Thus, methacholine, carbachol, or histamine reduce Kv7 currents in guinea pig, mouse, and rat ASMCs (Brueggemann et al., 2011, 2014; Haick et al., 2017). On the other hand, structurally unrelated Kv7 channel activators cause a concentration-dependent relaxation of small bronchioles (Brueggemann et al., 2014b) and tracheal rings (Evseev et al., 2013). Of note, *in vivo* administration of the Kv7 channel opener retigabine was proven to transiently reduce bronchoconstriction induced by methacholine in mice (Evseev et al., 2013).

Chronic administration of long-acting β_2-adrenergic receptor agonist elicits a time- and concentration-dependent relaxation of rat airways, with remarkable desensitization to short-term or with repeated treatments. Co-administration of retigabine plus the long-acting β_2-adrenergic receptor agonist formoterol was shown to cause a sustained reduction of methacholine-induced bronchoconstriction and reduced the desensitization observed with formoterol alone (Brueggemann et al., 2014b). These findings suggest that using a Kv7 channel enhancer together a β_2-adrenergic receptor agonist may improve bronchodilator therapy.

In addition to exaggerated bronchoconstriction, asthma is characterized by a chronic inflammatory response in the airways that is targeted primarily with inhaled corticosteroids. As stated in the previous section basolateral membrane K^+ channels play a key role in stimulating Cl^- transport across AEPs (Frizzell and Hanrahan, 2012). Unlike in cystic fibrosis, a decrease in airway mucus secretion ameliorates the symptomatology of asthma. Very recently, Hynes and Harvey have demonstrated that dexamethasone produces an inhibition of transepithelial chloride ion secretion though a rapid non-genomic inhibition of Kv7.1 channels and KCNN4 ($K_{Ca}3.1$) (Hynes and Harvey, 2019). Thus, it is plausible that down-regulation of Kv7.1 channel activity might contribute to the potential benefit of corticosteroids to reverse airway hypersecretion.

Chronic Obstructive Pulmonary Disease (COPD)

COPD is a progressive life-threatening lung disease characterized by airway obstruction due to inflammation of the small airways. The disease evolves with irreversible limitation of airflow, loss of lung function and severe acute exacerbations which significantly deteriorate the patient's quality of life (Barnes et al., 2003). β-adrenergic receptors (β-AR) agonists promote airway smooth muscle relaxation and are the drugs of choice for rescue from acute bronchoconstriction in asthma and COPD. A recent study has shown that the long-acting β-AR formoterol robustly enhanced Kv7 currents in human ASMCs (Brueggemann et al., 2018). Furthermore, these authors provided evidence that Kv7.5 is the predominant Kv7 α-subunit in human ASMCs and identified a PKA-dependent phosphorylation of S53 on the N-terminus of Kv7.5 channel subunits as a potential mechanism of activation following the stimulation of the βAR/cAMP pathway (Brueggemann et al., 2018).

The exposure to cigarette smoking is the principal cause of COPD (Salvi, 2014). Recently Sevilla-Montero et al. have analyzed the impact of cigarette smoke exposure on the pulmonary vasculature (Sevilla-Montero et al., 2019). Authors showed that cigarette smoke extract exposure had direct effects

on human fibroblasts and PASMCs, promoting a senescent phenotype which contributed to the secretion of inflammatory molecules and increase the proliferation of non-exposed cells. Furthermore, cigarette smoke extract exposure affected cell contractility attenuated the pulmonary vascular responses to Kv7 channel modulators and dysregulated the expression and activity of Kv7.4 channels. The results suggest that cigarette smoke may impair pulmonary vascular tone atleast in part through Kv7.4 downregulation (Sevilla-Montero et al., 2019).

A sequence polymorphism of the human *KCNE2* gene has been found to be associated with altered lung function (Soler-Artigas et al., 2011; Sabater-Lleal et al., 2014). The deletion of *KCNE2* reduced pulmonary expression of the Kv7.1 α-subunit suggesting the formation of pulmonary KCNQ1-KCNE2 complexes (Zhou et al., 2019). *KCNE2*$^{(-/-)}$ mice had reduced blood O_2, increased CO_2, increased pulmonary apoptosis, and increased inflammatory mediators TNF-α, IL-6, and leukocytes in bronchoalveolar lavage fluids (Zhou et al., 2019). These data indicated KCNE2 regulates *KCNQ1* in the lungs and is required for normal lung function.

Chronic Cough

Airway sensory afferent nerves are essential for triggering different reflex responses to potential injurious stimuli (Undem and Kollarik, 2005). Most vagal sensory afferent nerves which terminate in the respiratory tract are C-fibers (Undem and Kollarik, 2005). The excessive activity of VACF induces different symptoms associated with inflammatory lung diseases, such as nonproductive coughing, dyspnea, parasympathetic reflex mucus secretion and bronchoconstriction (Mazzone and Undem, 2016). Consequently, ion channels that regulate the excitability of C-fibers in the airways can be an attractive therapeutic target aimed at reducing the symptoms of various inflammatory diseases of the airways. Using single-cell mRNA analysis, Sun and collaborators have very recently shown that *KCNQ2*, *KCNQ3*, and *KCNQ5* are consistently expressed in mouse lung specific nodose neurons with *KCNQ3* being the most predominant (Sun et al., 2019). This study suggested that Kv7.3 channels are the major contributors to the Kv7-M current in the nodal neurons of the mouse lungs. The Kv7 channel activator retigabine increased the amplitude and accelerated the activation rate of the M-current, caused a prominent hyperpolarization, and suppressed the excitability of mouse nodose neurons. These effects were reversed or prevented in the presence of XE991, a Kv7 channel inhibitor. Remarkably, nebulized retigabine was found to attenuate cough evoked by inhalation of irritant gases (SO_2 and NH_3) in awake mice (Sun et al., 2019). Although future studies are needed to confirm their role in the regulation of C-fiber excitability of the airways in humans, these data indicate that Kv7.3 channels may provide a novel therapeutic target to reduce the conditions associated with pulmonary inflammatory diseases such as chronic coughing.

Non-small Cell Lung Cancer

K^+ channels regulate a number of processes of key relevance in cancer biology such as cell volume control, pH regulation, migration, proliferation, differentiation, apoptosis, and drug resistance (Rao et al., 2015; Prevarskaya et al., 2018; Haworth and Brackenbury, 2019; Serrano-Novillo et al., 2019). Thus, there is increasing evidence that K^+ channels play an important role in cancer; but a clear scenario is still far due to the plethora of K^+ channel isoforms and their diverse repertoire of effects. Notably, K^+ channels regulate cell cycle, but according to the channel isoform, its level of expression, the cell type, and the stage of differentiation the effects are diverse. For instance, Kv channels may exhibit proliferative or anti-proliferative roles, depending on the channel subtype and the phase of the cell cycle (Rao et al., 2015). Moreover changes in the phenotypes, signaling pathways, and pattern of expression of K^+ channels may be observed in cancer, which increases the complexity of K^+ channels regulation on cell viability. In addition to the well-characterized involvement of several Kv channels (most notably Kv1.1, Kv1.3, Kv1.5, Kv10.1, Kv10.2, and Kv11.1) in cancer (Rao et al., 2015), some members of the Kv7 family have also been suggested to participate in cell proliferation and cancer (Serrano-Novillo et al., 2019).

K^+ channels participate in tumor cell invasion and metastasis propagation *via* regulation of cell migration and growth (O'Grady and Lee, 2005; Schwab et al., 2008; Arcangeli et al., 2009; Prevarskaya et al., 2010). Indeed, inhibiting or silencing K^+ channels reduces the proliferation of cancer cells (Pancrazio et al., 1993; Jang et al., 2011). Kv7.1 channels have been highlighted in cell migration, proliferation, and repair of AECs (Trinh et al., 2007). In 2014, Girault and colleagues showed an increased expression (by 1.5- to 7-fold) of Kv7.1 channels in tumor lung adenocarcinoma samples, compared to non-neoplastic tissues from 17 of 26 patients (Girault et al., 2014). Authors found that the proliferation rates of lung adenocarcinoma cell monolayers were reduced by the Kv7.1 channel blockers clofilium and chromanol, or after silencing Kv7.1 channels using a specific siRNA. These data suggest that Kv7.1 channels could be a potential therapeutic target in lung cancer (Girault et al., 2014).

On the other hand, K^+ channels have also been implicated in both early and late stages of apoptosis. The decrease in cell volume is one of the earliest and necessary morphological changes observed in cells undergoing apoptosis. Active K^+ channels in the plasma membrane leads to K^+ and Cl^- outflow through K^+ and Cl^- channels, this generates an osmotic gradient leading to an outward water transport through aquaporins which results in a decrease in cell volume (Maeno et al., 2000). In later stages of apoptosis, the augmented intracellular K^+ concentration ($[K^+]_{int}$) suppresses caspase and nuclease activity and, conversely, the decreased $[K^+]_{int}$ caused by K^+ channel activation, promotes apoptosis (Burg et al., 2008). There is a wealth of literature supporting that the M- channel activators have a neuroprotective role *via* Kv7 activation and reduction of hyperactivity and, hence, suppression of glutamate excitotoxicity (Gamper et al., 2006; Bierbower et al., 2015; Vigil et al., 2020). It is likely that flupirtine would work the same way (Panchanathan et al., 2013; Li et al., 2014a). Nevertheless, its ability to inhibit cell viability has also been reported. Thus, Lee et al. showed that flupirtine exerted anti-proliferative effects in canine osteosarcoma cells by enhancing Kv7.5 function and arresting cells in the G_0/G_1 phases (Lee et al., 2014).

Similarly, retigabine was found to exhibit anti-proliferative effects in *C2C12* myoblasts (Iannotti et al., 2010).

Non-small-cell lung cancer (NSCLC) accounts for most cancer deaths. Aberrant activation of the epidermal growth factor receptor (EGFR) is involved in the pathogenesis and progression of several human cancers, including NSCLC (Melosky, 2014). Indeed, approximately 10–38% of NSCLC patients have EGFR gene mutations (Lin et al., 2014). EGFR tyrosine kinase inhibitors, such as Gefitinib have become standard first-line treatment for these patients (Cohen et al., 2003). However, acquired resistance represents a major limitation for drug efficacy. Cancer stem cells have self-renewal properties in various solid tumors, and play a key role in tumor growth and progression (Wang et al., 2013) and in therapy resistance (Steinbichler et al., 2018). A subset of cancer stem cells, termed the "side population" (SP), have elevated clonogenic potential and higher expression levels of ABC-transporters than main-population cells known as non-SP cells (Richard et al., 2013) and are considered to be responsible for anti-cancer drug resistance (Singh et al., 2010; Leon et al., 2016). Choi and colleagues have demonstrated a reduced mRNA expression of *KCNQ3* and *KCNQ5* in SP cells compared to non-SP cells (Choi et al., 2017). The treatment of flupirtine (a Kv7.2–7.5 channel enhancer) decreased the viability of SP and non-SP cells. In addition, the combination treatment of gefitinib plus flupirtine was more effective, compared to the individual treatment of both drugs, in decreasing the viability of both non-SP cells, and gefitinib-resistant SP cells. According to their results, the combination treatment of gefitinib plus flupirtine was proposed to reduce the viability of gefitinib-resistant cells through inhibition of the EGFR-Ras-Raf-ERK pathway *via* (Choi et al., 2017). These results suggest that the activation of Kv7.3 and Kv7.5 channels could play an important role in the induction of apoptosis in SP cells, either by participating in the decrease of the cellular volume necessary for apoptosis initiation; or by decreasing $[K^+]_{int}$, which results in the activation of caspases and nucleases.

Pulmonary Hypertension

Pulmonary hypertension is defined by an increase in mean pulmonary arterial pressure at rest, as assessed by right heart catheterization. Based on pathophysiological findings, clinical presentation, hemodynamic characteristics, and therapeutic considerations, pulmonary hypertension is categorized into five groups by WHO: pulmonary arterial hypertension (PAH), pulmonary hypertension due to left-sided heart disease, pulmonary hypertension due to lung disease or hypoxia, chronic thromboembolic pulmonary hypertension, and multifactorial pulmonary hypertension (Simonneau et al., 2019). PAH is characterized by excessive pulmonary vasoconstriction and progressive remodeling of the distal PAs, resulting in elevated pulmonary vascular resistance and, eventually, in right ventricular failure (McLaughlin et al., 2015). Reduced expression or function of K^+ channels (notably Kv1.5 and TASK-1 channels) in PASMCs results in a more depolarized Em, and is considered to contribute to the enhanced vasoconstriction and proliferation (Moudgil et al., 2006; Boucherat et al., 2015; Hayabuchi, 2017). In 2009, Joshi et al. demonstrated that Kv7 channel blockers, linopirdine, and XE991, inhibited the I_{KN} current in PASMCs and raised mean pulmonary arterial pressure (mPAP); while the Kv7 channel openers, retigabine and flupirtine, had the opposite effects (Joshi et al., 2009). Linopirdine can also increase mesenteric vascular resistance and systemic arterial pressure (Mackie et al., 2008). Functional expression of Kv7 channels in the pulmonary circulation was later confirmed by others (Morecroft et al., 2009; Eid and Gurney, 2018; Mondejar-Parreño et al., 2018). As in the systemic circulation, *KCNQ1* and especially *KCNQ4* and *KCNQ5* appear the most predominant *KCNQ* channels in the pulmonary circulation (Joshi et al., 2009; Morales-Cano et al., 2014; Sedivy et al., 2015; Mondejar-Parreño et al., 2018). The lack of selective drugs for the different Kv7 subunits hinders identification of the functional impact of individual Kv7 members (Barrese et al., 2018). Structurally related Kv7 enhancers such as retigabine and flupirtine (which activate all Kv7 isoforms except Kv7.1 channels) (Barrese et al., 2018) and zinc pyrithione (which activate all Kv7 members but Kv7.3) have been shown to induce pulmonary vasodilation (Joshi et al., 2009; Morecroft et al., 2009; Sedivy et al., 2015; Eid and Gurney, 2018; Mondejar-Parreño et al., 2018). In addition, drugs that block all Kv7 channels (i.e., linopirdine and XE991) are able to contract PAs previously primed (Chadha et al., 2012; Sedivy et al., 2015) or not (Joshi et al., 2009), whereas selective Kv7.1 blockers HMR1556, L768, L673, and JNJ39490282 (Barrese et al., 2018) have no contractile effects in PAs (Chadha et al., 2012). On the other hand, the application of R-L3, a compound purported to activate Kv7.1 channels, relaxed efficiently precontracted intrapulmonary arteries (Chadha et al., 2012; Mondejar-Parreño et al., 2018). These data suggest that even when Kv7.4 and Kv7.5 appear to be the most relevant Kv7 channels in controlling resting Em in PASMC, Kv7.1 channels are also functionally expressed.

The NO/cGMP pathway represents a key physiological signaling controlling tone in PAs, and drugs stimulating this pathway are used to treat PAH (Barnes and Liu, 1995), and drugs activating this pathway are used to treat pulmonary arterial hypertension. Recently, we have found that NO donors and riociguat, a stimulator of sGC used for the treatment of pulmonary arterial hypertension, enhance Kv7 current and induce membrane hyperpolarization in fresh isolated PASMCs contributing to pulmonary vasodilation (Mondejar-Parreño et al., 2019). Further, we identified Kv7.5 channels as a potential member of the Kv7 family targeted by these drugs.

The expression of Kv7 channels has been explored in several experimental models of pulmonary hypertension. While the hypoxic pulmonary vasoconstriction (HPV) reflects a physiological intrinsic property of PASMCs, which allows shifting blood flow from hypoxic to normoxic lung areas, coupling ventilation and perfusion, the chronic exposure to hypoxic air causes the pulmonary circulation to become hypertensive (Sylvester et al., 2012). Sedivy et al. found a significant loss of *KCNQ4* mRNA expression in PA from rats maintained for 3 days in a hypoxic environment, even when a significant reduction of the Kv7.4 protein expression could not be confirmed (Sedivy et al., 2015). On the other hand, the expression of *KCNQ1* and *KCNQ5* was unaffected. However, Li et al. reported that longer incubation on hypoxia (9 days) was associated with a downregulation of

KCNQ5 in the PAs (Li et al., 2014b). These authors also demonstrated that microRNA-190 (miR-190), whose expression is increased following the hypoxic exposure, directly targeted *KCNQ5*. This led the authors to propose the increased in miR-190 as a mechanism involved in the *KCNQ5* downregulation and enhanced vasoconstriction induced by hypoxia. In addition to the hypoxic pulmonary hypertension models, a slight reduction of *KCNQ5* expression has also been observed in rat lungs from monocrotaline-induced pulmonary hypertension (Morales-Cano et al., 2014). On the other hand, we found the expression of *KCNQ1*, *KCNQ4*, and *KCNQ5* mRNAs unaltered in lungs from Zucker rats, a model of type 2 diabetes, developing increased pulmonary arterial pressure, and right ventricular hypertrophy (Morales-Cano et al., 2019).

The potential impairment of *KCNQ* channels has been also explored in conditions associated with pulmonary hypertension. The HIV-1 infection is an established risk factor for PAH, although the pathological mechanisms underlying the development of HIV-related PAH remain unclear (Butrous, 2015). In a recent study using HIV-transgenic mice expressing seven of the nine HIV viral proteins and wild-type mice we observed that HIV mice had reduced lung expression of *KCNQ1* and *KCNQ5* channels (Mondejar-Parreño et al., 2018). By using vascular reactivity and patch-clamp experimental approaches we found decreased responses to the Kv7.1 channel activator L-364,373 but unaltered responses to retigabine (Mondejar-Parreño et al., 2018). These results suggest that Kv7 channels (especially Kv7.1) are impaired in mice expressing HIV-1 proteins.

Congenital diaphragmatic hernia is a rare congenital anomaly commonly associated with persistent pulmonary hypertension. A recent study showed that the expression of *KCNQ5*, but not that of *KCNQ1* or *KCNQ4*, was significantly downregulated at the mRNA and protein levels in rat lungs from nitrofen-induced congenital diaphragmatic hernia (Zimmer et al., 2017). Likewise, a markedly diminished *KCNQ5* expression was found in the pulmonary vasculature of congenital diaphragmatic hernia fetuses. Since Kv7.5 channels have shown to contribute to NO donors-induced pulmonary vasodilation (Mondejar-Parreño et al., 2019), its reduced availability in congenital diaphragmatic hernia could partly explain the poor therapeutic response to inhaled NO in these patients (Group (NINOS), 1997; Putnam et al., 2016).

Studies in experimental models have shown evidence that drugs activating Kv7 channels may be of benefit in the treatment of pulmonary hypertension with different etiologies (Morecroft et al., 2009; Sedivy et al., 2015). Morecroft et al. also demonstrated that flupirtine attenuated the development of chronic hypoxia-induced pulmonary hypertension in mice and reversed established pulmonary hypertension in mice that over-express the 5-HT transporter (SERT), apparently *via* Kv7 activation (Morecroft et al., 2009). In this study authors showed that the chronic treatment with flupirtine reduced mean right ventricle pressure (mRVP), pulmonary remodeling, and RV hypertrophy (RVH). More recently, Sedivy et al. confirmed the potential benefit of Kv7 channel openers showing the pulmonary antihypertensive effects of flupirtine in rats exposed to short-term (3–5 days) hypoxia (Sedivy et al., 2015). In line with this, we have found that retigabine-induced vasodilation is increased in PAs from pulmonary hypertensive animals and this is associated with an increased contribution of Kv7 channels to the net K^+ current present in pulmonary arterial myocytes (unpublished data from the author's laboratory).

Altogether, these findings suggest that Kv7 channels play a protective role in the pulmonary circulation limiting pulmonary vasoconstriction, and their activation may represent a promising therapeutic strategy in pulmonary hypertension.

Kv7 CHANNELS: NEW PHARMACOLOGICAL TARGETS IN RESPIRATORY DISEASES?

In addition to their well-established role in neural and cardiac tissue, studies over the last 20 decades have evidenced the expression and the plethora of cellular functions of Kv7 channels in numerous cell types. Thus, Kv7 channels are now recognized as playing relevant physiological roles in many tissues, which has encouraged the search for the therapeutic potential of Kv7 channel modulators in many diseases including those affected the lung. As stated in the previous section activation of Kv7 channels has been proposed to provide beneficial effects in a number of lung conditions. Therefore, Kv7 channel openers/enhancers or drugs acting partly through these channels could be proposed as bronchodilators, expectorants, antitussives, chemotherapeutics, and pulmonary vasodilators, as summarized below.

Flupirtine and retigabine are structurally related Kv7.2–7.5 channel activators. The discovery of Kv7 channels came later than the extensive use of flupirtine and retigabine as an analgesic and anticonvulsant, respectively, and whose mechanism of action was unknown until then (Schenzer et al., 2005). Retigabine has been shown to increase the open probability and cause a hyperpolarizing shift in the voltage dependence of activation of *KCNQ* channels by binding at the intracellular side of the pore-forming S5 helix around a key tryptophan (Trp) Trp236 in Kv7.2 and analogous residues in Kv7.3, 4, and 5 which is absent in Kv7.1 channels (Schenzer et al., 2005; Lange et al., 2009; Kim et al., 2015). Kim et al. have suggested that the retigabine interaction with this Trp residue depends largely on the formation of a hydrogen bond (Kim et al., 2015).

As the relevant role of Kv7 channels was discovered, the idea that these channels could constitute a new therapeutic strategy became more attractive, leading to investment in the development of new activators. The key residues for retigabine-binding have also been recognized to be also essential for the binding of other compounds such as S1 and BMS-204352; though these last drugs have a greater efficacy enhancing Kv7.4 and Kv7.5 isoforms rather than Kv7.2 or/and Kv7.3 channels (Dupuis et al., 2002; Bentzen et al., 2006). Zinc pyrithione activates the Kv7 channel by interacting with a binding site different to that of retigabine (Xiong et al., 2008). Hence, zinc pyrithione activates all Kv7 channels except for Kv7.3 channels, which is the most retigabine-sensitive. It is worth noting that the active compound in the action of zinc pyrithione on Kv7 channels is zinc, as

administration of free zinc also activates these channels by reducing their dependence on PIP_2 (Gao et al., 2017). The activity of ICA-27243, a drug more potent at activating Kv7.2/7.3 heteromeric channels than Kv7.4 homomeric channels or Kv7.3/7.5 heteromeric channels, is determined by a novel site inside the Kv7 channel voltage-detection domain (Wickenden et al., 2008; Blom et al., 2010). R-L3 is a benzodiazepine that activates Kv7.1 homomeric channels, but is considerably weaker when Kv7.1 is coexpressed with KCNE1 subunit, suggesting that R-L3 and KCNE1 subunits compete for the same interaction site on *KCNQ1* subunits (Salata et al., 1998).

Kv7 channels are currently new therapeutic targets in the field of lung diseases; consequently, there is still much to be learned about their physiological role, regulation, and possible impairment by pathological processes. The specific expression of certain Kv7 channel in different types of cells is of great pharmacological interest, since their selective modulation could provide beneficial therapeutic effects while avoiding the adverse effects associated with other isoforms (Stott et al., 2014). A clear example comes from the previous experience with retigabine and flupirtine, which have been used as analgesic and anticonvulsant, respectively, for many years but were withdrawn from the marked due to their adverse effects including the risk of serious liver injury, pigment changes in the retina and urinary retention (Herrmann et al., 1987; Brickel et al., 2012). While, as mentioned, both drugs activate Kv7.2–Kv7.5, they appear to display greater efficacy for Kv7.2 and Kv7.3 channels over Kv7.4 and Kv7.5 channels, which predominate in smooth muscle. Thus, it is expected that compounds selectively activate the Kv7.4 and/or the Kv7.5 subunit may be used in the treatment of vascular smooth muscle disorders with few neurological side effects. For instance, selective activation of Kv7.4 and Kv7.4/Kv7.5 channels (but not Kv7.1, Kv7.2, and Kv7.2/Kv7.3) has been implicated in the vasorelaxant effects of the Rho kinase inhibitor fasudil (Zhang et al., 2016). Thus, it is likely that, in addition to Rho kinase inhibition, activation of these channels may contribute to the beneficial effects of fasudil in cardiovascular conditions such as hypertension, vasospasm, stroke arteriosclerosis, pulmonary hypertension, and heart failure (Shi and Wei, 2013; Shimokawa and Satoh, 2015). Likewise, it is expected that selective Kv7.2 and Kv7.3 channel enhancers may be used to the treatment of neurological disorders with few adverse effects in smooth muscle cells (Stott et al., 2014; Barrese et al., 2018). Remarkably, Manville et al. have recently reported that the neurotransmitter γ-aminobutyric acid (GABA) and various metabolites (β-hydroxybutyric acid and γ-amino-β-hydroxybutyric acid) directly activate Kv7.3 and Kv7.5 but not Kv7.1, 2, and 4 (Manville et al., 2018). Manville and colleagues found that Trp residues in Kv7.3 (Trp265) and Kv7.5 (Trp270) were required for binding but not sufficient for GABA-induced activation. Thus, while Kv7.2 and Kv7.4 share analogous Trp residues and bind GABA, they are not activated by GABA (Manville and Abbott, 2018). Likewise, the synthetic GABA analog gabapentin, but not pregabalin, was also found to activate Kv7.3 and Kv7.5 (Manville and Abbott, 2018). Very recently, these authors have proposed a model in which the sodium-coupled myo-inositol transporter 1 (SMIT1) interacts with KCNQ2/3 and tunes the channel response to GABA and related metabolites (Manville and Abbott, 2020).

The search for Kv7 activators with a better safety profile than retigabine and flupirtine as well as the identification of novel potential therapeutic applications, has driven the development of a large number of compounds with Kv7 enhancing properties in the last decade (Jepps et al., 2013; Stott et al., 2014; Haick and Byron, 2016; Barrese et al., 2018).

Given the functional roles and ubiquitous expression of Kv7 channels in many tissues, drugs modulating Kv7 channels are presumed to cause off-target effects including alterations of the QT interval at the heart level, systemic hypotension, urinary retention or neuronal effects. This could be largely avoided by using localized drug delivery to the lungs by oral inhalation. Inhaled administration is the route of choice for many drugs (i.e., corticosteroids; anticholinegics, or β2-adrenergic receptor agonists) to treat respiratory diseases. Similarly, targeting lung Kv7 channels specifically using inhaled Kv7 activators may be an innovative approach for the treatment of COPD, cystic fibrosis, pulmonary hypertension, asthmatic events, lung cancer, bronchitis, and pulmonary edema.

Progress in the treatment of respiratory diseases is a global challenge because of its health and socio-economic implications. The optimal management and treatment of these diseases should be aimed at achieving adequate control of the disease, improving the quality of life of patients, and reducing hospitalizations and the enormous financial burden on healthcare systems. Targeting Kv7 channels in the treatment of lung disorders could be a promising therapeutic strategy. Here, we summarized the potential usefulness of Kv channel modulators in treating respiratory diseases.

Bronchodilators

Asthma and COPD are characterized by hyper-contraction of ASMC attributed to abnormally high concentrations of different bronchoconstrictor agonists and/or increased sensitivity to them, leading to pathological narrowing of the airways (Penn, 2008). K^+ channels are key regulators of Em in ASMC and their impairment causes membrane depolarization and bronchoconstriction (Hall, 2000). Among them, Kv7 channels contribute significantly to the negative resting Em in ASMC (Brueggemann et al., 2011). By acting on a different target, Kv7 channel openers offer the potential of being combined with standard bronchodilators such as β2-adrenergic receptor agonists and antimuscarinics as an adjuvant therapy to increase the efficacy or overcome desensitization (Brueggemann et al., 2014b).

Expectorants

The function of Kv7.1 together with KCNE1/E2/E3 subunits seems to play a key role in secretions in airway epithelial cells. The co-activation of Cl^- channels in the luminal surface and K^+ channels in the basolateral membrane in AEC is an essential requirement for Cl^- secretion (Frizzell and Hanrahan, 2012). The activation of Kv7.1 channels has an expectorant effect provoking or promoting the expulsion of accumulated bronchial secretions and the productive cough helping to airway clearance

(Mall et al., 2000; Al-Hazza et al., 2016; Kroncke et al., 2016; Hynes and Harvey, 2019). Kv7.1 channel openers or drugs that indirectly activate Kv7.1 channels might be beneficial in the treatment of bronchitis, COPD, cystic fibrosis and asthma (Boucherot et al., 2001; Athanazio, 2012; Hynes and Harvey, 2019).

Antitussives

Stimulation of airway C-fibers can be activated by inflammatory factors leading to the impulse to unproductive cough, dyspnea, mucus secretion and bronchoconstriction (Mazzone and Undem, 2016). Activation of Kv7.3 channels in VACF by retigabine has been shown to increase the M-current amplitude and to inhibit unproductive coughing (Sun et al., 2019). Thus, Kv7 activators could be useful as chronic coughing relievers in pulmonary inflammatory diseases.

Chemotherapy

Although, the role of Kv7 channel modulators as chemotherapy agents has been only barely studied, flupirtine has been shown to attenuate the resistance to gefitinib in a human lung cancer cell line (Choi et al., 2017). The anti-proliferative effect of flupirtine has also been proposed as potentially beneficial in other types of cancer (Lee et al., 2014). Additional studies are required to evaluate the potential of Kv7 channel activators with selectivity for particular isoforms as chemotherapy agents.

Pulmonary Vasodilators

K^+ channels are also key regulators of cellular Em in PASMCs, controlling the activity of voltage-dependent Ca^{2+} channels and, subsequently, pulmonary artery smooth muscle contraction. Pulmonary vascular tone is controlled by the interaction of different circulating or locally released vasoactive factors with vasoconstrictor or vasodilating properties. Acute modulation of the activity of K^+ channels has been extensively shown to contribute to the vascular effects of these vasoactive factors. Thus, different vasodilators such as nitric oxide, prostacyclin and calcitonin gene-related activate several K^+ channels and, conversely, vasoconstrictors as serotonin, angiotensin II, thromboxane A_2, noradrenaline and endothelin-1 have been shown to inhibit K^+ channels (Cogolludo et al., 2007; Stott et al., 2014). With such a crucial role in modulating and maintaining pulmonary arterial tone, changes in function and expression of K^+ channels during the development and pathogenesis of pulmonary hypertension results in aberrations of normal physiological functions. Consequently, K^+ channels openers are presumed to have a beneficial therapeutic effect, especially if their action can be directed to the pulmonary circulation (Gurney et al., 2010). Up to the present time, the availability of drugs modulating Kv7 channels has been relatively scarce. Advances in the understanding of the role of Kv7 channels in the pulmonary vasculature and the development of new selective drugs for these channels indicate that they may represent a useful therapeutic target for the treatment of pulmonary arterial hypertension. As detailed above, different studies have provided evidence that treatment with Kv7 channel activators such as flupirtine, retigabine, or fasudil is beneficial in different pulmonary hypertension models (Morecroft et al., 2009; Duong-Quy et al., 2013; Sedivy et al., 2015; Zhang and Wu, 2017). In addition, Kv7 channel activation has been shown to contribute to the pulmonary vasodilating effect induced by drugs approved for the pulmonary hypertension treatment such as nitric oxide or riociguat (Mondejar-Parreño et al., 2019).

CONCLUSIONS

In the last decade, a significant progress in the understanding of the role of Kv7 channels in lung physiology has been made. The recognition of the involvement of these channels in cellular functions of crucial relevance in the normal physiology of the lung, such as the control of water and salt transport across the epithelial cell membrane, the excitability of airway afferent nerves, and the regulation of airway and lung vascular smooth muscle tone, makes them attractive therapeutic targets in lung diseases. In this regard, deciphering the involvement and importance of specific Kv7 subunits in these cellular functions will lead to a significant advance that may constitute the basis for the therapeutic potential of selective Kv7 channel modulators in lung diseases.

AUTHOR CONTRIBUTIONS

GM-P, FP-V, and AC wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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