Received: 20 July 2022 | Revised: 13 March 2023 | Accepted: 3 April 2023

DOI: 10.1111/bph.16087

RESEARCH ARTICLE

Oxidized soluble guanylyl cyclase causes erectile dysfunction in alcoholic mice

Miguel A. Olivencia[1,2,3] | Leticia Gil de Biedma-Elduayen[1,4,5,6] | Pablo Giménez-Gómez[1,4,5,6] | Bianca Barreira[1,2,3] | Argentina Fernández[7,8] | Javier Angulo[7,8] | Maria Isabel Colado[1,4,5,6] | Esther O'Shea[1,4,5,6] | Francisco Perez-Vizcaino[1,2,3]

[1]Departamento de Farmacologia y Toxicologia, Facultad de Medicina, Universidad Complutense, Madrid, Spain

[2]CIBER Enfermedades Respiratorias, Madrid, Spain

[3]Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain

[4]Instituto de Investigación Sanitaria Hospital 12 de Octubre, Madrid, Spain

[5]Red de Investigación en Atención Primaria de Adicciones del Instituto de Salud Carlos III, Madrid, Spain

[6]Instituto Universitario de Investigación Neuroquímica (IUIN), Facultad de Medicina, Universidad Complutense, Madrid, Spain

[7]Centro de Investigación Biomédica en Red de Fragilidad y Envejecimiento Saludable (CIBERFES), Instituto de Salud Carlos III, Madrid, Spain

[8]Servicio de Histología-Investigación, Unidad de Investigación Traslacional en Cardiología (IRYCIS-UFV), Hospital Universitario Ramón y Cajal, Madrid, Spain

Correspondence
Francisco Perez-Vizcaino and Esther O'Shea, Departamento de Farmacología y Toxicología, Facultad de Medicina, Universidad Complutense, Pza. Ramón y Cajal s/n, 28040 Madrid, Spain.
Email: fperez@med.ucm.es and estheros@med.ucm.es

Funding information
This study was supported by grants from Ministerio de Economía y Competitividad and Ministerio de Ciencia e Innovación (PID2019-105847RB-I00 and PID2019-107363RB-I00), Ministerio de Sanidad, Servicios Sociales e Igualdad (Plan Nacional Sobre Drogas [PNSD] Grants 2019I025 and 2022I033), Instituto de Salud Carlos III RICORS - RIAPAd Grants RD21/0009/0027 and RD16/0017/0021, with funds from the European Union (Fondo Europeo de Desarrollo Regional FEDER). M. A. O. is funded by Universidad Complutense/Banco Santander CT63/19-CT64/19, and L.G.B. by a

Background and purpose: Alcohol abuse has been associated with erectile dysfunction (ED), but the implicated molecular mechanisms are unresolved. This study analyses the role of alterations in soluble guanylyl cyclase (sGC) in ED.

Experimental approach: ED was analysed in adult male C57BL/6J mice subjected to the Chronic Intermittent Ethanol (CIE) paradigm. Erectile function was assessed in anaesthetised mice in vivo by evaluating intracavernosal pressure (ICP) and in vitro in isolated mice corpora cavernosa (CC) mounted in a myograph. Protein expression and reactive oxygen species were analysed by western blot and dihydroethidium staining, respectively.

Key results: In CIE mice, we observed a significant decrease in the relaxant response of the CC to stimulation of NO release from nitrergic nerves by electrical field stimulation, to NO release from endothelial cells by acetylcholine, to the PDE5 inhibitor sildenafil, and to the sGC stimulator riociguat. Conversely, the response to the sGC activator cinaciguat, whose action is independent of the oxidation state of sGC, was significantly enhanced in these CC. The responses to adenylyl cyclase stimulation with forskolin were unchanged. We found an increase in reactive oxygen species in

Abbreviations: CC, corpora cavernosa; CIE, chronic intermittent ethanol; CYB5R3, NADH-cytochrome b5 reductase 3; CYP2E1, cytochrome P450 2E1; DEA-NO, diethylamine nonoate; DID, drinking in the dark; ED, erectile dysfunction; ICP, intracavernosal pressure; PDE5i, phosphodiesterase 5 inhibitors; sGC, soluble guanylyl cyclase.

Miguel A Olivencia, Leticia Gil de Biedma-Elduayen and Pablo Giménez-Gómez contributed equally. Esther O'Shea and Francisco Perez-Vizcaino also contributed equally.

FPI grant (BES-2017-083033) from Ministerio de Ciencia e Innovación.

the CC from CIE mice as well as an increase in CYP2E1 and NOX2 protein expression. In vivo pre-treatment with tempol prevented alcohol-induced erectile dysfunction.

Conclusions and implications: Our results demonstrate that alcoholic mice show ED in vitro and in vivo due to an alteration in the redox state of sGC and suggest that sGC activators may be effective in ED associated with alcoholism.

KEYWORDS
alcohol, cinaciguat, erectile dysfunction, mice, soluble guanylyl cyclase

1 | INTRODUCTION

Erectile dysfunction (ED) is defined as the inability to achieve and maintain an erection of the penis sufficient to permit satisfactory sexual intercourse ("NIH Consensus Conference. Impotence. NIH Consensus Development Panel on Impotence", 1993). It constitutes an important health burden due to its high prevalence and impact on quality of life (Kessler et al., 2019), and because it is a risk factor for cardiovascular disease and all-cause mortality (Andersson, 2011; Wespes, 2002). The aetiologies of ED include vascular, hormonal, neurologic, and/or psychological dysfunctions which are often interlinked (Andersson, 2011; Wespes, 2002; Zhao et al., 2022). Several factors can either cause or worsen ED, including ageing, obesity, smoking, diabetes, cardiovascular diseases, depression, certain drugs, as well as **alcohol** (Seftel et al., 2004; Wespes, 2002). In fact, alcohol abuse has long been associated with ED (Julian et al., 2020; Lemere & Smith, 1973). Chronic alcohol may affect any of the aetiologies of ED. Thus, social and psychological factors and altered hypothalamic–pituitary–gonadal axis function with reduced **testosterone** and/or liver cirrhosis in alcoholics have a well-known negative influence on libido and sexual behaviour. Besides these and other possible indirect mechanisms, it has been suggested that chronic alcohol also has direct effects on vascular penile physiology (Julian et al., 2020).

There is some experimental evidence in animal models to support a causal link between alcohol and ED (Aydinoglu et al., 2008; Leite et al., 2017; Lizarte et al., 2009; Tiraboschi et al., 2021; Yazir et al., 2012). Corpora cavernosa (CC) isolated from mice, rats or rabbits chronically exposed to alcohol show an impaired relaxation to **acetylcholine** or other **muscarinic receptor** agonists which stimulate the endothelium to release **nitric oxide** (NO) (Aydinoglu et al., 2008; Lizarte et al., 2009; Yazir et al., 2012). The relaxant response of CC to electrical field stimulation (EFS), which induces the release of NO from nitrergic nerves, that is, an in vitro correlate of penile erection, also has been observed to be impaired in some models of chronic exposure to alcohol (Yazir et al., 2012). The effect develops after 1 week (Aydinoglu et al., 2008) to 4 weeks (Yazir et al., 2012) of treatment and is dose-dependent (Yazir et al., 2012). These models have used chronic continuous alcohol in drinking water (Lizarte et al., 2009; Tiraboschi et al., 2021; Yazir et al., 2012) or alcohol inhalation (Aydinoglu et al., 2008), but they do not induce alcohol-dependence. Validated and clinically relevant models to study alcohol dependence include the Chronic Intermittent Ethanol (CIE) paradigm in mice. It uses repeated cycles of vaporization of alcohol, voluntary consumption and withdrawal in order to produce an escalation in voluntary alcohol consumption (Lopez et al., 2017), anxiety-like behaviour (Patel et al., 2021) and deficits in sleep regulation (Huitron-Resendiz et al., 2018).

Notably, the molecular mechanisms involved in alcohol-induced ED are currently unclear. The role of altered expression of **NO synthases** does not seem to be consistent (Yazir et al., 2012). Chronic alcohol consumption reliably produces oxidative stress and inflammation in the liver and the brain (Osna et al., 2017; Perez et al., 2020) and thus might also contribute to ED. In line with this, **NADPH**

What is already known?

- Alcohol abuse has long been associated with erectile dysfunction (ED).
- The molecular mechanisms involved in alcohol-induced ED are currently unknown.

What does this study add?

- Alcohol abuse produces ED by impaired activity of the NO-cGMP pathway with unchanged cAMP pathway.
- Alcoholic mice show oxidized sGC in the corpora cavernosa.

What is the clinical significance?

- PDE5i show limited effectiveness in alcohol-induced erectile dysfunction.
- sGC activators emerge as potential alternative drugs to treat ED.

oxidase-derived reactive oxygen species are increased in the CC after chronic alcohol consumption in the rat (Leite et al., 2017).

The aim of the present study was to analyse ED in a model of alcoholism in mice, the responses of their CC to therapeutic drugs, and the mechanisms involved. We hypothesized that an oxidative alteration of soluble guanylyl cyclase (sGC) in the CC explains ED induced by alcohol. To our knowledge, this is the first report showing functional evidence of an oxidative alteration of sGC in a clinically relevant condition. The data provide not only a molecular mechanism for alcohol-induced ED, but also suggest that sCG activators may be an optimal pharmacological therapy for these patients.

2 | METHODS

2.1 | Materials

Unless otherwise specified, drugs and general reagents were obtained from Sigma-Aldrich (Merck, Darmstadt, Germany). Absolute ethanol was purchased from Panreac Applichem (Spain); riociguat was acquired from Medchem Express (Sollentuna, SWEDEN); protease and phosphatase inhibitor PhosSTOP cocktail tablets were obtained from Roche Diagnosis GmbH (Mannheim, Germany) and Testosterone ELISA kit DE1559 was acquired from Demeditec (Kiel, Germany). Ketamine hydrochloride was obtained from Pfizer, S.L. (Alcobendas Spain) and diazepam from Roche Farma, S.A. (Madrid, Spain).

Primary antibodies used in this study were purchased as follows: sGC α1 subunit (Abcam, Cambridge, UK; Cat# ab85445, RRID:AB_10675532; rabbit IgG, immunogen: Synthetic peptide corresponding to Rat Guanylyl Cyclase alpha 1/GUCY1A3 aa 1-100 conjugated to keyhole limpet haemocyanin; polyclonal), sGC β1 subunit (Merck, Darmstadt, Germany; Cat# G4405, RRID:AB_259906; rabbit IgG, immunogen: whole molecule; polyclonal), Cyp2E1 (Merck, Darmstadt, Germany; Cat# HPA009128, RRID:AB_1078613; rabbit IgG, immunogen: cytochrome P450 2E1 recombinant protein epitope signature tag [PrEST]; polyclonal), NADH-cytochrome b5 reductase 3, CYB5R3 (Proteintech, Rosemont, USA; Cat# 10894-1-AP, RRID:AB_2292715; rabbit IgG; immunogen: CYB5R3 Fusion Protein Ag1339 [1-301 aa encoded by BC004821]; polyclonal), NOX2 (Abcam, Cambridge, UK; Cat# ab129068, RRID:AB_11144496; rabbit IgG; immunogen: synthetic peptide within Human NOX2/gp91phox aa 150-250; monoclonal [EPR6991]), SOD1 (Abcam, Cambridge, UK; Cat# ab16831, RRID:AB_302535; rabbit IgG; immunogen: recombinant full length protein corresponding to Human Superoxide Dismutase 1; polyclonal), catalase (Calbiochem, LaJolla, USA; Cat# 219010, RRID:AB_2071738; rabbit IgG; immunogen: purified, human erythrocytes catalase; polyclonal), β-actin (Merck, Darmstadt, Germany; Cat# A5441, RRID:AB_476744; mouse IgG, immunogen: slightly modified β-cytoplasmic actin N-terminal peptide, Ac-Asp-Asp-Asp-Ile-Ala-Ala-Leu-Val-Ile-Asp-Asn-Gly-Ser-Gly-Lys, conjugated to KLH; monoclonal [AC-15]) and vinculin (Santa Cruz Biotechnology, Texas, USA; Cat# sc-25,336, RRID:AB_628438; mouse IgG; immunogen: raised against amino acids 1-300 of vinculin of human origin; monoclonal [H-10]). Secondary antibodies were purchased as follows: anti-rabbit (Merck, Darmstadt, Germany; Cat# 12–348, RRID:AB_390191; goat IgG; immunogen: highly purified whole rabbit IgG; polyclonal) and anti-mouse (LI-COR Biosciences, Lincoln, USA; Cat# 926-68070, RRID:AB_10956588; goat IgG; immunogen: Mouse IgG paraproteins; polyclonal).

All drugs were dissolved in water, except riociguat, cinaciguat and forskolin which were dissolved in DMSO. Further dilutions were performed using distilled water. Pyrazole, tempol and ethanol for i.p. injections were dissolved in 0.9% w/v NaCl.

2.2 | Animals

Animal experimentation was performed according to the use and care of animals, 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-066/17 and PROEX-70.3/20). Animal studies are reported in compliance with the ARRIVE guidelines and following European Union Directive 2010/63/EU (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All investigators understand the ethical principles.

Adult male C57BL/6J mice (Envigo, East Millstone, NJ, USA) weighing 20–25 g (8 wk) were chosen, because this strain consumes greater amounts of alcohol (Belknap et al., 1993) and more effectively reproduces alcohol dependence than others (Lopez et al., 2017). Mice were maintained in conditions of constant temperature (21 ± 2°C) and a 12-h reverse light cycle (lights on at 8:00 PM). Animals were housed in groups of eight with ad libitum access to food and water for 5 days. Mice were then individually housed, randomly allocated into control and EtOH groups and habituated for 7 days to drinking water ad libitum from a 25-ml serological pipette fitted with a drinking spout.

For this study, we used a total of 157 male mice. In vivo ICP experiments were performed with 16 mice (Control and Alcohol). We utilized 16 mice for the analysis of the effects of CIE model on the CC responses to EFS, acetylcholine, sildenafil and riociguat and a further 16 mice to analyse responses to cinaciguat and forskolin (Control and Alcohol groups). Another set of 24 mice were used to determine if these effects lasted over time (Control, Alcohol 24 h ABS and 2 WK ABS). Moreover, 14 mice were used for the experimental groups treated with tempol (a superoxide scavenger). Four more groups of eight mice were used to perform ODQ and Na_2S experiments (Control and Alcohol). For the study of the effects of a binge drinking model, DID, we used 16 mice (Control and Alcohol). Finally, for the in vitro or the acute intraperitoneal experiments, we used groups of 5 mice, making a total of 25 mice.

2.3 | Drug administration

Alcohol for oral consumption was diluted in water to a concentration of 15% v/v and for injection was prepared in 0.9% w/v NaCl (saline) at a concentration of 20% v/v. For the CIE model, it was administered i.p. at a dose of 1.6 g·kg^{-1} before introducing the mice in the inhalation chamber. To study the effects of the acute administration, it was injected i.p. at a dose of 2 g·kg^{-1} 1 h before the mice were killed. Pyrazole was dissolved in saline and injected i.p. in a volume of 10 ml·kg^{-1} at a dose of 1 mmol·kg^{-1}, 30 min before introducing the mice in the inhalation chambers. Tempol was dissolved in saline and injected i.p. in a volume of 5 ml·kg^{-1} at a dose of 100 mg·kg^{-1}, 30 min before consumption and inhalation sessions, or dissolved in drinking water at a concentration of 2 nM and provided during the days of no exposure to alcohol.

2.4 | Chronic intermittent ethanol exposure

The CIE paradigm is a validated alcohol dependence and relapse drinking model (Gil de Biedma-Elduayen et al., 2022; Lopez et al., 2017). Briefly, the model involves repeated cycles of chronic intermittent exposure to alcohol vapours in inhalation chambers and periods of withdrawal, generating a progressive escalation in voluntary alcohol consumption reproducing the transition from voluntary consumption to dependence (Figure 1a). First, mice were subjected to a two-bottle choice limited access procedure for 2 weeks to establish the baseline of alcohol and water intake. Mice had access to two serological pipettes fitted with a drinking spout, one containing alcohol (15% v/v in water) and the other water, for 2 h from Monday to Friday. The rest of the time, mice had access to water ad libitum. Water and alcohol intake were measured every day. The position of the pipettes in the

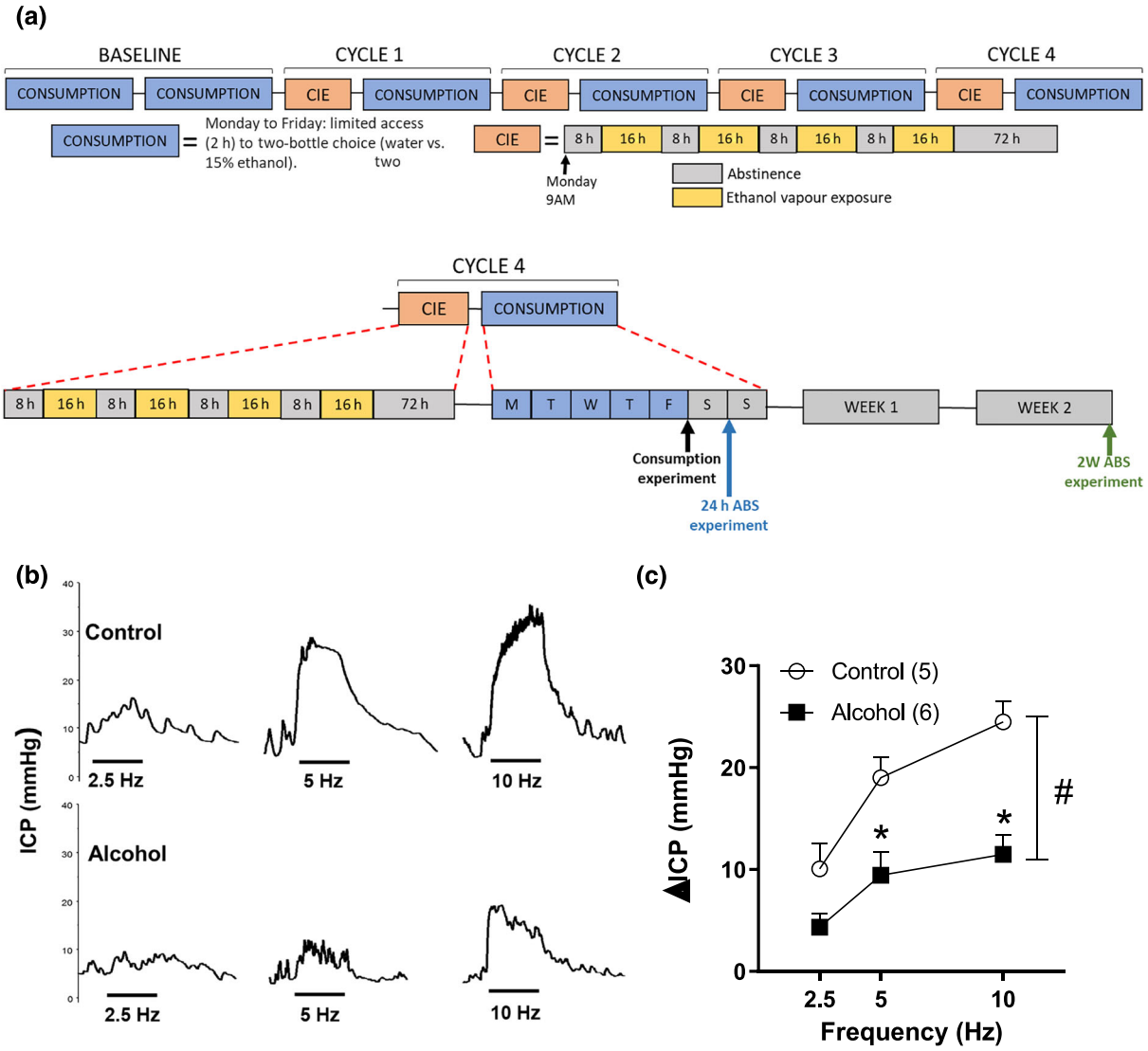

FIGURE 1 Chronic intermittent ethanol (CIE) impairs erectile function in vivo. (a) CIE protocol (see Section 2 for details). (b) Original traces of intracavernosal pressure recording after electrical stimulation of the cavernous nerve in anaesthetized mice with increasing frequencies; (c) averaged increases in pressure. Results are means ± standard error of the mean. #P < 0.05 versus control using a two-way (alcohol × electrical field stimulation [EFS] frequency) ANOVA test. * indicates P < 0.05 versus control by a Bonferroni post hoc test. The number of animals analysed is shown in parenthesis.

cages was alternated daily to avoid place preference. CIE groups received chronic intermittent exposure to alcohol vapour in inhalation chambers (16 h per day for 4 days) whereas control groups inhaled air alone. An additional group, no-CIE group, was created to validate the effect of alcohol vapours on alcohol consumption. This group did drink alcohol, but only inhaled air without alcohol vapour. Air flux and alcohol concentration in the inhalation chambers were adjusted each day to provide a plasmatic alcohol concentration between 150–250 mg·dl^{-1} (Gil de Biedma-Elduayen et al., 2022; Lopez et al., 2017). Before introducing mice in the chambers, they were injected i.p. with a dose of alcohol (1.6 g·kg^{-1}) and pyrazole (1 mmol·kg^{-1}; an alcohol dehydrogenase inhibitor), in order to maintain a stable level of intoxication during the 16 h of exposure to alcohol (Gil de Biedma-Elduayen et al., 2022; Lopez et al., 2017). Control animals received saline and pyrazole. After the fourth inhalation session of 16 h, mice were subjected to a 72-h forced abstinence period after which they were once again exposed for five consecutive days to the same voluntary alcohol consumption pattern. Mice were sacrificed by decapitation at three different points of the model just after the last consumption session of the fourth cycle (consumption experiment), 24 h after the last consumption session of the fourth cycle (24 h ABS experiment) and 2 weeks after the last consumption session of the fourth cycle (2 W ABS experiment) (Figure 1a). All further CIE experiments to characterize the in vivo effects and the mechanistic studies were performed using the consumption experiments. For the in vivo preventive model with the antioxidant tempol, mice were given an i.p. injection of 100 mg kg^{-1} 30 min before each session of alcohol consumption and before each inhalation session. Additionally, on the days of no exposure to alcohol (following the 5 days of alcohol consumption and before the start of the following inhalation cycle, and also during the 72-h forced abstinence period), mice had access to tempol 2 nM in the drinking water.

2.5 | Drinking in the dark

The Drinking in the Dark (DID) paradigm has been used as a model of binge-drinking alcohol consumption (Gimenez-Gomez et al., 2018; Rhodes et al., 2005). Following 5 days of group housing, mice were individually housed and habituated for 7 days to drinking water ad libitum from a 25 ml serological pipette fitted with a drinking spout. For four consecutive days, water was replaced by alcohol 20% (v/v), for 2 h during the first 3 days and for 4 h on the 4th day (Figure 2a). Alcohol and water intake values were recorded daily. Control mice drank water at all times. Mice were sacrificed immediately after the last alcohol exposure.

2.6 | Acute and in vitro exposure

The effects of acute administration of ethanol were tested in animals killed 1 h after a single i.p. dose of 2 g·kg^{-1}. Moreover, the effects of ethanol (1%) or **acetaldehyde** (50 μM) added to the myograph chamber were also analysed.

2.7 | Intracavernosal pressure recording

Mice were anaesthetized with ketamine (60 mg·kg^{-1}) and diazepam (4 mg·kg^{-1}). The right cavernous nerve was dissected and isolated through an abdominal midline incision, penile crura were exposed through a transverse perineal incision. Intracavernosal pressure (ICP) measurements were achieved by insertion into the right crus of a 25-gauge needle connected to a disposable pressure transducer (Abbott, Sligo, Ireland) and a data acquisition system (ADInstruments, Castle Hill, Australia) for continuous recording of ICP. Electrical stimulation of the cavernous nerve was applied by a delicate platinum bipolar hook electrode connected to a stimulator and current amplifier (Cibertec CS-9, Madrid, Spain). Parameters of electrical stimulation consisted of pulses with a duration of 1 ms and 1.5 mA of current intensity for 1 min. Frequency-response curves were performed by applying stimulation at 2.5, 5 and 10 Hz at 3-min intervals.

2.8 | Myography

The CC strips were dissected and suspended between two electrodes located in an adapted chamber of a wire myograph (Danish Myo Technology, Hinnerup, Denmmark) in Krebs–Henseleit solution (KHS) bubbled with a 95% O_2–5% CO_2 mixture (pH = 7.4, 37°C). A passive tension of 2.5 mN was applied to the CC strips, and they were allowed to equilibrate for 60 min. Contractility of the segments was then tested by an initial exposure to a high-K^+ solution (80 mmol·L^{-1} KCl). Segments were then washed and incubated with **guanethidine** (10 μmol·L^{-1}) and **atropine** (1 μmol·L^{-1}) for a period of 1 h with one wash after 30 min. The drugs were present throughout the period of electrical field stimulation (EFS) to block adrenergic neurotransmission and muscarinic receptors, respectively. The preparations were contracted with **phenylephrine** (1 μmol·L^{-1}). After a stable tone was reached, stimulation was performed with trains of 20 s of rectangular pulses of 1-ms duration at 1–16 Hz at 3 min intervals from a stimulator CS-9 (Cibertec, Madrid, Spain) with constant supramaximal current output adjusted to 75 mA. Next, a cumulative concentration-response curve to diethylamine nonoate (DEA-NO, 0.1 nmol·L^{-1} to 1 μmol·L^{-1}), acetylcholine, riociguat, cinaciguat or forskolin (1 nmol·L^{-1} to 10 μmol·L^{-1}) was obtained from the phenylephrine precontracted strips. The responses to a single concentration of sildenafil (1 μmol·L^{-1}) were analysed instead of a cumulative curve because the relaxation to this drug was slower and the contraction could not be properly sustained in parallel control experiments. In another set of experiments, after performing an initial concentration-response curve to riociguat or cinaciguat in phenylephrine-precontracted strips, the tissues were washed and incubated with either Na_2S (50 μmol·L^{-1}) or ODQ (1 μmol·L^{-1}) or vehicle for 25 min, then contracted again with phenylephrine and a second concentration-response curve to riociguat was performed. The effects of ethanol (1%) or acetaldehyde (50 μmol·L^{-1}) added to the myograph chamber were also analysed. Relaxation was expressed as a percentage of the reduction in the phenylephrine-induced contraction.

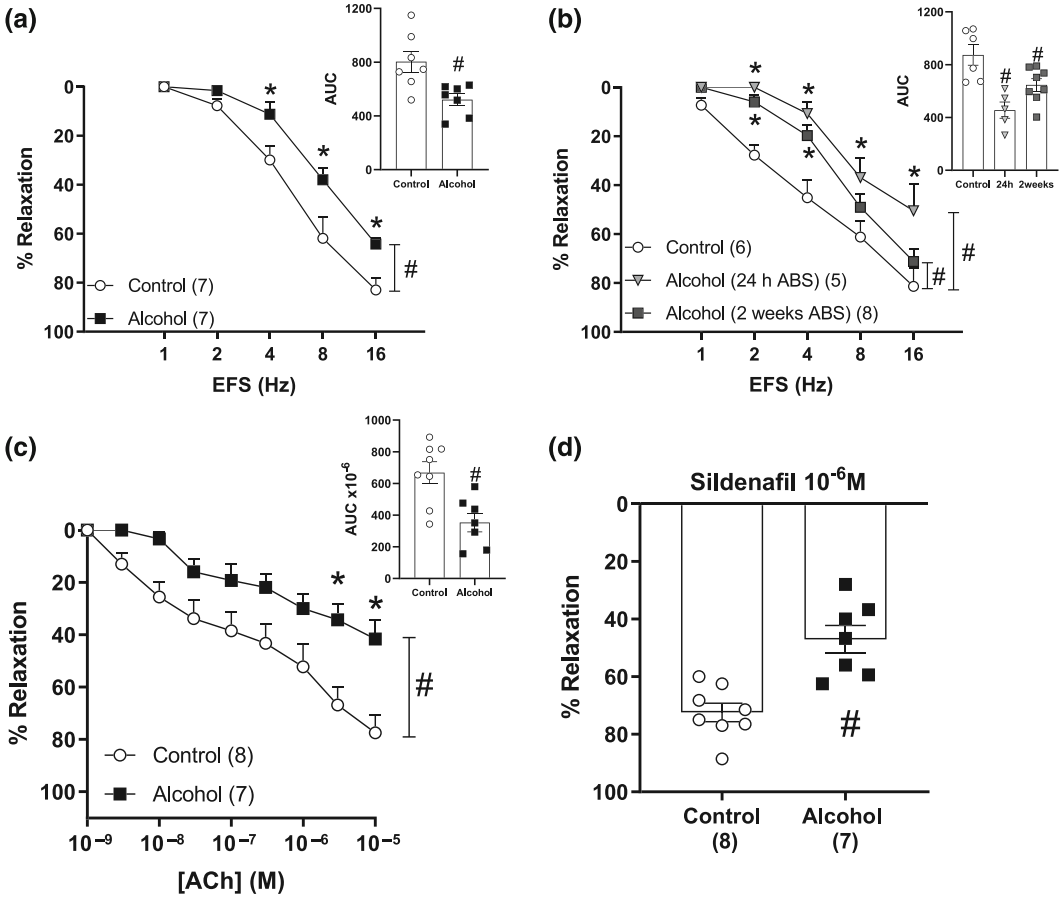

FIGURE 2 Chronic alcohol impairs erectile function in vitro. (a, b) Effects of chronic intermittent ethanol (CIE) on electrical field stimulation (EFS)-induced relaxant responses of mice corpora cavernosa (CC) analysed (a) immediately after the last period of voluntary consumption and (b) after 24 h or 2 weeks of alcohol abstinence. (c, d) Effects of alcohol on the relaxant responses of mice CC induced by (c) acetylcholine, (d) sildenafil. Area under the curve (AUC) analysis is shown as an insert in the panels (a)–(c); note that *Y* axes are inverted so that the AUC graphically corresponds to the area above the curve. Cumulative concentrations were studied in panel C but a single concentration of sildenafil was tested in panel (d) due to slower responses to this drug. Results are means ± standard error of the mean. #$P < .05$ versus control using *t* test, one-way ANOVA or a two-way (alcohol × concentration) ANOVA test. * indicates $P < 0.05$ versus control by a Bonferroni post hoc test. The number of animals analysed is shown in parenthesis.

Conductance pulmonary arteries (PA, 2–3 mm long, ~0.5 mm internal diameter) were mounted in a wire myograph with KHS maintained at 37°C and bubbled with 21% O_2 and 5% CO_2. Vessels were stretched to give a transmural pressure equivalent to 30 mmHg. After equilibration, PA rings were exposed to a high-K^+ solution to test the contractile capacity of the vessel. The preparations were contracted with phenylephrine (1 μmol·L^{-1}) and cumulative concentration-response curves to riociguat and cinaciguat (1 nmol·L^{-1} to 10 μmol·L^{-1}) were carried out. Relaxation was expressed as a percentage of the reduction in phenylephrine-induced contraction.

2.9 | Dihydroethidium staining

OCT-embedded penis sections of 6 μm also were obtained by means of a cryostat. Sections were stabilized at 37°C for 2 h before being washed with KHB-Hepes for 30 min. Penis sections were isolated with PAP pen for immunostaining for being randomly incubated with or without 4-hydroxy-tempo (4-tempol) 10 mmol·L^{-1} plus superoxide dismutase–polyethylene glycol (PegSOD) 30 U·mL^{-1} or clomethiazole hydrochloride 40 μmol·L^{-1} for 40 min. Afterwards, all slices were exposed to 3-μmol·L^{-1} dihydroethidium (DHE) for 20 min. Nuclei were counterstained with 1-μmol·L^{-1} DAPI for 5 min. All images were taken in a fluorescence microscope (Leica microsystems, Wetzlar, Germany). DHE intensity was obtained through ImageJ software and normalized with DAPI intensity.

2.10 | cGMP measurement

Penises from control and alcoholic mice were dissected and divided into two pieces, immersed in KHS at 37°C and aerated with 5% CO_2/95% O_2, pH 7.4 and incubated for 5 min with either vehicle

(0.001% DMSO) or 30 nM riociguat. Then, tissues were immediately frozen in liquid nitrogen and stored at −80°C. Tissues were extracted by homogenization into 8 volumes (ml of buffer/g of tissue) of 5% trichloroacetic acid, followed by ether (H_2O-saturated) extraction. The concentration of **cGMP** was determined by an ELISA assay kit (Cayman Chemical Company, Michigan, USA).

2.11 | Testosterone plasma measurement

Testosterone was measured in mouse citrated plasma using kit following manufacturer's instructions. The 25 µl of non-diluted plasmas were added to the different wells at room temperature (22–24°C), which was maintained for the duration of the entire experiment. Optical density of the final solution in each well at 450 nm was measured with Multiskan FC Microplate Photometer (Thermofisher, Massachusetts, USA).

2.12 | Protein expression by western blot

Frozen penises were homogenized with a potter containing RIPA lysis buffer supplemented with a protease and phosphatase inhibitor cocktail. Samples were sonicated for 20 s twice and centrifuged at 10000 rpm for 5 min. Proteins were separated on a sodium dodecyl sulphate-polyacrylamide electrophoresis and transferred to polyvinylidene difluoride membranes (Amersham™ Hybond® P Western blotting membranes, Sigma-Aldrich). They were then blocked with 5% BSA or milk TBST (0.5 M Tris pH 7.5; 1.5 M NaCl; 0.1%Tween© 20) and incubated with primary rabbit antibodies against sGC α1 subunit (1:1000), sGC β1 subunit (1:1000), NADH-cytochrome b5 reductase 3, CYB5R3 (1:1000), CYP2E1 (1:250), NOX2 (1:1000), SOD1 (1:1000), catalase (1:1000), β-actin (1:10000) and vinculin (1:1000) and then with the secondary peroxidase conjugated or fluorophore conjugated antibodies (1:10000). Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK). All antibodies were diluted in TBST. Blots were analysed using an Odyssey Fc System (LiCOR, Biosciences) and quantified using Image J software. Samples were normalized through expression of either β-actin or vinculin. For each control or experimental sample, the relative abundance of the protein of interest was normalized to the mean of the controls to minimize variations. All quantifications were carried out by a blind analyser. Western blot procedures were performed in compliance with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.13 | Data and statistical analyses

The data and statistical analysis comply with the recommendations the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022). Initial group size (n = 8) was selected for the CIE and DID protocols based on our previous experience, considering the possible loss of mice due to the application of the criteria of humane endpoints if physical signs other than alcohol intoxication were observed, loss of consumption data, failure during mounting the CC in the myograph or during the surgery for the in vivo, or outlier values (determined by the extreme studentized deviate method [ROUT test] with significance level of $Q = 1$). These conditions were the only exclusion criteria employed and account for differences in group sizes. A smaller group size of n = 5 was considered for the in vitro or the intraperitoneal acute experiments because of the lower intrinsic variability and hence lower number of expected outliers and lower expected loss of data due to simpler procedures. Analysis was performed using Prism v8 (GraphPad Software Inc., USA). The area under the curve (AUC) was analysed for each individual concentration-response curve. All data were tested for normal distribution using the Shapiro–Wilk test, and parametric or non-parametric statistics were used as appropriate. Data are presented either as scatter plots and means ± SEM or just means ± SEM for concentration-response or frequency-response curves. Comparisons between two groups were analysed by an unpaired Student's t test. Results defined by two factors were evaluated by two-way ANOVA. In the cases of interaction between factors, relevant differences were analysed by post hoc comparison using the Bonferroni or Sidak tests. Post hoc analyses were only carried out when the threshold for statistical significance had been reached and no significant variance in homogeneity was detected. *P* values of less than 0.05 were considered statistically significant.

2.14 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Pawson, Southan, Davies, Beuve, et al., 2021; Alexander, Fabbro, Kelly, Mathie, Peters, Veale, Armstrong, Faccenda, Harding, Pawson, Southan, Davies, Boison, et al., 2021; Alexander, Kelly, et al., 2021).

3 | RESULTS

3.1 | Model characteristics

As expected, mice exposed to CIE model, named alcoholic mice hereafter, consumed significantly more alcohol after four cycles of alcohol vaporization compared with mice that inhaled air alone, but there were no differences in water consumption or in body weight (Figure S1). Plasma alcohol levels after each vaporization session were between 150–250 $mg{\cdot}dl^{-1}$ and, after the last 2 h of consumption of the last day, alcohol levels in plasma reached 87.9 ± 4.4 $mg{\cdot}dl^{-1}$. Finally, testosterone plasma levels were similar between the two groups (Figure S1). In the DID paradigm, a model of acute intoxication (binge drinking), plasma alcohol levels reached 96.4 ± 11.7 $mg{\cdot}dl^{-1}$ after 4 h of alcohol exposure.

3.2 | In vivo effects of alcohol-dependence on intracavernosal pressure

We first analysed the in vivo impact of alcohol exposure in alcoholic mice (Figure 1a). Electrical stimulation of the cavernous nerve in anaesthetized mice produced frequency-dependent increases of ICP, which were strongly and significantly reduced in alcoholic mice compared with controls (Figure 1b,c), confirming the ED.

3.3 | Ex vivo effects of alcohol-dependence on the responses to EFS

In addition, we analysed the relaxation of isolated CC to EFS, that is, the in vitro surrogate for erectile function. In isolated CC from alcoholic mice, EFS-induced relaxant responses were significantly smaller than in controls, indicating ED (Figure 2a). This effect of CIE is long lasting because relaxations remained inhibited 24 h after consumption and still partially inhibited even after 2 weeks of alcohol abstinence (Figure 2b).

3.4 | Ex vivo and in vitro effects of acute ethanol on the responses to EFS

In contrast to chronic alcohol, the model of binge drinking (DID, Figure S2a) or a single intraperitoneal administration of ethanol to naïve mice (Figure S2b) did not induce any change of the EFS-induced relaxation. Similarly, neither ethanol (Figure S2b) nor its main metabolite acetaldehyde (Figure S2c), added directly to the CC in the myography chamber (in vitro), altered these relaxant responses.

3.5 | Effects of alcohol-dependence on the responses to drugs modulating the cGMP and cAMP pathways

EFS stimulates a **nNOS**-dependent release of NO by nitrergic nerves which then stimulates sGC in the CC to increase cGMP synthesis. Thus, we then studied the effects of alcohol on the relaxant responses induced by other agents that increase cGMP. Acetylcholine relaxes CC by an **eNOS**-dependent release of endothelial NO and sildenafil does so by an inhibitory effect on **PDE5**, which results in reduced degradation of cGMP and hence potentiates endogenous NO. Figure 2c,d show that CIE also impaired the relaxant response to both acetylcholine and sildenafil.

Next, we studied the effects of exogenous NO using DEA-NO as a NO donor and of forskolin that activates **adenylyl cyclase** to increase **cAMP** synthesis, as well as the effects of alternative modes of increasing cGMP with riociguat by direct NO-independent stimulation of intact sGC, and with cinaciguat also by activation of sGC but preferentially in its oxidized state (Figure 3). CIE reduced the relaxations to DEA-NO and riociguat (Figure 3a,c) but not those to forskolin (Figure 3b). In addition, the responses to riociguat for the different alcohol exposure protocols were similar to those of EFS (Figure S3). Notably, as opposed to the other agents increasing cGMP, CC from mice exposed to CIE showed an increase in the relaxant effect of the sGC activator cinaciguat (Figure 3d). In addition, we found that cGMP concentration was reduced in tissues from alcoholic mice and that incubation with riociguat at the concentration corresponding to the EC_{50} for vasodilation markedly increased cGMP levels in controls but not in alcoholic mice (Figure 3g). Finally, there was no difference in the expression of the α and β subunits of sGC in the CC between the two groups (averaged data in Figure 3e,f and full original blots in Figure S5a,b). The effects of riociguat or cinaciguat also were analysed in pulmonary arteries from CIE and control mice, but we found no differences in the relaxations induced by either drug when CC from CIE or controls were compared (Figure S4).

3.6 | Modulation of the effects of alcohol-dependence on EFS by agents oxidizing or reducing sGC

As discussed below, the opposite effects of riociguat and cinaciguat on EFS-induced relaxation suggested an oxidative alteration of sGC. Thus, we next analysed the effects of riociguat and cinaciguat after oxidizing or reducing sGC with ODQ and Na_2S, respectively. In control CC (Figure 4a), ODQ produced a strong inhibitory effect on riociguat-induced relaxation, but Na_2S was unable to modify the response. In contrast, in CC from alcoholic mice, ODQ produced a mild inhibition, but Na_2S significantly potentiated the responses of riociguat (Figure 4b). As opposed to riociguat, the relaxation induced by cinaciguat was potentiated by ODQ in control CC (Figure 4c) without changes after Na_2S incubation. Moreover, ODQ did not alter cinaciguat relaxation in CIE CC (Figure 4d) while Na_2S had a significant inhibitory effect in cinaciguat relaxant effect. Thus, there were marked differences in the effect of ODQ and Na_2S analysed as the shift in the concentration-response curve between CC from control and alcoholic mice and between riociguat and cinaciguat (Figure 4e).

3.7 | Effect of alcohol-dependence on ROS levels in the CC

Reactive oxygen species (ROS) levels were analysed in sections of CC using the DHE technique. Superoxide-dependent nuclear red staining was markedly elevated in the CC from alcoholic mice indicating elevated ROS levels (Figure 5a,b). Interestingly, excessive ROS production was prevented by the CYP2E1 inhibitor, clomethiazole.

3.8 | Effect of alcohol-dependence on protein expression

We then analysed the protein expression of enzymes regulating the oxidative status of sGC in the CC. In alcoholic mice we found an increased expression of NADPH oxidase 2 (NOX2) and cytochrome P450 2E1 (CYP2E1), two superoxide anion generating enzymes

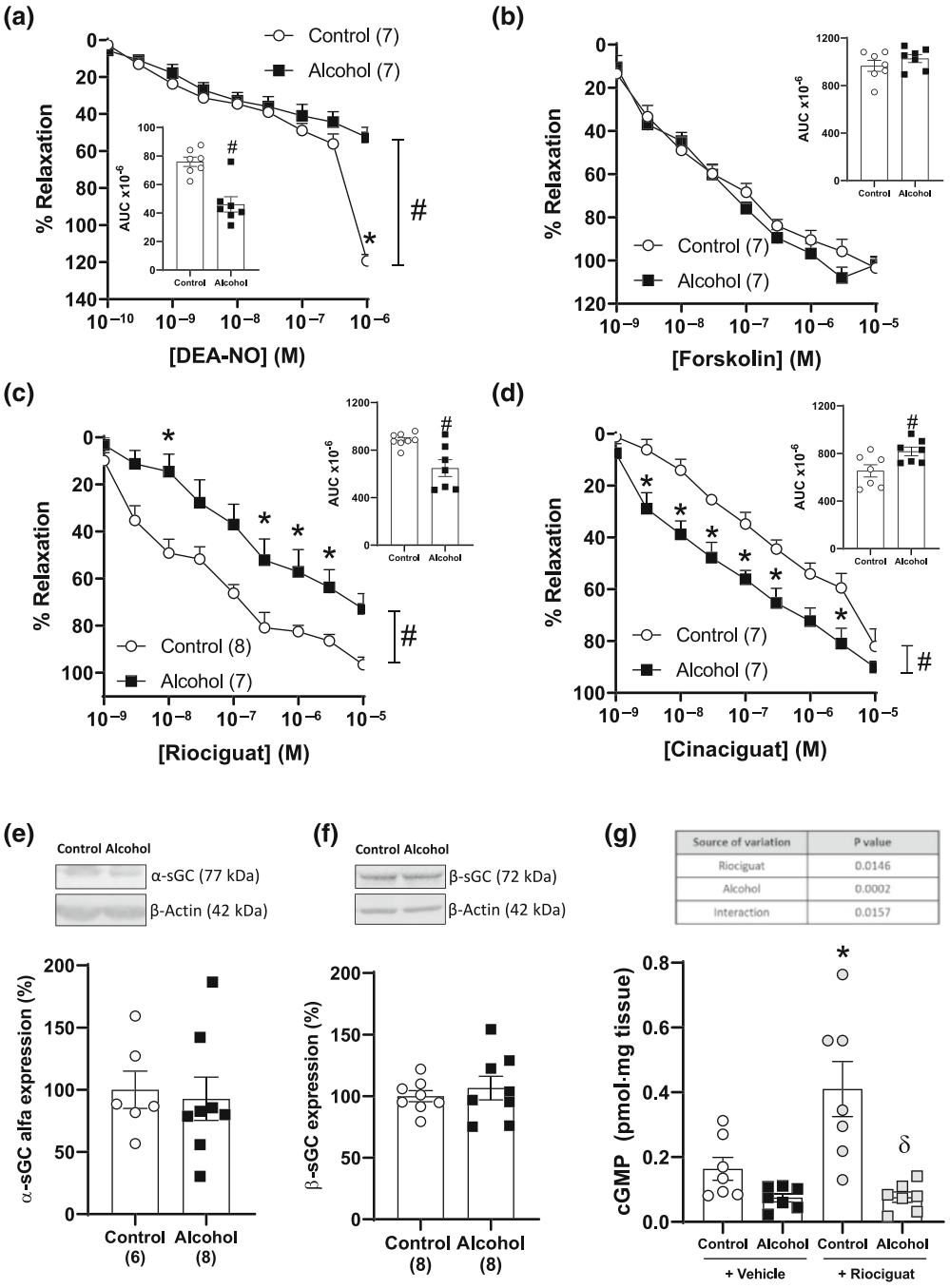

FIGURE 3 Effects of alcohol (chronic intermittent ethanol, CIE) on the responses to agents stimulating the NO-cGMP and cAMP signalling pathways ex vivo. (a–d) Effects of alcohol on the relaxant responses of mice corpora cavernosa (CC) induced by (a) diethylamine NONOate (DEA-NO), (b) forskolin, (c) riociguat and (d) cinaciguat. Area under the curve (AUC) analysis is shown as an inset; note that *Y* axes are inverted so that the AUC graphically corresponds to the area above the curve, (f) averaged densitometric protein expression of the (e) α subunit of sGC and (f) β subunit of sGC analysed by western blot and normalized to α-actin expression (original full blots are shown in Figure S4). (g) Effects of exposure to vehicle (0.001% DMSO) or riociguat (30 nM) on cGMP content in penis tissues from control and alcoholic mice (n = 8, two-way ANOVA results are shown in the table). Results are means ± standard error of the mean. The number of animals analysed is shown in parenthesis. # $P < 0.05$ versus control using a *t* test or two-way (alcohol × concentration) ANOVA test; * and δ $P < 0.05$ versus control or versus control + riociguat, respectively, by a Bonferroni post hoc test.

(Figure 5c,d and full original blots in Figure S5). In contrast, the antioxidant enzymes CYB5R3, SOD1 and catalase were similar in the two groups (Figure S6).

3.9 | In vivo preventive model with the antioxidant tempol

The findings of increased oxidative stress and the alteration of the redox state of sGC led us to perform an in vivo prevention study with the antioxidant tempol in alcoholic mice. Tempol did not interfere with the drinking behaviour of the alcoholic mice (alcohol consumption 4.84 ± 0.41, n = 7, and 5.04 ± 0.60 g·kg^{-1} for saline and tempol-treated mice, respectively). In tempol-treated mice, there were no differences in the relaxant response to EFS in alcoholic versus control mice, indicating that tempol prevented the in vitro ED (Figure 6a). Similarly, chronic treatment with this antioxidant prevented the effect of CIE on the acute in vitro relaxant response to sildenafil and riociguat (Figure 6b,c).

4 | DISCUSSION

The results of the present study using mice indicate that chronic exposure to alcohol decreased the relaxant responses of the CC in vitro via neurogenic and endothelial stimulation. It also markedly

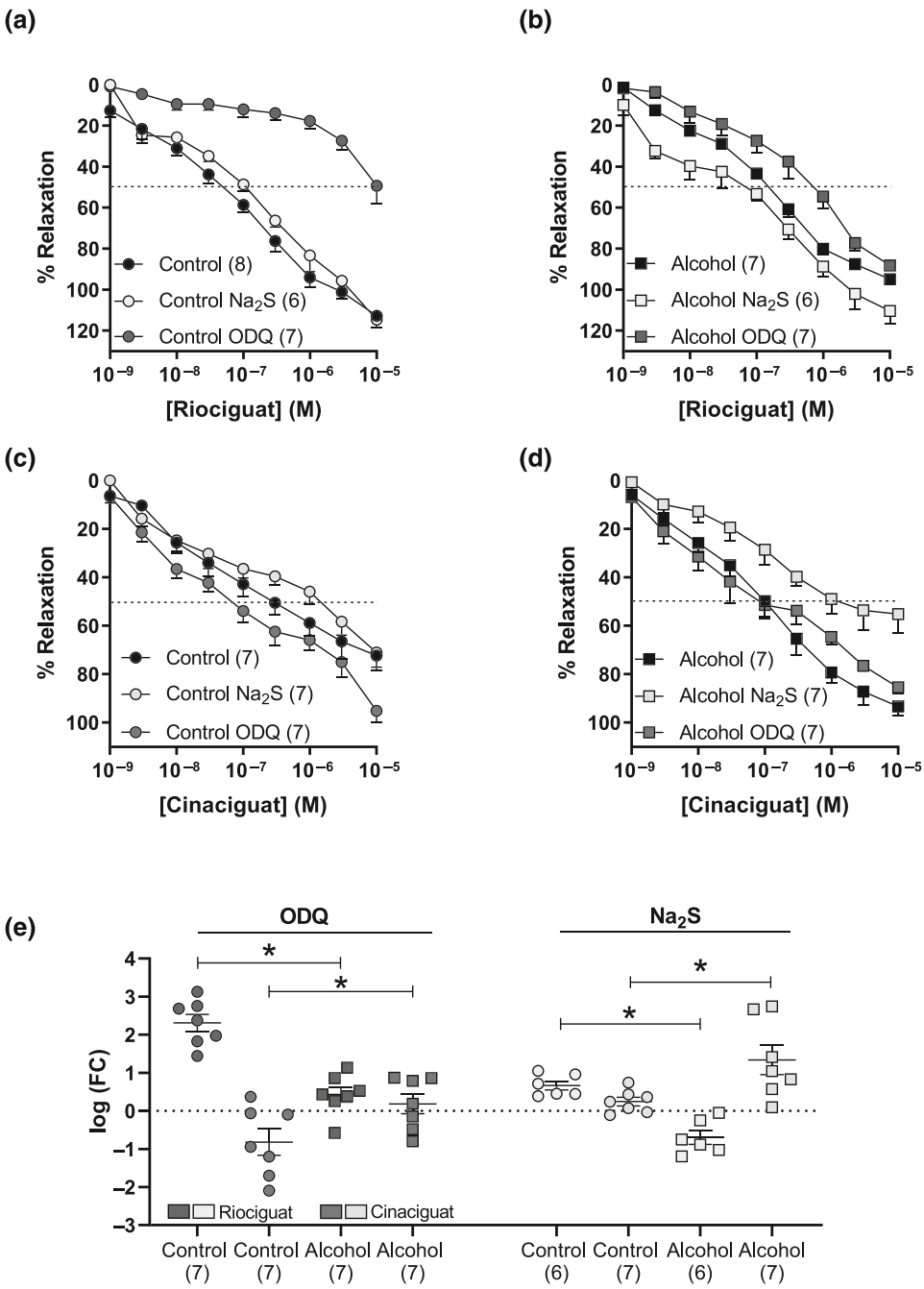

FIGURE 4 The responses of corpora cavernosa (CC) from control and alcoholic mice to riociguat and cinaciguat are differentially modulated by the oxidant ODQ and the reducing agent Na2S. An initial response curve to riociguat or cinaciguat was obtained in CC from control and alcoholic mice. Then CC were exposed to Na_2S (50 μmol·L^{-1}), ODQ (1 μmol·L^{-1}) or vehicle for 25 min and then the relaxant responses to riociguat (a and b) and cinaciguat (c and d) were re-analysed. In each CC the concentration producing a 50% relaxation (IC_{50}) was interpolated from the concentration-response curve. The ratio between the IC_{50} in the ODQ-treated vs the IC_{50} in the initial untreated CC or between to IC_{50} in the Na2S-treated vs the IC50 in the initial untreated CC is shown in panel C as log fold change (FC) for both riociguat and cinaciguat relaxant responses. Results are means ± standard error of the mean. * indicates $P < 0.05$ versus control using an unpaired Student's *t* test. The number of animals analysed is shown in parenthesis.

blunted the increase in intracavernosal pressure in response to electrical stimulation in vivo. The ED persisted partially at least 2 weeks after alcohol withdrawal. Moreover, we have demonstrated that neither acute intraperitoneal administration of alcohol nor a protocol of voluntary alcohol binge-like intoxication (DID) has any effect on erectile function ex vivo. In addition, neither alcohol nor acetaldehyde added in vitro, at relevant concentrations, directly into the bathing media of the CC had any effect on the response to EFS.

Neurogenic release of NO is considered the most important physiological mechanism for penile erection (Andersson, 2011). NO derived from the endothelium of sinusoids and blood vessels also may be involved. We showed that the responses mediated by stimulating the release of NO, either from nitrergic nerves with EFS or from endothelial cells with acetylcholine, were significantly attenuated by alcohol. To determine whether this was due to reduced synthesis or reduced sensitivity of the smooth muscle to NO, we analysed the relaxant responses of exogenously added NO using the NO donor DEA-NO, which also were reduced by CIE, supporting the latter hypothesis. NO diffuses from the nerve or endothelial cells towards the smooth muscle and activates sGC, which produces cGMP leading to relaxation of CC, facilitating penile erection. The responses to sildenafil which prevents the degradation of cGMP by PDE5 and hence potentiates the action of endogenous NO also were decreased. Moreover, riociguat, which directly stimulates sGC, was less effective in

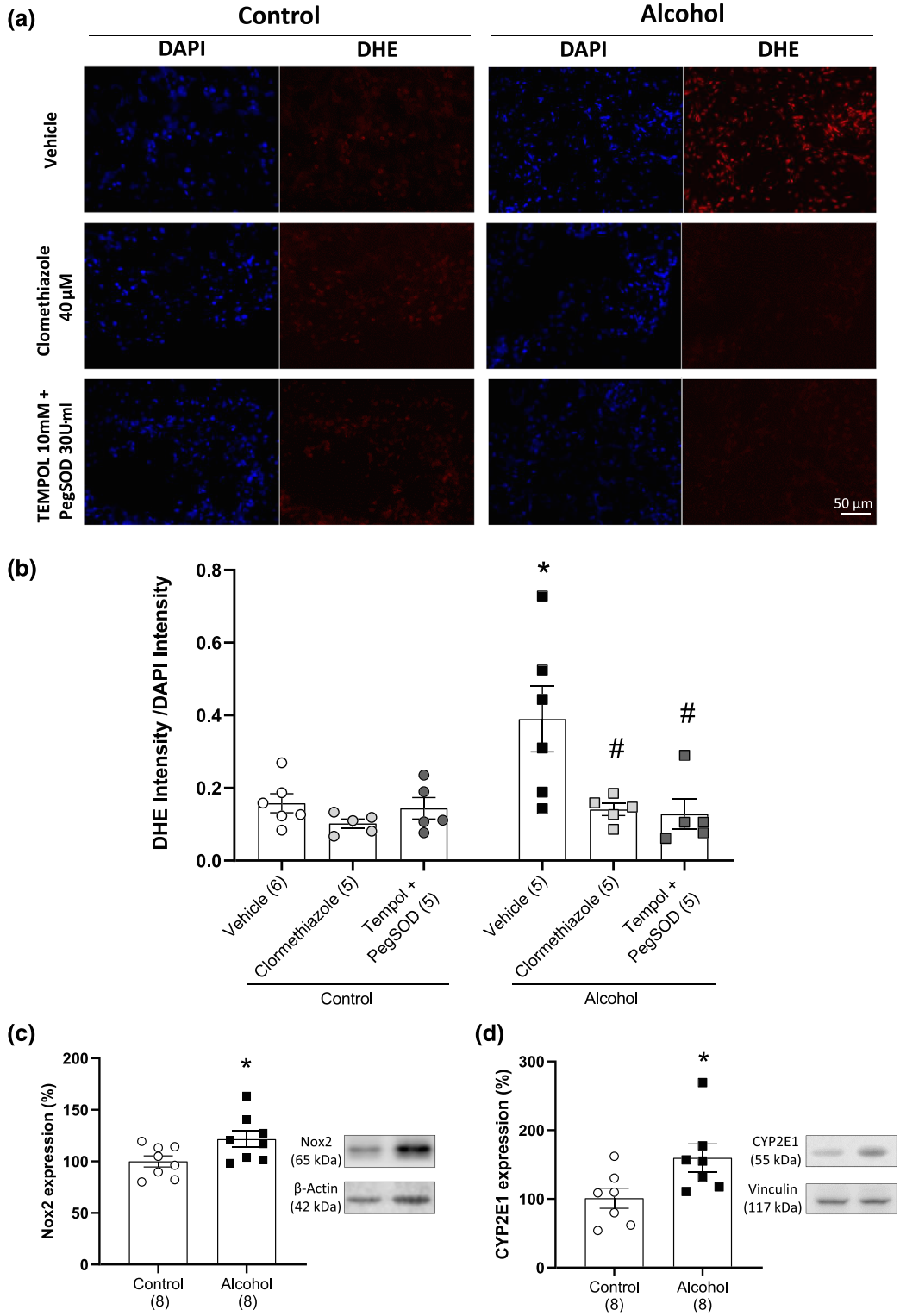

FIGURE 5 Reactive oxygen species (ROS) production and the ROS generating enzymes NOX2 and CYP2E1 are increased in alcoholic mice. (a) Left pictures show blue fluorescence of the nuclear stain DAPI, and right pictures show arteries incubated in the presence of DHE, which produces a red fluorescence when oxidized to ethidium by superoxide. Negative controls were obtained in the presence of the superoxide scavengers tempol plus PEG-SOD. (b) Values of red ethidium fluorescence normalized to the blue DAPI fluorescence. Results are means ± standard error of the mean of corpora cavernosa (CC) sections from five to six animals and were analysed using two-way ANOVA followed by Bonferroni test. * indicates $P < 0.05$ versus control-vehicle and # $P < 0.05$ versus alcohol-vehicle. (c, d) Averaged densitometric protein expression of NOX2 and CYP2E1 analysed by western blot and normalized to β-actin and vinculin expression, respectively (original blots are shown in Figure S5). Results are means ± standard error of the mean. * indicates $P < 0.05$ unpaired Student's *t* test. The number of animals analysed is shown in parenthesis.

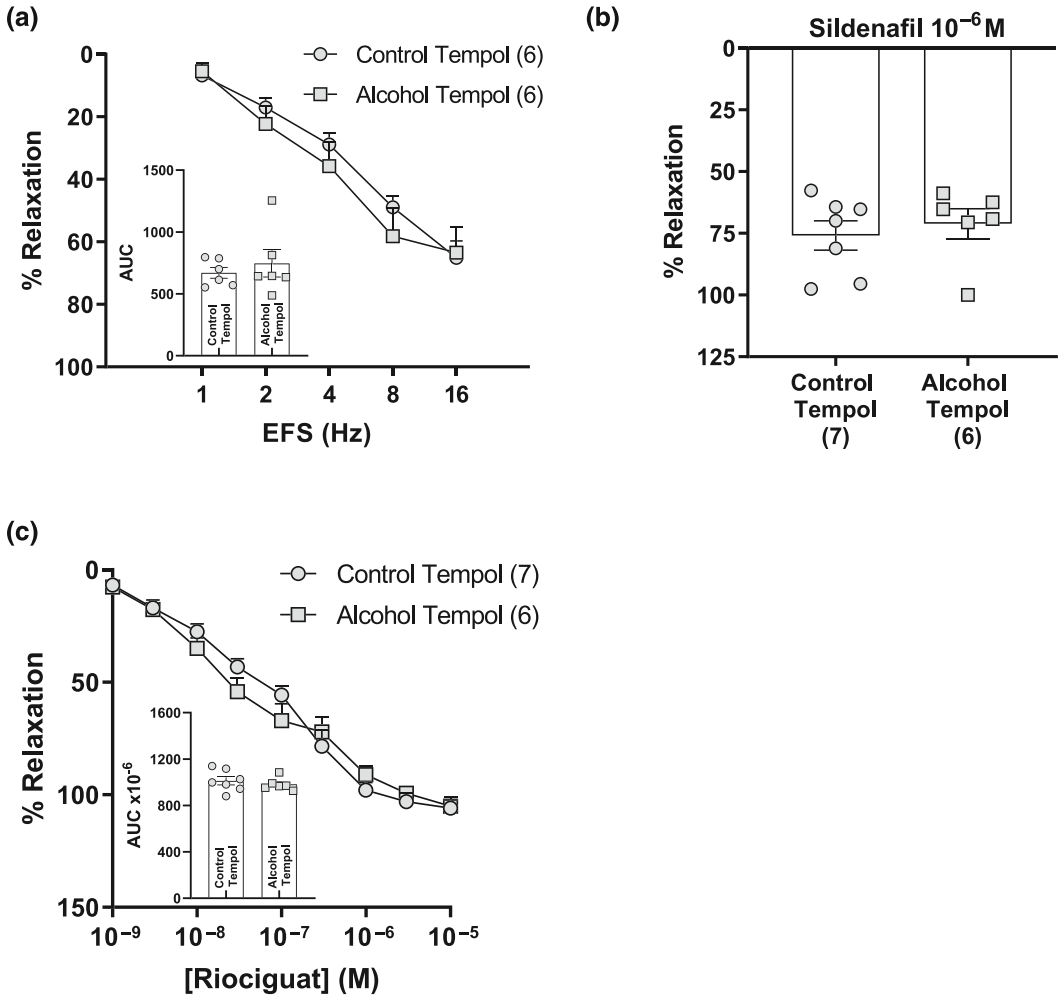

FIGURE 6 In vivo tempol treatment prevents chronic alcohol erectile function impairment. (a) Effect of chronic intermittent ethanol (CIE) on electrical field stimulation (EFS)-induced relaxant responses on corpora cavernosa (CC) of mice treated or not with tempol analysed immediately after the last period of voluntary consumption. (b, c) Effects of alcohol on the relaxant responses of mice CC, treated or not with tempol, induced by (b) sildenafil, (c) riociguat. Area under the curve (AUC) analysis is shown as an insert in panels (a) and (c); note that *Y* axes are inverted so that the AUC graphically corresponds to the area above the curve. Cumulative concentrations were studied in panel (c), but a single concentration of sildenafil was tested in panel (b) due to slower responses to this drug. Results are means ± standard error of the mean. $^{*}P < 0.05$ versus control and $\#P < 0.05$ versus alcohol using two-way (alcohol × concentration) ANOVA test followed by a Bonferroni post hoc test. The number of animals analysed is shown in parenthesis.

alcoholic mice. In fact, cGMP concentration was reduced in alcoholic mice and, in parallel to the results obtained in the CC relaxation experiments, incubation with riociguat increased cGMP synthesis by stimulating sGC in controls but not in alcoholic mice. Altogether, the results strongly suggest an alteration of the NO-sGC-GMP pathway of CC relaxation. The mechanism does not seem to involve changes in sGC expression as demonstrated by western blot experiments.

An alternative to the NO-sGC-cGMP pathway involves the activation of adenylate cyclase leading to increased levels of cAMP. Several vasoactive factors activating Gs-protein-coupled receptors including **PGE_2**, **VIP**, **adenosine**, **adrenomedullin** and **calcitonin gene-related peptide** (CGRP) increase intracavernosal pressure and penile length via increasing cAMP (Andersson, 2011), which is the main mechanism of penile erection induced by therapeutical intracavernosal injection of **alprostadil** (PGE_1) and papaverine (Angulo et al., 2000). The similar relaxant response in CC from controls and alcoholic mice to the adenylate cyclase activator forskolin suggests that the cAMP pathway remains unchanged and that the alteration is specific to the sGC-cGMP pathway. Therefore, the efficacies of endogenous mediators and therapies acting via cAMP to induce penile erection are presumably unaffected.

Critical to sGC function is its redox state which, if altered, can greatly influence the sensitivity and efficiency of cGMP production. sGC oxidation into its Fe^{3+} state results in a loss of the prosthetic heme group, and hence the NO binding site, yielding a NO-insensitive form (Shah et al., 2018). This oxidation also reduces the effects of phosphodiesterase 5 inhibitors (PDE5i), the first-line medication for ED, or to sGC stimulators, such as riociguat. In contrast, it leads to

enhanced responsiveness to sGC activators such as cinaciguat, a new family of drugs potentially useful in several conditions including ED (Monica & Antunes, 2018). According to this therapeutic principle, sGC activators are more potent activating oxidized compared to normal sGC (Sandner et al., 2021). Although there is no direct method to analyse oxidized or heme-free sGC in cellular systems (Sandner et al., 2021), a loss of the prosthetic group by oxidative stress can be corroborated by a simultaneous decreased effect of sGC stimulators and increased effect of sGC activators. Oxidized sCG is presumably present in several diseased vessels (Beuve, 2017). However, while reduced response to NO, **nitroprusside** and/or sGC stimulators associated with sGC S-nitrosylation has been reported following exposure to pathological stimuli including **angiotensin II**, **aldosterone**, **IL-1β** or **TNF-α** (Choi et al., 2011; Crassous et al., 2012; Maron et al., 2009; Rajagopal et al., 2015), increased response to sGC activators has not been shown. sGC oxidation associated with a reduced response to NO, NO donors or sGC stimulators with concomitant increased response to sGC activators has only been shown so far in artificial systems by oxidizing agents (Tawa et al., 2019) or in genetically modified animals such as the heme-deficient sGC mice (Thoonen et al., 2015) or the CYB5R3−/− mice (Rahaman et al., 2017). There is, however, a report showing reduced response to sCG stimulators with unchanged response to sGC activators after inducing **nitroglycerin** tolerance in the rat aorta, which may reflect a partial oxidation of sGC (Jabs et al., 2015). Notably, we show in the present study that alcohol-dependence in mice reduced not only the relaxant responses to riociguat but also increased those to cinaciguat. Thus, to our knowledge this is the first clear evidence of sGC sensitization to sGC activators in a clinically relevant condition. We also found that the effects of alcohol seem to be specific for the CC, because no differences were observed in the NO-sCG-cGMP pathway in the pulmonary vascular bed. This is consistent with a general view that the CC is more sensitive to impairments of the NO pathway than other vascular beds and, therefore, ED is considered a sentinel marker for cardiovascular disease (Assar et al., 2022).

In order to confirm that differences in the response to sCG modulators were due to changes in the oxidative status of sGC, we analysed the response to riociguat in the presence of the sGC oxidative agent ODQ and the reducing agent Na_2S (Zhou et al., 2016). The rationale for these experiments is that naive sGC is in a reduced state and should be more sensitive to being oxidized. In contrast, diseased sGC is already oxidized being less affected by oxidants. As expected, ODQ produced a very strong inhibitory effect on the relaxant response of riociguat in controls but only a minor effect in alcoholic mice. Following this reasoning, a reducing agent should have no effect on a reduced sGC but potentiate the activity of an oxidized sGC. Thus, as hypothesized, Na_2S potentiated the response to riociguat in alcohol-dependent mice but not in controls.

We confirmed the increased ROS in the CC from alcoholic mice by measuring the DHE staining sensitive to superoxide scavengers, which selectively measures tissue superoxide. Increased ROS may result from an increase in their synthesis and/or a decrease in their degradation. The classical ROS degrading enzymes SOD1 and catalase were unaffected. An antioxidant enzyme which might potentially be involved is the reductase CYB5R3 because it is responsible for preserving the oxidative status of sGC and whose downregulation leads to impaired NO-sGC-cGMP (Rahaman et al., 2017). However, CYB5R3 also was unaffected. In contrast, we found an increase in the NADPH oxidase subunit NOX2, a well-known ROS generating enzyme involved in impaired NO-cGMP pathway which confirmed previous results in CC from alcoholic mice (Leite et al., 2017). Interestingly, we also found up-regulated CYP2E1 in the CC. This cytochrome P450-dependent oxidase generates superoxide as a by-product in the ethanol metabolism reaction. CYP2E1 also can catalyse lipid peroxidation, metabolize acetone, acetol, steroids, polyunsaturated fatty acids and other short chain alcohols using **NADPH** as cofactor and releasing superoxide; CYP2E1 is highly expressed in the liver, is up-regulated after chronic alcohol and is involved in alcohol-induced oxidative hepatic injury (Bansal et al., 2010; Leung & Nieto, 2013). Accordingly, the increase in ROS in the alcoholic CC was inhibited by the CYP2E1 inhibitor clomethiazole, a preliminary result which suggests that it might also play a role in alcohol-induced ED.

PDE5i refractoriness in ED can develop for several reasons, including reduced androgen levels, nitrergic nerve damage, decreased NO production, or inflammation-related oxidation of the sGC haem group. Androgen supplementation has been reported to be effective as an add-on therapy in some patients but there is largely an unmet need for oral treatment of ED in non-responders to PDE5i treatment (Munk et al., 2019). Herein we show, for the first time, a reduced response to PDE5i in alcoholic mice. sGC activators have been developed to enhance sGC activity in the absence of NO or when sGC is oxidized (Stasch et al., 2002). Therefore, modulation of sGC has been proposed as a strategy to overcome therapeutic resistance to PDE5i in some diseases including ED (Oudot et al., 2011). The present results suggest that sGC activators not only serve as pharmacological tools to identify the mechanism involved in alcohol-induced ED but also as potential drugs to treat this condition. The experience with this kind of drugs in ED is limited to preclinical studies. Thus, the sGC activator BAY 60-2770 produces erectile responses in the rat (Lasker et al., 2013) and after 2-weeks of administration reverses the impairment of erectile responses in obese mice (Silva et al., 2014).

Taken together, the results indicate an increase in oxidative stress induced by chronic alcohol consumption resulting in an altered sGC redox state in the CC. This hypothesis was reinforced with the experiments with tempol. The antioxidant treatment prevented ED, measured as reduced response to EFS and sildenafil. Interestingly, the reduced response to riociguat also was prevented, suggesting that tempol may have avoided sGC oxidation.

In conclusion, alcoholic mice show an oxidative dysfunction of sGC in the CC. This effect results in impaired erectile function when stimulated via the classic NO-cGMP pathway but is preserved when stimulated via cAMP. PDE5i, the first-line current treatment for ED, show limited effectiveness in this condition whereas sGC activators emerge as potential alternative drugs to treat ED in alcoholics.

AUTHOR CONTRIBUTIONS

Miguel A. Olivencia and Leticia Gil de Biedma-Elduayen coordinated the in vitro and biochemical studies, did the animal model, and analysed the data. Bianca Barreira performed the pulmonary artery experiments. Javier Angulo and Argentina Fernández were responsible for the in vivo study. Pablo Giménez-Gómez conceived the study. Francisco Perez-Vizcaino contributed to the study design with relevant contributions of Miguel A. Olivencia, Pablo Giménez-Gómez, Javier Angulo, Esther O'Shea and Maria Isabel Colado, Francisco Perez-Vizcaino and Esther O'Shea wrote the manuscript with significant contributions from all authors.

ACKNOWLEDGEMENTS

The authors thank Centro de Asistencia a la Investigación (CAI) Animalario, Universidad Complutense de Madrid (UCM) for the care and maintenance of the mice used in the study.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests related to the present study.

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 and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

ORCID

Javier Angulo https://orcid.org/0000-0002-3789-9465
Esther O'Shea https://orcid.org/0000-0001-5008-882X
Francisco Perez-Vizcaino https://orcid.org/0000-0001-6309-7418

REFERENCES

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Beuve, A., Brouckaert, P., Bryant, C., Burnett, J. C., Farndale, R. W., Friebe, A., Garthwaite, J., … Waldman, S. A. (2021). The concise guide to pharmacology 2021/22: Catalytic receptors. *British Journal of Pharmacology*, *178*, S264– S312.

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Boison, D., Burns, K. E., Dessauer, C., Gertsch, J., Helsby, N. A., Izzo, A. A., Koesling, D., … Wong, S. S. (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Enzymes. *British Journal of Pharmacology*, *178*(S1), S313–S411. https://doi.org/10.1111/bph.15542

Alexander, S. P. H., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Buneman, O. P., Cidlowski, J. A., Christopoulos, A., Davenport, A. P., CGTP Collaborators, Fabbro, D., Spedding, M., Striessnig, J., Davies, J. A., … Zolghadri, Y. (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Introduction and Other Protein Targets. *British Journal of Pharmacology*, *178*(S1), S1–S26. https://doi.org/10.1111/bph.15537

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the British Journal of pharmacology. *British Journal of Pharmacology*, *175*(3), 407–411. https://doi.org/10.1111/bph.14112

Andersson, K. E. (2011). Mechanisms of penile erection and basis for pharmacological treatment of erectile dysfunction. *Pharmacological Reviews*, *63*(4), 811–859. https://doi.org/10.1124/pr.111.004515

Angulo, J., Cuevas, P., Moncada, I., Martín-Morales, A., Allona, A., Fernández, A., Gabancho, S., Ney, P., & Sáenz de Tejada, I. (2000). Rationale for the combination of PGE(1) and S-nitroso-glutathione to induce relaxation of human penile smooth muscle. *The Journal of Pharmacology and Experimental Therapeutics*, *295*(2), 586–593.

Assar, M. E., Angulo, J., Garcia-Rojo, E., Sevilleja-Ortiz, A., Garcia-Gomez, B., Fernandez, A., Sanchez-Ferrer, A., La Fuente, J. M., Romero-Otero, J., & Rodriguez-Manas, L. (2022). Early manifestation of aging-related vascular dysfunction in human penile vasculature-a potential explanation for the role of erectile dysfunction as a harbinger of systemic vascular disease. *Geroscience*, *44*(1), 485–501. https://doi.org/10.1007/s11357-021-00507-x

Aydinoglu, F., Yilmaz, S. N., Coskun, B., Daglioglu, N., & Ogulener, N. (2008). Effects of ethanol treatment on the neurogenic and endothelium-dependent relaxation of corpus cavernosum smooth muscle in the mouse. *Pharmacological Reports*, *60*(5), 725–734.

Bansal, S., Liu, C. P., Sepuri, N. B., Anandatheerthavarada, H. K., Selvaraj, V., Hoek, J., Milne, G. L., Guengerich, F. P., & Avadhani, N. G. (2010). Mitochondria-targeted cytochrome P450 2E1 induces oxidative damage and augments alcohol-mediated oxidative stress. *The Journal of Biological Chemistry*, *285*(32), 24609–24619. https://doi.org/10.1074/jbc.M110.121822

Belknap, J. K., Crabbe, J. C., & Young, E. R. (1993). Voluntary consumption of ethanol in 15 inbred mouse strains. *Psychopharmacology*, *112*(4), 503–510. https://doi.org/10.1007/BF02244901

Beuve, A. (2017). Thiol-based redox modulation of soluble guanylyl cyclase, the nitric oxide receptor. *Antioxidants & Redox Signaling*, *26*(3), 137–149. https://doi.org/10.1089/ars.2015.6591

Choi, H., Allahdadi, K. J., Tostes, R. C., & Webb, R. C. (2011). Augmented S-nitrosylation contributes to impaired relaxation in angiotensin II hypertensive mouse aorta: Role of thioredoxin reductase. *Journal of Hypertension*, *29*(12), 2359–2368. https://doi.org/10.1097/HJH.0b013e32834d2554

Crassous, P. A., Couloubaly, S., Huang, C., Zhou, Z., Baskaran, P., Kim, D. D., Papapetropoulos, A., Fioramonti, X., Durán, W. N., & Beuve, A. (2012). Soluble guanylyl cyclase is a target of angiotensin II-induced nitrosative stress in a hypertensive rat model. *American Journal of Physiology. Heart and Circulatory Physiology*, *303*(5), H597–H604. https://doi.org/10.1152/ajpheart.00138.2012

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, 179, 3907–3913. https://doi.org/10.1111/bph.14153

Gil de Biedma-Elduayen, L., Gimenez-Gomez, P., Morales-Puerto, N., Vidal, R., Nunez-de la Calle, C., Gutierrez-Lopez, M. D., O'Shea, E., & Colado, M. I. (2022). Influx of kynurenine into the brain is involved in the reduction of ethanol consumption induced by Ro 61-8048 after chronic intermittent ethanol in mice. *British Journal of Pharmacology*, *179*, 3711–3726. https://doi.org/10.1111/bph.15825

Gimenez-Gomez, P., Perez-Hernandez, M., Gutierrez-Lopez, M. D., Vidal, R., Abuin-Martinez, C., O'Shea, E., & Colado, M. I. (2018). Increasing kynurenine brain levels reduces ethanol consumption in mice by inhibiting dopamine release in nucleus accumbens. *Neuropharmacology*, *135*, 581–591. https://doi.org/10.1016/j.neuropharm.2018.04.016

Huitron-Resendiz, S., Nadav, T., Krause, S., Cates-Gatto, C., Polis, I., & Roberts, A. J. (2018). Effects of withdrawal from chronic intermittent ethanol exposure on sleep characteristics of female and male mice. *Alcoholism, Clinical and Experimental Research*, *42*(3), 540–550. https://doi.org/10.1111/acer.13584

Jabs, A., Oelze, M., Mikhed, Y., Stamm, P., Kröller-Schön, S., Welschof, P., Jansen, T., Hausding, M., Kopp, M., Steven, S., Schulz, E., Stasch, J. P., Münzel, T., & Daiber, A. (2015). Effect of soluble guanylyl cyclase activator and stimulator therapy on nitroglycerin-induced nitrate tolerance in rats. *Vascular Pharmacology*, *71*, 181–191. https://doi.org/10.1016/j.vph.2015.03.007

Julian, T. H., Syeed, R., Glascow, N., & Zis, P. (2020). Alcohol-induced autonomic dysfunction: A systematic review. *Clinical Autonomic Research*, *30*(1), 29–41. https://doi.org/10.1007/s10286-019-00618-8

Kessler, A., Sollie, S., Challacombe, B., Briggs, K., & Van Hemelrijck, M. (2019). The global prevalence of erectile dysfunction: A review. *BJU International*, *124*, 587–599. https://doi.org/10.1111/bju.14813

Lasker, G. F., Pankey, E. A., Frink, T. J., Zeitzer, J. R., Walter, K. A., & Kadowitz, P. J. (2013). The sGC activator BAY 60-2770 has potent erectile activity in the rat. *American Journal of Physiology. Heart and Circulatory Physiology*, *304*(12), H1670–H1679. https://doi.org/10.1152/ajpheart.00062.2013

Leite, L. N., do Vale, G. T., Simplicio, J. A., De Martinis, B. S., Carneiro, F. S., & Tirapelli, C. R. (2017). Ethanol-induced erectile dysfunction and increased expression of pro-inflammatory proteins in the rat cavernosal smooth muscle are mediated by NADPH oxidase-derived reactive oxygen species. *European Journal of Pharmacology*, *804*, 82–93. https://doi.org/10.1016/j.ejphar.2017.03.024

Lemere, F., & Smith, J. W. (1973). Alcohol-induced sexual impotence. *The American Journal of Psychiatry*, *130*(2), 212–213. https://doi.org/10.1176/ajp.130.2.212

Leung, T. M., & Nieto, N. (2013). CYP2E1 and oxidant stress in alcoholic and non-alcoholic fatty liver disease. *Journal of Hepatology*, *58*(2), 395–398. https://doi.org/10.1016/j.jhep.2012.08.018

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*(16), 3611–3616. https://doi.org/10.1111/bph.15178

Lizarte, F. S., Claudino, M. A., Tirapelli, C. R., Morgueti, M., Tirapelli, D. P., Batalhao, M. E., Carnio, E. C., Queiroz, R. H., Evora, P. R., Tucci, S. Jr., Cologna, A., Antunes, E., Martins, A. C., & Tirapelli, L. F. (2009). Chronic ethanol consumption induces cavernosal smooth muscle dysfunction in rats. *Urology*, *74*(6), 1250–1256. https://doi.org/10.1016/j.urology.2009.04.043

Lopez, M. F., Miles, M. F., Williams, R. W., & Becker, H. C. (2017). Variable effects of chronic intermittent ethanol exposure on ethanol drinking in a genetically diverse mouse cohort. *Alcohol*, *58*, 73–82. https://doi.org/10.1016/j.alcohol.2016.09.003

Maron, B. A., Zhang, Y. Y., Handy, D. E., Beuve, A., Tang, S. S., Loscalzo, J., & Leopold, J. A. (2009). Aldosterone increases oxidant stress to impair guanylyl cyclase activity by cysteinyl thiol oxidation in vascular smooth muscle cells. *The Journal of Biological Chemistry*, *284*(12), 7665–7672. https://doi.org/10.1074/jbc.M809460200

Monica, F. Z., & Antunes, E. (2018). Stimulators and activators of soluble guanylate cyclase for urogenital disorders. *Nature Reviews. Urology*, *15*(1), 42–54. https://doi.org/10.1038/nrurol.2017.181

Munk, N. E., Knudsen, J. S., Comerma-Steffensen, S., & Simonsen, U. (2019). Systematic review of Oral combination therapy for erectile dysfunction when phosphodiesterase type 5 inhibitor monotherapy fails. *Sexual Medicine Reviews*, *7*(3), 430–441. https://doi.org/10.1016/j.sxmr.2018.11.007

NIH Consensus Conference. (1993). Impotence. NIH consensus development panel on impotence. *Jama*, *270*(1), 83–90Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/8510302. https://doi.org/10.1001/jama.270.1.83

Osna, N. A., Donohue, T. M. Jr., & Kharbanda, K. K. (2017). Alcoholic liver disease: Pathogenesis and current management. *Alcohol Research: Current Reviews*, *38*(2), 147–161.

Oudot, A., Behr-Roussel, D., Poirier, S., Sandner, P., Bernabe, J., Alexandre, L., & Giuliano, F. (2011). Combination of BAY 60-4552 and vardenafil exerts proerectile facilitator effects in rats with cavernous nerve injury: A proof of concept study for the treatment of phosphodiesterase type 5 inhibitor failure. *European Urology*, *60*(5), 1020–1026. https://doi.org/10.1016/j.eururo.2011.07.052

Patel, R. R., Wolfe, S. A., Bajo, M., Abeynaike, S., Pahng, A., Borgonetti, V., D'Ambrosio, S., Nikzad, R., Edwards, S., Paust, S., Roberts, A. J., & Roberto, M. (2021). IL-10 normalizes aberrant amygdala GABA transmission and reverses anxiety-like behavior and dependence-induced escalation of alcohol intake. *Progress in Neurobiology*, *199*, 101952. https://doi.org/10.1016/j.pneurobio.2020.101952

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Wurbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *British Journal of Pharmacology*, *177*(16), 3617–3624. https://doi.org/10.1111/bph.15193

Perez, M. J., Loyola, R., Canelo, F., Aranguiz, A., Tapia-Monsalves, C., Osorio-Fuentealba, C., & Quintanilla, R. A. (2020). NADPH oxidase contributes to oxidative damage and mitochondrial impairment induced by acute ethanol treatment in rat hippocampal neurons. *Neuropharmacology*, *171*, 108100. https://doi.org/10.1016/j.neuropharm.2020.108100

Rahaman, M. M., Nguyen, A. T., Miller, M. P., Hahn, S. A., Sparacino-Watkins, C., Jobbagy, S., Carew, N. T., Cantu-Medellin, N., Wood, K. C., Baty, C. J., Schopfer, F. J., Kelley, E. E., Gladwin, M. T., Martin, E., & Straub, A. C. (2017). Cytochrome b5 reductase 3 modulates soluble guanylate cyclase redox state and cGMP signaling. *Circulation Research*, *121*(2), 137–148. https://doi.org/10.1161/circresaha.117.310705

Rajagopal, S., Nalli, A. D., Kumar, D. P., Bhattacharya, S., Hu, W., Mahavadi, S., Grider, J. R., & Murthy, K. S. (2015). Cytokine-induced S-nitrosylation of soluble guanylyl cyclase and expression of phosphodiesterase 1A contribute to dysfunction of longitudinal smooth muscle relaxation. *The Journal of Pharmacology and Experimental Therapeutics*, *352*(3), 509–518. https://doi.org/10.1124/jpet.114.221929

Rhodes, J. S., Best, K., Belknap, J. K., Finn, D. A., & Crabbe, J. C. (2005). Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. *Physiology & Behavior*, *84*(1), 53–63. https://doi.org/10.1016/j.physbeh.2004.10.007

Sandner, P., Zimmer, D. P., Milne, G. T., Follmann, M., Hobbs, A., & Stasch, J. P. (2021). Soluble guanylate cyclase stimulators and activators. *Handbook of Experimental Pharmacology*, *264*, 355–394. https://doi.org/10.1007/164_2018_197

Seftel, A. D., Sun, P., & Swindle, R. (2004). The prevalence of hypertension, hyperlipidemia, diabetes mellitus and depression in men with erectile dysfunction. *The Journal of Urology*, *171*(6 Pt 1), 2341–2345. https://doi.org/10.1097/01.ju.0000125198.32936.38

Shah, R. C., Sanker, S., Wood, K. C., Durgin, B. G., & Straub, A. C. (2018). Redox regulation of soluble guanylyl cyclase. *Nitric Oxide*, *76*, 97–104. https://doi.org/10.1016/j.niox.2018.03.013

Silva, F. H., Leiria, L. O., Alexandre, E. C., Davel, A. P., Mónica, F. Z., De Nucci, G., & Antunes, E. (2014). Prolonged therapy with the soluble guanylyl cyclase activator BAY 60-2770 restores the erectile function in obese mice. *The Journal of Sexual Medicine*, *11*(11), 2661–2670. https://doi.org/10.1111/jsm.12682

Stasch, J. P., Schmidt, P., Alonso-Alija, C., Apeler, H., Dembowsky, K., Haerter, M., Heil, M., Minuth, T., Perzborn, E., Pleiss, U., Schramm, M., Schroeder, W., Schroder, H., Stahl, E., Steinke, W., & Wunder, F. (2002). NO- and haem-independent activation of soluble guanylyl cyclase: Molecular basis and cardiovascular implications of a new pharmacological principle. *British Journal of Pharmacology*, *136*(5), 773–783. https://doi.org/10.1038/sj.bjp.0704778

Tawa, M., Yamashita, Y., Masuoka, T., Nakano, K., Yoshida, J., Nishio, M., & Ishibashi, T. (2019). Responsiveness of rat aorta and pulmonary artery to cGMP generators in the presence of thiol or heme oxidant. *Journal of Pharmacological Sciences*, *140*(1), 43–47. https://doi.org/10.1016/j.jphs.2019.04.002

Thoonen, R., Cauwels, A., Decaluwe, K., Geschka, S., Tainsh, R. E., Delanghe, J., Hochepied, T., De Cauwer, L., Rogge, E., Voet, S., Sips, P., Karas, R. H., Bloch, K. D., Vuylsteke, M., Stasch, J. P., Van de Voorde, J., Buys, E. S., & Brouckaert, P. (2015). Cardiovascular and pharmacological implications of haem-deficient NO-unresponsive soluble guanylate cyclase knock-in mice. *Nature Communications*, *6*, 8482. https://doi.org/10.1038/ncomms9482

Tiraboschi, R. B., Neto, F. S. L., da Cunha Tirapelli, D. P., de Bessa, J. Jr., Miranda, E. P., de Assis Cirino, M. L., Tirapelli, L. F., Tucci, S. Jr., & Molina, C. A. F. (2021). Expression of MicroRNAs (miR-15b, miR-16, miR-138, miR-221, and miR-222) as biomarkers of endothelial corpus Cavernosum dysfunction in a diabetic alcoholic murine model. *Sexual Medicine*, *9*(2), 100326. https://doi.org/10.1016/j.esxm.2021.100326

Wespes, E. (2002). Smooth muscle pathology and erectile dysfunction. *International Journal of Impotence Research*, *14*(Suppl 1), S17–S21. https://doi.org/10.1038/sj.ijir.3900792

Yazir, Y., Gocmez, S. S., Utkan, T., Komsuoglu-Celikyurt, I., Gacar, N., & Sarioglu, Y. (2012). Effects of chronic low- and high-dose ethanol intake on the nitrergic relaxations of corpus cavernosum and penile nitric oxide synthase in the rabbit. *International Journal of Impotence Research*, *24*(5), 185–190. https://doi.org/10.1038/ijir.2012.14

Zhao, W., Sun, J., Yao, L.-Y., Hang, D., Li, Y.-Q., Chen, C.-P., Zhou, Y.-W., Chen, X., Tao, T., Wei, L.-S., Zheng, Y.-Y., Ge, X., Li, C.-J., Xin, Z.-C., Pan, Y., Wang, X.-Z., He, W.-Q., Zhang, X.-N., Yao, B., & Zhu, M.-S. (2022). MYPT1 reduction is a pathogenic factor of erectile dysfunction. *Communications Biology*, *5*(1), 744. https://doi.org/10.1038/s42003-022-03716-y

Zhou, Z., Martin, E., Sharina, I., Esposito, I., Szabo, C., Bucci, M., Cirino, G., & Papapetropoulos, A. (2016). Regulation of soluble guanylyl cyclase redox state by hydrogen sulfide. *Pharmacological Research*, *111*, 556–562. https://doi.org/10.1016/j.phrs.2016.06.029

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Olivencia, M. A., Gil de Biedma-Elduayen, L., Giménez-Gómez, P., Barreira, B., Fernández, A., Angulo, J., Colado, M. I., O'Shea, E., & Perez-Vizcaino, F. (2023). Oxidized soluble guanylyl cyclase causes erectile dysfunction in alcoholic mice. *British Journal of Pharmacology*, *180*(18), 2361–2376. https://doi.org/10.1111/bph.16087