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Mecanismos de acción de los flavanoles del cacao en las células hepáticas durante la resistencia a la insulina y la diabetes: estudio en cultivos celulares y en animales de experimentación

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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EUROPEAN DOCTORAL THESIS

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Trabajo de investigación presentado por Isabel Cordero Herrera para optar al grado de Doctor Europeo por la Universidad Complutense de Madrid (UCM)

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La **Dra. Sonia Ramos Rivero**, Científico titular del Departamento de Metabolismo y Nutrición del Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), Consejo Superior de Investigaciones Científicas (CSIC), certifica que el trabajo de investigación titulado:

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Índice

Abreviaturas

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ACC	Acetil Coenzima A carboxilasa
ACS	Acil Coenzima A sintetasa
ADN	Ácido desoxirribonucleico
AKT/PKB	Proteína quinasa B
AMPK	Proteína quinasa activada por 5´-adenosín-monofosfato
ARE	Elemento de respuesta antioxidante
ARN	Ácido ribonucleico
ATP	Adenosín-trifosfato
CACT	Carnitina/acetilcarnitina translocasa
CAT	Catalasa
CBG	β-glucosidasa citosólica
ChREBP	Proteína de unión al elemento de respuesta a carbohidratos
CPE	Extracto polifenólico de cacao
CPT	Carnitina palmitoil transferasa
CREB	Proteína de unión al elemento de respuesta de AMP cíclico
DAG	Diacilglicerol
DMT1	Diabetes mellitus tipo 1
DMT2	Diabetes mellitus tipo 2
EC	Epicatequina
EGCG	Epigalocatequina-3-galato
EpRE	Elemento de respuesta electrofílica
ERK	Quinasas reguladas por señales extracelulares
FAS	Ácido graso sintasa
FOXO	Factor de transcripción *forkhead*
GAPDH	Gliceraldehído-3-fosfato deshidrogenasa
GLP-1	Péptido 1 similar al glucagón
GLUT	Transportador de glucosa
GK	Glucoquinasa hepática
G6Pasa	Glucosa-6-fosfatasa
GPx	Glutation peroxidasa
GR	Glutation reductasa
GS	Glucógeno sintasa
GSH	Glutation reducido
GSK3	Glucógeno sintasa quinasa 3

Abreviaturas

GSSG	Glutation oxidado
GST	Glutation S transferasa
HDL-Cho	Lipoproteínas de alta densidad
HMGCR	3-hidroxi-3-metilglutaril coenzima A reductasa
HO-1	Hemooxigenasa-1
IκB	Inhibidor del elemento kappa B
IKK	Quinasa del inhibidor del elemento kappa B
IL	Interleuquina
IR	Receptor de la insulina
IRS	Sustrato del receptor de la insulina
JNK	Quinasa c-Jun NH2-terminal
Keap-1	Proteína represora asociada a ECH
LXR	Receptor X hepático
MAPKs	Proteínas quinasas activadas por mitógenos
MCP-1	Proteína quimioatractante de monocitos 1
MODY	Maturity onset diabetes of the young
mTORC1	Complejo diana de rapamicina en mamíferos 1
NADPH	Nicotinamida-adenina-dinucleótido-fosfato reducida
NEFA	Ácido graso no esterficado
NF-κB	Factor de transcripción nuclear kappa B
Nrf2	Factor de transcripción relacionado con el factor nuclear eritroide 2p45
LDL-Cho	Lipoproteínas de baja densidad
LPH	Lactasa floridzina hidrolasa
p38	Quinasa p38
PDK	Proteína quinasa dependiente del 3-fosfoinositol
PEPCK	Fosfoenolpiruvato carboxiquinasa
PGC-1α	Coactivador del receptor de proliferación activado por peroxisomas 1 α
PI3K	Fosfatidil-inositol-3-quinasa
PKCa	Proteína quinasa C atípica
PPAR	Receptor activado por proliferación de peroxisomas
PTEN	Homólogo a fosfatasa y tensina
PTP1B	Proteína fosfatasa de tirosina 1B

RNS	Especies reactivas del nitrógeno
ROS	Especies reactivas del oxígeno
SOCS	Proteínas supresoras de la señalización por citoquinas
SOD	Superóxido dismutasa
SREBP	Proteína de unión al elemento regulado por esteroles
T-Cho	Colesterol total
TG	Triglicérido
TNF-α	Factor de necrosis tumoral-alfa
UCP	Proteína desacoplante
VLDL	Lipoproteína de muy baja densidad
ZDF	Ratas Zucker Diabetic Fatty (Ratas Zucker diabéticas obesas)
ZL	Ratas Zucker Lean (Ratas Zucker delgadas).

Resumen

Resumen

Introducción.

La diabetes mellitus tipo 2 es una enfermedad con gran incidencia en nuestra sociedad y supone una gran carga socioeconómica y médica. Durante la resistencia a la insulina y la diabetes se alteran el metabolismo glucídico y lipídico, y el equilibrio redox por la aparición del estrés oxidativo. Estos y otros cambios que se producen en esta enfermedad están directamente relacionados con la desregulación de la señalización de la insulina y el desarrollo y progreso de esta patología.

En la actualidad, la prevención nutricional se considera una de las estrategias clave para la prevención de la diabetes tipo 2, por lo que en los últimos años ha aumentado el interés por identificar compuestos de la dieta con actividad antidiabética, ya que suponen una alternativa prometedora para la prevención y/o tratamiento de esta enfermedad al presentar baja o nula toxicidad, ser abundantes en la naturaleza y baratos de producir.

El cacao y sus derivados como el chocolate se consumen comúnmente en un gran número de países de todo el mundo. Este alimento es una fuente importante de flavanoles, entre los que se podría destacar a la epicatequina, y parece presentar distintas propiedades potencialmente beneficiosas para la salud, como la actividad antidiabética. Sin embargo, hasta el momento son pocos los estudios que investigan los mecanismos de acción molecular responsables del efecto antidiabético de los flavanoles del cacao en el hígado, órgano clave para la homeostasis metabólica.

Objetivos.

Con el fin de estudiar los mecanismos de acción responsables del efecto antidiabético de los flavanoles del cacao en el hígado, se plantearon los siguientes objetivos específicos, que se han desarrollado en un modelo celular (células hepáticas humanas HepG2) y un modelo animal de DMT2 (ratas ZDF):

Objetivo 1. Evaluar el efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico en condiciones fisiológicas y durante una situación de resistencia a la insulina y diabetes en las células hepáticas (***Capítulo 1***).

Objetivo 2. Analizar el posible efecto protector del cacao y la epicatequina y sus mecanismos moleculares de acción frente al estrés oxidativo durante la resistencia a la insulina y diabetes en las células hepáticas (***Capítulo 2***).

Objetivo 3. Estudiar el posible efecto beneficioso de los flavanoles del cacao sobre el metabolismo lipídico y sus mecanismos de acción en una situación de resistencia a la insulina y diabetes en las células hepáticas (***Capítulo 3*****)**.

Resultados.

Capítulo 1: Efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico hepático en condiciones fisiológicas y durante la resistencia a la insulina y la diabetes.

En condiciones fisiológicas, las distintas concentraciones de epicatequina (EC, 1-10 µM) y el extracto polifenólico de cacao (CPE, 1-10 µg/mL) no afectaron a la proliferación ni a la viabilidad de las células HepG2, y activaron la ruta de señalización de la insulina, ya que incrementaron los niveles totales y fosforilados en tirosina de IR, IRS-1 e IRS-2, los valores fosforilados de AKT y GSK3 y disminuyeron los de p-GS. Todas las concentraciones de EC y CPE analizadas aumentaron los niveles fosforilados de AMPK, aunque sólo CPE (10 µg/mL) incrementó los niveles de GLUT2. EC y CPE modularon la expresión de PEPCK, lo que condujo a un descenso de la producción de glucosa. Además, la inhibición de la gluconeogénesis hepática inducida por EC y CPE se previno por el bloqueo de AKT y AMPK **(Artículo 1).**

En la resistencia a la insulina, la suplementación de la dieta con cacao, al igual que la incubación de las células HepG2 insulino-resistentes (incubadas con 30 mM de glucosa durante 24 horas) con EC y CPE, protegieron del bloqueo de la ruta de señalización de la insulina en ambos modelos experimentales (hígado de las ratas ZDF y células HepG2) desde los primeros eslabones de la vía, ya que previnieron la inhibición de IR, IRS-1 y -2, la ruta PI3K/AKT y la AMPK. Además, la dieta rica en cacao en los animales, y EC y CPE en las células, mejoraron la funcionalidad de los hepatocitos, dado que impidieron el incremento de los niveles de PEPCK y restauraron el contenido de glucógeno y los niveles de GLUT2 a valores similares a los de los controles. En este sentido, en las células HepG2, CPE y EC previnieron el descenso en la captación de glucosa y el aumento en la producción de glucosa causado por la alta dosis de glucosa,

mientras que la dieta rica en cacao incrementó los niveles de GK en el hígado de los animales ZDF. Además, la dieta rica en cacao suprimió la activación de las JNK y p38-MAPK causada por la resistencia a la insulina **(Artículos 2 y 3).**

Capítulo 2: Efecto de los flavanoles del cacao sobre el estrés oxidativo hepático durante la resistencia a la insulina y la diabetes.

El pretratamiento con EC y CPE en las células HepG2 y la administración de una dieta enriquecida en cacao a las ratas ZDF protegieron frente al estrés oxidativo. Todos los tratamientos disminuyeron la generación de ROS y previnieron el aumento en los niveles de los grupos carbonilos, que mostraron valores similares a los de los controles en ambos modelos experimentales. En las células resistentes a la insulina, EC y CPE reprimieron el descenso de los niveles de GSH y la alteración de la actividad de GPx, y CPE restauró la actividad de GR a los valores control, aunque EC y CPE no fueron capaces de prevenir la disminución en la GST inducida por la dosis alta de glucosa. En los animales, el cacao restauró la actividad de SOD y de HO-1, pero no la de GST, que permaneció disminuida. La actividad de la CAT en las células, y la de GPx, GR y CAT, así como el contenido de GSH en las ratas, no se modificaron por ningún tratamiento. Además, en los animales ZDF, la dieta rica en cacao suprimió el aumento en los niveles de Nrf2 y NF-κB. Por su parte, en las células HepG2, EC y CPE activaron a las ERK y el Nrf2, situación que se mantuvo cuando las células fueron pretratadas con ambas sustancias, a la vez que previnieron la activación de p38 y JNK inducida por la dosis alta de glucosa. En estas condiciones de resistencia, los inhibidores específicos de las MAPKs indujeron cambios en el estado redox, la captación de glucosa y los niveles totales y fosforilados en serina de IRS-1, lo que sugiere la implicación de las MAPKs en los efectos protectores mediados por CPE **(Artículos 4 y 5).**

Capítulo 3: Efecto de los flavanoles del cacao sobre el metabolismo lipídico hepático durante la resistencia a la insulina y la diabetes.

La dieta enriquecida en cacao redujo el peso final de las ratas ZDF respecto a los animales ZDF que recibieron la dieta estándar sin modificar la ingesta de ambos grupos. Además, dicha dieta rica en cacao mejoró los perfiles lipídicos sérico y hepático: se produjo un descenso en los niveles de triglicéridos (TG), colesterol LDL, ácidos grasos no esterificados (NEFA) y la acumulación de gotas lipídicas, y un aumento en los

niveles de colesterol HDL. De manera similar, la EC restauró los valores lipídicos alterados (TG, colesterol total y NEFA) por la alta concentración de glucosa en las células HepG2. El cacao y la EC también previnieron el descenso en los niveles fosforilados de AKT, AMPK y PPARα, así como el aumento de p-PKCζ, SREBP1-c y FAS, lo que se asoció con la estimulación de la lipolisis y la inhibición de la lipogénesis. La regulación de la PKCζ por la EC contribuyó a mejorar el metabolismo lipídico a través de la AKT y la AMPK **(Artículo 6).**

Conclusiones.

En relación con los objetivos planteados y los resultados obtenidos, ha sido posible extraer las siguientes conclusiones:

1. El tratamiento de las células hepáticas HepG2 con dosis realistas del flavanol epicatequina (1-10 µM) y del extracto polifenólico de cacao (1-10 µg/mL) ejerce un efecto similar al de la insulina que contribuye a fortalecer la señalización de la hormona. Este efecto se relacionó con la activación del receptor de la insulina, los sustratos de dicho receptor (IRS-1 y -2), la vía PI3K/AKT y AMPK, y la inhibición de la producción de glucosa.

   El pretratamiento de las células HepG2 insulino-resistentes con epicatequina (10 µM) o el extracto polifenólico de cacao (1 µg/mL) y la administración de una dieta rica en cacao a las ratas ZDF mejoraron la homeostasis glucídica y la sensibilidad hepática a la hormona. Este retraso o prevención de la potencial disfunción hepática se asoció con la atenuación del bloqueo de la ruta de la insulina que tiene lugar durante la resistencia a la insulina y la diabetes, dado que se previno la inhibición de IR, IRS-1 y -2, la ruta PI3K/AKT y de la AMPK. Todos los tratamientos administrados también contribuyeron a mantener la funcionalidad de las células hepáticas, puesto que impidieron el incremento de los niveles de PEPCK y restauraron el contenido de glucógeno y los niveles de GLUT2. Además, en las células HepG2, la epicatequina y el extracto polifenólico de cacao previnieron el descenso en la captación de glucosa y el aumento en la producción de glucosa causado por la alta dosis de glucosa, mientras que la dieta rica en

cacao incrementó los niveles de GK y suprimió la activación de las JNK y p38-MAPK en el hígado de los animales ZDF.

2. El pretratamiento de las células HepG2 insulino-resistentes incubadas con epicatequina (10 µM) o el extracto polifenólico de cacao (1 µg/mL) y la administración de una dieta enriquecida en cacao a las ratas ZDF protegieron frente al estrés oxidativo. Este efecto protector se relacionó con el refuerzo de los niveles de las defensas antioxidantes celulares (GPx y GR en las células HepG2 insulino-resistentes, y SOD y HO-1 en las ratas ZDF), así como con la modulación de factores de trascripción clave relacionados con el estado redox (Nrf2 y NF-κB). Además, en las células HepG2 insulino-resistentes, la epicatequina y el extracto polifenólico de cacao previnieron la activación de p38 y JNK inducida por la dosis alta de glucosa, sugiriendo que los efectos beneficiosos del extracto de cacao se deben en parte a su acción moduladora sobre las MAPKs.

3. El pretratamiento de las células HepG2 insulino-resistentes con epicatequina (10 µM) y la administración de una dieta rica en cacao a las ratas ZDF aliviaron la hiperlipidemia y la esteatosis hepática mediante la regulación de múltiples vías de señalización. El descenso de los niveles lipídicos se relacionó con la inhibición de proteínas clave para la lipogénesis *de novo* (SREBP1-c y FAS) y la activación de la oxidación de los ácidos grasos (PPARα); estos efectos parecen depender de AMPK, AKT y PKCζ. Además, en las células HepG2, la activación de AKT y AMPK inducida por la epicatequina parece estar mediada por PKCζ.

Conclusión general

En resumen, los resultados obtenidos en cultivos celulares y en animales de experimentación han puesto de manifiesto que los flavanoles del cacao en las células hepáticas parecen capaces de modular el metabolismo glucídico y lipídico, lo que se traduce en una mejora de la sensibilidad a la insulina y de la tolerancia a la glucosa en una situación de resistencia a la insulina y diabetes. Además, los flavanoles del cacao

son capaces de proteger el potencial antioxidante del hepatocito para mantener el equilibrio redox durante el estrés oxidativo que tiene lugar durante la resistencia a la insulina y diabetes.

Abstract

Abstract

Mechanism of action of cocoa flavanols in hepatic cells during insulin resistance and diabetes. Study in cultured cells and experimental animals.

Background.

Prevalence of type 2 diabetes is increasing globally and it is one of the most costly and burdensome chronic diseases. During insulin resistance and diabetes, an alteration of carbohydrate and lipid metabolism, as well as a redox imbalance due to oxidative stress, occurs. All these alterations are related to the insulin signalling deregulation and play a critical role in the development and progression of this disease.

Nowadays, it is assumed that the most efficient approach to prevent or delay the onset of type 2 diabetes at the lowest cost is at nutritional level. Therefore there is a raising interest in the identification of dietary compounds with antidiabetic properties, which constitutes a promising approach for prevention and/or treatment of type 2 diabetes, and may benefit because of their safety, they are abundant in nature and inexpensive to produce.

Cocoa and its derived products, such as chocolate, are world-wide consumed. Cocoa is a rich source of flavanols, such as epicatechin, and it seems to exert beneficial effects on health, including antidiabetic activity. However, limited studies have evaluated the molecular mechanisms related to the cocoa antidiabetic effect in the liver, which is a key organ for the metabolic homeostasis.

Aims.

In order to analyse the molecular mechanisms related to the antidiabetic effect of cocoa flavanols in the liver, two experimental models have been used: a cellular model (HepG2 cells) and an animal model for type 2 diabetes (ZDF rats).

List below are the specific aims studied in the present thesis:

Aim 1. Evaluation of the effect of cocoa flavanols on insulin signalling and glucose metabolism in hepatic cells in physiological conditions and during insulin resistance situation and diabetes ***(Chapter 1***).

Aim 2. Analysis of the potential protective effect of cocoa flavanols and epicatechin against the oxidative stress induced by insulin resistance and diabetes in hepatic cells, and molecular mechanisms related to this effect (***Chapter 2***).

Aim 3. Study of the potential beneficial effect of cocoa flavanols on lipid metabolism and their molecular mechanism of action in the liver during insulin resistance and diabetes (***Chapter 3***).

Results.

Chapter 1: Effect of cocoa flavanols on insulin signalling and glucose metabolism in hepatic cells in physiological conditions and during insulin resistance situation and diabetes

Treatment of HepG2 cells during 24 h with different concentrations of epicatechin (EC, 1-10 µM) or a cocoa polyphenolic extract (CPE, 1-10 µg/mL) did not evoke changes in cell viability and proliferation. Both substances activated insulin signalling pathway as increased total and phosphorylated IR, IRS-1 and IRS-2 levels, as well as phosphorylated AKT and GSK3 values, and decreased p-GS levels. All EC and CPE concentrations tested enhanced phosphorylated AMPK levels, and just CPE (10 µg/mL) increased GLUT2 levels. EC and CPE modulated PEPCK expression, leading to a diminished glucose production. Moreover, inhibition of gluconeogenesis induced by EC and CPE was prevented by AKT and AMPK blockage in hepatic cells **(Paper 1).**

During insulin resistance cocoa rich diet in ZDF rats and EC and CPE preincubation of insulin-resistant HepG2 cells (induced by adding 30mM glucose for 24 h) prevented the blockage of the insulin signalling pathway on both experimental models (ZDF rat's liver and HepG2 cells). EC and CPE prevented IR, IRS-1 and -2 inhibition, and PI3K/AKT and AMPK pathways downregulation. Moreover, cocoa-rich diet, EC and CPE preserved hepatocyte functionality, as prevented the enhancement in PEPCK levels, and restored glycogen content and GLUT2 values to similar levels to those of controls. In this regard, in HepG2 cells CPE and EC prevented the diminished glucose uptake and the increased glucose production evoked by the high glucose concentration. Cocoa-rich diet increased GK levels in ZDF rat's liver and inhibited JNK and p38-MAPK activation induced by insulin resistance situation. **(Papers 2 and 3).**

Chapter 2: Effect of cocoa flavanols on hepatic oxidative stress during insulin resistance and diabetes.

EC and CPE pretreatment and cocoa-rich diet supplementation protected HepG2 cells and ZDF rats against oxidative stress, respectively. All treatments decreased ROS generation and prevented the enhancement of carbonyl group levels, showing similar levels to those of control condition in both experimental models. EC and CPE avoided the decrease of GSH and the alteration of GPx activity induced by the high-glucose-challenge in HepG2 cells, and CPE restored GR activity to control values. However, EC and CPE were not able to revert the high-glucose induced GST decrease. In ZDF rats, cocoa-rich diet restored SOD and HO-1 altered activities, although GST activity remained reduced. CAT activity in HepG2 cells, as well as GPx, GR, CAT activity and GSH levels in ZDF rat's liver were not modified by any treatment. Moreover, cocoa-rich diet abolished Nrf2 and NF-κB levels enhancement in ZDF rat's liver. Additionally, EC and CPE alone activated ERK and Nrf2 in HepG2 cells, which was maintained in EC- and CPE-pre-treated cells, whereas both substances prevented p38 and JNK activation-induced by a high dose of glucose. MAPKs specific inhibitors induced changes in the redox status, glucose uptake, and total and serine phosphorylated levels of IRS-1during insulin resistance, suggesting that CPE beneficial effects seem to be mediated at least in part by its ability to target MAPKs. **(Papers 4 and 5).**

Chapter 3: Effect of cocoa flavanols on hepatic lipid metabolism during insulin resistance and diabetes.

Body weight was reduced by cocoa-rich diet in ZDF rats without modifying total food intake. Moreover, cocoa-rich diet improved serum and hepatic lipid profile in ZDF rats, as it decreased triglycerides (TG), LDL cholesterol (LDL-Cho), non-esterified fatty acid (NEFA) and lipid drops levels, and increased HDL cholesterol (HDL-Cho) levels. Similarly, EC restored the altered lipid values (TG, total cholesterol and NEFA) to control levels in HepG2 cells exposed to high glucose concentration. Cocoa and EC also prevented the decrease in the phosphorylated levels of AKT, AMPK and PPARα, as well as the enhanced p-PKCζ, SREBP1-c and FAS values, which could be associated with lipolysis stimulation and lipogenesis inhibition. PKCζ regulation induced by EC contributed to improve the lipid metabolism through AKT and AMPK. **(Paper 6).**

Conclusions.

According to the aims proposed and the results obtained, the following conclusions may be drawn:

1. Treatment of HepG2 cells with realistic concentrations of epicatechin (1-10 µM) and a cocoa polyphenolic extract (1-10 µg/mL) exerts an insulin-like activity that contributes to strengthen the hormone pathway. This effect was associated to the activation of insulin receptor, insulin substrates (IRS-1 and -2), PI3K/AKT and AMPK pathways and the inhibition of glucose production.

   Pretreatment of insulin-resistant HepG2 cells with epicatechin (10 µM) or cocoa polyphenolic extract (1 µg/mL), as well as administration of a cocoa-rich diet to ZDF rats improve glucose homeostasis and hepatic insulin sensitivity. Cocoa and EC could prevent or ameliorate the hepatic dysfunction associated with the insulin signalling blockage that occur during insulin resistance and diabetes by preventing inhibition of IR, IRS-1 y -2, PI3K/AKT and AMPK pathways. All treatments contribute to maintain hepatic functionality as they avoided the increase of hepatic PEPCK values and restored glycogen content and GLUT2 levels. Moreover, epicatechin and the cocoa polyphenolic extract prevented the diminution of glucose uptake and the enhanced glucose production induced by the high glucose concentration in HepG2 cells. In addition, cocoa- rich diet increased GK levels and suppressed hepatic JNK and p38-MAPK activation in the liver of ZDF rats.

2. Pretreatment of insulin-resistant HepG2 cells with epicatechin (10 µM) or cocoa polyphenolic extract (1 µg/mL), as well as the administration of a cocoa-rich diet in ZDF rats protected against the oxidative stress. This protective effect was related to a reinforcement of the antioxidant defences (GPx y GR activities in insulin-resistant HepG2 cells and SOD y HO-1 activities in ZDF rats), and to the modulation of relevant close-related redox transcriptional factors (Nrf2 and NF-κB). Besides, epicatechin and the cocoa

polyphenolic extract prevented p38 and JNK activation in insulin resistance HepG2 cells, suggesting that the cocoa extract beneficial effects seem to be mediated at least in part by its ability to target MAPKs.

3. Epicatechin (10 μM) pretreatment of insulin resistant HepG2 cells and cocoa-rich diet administration to ZDF rats alleviated the hyperlipidemia and hepatic steatosis through the modulation of multiple signalling pathways. Cocoa- and epicatechin-treatment decreased hepatic lipid levels, which was associated to the inhibition of *de novo* key lipogenic proteins (SREBP1-c and FAS) and the activation of fatty acid oxidation (PPARα). Likewise, cocoa- and EC-induced changes in hepatic lipid content were regulated by AMPK, AKT and PKCζ. Indeed, EC-induced AKT and AMPK regulation seemed to be mediated by PKCζ in HepG2 cells.

General conclusion.

In summary, the results obtained in cell culture and experimental animals demonstrate that cocoa flavanols might modulate glucose and lipid metabolism in hepatic cells, which leads to an improved insulin sensitivity and glucose tolerance during insulin resistance and diabetes. Moreover, cocoa flavanols seem to be able to protect the hepatocyte antioxidant capacity to maintain the redox homeostasis against the oxidative stress that occurs during insulin resistance and diabetes.

Abstract

Introducción

1. Compuestos fenólicos.

1.1. Clasificación y estructura de los compuestos fenólicos de la dieta.

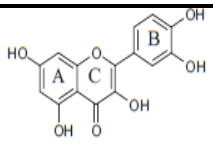
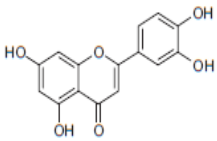
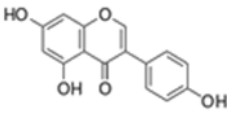
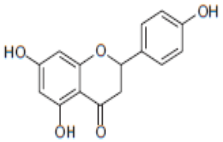
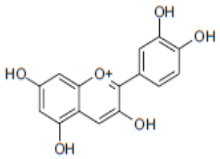
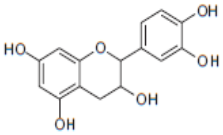
Los compuestos fenólicos están presentes de manera habitual en la dieta, ya que se encuentran en abundancia en las plantas (frutas, verduras, semillas) y en sus derivados [1]. Se caracterizan por tener al menos un anillo aromático con uno o más grupos hidroxilo en el anillo de benceno [2, 3]. La mayoría de los compuestos fenólicos proceden del aminoácido fenilalanina y se forman a través de la ruta metabólica de los fenilpropanoides, que está presente en casi todas las plantas [4]. Los compuestos fenólicos son metabolitos secundarios no esenciales para el crecimiento y desarrollo satisfactorio de la mayoría de las plantas, si bien realizan funciones importantes para la supervivencia de las mismas como la protección frente a agresiones externas (luz ultravioleta, herbívoros e infección de microorganismos), favorecen la atracción de polinizadores y animales que dispersan las semillas, y actúan como agentes alelopáticos y como señales moleculares en la formación de nódulos fijadores de nitrógeno en las raíces [2].

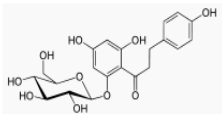
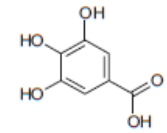
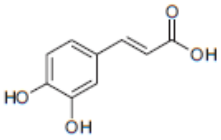
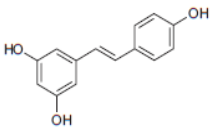
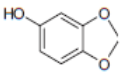
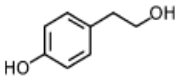
Los compuestos fenólicos se pueden clasificar según su fuente de origen, función biológica o estructura química. La clasificación estructural divide los compuestos fenólicos en diferentes clases según el número de anillos que contengan y los sustituyentes que lleven unidos [3]. De acuerdo con esta clasificación, los principales grupos de compuestos fenólicos de la dieta son: flavonoides, ácidos fenólicos, alcoholes fenólicos, estilbenos y lignanos (Tabla 1). En las plantas, la mayoría de estos compuestos fenólicos se encuentran glicosilados o unidos a azúcares acetilados en diferentes posiciones del esqueleto polifenólico, lo que les confiere diferentes actividades biológicas. No obstante, para simplificar, la clasificación se ha hecho de acuerdo a la estructura de las agliconas [5].

1.1.1. Flavonoides.

Los flavonoides tienen un segmento central de 15 átomos de carbono que consta de dos anillos de benceno unidos por un puente de 3 carbonos (C6-C3-C6) para formar un anillo heterocíclico [3]. Los flavonoides se subdividen dependiendo del estado de oxidación del anillo central en flavonoles, flavonas, isoflavonas, flavanonas, antocianidinas, flavanoles, chalconas y dihidrochalconas (Tabla 1).

***Tabla 1**: Clasificación de los compuestos fenólicos según su estructura química, compuestos representativos de cada grupo y alimentos donde se encuentran mayoritariamente.*

Clase	Subclase	Compuestos representativos	Estructura	Alimento
Flavonoides	Flavonoles	Quercetina Kaempferol Miricetina	 Quercetina	Cebolla Col rizada Brócoli Arándano Té Vino tinto Manzana
	Flavonas	Luteolina Apigenina	 Luteolina	Apio Perejil Té rooibos
	Isoflavonas	Genisteína Daidzeína Gliciteína	 Genisteína	Soja Tofu
	Flavanonas	Hesperidina Neohesperidina Naringenina	 Narangenina	Naranja amarga Pomelo Tomate
	Antocianidinas	Malvidina Cianidina Pelargonidina	 Cianidina	Frutos rojos Repollo Judías Cereales Vino tinto
	Flavanoles	Catequina Epicatequina Epigalocatequina Teaflavinas	 Catequina	Cacao Chocolate Té Uvas

		Epigalocatequina -3-galato Procianidina B2		Vino tinto
	Chalconas y dihidrochalconas	Floridzina Aspalatina Notofagina	 Florizidina	Manzana Té rooibos
Ácidos fenólicos	Ácidos hidroxibenzoicos	Ácido gálico Ácido elágico Ácido protocatéquico	 Ácido gálico	Mora Frambuesa Fresa Nuez Avellana
	Ácidos hidroxicinámicos	Ácido cafeico Ácido ferúlico Ácido caftárico	 Ácido cafeíco	Kiwi Café verde Uva Vino
Estilbenos		Resveratrol	 Resveratrol	Uva Vino tinto
Lignanos		Sesamol Secoisolaricire-sinol	 Sesamol	Linaza Sésamo
Alcoholes fenólicos		Tirosol Hidroxitirosol	 Tirosol	Aceite de oliva Vino Cerveza

Los flavonoles son el grupo de flavonoides más abundantes de la dieta, ya que se encuentran de forma ubicua en las plantas. Se caracterizan por tener un doble enlace entre C2 y C3 con un grupo hidroxilo en la posición C3. Los flavonoles más comunes son quercetina, kaempferol y miricetina, y entre los alimentos donde son más abundantes cabe citar la cebolla amarilla y roja, col rizada, puerro, brócoli, tomate,

manzana y arándano [3, 6], y todos los tipos de mieles [7]. También contienen flavonoles algunas bebidas como el té verde, el té negro y el vino tinto [3, 6].

La estructura química de las flavonas es muy similar a la de los flavonoles, y se caracteriza por tener un doble enlace entre C2 y C3. Los compuestos más representativos del grupo son apigenina, luteolina, wogonina y baicaleína. Son los flavonoides menos distribuidos en las plantas, aunque se encuentran en grandes cantidades en el apio y el perejil [2, 3]. Además se han encontrado flavonas polimetoxiladas en la piel de cítricos como la mandarina, y también pequeñas cantidades de las formas glucoconjugadas de apigenina y luteolina en las hojas del arbusto sudafricano *Aspalathus linearis*, que se emplea para hacer el té rooibos [2, 3].

Las isoflavonas se caracterizan por tener el anillo B unido a C3. Tienen una estructura muy similar al estradiol y pueden unirse al receptor de estrógenos, por lo que también reciben el nombre de fitoestrógenos. La genisteína, daidzeína y gliciteína son los compuestos más representativos del grupo [3, 5]. Las isoflavonas están presentes casi de manera exclusiva en las plantas leguminosas, como en la soja, donde se encuentran grandes cantidades de daidzeína y genisteína en su forma libre y glicosilada [3, 5].

Las flavanonas se caracterizan por la ausencia de doble enlace entre el C2 y C3 y por tener un centro quiral en C2. En las plantas suele estar presente el enantiómero con configuración α. Se encuentran en los cítricos, el tomate y también en algunas hierbas aromáticas como la menta [2, 3]. El glucósido más abundante es la hesperidina, que carece de sabor, mientras que los glucósidos neohesperidina y naringina, presentes en la naranja amarga y el pomelo respectivamente, poseen un intenso sabor amargo característico de estos frutos [2, 3].

Las antocianidinas son las responsables del color de la mayoría de los frutos, las flores y otros tejidos de las plantas. La gama de colores va desde el naranja y el rojo hasta al azul y el morado, y la cantidad de antocianidinas suele ser proporcional a la intensidad del color [3]. En este grupo se podrían destacar la pelargonidina, cianidina, delfinidina, peonidina, petunidina y malvidina. Los antocianos, los glucósidos de las antocianidinas, son especialmente abundantes en los frutos rojos como los arándanos, las grosellas, las fresas, las moras y las cerezas [6], pero también están presentes en

verduras como el repollo, las judías, las cebollas y el rábano, y en algunos tipos de cereales, así como en el vino tinto [3].

Los flavanoles son el grupo más complejo dentro de los flavonoides, ya que se presentan como estructuras monoméricas, oligómeros y polímeros complejos conocidos como proantocianidinas o taninos condensados [5]. Al contrario que otros flavonoides, las formas glicosiladas no se encuentran en los alimentos [3], y se caracterizan por poseer dos centros quirales en C2 y C3, lo que hace que un flavanol monomérico tenga cuatro isómeros, dos de los cuales (+)-catequina y (-)-epicatequina (EC) son los más abundantes en los alimentos junto a sus derivados (-)-epigalocatequina, (-)-epigalocatequina-3-*O*-galato (EGCG) y epicatequina-3-*O*-galato [2]. Las proantocianidinas son responsables del carácter astringente de los alimentos y pueden formar polímeros de hasta 50 unidades. Las proantocianidinas constituidas exclusivamente por (epi)catequina se llaman procianidinas y son las más abundantes en los alimentos [2]. Los flavanoles monoméricos y proantocianidinas están muy extendidos en frutas y verduras, principalmente en el té verde, el cacao y el chocolate negro, la manzana, el albaricoque, la pera, la uva, la cereza, el arándano, la avellana, el vino tinto y blanco y la sidra [3, 6, 8].

Las chalconas y dihidrochalconas se caracterizan por tener el anillo C abierto. Son un grupo minoritario de flavonoides, y sus principales representantes son floridzina, aspalatina y notofagina, que se encuentran en la manzana, el té rooibos, el lúpulo y la cerveza [2, 5].

1.1.2. Ácidos fenólicos.

Tienen estructura C1-C6 y son los fenoles no flavonoides más abundantes y extendidos de la dieta [6]. Se dividen en dos subgrupos: los derivados del ácido benzoico y los derivados del ácido cinámico.

En cuanto a los ácidos hidroxibenzoicos destacan el ácido gálico, el ácido elágico y sus derivados, los elagitaninos y el ácido protocatéquico. Están presentes en el té y en frutas como las moras, las frambuesas, las fresas, la granada y el caqui, así como en las nueces, las avellanas y en los vinos madurados en barriles con madera de roble [2, 3, 6]. Sin embargo, la concentración de ácido protocatéquico puede ser mayor *in vivo*, ya que es el principal metabolito de los antocianos [3].

Los ácidos hidroxicinámicos suelen encontrase glicosilados o en forma de ésteres en los alimentos. Destacan el ácido cafeico, el ácido ferúlico y el ácido caftárico, siendo muy abundantes en el kiwi, los cereales, las uvas blancas y rojas y en los vinos blancos y tintos [2]. Los conjugados con el ácido tartárico y quínico se conocen como ácidos clorogénicos, y son muy abundantes en el café [2], las manzanas, el té y los frutos rojos, especialmente los arándanos [6].

1.1.3. Alcoholes fenólicos.

En este grupo destacan el tirosol y el hidroxitirosol, presentes en el aceite de oliva [3]. El tirosol también se encuentra en el vino blanco y la cerveza, y el hidroxitirosol está presente en el vino tinto [3].

1.1.4. Estilbenos.

Tienen una estructura C6-C2-C6 y el estilbeno mayoritario es el resveratrol, presente en las uvas rojas, el vino y el zumo de uva [3].

1.1.5. Lignanos.

Se forman por la dimerización de dos unidades de fenolpiruvato, y al igual que las isoflavonas, son fitoestrógenos, ya que su estructura química es similar a la de la hormona estradiol [2]. Los compuestos más abundantes son el sesamol y el secoisolariciresinol cuyas principales fuentes en la dieta son la linaza y el sésamo [3, 5].

1.2. Ingesta, biodisponibilidad y metabolismo de los compuestos fenólicos.

En la actualidad, la ingesta diaria de polifenoles se estima principalmente a través de cuestionarios de frecuencia de consumo de alimentos, lo que a su vez refleja las preferencias y hábitos de la población. Así, se ha estimado que la ingesta de compuestos fenólicos *per capita* en Estados Unidos es alrededor de 1-1,1 g/día [8] y en Europa de 1 g/día [9]. De manera más concreta, en Finlandia la ingesta diaria de compuestos fenólicos es de 0,8 ± 0,4 g/día [8], en Alemania oscila entre 0,1 y 2 g/día [9] y en España entre 2,6 y 3,0 g/día [10]. Además, se ha de destacar que de todos los

compuestos fenólicos ingeridos, los ácidos fenólicos representan el 50-75% y los flavonoides el 25-50% [11]. De esta manera, en España sólo el consumo de flavonoides oscila entre 0,27 y 0,31 y mg/día [12].

El posible impacto beneficioso de estos compuestos en el organismo está condicionado por la cantidad y la biodisponibilidad de los compuestos ingeridos. La biodisponibilidad se define como la fracción de nutriente o no nutriente que llega al cuerpo humano para desempeñar funciones fisiológicas o para su almacenamiento [1, 8]. Así pues, la biodisponibilidad incluye el metabolismo, la absorción y el transporte de los nutrientes o no nutrientes hasta los distintos órganos. Además, existen otros factores que van a determinar la biodisponibilidad de los compuestos fenólicos, como son la matriz alimentaria donde se encuentran, el procesado del alimento (fritura, cocción, etc.), los procesos digestivos (saliva, enzimas digestivas) y la variabilidad individual (edad, sexo, microflora intestinal, etc.) [1].

La digestión comienza en la cavidad oral donde se liberan de la matriz alimentaria algunos fenoles (principalmente flavanoles) (Fig. 1) [1]. Dichos compuestos fenólicos forman agregados con algunas proteínas de la saliva como la prolina, aunque este hecho no altera su biodisponibilidad. Además, dado el poco tiempo de exposición, se asume que el impacto de dicha digestión sobre la biodisponibilidad es muy bajo [1, 8].

La digestión continúa en el estómago, donde se liberan el resto de los fenoles de la matriz alimentaria. Algunos estudios indican que ciertos polifenoles, como la malvidina-3-glucósido y los ácidos fenólicos, pueden absorberse en el estómago y aparecer rápidamente en la sangre [1, 8], si bien, en términos generales, los polifenoles continúan hasta el intestino delgado donde se absorberán [1].

En el intestino delgado, las enzimas β-glucosidasas, como la lactasa floridzina hidrolasa (LPH) y β-glucosidasa citosólica (CBG), eliminan los residuos de azúcares y aumentan la lipofilia y solubilidad de los polifenoles. Así, la enzima LPH, que se encuentra en el borde en cepillo de los enterocitos, hidroliza el enlace con el azúcar y deja libre la aglicona que atraviesa el enterocito por difusión facilitada. Esta es la vía de absorción más común para los compuestos fenólicos de bajo peso molecular, como los ácidos fenólicos y algunas agliconas como las del té y el cacao (EC y catequina) [8]. Sin embargo, en algunos casos, los glucósidos polares son transportados al interior del

enterocito por transporte activo mediante el transportador acoplado a sodio-glucosa y el trasportador de ácido monocarboxílico, y una vez en el interior de la célula, la enzima CBG hidroliza el residuo de azúcar [1, 8]. En el enterocito también comienzan las reacciones de metabolismo de fase II: glucuronidación, metilación y sulfatación por las enzimas uridinadifosfato-glucuronosiltranferasas, catecol-O-metiltransferasas y sulfotransferasas, respectivamente [1, 2, 8]. A continuación, los metabolitos pasan al torrente sanguíneo, aunque en algunos casos se produce la vuelta al lumen del intestino de algunos compuestos a través de los transportadores de la familia ABC (del inglés ATP binding cassette) [2].

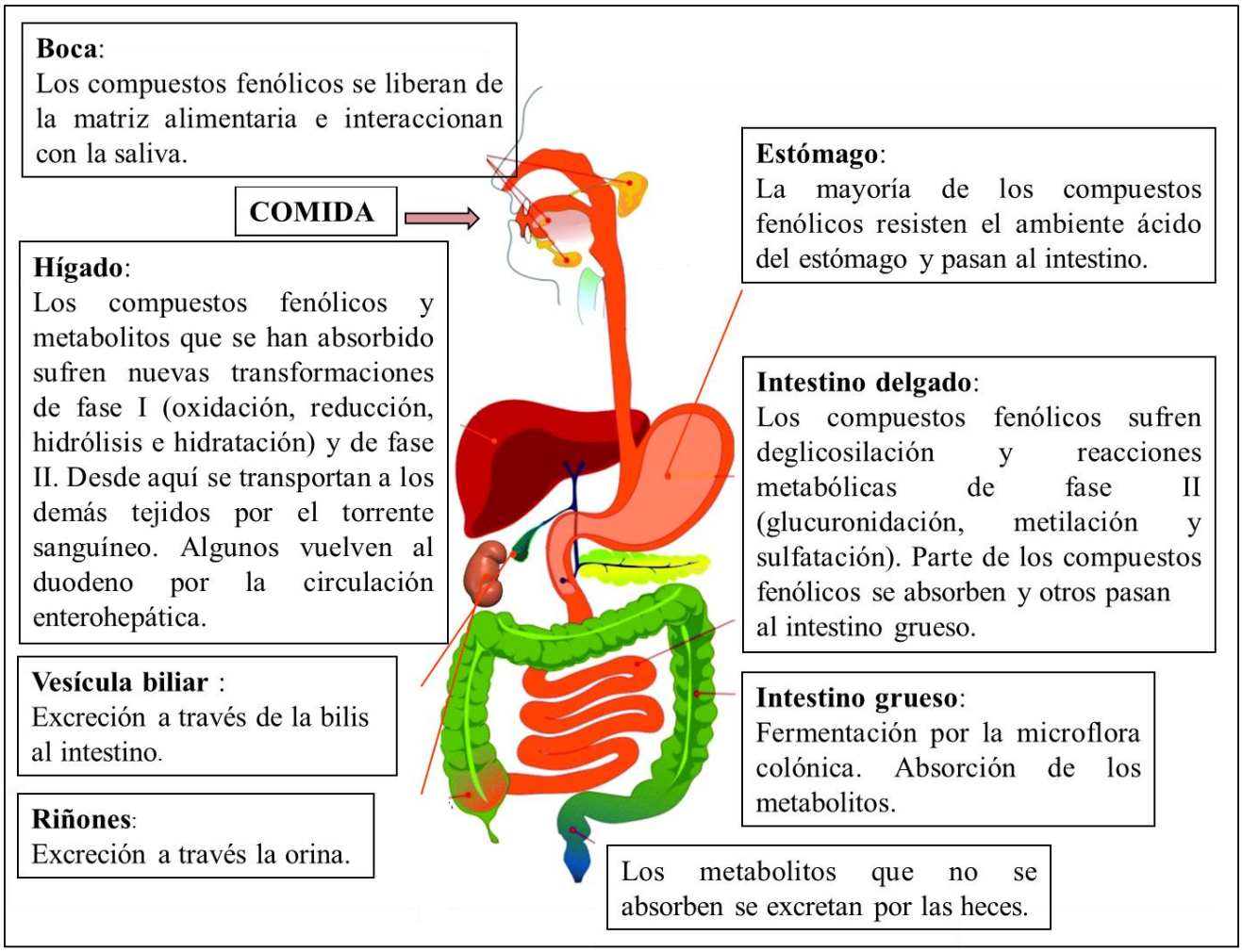


Figura 1: *Biodisponibilidad y metabolismo de los compuestos fenólicos*.

En el torrente sanguíneo, los metabolitos y los compuestos fenólicos se transportan de forma libre, unidos a lipoproteínas, o mayoritariamente a albúmina, y rápidamente llegan al hígado a través de la vena porta. En el hígado, los compuestos

fenólicos y sus metabolitos son sometidos a nuevas reacciones de conjugación y, en menor medida, a reacciones catalizadas por enzimas de fase I (oxidación, reducción, hidrólisis e hidratación) [1]. Posteriormente, desde el hígado son transportados por el torrente sanguíneo a los demás tejidos, incluyendo el cerebro, ya que algunos compuestos fenólicos son capaces de atravesar la barrera hematoencefálica [8].

La concentración de polifenoles en plasma es del orden nM-µM, y la concentración en los diferentes tejidos varía según el tejido y el compuesto [13]. La mayoría de los metabolitos se absorben conjugados y así llegan a los tejidos, aunque también se absorbe un porcentaje como aglicona libre [8]. Además, Perez-Vizcaíno y colaboradores recientemente han mostrado que dentro del tejido algunos polifenoles entran en un ciclo de conjugación-deconjugación, que es necesario para la actividad de compuesto, y por el cual los metabolitos se transforman de nuevo en la aglicona en el tejido diana, siendo ésta última el efector final [14].

Los polifenoles se excretan en la orina a través de los riñones, y a través de la bilis, siendo este último el mecanismo de excreción más común para los polifenoles apolares, como la genisteína.

El resto del total de los polifenoles ingeridos (95%) no se absorbe fácilmente en el intestino delgado, y se consideran no biodisponibles, como la procianidina B2 del cacao, que se absorbe en torno a un 1% frente al 45% de absorción del monómero de EC [8], y se pueden acumular en el intestino grueso donde, junto con los conjugados excretados al intestino a través de la bilis, son fermentados por la microflora intestinal [15]. Los metabolitos microbianos que se absorben aparecen como derivados conjugados en el plasma, y los remanentes que no se absorben se eliminan por las heces. No obstante, una vez absorbidos y metabolizados, los polifenoles pueden volver al duodeno a través de la circulación enterohepática, prolongando su presencia en el organismo [2].

1.3. Actividad biológica de los compuestos fenólicos.

Numerosos estudios epidemiológicos muestran una relación entre el consumo de una dieta rica en frutas y verduras, alimentos ricos en compuestos fenólicos, y una reducción del riesgo de padecer enfermedades crónicas como el cáncer, enfermedades

neurodegenerativas, enfermedad cardiovascular y diabetes mellitus, así como un retraso en la aparición del envejecimiento [16, 17]. Durante muchos años se ha pensado que los efectos beneficiosos para la salud de los polifenoles se debían a su actividad antioxidante; Sin embargo, hoy en día esta parece una visión muy simplificada de su modo de acción. Actualmente se sabe que las células responden a los efectos de los polifenoles principalmente a través de las interacciones directas con receptores o con proteínas que intervienen en la transducción de señales. De esta manera, los polifenoles modulan estas vías de señales desencadenando toda una serie de efectos beneficiosos, por lo que en los últimos años ha aumentado el interés por estos compuestos naturales y por su posible papel beneficioso en la prevención y tratamiento de enfermedades. De hecho, los compuestos naturales se han utilizado como fuente de nuevos fármacos desde hace años, y aproximadamente el 50% de los fármacos aprobados por la Administración de Alimentos y Medicamentos (FDA) proceden de fitoquímicos o de sus derivados [18].

1.3.1. Actividad antioxidante.

Los compuestos fenólicos son agentes reductores capaces de retrasar o prevenir la oxidación de otras moléculas como el ácido desoxirribonucleico (ADN), las lipoproteínas de baja densidad (LDL-Cho) y las membranas biológicas, entre otros [19]. Ejercen su poder antioxidante de diferentes maneras: neutralizan radicales libres como las especies reactivas de oxígeno (ROS) y de nitrógeno (RNS), quelan metales y/o estimulan los sistemas de defensa endógena, como enzimas antioxidantes/detoxificantes y factores de transcripción [2]. La eficacia de los polifenoles como antioxidantes depende de su estructura química, y se considera que, dentro de los polifenoles, los flavonoides son los antioxidantes más potentes [16].

1.3.2. Actividad antiinflamatoria.

Los compuestos fenólicos ejercen efectos antiinflamatorios *in vitro* e *in vivo*, ya que son capaces de actuar sobre la vía de señalización del factor nuclear kappa B (NF-κB) y de las proteínas quinasas activadas por mitógenos (MAPK) para modular la expresión de genes proinflamatorios como ciclooxigenasa, lipooxigenasa, óxido nítrico sintetasa [16, 17, 20]. Los polifenoles son capaces también de interferir y disminuir la producción de citoquinas y quimioquinas en situaciones de inflamación [16, 17, 20].

1.3.3. Actividad anticancerígena.

El efecto anticancerígeno de los polifenoles se ha demostrado sobre una gran variedad de tipos de cáncer (boca, estómago, duodeno, colon, hígado, pulmón, mama, piel, etc.) [16, 19]. Además, distintos estudios clínicos han puesto de manifiesto la existencia de una correlación inversa entre la ingesta de polifenoles y el riesgo de desarrollar distintos tipos de cáncer o su reaparición [2, 19].

Los compuestos fenólicos pueden interferir en las distintas etapas del desarrollo del cáncer, actuando sobre el inicio, el desarrollo y el progreso (angiogénesis y metástasis) de la enfermedad [19]. Este efecto anticancerígeno se puede explicar por la actividad antioxidante de estos compuestos fenólicos, que puede prevenir la oxidación del ADN y activar la maquinaria antioxidante de la célula, pero también por la modulación de diferentes vías de señalización celular relacionadas con la apoptosis, el ciclo celular, proliferación-supervivencia e inflamación como son las rutas del NF-κB, MAPK y fosfatidilinositol-3 quinasa (PI3K) y la proteína quinasa B (PKB o AKT), entre otras [19].

1.3.4. Actividad en enfermedad cardiovascular.

Diversos estudios han demostrado el efecto positivo de los compuestos fenólicos, principalmente los flavonoides, sobre la enfermedad cardiovascular [6, 16, 21]. Los compuestos fenólicos favorecen la vasodilatación, lo que reduce la hipertensión, disminuyen la oxidación de las LDL y protegen frente a la disfunción endotelial y la formación de la placa de ateroma [2]. Además, regulan el metabolismo lipídico, aumentan los niveles de las lipoproteínas de alta densidad (HDL-Cho) y disminuyen los triglicéridos (TG) [2] y la activación plaquetaria, lo que reduce el riesgo de trombosis [2, 16].

1.3.5. Actividad en enfermedades neurodegenerativas.

El cerebro es especialmente sensible al estrés oxidativo. Estudios preclínicos y epidemiológicos sugieren que los polifenoles podrían ayudar a mejorar la patología neurodegenerativa y el empeoramiento cognitivo asociado a la edad [2, 22]. En este sentido, los fenoles mejoran distintos aspectos relacionados con la memoria y el aprendizaje, como la adquisición de memoria de corto y largo plazo, la memoria de

trabajo y la memoria de retención [22]. Igualmente, los compuestos fenólicos ejercen efectos positivos sobre la actividad psicomotora de animales viejos, por lo que se podría relacionar con un potencial beneficio en la prevención de enfermedades neurodegenerativas como el Alzheimer y el Parkinson [23]. Además, los compuestos fenólicos favorecerían la salud neurológica a través de diferentes mecanismos para reducir el daño y la pérdida neuronal inducida por neurotoxinas y la neuroinflamación, así como mediante la interacción con las vías de señalización de las neuronas y de la glía, y la regulación del riego sanguíneo cerebrovascular y periférico [2, 22].

1.3.6. Actividad antidiabética.

En los últimos años se ha sugerido que los polifenoles pueden mejorar la hiperglucemia en ayunas y postprandial, características de la diabetes tipo 2, mediante la inhibición de las enzimas disacaridasas del intestino (α-amilasa y α-glucosidasa) [24]; este hecho limitaría la digestión de los polisacáridos de la dieta y, por tanto, reduciría la absorción de los azúcares simples [13, 24]. Además, los compuestos fenólicos son capaces de actuar sobre tejidos clave en el desarrollo de la diabetes y la resistencia a la insulina como el páncreas, músculo esquelético, hígado y tejido adiposo [24, 25]. De esta manera, los compuestos fenólicos protegen a las células beta pancreáticas frente a la glucotoxicidad, mejoran la captación de glucosa en el músculo y los adipocitos, incrementan el almacenaje de la glucosa en forma de glucógeno (glucogénesis) y disminuyen la producción de glucosa en el hígado [13, 24, 25].

1.3.7. Actividad antiobesidad.

Diferentes estudios han mostrado que los polifenoles pueden incrementar la termogénesis y el gasto de energía, a la vez que disminuyen la inflamación y el estrés oxidativo, lo que puede resultar en pérdida de peso y/o mejora de los desórdenes metabólicos [26, 27]. Los efectos antiobesidad de los alimentos ricos en polifenoles se han relacionado con la interacción directa o indirecta con el tejido adiposo (preadipocitos, células madre adiposas y células del sistema inmune) para inhibir la diferenciación de los preadipocitos y disminuir la proliferación [27]. Además, los compuestos fenólicos modulan el metabolismo lipídico para favorecer la beta oxidación de los ácidos grasos e inhibir la lipogénesis [27].

1.3.8. Otras actividades biológicas.

Los compuestos fenólicos han demostrado tener actividad antimicrobiana, ya que limitan la actividad de bacterias, hongos y virus. Así, suprimen factores de virulencia como la adhesión al hospedador, las toxinas bacterianas, el crecimiento de patógenos (efecto barrera) e inducen cambios en la actividad de los linfocitos [13, 16, 28]. Además, los polifenoles muestran un efecto sinérgico con los antibióticos y potencian su eficacia e incrementan la actividad de los fungicidas, por lo que estos compuestos naturales podrían ser usados para desarrollar nuevas terapias para tratar infecciones resistentes a los antibióticos tradicionales [17]. De manera adicional, los polifenoles ejercen un efecto prebiótico o estimulador del crecimiento y/o la actividad de ciertas bacterias de la flora intestinal. Así, el consumo de polifenoles puede modificar la ecología microbiana para favorecer el crecimiento de la población de especies de los géneros *Lactobacillus* y *Bifidobacterium* e inhibir el de las bacterias *Bacteroidaceae,* lo que influye en la salud del hospedador [15].

Los compuestos fenólicos parecen jugar un papel inmunomodulador para disminuir la respuesta alérgica, ya que afectan a las dos fases críticas de dicha respuesta (sensibilización a un alérgeno y nueva exposición a dicho alérgeno) [28, 29]. De esta manera, los polifenoles forman complejos insolubles con las proteínas alergénicas, actúan sobre las células dendríticas para inhibir la producción de citoquinas o la presentación del antígeno y la producción de inmunoglobulinas E [28, 29].

2. Diabetes mellitus.

2.1. Generalidades.

La incidencia global de la diabetes ha aumentado exponencialmente en los últimos años y ha alcanzado niveles epidémicos. Se prevé que en 2030 el número de personas afectadas en el mundo por esta enfermedad sea de unos 439 millones, lo que supone una gran carga socioeconómica y médica [30, 31].

La diabetes es un conjunto de alteraciones metabólicas complejas que se caracteriza por una hiperglucemia prolongada. De manera general, se distinguen cuatro

tipos de diabetes: diabetes mellitus tipo 1 (DMT1), diabetes mellitus tipo 2 (DMT2), diabetes gestacional y diabetes tipo MODY (Maturity Onset Diabetes of the Young).

La DMT1, o insulinodependiente, se produce como consecuencia de la destrucción autoinmune de las células β pancreáticas y suele manifestarse en la infancia o adolescencia [32]. Su aparición parece estar relacionada con una predisposición genética y con factores ambientales que aún no están muy bien definidos [32]. La diabetes idiopática es una forma de DMT1 no autoinmune poco común que se ha encontrado en algunas poblaciones africanas y asiáticas. Estos enfermos no producen insulina y tienen tendencia a padecer cetoacidosis, aunque su etiología es desconocida [32].

La DMT2 se produce como consecuencia de un fallo en la secreción de la insulina por parte de las células β, la aparición de resistencia a la insulina en los tejidos periféricos, o de una combinación de ambos factores [32]. El porcentaje de enfermos afectados por la DMT2 supone el 90-95% del total de los diabéticos frente a 5-10% que representa los diabéticos tipo 1 [32].

La aparición de la DMT2 está asociada a factores genéticos, estilo de vida y factores ambientales y socioeconómicos [33]. La contribución genética parece predisponer a la DMT2, ya que los individuos que tienen un familiar de primer grado con DMT2 presentan de 2 a 4 veces más probabilidades de desarrollar la enfermedad a lo largo de su vida [30, 34]. Así pues, en las últimas décadas se ha intentado identificar genes candidatos como factores causantes de la DMT2, ya que podrían ser de gran utilidad para reconocer personas "de alto riesgo" y para encontrar nuevas dianas terapéuticas. Entre los genes propuestos se encuentran los del sustrato del receptor de la insulina 1 y 2 (*irs1* e *irs2)*, el receptor β-adrenérgico (*adrb2* y *adrb3*), las proteínas desacoplantes 1 y 3 (*ucp1* y *ucp3*), el receptor activado por proliferación de peroxisomas γ (*pparγ*), las proteínas de unión de ácidos grasos, las apolipoproteínas y la proteína lipasa [30, 35]. No obstante, parece claro también que una mutación en un gen no sería la única causa de la aparición de la enfermedad, y en este sentido se le atribuye una gran responsabilidad e importancia a la relación entre genes y factores ambientales/nutricionales [36]. Así, los factores ambientales y el estilo de vida, la obesidad, el sedentarismo y la dieta juegan un papel clave en la aparición y desarrollo

de la DMT2 [31]. Otra condición relacionada con la aparición de la DMT2 es el exceso o la falta de nutrientes en el ambiente intrauterino [33].

La diabetes gestacional se define como la intolerancia a la glucosa que se desarrolla, o que se detecta por primera vez, durante el embarazo, y que en la mayoría de los casos vuelve a la normalidad tras el parto. Esta complicación del embarazo aumenta la morbilidad materna y de la descendencia durante el parto, y predispone a las mujeres para desarrollar DMT2 después del embarazo.

La diabetes tipo MODY se asocia con defectos en la función de la célula β y suele aparecer antes de los 25 años. Es una enfermedad heredada y normalmente asociada con mutaciones en el factor nuclear hepático 1-α o en la glucoquinasa hepática (GK) [32]. Se caracteriza por un fallo en la secreción de insulina sin defecto en la acción de la hormona.

2.2. Patogénesis.

La DMT2 es una enfermedad subdiagnosticada que plantea desafíos para su tratamiento. La introducción de nuevos fármacos orales en los últimos años ha ampliado la gama de opciones disponibles para su tratamiento. Sin embargo, y a pesar de la mayor selección de agentes farmacológicos, especialmente hipoglucemiantes (biguanidinas, tiazolidinedionas, etc.), es necesario destacar que el tratamiento de primera elección son los enfoques no farmacológicos, incluyendo la modificación de la dieta, control de peso y ejercicio regular [33]. Además, los fármacos disponibles en la actualidad no son capaces de mantener el control glucémico a largo plazo, y su consumo prolongado produce efectos secundarios, lo que hace que sea importante seguir trabajando en la prevención y control de la diabetes. Por todo ello, hoy día el tratamiento que se considera más eficaz para prevenir y frenar el desarrollo de esta enfermedad a un menor coste es la actuación a nivel nutricional mediante la adecuación de las recomendaciones dietéticas.

En la DMT2, de manera previa a la manifestación de la enfermedad aparece un estado intermedio llamado prediabetes, que se define como un estado anormal de la homeostasis glucídica caracterizado por la alteración de la glucosa en ayunas, la intolerancia a la glucosa, o ambas cosas [31, 33]. Este estado intermedio puede durar

años y muchas veces pasa desapercibido, a pesar de que distintos estudios muestran que los daños micro- y macro-vasculares comienzan durante esta etapa de prediabetes, y contribuyen a acelerar el progreso de la enfermedad [33]. Dichas complicaciones micro- y macro-vasculares contribuyen al incremento de la mortalidad y la morbilidad [33]. Así, las complicaciones macrovasculares conducen a la enfermedad cardiovascular, y pueden provocar infarto cerebrovascular, enfermedad coronaria, angina de pecho, fallo cardíaco y enfermedad vascular periférica, que está relacionada con la formación de úlceras y amputación de las extremidades inferiores [37]. Las complicaciones microvasculares incluyen la retinopatía, que puede provocar ceguera, la nefropatía, que puede derivar en fallo renal, y la neuropatía, que provoca debilidad y pérdida de sensibilidad en las extremidades [37].

2.3. Funciones metabólicas de la insulina y ruta de señalización.

La insulina es la hormona anabólica que regula el metabolismo de los hidratos de carbono, lípidos y proteínas, además de otros procesos celulares como la mitogénesis [38]. La insulina se produce en las células β de los islotes de Langerhans del páncreas y su secreción está regulada por los niveles de glucosa en sangre. Así, cuando aumenta la glucosa plasmática, los transportadores de glucosa (GLUT)-2 ingresan la hexosa hacia el interior de las células β donde es metabolizada rápidamente, y aumentan los niveles intracelulares de adenosinatrifosfato (ATP). A continuación, se cierran los canales de K^+ (regulados por ATP), se reduce la salida de K^+ y la consecuente despolarización de la membrana plasmática, apertura de los canales de Ca^{2+} y estimulación de la secreción de la insulina por exocitosis [39]. Esta liberación de la hormona está controlada por un bucle de retroalimentación, de forma que al disminuir los niveles de glucosa sanguínea, la secreción de insulina disminuye o cesa [36, 39]. La glucosa es el principal secretagogo de la insulina, pero también hay otras sustancias que pueden estimular la secreción de la hormona como la arginina y los ácidos grasos [36].

Durante el periodo postprandial se libera insulina a la sangre, y ésta va a actuar sobre los tejidos sensibles a la insulina (hígado, músculo esquelético y tejido adiposo) para que se capte la glucosa y se metabolice [38]. En el hígado, la insulina estimula la síntesis de glucógeno e inhibe la gluconeogénesis y la glucogenolisis para frenar la

salida de glucosa [40]. En el músculo esquelético y los adipocitos, la insulina promueve la captación de glucosa mediante la translocación de los transportadores de glucosa GLUT4 a la membrana plasmática [38]. En el caso del músculo esquelético, el exceso de glucosa se almacena en la célula como glucógeno para su posterior uso durante el ayuno, mientras que en los adipocitos se convierte en glicerofosfato que puede esterificarse y almacenarse como TG [38]. De manera adicional, la insulina estimula la lipogénesis, inhibe la oxidación de los ácidos grasos, y aumenta la salida de ácidos grasos libres a la sangre desde el hígado y el tejido adiposo [38, 41, 42].

La insulina tiene además otros tejidos diana como el epitelio vascular, el cerebro, el riñón y el estómago, y contribuyen también a mantener la homeostasis metabólica [38].

En la célula, el primer paso en la ruta de señalización de la insulina es su unión a la subunidad α del receptor de insulina (IR), lo que estimula la autofosforilación del IR en la subunidad β [40]. A continuación, al IR fosforilado se unen los IRS, que son piezas clave en la transducción de la señal al interior celular, ya que su activación (fosforilación en tirosina) permite el anclaje y la activación de las proteínas posteriores de la ruta (Fig. 2). Esta fosforilación en tirosina de los IRS permite la unión de proteínas con dominios de homología Src 2 (SH2), como la proteína PI3K a través de su subunidad reguladora p85 [40, 43]. Esta unión activa a la PI3K y el producto de la enzima, el fosfatidilinositol-3,4,5-trifosfato, promueve la translocación de la proteína AKT y la proteína quinasa dependiente del 3-fosfoinositol (PDK) a la membrana plasmática, donde PDK activa a AKT [40].

La insulina también actúa sobre la proteína Ras-Guanosina 5'-trifosfato (Ras-GTP), y activa una cascada de fosforilación en la que se incluyen Raf y las MAPK, que median efectos sobre el crecimiento, la diferenciación y el ciclo celular, y que a veces también implican a los IRS. Además, la insulina también activa a las proteína quinasa C atípicas (PKCa) a través de PI3K [44]. Las PKCa favorecen la captación de glucosa en músculo esquelético y en tejido adiposo, mientras que en el hígado están implicadas en la síntesis lipídica [44]. Así, PKCζ, que es una PKCa, ejerce un papel importante al intervenir en algunos efectos de la insulina, como la captación de glucosa por el músculo y la regulación del metabolismo lipídico en el hígado [44].

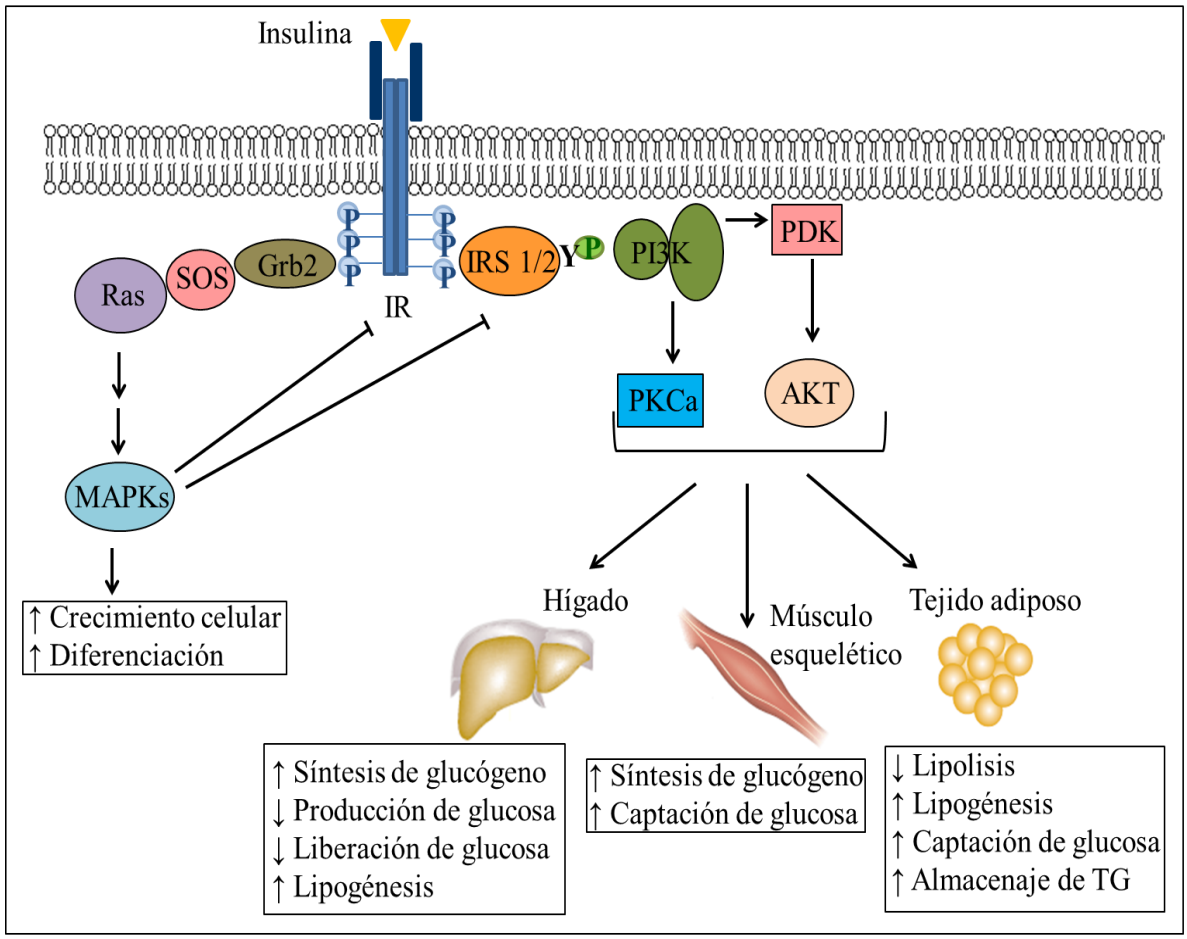


Figura 2: *Vía de señalización de la insulina y efectos metabólicos de la hormona en el hígado, músculo esquelético y tejido adiposo.*

2.4. Resistencia a la insulina.

La resistencia a la insulina se define como una situación en la que una concentración normal de insulina no produce una respuesta adecuada en los tejidos sensibles a la hormona [45]. En este estado, inicialmente la célula β secreta más insulina para intentar compensar la falta de sensibilidad a la hormona, por lo que aparece la hiperinsulinemia, pero se mantiene la hiperglucemia, con lo que se produce un daño en los tejidos (glucotoxicidad). Posteriormente, si la enfermedad progresa, en las células β, se reduce la secreción de insulina [46, 47], y en el hígado, músculo esquelético y tejido adiposo, se disminuye la captación de glucosa, y se incrementa la producción hepática de glucosa por la falta de inhibición de la insulina sobre la gluconeogénesis, lo que

contribuye a agravar la situación de hiperglucemia (Fig. 3) [30]. Se crea así un bucle en el que los tejidos no responden a la hormona y la célula β no es capaz de producir suficiente insulina, lo que conduce a hiperglucemia y al desarrollo de DMT2 [30, 45]. Además, en esta situación, la estimulación de la lipogénesis por parte de la insulina no está bloqueada, de hecho está incrementada, y hay una inadecuada oxidación de los ácidos grasos [41, 42, 48]. Este aumento de la lipogénesis produce la acumulación de grasa ectópica (intramuscular, hepática…), lo que en el hígado puede causar esteatosis, hígado graso, fibrosis, esteatohepatitis no alcohólica, cirrosis y fallo hepático [41]. Además, durante la resistencia a la insulina se estimula la producción hepática de lipoproteínas de muy baja densidad (VLDL), disminuyen los niveles de HDL-Cho y aumentan los de los TG (hipertrigliceridemia), lo que contribuye a la lipotoxicidad [41, 46, 49].

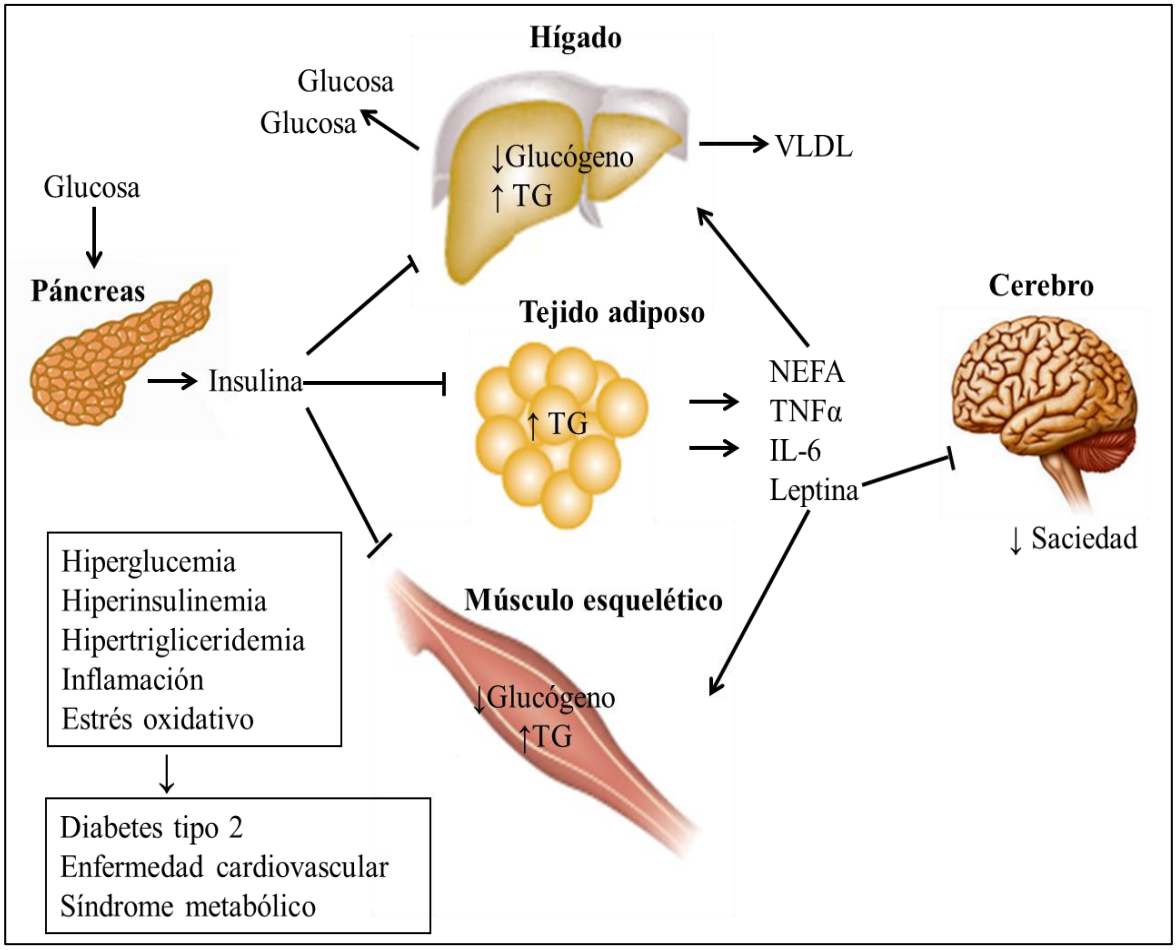


Figura 3: *Alteraciones del metabolismo durante la resistencia a la insulina en el periodo postprandial.*

Por su parte, en el músculo y tejido adiposo se produce el acúmulo de grasa, y se alteran los niveles de distintas adipoquinas producidas por los adipocitos; así disminuyen los niveles de adiponectina, que activa a la proteína quinasa activada por 5´-adenosín-monofosfato (AMPK), y se favorece la oxidación de la glucosa y los ácidos grasos [30]. Además, en el músculo esquelético se disminuye la captación de glucosa dada la alteración de la ruta de señalización de la insulina, la AMPK y la translocación del GLUT4 a la membrana plasmática [38]. Así pues, la resistencia a la insulina afecta los tejidos insulino-sensibles, lo que conduce a hiperglucemia, hiperinsulinemia, hipertrigliceridemia, dislipemia e inflamación, que son características de la DMT2 [30, 46].

En la célula se produce un fallo en la ruta de señalización de la insulina que impide que se transmita la señal correctamente al interior de la célula. Este hecho parece relacionarse con la disminución en la autofosforilación en tirosina del IR por aumento de los niveles de las proteínas fosfatasas, como la proteína fosfatasa de tirosina 1B (PTP1B) y la homóloga de fosfatasa y tensina (PTEN) [38, 40]. El bloqueo de la ruta también puede deberse a la inhibición por la fosforilación en posiciones de serina del lRS-1 y-2 por diversas serina/treonina quinasas como la quinasa c-Jun NH2-terminal (JNK) y las PKCa, entre otras [30]. Además, en la resistencia a la insulina se produce la inhibición de la vía de la hormona por la internalización y degradación de las proteínas IRS, dado que las proteínas supresoras de la señalización por citoquinas (SOCS) compiten con el IRS-1 por la asociación con el IR y promueven su degradación por el proteasoma [30, 46].

El incremento del tejido adiposo subcutáneo y visceral, que se produce durante la resistencia a la insulina, conlleva un aumento de la secreción de gran cantidad de ácidos grasos libres no esterificados (NEFA) y de citoquinas proinflamatorias [factor de necrosis tumoral α (TNF-α), interleuquina-1β (IL-1β), interleuquina 6 (IL-6)], lo que a su vez contribuye a agravar la situación [30]. Se activan diferentes proteínas quinasas como el complejo proteico quinasa IκB (IKK), JNK y PKCa, entre otras [49], con lo que se favorece la fosforilación en serina de los IRS y se produce la activación de la respuesta inflamatoria. Dicha respuesta inflamatoria se exacerba por el aumento en los niveles de resistina [50] y puede aparecer una resistencia a la leptina [30, 46].

2.5. Estrés oxidativo.

Las ROS y RNS son productos normales del metabolismo. En condiciones no patológicas, las ROS y RNS están implicadas en respuestas fisiológicas, como la defensa frente a agentes infecciosos o la mitosis, y pueden actuar como segundos mensajeros [51, 52]. Las células tienen una serie de mecanismos de defensa antioxidante (enzimáticos y no enzimáticos) cuya función es neutralizar los radicales libres [51, 52]. Sin embargo, cuando se pierde este delicado equilibrio redox debido a una sobreproducción de ROS/RNS y/o una deficiencia en las defensas antioxidantes de la célula, aparece el estrés oxidativo [51]. Este exceso de radicales libres puede dañar a los lípidos, las proteínas y el ADN e inhibir su función normal, alterar la estructura y fluidez de las membranas celulares y limitar gravemente la actividad metabólica [47, 51, 53]. De hecho, todas estas posibles alteraciones inducidas por el estrés oxidativo se han relacionado con la aparición, desarrollo y progreso de un gran número de enfermedades crónicas como el cáncer, enfermedades neurodegenerativas, diabetes y, también con el envejecimiento [51].

Los radicales libres se definen como moléculas o fragmentos de moléculas que contienen uno o más electrones desapareados, lo que les confiere una gran reactividad [47, 51]. El anión superóxido ($O_2^{\bullet-}$), que aparece durante las reacciones metabólicas o por la irradiación, se considera el "radical libre primario" y puede interaccionar con otras moléculas para originar "radicales libres secundarios". El principal punto de generación del anión superóxido en las células es la cadena de trasporte de electrones mitocondrial, donde se pierden algunos electrones del oxígeno prematuramente formando dicho radical libre [51, 54]. El anión superóxido puede convertirse en el radical hidroxilo ($^{\bullet}OH$), que tiene una gran reactividad [55] y reacciona con el ADN dañándolo, lo que representa el primer paso de la mutagénesis, la carcinogénesis y el envejecimiento [51]. Además, los peroxisomas y las mitocondrias generan peróxido de hidrógeno (H_2O_2), que se libera al citoplasma, y contribuye a exacerbar la situación de estrés oxidativo [55]. Por su parte, las células del músculo liso y los macrófagos producen el radical óxido nítrico ($NO^{\bullet}$) que puede reaccionar con $O_2^{\bullet-}$ y generar el anión peroxinitrito ($ONOO^-$), que es altamente reactivo, y favorece el incremento de la peroxidación lipídica, la nitración proteica, la fragmentación del ADN y la oxidación de las LDL-Cho [55].

2.5.1. Estrés oxidativo y diabetes.

Estudios recientes han puesto de manifiesto que las alteraciones que se producen durante la resistencia a la insulina y la diabetes son consecuencia, al menos en parte, del estrés oxidativo, y que dicho estrés juega un papel crítico en el desarrollo de las complicaciones de la diabetes [47, 55]. Así, se ha demostrado que la hiperglucemia induce estrés oxidativo por diferentes mecanismos [47, 53, 56]:

- el aumento de la oxidación de la glucosa;

- la inducción de la vía de los polioles activa a la enzima aldosa reductasa y reduce la glucosa a sorbitol a expensas del nicotinamida adenina dinucleótido fosfato reducido (NADPH), que es necesario para regenerar el antioxidante glutation reducido (GSH). Se produce así una disminución del poder antioxidante de la célula;

- el aumento de la glicación no enzimática de proteínas, que por su unión a sus receptores conduce a un aumento en la generación de ROS;

- la activación de la PKC motivada por un descenso en la actividad de la enzima gliceraldehído-3-fosfato deshidrogenasa (GAPDH) provoca el aumento de diacilglicerol (DAG). Esta estimulación de la PKC conduce a la activación de la NADPH oxidasa, con lo que disminuye la relación NADPH/$NADP^+$ y se incrementa el estrés oxidativo. Además, PKC puede activar también la vía de NF-κB;

- la activación de la vía de las hexosaminas genera glucosamina-6-fosfato que inhibe a la enzima glucosa-6-fosfato deshidrogenasa, acoplada a la reducción de $NADP^+$, lo que favorece aún más el descenso de la relación NADPH/$NADP^+$;

- la inducción de citocromo P4502E1, que aumenta la generación de radicales libres en la cadena de transporte de electrones.

Todas estas alteraciones conducen a la generación de un estrés oxidativo crónico y están implicadas en la aparición de la resistencia a la insulina, la disfunción de la célula β, el desarrollo de la diabetes y sus complicaciones micro- y macro-vasculares [53-55].

2.5.2. Mecanismos de defensa antioxidante.

Las células han desarrollado mecanismos de defensa frente a los radicales libres para mantener la homeostasis redox. Estos mecanismos protectores, que neutralizan los ROS/RNS y/o bloquean su producción, incluyen defensas enzimáticas y no enzimáticas producidas por el cuerpo (endógenas) o aportadas por la dieta (exógenas).

2.5.2.1. Defensas antioxidantes no enzimáticas.

Dentro de las defensas antioxidantes no enzimáticas cabe destacar al GSH, ácido ascórbico (vitamina C), α-tocoferol (vitamina E), carotenoides y polifenoles de la dieta.

El GSH es la principal defensa antioxidante no enzimática celular que en condiciones fisiológicas se encuentra en concentraciones del orden de mM dentro la célula [52]. El GSH puede unirse covalentemente a las proteínas y actúa como coenzima de numerosas enzimas implicadas en la defensa antioxidante, oxidándose [glutation oxidado o glutation disulfóxido (GSSG)] (Fig. 4). Así, en una situación de estrés oxidativo los niveles de GSH disminuyen rápidamente y aumentan los de GSSG [51, 52]. Esta alteración de la relación GSH/GSSG activa elementos transcripcionales de respuesta antioxidante y estimula la síntesis *de novo* de GSH. Dicha síntesis depende de los niveles intracelulares de cisteína (precursor del GSH), y se detiene cuando se restablecen los niveles del GSH, ya que el propio GSH actúa como un regulador negativo de su síntesis mediante un mecanismo de retroalimentación [52].

Algunas vitaminas y los polifenoles de la dieta tienen un papel muy importante como antioxidantes. Las vitaminas C y E protegen a las membranas lipídicas de la peroxidación [47]. Los carotenoides, precursores del retinol (vitamina A), son capaces de neutralizar de manera eficiente los radicales libres y tienen un importante papel en la fotoprotección [57]. Del mismo modo, los polifenoles de la dieta minimizan o impiden el daño a las macromoléculas, ya que son capaces de captar directamente los radicales libres y quelar cationes divalentes involucrados en la reacción de Fenton, y también pueden estimular la transcripción de las enzimas antioxidantes [52].

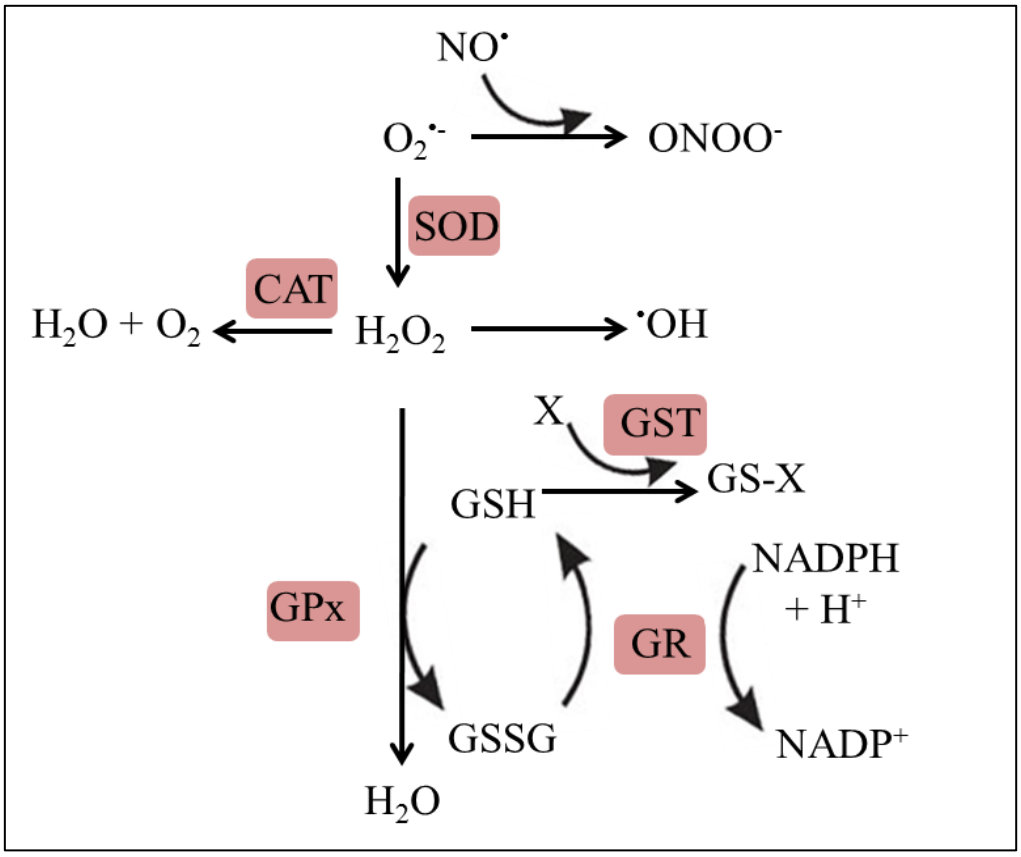


***Figura 4**: Esquema de las defensas antioxidantes celulares más importantes y sus reacciones para neutralizar los radicales libres. Xenobiótico (X).*

2.5.2.2. Defensas antioxidantes enzimáticas.

Las defensas antioxidantes enzimáticas están formadas por un conjunto de enzimas que actúan de manera coordinada para preservar el estado redox celular frente a posibles agentes pro-oxidantes y xenobióticos.

Las enzimas superóxido dismutasa (SOD), catalasa (CAT), tiorredoxina reductasa y glutation peroxidasa (GPx) son enzimas de fase I, y constituyen la primera línea de defensa celular frente a los radicales libres [52]. Sin embargo, si se ven sobrepasadas en su actividad por la intensidad o duración del estrés oxidativo, los radicales libres pueden interaccionar con las macromoléculas y generar compuestos altamente reactivos [52]. En esta situación, pueden actuar las enzimas llamadas de fase II o detoxificantes, donde se incluyen la glutation-S-transferasa (GST), aldo-ceto

reductasa y aldehído deshidrogenasa, que ayudan a eliminar estos compuestos para evitar mayores daños celulares (Fig. 4) [52].

Las enzimas GPx constituyen una familia de selenoproteínas que se encuentran distribuidas de forma ubicua en la célula. Esta familia de enzimas reduce los hidroperóxidos a sus correspondientes compuestos hidróxidos a expensas del GSH que actúa como sustrato [52].

La enzima glutation reductasa (GR) se encarga de restaurar el GSSG, producido generalmente por la GPx, a GSH, y utiliza como cofactor de reducción a la NADPH. La acción de esta enzima es fundamental para el metabolismo del GSH y para aumentar el sustrato de las enzimas detoxificantes dependientes de GSH [52].

La enzima SOD cataliza la reacción de descomposición del anión superóxido a H_2O_2, sobre el que actuarán otras enzimas como GPx y CAT para convertir el peróxido de hidrógeno en agua y oxígeno [58]. Existen dos tipos de SOD: la enzima CuZnSOD (SOD1), que posee cobre y zinc en el centro activo y se localiza en el citoplasma, y la enzima MnSOD (SOD2), que posee manganeso en el centro activo y está localizada en la mitocondria [58].

La CAT cataliza el paso del peróxido de hidrógeno a agua y oxígeno para evitar el daño a macromoléculas [58]. Contiene hierro en el centro activo y se localiza principalmente en los peroxisomas.

La enzima GST comprende una familia de proteínas detoxificantes que se encarga de eliminar xenobióticos mediante su conjugación con GSH [52], y su expresión está modulada por la insulina [59].

La hemooxigenasa (HO-1) es una proteína de estrés celular inducible cuya principal función metabólica es su participación en el paso limitante de la oxidación del grupo hemo para dar lugar a biliverdina, que posteriormente se transforma en bilirrubina [60]. La HO-1 tiene una función crucial en la protección celular, y entre sus actividades biológicas cabe mencionar su papel como defensa antioxidante, y su participación en procesos antiinflamatorios y antiapoptóticos [60].

Numerosos estudios han relacionado las alteraciones que se producen en las defensas antioxidantes con una amplia variedad de patologías implicadas en

enfermedades inflamatorias y la diabetes [47, 51]. En este sentido se ha de mencionar que la modulación de las defensas antioxidantes en la diabetes está alterada debido al aumento del estrés oxidativo que tiene lugar en la enfermedad. Algunos autores han mostrado un descenso de las defensas enzimáticas (GPx, CAT, GST y SOD) y no enzimáticas (vitaminas C y E y GSH) en pacientes con DMT2 [47, 56, 61], mientras que otros estudios han demostrado el aumento de la actividad de las enzimas antioxidantes (GPx, GR, CAT, SOD y HO-1) en el suero de los pacientes DMT2 [62, 63].

2.5.3. Vías de señalización relacionadas con el estrés oxidativo.

En una situación de estrés oxidativo se puede producir la activación de la vía de las MAPKs, lo que puede ir acompañado de una estimulación del sistema de defensa antioxidante, y de factores de transcripción sensibles al estado redox de gran relevancia para la respuesta antioxidante celular, como son el factor nuclear relacionado con el factor de trascripción eritroide 2 (Nrf2) y NF-κB.

2.5.3.1. Vía de señalización de las MAPKs.

Las MAPKs son un grupo de proteínas que trasmiten la señal de estímulos (ROS, hiperglucemia, estrés osmótico, inflamación, radiación UV) mediante la fosforilación de proteínas [51]. Así, las MAPKs interaccionan con receptores de factores de crecimiento, proteínas G, proteínas tirosina quinasas o factores de transcripción [51].

Las MAPKs están formadas por tres familias: las quinasas regulada por señales extracelulares (ERK), JNK y la quinasa p38 (p38). Las MAPKs se encargan de coordinar la regulación de la proliferación, la diferenciación, la adaptación al estrés y la apoptosis [51]. De manera más concreta, las ERK están muy relacionadas con la proliferación celular, mientras que las JNK se asocian con la apoptosis, por lo que el equilibrio entre ambas proteínas es un factor clave para la supervivencia celular [51]. Además, las ERK y JNK se estimulan por citoquinas proinflamatorias [64]. La proteína p38-MAPK está involucrada en procesos relacionados con la inflamación, la inmunidad, el crecimiento celular y la apoptosis [64]. De esta manera, una disfunción en estas vías de señales puede estar implicada en la patogénesis de numerosas enfermedades [51, 64].

En la diabetes, el estrés derivado de la hiperglucemia activa a las JNK, de manera que su activación crónica se ha relacionado con el desarrollo de la nefropatía diabética [64]. Por su parte, la activación crónica de p38 está asociada con la aparición de complicaciones de la diabetes como la neuropatía [64]. En este sentido, la fosforilación en serina de IRS-1 se relaciona con la desensibilización a la insulina, y la activación de las ERK y JNK induce dichas fosforilaciones [64, 65].

2.5.3.2. Vía de señalización de Nrf2.

El Nrf2 es esencial para el mantenimiento de la homeostasis celular, pues protege a las células frente al estrés oxidativo y la inflamación [52, 66]. Este factor de transcripción se induce en respuesta al estrés oxidativo, uniéndose a secuencias del ADN conocidas como elementos de respuesta antioxidante (ARE) o elementos de respuesta electrofílica (EpRE). Estas secuencias codifican para diversas enzimas que directa o indirectamente pueden ejercer funciones antioxidantes y detoxificantes, aumentar la síntesis y la regeneración del glutation o ejercer un efecto antiinflamatorio [66]. Dichas secuencias codifican también para proteínas de reconocimiento, reparación y eliminación de proteínas y nucleótidos dañados, así como para receptores, proteínas transportadoras y chaperonas que contribuyen a restablecer la homeostasis celular a través de sus funciones [66].

En condiciones fisiológicas, el factor de transcripción Nrf2 está secuestrado en el citoplasma, ya que se encuentra unido a la proteína asociada a ECH 1 similar a Kelch (Keap1), lo que permite la ubiquitinación del Nrf2 y, por tanto, su degradación por el proteasoma (Fig. 5A) [66]. Sin embargo, en una situación de estrés se produce la oxidación de Keap-1 en residuos de cisteína, lo que provoca un cambio conformacional y la liberación del Nrf2. Además, la fosforilación de Nrf2 en serina también promueve la disociación del factor de transcripción de su proteína represora Keap-1. A continuación, el Nrf2 se transloca al núcleo donde se une a los ARE, presentes en los promotores génicos de las proteínas encargadas de la síntesis *de novo* de glucógeno y de enzimas antioxidantes y detoxificantes, para estimular la trascripción de los genes [66]. Además, el *nrf2* contiene secuencias ARE que autorregulan al propio factor de transcripción y contribuyen a mantener la expresión génica de estas proteínas de manera más prolongada [67].

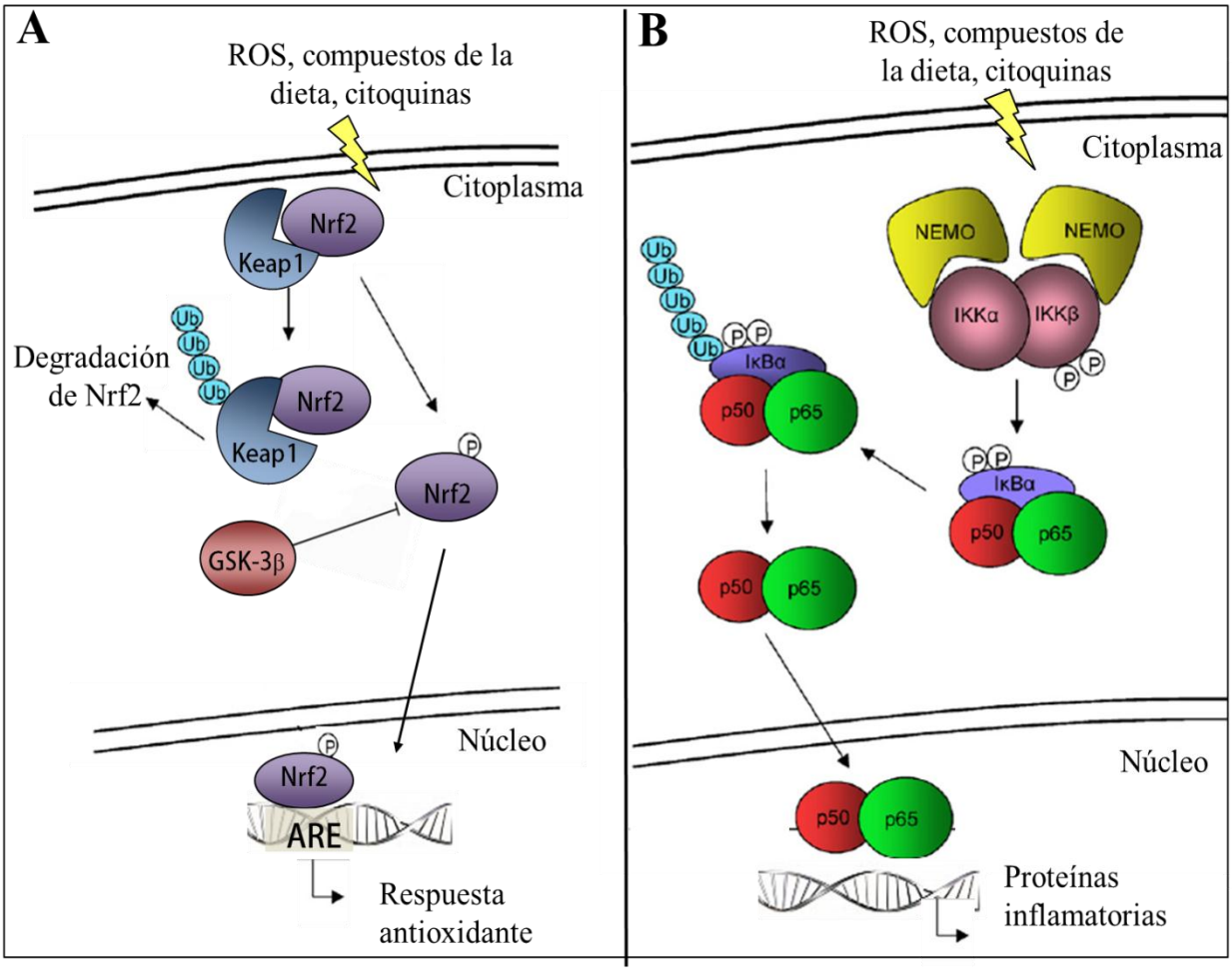


Figura 5: *Regulación de las vías de señalización del (A) Nrf2 y (B) NF-κB (vía clásica). Adaptado de Glorie et al [68]*.

Estudios recientes muestran que la proteína glucógeno sintasa quinasa 3 β (GSK3β) puede inhibir la actividad de Nrf2; así, cuando GSK3β está inhibida por la señalización de la insulina, aumenta el Nrf2. Por tanto, la señalización del Nrf2 estaría acoplada a la señalización de la insulina y podría tener un papel durante la resistencia a la insulina [67].

2.5.3.3. Vía de señalización de NF-κB.

El factor de trascripción NF-κB está formado por homo- o hetero-dímeros de grupos de cinco proteínas: p50 ó NF-κB1 (y su precursor p105), p52 ó NF-κB2 (y su precursor p100), p65/RelA, RelB y c-Rel [69]. En una situación fisiológica, el NF-κB está secuestrado en el citoplasma, y aparece asociado a la proteína inhibitoria kappa B (IκB) (Fig. 5B) [69]. No obstante, si se produce la activación de la vía clásica por

citoquinas inflamatorias (TNFα, IL-1β), estrés oxidativo u otro estímulo, se estimula el complejo proteico IKK, que está formado por NEMO (también llamado IKKɣ), IKKα e IKKβ. A continuación, IKK fosforila a IκB, que se degrada por el proteasoma y libera al NF-κB. Después, el NF-κB se transloca al núcleo donde regula la actividad de múltiples genes implicados en diferentes procesos como la inflamación, la inmunidad, la proliferación y la apoptosis [68, 69].

Durante la diabetes, la vía de señalización del NF-κB está activada debido al aumento del estrés oxidativo y al estado pro-inflamatorio con que cursa la enfermedad [68].

3. Papel del hígado en la resistencia a la insulina.

El hígado juega un papel central en el metabolismo de los hidratos de carbono, lípidos y proteínas, y en el mantenimiento del equilibrio metabólico de acuerdo con la disponibilidad y demanda de los nutrientes [36, 40]. El control hepático de estos procesos está regulado por hormonas y estímulos nutricionales, y la insulina y el glucagón juegan un papel clave [40]. La pérdida de este control hepático es una característica de la resistencia a la insulina y de la DMT2 que tiene consecuencias en todo el organismo [40].

3.1. Metabolismo de hidratos de carbono.

Los hepatocitos intervienen en el mantenimiento de la homeostasis glucídica, ya que controlan los niveles de la glucosa plasmática almacenando o produciendo glucosa según las necesidades del organismo.

Durante el periodo postprandial, los niveles elevados de glucosa en plasma inducen la secreción de insulina por las células β pancreáticas. En los hepatocitos, la unión de la insulina a su receptor (IR) estimula, mediante fosforilación en tirosina, a los IRS, que a su vez activan la vía de la PI3K/AKT [40] (Fig. 6). Así, la AKT fosforila e inhibe a GSK3 que, a su vez, fosforila e inhibe a la glucógeno sintasa (GS), enzima implicada en la glucogenogénesis, lo que conduce al aumento de la síntesis de glucógeno (glucogenosíntesis) e inhibición de la glucogenolisis [40, 46]. AKT además fosforila al factor de transcripción “forkhead box” (FOXO) con lo que sale del núcleo

[40]. Este factor de transcripción regula al coactivador del receptor de proliferación activado por peroxisomas 1 α (PGC-1α) que a su vez estimula la expresión de enzimas gluconeogénicas como la glucosa-6-fosfatasa (G6Pasa) y la fosfoenolpiruvato carboxiquinasa (PEPCK) [40, 70]. Por tanto, la inactivación de FOXO en respuesta a la insulina inhibe la gluconeogénesis y la síntesis neta de glucosa, procesos clave para mantener la homeostasis glucídica [40]. Además, se ha descrito que la sobreexpresión de PEPCK produce hiperglucemia e hiperinsulinemia, y está relacionada con la DMT2, mientras que la supresión de la enzima conduce a una hipoglucemia severa y cambios en el metabolismo de los lípidos y las proteínas [36].

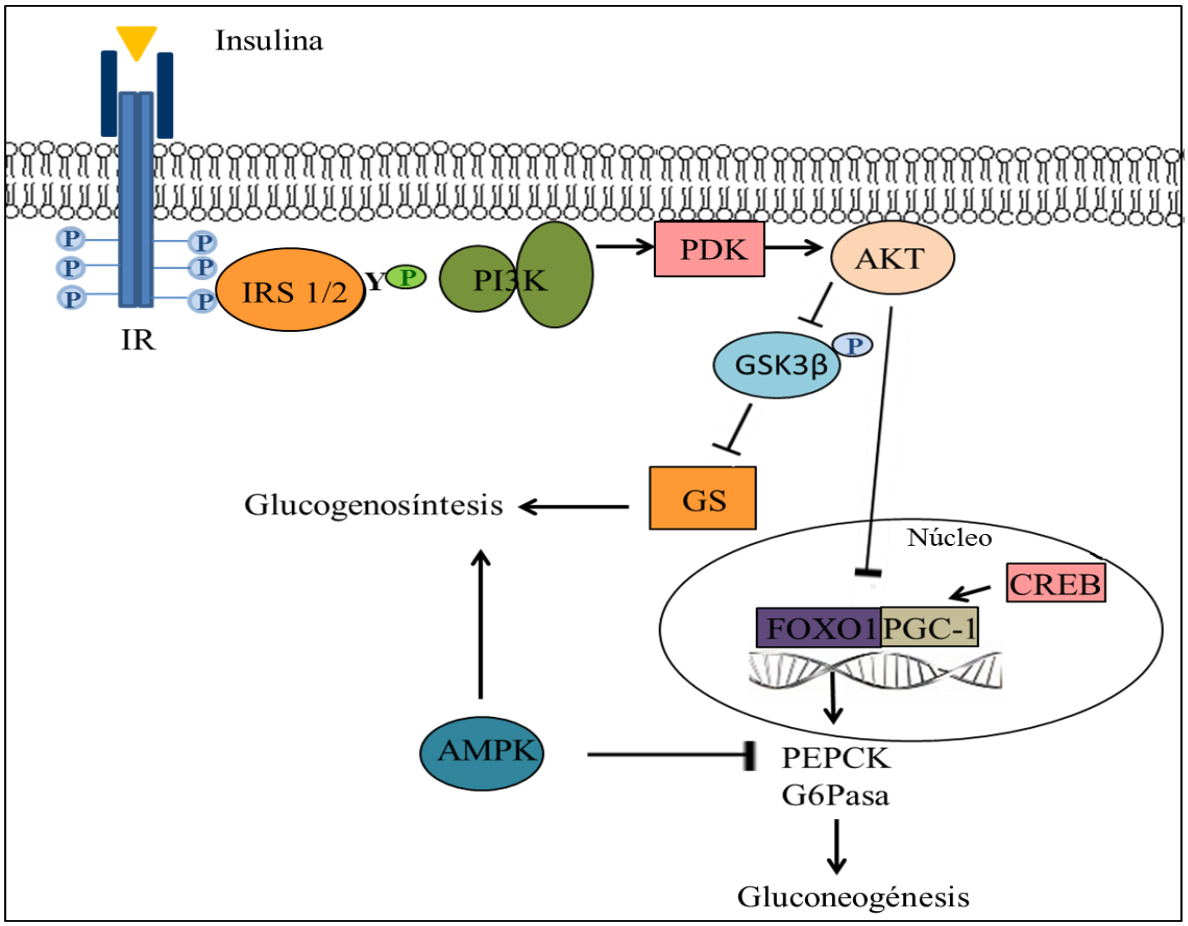


Figura 6: *Ruta de señalización de la insulina y metabolismo glucídico en el hígado. Proteína de unión al elemento de respuesta a AMP cíclico (CREB).*

La AMPK es una proteína de gran relevancia para el control metabólico, el crecimiento y la diferenciación celulares, y se considera un sensor energético altamente conservado en la evolución [71]. La AMPK se activa por el incremento de la relación

AMP/ADP provocada por la bajada de nutrientes y el ejercicio prolongado, así como por algunos fármacos antidiabéticos, como la metformina [71]. En relación con el metabolismo hepático, la AMPK inhibe la lipogénesis y la gluconeogénesis, ya que impide la expresión de los genes diana de FOXO, PEPCK y G6Pasa (Fig. 6) [71].

En los hepatocitos, la insulina también estimula la captación de glucosa a través del trasportador de glucosa GLUT2 [40, 72], que en el interior de la célula se trasforma en glucosa-6-fosfato por la acción de la enzima GK, lo que permite la entrada de la glucosa en la glicolisis para obtener ATP (Fig. 6) [40]. En el hígado, la activación de la enzima GK por parte de la insulina es clave para el metabolismo de los hidratos de carbono, ya que participa en el control del flujo de glucosa en la célula [40]. En este sentido se ha descrito el descenso progresivo en los niveles de GK durante el progreso de la DMT2 [73].

Durante el ayuno, el hígado se encarga de mantener la homeostasis glucídica liberando glucosa a la sangre para que esté disponible para los demás tejidos [40]. PGC-1α interacciona con FOXO y permite la trascripción de las enzimas gluconeogénicas PEPCK y G6Pasa, lo que estimula la síntesis *de novo* de glucosa a partir de precursores no glucídicos y se favorece la glucogenosíntesis [40, 74].

Durante la resistencia a la insulina se produce un bloqueo de la ruta de la hormona. En la célula, la autofosforilación del IR es menos sensible, lo que conduce a la mala regulación de proteínas de esta vía como los IRS-1 y -2, GSK3 y GS, y se traduce en una disminución en la síntesis de glucógeno [40]. Además, tiene lugar un fallo en el control de la gluconeogénesis y la glucolisis causado por la desregulación de enzimas clave para estos procesos, como PEPCK y GK, respectivamente [40], y se altera el flujo neto de glucosa al modificarse los niveles de GK y del transportador GLUT2 [72]. La hiperglucemia también contribuye a aumentar el estrés oxidativo [64].

3.2. Metabolismo lipídico.

El hígado coordina la síntesis de ácidos grasos *de novo* (lipogénesis) y su degradación (β-oxidación lipídica) [48, 70].

Durante el periodo postprandial, la insulina estimula la biosíntesis de ácidos grasos en el hígado e inhibe su oxidación, lo que conduce a un aumento de ácidos grasos y TG que se almacenan localmente y se exportan [48]. La principal fuente de acetil-CoA para la síntesis de ácidos grasos es la glucosa, si bien la galactosa, la fructosa, el lactato, el piruvato y los aminoácidos también contribuyen a la reserva celular de acetil-CoA [48, 70]. La enzima acetil-CoA carboxilasa (ACC), que es una enzima limitante de la biosíntesis de ácidos grasos, trasforma el acetil-CoA en malonil-CoA (inhibidor alostérico de la β-oxidación), y se activa por distintos estímulos, como el citrato, que es un producto de la glucolisis. A continuación, el malonil-CoA sirve de sustrato a la enzima ácido graso sintasa (FAS) para generar el ácido palmítico (C16:0) a expensas de NADPH y diferentes enzimas elongasas y desaturasas, que catalizan la síntesis de los diferentes ácidos grasos [48, 70]. Después, los ácidos grasos se esterifican con glicerol para formar TG, o con colesterol para dar lugar a los ésteres de colesterol, que se almacenan en el hígado como gotas lipídicas y se exportan en forma de VLDL [48, 75]. Además, los ácidos grasos pueden incorporarse a los fosfolípidos para formar membranas biológicas, o se pueden esterificar con esfingosina para formar ceramidas que, junto a otros intermediarios lipídicos como DAG, actúan como segundos mensajeros y activan a las PKCa [42].

Durante el ayuno, disminuyen los niveles de insulina y glucosa en sangre, y se estimula el catabolismo de los ácidos grasos para obtener energía y producir cuerpos cetónicos [70]. En la β-oxidación, los ácidos grasos libres se transportan a la matriz mitocondrial y se esterifican con la coenzima A mediante un sistema de trasporte que incluye diferentes proteínas, como la acil-CoA sintetasa (ACS), la carnitina palmitoil transferasa I y II (CPT-I, CPT-II) y la carnitina/acetilcarnitina translocasa (CACT) [70]. Una vez en la matriz, el ácido graso acil-CoA se oxida hasta acil-CoA para obtener energía. Además, en situaciones de ayuno se puede producir la síntesis de cuerpos cetónicos (acetona, acetoacetato y β-hidroxibutirato) desde acil-CoA, que se secretan a la sangre para ser usados como fuente de energía alternativa a la glucosa en el cerebro y los músculos [70].

Como se muestra en la figura 7, la síntesis de ácidos grasos está ligada al metabolismo de los hidratos de carbono, ya que la oxidación completa de la glucosa produce acetil-CoA, cuyo exceso se desvía hacia la síntesis de lípidos *de novo* [74]. Además, el exceso de glucosa, que no se ha metabolizado ni almacenado como

glucógeno, se trasforma en TG que se almacenan en el hígado o se transportan en las VLDL al tejido adiposo [74].

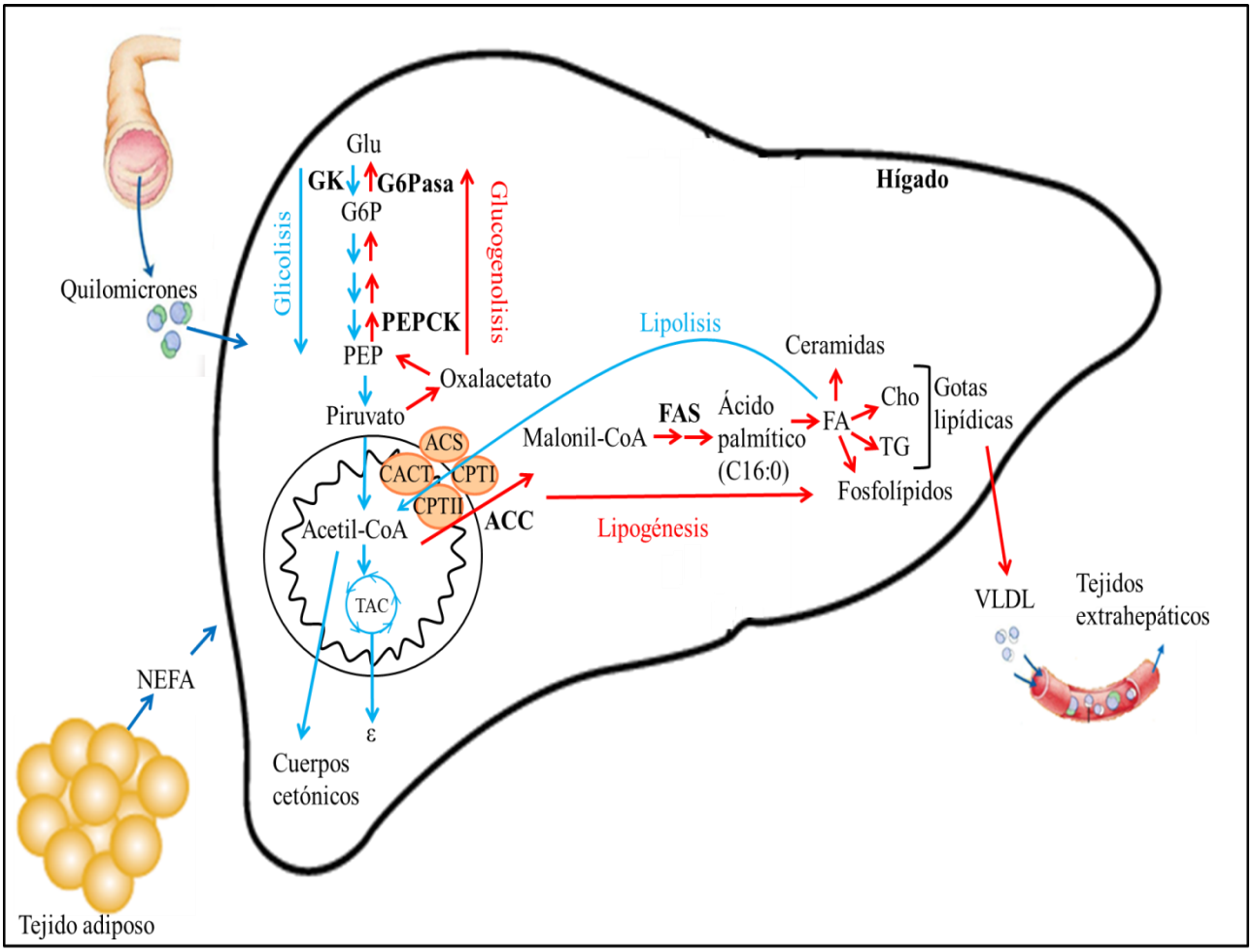


Figura 7: *Regulación del metabolismo lipídico y relación con el metabolismo de hidratos de carbono en el hígado. Las flechas azul claro representan rutas catabólicas y las fechas rojas rutas anabólicas.*

El metabolismo lipídico está modulado de manera destacada por la familia de factores de transcripción de unión regulado por esteroles (SREBP) y por el receptor nuclear activado por proliferación de los peroxisomas α (PPARα), que regulan la expresión de las enzimas lipogénicas o lipolíticas, respectivamente (Fig. 8) [36].

La familia de proteínas SREBP es una familia de factores de trascripción implicados en la regulación del metabolismo de ácidos grasos y colesterol, y está formada por SREBP1 y SREBP2 [48, 70]. La proteína SREBP1 es la principal implicada en la expresión de enzimas lipogénicas, y responde al incremento de insulina

y nutrientes que se produce después de una comida [48, 70, 76]. Se han descrito dos isoformas de SREBP1 que se generan por splicing alternativo: SREBP1-a y SREBP1-c. SREBP1-a es más activa y se expresa más en las células en cultivo, mientras que SREBP1-c es más abundante en el hígado [48]. SREBP1-c se sintetiza como un precursor en el retículo endoplasmático, y ciertos estímulos, como la insulina, impulsan la rotura de la región N-terminal del precursor para generar la forma madura de SREBP1-c.

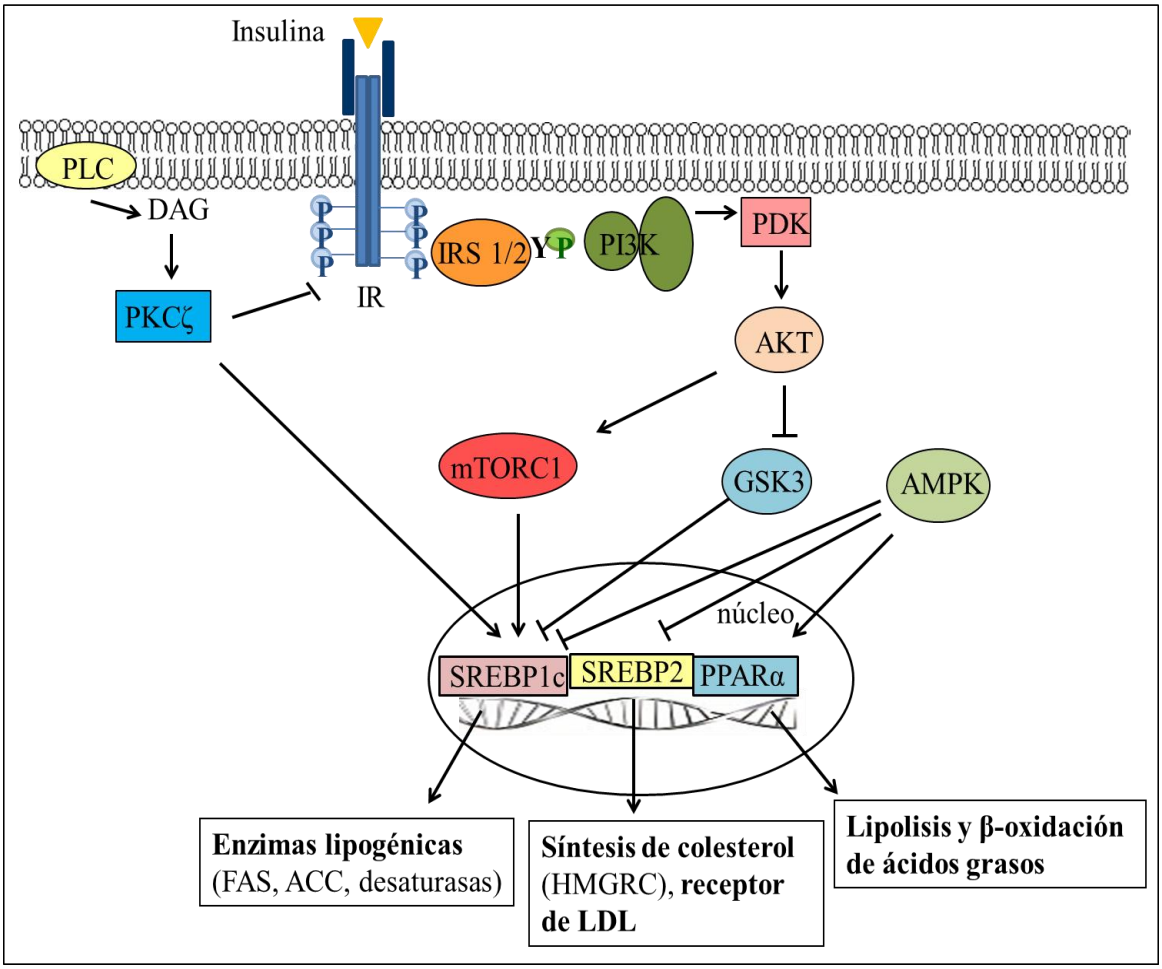


Figura 8: *Ruta de señalización de la insulina y metabolismo lipídico en el hígado. Fosfolipasa C (PLC).*

En el núcleo, SREBP1-c promueve la expresión de las enzimas lipogénicas ACC, FAS, Δ9-desaturasa, Δ6-desaturasa y Δ5-desaturasa [70]. Además, la AKT activa al complejo diana de rapamicina en mamíferos 1 (mTORC1), que es necesario para la activación de SREBP1-c [48, 70, 76], e inhibe a la proteína GSK3, que regula

negativamente SREBP1-c y favorece su degradación por el proteasoma [70]. Aunque SREBP1-c juega un papel clave en la lipogénesis, otros factores de trascripción están implicados en la regulación de este proceso, como son el receptor X hepático (LXR) y la proteína de unión al elemento de respuesta a carbohidratos (ChREBP) [70, 76]. La isoforma SREBP2 activa genes implicados en la síntesis y captación del colesterol, como la enzima 3-hidroxi-3-metilglutaril coenzima A reductasa (HMGCR), que cataliza el paso limitante de la biosíntesis de colesterol, y el receptor de las proteínas LDL-Cho, que ingresa colesterol desde la sangre [76].

El PPARα es el principal regulador del catabolismo de los ácidos grasos, y se encarga de estimular la expresión de las enzimas implicadas en el trasporte de los ácidos grasos, en el tráfico intracelular de los ácidos grasos y en la β-oxidación [70]. Así, los ratones knock-out para PPARα muestran un defecto en la oxidación de los ácidos grasos y acumulan TG y colesterol en el hígado [70]. La insulina inhibe a PPARα, mientras que el glucagón, los ácidos grasos polinsaturados y sus productos de oxidación intermedios, y los eicosanoides lo activan [70].

El metabolismo lipídico está regulado también por la AMPK, que fosforila e inhibe a la ACC, lo que provoca el descenso en los niveles de malonil-CoA, activa la β-oxidación y disminuye la acumulación de los lípidos en el hígado [71, 77]. Además, la AMPK inhibe a HMGCR, disminuyendo la síntesis de colesterol y a SREBP1-c, lo que conduce a la inhibición de la lipogénesis [76, 77]. En este sentido, se ha descrito que la sobreexpresión de AMPK en el hígado o el tratamiento con 5-aminoimidazol-4-carboxamida ribonucleótido (AICAR) o metformina en roedores delgados y obesos incrementan la oxidación lipídica, disminuye los niveles de TG y, por tanto, mejora el perfil lipídico [77].

La acción de las PKCa depende de la isoforma y del tejido. En el hígado está presente de manera abundante la isoforma PKCζ que inhibe a la AKT, lo que promueve la activación de las enzimas gluconeogénicas PEPCK y G6Pasa [42], estimula la vía del NF-κB y también a SREBP1-c para favorecer la lipogénesis [44]. En condiciones patológicas, la activación excesiva de PKCζ hepática contribuye a la resistencia a la insulina, como se ha visto en modelos animales y pacientes diabéticos [44].

Durante la resistencia a la insulina, al contrario que en el metabolismo de hidratos de carbono, la insulina continúa ejerciendo su acción anabólica, de manera que

la lipogénesis continúa activa y la lipolisis se inhibe [41]. Además, aumenta el flujo de ácidos grasos desde el tejido adiposo, con lo que se incrementan sus niveles en el hígado, su acumulación como TG y la secreción de VLDL desde el hígado [42]. Esta alteración del metabolismo lipídico hepático durante la resistencia a la insulina provoca la inactivación de la AMPK y PPARα, y la activación de FAS y SREBP1, y de vías de señalización, como la PKCζ o NF-κB que, a su vez favorecen o exacerban la resistencia a la insulina [44]. Además, el exceso de ácidos grasos sin esterificar en el citoplasma contribuye a aumentar el estrés oxidativo y de retículo plasmático agravando la situación [44].

4. Polifenoles del cacao y resistencia a la insulina.

4.1. El cacao.

El cacao procede de la semilla del árbol tropical *Theobroma cacao* (alimento de los dioses), y forma parte del chocolate, alimento que en la actualidad se consume en grandes cantidades en un gran número de países occidentales [78].

El primer indicio de consumo de cacao data de unos cuencos de 1600 a.C. donde se cree que los aztecas tomaban una bebida hecha con este alimento [79]. Los indígenas olmecas, mayas, aztecas e incas consumían el cacao con fines rituales y medicinales, incluyendo la prevención de infecciones, inflamación y palpitaciones, y lo utilizaban como moneda de cambio [80-82]. Además, consideraban que el cacao tenía un origen divino y su consumo estaba reservado a las personas más importantes (sacerdotes, oficiales de gobierno más altos y guerreros) [81]. Cristóbal Colón en 1502 se refirió a las semillas del cacao como unas “almendras” de forma misteriosa [81], y los primeros manuscritos donde se describe al cacao y sus usos proceden de Hernán Cortés (1519) y Díaz del Castillo (1560), que explican en la narración de la conquista de Méjico el recibimiento por el emperador Moctezuma y la preparación de la bebida de chocolate [81, 83].

El cacao se introdujo en la corte española en 1544 desde Mesoamérica a través de unos frailes dominicos, y en menos de un siglo, sus usos culinarios y medicinales se extendieron desde España a Francia, Inglaterra y Europa del este [81]. Debido al

aumento de la demanda de cacao, los franceses y los españoles establecieron plantaciones de cacao en sus colonias del caribe y Filipinas respectivamente y en 1880 los ingleses lo llevaron a África occidental [81]. Entre los siglos XVI y XX se determinaron más de cien usos medicinales del cacao y el chocolate [26, 81]. En los últimos años, mediante estudios de meta-análisis se han confirmado varios de estos efectos beneficiosos como el descenso de la presión sanguínea, los niveles de colesterol y de marcadores de inflamación [80].

4.2. Composición del cacao.

Algunos de los principales nutrientes del cacao son los hidratos de carbono, lípidos (ácidos grasos saturados e insaturados), vitaminas, fibra y minerales (magnesio, cobre, potasio, hierro) [84].

El cacao también contiene numerosos fitoquímicos entre los que se puede destacar a los polifenoles y metilxantinas por ser los más abundantes [85] (Tabla 2). Los polifenoles suponen hasta 12-18% del peso seco de la semilla, y son mayoritariamente flavanoles [80]. Así, los flavanoles más abundantes son la EC (328,1 mg/100 g de semilla), las procianidinas (108,7 mg/100 g de semilla cacao) y la catequina (72,4 mg/100 g de semilla) [86], además de contener trazas de otros flavanoles como (+)-galocatequina y (-)-epigalocatequina; por tanto, la EC es el flavanol monomérico más abundante del cacao (35% del contenido total de polifenoles) [87]. Además, el cacao contiene otros polifenoles que aparecen como minoritarios (trazas), y entre los que cabe mencionar a las antocianinas (3-α-L-arabinosidil cianidina y 3-β-L-galactosidil cianidina), flavonoles (quercetina, isoquercetina y quercetina-3-*O*-arabinosido), flavonas (luteolina) y flavanonas (naringenina) [85]. El cacao también contiene metilxantinas como teobromina (2,5% del peso seco) y cafeína (0,24% del peso seco) [84, 85].

4.3. Efectos biológicos de los polifenoles del cacao.

Los mecanismos de acción implicados en algunos de los efectos saludables producidos por el cacao parecen estar relacionados con su potente actividad antioxidante y con su capacidad para modular las vías de señales celulares.

***Tabla 2**: Fitoquímicos presentes en el cacao (Tabla adaptada de Kim et al [85])*

Flavanoles	(-)-Epicatequina (-)-Catequina (+)-Catequina (-)-Epicatequina-3-*O*-galato (+)-Galocatequina Procianidina B1 Procianidina B2 Procianidina B2-*O*-galato Procianidina B2-3,3-di-*O*-galato Procianidina B3 Procianidina B4 Procianidina B4-3-*O*-galato Procianidina C1 Procianidina D
Antocianinas	3-α-L-arabinosidil cianidina 3-β-D-galactosidil cianidina
Flavonoles	Quercetina Quercetina-3-*O*-arabinósido Quercetina-3-*O*-galactósido Isoquercetina
Flavonas	Luteolina Luteolina-7-*O*-hiperósido Orientina Isoorientina Vitexina Isovitexina
Flavanonas	Naringenina Naringenina-7-glucósido
Ácidos fenólicos	Ácido clorogénico Ácido vanílico Ácido cumárico Ácido cafeico Ácido felúrico Ácido fenilacético Ácido siríngico
	Clovamida (ácido fenólico) Deoxiclovamida (ácido fenólico) Dideoxiclovamida (ácido fenólico)
Metilxantinas	Teobromina Cafeína

Como consecuencia de dicha modulación en las vías de señales, los flavanoles del cacao pueden modificar algunas de las funciones celulares para producir efectos beneficiosos en ciertas enfermedades como puede ser la enfermedad cardiovascular, cáncer, diabetes, etc. [85].

4.3.1. Efectos del cacao sobre salud cardiovascular.

Los flavanoles del cacao parecen estar implicados en la reducción del riesgo cardiovascular a través de la inhibición de la agregación plaquetaria y de la mejora en el estado antioxidante, dado que disminuyen biomarcadores de estrés oxidativo como la oxidación de las LDL-Cho [84, 85]. Además, se ha demostrado que los compuestos fenólicos del cacao, y especialmente su grupo más abundante, los flavanoles, pueden reducir la producción del anión superóxido e incrementar la producción del óxido nítrico, lo que contribuye a aumentar la vasodilatación y, por tanto, a disminuir la hipertensión [84, 85]. En este sentido, en un estudio llevado a cabo con personas de la tribu de los indios *Kuna*, que probablemente son la comunidad que toma las mayores cantidades de cacao en todo el mundo, se observó que la dieta rica en cacao disminuía la presión arterial en los individuos de la población de esta tribu aislada. Sin embargo, cuando estos individuos emigraban a poblaciones urbanas, modificando su dieta, sus parámetros cardiovasculares se veían aumentados [88].

4.3.2. Efectos neuroprotectores del cacao.

Cho y colaboradores han demostrado que un extracto polifenólico obtenido a partir del cacao y la procianidina B2 protegen a las células neuronales PC12 frente la apoptosis inducida por hidroxinonenal al prevenir el aumento de la actividad caspasa-3 y de las JNK [89]. De manera similar, los flavonoides del cacao, como la EC, también parecen ejercer efectos neuroprotectores mediante la regulación de las MAPKs [90]. Un estudio reciente con personas sanas y jóvenes ha mostrado que la ingesta de cacao aumenta el flujo sanguíneo cerebral con una mejora cognitiva, lo que sugiere que este alimento podría jugar un papel en el tratamiento de la demencia y otras enfermedades neurodegenerativas asociadas [91, 92].

4.3.3. Efectos del cacao sobre la respuesta inmune y la inflamación.

El cacao es capaz de modular la respuesta inmunológica, ya que disminuye la activación de macrófagos y linfocitos y, como consecuencia, la secreción de sus mediadores inflamatorios [93]. En este sentido, se ha descrito que un extracto polifenólico de cacao inhibió la secreción de la proteína quimioatractante de monocitos (MCP-1) y TNFα, y este efecto fue mayor que el producido por una concentración equivalente de EC [94]. Además, el extracto de cacao y la EC disminuyeron los niveles de expresión génica de TNFα, IL-1 e IL-6 [94]. Más recientemente, se ha mostrado que un extracto polifenólico de cacao redujo los valores de expresión de varios marcadores de inflamación (secreción de IL-8, niveles de ciclooxigensas 2 y óxido nítrico sintasa inducible) inducidos por TNFα, a la vez que el extracto de cacao disminuyó la fosforilación de JNK y la translocación de NF-κB [95].

4.3.4. Efectos del cacao sobre la carcinogénesis.

Diversos estudios han puesto de manifiesto que los extractos polifenólicos obtenidos a partir del cacao son capaces de inhibir la transformación neoplásica inducida por diversos mitógenos [96, 97]. La EC también se relaciona con la modulación de vías de señales y factores de transcripción sensibles a cambios redox. Así, se ha descrito que este monómero mayoritario en el cacao activa señales de supervivencia en las células de hígado HepG2 a través de la modulación de las vías de las ERK y PI3K/AKT, y de los factores de transcripción Nrf2 y NF-κB [98]. En experimentos con ratas se ha comprobado que una dieta rica en cacao atenúa parcialmente el daño hepático inducido por el carcinógeno dietil-N-nitrosamina, así como el daño colónico causado por el carcinógeno azoximetano, a través de la modulación de las enzimas antioxidantes y de proteínas clave implicadas en la cascada de señalización celular asociadas a la muerte celular y la proliferación/supervivencia [99, 100].

4.3.5. Efectos del cacao sobre la obesidad.

En los últimos años se ha sugerido que los polifenoles del cacao podrían tener un efecto antiobesidad, dado que parecen afectar al peso corporal, el contenido de grasa corporal, los niveles séricos de lípidos y lipoproteínas, y las enzimas que regulan el metabolismo lipídico [27]. En este sentido, en un estudio en el que los animales de

experimentación recibían una dieta rica en grasa, aquellas ratas que se alimentaron con la dieta rica en cacao presentaron un descenso del peso corporal, del tejido adiposo blanco mesentérico y de los niveles de TG circulantes [101]. Además, los animales que recibían la dieta rica en cacao disminuyeron la expresión de varios genes relacionados con la síntesis y el transporte de los ácidos grasos en el hígado, e incrementaron la expresión de genes asociados con la termogénesis [101].

4.4. Efectos de los polifenoles del cacao en la resistencia a la insulina.

Diferentes estudios muestran que los flavanoles del cacao podrían mejorar la resistencia a la insulina y disminuir el riesgo de padecer diabetes y otras enfermedades relacionadas, ya que reducen el estrés oxidativo, modulan el metabolismo de la glucosa y la señalización de la insulina y/o regulan el metabolismo lipídico [26].

4.4.1. Efecto antioxidante de los polifenoles del cacao.

El cacao posee una cantidad superior de flavonoides por ración en comparación con otras bebidas ricas en polifenoles como el té verde (2-3 veces más), el té negro (4-5 veces más) y el vino tinto (2 veces más), lo que hace que su capacidad antioxidante sea mayor [102]. De acuerdo con esto, y dado su alto consumo, se considera que el chocolate negro constituye uno de los principales aportes de antioxidantes a la dieta [27, 102]. De hecho, España es el país donde se consume más cacao por persona (1668 g/persona/año), seguido de Noruega (1647 g/persona/año) y de Suecia (1288 g/persona/año) [87].

Numerosos estudios *in vitro* e *in vivo* muestran que el cacao y sus flavonoides ejercen un efecto antioxidante al neutralizar directamente los radicales libres [79, 86] y activar a las enzimas antioxidantes [90]. Así, el consumo de cacao y chocolate negro aumenta la capacidad antioxidante plasmática en animales y humanos [78, 103, 104] y la vasodilatación [79]. Además, la suplementación de la dieta con cacao o chocolate negro disminuye el estrés oxidativo en ratones obesos y diabéticos [105] y la oxidación de LDL-Cho en animales y humanos, lo que se ha relacionado con un retardo en el desarrollo de la ateroesclerosis [104, 106]. En este sentido, un estudio muestra que la EC podría ser la responsable de atenuar la oxidación de las LDL-Cho y reducir el daño

al endotelio [43]. También se ha descrito que un extracto polifenólico del cacao (CPE) protege a las células hepáticas humanas HepG2 frente al estrés oxidativo inducido por un potente pro-oxidante al disminuir la generación de ROS, la lipoperoxidación y restaurar las defesas antioxidantes (GSH, GPx y GR) [107]. Igualmente, CPE y EC parece que son capaces de proteger frente a la apoptosis, pues activan vías de supervivencia (ERK y PI3K/AKT) e inhiben rutas relacionadas con la muerte celular (JNK) [108-110]. Además, en una situación no patológica, la EC estimula la defensas antioxidantes mediante la regulación de factores de transcripción íntimamente relacionados con el estrés oxidativo (Nrf2 y NF-κB), lo que parece preparar a las células para hacer frente a un posible daño oxidativo en mejores condiciones [98].

4.4.2. Efectos sobre la señalización de la insulina y el metabolismo glucídico.

Los compuestos fenólicos pueden modular el metabolismo glucídico de varias maneras [24, 27]:

- inhibición de la digestión y absorción de los carbohidratos en el intestino;
- estimulación de la secreción de insulina por parte de las células β;
- modulación de la producción de glucosa desde el hígado;
- activación de los receptores de glucosa y de la captación de glucosa en los tejidos insulinodependientes.

En concreto, los flavanoles del cacao pueden inhibir las enzimas digestivas lipasa pancreática, α-amilasa pancreática, fosfolipasa A_2 y α-glucosidasa implicadas en la digestión de hidratos de carbono y lípidos *in vitro* [111, 112], y por tanto, disminuir la absorción de macronutrientes y la ingesta de energía en conjunto.

Distintos estudios *in vivo* muestran que el cacao tiene propiedades similares a las de la insulina, ejerce un efecto hipoglucemiante en ratas obesas y diabéticas [105, 113], y disminuye la inflamación relacionada con el síndrome metabólico [80]. De manera similar, otros estudios realizados con los componentes mayoritarios del cacao, como las procianidinas y la EC, muestran efectos antidiabéticos. La suplementación con un licor de procianidinas a ratones obesos y diabéticos disminuyó los niveles de glucosa plasmática de forma dosis dependiente [114]. También las procianidinas oligoméricas del cacao actuaron en el estómago de ratas alimentadas con una dieta grasa, ya que

inhibieron la digestión y la absorción de los macronutrientes, o activaron al péptido 1 similar al glucagón (GLP-1) y estimularon la función de la célula β y la señalización de la insulina [115]. Además, la suplementación de la dieta con EC disminuyó la infiltración de células del sistema inmune en los islotes de Langerhans en ratones no obesos, lo que previno la destrucción autoinmune de las células β pancreáticas, mejoró la tolerancia a la glucosa y disminuyó la hemoglobina glicosilada [116].

Distintos estudios en humanos han demostrado la capacidad del cacao para aumentar la secreción de insulina postprandial en sujetos sanos [117], y la mejora de la sensibilidad a la insulina, la presión sanguínea y la función vascular en personas sanas e intolerantes a la glucosa [21, 118].

4.4.3. Efectos sobre el metabolismo lipídico.

El consumo de cacao y chocolate negro está relacionado con la mejora del perfil lipídico y la reducción del riesgo de enfermedad cardiovascular [26, 84]. Diferentes estudios muestran que el cacao tiene un efecto hipocolesterolémico, ya que disminuye los niveles de TG, colesterol total (T-Cho) y colesterol LDL-Cho, y aumenta los de HDL-Cho en ratas diabéticas [105, 113]. Además, en voluntarios hipertensos, el consumo de 100 g/día de chocolate rico en flavanoles (88 mg) durante 2 semanas redujo los niveles de colesterol total y LDL-Cho [118], y la ingesta de cacao en polvo o chocolate negro aumentó los niveles de HDL-Cho y disminuyó la oxidación de LDL-Cho en voluntarios normo- e hiper-colesterolémicos [104] y en individuos sanos [106].

El cacao y los flavanoles del cacao pueden regular el metabolismo lipídico, ya que se pueden disminuir la expresión de genes implicados en la síntesis y el transporte de ácidos grasos, además de aumentar la lipolisis y la termogénesis en el hígado y el tejido adiposo [27, 101, 119, 120]. Igualmente, el cacao parece modular la síntesis de las apolipoproteínas en las células hepáticas e intestinales en cultivo, incrementando la síntesis de ApoA1 (principal proteína de las HDL-Cho) y disminuyendo la síntesis de ApoB (principal proteína de LDL-Cho) [121].

Todos estos estudios muestran que los flavanoles del cacao podrían mejorar la resistencia a la insulina al disminuir el estrés oxidativo y modular el metabolismo glucídico y lipídico en tejidos diana para la diabetes, lo que podría traducirse en un retraso en la aparición de la enfermedad. Además, ponen de manifiesto la importancia

de los compuestos fenólicos del cacao en la modulación de la expresión génica a través de la regulación de vías de señales celulares y de factores de transcripción en tejidos insulino-sensibles. Sin embargo, se han realizado pocos estudios que expliquen cómo los polifenoles del cacao modulan las vías de señales intracelulares para ejercer sus efectos.

Objetivos generales

Objetivos

La DMT2 es una enfermedad con gran incidencia en nuestra sociedad y que supone una gran carga socioeconómica y médica. Durante la resistencia a la insulina y la diabetes se alteran el metabolismo glucídico y lipídico, y el equilibrio redox por la aparición del estrés oxidativo. Estos y otros cambios que se producen en esta enfermedad están directamente relacionados con la desregulación de la señalización de la insulina y el desarrollo y progreso de esta patología.

En la actualidad, la prevención nutricional se considera una de las estrategias clave para la prevención de la DMT2, por lo que en los últimos años ha aumentado el interés por identificar compuestos de la dieta con actividad antidiabética, ya que suponen una alternativa prometedora para la prevención y/o tratamiento de esta enfermedad al presentar baja o nula toxicidad, ser abundantes en la naturaleza y baratos de producir.

El cacao y sus derivados como el chocolate se consumen comúnmente en un gran número de países de todo el mundo. Este alimento es una fuente importante de flavanoles, entre los que se podría destacar a la epicatequina, y parece presentar distintas propiedades potencialmente beneficiosas para la salud, como la actividad antidiabética. Sin embargo, hasta el momento son pocos los estudios que investigan los mecanismos de acción molecular responsables del efecto antidiabético de los flavanoles del cacao en el hígado, órgano clave para la homeostasis metabólica. Con este fin se plantearon los siguientes objetivos específicos, que se han desarrollado en un modelo celular (células hepáticas humanas HepG2) y un modelo animal de DMT2 (ratas ZDF):

1. Evaluar el efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico en condiciones fisiológicas y durante una situación de resistencia a la insulina y diabetes en las células hepáticas.
2. Analizar el posible efecto protector del cacao y la epicatequina y sus mecanismos moleculares de acción frente al estrés oxidativo durante la resistencia a la insulina y diabetes en las células hepáticas.
3. Estudiar el posible efecto beneficioso de los flavanoles del cacao sobre el metabolismo lipídico y sus mecanismos de acción en una situación de resistencia a la insulina y diabetes en las células hepáticas.

Resultados

Capítulo 1: Efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico hepático en condiciones fisiológicas y durante la resistencia a la insulina y la diabetes

Resumen

Antecedentes: El hígado es un órgano clave en la regulación del metabolismo y juega un papel determinante en la resistencia a la insulina y la DMT2. El cacao y la EC tienen propiedades antidiabéticas, y podrían ejercer un efecto beneficioso en el hígado frente la resistencia a la insulina. Sin embargo, se desconoce el mecanismo de acción molecular responsable de su efecto antidiabético en relación con una posible mejora de la sensibilidad a la insulina.

Objetivos: Determinar el efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico hepático en condiciones fisiológicas y durante una situación de resistencia a la insulina y diabetes.

Metodología: El estudio del efecto de los flavanoles del cacao en condiciones fisiológicas se realizó en las células hepáticas humanas HepG2. Las células se incubaron durante 24 horas con distintas concentraciones de EC (1-10 µM), que es el principal flavanol del cacao, y de un extracto polifenólico del cacao (CPE, 1-10 µg/mL), y se estudió el efecto sobre la viabilidad y proliferación celular, los niveles de proteínas clave de la ruta de la insulina y la producción de glucosa.

El estudio del posible efecto protector de los flavanoles del cacao durante la resistencia a la insulina se llevó a cabo mediante el uso de un modelo de resistencia a la hormona en las células HepG2 y en las ratas Zucker Diabetic Fatty (ZDF). Las células se preincubaron durante 24 horas con EC (10 µM) y CPE (1 µg/mL) y posteriormente con glucosa (30 mM, 24 horas). Las ratas se alimentaron con una dieta estándar (ZDF-C y Zucker Lean, ZL) y con una dieta enriquecida con un 10% de cacao (ZDF-Co) durante 9 semanas (6-15 semanas de vida). En ambos modelos experimentales se analizaron los niveles de proteínas clave de la ruta de señalización de la insulina y del metabolismo glucídico, así como la producción y captación de glucosa en las células. Además, en los animales, se monitorizaron la ingesta de comida y el peso, se analizaron parámetros bioquímicos (glucemia e insulinemia), y una semana antes del sacrificio se realizó el test de tolerancia a la glucosa.

Resultados: En condiciones fisiológicas, las distintas concentraciones de EC (1-10 µM) y CPE (1-10 µg/mL) no afectaron a la proliferación ni a la viabilidad celular, y activaron la ruta de señalización de la insulina, ya que incrementaron los niveles totales

y fosforilados en tirosina de IR, IRS-1 e IRS-2, los niveles fosforilados de AKT y GSK3 y disminuyeron los niveles fosforilados de GS. Todas las concentraciones de EC y CPE analizadas aumentaron los valores fosforilados de AMPK, aunque sólo CPE (10 µg/mL) incrementó los niveles de GLUT2. EC y CPE modularon la expresión de PEPCK, lo que condujo a un descenso de la producción de glucosa. Además, la inhibición de la gluconeogénesis hepática inducida por EC y CPE se previno por el bloqueo de AKT y AMPK.

En la resistencia a la insulina, la suplementación de la dieta con cacao, al igual que la incubación de las células con EC y CPE, protegieron del bloqueo de la ruta de señalización de la insulina en ambos modelos experimentales (hígado de las ratas ZFD y células HepG2 resistentes a la insulina) desde los primeros eslabones de la vía, ya que previnieron la inhibición de IR, IRS-1 y -2, la ruta PI3K/AKT y de la AMPK. Además, la dieta rica en cacao en los animales, y EC y CPE en las células, mejoraron la funcionalidad de los hepatocitos, dado que impidieron el incremento de los niveles de PEPCK y restauraron el contenido de glucógeno y los niveles de GLUT2 a valores similares a los de los controles. En este sentido, en las células HepG2, CPE y EC previnieron el descenso en la captación de glucosa y el aumento en la producción de glucosa causado por la alta dosis de glucosa, mientras que la dieta rica en cacao incrementó los niveles de GK en el hígado de los animales ZDF. Además, la dieta rica en cacao suprimió la activación de las JNK y p38-MAPK causada por la resistencia a la insulina.

Conclusión: Estos resultados demuestran que los flavanoles del cacao poseen un efecto similar a la insulina que contribuye a fortalecer la señalización de la hormona al activar su receptor, los IRS, la vía PI3K/AKT y AMPK e inhibir la producción de glucosa en situaciones fisiológicas y con concentraciones fácilmente alcanzables con la dieta. Además, en condiciones de resistencia a la insulina, el cacao y la EC mejoran la homeostasis glucídica y la sensibilidad hepática a la hormona al prevenir o retrasar el bloqueo de la ruta de la insulina y mantener la funcionalidad de las células hepáticas. Estos estudios muestran nuevos conocimientos sobre el mecanismo molecular de acción de los flavanoles del cacao en relación con su efecto antidiabético, y sugieren que podrían ser útiles para prevenir la DMT2 o mejorar la enfermedad.

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RESEARCH ARTICLE

Cocoa flavonoids improve insulin signalling and modulate glucose production via AKT and AMPK in HepG2 cells

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Scope: Cocoa and (–)-epicatechin (EC), a main cocoa flavanol, have been suggested to exert beneficial effects in diabetes, but the mechanism for their insulin-like effects remains unknown. In this study, the modulation of insulin signalling by EC and a cocoa phenolic extract (CPE) on hepatic HepG2 cells was investigated by analysing key proteins of the insulin pathways, namely insulin receptor, insulin receptor substrate (IRS) 1 and 2, PI3K/AKT and 5′-AMP-activated protein kinase (AMPK), as well as the levels of the glucose transporter GLUT-2 and the hepatic glucose production.

Methods and results: EC and CPE enhanced the tyrosine phosphorylation and total insulin receptor, IRS-1 and IRS-2 levels and activated the PI3K/AKT pathway and AMPK in HepG2 cells. CPE also enhanced the levels of GLUT-2. Interestingly, EC and CPE modulated the expression of phosphoenolpyruvate carboxykinase, a key protein involved in the gluconeogenesis, leading to a diminished glucose production. In addition, EC- and CPE-regulated hepatic gluconeogenesis was prevented by the blockage of AKT and AMPK.

Conclusion: Our data suggest that EC and CPE strengthen the insulin signalling by activating key proteins of that pathway and regulating glucose production through AKT and AMPK modulation in HepG2 cells.

Keywords:
Cocoa / Epicatechin / Glucose production / HepG2 cells / Insulin signalling pathway

1 Introduction

Diabetes is one of the most common chronic diseases in nearly all countries, and it is continuing to be an increasing international health burden [1]. Current medications are not adequately effective in maintaining long-term glycemic control in most patients. Therefore, there is an urgent need to continue working on the prevention and control of diabetes. In this regard, a promising approach is the use of natural compounds with insulin-like activity, which have been proposed as potential therapeutic agents in the prevention and/or treatment of this disease [2].

The liver has an important role in the control of the whole body metabolism of energy nutrients. In diabetes, the insulin target tissues are damaged, which aggravates the ability of insulin to trigger downstream metabolic actions resulting in insulin resistance. In this regard, one of the hallmarks of diabetes is the alteration of the hepatic metabolism: the liver is not able to control the glucose homeostasis, which is one of the causes for the hyperglycemia, and there is a miss-regulation of the insulin pathway in this organ [3].

Insulin signal transduction is initiated when insulin binds to the insulin receptor (IR), which leads to the stimulation of several intracellular protein substrates including

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Abbreviations: AMPK, 5′-AMP-activated protein kinase; **CPE**, cocoa phenolic extract; **EC**, (–)-epicatechin; **EGCG**, epigallocatechin gallate; **G6Pase**, glucose-6-phosphatase; **GS**, glycogen synthase; **GSK-3**, glycogen synthase kinase-3; **IR**, insulin receptor; **IRS**, insulin receptor substrate; **PEPCK**, phosphoenolpyruvate carboxykinase; **PI3K**, phosphatidylinositol-3-kinase

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insulin receptor substrate (IRS)-1 and IRS-2. This triggers the phosphatidylinositol-3-kinase (PI3K) pathway that stimulates AKT and leads to the inhibition of glycogen synthase kinase-3 (GSK-3) by phosphorylation, which subsequently phosphorylates and inactivates glycogen synthase (GS), and conducts the modulation of other proteins necessary for the acute metabolic effects of insulin [3]. In addition, in the hepatocyte, AKT might phosphorylate the transcription factor forkhead box protein O1, restraining the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and inhibiting the gluconeogenesis [3]. The 5′-AMP-activated protein kinase (AMPK) also constitutes a central regulator of the cellular metabolism, able to suppress the hepatic gluconeogenesis [4]; in fact numerous drugs that are in clinical use for treatment of type 2 diabetes activate AMPK [4, 5].

The (–)-epicatechin (EC) is one of the most abundant flavonoids in human diet, being present in high concentrations in grapes, cocoa, tea, and many other fruits and vegetables [6, 7]. Different studies have shown that EC is able to interfere with the oxidative/antioxidative potential of hepatic cells [6, 8], induces survival/proliferation pathways [8–10], exerts insulin-like activities [11], and improves insulin sensitivity [12], blood glucose levels [13] and parameters related to the inflammation in cardiovascular disease and diabetes [12, 14, 15]. Similarly, dark chocolate or cocoa, which are widely consumed [16] and are a rich source of flavonoids, especially EC [6], have been reported to protect against carcinogenesis [7,17], improve the allergy process [18], cardiovascular status [16, 19, 20], insulin sensitivity and hyperglycemia [19, 21] in humans and experimental animals. However, the precise mechanism for the preventive activities of EC and cocoa related to glucose metabolism and insulin signalling in the liver remains largely unknown.

The present work evaluates the effects of EC and a cocoa phenolic extract (CPE) on insulin signalling pathways and glucose production in HepG2 cells. This study demonstrates that EC and CPE have insulinomimetic activities in the human hepatic HepG2 cells, as EC and CPE enhance the tyrosine phosphorylation and total IR, IRS-1 and IRS-2 levels, which are associated to activation of the PI3K/AKT pathway and AMPK. We have also found that AKT and AMPK are required to modulate the hepatic glucose production.

2 Materials and methods

2.1 Materials and chemicals

(–)-EC (>95% of purity), Compound C (6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), anti-mouse IgG-agarose, sodium lactate, sodium pyruvate, gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical (Madrid, Spain). Cell proliferation ELISA BrdU (colorimetric) assay kit was from Roche Diagnostics (Barcelona, Spain). Anti-AKT and anti-phospho-Ser473-AKT detecting levels of phospho- and total AKT 1–3, anti-AMPK and anti-phospho-Thr172-AMPK, as well as anti-GSK-3 α/β and anti-phospho-GSK-3 α/β recognising phosphorylated Ser21/9 of GSK-3, anti-GS and anti-phospho-GS recognising phosphorylated Ser641 of GS, anti-IRS-2 and anti-β-actin were obtained from Cell Signalling Technology (Izasa, Madrid, Spain). Anti-IR β, anti-PEPCK and anti-Tyr(P) (PY20) were purchased from Santa Cruz (sc-711, sc-32879 and sc-508, respectively, Quimigen, Madrid, Spain). Anti-IRS-1 and anti-GLUT-2 were from Millipore (Madrid, Spain). Materials and chemicals for electrophoresis were from BioRad (BioRad Laboratories S.A., Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively.

2.2 Cocoa polyphenol extraction

Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. Soluble polyphenols were extracted by sequentially washing 1 g of sample with 40 mL of 16 mM hydrochloric acid in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 mL of acetone:water (70:30, v/v, 1 h at room temperature, constant shaking). After centrifugation (15 min, 3000 g), supernatants from each extraction step were combined and made up to 100 mL. The desiccated extract was dissolved in distilled water and kept frozen until assay. A detailed description of this CPE is given elsewhere [6, 22]. Accordingly, the amount of EC and polyphenols present in the CPE were 383.5 mg/100 g (determined by LC-MS) and 2 g/100 g on dry matter basis (determined by Folin-Ciocalteu) [6]. Concentrations of EC in tested doses of CPE range from 13.2 nM (in the dose of 1 μg CPE/mL) to 132 nM (in that of 10 μg CPE/mL) [6].

2.3 Cell culture and treatments

Human HepG2 cells were grown in DMEM-F12 medium supplemented with 2.5% foetal bovine serum and the following antibiotics: gentamicin, penicillin and streptomycin (50 mg/L). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. One day after plating, the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and foetal bovine serum, and the culture was continued. Subsequently, the experimental treatment was carried out for the indicated periods with various concentrations of EC or CPE in serum-free media containing 5.5 mM D-glucose, 2 mM glutamine.

Cells were treated with different concentrations of EC (1–20 μM) or CPE (1–20 μg/mL) diluted in serum-free culture medium during 24 h. In the experiments with the pharmacological inhibitors, cells were preincubated with 25 μM

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LY294002 or 40 μM Compound C for 1 h prior to 24 h of EC or CPE treatment.

2.4 Cell viability assay

Cell viability was determined by using the crystal violet assay [8]. HepG2 cells were seeded at low density (10^4 cells per well counted in a Neubauer chamber) in 96-well plates, grown for 20 h and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with water, allowed to dry, and 1% SDS added. The absorbance of each well was measured using a microplate reader at 570 nm.

2.5 Cell proliferation assay (5-bromo-2′-deoxyuridine assay (BrdU))

A colorimetric immunoassay (ELISA) was used for the quantification of cell proliferation [8]. This method is based on the measurement of BrdU incorporation into genomic DNA during DNA synthesis of proliferating cells. HepG2 cells were seeded (10^4 cells per well counted in a Neubauer chamber) in 96-well plates, grown 20 h and labelled with the addition of BrdU for 4 h. Then the anti-BrdU antibody was added and the immune complexes were quantified by measuring the absorbance at 620 nm in a microplate reader.

2.6 Preparation of cell lysates

Cells were lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin and 1 mM PMSF. The supernatants were collected, assayed for protein concentration by using the Bio-Rad (Bio-Rad) protein assay kit according to the manufacturer's specifications, aliquoted and stored at −80°C until used for immunoprecipitation and/or Western blot analyses.

2.7 Immunoprecipitation

Protein extracts containing 200 μg of protein were immunoprecipitated overnight at 4°C with gentle rotation in the presence of 2–5 μg of anti-Tyr(P) (PY20) antibody, followed by the addition of anti-mouse IgG-agarose. After mixing for 2 h, the pellets were collected by centrifugation, and the supernatants were discarded. Then the pellets were washed and saved for Western blot analyses.

2.8 Western blot analysis

Equal amounts of proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters (Bio-Rad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare, Madrid, Spain) or anti-mouse (Sigma, Madrid, Spain) immunoglobulin. Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin and bands were quantified using a scanner and accompanying software.

2.9 Glucose production assay

HepG2 cells were seeded in 24-well plates (2×10^5 cells per well counted in a Neubauer chamber) and treated in serum-free DMEM with 10 μM EC or 1 μg/mL CPE for 24 h. The medium was then replaced with glucose production buffer consisting of glucose-free DMEM (pH 7.4), without phenol red (Invitrogen, Madrid, Spain), supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate, as previously described [23,24]. After a 3-h incubation, medium was collected and glucose concentration measured with a colorimetric glucose assay kit (Sigma). The readings were then normalized to the total protein content determined from the whole-cell lysates.

2.10 Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $p < 0.05$ was considered significant. An SPSS version 19.0 program has been used.

3 Results

3.1 Cell viability and proliferation

To determine the potential effects on cell viability and proliferation of EC and CPE in a human hepatic cell line (HepG2), cells were exposed to a range of concentrations (0–20 μM and 0–20 μg/mL, respectively) for 24 h.

Treatment of HepG2 cells for 24 h with EC or CPE did not evoke changes in cell viability, as determined by the crystal violet assay, indicating that the concentrations selected for the study did not damage cell integrity during the period of incubation (Table 1). Similarly, treatment with EC or CPE did not affect cell growth, indicating no impairment of cell proliferative machinery and preservation of a regular cell cycle (Table 1). Since none of the tested doses induced cell toxicity,

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Table 1. Effects of EC and CPE on cell viability and cell proliferation. Cell viability was determined as relative percent of Crystal Violet stained control cells. Cell proliferation was calculated as percentage of the relative increase over the control values of BrdU incorporated into genomic DNA.

	Cell viability (percentage of viable cells)	Cell proliferation (percentage of controls)
C	100.5 ± 10.1[a]	100.0 ± 7.8[a]
EC (μM)		
1	92.2 ± 6.3[a]	100.8 ± 9.2[a]
5	103.7 ± 5.3[a]	100.2 ± 7.8[a]
10	104.7 ± 10.9[a]	104.1 ± 8.7[a]
20	108.7 ± 7.2[a]	102.4 ± 8.2[a]
CPE (μg/mL)		
1	95.0 ± 4.8[a]	93.2 ± 7.5[a]
5	97.3 ± 6.3[a]	93.9 ± 12.6[a]
10	98.0 ± 9.4[a]	97.5 ± 13.3[a]
20	107.2 ± 7.8[a]	91.3 ± 6.4[a]

Data represent means ± SD of eight to ten samples. Means for each antioxidant with a common letter are equal, $p < 0.05$.

the lowest and realistic range of concentrations was selected for further studies [6, 22].

3.2 Effects of EC and CPE on tyrosine phosphorylation and protein levels of IR and its substrates IRS-1 and IRS-2

The modulation of IR and its downstream substrates IRS-1 and IRS-2 is essential for recruiting and activating downstream pathways [25]. In fact, in hepatic insulin resistance, the insulin-stimulated-IR and IRS tyrosine phosphorylation is defective and results in reduced IRS-associated PI3K activities [3]. To test the effect of EC and CPE on tyrosine phosphorylation and total levels of IR and its substrates, HepG2 cells were exposed for 24 h to various concentrations of these substances.

As shown in Fig. 1, IR and IRS-2 phosphorylated and total protein levels equally increased with all concentrations of EC and CPE tested, as well as the phosphorylated and total protein levels of IRS-1 when cells were incubated with CPE. EC dose dependently increased the phosphorylated and total levels of IRS-1 (Fig. 1A and C). All these results suggest that EC and CPE could strengthen the insulin signalling cascade by upregulating the early steps of this pathway.

3.3 Effects of EC and CPE on AKT, GSK-3 and GS phosphorylation

AKT is the molecular key in mediating the metabolic effects of insulin signalling [25]. It lays downstream of PI3K and facilitates glucose uptake and glycogen synthesis in the liver [25]. To test the modulation of AKT by EC and CPE, phosphorylated and total AKT were evaluated in cell lysates by Western blot analysis. As shown in Fig. 2A and B, the treatment of cells with EC and CPE for 24 h increased the phosphorylation of AKT with all concentrations tested. EC (10 μM) showed higher levels of p-AKT than lower concentrations of EC, whereas all doses of CPE induced a similar enhancement in p-AKT values.

Since AKT directly contributes to the activity of GS [25], the effect of EC and CPE on the phosphorylated and total protein expression levels of GSK-3 and GS was assayed. Western blot analysis of GSK-3 and p-GSK-3 proteins showed two bands corresponding to the α and β isoforms. EC and CPE treatment enhanced the phosphorylated isoforms, as illustrated in Fig. 2A and C. EC (10 μM) induced the highest increase in the levels of p-GSK-3 in comparison to the other doses of EC, and CPE equally enhanced the levels of p-GSK-3 with all concentrations assayed, in agreement to what was observed for AKT (Fig. 2A and B).

As shown in Fig. 2A and D, p-GS levels decreased in the presence of EC or CPE with all concentrations tested. As it could be expected according to the previous results, CPE equally diminished the phosphorylated levels of GS and 10 μM EC induced the most remarkable diminution of p-GS of all EC concentrations assayed. There was no difference in the total levels of AKT, GSK-3 and GS.

3.4 Effects of EC and CPE on AMPK phosphorylation

To continue the study of the potential effect of EC and CPE on key proteins of the insulin signalling, AMPK, which is a sensor of energy status for maintaining cellular energy homeostasis [5], was evaluated by analysing the total and phosphorylated levels in total cell lysates by Western blot analysis. Figure 3 illustrates that a 24-h treatment with EC or CPE induced an increase in AMPK phosphorylated levels. EC (5 and 10 μM) showed higher levels of p-AMPK than the lowest concentration of EC, whereas CPE induced a similar activation of AMPK with all concentrations tested. The protein levels of total AMPK were not modified by EC or CPE treatment.

3.5 Effects of EC and CPE on GLUT-2 protein levels

The GLUT-2 transporter mediates the diffusion of glucose across the plasma membrane of the hepatocyte, maintaining intracellular glucose in equilibrium with extracellular glucose [3]. To test whether this transporter was affected by EC and CPE, HepG2 cells were incubated with the selected concentrations for 24 h.

Treatment of HepG2 cells with any of the three concentrations of EC did not affect the expression levels of GLUT-2, and only 10 μg/mL CPE increased the levels of GLUT-2 after a 24-h incubation (Fig. 4).

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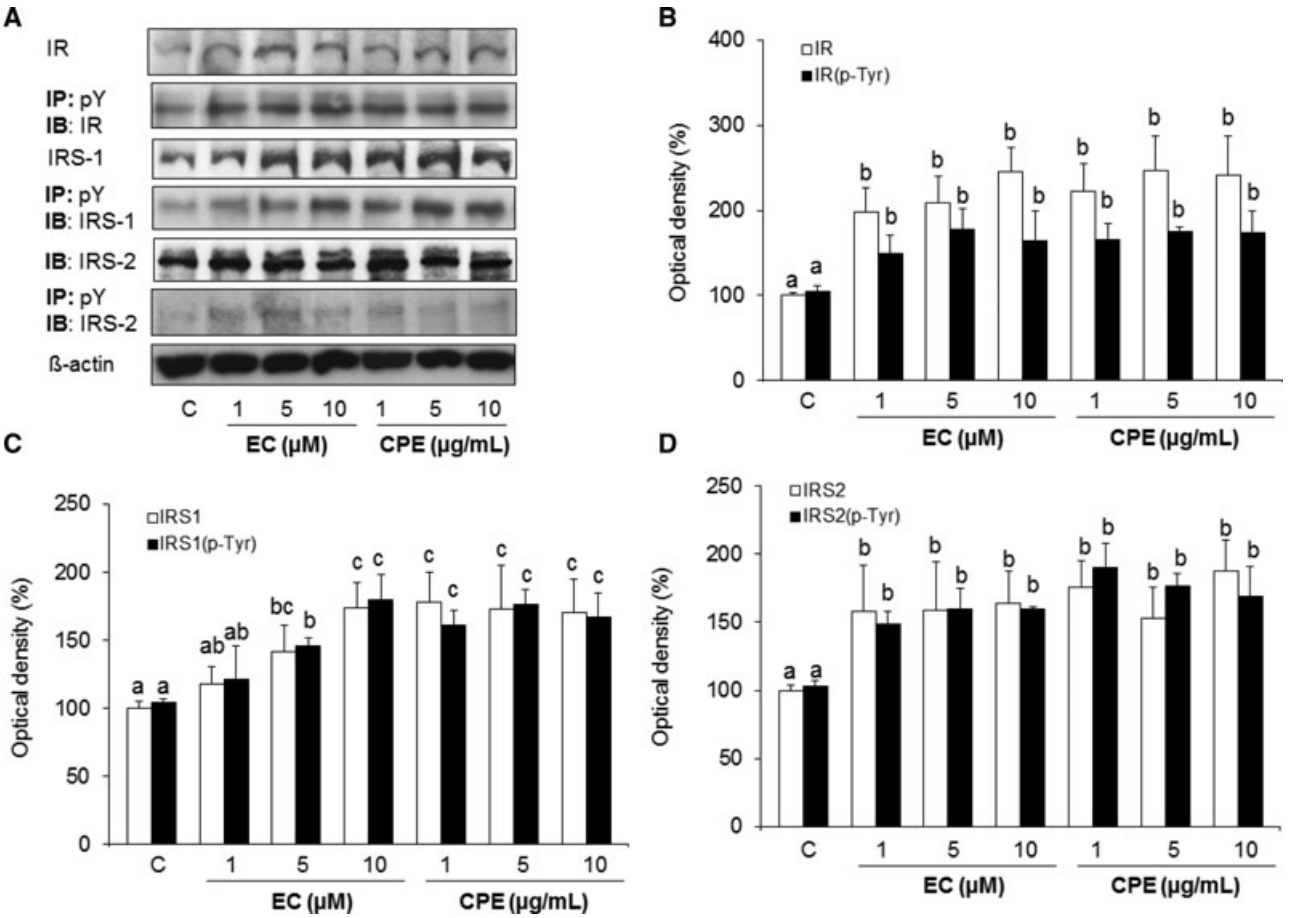

Figure 1. Effect of EC and CPE on phosphorylated and total levels of insulin receptor (IR), IRS-1 and IRS-2 in HepG2 cells after 24 h of treatment. (A) Bands of representative experiments. Densitometric quantification of (B) p-IR and total IR, (C) p-IRS-1 and total IRS-1 and (D) p-IRS-2 and total IRS-2. Protein extracts were subjected to immunoprecipitation with the anti-phospho-tyrosine (P-Tyr) antibody. The resulting immunocomplexes were analysed by Western blot (WB) with the anti-IR or IRS-1 or IRS-2 antibody. Values are expressed as a percentage relative to the control condition (means ± SD, $n = 7$–9). Equal loading of Western blots was ensured by β-actin. Means (for the phosphorylated or total protein levels) without a common letter differ significantly ($p < 0.05$).

3.6 Effect of EC and CPE activation of AKT on hepatic gluconeogenesis

In the liver, insulin-activated AKT inhibits the expression of PEPCK and G6Pase and, therefore, gluconeogenesis [3]. In view of the increased levels of p-AKT induced by EC and CPE, it was studied whether both substances were able to modulate the expression of a major enzyme responsible of the regulation of gluconeogenesis, such as PEPCK, as well as the production of glucose. To this end, HepG2 cells were exposed to a selective inhibitor of AKT (LY294002) and EC or CPE and the levels of PEPCK and the novo production of glucose were assayed. The concentrations selected for these analyses were the doses that exhibited a prominent effect on the activation of the PI3K/AKT pathway, i.e.: 10 μM for EC and 1 μg/mL for CPE.

Treatment of HepG2 cells with EC or CPE showed a comparable decrease in the expression of PEPCK (Fig. 5A and B). LY294002 alone increased the levels of PEPCK, although these values decreased by the addition of EC and CPE (Fig. 5A and B). LY294002 treatment decreased p-AKT levels in all cells incubated with this inhibitor, although p-AKT values were partly recovered in the presence of EC or CPE when compared to untreated cells (Fig. 5A and C). p-AMPK and total expression of AKT and AMPK were not affected by LY294002 (Fig. 5A, C and D). EC and CPE decreased the production of glucose (Fig. 5E). In addition, the presence of the AKT selective inhibitor alone increased the hepatic glucose production, and this enhancement was less prominent in LY+EC- and LY+CPE-treated cells (Fig. 5E). Conversely, LY294002 could not totally block the inhibitory effect of EC and CPE on PEPCK and glucose production, indicating that other pathways may also be involved in EC- and CPE-induced effects.

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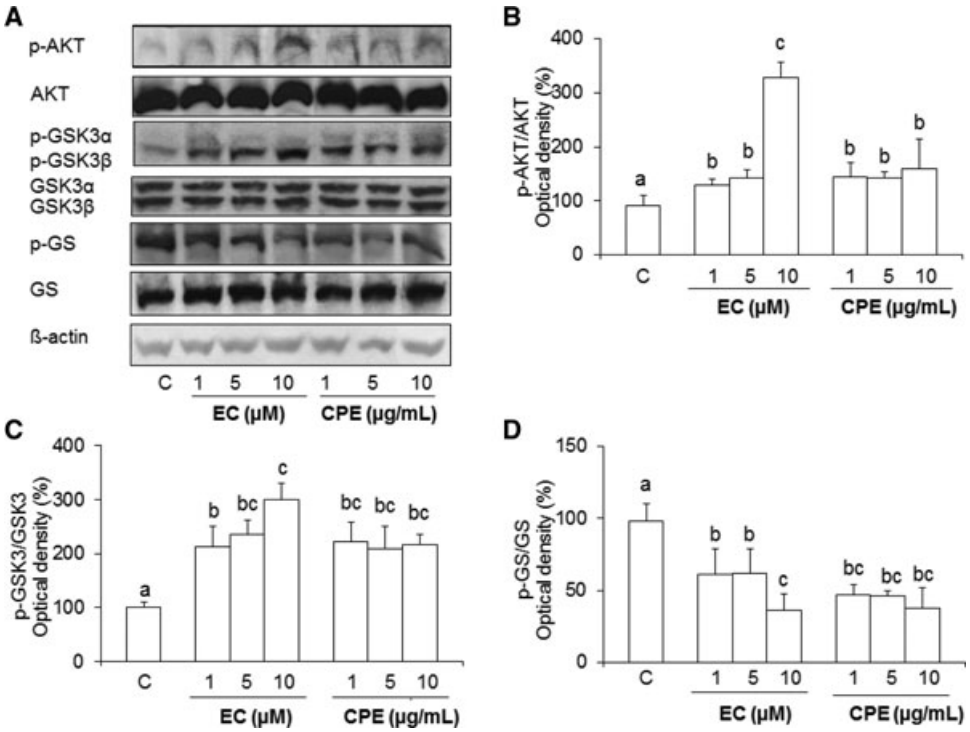

Figure 2. Effect of EC and CPE on levels of phosphorylated and total AKT, glycogen synthase kinase-3 (GSK-3) and glycogen synthase (GS) in HepG2 cells. (A) Bands of representative experiments. Percentage data of (B) p-AKT/AKT, (C) p-GSK-3/GSK-3 and (D) pGS/GS ratios relative to controls. Values are expressed as a percentage relative to the control condition and are means ± SD, $n = 6–9$. Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($p < 0.05$).

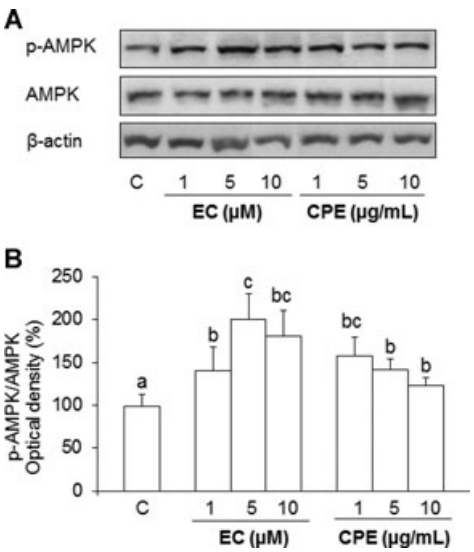

Figure 3. Effect of EC and CPE on phosphorylated and total 5′-AMP-activated protein kinase (AMPK) levels in HepG2 cells. (A) Bands of representative experiments. (B) Percentage values of p-AMPK/AMPK ratio relative to the control condition (means ± SD, $n = 7–8$). Equal loading of Western blots was ensured by β-actin. Different letters over bars indicate statistically significant differences ($p < 0.05$).

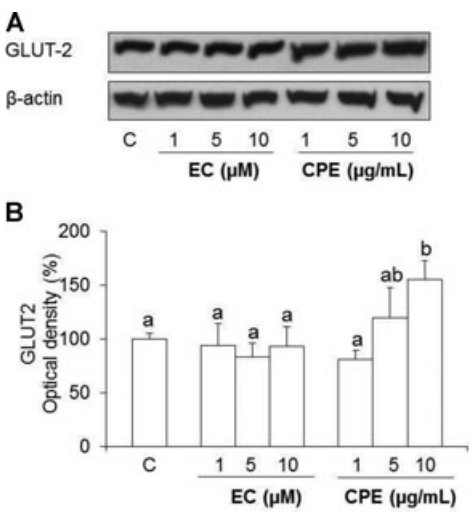

Figure 4. Effect of EC and CPE on GLUT-2 levels in HepG2 cells. (A) Bands of representative experiments. (B) Densitometric quantification of GLUT-2. Values are expressed as a percentage relative to the untreated control condition and are means ± SD, $n = 7–8$. Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($p < 0.05$).

3.7 Effect of EC and CPE activation of AMPK on hepatic gluconeogenesis

AMPK is another known suppressor of hepatic gluconeogenesis [3]. To examine the role of AMPK on the modulation of the levels of PEPCK and the production of glucose, AMPK was blocked by a selective chemical inhibitor (Compound C) prior to the treatment with EC or CPE. As mentioned above, the concentrations selected for these analyses were the previously chosen (10 μM for EC and 1 μg/mL for CPE), since they showed a prominent effect on the activation of AMPK.

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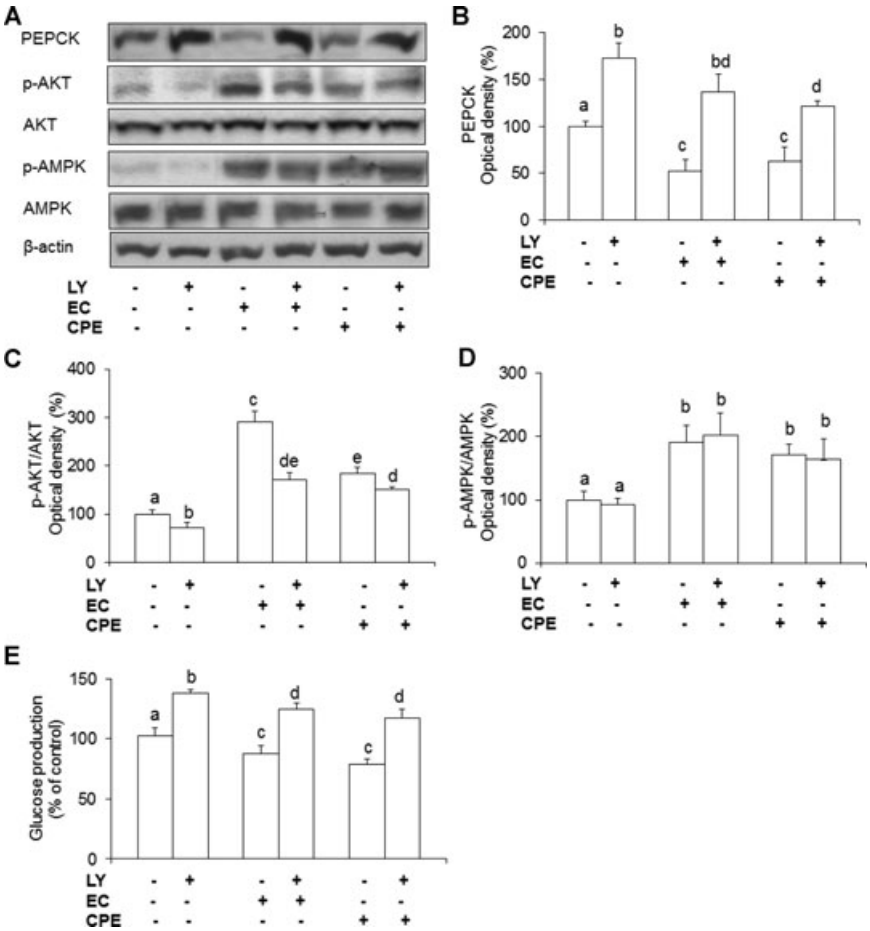

Figure 5. Effect of EC and CPE and selective inhibitor LY (LY294002) on levels of PEPCK, AKT and AMPK, and glucose production. HepG2 cells were incubated in the presence or absence of 25 μM LY for 1 h and later with 10 μM EC or 1 μg/mL CPE for 24 h. (A) Bands of representative experiments. Percentage data of (B) PEPCK, (C) p-AKT/AKT and (D) p-AMPK/AMPK relative to the control condition (means ± SD, n = 6–9). Equal loading of Western blots was ensured by β-actin. (E) Glucose production was expressed as percent of control and are means ± SD of 10–15 different samples per condition. Different letters over bars indicate statistically significant differences ($p < 0.05$).

As illustrated in Fig. 6A and B, EC and CPE decreased the levels of PEPCK, and the blockage of AMPK induced a significant increase in the expression of the mentioned gluconeogenic enzyme. However, this enhancement was less pronounced in the presence of EC and CPE in comparison to that induced by the compound C alone. Treatment with compound C alone decreased p-AMPK levels, whereas incubation with EC or CPE and compound C partly recovered p-AMPK levels (Fig. 6A and D). p-AKT and total AKT and AMPK remained unaltered after incubating the cells with compound C (Fig. 6A, C and D). In addition, compound C enhanced the glucose production via hepatic gluconeogenesis (Fig. 6E). Similarly, the increase in glucose production was higher in compound C-treated cells than in C+EC- and C+CPE-treated cells (Fig. 6E). However, compound C could not completely block the inhibitory effect of EC and CPE on PEPCK and glucose production, indicating that other pathways may also contribute to the beneficial effects of EC and CPE.

4 Discussion

Flavonoids have been found to possess beneficial effects on health and have drawn attention because of their safety and accumulating evidence on their antidiabetic effects in animals and humans [2]. In the current study, we have demonstrated for the first time that EC and CPE enhanced the tyrosine phosphorylated and total IR, IRS-1 and IRS-2 levels together with an activation of the PI3K/AKT pathway and AMPK (Fig. 7). We have also provided evidence on the insulin-like activity of EC and CPE, which were able to downregulate the levels of the key gluconeogenic enzyme PEPCK and modulate the hepatic glucose production through AKT and AMPK (Fig. 7). HepG2 cells are widely used for biochemical and nutritional studies as a cell culture model of human hepatocytes since they retain their morphology and most of their function in culture [26–28]. Thus, this cell line has been extensively used to study the hepatic glucose production and the modulation of the insulin pathway in vitro [27–30].

The liver plays a critical role in maintaining blood glucose concentration both through its ability to supply glucose to the circulation via glycogenolysis and gluconeogenesis in the postabsorptive state and to remove glucose from the circulation after meal ingestion [3]. However, in diabetes, the gluconeogenic pathway is aberrantly activated, supplies a relatively larger amount of glucose into the circulation [2, 3], and there is also hepatic insulin resistance [3]. Therefore, the modulation of the mentioned targets could be beneficial for

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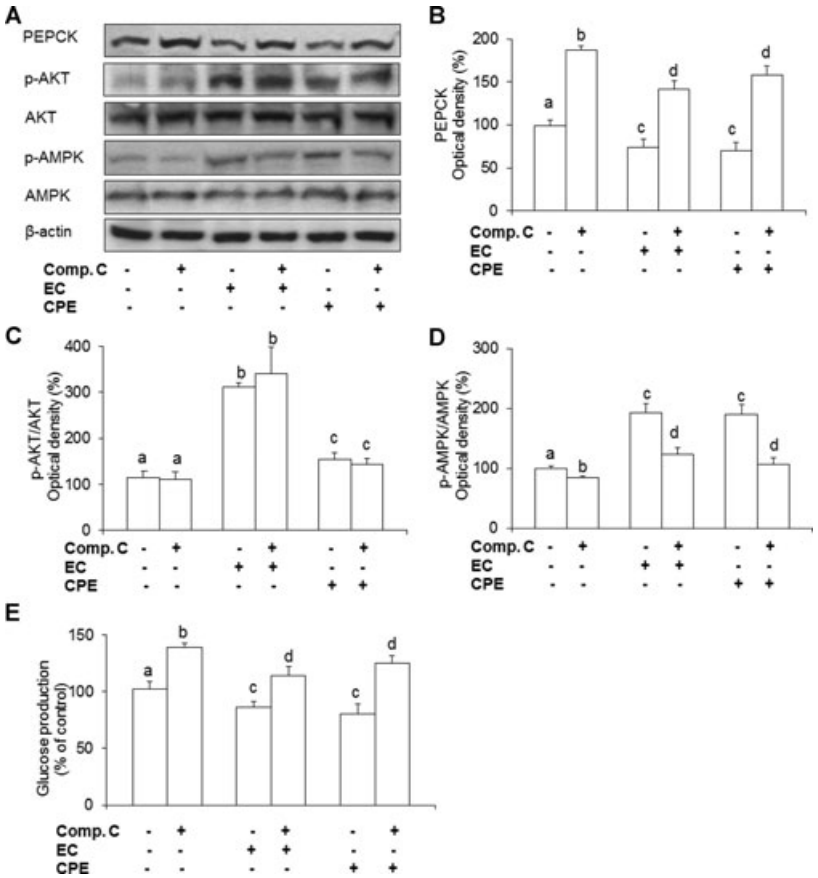

Figure 6. Effect of EC and CPE and selective inhibitor Compound C (Comp. C) on levels of PEPCK, AKT and AMPK, and glucose production. HepG2 cells were incubated in the presence or absence of 30 μM Comp. C for 1 h and later with 10 μM EC or 1 μg/mL CPE for 24 h. (A) Bands of representative experiments. Densitometric quantification of (B) PEPCK levels, (C) p-AKT/AKT and (D) p-AMPK/AMPK. Values are expressed as a percentage relative to the control condition and are means ± SD, $n = 6$–10. Equal loading of Western blots was ensured by β-actin. (E) Glucose production was expressed as percent of control and are means ± SD of 10–16 different samples per condition. Means without a common letter differ significantly ($P < 0.05$).

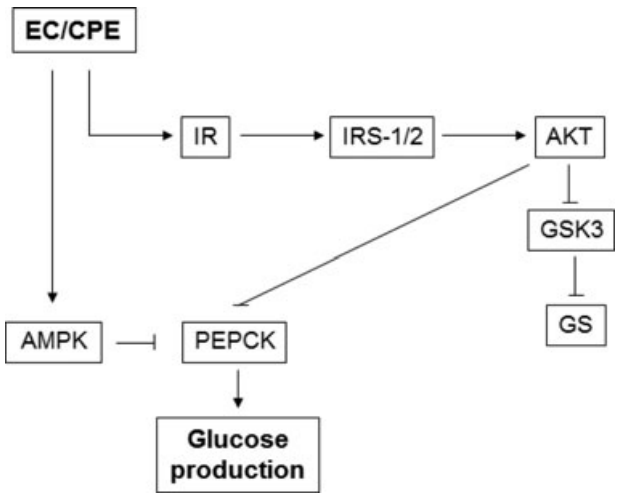

Figure 7. Schematic overview showing the EC- and CPE-induced insulin and glucose signalling pathways analysed in HepG2 cells. Arrows indicate positive inputs (activation), whereas perpendicular lines show negative inputs (inhibition).

the prevention and control of this disease; in this line, natural compounds such as flavonoids could play a major role, although little investigation at the molecular level has been performed.

In the present study, it is shown that EC and CPE increased tyrosine phosphorylation and total levels of IR and IRS in HepG2 cells. In concert, epigallocatechin gallate (EGCG) and naringenin attenuated high glucose-induced signalling blockage by reducing IRS-1 serine phosphorylation in HepG2 cells [27] and by activating IRS-1 in primary hepatocytes of mice with metabolic syndrome [31]. In addition, green tea polyphenols increased IRS-2 mRNA levels in the liver [32], as well as IR and IRS-1 and -2 levels in the myocardium of insulin-resistant rats [33]. Similarly, oligomers of a grape-seed procyanidin extract increased IR and IRS levels in preadipocytes [34]. In this line, the oligomeric structures of the extract activated the IR by interacting with and inducing its tyrosine phosphorylation [34]. ECGC and rutin stimulated the IRS2 signalling by enhancing tyrosine phosphorylation under high glucose condition on pancreatic β cells [35], and in 3T3-L1 preadipocytes a CPE did not affect the levels of IR but it modulated its IR kinase activity via direct binding [36].

The activation of IRS-1/-2 initiates the stimulation of the PI3K/AKT pathway, which is needed for the metabolic effects of insulin in the liver and is responsible of the inhibition of GSK-3 and activation of GS [3]. In this regard, EC and CPE seemed to mimic the metabolic actions of insulin, as they increased the phosphorylated levels of AKT and GSK-3, and decreased p-GS values. Previous studies have also

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demonstrated the stimulation of AKT by EC and CPE in HepG2 cells [10,22], and EC was reported to partly reverse the inhibition of AKT phosphorylation induced by high glucose levels [27]. Similarly, naringenin activated the AKT pathway in HepG2 cells [29] and green tea polyphenols increased the RNA expression levels of PI3K/AKT in the liver of insulin-resistant rats [32]. In this line, green tea polyphenols also increased the mRNA levels of GSK-3 in the liver of insulin-resistant rats [32]. However, a decrease in the expression of GSK-3 and an increase in the mRNA levels of GS have been reported in the myocardium of insulin-resistant rats [33]. All together indicates that polyphenols reinforced the signal pathways responsive to insulin.

In the liver, GLUT-2 is a glucose-sensitive gene that mediates both influx and efflux of glucose across the plasma membrane [3]. In the present study, levels of GLUT-2 were induced by the highest concentration of CPE tested, whereas EC did not show any effect on the modulation of the protein levels of this transporter. Although the influence of the phenolic compounds on the GLUTs has been scarcely evaluated, especially in the liver, in agreement with the present results, no effects on the mRNA GLUT-2 levels in the liver of insulin-resistant rats fed with green tea polyphenols have been reported [32]. However, it should be mentioned that, as occurs for 10 μg/mL CPE, the grape polyphenol resveratrol and the antidiabetic drugs pioglitazone and rosiglitazone, which are peroxisome proliferator-activated receptor-γ agonists, were able to increase GLUT-2 expression in β-cells [37, 38]. In this line, our results could suggest that CPE could more efficiently contribute to glucose uptake from the blood when glucose levels are postprandially elevated in comparison with EC, as described for rosiglitazone [37]. This effect has been related to the regulation of peroxisome proliferator-activated receptor-γ for rosiglitazone [37], as well as to the activation of AKT or AMPK in diabetic animals [39, 40], but further studies are needed to elucidate how GLUT-2 is modulated by CPE in hepatic cells.

AMPK is an important therapeutic target for diabetes as it is one of the central regulators of cellular metabolism, which can be activated at least by two pathways: liver kinase B1 and Ca^{2+}/calmodulin-dependent protein kinase kinase [5]. In the present work, the phosphorylated levels of AMPK increased after incubating HepG2 cells with EC and CPE. Interestingly, the major green tea compound EGCG has previously been shown to activate AMPK in hepatic cells via Ca^{2+}/calmodulin-dependent protein kinase kinase [23, 27]. In addition, other polyphenols such as theaflavins, naringin, anthocyanins, resveratrol and apigenin have proved to activate AMPK in the liver of mice and rats and, consequently, modulate cellular metabolism [30, 31, 41, 42]. Similarly, other natural compounds such as berberine, ginsenosides obtained from ginseng and extracts from the plant *Artemisia sacrorum* have been demonstrated to stimulate AMPK in HepG2 cells [43–46].

PEPCK is one of the major enzymes responsible of the regulation of gluconeogenesis [3]. In the present work, EC and CPE decreased PEPCK levels in HepG2 cells. In agreement with our results, other phenolic compounds such as EGCG, naringenin, catechin-rich green tea, naringin, genistein, daidzein and anthocyanins diminished the levels of this gluconeogenic enzyme in hepatic cells and mouse liver during an induced insulin-resistant situation and, consequently, reduced glucose production [23, 31, 41, 47–50].

AKT and AMPK are known to suppress the gluconeogenesis in the liver [3, 4, 25]. Suppression of hepatic gluconeogenesis by AKT-dependent insulin signalling is reduced or lost in type 2 diabetes due to insulin resistance plus a relatively insufficient insulin production [25]. In addition, activation of AMPK results in enhanced fatty acid oxidation and decreased production of glucose, cholesterol and triglycerides in the liver [3, 4]. Thus, activation of AMPK supresses G6Pase and PEPCK and then decreases hepatic glucose production [3, 4]. In agreement, we have shown that both kinases are involved in the modulation of PEPCK levels and the production of glucose in HepG2 cells treated with EC and CPE (Fig. 7). As previously mentioned, EGCG activated AMPK and consequently inhibited hepatic gluconeogenesis via AMPK, although AKT seemed not to be involved in this process since it was not stimulated by the flavanol [23]. Similarly, naringenin did not modify AKT phosphorylation, and the incubation of the cells with LY294002 did not suppress the hepatic glucose production [48]. In addition, AMPK also mediated naringin repression of hepatic gluconeogenesis [31]. In this line, other natural compounds such as berberine, ginsenosides and an extract from *A. sacrorum* suppressed the hepatic glucose production and/or downregulated the levels of PEPCK via AMPK, although their effects on the mentioned parameters in the presence of compound C were weaker than the effect showed by the natural compound alone [43–46]. Interestingly, clove extract acted like insulin in HepG2 cells by reducing PEPCK gene expression and this feature was reverted by LY294002 [51]. It has also been shown that LY294002 inhibited the expression of PEPCK, but AKT did not modulate the hepatic production of glucose via gluconeogenesis [23, 49]. Likewise, in the present study p-AMPK and p-GSK-3-β levels were increased by EC and CPE incubation. Accordingly, it has been shown that in the liver, the activated form of AMPK is responsible for metabolic changes via phosphorylation of downstream substrates such as GSK-3-β and cAMP response element-binding protein, which are directly or indirectly related to glucose production [43, 46]. In this regard, EGCG promotes phosphorylation of CaMKK, and blockade of CaMKK activity prevents EGCG activation of AMPK and mitigates the inhibitory role of EGCG in hepatic gluconeogenesis [23].

As mentioned above, cocoa is a rich source of flavonoids such as (−)-EC, (+)-catechin, and procyanidins, and EC is the most abundant flavanol in the CPE employed in this study [6]. Considering that concentrations of EC in tested doses of CPE range from 13.2 nM (in the dose of 1 μg CPE/mL) to 132 nM (in that of 10 μg CPE/mL) [6], and CPE effects are equivalent to those of the pure flavanol, it is reasonable to assume that EC is just one of the many bioactive substances present

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in CPE and that the synergic effect of phenolic compounds in foodstuffs should be taken into account, as previously shown [52].

It is worth mentioning that the range of concentrations used in the study is not far from realistic. In this regard, rats fed with EC showed plasma concentrations of EC and EC metabolites of about 35 μM 1 h after oral administration of 172 μmol EC/kg body weight [53]. In humans, levels of 6 μM EC and 41 nM procyanidin B2 have been reported after ingestion of 26 g cocoa [54]. Similarly, levels of 0.2–0.4 μM EC have been observed after ingestion of 50 g [55] and 80 g [56] chocolate. In addition, one should not underestimate the potential contribution of EC and flavanol metabolites to the biological activity, which is unclear at present; their evaluation will require further studies. In this regard, more accurate approaches used recently are the incubation of cultured cells with plasma obtained from volunteers consuming the molecule or food of interest [57] and the use of a system based on co-culture of human enterocytes with human hepatocytes, which has been proved to resemble a human physiological system useful for assaying the bioactivity of extracts [58].

In summary, EC and CPE possess an insulin-like activity, as they enhance tyrosine phosphorylation and total levels of IR, IRS-1 and IRS-2 and activated PI3K/AKT pathway and AMPK at concentrations that are not toxic to hepatic cells and are reachable through the diet (Fig. 7). We have also revealed a new mechanism by which EC and CPE modulate the hepatic gluconeogenesis and PEPCK expression via AKT and AMPK (Fig. 7). Although, further efforts are needed to define the precise role of EC and cocoa in the regulation of the insulin pathways in liver cells, a diet rich in EC and/or cocoa may be a potential chemopreventive tool useful for the management of diabetes.

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The authors have declared no conflict of interest.

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Cocoa flavonoids attenuate high glucose-induced insulin signalling blockade and modulate glucose uptake and production in human HepG2 cells

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ABSTRACT

Insulin resistance is the primary characteristic of type 2 diabetes. Cocoa and its main flavanol, (−)-epicatechin (EC), display some antidiabetic effects, but the mechanisms for their preventive activities related to glucose metabolism and insulin signalling in the liver remain largely unknown. In the present work, the preventive effect of EC and a cocoa polyphenolic extract (CPE) on insulin signalling and on both glucose production and uptake are studied in insulin-responsive human HepG2 cells treated with high glucose. Pre-treatment of cells with EC or CPE reverted decreased tyrosine-phosphorylated and total levels of IR, IRS-1 and -2 triggered by high glucose. EC and CPE pre-treatment also prevented the inactivation of the PI3K/AKT pathway and AMPK, as well as the diminution of GLUT-2 levels induced by high glucose. Furthermore, pre-treatment of cells with EC and CPE avoided the increase in PEPCK levels and the diminished glucose uptake provoked by high glucose, returning enhanced levels of glucose production and decreased glycogen content to control values. These findings suggest that EC and CPE improved insulin sensitivity of HepG2 treated with high glucose, preventing or delaying a potential hepatic dysfunction through the attenuation of the insulin signalling blockade and the modulation of glucose uptake and production.

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1. Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is becoming a health burden that is reaching epidemic proportions worldwide (Whiting et al., 2011). Despite the available number of hypoglycaemic agents, nowadays, it is assumed that the most efficient approach to prevent or delay the onset of T2DM at the lowest cost is at nutritional level. Important candidates are plant flavonoids, which are naturally occurring compounds widely distributed in vegetables, fruits and beverages such as tea and wine, which have drawn attention because their beneficial effects on health and their safety (Hanhineva et al., 2010).

Sustained hyperglycaemia is a major contributor to insulin resistance, which is the hallmark of T2DM and a central component in the so-called metabolic syndrome (Klover and Mooney, 2004). In this pathology, the liver, which plays a crucial role in the control of the whole body metabolism of energy nutrients, presents an altered metabolism. Thus, this organ is not able to control glucose homeostasis and there is a miss-regulation of the insulin pathway (Klover and Mooney, 2004). At the cellular level, insulin binds and activates the insulin receptor (IR) by phosphorylating key tyrosine residues. This is followed by tyrosine phosphorylation of insulin receptor substrates (IRS) and subsequent activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/AKT) pathway (Klover and Mooney, 2004). AKT stimulation leads to the inhibition of glycogen synthase kinase-3 (GSK-3) by phosphorylation, which subsequently phosphorylates and inactivates glycogen synthase (GS). This pathway mediates the metabolic effects of

Abbreviations: AKT/PKB, protein kinase B; AMPK, 5′-AMP-activated protein kinase; BrdU, 5-bromo-2′-deoxyuridine; CaMMK, Ca^{2+}/calmodulin-dependent protein kinase kinase; CPE, cocoa phenolic extract; EC, (−)-epicatechin; EGCG, epigallocatechin gallate; ERK, extracellular regulated kinase; FBS, fetal bovine serum; FOXO1, forkhead box protein O1; GLUT, glucose transporter; G6Pase, glucose-6-phosphatase; GS, glycogen synthase; GSK-3, glycogen synthase kinase-3; GSPE, grape-seed procyanidin extract; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal Kinase; LKB1, liver kinase B1; 7-*o*-MA, 7-*o*-methylaromadendrin; 2-NBDG, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino); MAPK, mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphatidylinositol-3-kinase; PSP, purple sweet potato; PTP-1B, phosphatase 1B; T2DM, type 2 diabetes mellitus.

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insulin, including glucose transport and metabolism, as well as lipid and protein metabolism in target tissues (Klover and Mooney, 2004). In addition, in the hepatocyte AKT phosphorylates the forkhead box protein O1 (FOXO1), which inhibits the expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), and represses gluconeogenesis (Klover and Mooney, 2004). 5′-AMP-activated protein kinase (AMPK) is also a key regulator of the cellular metabolism that is able to suppress the hepatic gluconeogenesis through the modulation of PEPCK and G6Pase in the liver (Mihaylova and Shaw, 2011).

Flavonoids such as (−)-epicatechin (EC) constitute an important part of the human diet, and it can be found in green tea, grapes and especially in cocoa. EC and cocoa have a variety of properties, including antioxidant (Granado-Serrano et al., 2009a,b, 2010, 2007; Martín et al., 2010, 2008), anti-carcinogenic (Granado-Serrano et al., 2009a,b, 2010, 2007; Martín et al., 2013b), anti-allergic (Abril-Gil et al., 2012) and anti-atherogenic (Grassi et al., 2008; Hooper et al., 2012; Vinson et al., 2006) activities. The antidiabetic effect of EC and cocoa is assumed to be due to their ability to improve the insulin sensitivity (Grassi et al., 2008; Ruzaidi et al., 2008; Vazquez-Prieto et al., 2012) and secretion (Martín et al., 2013a), reduce blood glucose levels (Grassi et al., 2008; Igarashi et al., 2007; Ruzaidi et al., 2008) and regulate parameters related to the inflammation in cardiovascular disease and diabetes (Kim et al., 2003; Taub et al., 2012; Vazquez-Prieto et al., 2012). Previous work by our group has demonstrated that EC and a cocoa phenolic extract (CPE) strengthen the insulin signalling by activating key proteins of that pathway and regulating glucose production through AKT and AMPK modulation in HepG2 cells (Cordero-Herrera et al., 2013). These properties might indicate that EC and CPE may have interesting health protective benefits against the blockage of the hepatic insulin resistance. However, the precise mechanism for the preventive activities of EC and cocoa related to glucose metabolism and insulin signalling in the liver remains largely unknown.

The aim of the study was to test the potential chemoprotective effect of EC and CPE against insulin signalling restraint induced by a high glucose challenge in HepG2 cultured cells. Thus, key

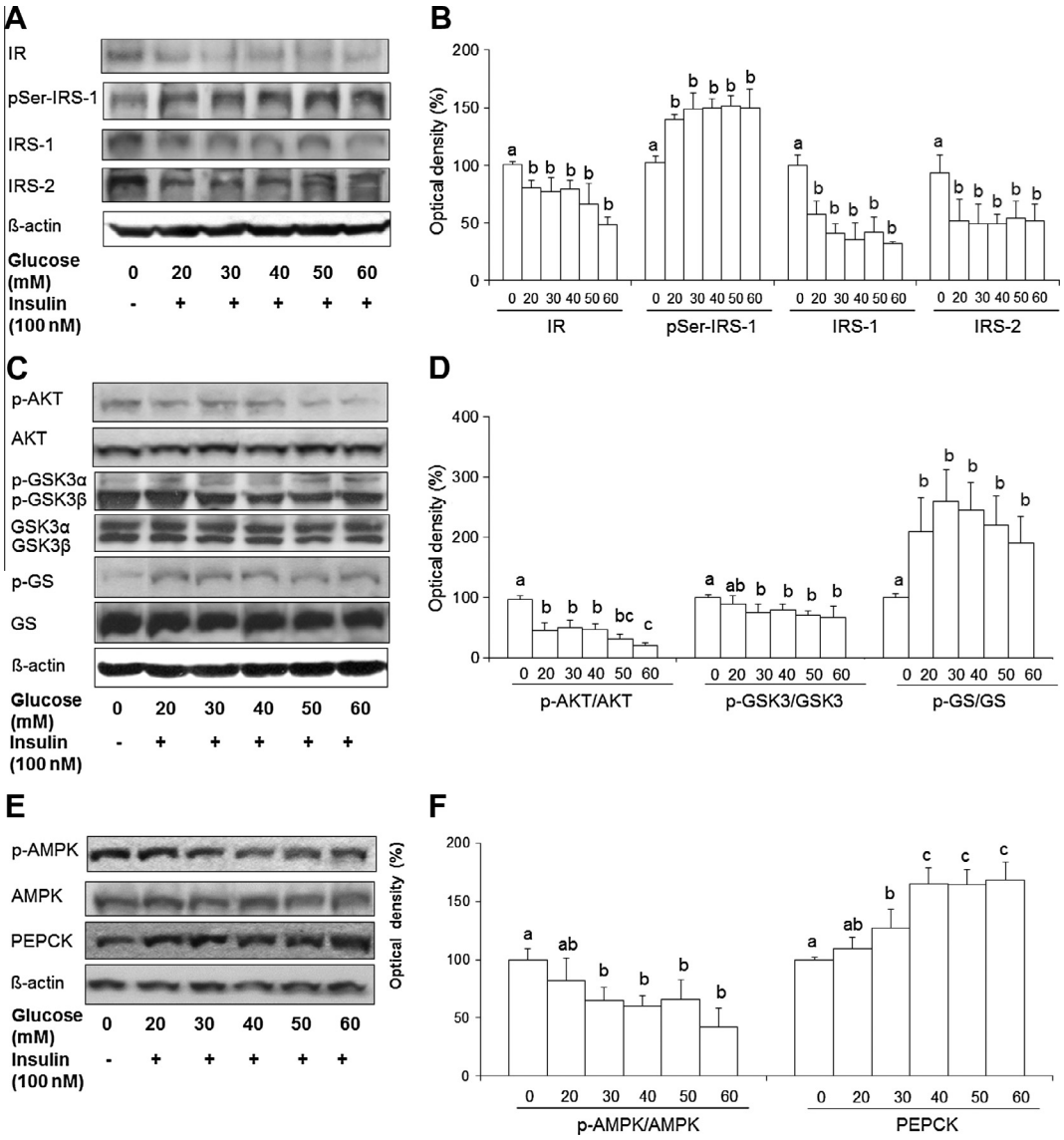

Fig. 1. Effect of glucose concentrations on phosphorylated and total levels of IR and its substrates, PI3K/AKT pathway, AMPK and PEPCK in HepG2 cells after 24 h of treatment and 10 min of stimulation with 100 nM insulin. (A) Bands of representative experiments. (B) Densitometric quantification of IR, p-IRS-1 (Ser), IRS-1 and IRS-2. Values are expressed as a percentage relative to the control condition. (C) Blots of representative experiments (D) Percentage values of p-AKT/AKT, p-GSK3/GSK3 and p-GS/GS ratios relative to controls. (E) Bands of representative experiments. (F) Percentage data of p-AMPK/AMPK ratio and PEPCK relative to controls. Equal loading of Western blots was ensured by β-actin ($n = 6–8$). Means without a common letter differ ($P < 0.05$).

proteins in the signalling transduction pathway of the insulin, as well as glucose production, glucose uptake and glycogen content were evaluated.

2. Materials and methods

2.1. Materials and chemicals

(−)-EC (>95% of purity), D-glucose, anti-mouse IgG-agarose, sodium lactate, sodium pyruvate, gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical (Madrid, Spain). The fluorescent probe D-glucose, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino) (2-NBDG) was from Molecular Probes (Invitrogen, Madrid, Spain). Anti-phospho-IRS-1 recognizing levels of phosphorylated Ser636/639 of IRS1, anti-AKT and anti-phospho-Ser473-AKT detecting levels of total and phospho-AKT, anti-AMPK and anti-phospho-Thr172-AMPK, as well as anti-GSK3 α/β and anti-phospho-GSK3 α/β detecting phosphorylated Ser21/9 of GSK3, anti-GS and anti-phospho-GS recognizing phosphorylated Ser641 of GS, anti-IRS-2 and anti-β-actin were obtained from Cell Signalling Technology (Izasa, Madrid, Spain). Anti-IR β, anti-PEPCK and anti-Tyr(P) (PY20) were purchased from Santa Cruz (sc-711, sc-32879 and sc-508, respectively, Qimigen, Madrid, Spain). Anti-IRS-1 and anti-GLUT-2 were from Millipore (Madrid, Spain). Materials and chemicals for electrophoresis were from BioRad (BioRad Laboratories S.A., Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively.

2.2. Cocoa polyphenol extraction

Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. Soluble polyphenols were extracted by sequentially washing 1 g of sample with 40 mL of 16 mM hydrochloric acid in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 mL of acetone:water (70:30, v/v, 1 h at room temperature, constant shaking). After centrifugation (15 min, 3000g), supernatants from each extraction step were combined and made up to 100 mL. The desiccated extract was dissolved in distilled water and kept frozen until assay. A detailed description of this cocoa polyphenol extract (CPE) is given elsewhere (Martín et al., 2010, 2008). The amount of EC and polyphenols present in the CPE were 383.5 mg/100 g (determined by LC-MS) and 2 g/100 g on dry matter basis (determined by Folin–Ciocalteu) (Martín et al., 2008).

2.3. Cell culture and treatments

Human HepG2 cells were grown in DMEM-F12 medium supplemented with 2.5% foetal bovine serum (FBS) and the following antibiotics: gentamicin, penicillin and streptomycin (50 mg/L). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. One day after plating, the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and FBS, and the culture was continued. Subsequently, the experimental treatment was carried out for the indicated periods with various concentrations of glucose in serum-free media for 24 h. At the end of the treatment, cells were incubated with 100 nM insulin for 10 min and then harvested, as previously reported (Lin and Lin, 2008; Nakajima et al., 2000; Zang et al., 2004; Zhang et al., 2010). In the experiments with EC and CPE, cells were preincubated for 24 h with 10 μM EC or 1 μg/mL CPE prior to 24 h of glucose (30 mM) treatment. At the end of the treatment, the response to insulin was tested by incubating the cells with 100 nM insulin for 10 min and then, cells were harvested.

2.4. Preparation of cell lysates

Cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1% Trition X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride. The supernatants were collected, assayed for protein concentration by using the Bio-Rad (Bio-Rad, Madrid, Spain) protein assay kit according to the manufacture's specifications, aliquoted and stored at −80 °C until used for immunoprecipitation and/or Western blot analyses.

2.5. Immunoprecipitation

Protein extracts containing 200 μg of protein were immunoprecipitated overnight at 4 °C with gentle rotation in the presence of 2–5 μg of anti-Tyr(P) (PY20) antibody, followed by the addition of anti-mouse IgG-agarose. After mixing for 2 h, the pellets were collected by centrifugation, and the supernatants were discarded. Then the pellets were washed and saved for Western blot analyses.

2.6. Western blot analysis

Equal amounts of proteins were separated by SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters (Bio-Rad, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare, Madrid, Spain) or anti-mouse (Sigma, Madrid, Spain) immunoglobulin. Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was ensured by β-actin and bands were quantified using a scanner and accompanying software.

2.7. Determination of glycogen content

Glycogen content was determined by using a commercial glycogen fluorometric assay kit from Biovision Research Products (Deltaclon, Madrid, Spain). Treated cells were homogenized in distilled water, boiled samples and later centrifuged at 12,000g for 5 min, and glycogen was measured in the supernatants. In the assay, glucoamylase hydrolyzes the glycogen to glucose, which is then specifically oxidized to produce a product that reacts with the probe to generate fluorescence. Glycogen levels in samples were detected in a fluorescent microplate reader (Bio-Tek, Winooski, VT, USA) at an excitation wavelength of 535 nm and an emission wavelength of 587 nm. A standard curve of glycogen (0.4–2 μg) was used, and protein was measured by the Bradford reagent.

2.8. Glucose uptake

Cellular glucose uptake was quantified by the 2-NBDG assay using a microplate reader. Cells were plated in 24-well plates at a rate of 2×10^5 cells per well and after the treatments, 2-NBDG was added at 10 μM final concentration and incubated for 1 h at 37 °C. Then, cells were washed twice with PBS, serum-free medium was added and the fluorescence intensity immediately measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. After being taken by the cells, 2-NBDG was converted to a non-fluorescent derivative (2-NBDG metabolite). A fair estimation of the overall glucose uptake was obtained by quantifying the fluorescence. The assay has been described elsewhere (Zou et al., 2005).

2.9. Glucose production assay

HepG2 cells were seeded in 24-well plates (2×10^5 cells per well) and the day of the assay, the medium was then replaced with glucose production buffer consisting of glucose-free DMEM (pH 7.4), without phenol red (Invitrogen, Madrid, Spain),

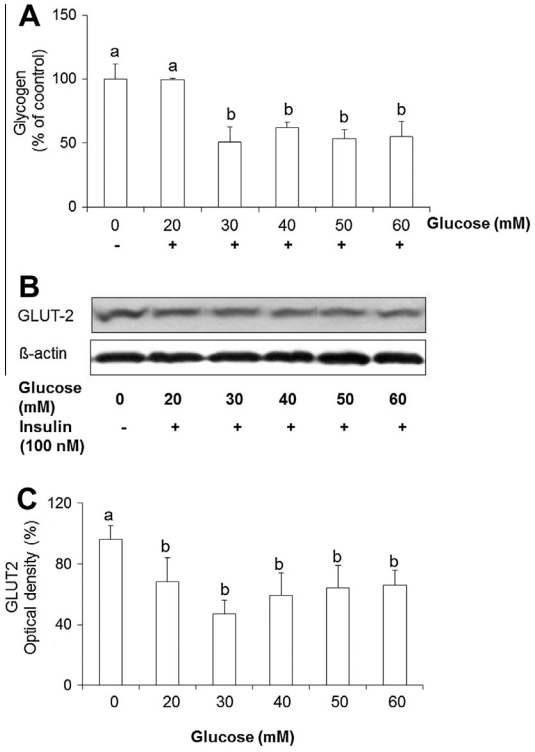

Fig. 2. Effect of glucose concentrations on glycogen content and levels of GLUT-2 in HepG2 cells after 24 h of treatment. Cells were incubated with 100 nM insulin for 10 min before the harvest. (A) Glycogen content expressed as percent of control are means ± SD of 8–10 different samples per condition. (B) Bands of representative experiments for GLUT-2. (C) Densitometric quantification of GLUT-2. Values are expressed as a percentage relative to the control condition (n = 7–9). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ ($P < 0.05$).

supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate, as previously described (Collins et al., 2007; Cordero-Herrera et al., 2013). After a 3 h-incubation, medium was collected and glucose concentration measured with a colorimetric glucose assay kit (Sigma, Madrid, Spain). The readings were then normalized to the total protein content determined from the whole-cell lysates.

2.10. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P < 0.05$ was considered significant. A SPSS version 19.0 program has been used.

3. Results

3.1. High glucose concentrations alters insulin signalling and glycogen content

In order to develop a model of insulin resistance induced by high concentrations of glucose in hepatic cells, HepG2 cells were exposed to rising doses of glucose for 24 h followed by a chase for 10 min with 100 nM insulin, and the modulation of key proteins related to the insulin signalling pathway were evaluated.

Treatment of HepG2 cells for 24 h with all glucose concentrations tested decreased IR, IRS-1 and IRS-2 levels, and increased p-(Ser636/639)-IRS-1 values, which are related to the inhibition of insulin signalling (Fig. 1A). Moreover, doses of glucose higher than 20 mM caused a reduction in the phosphorylated levels of AKT, GSK3 and AMPK, whereas p-GS and PEPCK expression values increased (Fig. 1B and C). In line with these results, glucose (30–60 mM) provoked a decrease in the glycogen content and diminished GLUT-2 levels were already observed with 20 mM glucose (Fig. 2). All these data suggest that high doses of glucose (30–60 mM) are able to alter the insulin signalling and glycogen content in HepG2 cells to simulate a situation that resembles insulin resistance in hepatic cells.

Since 30 mM glucose was the lowest concentration that altered the levels of the insulin pathway-related proteins and glycogen content in HepG2 cells, this was the concentration selected for

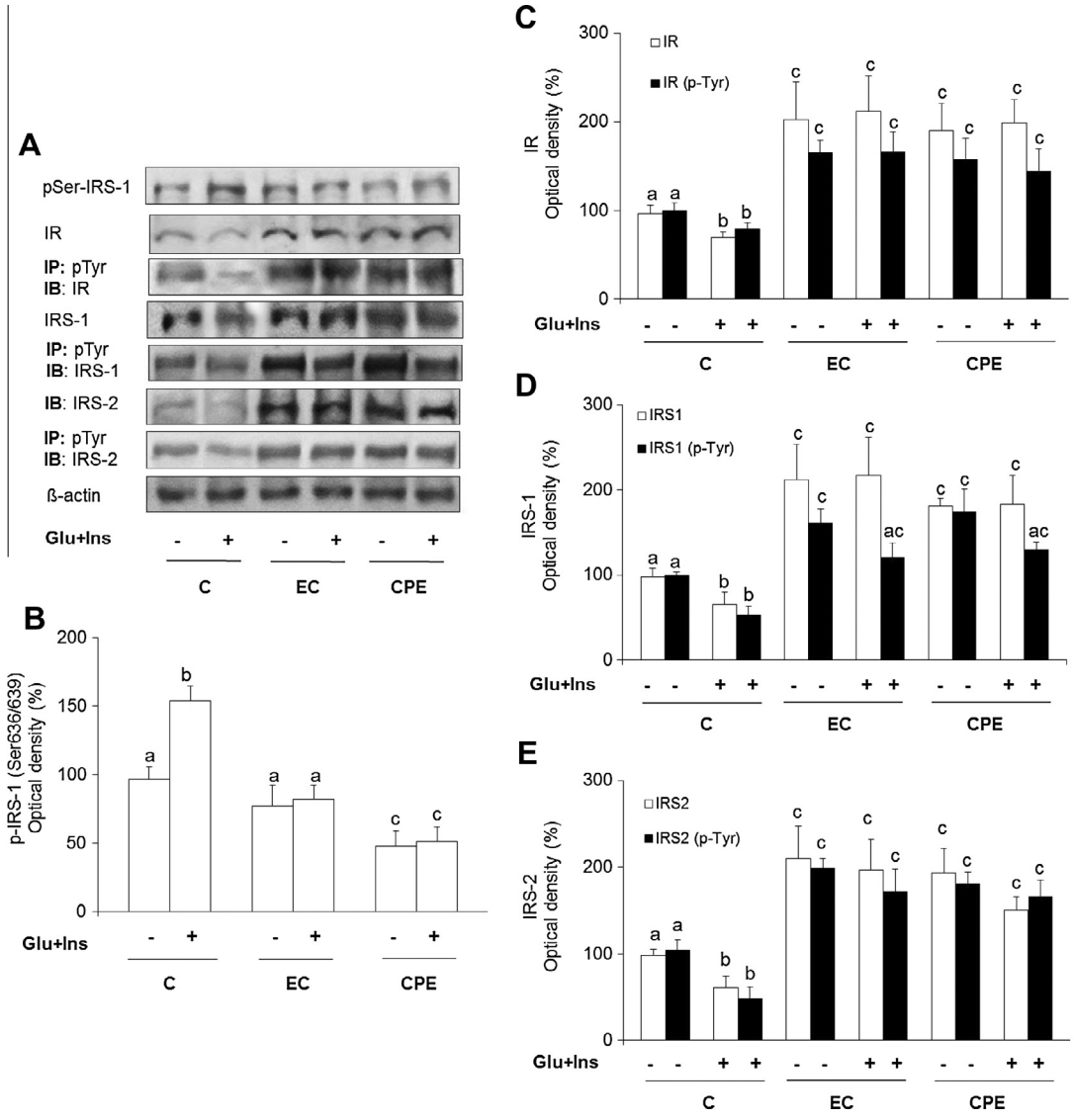

Fig. 3. Protective effect of EC and CPE on the decreased phosphorylated and total levels of IR, IRS-1 and IRS-2 induced by 30 mM glucose. HepG2 cells incubated with 10 μM EC or 1 μg/mL CPE for 24 h were exposed to 30 mM glucose (Glu) for additional 24 h and then treated with 100 nM insulin (Ins) for 10 min. (A) Bands of representative experiments. Densitometric quantification of (B) p-IRS-1 (Ser), (C) p-IR and total IR, (D) p-IRS-1 (Tyr) and total IRS-1 and (E) p-IRS-2 (Tyr) and total IRS-2. Protein extracts were subjected to immunoprecipitation (IP) with the anti-phospho-tyrosine (P-Tyr) antibody. The resulting immunocomplexes were analyzed by Western blot (WB) with the anti-IR or IRS-1 or IRS-2 antibody. Values are expressed as a percentage relative to the control condition (means ± SD, $n = 7–9$). Equal loading of Western blots was ensured by β-actin. Means (for the phosphorylated or total protein levels) without a common letter differ ($P < 0.05$).

studying the protective effects of EC and CPE on the mentioned parameters.

3.2. EC and CPE prevent high-glucose induced downregulation of tyrosine phosphorylated and total levels of IR and its substrates 1 (IRS-1) and 2 (IRS-2), and avoid upregulation of IRS-1 serine phosphorylation

To analyse the effect of EC and CPE on tyrosine phosphorylation and total levels of IR and its substrates, as well as on the serine phosphorylation of IRS-1, HepG2 cells were exposed for 24 h to 10 μM EC or 1 μg/mL CPE followed by a 24 h-incubation with 30 mM glucose and then, stimulated with 100 nM insulin for 10 min. On the non-insulin resistance state, IRS-1/-2 are readily in tyrosine phosphorylated by IR upon stimulation with insulin (Klover and Mooney, 2004). However, it has been reported the involvement of serine phosphorylation of IRS-1 in the desensitization of insulin by chronic high glucose treatment (Nakajima et al., 2000). In this regard, EC and CPE pretreatment prevented the increase in p-(Ser636/639)IRS-1 induced by the high glucose dose, showing comparable values to those of controls when cells were incubated with EC or CPE, respectively (Fig. 3A and B). In addition, total and tyrosine phosphorylated levels of IR and IRS-1 and -2 were diminished when cells were treated with 30 mM glucose for 24 h (Fig. 3A and C–E). As previously reported (Cordero-Herrera et al., 2013), treatment with 10 μM EC and 1 μg/mL CPE for 24 h activated key proteins at the early stages of the insulin pathway (IR and IRS). In this line, EC and CPE pretreatment totally restrained the decrease in both tyrosine phosphorylated and total levels of IR, IRS-1 and -2 values induced by the high dose of glucose, showing comparable levels to those of cells treated with EC or CPE alone (Fig. 3). All these results suggest that EC and CPE could prevent the blockage of the insulin signalling cascade induced by a high dose of glucose by modulating the early steps of this pathway.

3.3. EC and CPE restrain downregulation of AKT and GSK3, and upregulation of GS phosphorylation induced by high-glucose

AKT lays downstream of PI3K and facilitates glucose uptake and glycogen synthesis in the liver, and directly contributes to the activity of GS, which is the key molecular mediating the metabolic effects of insulin signalling (Whiteman et al., 2002). To evaluate the potential protective effect of EC and CPE against the alterations caused on AKT, GSK3 and GS by a high glucose concentration, the phosphorylated and total levels of the mentioned proteins were analyzed in cell lysates by Western blot analysis.

Treatment of HepG2 cells with EC and CPE during 24 h evoked a significant increase in the phosphorylated levels of AKT and GSK3, whereas both substances induced a decrease in p-GS values (Fig. 4), as previously reported (Cordero-Herrera et al., 2013). Likewise, pre-treatment of HepG2 cells with EC and CPE prevented the diminution in the p-AKT and p-GSK3 levels caused by 30 mM glucose (Fig. 4A–C). Accordingly, EC and CPE reverted the increased p-GS values induced by the high glucose concentration (Fig. 4A and D). There was no difference in the total levels of AKT, GSK3 and GS. All these results suggest that EC and CPE restrained the inhibition of the PI3K/AKT pathway, which constitutes a key route in the insulin signalling cascade.

3.4. EC and CPE prevent high-glucose induced downregulation on AMPK phosphorylation

AMPK has been proved to be required for antidiabetic effects of some clinical drugs in insulin-resistant human HepG2 cells (Hardie, 2011). Thus, to continue the study of the potential protective effect of EC and CPE on key proteins of the insulin signalling, total and phosphorylated levels of AMPK were evaluated by Western blot in cell lysates. Fig. 5 illustrates that a 24 h-treatment with 30 mM glucose decreased p-AMPK levels, whereas EC or CPE

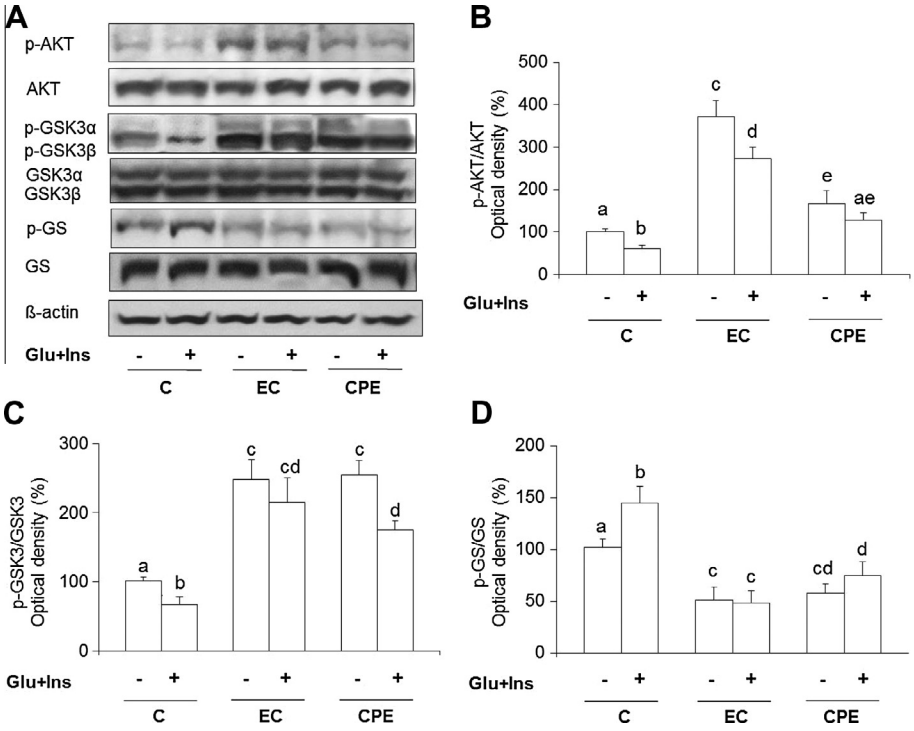

Fig. 4. Preventive effect of EC and CPE on the decreased phosphorylated and total levels of AKT, GSK3 and on the enhanced levels of phosphorylated and total GS induced by 30 mM glucose in HepG2 cells. Cells treated with 10 μM EC or 1 μg/mL CPE for 24 h were later incubated with 30 mM glucose (Glu) for 24 h and further exposed to 100 nM (Ins) for 10 min. (A) Bands of representative experiments. Percentage data of (B) p-AKT/AKT, (C) p-GSK3/GSK3 and (D) pGS/GS ratios relative to controls. Values are expressed as means ± SD, $n = 6–8$. Equal loading of Western blots was ensured by β-actin. Means without a common letter differ ($P < 0.05$).

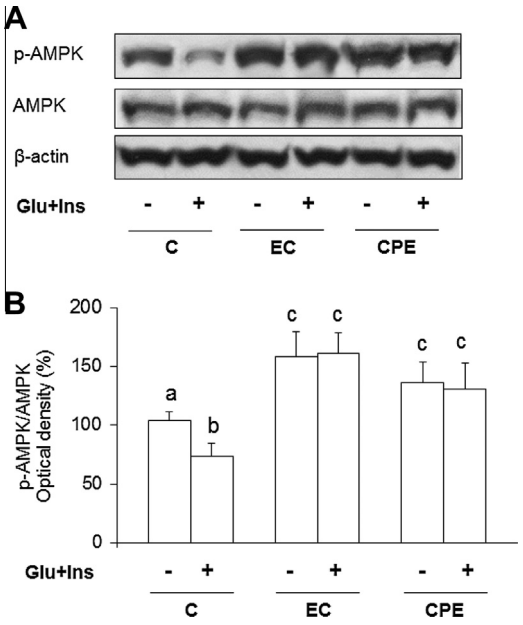

Fig. 5. Protective effect of EC and CPE on the diminished phosphorylated and total AMPK levels evoked by 30 mM glucose in HepG2 cells. Cells treated with 10 μM EC or 1 μg/mL CPE for 24 h were exposed to 30 mM glucose (Glu) for 24 h and then incubated with 100 nM (Ins) for 10 min. (A) Bands of representative experiments. (B) Percent values of p-AMPK/AMPK ratio relative to the control condition (means ± SD, $n = 7–8$). Equal loading of Western blots was ensured by β-actin. Different letters over bars indicate statistically significant differences ($P < 0.05$).

alone induced an increase in the AMPK phosphorylated levels (Fig. 5). The diminution in the p-AMPK values induced by the high concentration of glucose was counteracted by the EC and CPE pretreatment, and cells showed comparable levels to those of cells treated with EC or CPE alone (Fig. 5). The protein levels of total AMPK were not modified by any treatment.

3.5. EC and CPE prevent high-glucose induced downregulation on GLUT-2 levels and glucose uptake

GLUT-2 transporter mediates the diffusion of glucose across the plasma membrane of the hepatocyte and maintains intracellular glucose in equilibrium with extracellular glucose (Klover and Mooney, 2004). To test the potential preventive effect of EC and CPE against the alterations evoked by a high glucose concentration on this transporter, HepG2 cells were incubated with the selected concentrations of both natural substances for 24 h, later treated with 30 mM glucose for additional 24 h, and then stimulated for 10 min with 100 nM insulin.

Treatment of HepG2 cells with EC or CPE alone did not affect the levels of GLUT-2 (Cordero-Herrera et al., 2013), and both substances were able to restrain the diminution in the GLUT-2 levels triggered by 30 mM glucose challenge (Fig. 6A and B). In addition, EC alone increased the basal cell glucose uptake and EC and CPE were able to avoid the inhibited glucose uptake caused by the high concentration of glucose, showing comparable levels to those of EC- and CPE-treated cells (Fig. 6C). All these results indicate that both natural substances protect HepG2 responsiveness of one of the most important hepatic cell functions, glucose uptake.

3.6. EC and CPE prevent high-glucose induced hepatic gluconeogenesis

In the hepatocyte, in a situation of insulin resistance, inhibition of AKT stimulates PEPCK and G6Pase levels and gluconeogenesis, as well as restrains the synthesis of glycogen (Klover and Mooney, 2004). In view of the protective effects showed by EC and CPE on p-AKT levels in cells incubated with 30 mM glucose, it was studied whether both substances were able to modulate the expression of a major enzyme responsible of the regulation of gluconeogenesis, such as PEPCK, as well as the production of glucose and the glycogen content. To this end, HepG2 cells were pretreated with EC or CPE for 24 h, later exposed to 30 mM glucose for 24 h, stimulated with 100 nM insulin for 10 min and then, the levels of PEPCK, the

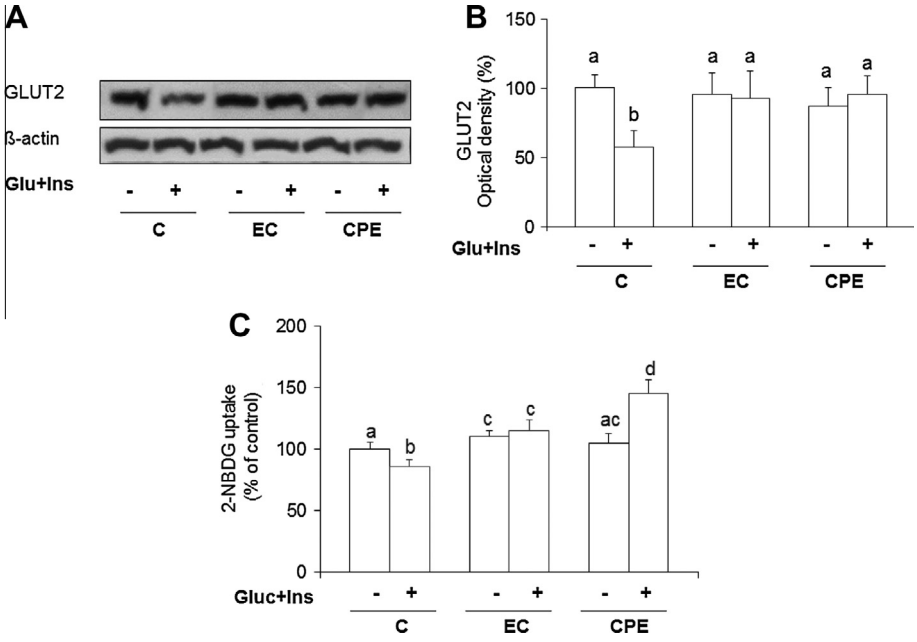

Fig. 6. Protective effect of EC and CPE on the reduced GLUT-2 levels and decreased glucose uptake. HepG2 cells incubated with 10 μM EC or 1 μg/mL CPE for 24 h were exposed to 30 mM glucose (Glu) for additional 24 h and then treated with 100 nM insulin (Ins) for 10 min. (A) Bands of representative experiments. (B) Densitometric quantification of GLUT-2. Values are expressed as a percentage relative to the untreated control condition and are means ± SD, $n = 7–8$. Equal loading of Western blots was ensured by β-actin. (C) Glucose uptake expressed as percent of control are means ± SD of 8–12 different samples per condition. Means without a common letter differ ($P < 0.05$).

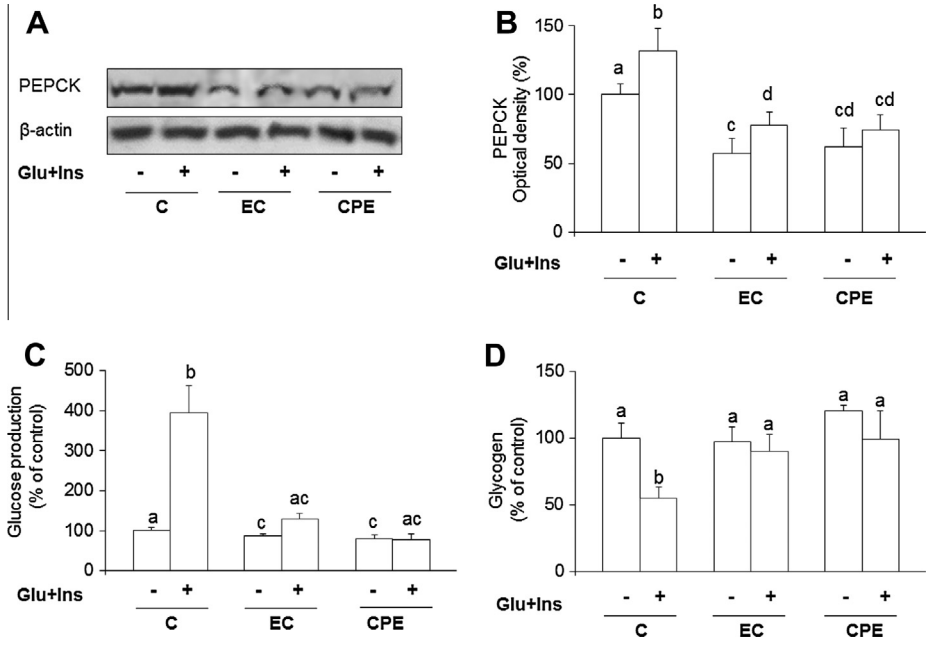

Fig. 7. Protective effect of EC and CPE on the increased PEPCK levels and glucose production, and decreased glycogen content induced by 30 mM glucose in HepG2 cells. Cells incubated with 10 μM EC or 1 μg/mL CPE for 24 h were exposed to 30 mM glucose (Glu) for additional 24 h and further treated with 100 nM (Ins) for 10 min. (A) Bands of representative experiments. (B) Percentage data of PEPCK relative to the control condition (means ± SD, $n = 6–9$). Equal loading of Western blots was ensured by β-actin. (C) Glucose production expressed as percent of control are means ± SD of 10–14 different samples per condition. (D) Glycogen content expressed as percent of control are means ± SD of 8–10 different samples per condition. Different letters over bars indicate statistically significant differences ($P < 0.05$).

novo production of glucose and the content of glycogen were assayed.

Treatment of HepG2 cells with EC or CPE alone led to a comparable decrease in the expression of PEPCK (Fig. 7A and B). High glucose incubation increased the levels of PEPCK, but this effect was totally repressed in cells previously treated with EC or CPE, and cells showed comparable levels to those of cells treated with EC and CPE alone (Fig. 7A and B). In this line, EC and CPE pre-treatment counteracted the enhanced glucose production evoked by the high glucose incubation (Fig. 7C). In addition, high glucose treatment decreased glycogen levels, although its values were recovered when cells were previously treated with EC or CPE (Fig. 7D). EC and CPE alone did not modify the glycogen content. All this suggested that EC and CPE might also contribute to preserve HepG2 functionality and modulate the glucose homeostasis.

4. Discussion

Natural occurring compounds have been proposed to exert beneficial effects on health and have drawn attention because of their safety (Hanhineva et al., 2010). Thus, there is accumulating evidence that suggest the antidiabetic activity of flavonoids (Hanhineva et al., 2010), although insufficient investigation at molecular level has been performed to support these observations. Recently, we have reported that EC and CPE stimulate key proteins of the insulin route, such as IR/IRS, PI3K/AKT pathway and AMPK, and regulate the glucose production through AKT and AMPK modulation in HepG2 cells (Cordero-Herrera et al., 2013). In the present study, we show that EC and CPE attenuate the insulin signalling blockade induced by a high dose of glucose by preventing the decrease of tyrosine phosphorylated and total IR, IRS-1 and IRS-2 levels, the inhibition of PI3K/AKT and AMPK pathways, and the increase of IRS-1 Ser636/639 phosphorylation values. Furthermore, we have demonstrated that EC and CPE restore the levels of GLUT-2 to control levels, and protect HepG2 functionality by modulating glucose production and uptake, as well as glycogen content, which are also altered by the exposure to the high dose of glucose.

Cocoa is a rich source of flavonoids such as (−)-EC, (+)-catechin, and procyanidins, and EC is the most abundant flavanol in the CPE employed in this study (Martín et al., 2008). It is worth mentioning that the concentrations used in the study are not far from realistic since steady-state concentrations around 35–50 μM of EC have been reported in rat serum 1 h after oral administration of 172 μM of EC per kg of body weight (Baba et al., 2001). It has also been observed in humans serum levels of 6 μM EC and 41 nM procyanidin B2 after ingestion of 26 g cocoa (Holt et al., 2002).

The liver plays a key role in maintaining blood glucose concentration both through its ability to supply glucose to the circulation via glycogenolysis and gluconeogenesis and to remove glucose from the circulation to increase glycogen synthesis (Klover and Mooney, 2004). However, the hepatic insulin resistance is characterized by a reduced capacity of insulin to increase glycogen synthesis and an impaired insulin signalling (Klover and Mooney, 2004). Consequently, interventions to prevent insulin resistance are of great protective and therapeutic interest.

The modulation of IR and its downstream substrates IRS-1 and IRS-2 is essential for recruiting and activating downstream pathways (Whiteman et al., 2002). In fact, in hepatic insulin resistance the insulin-stimulated-IR and IRS tyrosine phosphorylation is defective and results in reduced IRS-associated PI3K activities (Klover and Mooney, 2004; Nakajima et al., 2000), as we have shown in the present study. However, we have demonstrated that EC and CPE prevent the decrease in the tyrosine phosphorylation of IR and IRS-1/2 and the increase in the serine phosphorylation of IRS-1 induced by high glucose exposure, which is critical for the development of insulin resistance (Nakajima et al., 2000). In this line, it has been reported in preadipocytes that CPE did not affect the IR levels, but it modulated the activity of IR kinase via direct binding (Min et al., 2013), and that oligomeric structures of a grape-seed procyanidin extract (GSPE) activated IR by interacting with and inducing its tyrosine phosphorylation (Montagut et al.,

2010). In addition, epigallocatechin gallate (EGCG), berberine and anthocyanins derived from purple sweet potato (PSP) attenuated insulin resistance by reducing IRS-1 serine phosphorylation in HepG2 cells (Lin and Lin, 2008; Lou et al., 2011) and in mice (Zhang et al., 2013). Berberin, naringenin and EGCG also alleviated the insulin resistance by activating IRS-1 and increasing the tyrosine phosphorylated levels in HepG2 cells (Lou et al., 2011), primary hepatocytes of mice fed with a high-fat diet (Pu et al., 2012), and liver of obese mice (Ueno et al., 2009). Similarly, green tea polyphenols increased IRS-2 mRNA levels in the liver and myocardium of insulin resistant rats (Cao et al., 2007; Qin et al., 2010), whereas IR and IRS-1 levels were restored to controls in the myocardium of insulin-resistant rats (Qin et al., 2010).

The activation of IRS-1/-2 leads to the stimulation of other signalling cascades such as the PI3K/AKT pathway (Klover and Mooney, 2004). In agreement with our results, in a situation of hepatic insulin resistance phosphorylation of AKT and its downstream effector GSK3 decreased, and GS phosphorylated levels increased (Klover and Mooney, 2004). However, this effect was abolished when cells were previously treated with EC or CPE. In this line, different natural compounds have shown to induce the reactivation of the PI3K/AKT pathway in a situation of insulin resistance. Thus, berberine and 7-*o*-methylaromadendrin (7-*o*-MA) improved insulin-mediated AKT activation in insulin resistant HepG2 cells (Lou et al., 2011; Zhang et al., 2010). Similarly, green tea polyphenols, EGCG and anthocyanins derived from PSP also increased the expression levels of PI3K/AKT and GSK3 in the liver of insulin-resistant rats (Cao et al., 2007; Zhang et al., 2013) and mice (Ueno et al., 2009). All together indicates that in a situation of insulin resistance polyphenols might improve the cell sensitivity to the hormone.

AMPK is an intracellular energy sensor implicated in the regulation of cellular metabolism, which phosphorylation is suppressed in insulin-resistant hepatic cells (Hardie, 2011; Zang et al., 2004). We have demonstrated that EC and CPE increase the phosphorylated levels of AMPK (Cordero-Herrera et al., 2013), and this situation remains unaltered in cells pretreated with both natural substances and later exposed to a high glucose concentration. In this line, EC, a cacao liquor procyanidin extract and cyanidin-3-glucoside contribute to restore the diminished p-AMPK levels in the liver of insulin-resistant mice and in high-glucose-incubated adipocytes, respectively, which helped to prevent the hyperglycaemia and insulin resistance (Guo et al., 2012, Si et al., 2011; Yamashita et al., 2012). EGCG and 7-*o*-MA have also been shown to activate AMPK in hepatic cells and in high glucose-induced insulin resistant HepG2 cells (Lin and Lin, 2008; Zhang et al., 2010) and, consequently, to modulate cellular metabolism. Similarly, naringin reversed the reduced phosphorylated levels of AMPK in primary hepatocytes cultured in high glucose as well as in the liver of mice fed with a high-fat diet (Pu et al., 2012). Therefore, AMPK reactivation in insulin resistant state of cells could be associated to insulin responsiveness (Lin and Lin, 2008; Pu et al., 2012; Zhang et al., 2010).

In the liver, GLUT2 maintains intracellular glucose in equilibrium with extracellular glucose, although this balance could be altered during insulin resistance (Klover and Mooney, 2004; Nakajima et al., 2000). In this line, the antidiabetic drugs telmisartan, sitagliptin and metformin recovered the diminished levels of hepatic GLUT-2 on insulin-resistant mice, suggesting a normalization of post-receptor insulin signalling and a restoration of the hepatic insulin sensitivity (Souza-Mello et al., 2010). Interestingly, an improvement in the decreased glucose uptake of HepG2 insulin-resistant cells have been reported when cells were co-treated with the major green tea compound EGCG (Lin and Lin, 2008). Similarly, different polyphenols such as 7-*o*-MA and GSPE stimulated the glucose uptake in HepG2 cells (Zhang et al., 2010) and adipocytes (Montagut et al., 2010), respectively. In agreement with all of the above, GLUT-2 levels and glucose uptake decreased in HepG2 cells exposed to a high dose of glucose, but this was reverted when cells were previously treated with EC or CPE.

PEPCK is one of the major enzymes responsible for the regulation of gluconeogenesis; thus, PEPCK and glucose production increase in the hepatic insulin resistance (Klover and Mooney, 2004). We have previously demonstrated that EC and CPE decreased PEPCK levels and glucose production in HepG2 cells through AMPK and AKT (Cordero-Herrera et al., 2013). In the present work, EC and CPE prevented the high-glucose enhancement of PEPCK values, which was associated to a suppression of the hepatic gluconeogenesis. Accordingly other phenolic compounds such as EGCG, areca nut procyanidins and naringin diminished the levels of this gluconeogenic enzyme in hepatic cells and in the liver of mice during an induced insulin-resistant situation and, consequently, reduced glucose production (Collins et al., 2007; Huang et al., 2013; Pu et al., 2012; Waltner-Law et al., 2002).

The main insulin action in the liver is to increase glycogen synthesis, and hepatic insulin resistance is characterized by a reduced insulin capacity to build up glycogen (Klover and Mooney, 2004). Thus, the enhanced gluconeogenesis coexists with reduced accumulation of hepatic glycogen in diabetes, and a liver-specific activation of PEPCK (Klover and Mooney, 2004). In the current study, EC and CPE alone did not altered the glycogen content, but were able to prevent the diminution of the glycogen content induced by the high glucose challenge. In this line, it has been reported that EGCG, berberine and extracts rich in phenolic compounds from fruits and plants reversed induced inhibition of glycogen synthesis in insulin-resistant hepatic cells (Lin and Lin, 2008; Lou et al., 2011) and in the liver of diabetic rodents (Gandhi et al., 2011; Jung et al., 2007).

AKT and AMPK are known to suppress gluconeogenesis in the liver (Klover and Mooney, 2004; Mihaylova and Shaw, 2011; Whiteman et al., 2002). EC and CPE inactivated GSK3 in high glucose-induced insulin resistant HepG2 cells. In order to maintain glucose homeostasis, GSK3β interacts with the regulation of PEPCK and G6Pase (Lochhead et al., 2001), and in a situation of hyperglycaemia insulin-phosphorylated AKT phosphorylates and inactivates GSK3β, which inhibits the synthesis of glycogen (Whiteman et al., 2002). In addition, AMPK also phosphorylates and inactivates GSK3β, which suppresses G6Pase and PEPCK and then decreases hepatic glucose production. Therefore, both AKT and AMPK phosphorylate GSK3β and consequently modulate gluconeogenesis, as we have previously shown (Cordero-Herrera et al., 2013). Interestingly, phosphatase 1B (PTP-1B), IkB kinase and mitogen-activated protein kinases (MAPKs) have been identified as important proteins in the development of insulin resistance via phosphorylation of serine residues of IRS proteins (Klover and Mooney, 2004). In this regard, activation of extracellular regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) induces negative regulators of insulin sensitivity such as PTP-1B and p-Ser307-IRS-1, and their suppression by grape powder extract attenuated TNFα-induced insulin resistance (Chuang et al., 2011). AKT and MAPKs also seem to be key proteins for the stimulation of the insulin signalling pathway by GSPE (Montagut et al., 2010). Similarly, PKC/JNK inhibition and AMPK activation could also mediate the inhibition of IRS-1 serine phosphorylation (Lin and Lin, 2008), which also contributes to the stimulation of the hepatic insulin sensitivity. The present study demonstrates that EC and CPE inhibit glucose production and maintain glycogen content in high glucose-exposed HepG2 cells, which correlates with changes in the levels of AKT, AMPK, GSK3β and p-IRS-1(Ser). However, a relevant role for serine/threonine kinases related to the development of insulin resistance such as MAPKs and phosphatases should not be ruled out. This subject deserves further studies.

In a clinical situation, the diabetic patient maintains the glycaemia with difficulty and this risk might lead to severe complications. Here, we report for the first time that EC and CPE improve insulin sensitivity in cultured HepG2 cells preventing or delaying a potential hepatic dysfunction. In this line, it could be highlighted that antioxidant treatments, such as N-acetylcysteine incubation, decreased the expression of inflammatory mediators but did not improve insulin resistance in the liver (Setshedi et al., 2011). Thus, EC and CPE could offer another choice in term of nutritional intake and play a role in the protection afforded by fruits, vegetables and plant-derived beverages against diseases such as type 2 diabetes.

In summary, EC and CPE alleviate the hepatic insulin resistance, as they decreased IRS-1 Ser636/639 phosphorylation, enhanced tyrosine phosphorylated and total levels of IR, IRS-1 and IRS-2 and activated PI3K/AKT pathway and AMPK at concentrations that are not toxic to hepatic cells and are reachable through the diet. In addition, EC and CPE preserve HepG2 cell functionality by restoring the levels of GLUT-2, increasing glucose uptake, maintaining glycogen synthesis and decreasing glucose production. Although the understanding of high glucose-induced insulin resistance has recently progressed, effective therapeutic strategies to prevent or delay the development of this damage remain limited. Further experiments to define in detail the mechanism of EC and CPE or other novel phytochemicals action may lead to the identification of molecular targets for the generation of therapeutic agents useful in the management of insulin resistance disease like diabetes.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Cocoa-rich diet ameliorates hepatic insulin resistance by modulating insulin signaling and glucose homeostasis in Zucker diabetic fatty rats☆

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Abstract

Insulin resistance is the primary characteristic of type 2 diabetes and results from insulin signaling defects. Cocoa has been shown to exert anti-diabetic effects by lowering glucose levels. However, the molecular mechanisms responsible for this preventive activity and whether cocoa exerts potential beneficial effects on the insulin signaling pathway in the liver remain largely unknown. Thus, in this study, the potential anti-diabetic properties of cocoa on glucose homeostasis and insulin signaling were evaluated in type 2 diabetic Zucker diabetic fatty (ZDF) rats. Male ZDF rats were fed a control or cocoa-rich diet (10%), and Zucker lean animals received the control diet. ZDF rats supplemented with cocoa (ZDF-Co) showed a significant decrease in body weight gain, glucose and insulin levels, as well as an improved glucose tolerance and insulin resistance. Cocoa-rich diet further ameliorated the hepatic insulin resistance by abolishing the increased serine-phosphorylated levels of the insulin receptor substrate 1 and preventing the inactivation of the glycogen synthase kinase 3/glycogen synthase pathway in the liver of cocoa-fed ZDF rats. The anti-hyperglycemic effect of cocoa appeared to be at least mediated through the decreased levels of hepatic phosphoenolpyruvate carboxykinase and increased values of glucokinase and glucose transporter 2 in the liver of ZDF-Co rats. Moreover, cocoa-rich diet suppressed c-Jun N-terminal kinase and p38 activation caused by insulin resistance. These findings suggest that cocoa has the potential to alleviate both hyperglycemia and hepatic insulin resistance in type 2 diabetic ZDF rats.
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Keywords: Cocoa; Glucose homeostasis; Glucose tolerance; Insulin resistance; Insulin signaling pathway; Type 2 diabetic ZDF rats

1. Introduction

Prevalence of type 2 diabetes mellitus (T2DM) is increasing globally and it has acquired epidemic proportions [1,2]; in fact, diabetes is currently one of the most costly and burdensome chronic diseases [3]. Despite that several drugs are available for the treatment of diabetes, adverse effects and drug resistance are of great concern. Therefore, there is an urgent need to continue working on the prevention and control of this pathology, and as a promising alternative, researchers are seeking natural products to prevent or treat diabetes because of their potential beneficial effects on health and their safety [2,4].

T2DM is a complex metabolic disorder characterized by sustained hyperglycemia that results from defects in insulin secretion, action or a combination of both [2]. Previous to the onset of T2DM, it appears a transitional state called pre-diabetes, characterized by an abnormal glucose homeostasis because of the impaired glucose tolerance and insulin sensitivity [5,6]. In this situation, the liver, which plays a critical role as it coordinates the whole body metabolism, is not able to control the glucose homeostasis [6]. At cellular level, insulin receptor (IR) autophosphorylation is less responsive to insulin, leading to a deregulation of downstream proteins of this pathway, such as insulin receptor substrates (IRS-1 and IRS-2), glycogen synthase kinase 3 (GSK3) and glycogen synthase (GS), and that is followed by a reduced synthesis of glycogen [6]. In addition, there is a failure in the control of the hepatic gluconeogenesis and glycolysis because of the misregulation of key enzymes for these processes, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucokinase (GK), respectively [2]. Similarly, net hepatic glucose flux and levels of the most expressed glucose transporter (GLUT) in the liver, GLUT-2, are also altered [6–8]. Mitogen-activated protein kinases (MAPKs) also contribute to the insulin desensitization; in fact, an increased phosphorylation of IRS-1, in particular, serine residues, has been reported during insulin resistance [9–11].

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Cocoa and its derived products are widely consumed in Europe and in the United States [12] and, together with its phenolic compounds, have increasingly attracted attention because of their potential ability to act as highly effective chemopreventive agents [2,4,13]. As anti-diabetic, cocoa flavanols have been shown to protect β-cells and decrease levels of blood glucose, limiting the risk factors for diabetes and other chronic diseases [2,13]. Accordingly, we have recently shown that a cocoa phenolic extract and its main flavanol, (−)-epicatechin, improve insulin sensitivity, as well as the glucose uptake and production in insulin-resistant hepatic HepG2 cells [14]. Indeed, both natural substances alone stimulate key proteins of the insulin pathway and regulate the glucose production in HepG2 cells [15]. In this regard, different studies have demonstrated that cocoa or its main components, such as epicatechin or procyanidins, exert a hypoglycemic effect on several animal models of diabetes and insulin resistance [16–20]. Moreover, cocoa and dark chocolate have been reported to lower glucose levels and improve insulin sensitivity in humans [21,22]. However, to the best of our knowledge, limited studies have evaluated the effects of cocoa on an animal model that mimics human T2DM; in fact, the precise mechanism for the potential anti-diabetic effect of cocoa related to glucose metabolism and insulin signaling in the liver remains largely unknown.

The aim of this study was to explore the contribution of cocoa on glucose homeostasis and insulin resistance in the liver of a widely used model for type 2 diabetes, Zucker diabetic fatty [ZDF; ZDF/*crl-lepr* (fa/fa)] rats, during the pre-diabetic stage (6–15 weeks of life). These animals possess a mutation in the leptin receptor and spontaneously develop severe obesity, hyperglycemia, hyperlipidemia and insulin resistance [23]. Here, we report that cocoa could act as an insulin sensitizer as it reduces hyperglycemia and hyperinsulinemia, ameliorates glucose intolerance and alleviates insulin resistance by attenuating the insulin signaling blockade in the liver of ZDF rats.

2. Materials and Methods

2.1. Materials and chemicals

Anti-extracellular regulated kinases (ERK1/2) and anti-phospho-ERK1/2 recognizing levels of phosphorylated Thr202/Thr204 of ERK1/2; anti-c-Jun N-terminal kinases (JNK1/2) and anti-phospho-JNK1/2 detecting phosphorylated Thr183/Thr185 of JNK1/2; anti-phospho-(Thr180/Thr182)-p38, anti-phospho-(Ser307)-IRS-1, anti-phospho-(Ser636/639)-IRS-1, anti-GSK3α/β and anti-phospho-GSK3α/β recognizing phosphorylated Ser21/Ser9 of GSK3; and anti-GS and anti-phospho-GS detecting phosphorylated Ser641 of GS, anti-IRS-2 and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-p38α, anti-IRβ, anti-PEPCK and anti-GK were purchased from Santa Cruz (sc-535, sc-711, sc-32879 and sc-7908, respectively; Quimigen, Madrid, Spain). Anti-IRS-1 and anti-GLUT-2 were obtained from Millipore (Madrid, Spain). Materials and chemicals for electrophoresis were from BioRad Laboratories S.A. (Madrid, Spain).

2.2. Cocoa

Natural Forastero cocoa powder (a kind gift from Nutrexpa S. L., Barcelona, Spain) was used for this study. A detailed description of this cocoa is given elsewhere [24,25].

Diets were prepared from an AIN-93G formulation (Panlab S. L., Barcelona, Spain) providing all nutrients required by adult rats. AIN-93G formulation lacking cellulose, starch and sucrose was prepared by adding the mentioned ingredients to adjust the quantities of carbohydrate and fiber according to the amount of cocoa added. Thus, the 10% cocoa diet was produced by adding 100 g/kg cocoa to AIN-93G; as a result, the cocoa diet was slightly richer in proteins (2.2%) and lipids (1.1%), but in order to make both diets isoenergetic (3612 kcal/kg diet), the content in carbohydrate was slightly reduced (4.2%). The total polyphenol content of the cocoa was determined by the spectrophotometric method of Folin–Ciocalteu using gallic acid as the standard. A Beckman DU640 spectrophotometer (Beckman Instruments, Inc.) was used. The composition of the diets is given in Table 1.

2.3. Animals and diets

Eight male Zucker lean (ZL) rats and sixteen ZDF/*crl-lepr fa* (5 weeks old) were purchased from Charles River Laboratories (L'Arbresle, France). Animals were caged in groups under controlled conditions (19–23°C, 50–60% humidity and 12-h light–dark cycles). After 1 week of acclimatization, that is, at 6 weeks of life, ZDF rats were randomly assorted into two different experimental groups: one group received a standard diet (ZDF-C) and the other one was fed with the cocoa-rich diet (ZDF-Co). ZL rats remained as a unique group and were fed with the standard control diet. Both diets were isocaloric and all animals were provided with food and water *ad libitum*.

Table 1
Composition of the experimental control (C) and cocoa-rich (Co) diets (g/kg dry weight)

	C	Co
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ[a]	0.008	0.008
Mineral mix	35	35
Vitamin mix	10	10
L-Cys	1.8	1.8
Cholin bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa powder	–	100
Protein (%)	–	22
Lipid (%)	–	11
Carbohydrate (%)	–	16
Fiber (%)	–	34
Total cocoa polyphenols[b]		2

[a] *tert*-Butylhydroquinone.
[b] Cocoa polyphenols were determined by the Folin–Ciocalteu method.

Food intake was monitored daily and animal weight was weekly followed. After 9 weeks of treatment (15 weeks old), blood was harvested from the trunk after decapitation in the absence of anesthesia from overnight fasted rats, and serum was separated by centrifugation at 1000*g*, 10 min, 4°C for further biochemical analysis. Livers were collected, weighted and frozen in liquid N_2 and stored at −80°C. Animals were treated according to Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas).

2.4. Glucose tolerance test (GTT)

One week before the sacrifice, overnight fasted rats were administered 35% glucose solution via saphenous vein (1 g/kg of body weight) and blood samples were obtained from the tail vein before the glucose load (t=0) and at 15, 30, 60, 90 and 120 min after glucose administration. Blood glucose levels were measured with a glucometer (LifeScan). Blood samples were centrifuged (1000*g* at 4°C) and serum was stored at −80°C until insulin estimation. Overall changes in glucose and insulin during GTT were calculated as the area under the curve (AUC) above the basal levels.

2.5. Preparation of liver lysates

Samples of frozen liver were homogenized 1:5 (w:v) in extraction buffer [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na_3VO_4, 5 μg/ml leupeptin, 20 μg/ml aprotinin, 2 mM benzamidine and 2 mM phenylmethylsulfonyl fluoride]. Homogenates were centrifuged at 14,000*g* for 60 min and the supernatants were collected, assayed for protein concentration by using the Bradford reagent and stored at −80°C until use for Western blot analyses.

2.6. Biochemical analysis

Blood glucose was determined using an Accounted Glucose Analyzer (LifeScan España, Madrid, Spain). Serum insulin was analyzed with a rat insulin enzyme-linked immunosorbent assay kit with a detection limit lower than 0.15 ng/ml (Mercodia, AD Bioinstruments, Barcelona, Spain). HbA1c was measured in blood using a latex turbidimetry kit following the manufacturer's instructions (Spinreact, BioAnalitica, Madrid, Spain). The minimum detectable amount was 0.1%.

Insulin sensitivity from final fasting insulin and glucose values was estimated by the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) according to the following formula: [fasting glucose (mM)×fasting insulin (mUI/L)]/22.5.

To quantify hepatic glycogen content, frozen liver samples were dissolved in 30% KOH, boiled and later centrifuged at 12,000*g* for 5 min, and glycogen was measured in the supernatants. Glycogen content was measured using a commercial glycogen fluorometric kit (BioVision, Deltaclon, Madrid, Spain) [14].

2.7. Western blot analysis

To detect IR, IRS-1, p-(Ser307)-IRS-1, p-(Ser636/639)-IRS-1, IRS-2, GSK3, p-GSK3, GS, p-GS, GCK, PEPCK, GLUT-2, ERK1/2, p-ERKs, JNK1/2, p-JNKs, p38 and p-p38, equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Millipore, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare, Madrid, Spain) or anti-mouse (Sigma, Madrid, Spain)

immunoglobulin. Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was ensured by β-actin and band quantification was carried out with a scanner and accompanying software.

2.8. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way analysis of variance (ANOVA) was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous.

Variables repeated over time were previously analyzed by a lineal mixed model considering a repetition factor and effect of time and diet treatment were evaluated, as well as the interactions between them. The analysis was followed by a one-way ANOVA. $P<.05$ was considered significant. A SPSS version 19.0 program has been used.

3. Results

3.1. Characteristics of the animals

Initial and final body weight and total food intake were significantly enhanced in both groups of ZDF rats in comparison to ZL animals (Table 2). Interestingly, the final body weight of animals fed with cocoa diet (ZDF-Co rats) was reduced as compared with ZDF-C group. In concert with this, both relative and body weight gain increased more in ZDF-C rats than in ZDF-Co animals, which showed similar values to those of ZL group. Moreover, total food intake was diminished in ZL rats when compared to ZDF-C and ZDF-Co animals, although food efficiency was equal among all groups (Table 2). ZDF-C and ZDF-Co rats had greater liver weight than ZL animals, and this was also maintained for the liver weight-to-body weight ratio, although there were no differences on this relation between ZDF-C and ZDF-Co groups (Table 2).

Glucose and insulin levels were elevated in ZDF groups in comparison to ZL rats, and those values were greater in ZDF-C animals than in ZDF-Co rats (Table 3). Additionally, ZDF-C rats displayed the highest values of post-prandial plasma glucose (measured after 1 h of food intake), whereas in ZL and ZDF-Co levels, they were comparable. Insulin concentration increased in both ZDF groups when compared to ZL rats, and ZDF-Co animals exhibited lower levels than ZDF-C rats.

As indicator of long-term glucose metabolism status, HbA1c levels were assayed. This parameter was maintained within a range of normal values, although it was slightly but significantly increased in ZDF-C rats, whereas ZDF-Co rats stayed at control levels.

All of these results could suggest that cocoa-rich diet ameliorates hyperglycemia and hyperinsulinemia in ZDF rats.

3.2. Cocoa-rich diet improves glucose tolerance and insulin sensitivity in ZDF rats

After 8 weeks of treatment (14 weeks of life), GTT was performed. There was a significant effect of time ($P=.002$) and diet ($P=.001$) on glucose and insulin levels (Fig. 1). Glucose levels were elevated in both groups of ZDF rats as compared with ZL animals during the 120-min follow-up (Fig. 1A). However, ZDF-Co rats showed intermediate values of blood glucose to those of ZL and ZDF groups during the GTT. These results were further supported by the AUC for glucose as increased the most in ZDF-C rats followed by ZDF-Co group, and ZL animals showed the lowest value (Fig. 1A). Additionally, insulin needed to cope with the glucose load was significantly reduced in ZL rats when compared to ZDF groups, and these levels were smaller in ZDF-Co than in ZDF-C animals during the 120-min follow-up (Fig. 1B). In concert, insulin sensitivity index (HOMA-IR index) increased in ZDF rats *vs.* ZL, but a significant decrease was observed in ZDF-Co animals in comparison to ZDF-C group (Fig. 1C). Thus, it could be suggested that cocoa-rich diet alleviates glucose intolerance and insulin resistance of ZDF rats.

Table 2
Body weight data, food intake and liver weight of rats fed with standard (ZL and ZDF-C) and cocoa-rich (ZDF-Co) diets

	ZL	ZDF-C	ZDF-Co
Initial body weight (g)	124.50±5.21[a]	146.75±12.98[b]	145.87±14.46[b]
Final body weight (g)	328.67±18.46[a]	444.13±13.47[c]	363.00±22.08[b]
Body weight gain (g in 9 weeks)	204.17±17.86[a]	297.38±12.63[b]	217.13±33.03[a]
Relative body weight gain [(body weight gain/initial body weight)×100] (%)	64.23±16.19[a]	104.32±22.77[b]	68.53±13.43[a]
Total food intake (g in 9 weeks)	983.97±54.58[a]	1353.54±96.01[b]	1279.10±58.49[b]
Food efficiency (body weight gain/food intake)	0.21±0.02[a]	0.22±0.01[a]	0.18±0.02[a]
Liver weight (g)	8.10±0.66 [a]	17.26±2.40[c]	13.26±1.85[b]
Liver to body weight [(liver weight/final body weight)×100] (%)	2.46±0.12[a]	3.89±0.47[b]	3.65±0.42[b]

Data represent the means±S.D. Means in a row without a common letter differ, $P<.05$.

Table 3
Glucose, insulin and HbA1c levels of rats fed with standard (ZL and ZDF-C) and cocoa-rich (ZDF-Co) diets

	ZL	ZDF-C	ZDF-Co
Post-prandial glucose (mmol/L)	8.18±1.13[a]	12.51±1.58[b]	8.91±1.72[b]
HbA1c (%)	4.20±0.07[a]	4.63±0.18[b]	4.23±0.12[a]
Glucose (mmol/L)	4.81±0.30[a]	11.22±1.48[c]	8.92±0.92[b]
Insulin (pmol/L)	67.80±7.39[a]	811.10±83.7[c]	613.75±63.46[b]

Data represent the means±S.D. Means in a row without a common letter differ, $P<.05$.

3.3. Cocoa-rich diet improves insulin signaling in ZDF rat's liver

It is known that, during a pre-diabetic situation, there is impairment of liver insulin signaling pathway characterized by an alteration of key proteins of the route [6]. In view of the obtained results, we studied whether a cocoa-rich diet modulates the insulin signaling in the liver, and total and phosphorylated levels of main proteins of this pathway were analyzed in the different groups of rats by Western blot. There were no significant differences in the content of IR and its substrates IRS-1 and IRS-2 among the animal groups (Fig. 2A–D). However, p-(Ser307)-IRS-1 and p-(Ser636/639)-IRS-1 levels, involved in the desensitization of insulin by diabetes [9–11], were enhanced in ZDF-C rats in comparison with ZDF-Co animals, which showed similar values to those of ZL group (Fig. 2A and E).

GSK3 is a rate-limiting enzyme the glycogen synthesis [6]. As shown in Fig. 3A and B, p-GSK3β, the most abundant isoform in the liver, decreased in ZDF-C rats when compared to ZL and ZDF-Co animals (Fig. 3A and B). In line with these results, ZDF-C animals presented increased p-GS levels that were diminished to achieve similar values to those of ZL group by feeding the animals with the cocoa-rich diet (Fig. 3A and C). The liver contents of total GSK3 and GS were not modified among groups. In addition, an enlargement in the liver glycogen content in ZDF-C rats was found, whereas ZDF-Co maintained similar values to those of their lean littermates (Fig. 3D). Altogether, it suggests that the cocoa-rich diet prevents the blockage of the insulin signaling cascade observed in ZDF rats by modulating main proteins of the insulin pathway from the early steps of this route, contributing to the glucose homeostasis.

3.4. Cocoa-rich diet modulates rate-limiting proteins of gluconeogenesis, transport and disposal of glucose in the liver of ZDF rats

To continue with the study of the glucose homeostasis, key proteins involved in hepatic glucose production as well as glucose transport across the plasma membrane and glucose utilization, namely PEPCK, GLUT-2 and GK, were evaluated by Western blot in all animal groups.

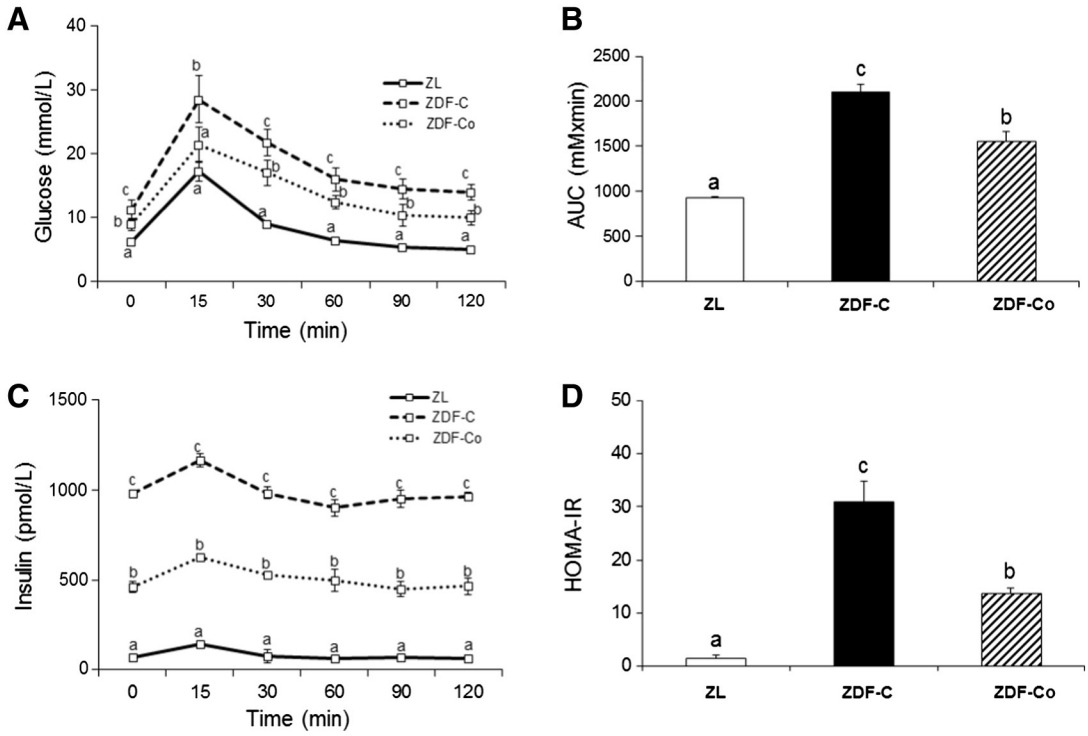

Fig. 1. Effect of the cocoa-rich diet on glucose tolerance and insulin resistance in ZDF rats. (A) Time-course changes in the basal level and after glucose loading (1 g/kg) of blood glucose. (B) AUC calculated from GTT data. (C) Time-course changes in the basal level and after glucose loading (1 g/kg) of serum insulin. Each point represents the mean±S.D. from 8 determinations. (D) HOMA-IR was determined as described in Materials and Methods. Values are expressed as means±S.D., n=6–8. Means without a common letter differ (P<.05).

Hepatic PEPCK levels increased in ZDF-C rats when compared to ZL animals, and this effect was totally repressed in animals receiving the cocoa rich-diet. Indeed, ZDF-Co rats showed comparable levels of PEPCK to those of ZL group (Fig. 4A and B). Additionally, GK and GLUT-2 levels were similar in ZL and ZDF-C animals, but both proteins significantly increased their content in ZDF-Co group (Fig. 4A, C and D). All of this suggested that cocoa-rich diet might also contribute to preserve the hepatic functionality and modulate the glucose homeostasis in ZDF rats.

3.5. Cocoa-rich diet modulates MAPKs signaling in ZDF rat's liver

MAPKs have been related to insulin resistance as they induce negative insulin sensitivity regulators such as the phosphorylation of Ser307 and Ser636/639 of IRS-1 [9–11,14]. Thus, phosphorylated and total levels of ERK, JNK and p38 were analyzed in the three groups of animals by Western blot.

Fig. 5 illustrates that phosphorylated levels of ERK, p38 and JNK increased in ZDF-C rats in comparison with ZL group. In this regard, p-ERK levels remained high in ZDF-Co animals, which showed comparable levels to those of ZDF-C rats. Likewise, the enhanced levels of p-p38 observed in ZDF-C rats were diminished by the cocoa-rich diet, as ZDF-Co animals presented intermediate values to those of ZL and ZDF-C rats. Phosphorylated levels of JNK were similar in ZDF-Co and ZL rats, showing that the enhancement in the values of p-JNK in ZDF-C animals was totally prevented by feeding the rats with the cocoa-rich diet. There was no difference in the total levels of ERK, JNK and p38 among groups. Therefore, the cocoa-rich diet partly or totally prevented the activation of p38 and JNK, although it did not modify the increased values of active ERK in ZDF rats; this modulation of MAPKs could contribute to attenuate the insulin resistance in ZDF-Co animals.

4. Discussion

Prevention and treatment of T2DM involves lifestyle modifications such as increased physical activity and intake of fruits and vegetables [5], which constitute a good source of flavonoids. Growing evidence suggests the anti-diabetic activity of flavonoids [2,4,13,21,22], although insufficient investigation at molecular level has been performed to support these observations.

This study shows that a cocoa-rich diet improves glycemic control and insulin sensitivity in ZDF rats. To our knowledge, this is the first demonstration that a cocoa-rich diet prevents or ameliorates the hepatic dysfunction that takes place in T2DM by restraining the increase of Ser307-IRS-1 and Ser636/639-IRS-1 phosphorylated levels, the inhibition of GSK3-GS pathway and the increase of JNK and p38 phosphorylated values (Fig. 6). Furthermore, we have shown that cocoa prevents the enhancement of PEPCK levels, increases GLUT-2 and GK content and protects the hepatic functionality by preserving glycogen content (Fig. 6).

Cocoa-rich diet resulted in a decreased body weight gain without influencing total food intake in comparison to ZDF-C group. This reduction of body weight is in agreement with previous studies [26,27], and it has been attributed to the cocoa polyphenolic fraction and its ability to reduce fat adipose tissue. Additionally, the lack of effect of the cocoa-rich diet on food intake observed in this study excludes the possibility that cocoa enhances glucose tolerance simply by reducing food intake, as previously shown for its main flavanol, epicatechin and a cocoa extract [17,20]. Assessment of the liver weight-to-body weight ratio was used to investigate potential changes in the liver size, and enhanced values were found in ZDF animals *vs*. ZL rats, which may indicate adverse health consequences, including liver steatosis and/or enlarged glycogen content [23]. However, cocoa-rich diet seemed not to exert any effect on this parameter, as shown in ZDF rats fed with diets enriched in natural

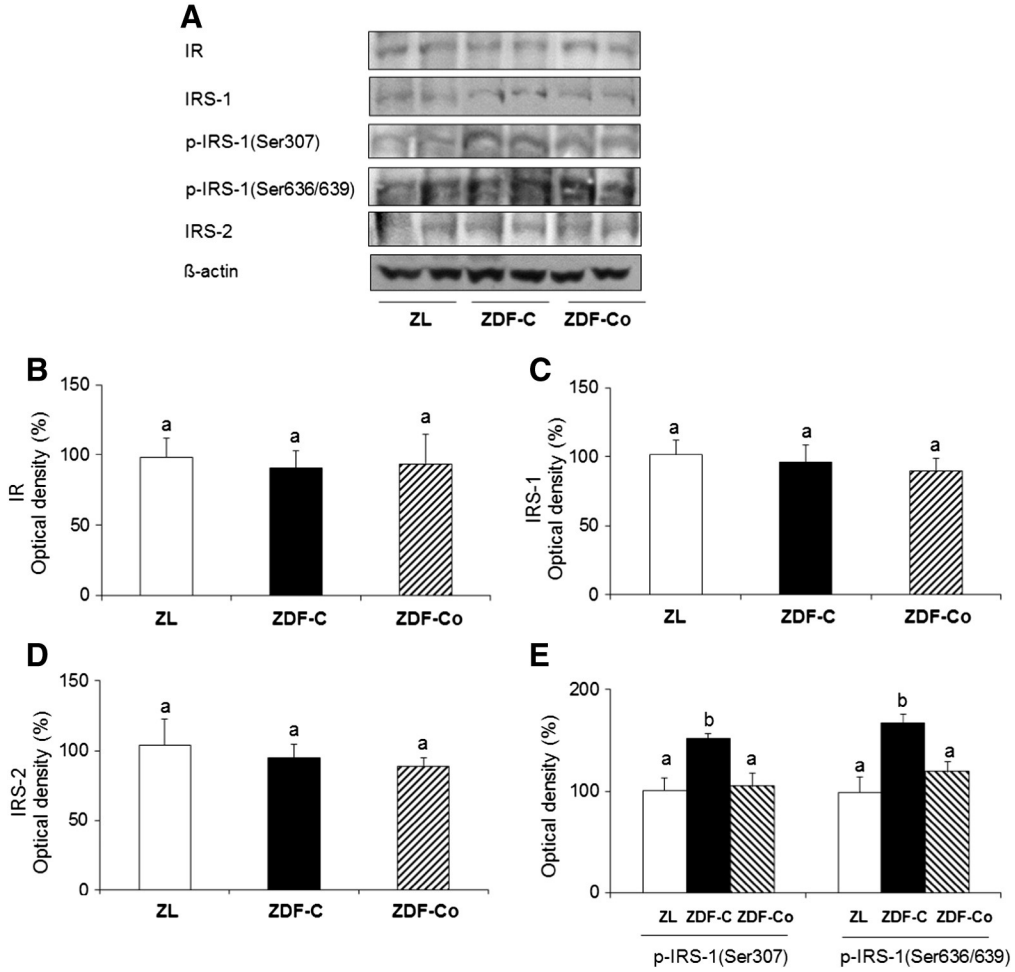

Fig. 2. Effect of the cocoa-rich diet on total levels of IR and its substrates and serine phosphorylated IRS-1 in liver of ZDF rats. (A) Bands of representative experiments. Densitometric quantification of (B) IR, (C) IRS-1, (D) IRS-2 and (E) p-IRS-1 (Ser). Values are expressed as a percentage relative to ZL rats. Equal loading of Western blots was ensured by β-actin (n=6–8). Means without a common letter differ (P<.05).

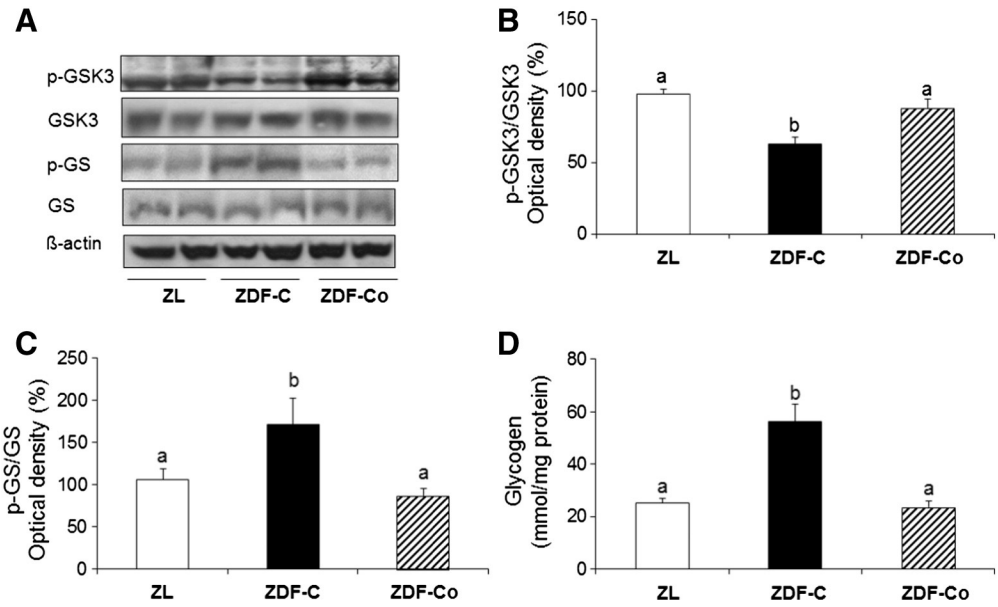

Fig. 3. Effect of the cocoa-rich diet on phosphorylated and total levels of GSK3β and GS and glycogen content in liver of ZDF rats. (A) Bands of representative blots. Percentage data of (B) p-GSK3β/GSK3β and (C) p-GS/GS ratios relative to ZL group. Values are expressed as means±S.D., n=6–8. Equal loading of Western blots was ensured by β-actin. (D) Glycogen content expressed as percent of control are means±S.D. of 6–8 different samples per animal group. Means without a common letter differ (P<.05).

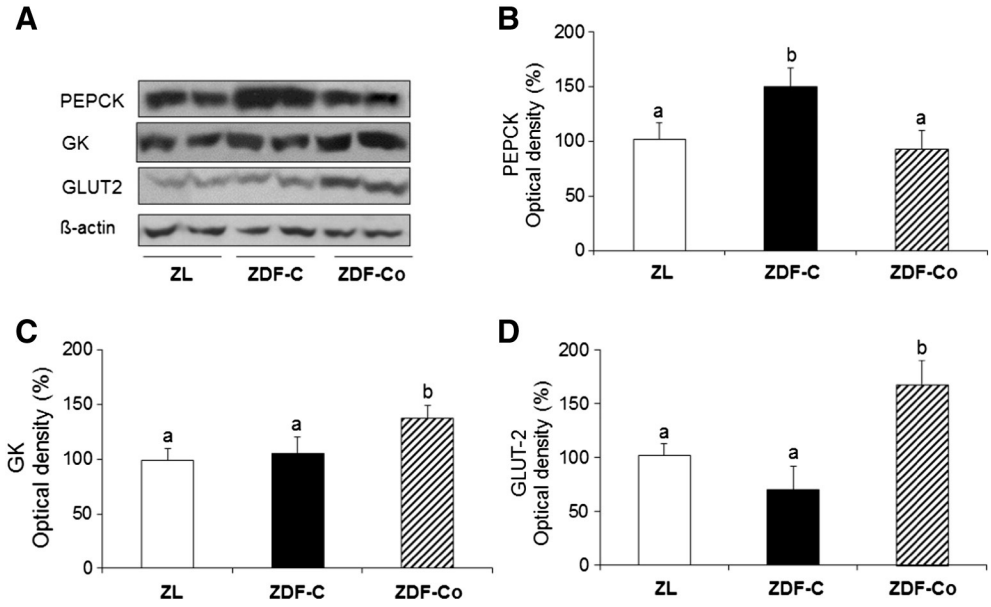

Fig. 4. Effect of the cocoa-rich diet on total levels of PEPCK, GK and GLUT-2 in liver of ZDF rats. (A) Bands of representative experiments. Densitometric quantification of (B) PEPCK, (C) GK and (D) GLUT-2. Values are expressed as a percentage relative to ZL animal group. Equal loading of Western blots was ensured by β-actin (n=6–8). Means without a common letter differ (P<.05).

compounds [28,29]. Nevertheless, the diminution in body weight has been related to reduced circulating glucose blood levels and improved lipid profiles in cocoa-fed diabetic rats [20]. It is worth mentioning that phenolic compounds in cocoa regulate lipid metabolism by inducing metabolic gene expression and/or activating transcription factors, which may play an important role in energy metabolism and insulin sensitivity [13]. In fact, activation of 5′-AMP-activated protein kinase by cocoa polyphenols stimulates downstream events that suppress hyperglycemia and fat deposition, leading to the prevention of hyperglycemia, hyperinsulinemia and insulin resistance [13]. Additionally, cocoa polyphenols have demonstrated to decrease fatty acid synthesis and transport systems, up-regulate the expression of genes for fatty acid β-oxidation and promote part of the thermogenesis mechanism to activate energy expenditure in the liver [13,30]. All of these features result in a reduction of intra-abdominal fat and improvement of hyperlipidemia and liver steatosis [13,30]. In this regard, it has been reported that oral ingestion of other natural compounds such as *Aloe ver*a phytosterols suppresses expressions of

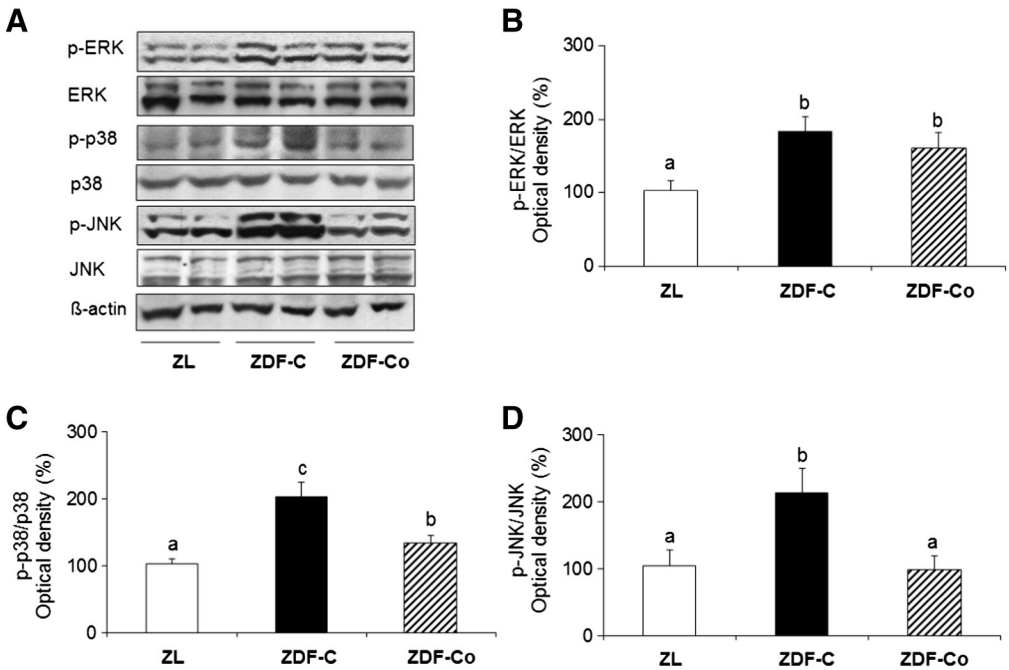

Fig. 5. Effect of the cocoa-rich diet on phosphorylated and total levels of ERK, p38 and JNK in liver of ZDF rats. (A) Bands of representative experiments. Percentage data of (B) p-ERK/ERK, (C) p-p38/p38 and (D) p-JNK/JNK ratios relative to ZL animals (means±S.D., n=6–8). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly (P<.05).

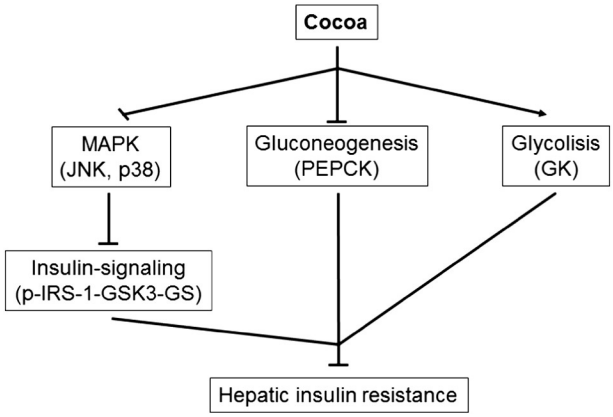

Fig. 6. Schematic diagram representing the hepatoprotective effects of cocoa-rich diet against insulin resistance in the ZDF rat liver.

gluconeogenic and lipogenic enzymes and increases enzymes related to glycolysis and lipolysis in the livers of ZDF rats, being all of these changes partially regulated by key transcription factors of lipid metabolism and contributing to the prevention of hyperglycemia, reduced hyperlipidemia, improved liver steatosis and visceral fat accumulation [28]. However, further studies are needed to clearly elucidate the mechanism of lipid regulation by cocoa phenolic compounds.

In ZDF rats, hyperglycemia develops between 7 and 12 weeks [23]. Interestingly, the present work demonstrates that cocoa supplementation reduces short- and long-term glycemia, as assessed by postprandial glucose and GTT (Fig. 1) and fasting glucose at the end of the experiment (Table 3), respectively. In line with this, epicatechin, oligomeric cocoa procyanidins and different cocoa extracts have proved to reduce the glucose levels on diabetic rodents [16–18,20]. This short-term effect has been related to the suppression of glucose intestinal absorption by catechins present in cocoa in rodents [16,17], which could contribute to the post-prandial glycemic control and body weight gain, but it has also been associated to an increased postprandial insulin secretion, as shown in humans fed with cocoa-supplemented diets [22]. In agreement with our results, a positive effect on long-term glucose and insulin levels was found in insulin-resistant rats fed with a grape seed procyanidin extract [31]. Consistently, the improved glucose tolerance induced by cocoa-rich diet in ZDF rats was accompanied by reduced insulin levels at short term and at long term, which points to the improved hormone sensitivity, as evidenced by a decreased HOMA-IR index. Diminished insulin values have also been reported in insulin-resistant rodents fed with procyanidins from cocoa or grapes [16,31]. Moreover, in our experimental conditions, cocoa reduced body weight of ZDF rats without affecting the food intake, which could contribute to the improved insulin sensitivity, as previously shown for high-fat fed mice supplemented with oligomeric cocoa procyanidins [16].

Insulin resistance is a crucial pathophysiological factor in the development and progression of diabetes [6,23], and the hepatic insulin resistance is characterized by an impaired insulin signaling. Thus, the modulation of IR and its downstream substrates IRS-1 and IRS-2, which is essential for recruiting and activating downstream pathways, is damaged [6]. Indeed, IRS phosphorylation in particular serine residues, such as Ser307 and Ser6363/639, is enhanced in hepatic insulin resistance [9–11], as we have shown in the present study. However, the cocoa-rich diet prevented this critical aspect for the development of insulin resistance in ZDF rats. In agreement with this, we have recently reported that a cocoa extract attenuated insulin resistance by reducing IRS-1 serine phosphorylation in HepG2 cells [14]. Similarly, anthocyanins derived from purple sweet potato color attenuated insulin resistance by reducing IRS-1 serine phosphorylation in the liver of mice treated with high-fat diet [32].

Several pathways are activated in response to IRS phosphorylation, such as PI3K/AKT [6]. GSK3β is a critical substrate of the PI3K/AKT signaling implicated in the regulation of glycogen synthesis, and its activity can be inhibited by phosphorylation at Ser9. Subsequently, GSK3β phosphorylates and inactivates GS, which plays an important role in insulin resistance [6]. Thus, ZDF-C rats showed decreased hepatic p-GSK3β and increased GS phosphorylated levels although enhanced glycogen content compared to ZL animals, as previously shown [23]. These effects were abolished when animals were fed the cocoa-rich diet. Interestingly, activation of GS by a GSK3 inhibitor improves glucose disposal during an oral GTT in ZDF rats, which indicated a defect in net glycogenesis [33]. In this line, different natural compounds have shown to reactivate this pathway in different insulin-resistant models. Thus, anthocyanins derived from purple sweet potato color and a green tea polyphenol extract increased the expression levels of GSK3 in the liver of insulin-resistant rats [32,34]. Similarly, we have previously demonstrated that a cocoa extract improved insulin-mediated GSK3 activation and restored the glycogen content in insulin resistant HepG2 cells [14]. Moreover, it has also been reported that extracts rich in phenolic compounds from fruits and plants reversed inhibition of glycogen synthesis in the liver of diabetic rodents [35–37] and in insulin-resistant hepatic cells [9,38]. Altogether, this indicates that cocoa could improve hepatic insulin resistance by inhibiting GSK3β activation mediated by impaired insulin signaling.

The roles of insulin signaling in the liver include activation of glycogen synthesis for energy storage and suppression of hepatic glucose output by inhibiting PEPCK and glucose-6-phosphatase [6]. In addition, hepatic GK plays a major role in controlling glucose utilization in this organ [6]. Therefore, increased glucose production, decreased glycogen synthesis and glycolysis are main symptoms in T2DM [6]. In the current study, ZDF-C rats showed increased levels of PEPCK and comparable values of GK to those of ZL animals. It has been reported that hepatic GK decreased progressively with the development of hyperglycemia in ZDF rats, showing equal levels to those of ZL animals at a young age [39], in agreement with our results. In contrast, GK values were enhanced in cocoa-fed ZDF rats, which could be related to an increased utilization of blood glucose for energy production [40]. Moreover, expression levels of PEPCK were reduced to ZL values by feeding the ZDF animals with the cocoa-rich diet. In agreement with this, we have previously shown in insulin-resistant HepG2 cells that a cocoa extract prevents the enhancement of PEPCK values [14]. Accordingly, other phenolic compounds such as hesperidin, naringin and phenolic extracts from plants, as well as food flavoring preservative agents, diminished the expression levels and activities of the gluconeogenic enzyme PEPCK and up-regulated GK in the liver of type 2 diabetic mice [36,40,41]. Interestingly, treatment of ZDF rats with anti-diabetic drugs, such as PPAR-γ agonists, decreases PEPCK and increases GK, which may lead to enhanced glycolysis and glycogen synthesis and decreased blood glucose and insulin levels [42]. However, overcompensation of GK activity to 2-fold above that for non-diabetics controls is needed to achieve glycemic control [39]. Therefore, all of these results suggest that cocoa might improve hyperglycemia via the restoration of PEPCK and the enhancement of GK in ZDF rats.

GLUT-2 maintains intracellular glucose in equilibrium with extracellular glucose in the liver, although this balance could be altered during insulin resistance [6,43]. In this line, natural substances such as the terpenoid borapetoside C and food flavoring preservative agents increase the expression of hepatic GLUT-2 in diabetic mice, which has been related to the hypoglycemic effect [41,44]. Similarly, the anti-diabetic drugs telmisartan, sitagliptin and metformin increase the levels of hepatic GLUT-2 on insulin-resistant mice, pointing to the

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normalization of post-receptor insulin signaling and the restoration of the hepatic insulin sensitivity [45]. In fact, we have recently demonstrated that a cocoa extract increased GLUT-2 levels and glucose uptake in insulin-resistant HepG2 cells [14]. In agreement with all of the above, comparable contents of hepatic GLUT-2 have been reported in mildly diabetic ZDF and ZL rats [46], whereas the transporter levels increased in ZDF rats fed with cocoa. Therefore, the increased GLUT-2 content could also contribute to the hypoglycemic effect and to improve the hepatic sensitivity to the hormone observed in ZDF-Co animals.

MAPKs are involved in the development of insulin resistance [6]. Indeed, MAPKs induce negative regulators of insulin sensitivity such as protein-tyrosine phosphatase-1B, p-Ser307-IRS-1 and p-(Ser636/639)-IRS-1 [10,11]. In concert with this, high phosphorylated levels of ERK, JNK and p38-MAPK have been demonstrated in ZDF rats [47,48]. In the current study, feeding ZDF animals with the cocoa-rich diet abolished the increased p-JNK and p38 values, although not for p-ERK levels. Accordingly, JNK blockage mediated the inhibition of IRS-1 serine phosphorylation in the liver of ZDF rats [48] and in human hepatic cells [9], which has been related to an improved insulin resistance. Moreover, in the brain of ZDF rats, JNK and p38 were suppressed by a peptide with anti-diabetic properties [47]. Interestingly, in our experimental conditions, p-ERK values remained increased in ZDF rats fed with cocoa, and we have previously shown that a cocoa extract enhanced p-ERK values in hepatic cells, which was associated to a protective effect against an induced oxidative stress [25]. Although further studies would be needed to clearly elucidate the role of ERK in cocoa-fed ZDF animals, it could be suggested that cocoa-rich diet modulates MAPKs, and this might contribute to improve the hepatic insulin resistance.

In summary, a cocoa-rich diet alleviates glycemic homeostasis and insulin resistance in ZDF rats. Therefore, cocoa ameliorates hyperglycemia through its ability to preserve the hepatic functionality by preserving the levels of GLUT-2 and glycogen content and to modulate gluconeogenic and glycolytic enzymes, as it decreases hepatic PEPCK and enhances hepatic GK. Furthermore, cocoa improves insulin resistance by decreasing IRS-1 Ser307 and Ser636/639 phosphorylation and activated GSK3/GS pathway. This study provides novel mechanistic insights into the preventive effects of cocoa on hepatic insulin resistance in T2DM and could indicate that cocoa's anti-diabetic effects may be helpful in preventing or ameliorating the development of T2DM.

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Capítulo 2: Efecto de los flavanoles del cacao sobre el estrés oxidativo hepático durante la resistencia a la insulina y la diabetes

Resumen

Antecedentes: Las alteraciones que se producen en la diabetes y la resistencia a la insulina son consecuencia, al menos en parte, del estrés oxidativo, que juega un papel crítico en el desarrollo y progreso de la DMT2. Recientemente ha aumentado el interés por la identificación de antioxidantes de la dieta con propiedades antidiabéticas, ya que suponen una alternativa prometedora para la prevención y/o tratamiento de la DMT2 al presentar baja o nula toxicidad, ser abundantes en la naturaleza y baratos de producir.

Objetivo: Analizar el posible efecto beneficioso del cacao y la EC, principal flavanol del cacao, sobre el estado redox hepático en una situación de resistencia a la insulina y diabetes en un modelo *in vitro* (células hepáticas humanas HepG2 tratadas con dosis altas de glucosa) e *in vivo* (ratas ZDF diabéticas).

Metodología: Las células HepG2 se expusieron a altas concentraciones de glucosa (20-60 mM) para establecer un modelo de resistencia a la insulina que indujera cambios en el estado redox. Posteriormente, para evaluar el posible efecto protector de EC (10 µM) y CPE (1 µg/mL), las células se pretrataron 24 horas con ambas sustancias naturales y, a continuación, con una alta dosis de glucosa (30 mM, 24 horas). Por su parte, las ratas se alimentaron con una dieta estándar (ZDF-C y ZL) o con dieta enriquecida con un 10% de cacao (ZDF-Ca) durante 9 semanas (6-15 semanas de vida). En los dos modelos experimentales se analizaron marcadores de daño oxidativo (producción de ROS, niveles de grupos carbonilo), el contenido de GSH, la actividad de defensas antioxidantes-detoxificantes (GPx, GR, CAT, GST), así como los niveles del factor de transcripción Nrf2. En el modelo *in vitro* también se evaluaron los niveles de las proteínas MAPKs y su papel en la protección del extracto de cacao en la resistencia a la insulina, mientras que en el estudio *in vivo* se analizó la actividad de las enzimas SOD y HO-1 y los niveles del factor de transcripción NF-κB.

Resultados: En las células HepG2, la concentración mínima de glucosa necesaria para inducir cambios en el estado redox y resistencia a la insulina fue 30 mM, y por tanto, fue la dosis seleccionada para los siguientes experimentos. El pretratamiento con EC y CPE en las células HepG2 y la administración de una dieta enriquecida en cacao a las ratas ZDF protegieron frente al estrés oxidativo. Todos los tratamientos disminuyeron la generación de ROS y previnieron el aumento en los niveles de los grupos carbonilos,

que mostraron valores similares a los de los controles en ambos modelos experimentales. En las células resistentes a la insulina, EC y CPE reprimieron el descenso de los niveles de GSH y la actividad de GPx, y CPE frenó el descenso de la actividad de GR, aunque EC y CPE no fueron capaces de prevenir la disminución en la GST inducida por la dosis alta de glucosa. En los animales, el cacao restauró la actividad de SOD y de HO-1, pero no la de GST, que permaneció disminuida. La actividad de la CAT en las células, y las de GPx, GR y CAT, así como el contenido de GSH en las ratas, no se modificaron por ningún tratamiento. Además, en los animales ZDF, la dieta rica en cacao suprimió el aumento en los niveles de Nrf2 y NF-κB. Por su parte, en las células HepG2, EC y CPE activaron a las ERK y el Nrf2, situación que se mantuvo cuando las células fueron pretratadas con ambas sustancias, a la vez que previnieron la activación de p38 y JNK inducida por la dosis alta de glucosa. En estas condiciones de resistencia, los inhibidores selectivos de las MAPKs indujeron cambios en el estado redox, la captación de glucosa y los niveles totales y fosforilados en serina de IRS-1, lo que sugiere la implicación de las MAPKs en los efectos protectores mediados por CPE.

Conclusión: Estos resultados muestran que el cacao y la EC alivian el desequilibrio redox provocado durante la resistencia a la insulina y mantienen la funcionalidad de los hepatocitos al mejorar las defensas antioxidantes y modular factores de transcripción clave sensibles al estado redox (Nrf2 y NF-κB). Además, estos efectos beneficiosos del cacao se deben en parte, por su acción sobre las MAPKs.

RESEARCH ARTICLE

Cocoa flavonoids protect hepatic cells against high-glucose-induced oxidative stress: Relevance of MAPKs

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Scope: Oxidative stress plays a main role in the pathogenesis of type 2 diabetes mellitus. Cocoa and (-)-epicatechin (EC), a main cocoa flavanol, have been suggested to exert beneficial effects in type 2 diabetes mellitus because of their protective effects against oxidative stress and insulin-like properties. In this study, the protective effect of EC and a cocoa phenolic extract (CPE) against oxidative stress induced by a high-glucose challenge, which causes insulin resistance, was investigated on hepatic HepG2 cells.

Methods and results: Oxidative status, phosphorylated mitogen-activated protein kinases (MAPKs), nuclear factor E2 related factor 2 (Nrf2) and p-(Ser)-IRS-1 expression, and glucose uptake were evaluated. EC and CPE regulated antioxidant enzymes and activated extracellular-regulated kinase and Nrf2. EC and CPE pre-treatment prevented high-glucose-induced antioxidant defences and p-MAPKs, and maintained Nrf2 stimulation. The presence of selective MAPK inhibitors induced changes in redox status, glucose uptake, p-(Ser)- and total IRS-1 levels that were observed in CPE-mediated protection.

Conclusion: EC and CPE recovered redox status of insulin-resistant HepG2 cells, suggesting that the functionality in EC- and CPE-treated cells was protected against high-glucose-induced oxidative insult. CPE beneficial effects on redox balance and insulin resistance were mediated by targeting MAPKs.

Keywords:
Antioxidant defences / Cocoa flavanols / HepG2 cells / Insulin resistance / Oxidative markers

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1 Introduction

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder and one of the most common chronic diseases [1]. This pathology is characterized by two hallmarks: insulin resistance and impaired insulin secretion, and both alterations have been associated to oxidative stress [2, 3]. Antioxidants have been shown to exert beneficial effects against hyper-glycaemia, insulin resistance and oxidative stress [4, 5], which draws the attention towards the identification of antioxidant components with these properties, and

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Abbreviations: 2-NBDG, D-glucose, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino); **CAT**, catalase; **CPE**, cocoa phenolic extract; **EC**, (-)-epicatechin; **ERK**, extracellular-regulated kinase; **GPx**, glutathione peroxidase; **GR**, glutathione reductase; **GSH**, glutathione; **GST**, glutathione-*S*-transferase; **IRS**, insulin receptor substrate; **JNK**, c-Jun amino-terminal kinase; **MAPKs**, mitogen-activated protein kinases; **Nrf2**, nuclear factor E2 related factor 2; **PI3K**, phosphatidylinositol 3-kinase; **T2DM**, type 2 diabetes mellitus

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constitutes a promising approach in the prevention and/or treatment of T2DM [4, 6].

During T2DM in the liver, there is a miss-regulation of the glucose homeostasis and of the insulin pathway, and a decrease in the levels of antioxidants, such as glutathione (GSH), because of the oxidative stress induced by the hyper-glycaemia [2, 7, 8]. To avoid the damage by an overproduction of reactive oxygen species (ROS), cellular antioxidant defences, such as glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), glutathione-*S*-transferase (GST), GSH and ascorbic acid [8, 9], play a crucial role. In this line, the redox-sensitive transcription factor nuclear factor E2 related factor 2 (Nrf2), which is regulated by dietary flavonoids, modulates critical genes of enzymatic defences [9, 10], and its primary control of function lies on its subcellular distribution and/or phosphorylation [9–11]. Moreover, a number of cellular kinases, including the mitogen-activated protein kinases (MAPKs), also modulated by dietary flavanols [12–14], may regulate Nrf2.

Cocoa is largely consumed and constitutes a rich source of biophenols with a wide array of biological activities [15, 16]. Its beneficial health effects have been mainly attributed to its elevated phenolic content, especially (-)-epicatechin (EC) that is the most abundant flavonoid in cocoa [17]. Accumulating data in non-insulin-resistant hepatic cells under basal or stress conditions have demonstrated that EC is an effective free radical scavenger and interferes with the cellular oxidative/antioxidative potential [13, 17–19], resulting in the induction of survival/proliferation pathways through the protein kinase B/phosphatidylinositol 3-kinase (AKT/PI3K) and extracellular-regulated kinase (ERK) pathways. Moreover, in a non-pathological situation in hepatic cells EC stimulates the antioxidant defence response by activating redox-regulated transcription factors [11, 12], and a cocoa phenolic extract (CPE) protects against oxidative stress by modulating the antioxidant enzyme activities through ERK [14, 17]. Additionally, we have recently reported in insulin-resistant HepG2 cells induced with high glucose that a CPE and EC improve insulin sensitivity, preventing or delaying a potential hepatic dysfunction through the attenuation of the insulin signalling blockade and the modulation of glucose uptake and production [20]. In fact, EC and CPE alone stimulate key proteins of the insulin route and regulate the glucose production through AKT and AMPK modulation in HepG2 cells [21]. In this regard, it should be mentioned that EC is just one of the many bioactive substances present in CPE and that the synergic effect of phenolic compounds in foodstuffs should be taken into account, as previously shown [19]. All the above indicates that EC and cocoa may have interesting health protective benefits, including insulinomimetic and antioxidant effects. It could be hypothesized that EC and CPE could exert an antidiabetic effect by reducing or even supressing the oxidative stress of T2DM through the modulation of the cellular antioxidant defences and close-related key proteins for that response. Nevertheless, there is currently a controversy about the enzymatic response of the antioxidant defences during insulin resistance, and enhanced, decreased and unchanged activities have been reported in serum of T2DM patients [8]. Similarly, an unclear role has been attributed to Nrf2 in T2DM. In this regard, and to the best of our knowledge this is the first time that the response and precise antioxidant defence molecular mechanism for the preventive activities of EC and cocoa during insulin resistance in the liver have been studied in terms of antioxidant defences, Nrf2 and regulation by MAPKs.

Thus, the aim of the study was to test the protective effects of EC and CPE against the oxidative stress induced by a high-glucose challenge in the mentioned model of insulin-resistant HepG2 cells. Markers of oxidative damage, antioxidant defences and related signals, as well as stress-related signalling pathways and keys features of insulin resistance were evaluated to assess the effect of EC and CPE in the cellular response to an induced oxidative stress.

2 Materials and methods

2.1 Materials and chemicals

(–)-EC (>95% of purity), D-glucose, gentamicin, penicillin G, streptomycin, PD98059, SB203580, SP600125, GR, GSH, glutathione oxidized, nicotine adenine dinucleotide phosphate reduced salt (NADPH), *tert*-butylhydroperoxide, *o*-phthaldehyde and dichlorofluorescin (DCFH) were purchased from Sigma Chemicals (Madrid, Spain). GST fluorometric activity kit was from BioVision (Deltaclon, Madrid, Spain). The fluorescent probe D-glucose, 2-deoxy-2-((7-nitro-2,1,3-benzoxadiazol-4-yl)amino) (2-NBDG) was purchased from Molecular Probes (Invitrogen, Madrid, Spain). Anti-ERK1/2 and anti-phospho-ERK1/2 recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2 (where JNK is c-Jun amino-terminal kinase) and anti-phospho-JNK1/2 recognizing phosphorylated Thr183/Tyr185 of JNK1/2, anti-phospho-Thr180/Tyr182-p38, anti-phospho-(Ser307)-IRS-1, anti-phospho-(Ser636/639)-IRS-1 and anti-β-actin were obtained from Cell Signalling Technology (Izasa, Madrid, Spain). Anti-p38α (sc-535), anti-Nrf2 (C-20, sc-722), anti-Nrf2 (H-300, sc-13032) were purchased from Santa Cruz (Qimigen, Madrid, Spain). Anti-lamin B1 (ab16048) was purchased from Abcam (Cambridge, UK). Bradfrod reagent, and materials and chemicals for electrophoresis were from Bio-Rad Laboratories S.A. (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively.

2.2 Cocoa polyphenol extraction

Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. Soluble polyphenols were extracted by sequentially washing 1 g of sample with 40 mL of 16 mM hydrochloric acid in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 mL of acetone:water (70:30, v/v, 1 h at room temperature,

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constant shaking). After centrifugation (15 min, 3000 × *g*), supernatants from each extraction step were combined and made up to 100 mL. The desiccated extract was dissolved in distilled water and kept frozen until assay. A detailed description of this CPE has been previously published [17]. The amount of EC and polyphenols present in the CPE were 383.5 mg/100 g (determined by LC-MS) and 2 g/100 g on dry matter basis (determined by Folin-Ciocalteu) [17].

2.3 Cell culture and treatments

Human HepG2 cells were grown in DMEM-F12 medium supplemented with 2.5% foetal bovine serum and 50 mg/L antibiotics (gentamicin, penicillin, and streptomycin). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. One day after plating, the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and foetal bovine serum, and the culture was continued.

Human hepatoma HepG2, a well-differentiated transformed cell line, is a reliable model, easy to culture, well characterized and widely used for biochemical and nutritional studies where many antioxidants and conditions can be assayed with minor inter-assay variations [11, 14, 17]. In addition, steady-state functioning of the antioxidant defences in human hepatoma HepG2 is relatively higher than that in hepatocytes and other non-transformed cells, therefore, variations of responses to different conditions are more easily detected.

To induce a condition of oxidative stress, cells were treated for 24 h with various concentrations of glucose in serum-free media. At the end of the treatment, cells were incubated with 100 nM insulin for 10 min and then harvested, as previously described [20, 22, 23] and tested for ROS production, GSH and carbonyl content, and enzymatic activities (GPx, GR, CAT and GST)

To evaluate the protective effect of EC and CPE against high-glucose challenge, 10 μM EC or 1 μg/mL CPE were added to the cells for 24 h; then, the medium was discarded and fresh medium containing 30 mM glucose was added for additional 24 h. Later, cells were incubated with 100 nM insulin for 10 min to test the response to insulin and harvested. The selection of the concentrations of glucose, EC, and CPE is explained below (see Sections 3 and 4).

In the experiments with the pharmacological inhibitors, cells were pre-incubated with 50 μM PD98059 (specific inhibitor of ERK), 10 μM SB203580 (specific inhibitor of p38) or 40 μM SP600125 (JNK-specific inhibitor) for 1 h prior to high-glucose challenge. The concentrations of the inhibitors employed have been selected according to our previously used doses in HepG2 cells [11, 13, 14, 24–26].

2.4 Determination of ROS production

Intra-cellular ROS were quantified by the DCFH assay [13]. After being oxidized by intra-cellular oxidants, DCFH becomes dichlorofluorescein and emits fluorescence. Briefly, at the end of the treatment 5 μM DCFH probe was added to cells cultured in 24-well plates (2×10^5 cells per well) for 30 min at 37°C. Then, the unabsorbed probe was removed, medium with the different treatments added and fluorescence immediately measured in a micro-plate reader (Bio-Tek, Winooski, VT) at 485 nm/50 nm (excitation/emission wavelengths, respectively).

2.5 Analysis of GSH content

GSH was quantified by Hissin and Hilf fluorimetric assay [27]. The method is based on the reaction of GSH with *o*-phthaldehyde at pH 8.0 and fluorescence was measured at excitation wavelength of 340 nm and emission wavelength of 460 nm. The results of samples were referred to those of a standard curve of GSH.

2.6 Determination of carbonyl groups

Protein oxidation of cells was measured as carbonyl groups content according to a published method [28]. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22 000 nmol/L/cm. Protein was measured by the Bradford reagent.

2.7 Determination of GPx, GR, CAT and GST activities

Determination of GPx activity is based on the oxidation of GSH by GPx, using *tert*-butylhydroperoxide as a substrate, coupled to the disappearance of NADPH by GR [14, 17]. GR activity was assayed by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [14, 17]. CAT activity was determined by the decomposition of H_2O_2 as a decrease in absorbance at 240 nm [28].

GST activity was analysed by a commercial fluorimetric activity assay kit [28]. The assay utilizes monochlorobimane as an artificial substrate and GSH to determine total GST activity. Fluorescence was measured using a fluorescent micro-plate reader at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Protein concentration was measured by the Bradford reagent.

2.8 Preparation of total cell lysates for Western blotting

To detect ERK, p-ERK, JNK, p-JNK, p38, p-p38, total Nrf2 and p-(Ser)-IRS-1, cells were lysed at 4°C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1% Triton X-100, 200 mM

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β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at −80°C until used for Western blot analyses.

2.9 Preparation of nuclear cell extracts for Western blotting

To analyse nuclear Nrf2, cells were re-suspended at 4°C in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$ 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF (buffer A) and centrifuged. Pellets were re-suspended in cold buffer B (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin). Supernatant fraction containing nuclear protein extract was stored at −80°C until used for Western blot analyses. Proteins were measured using the Bradford reagent.

2.10 Western blot analysis

Equal amounts of proteins were separated by SDS PAGE and transferred to poly-vinylidene difluoride filters (Merck, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare) or anti-mouse (Sigma) Ig. Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin or lamin B1 for nuclear protein extracts and band quantification was carried out with a scanner and the Scion Image software.

2.11 Glucose uptake

Cellular glucose uptake was quantified by the 2-NBDG assay, as described in [20]. Briefly, cells were plated in 24-well plates at a rate of 2×10^5 cells per well and after the treatments, 10 μM 2-NBDG was added for 1 h at 37°C. Then, the unabsorbed probe was removed and fluorescence intensity immediately measured in a micro-plate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. After being taken by the cells, 2-NBDG was converted to a non-fluorescent derivative (2-NBDG metabolite). A fair estimation of the overall glucose uptake was obtained by quantifying the fluorescence.

2.12 Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $p < 0.05$ was considered significant. An SPSS version 21.0 program has been used. According to that program, cell treatment was considered as the factor and data obtained from the measurement of ROS, GSH, protein carbonyl content, enzymatic activity, protein levels in Western blot and glucose uptake were considered as dependent variables.

3 Results

3.1 High-glucose concentrations induce oxidative stress

First, HepG2 cells were treated with a wide range of glucose concentrations (20–60 mM) for 24 h to simulate a long-term exposure as in T2DM. This was followed by an incubation with 100 nM insulin for 10 min and parameters related to redox status as well as the antioxidant response were evaluated to select the dose that induces oxidative stress.

Treatment with concentrations higher than 20 mM glucose increased ROS and carbonyl groups levels, and GSH content decreased with all tested doses of glucose (Supporting Information Fig. 1). Interestingly, GPx and GR activities increased with 30–60 mM glucose and CAT activity augmented with doses higher than 50 mM (Supporting Information Fig. 2). Additionally, 30–60 mM glucose reduced the activity of GST (Supporting Information Fig. 2). These results suggest that high doses of glucose, which have been demonstrated to induce insulin resistance [20], also evoked an imbalance of HepG2 redox status.

3.2 High-glucose concentrations up-regulate Nrf2 and MAPKs

Nrf2 and MAPKs are regulated by oxidative stress and insulin resistance, and Nrf2, which is upstream modulated by MAPKs [3, 10], regulates gene expression of antioxidant and detoxifying enzymes [8–10]. Therefore, it was evaluated whether high-glucose concentrations could alter these proteins.

Supporting Information Fig. 3A and B illustrates that all glucose concentrations tested (20–60 mM) increased nuclear (100 and 57 kDa) and total Nrf2 levels when compared to control cells. Moreover, phosphorylated levels of ERK, JNK and p38 also increased with high-glucose concentrations. ERK activation was similar with all doses of glucose, whereas higher levels of p-JNK and p-p38 were found with 40–60 and 60 mM glucose, respectively (Supporting Information Fig. 3A and B). Total levels of ERK, JNK and p38 were not modified by any treatment. Thus, it could be suggested that high-glucose concentrations induce a response to stress in HepG2 by activating Nrf2 and MAPK pathways.

In view of the obtained results, 30 mM glucose was the dose selected to perform further studies as it was the lowest

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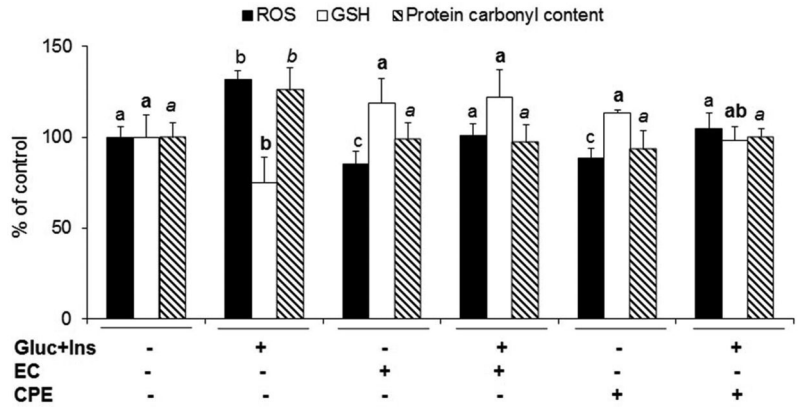

Figure 1. Protective effect of EC and CPE on ROS production, GSH levels and protein carbonyl content. HepG2 cells treated with 10 μM EC or 1 μg/mL CPE for 24 h were later incubated with 30 mM glucose (Gluc) for 24 h and further exposed to 100 nM (Ins) for 10 min. Data are expressed as percentage of controls. Values are means ± SD of six to nine different samples per condition. Different letters over bars indicate statistically significant differences ($p < 0.05$). Different styles of letters (normal, bold and italics) have been used for each parameter depicted within the same graph.

concentration that induces oxidative stress and, as previously shown, causes insulin resistance without cellular osmotic effect [20, 22, 23].

3.3 EC and CPE prevent high-glucose-induced oxidative stress

To test the long-term protective effect of EC and CPE on HepG2-cultured cells submitted to oxidative stress, cells were pre-treated for 24 h with 10 μM EC or 1 μg/mL CPE prior to 24 h of 30 mM glucose treatment followed by a 10 min chase with 100 nM insulin; then, parameters related to redox status and antioxidant response were evaluated. EC and CPE doses were selected based on our previous works, since these realistic concentrations were the lowest doses tested of both natural substances that did not induce cellular damage [11–14, 20, 21], exhibited a prominent effect on the activation of key proteins in the insulin signalling pathway [21], attenuated high-glucose-induced insulin signalling blockade and improved glucose uptake and production [20]. In this regard, EC and CPE alone decreased ROS production, and did not alter GSH or protein carbonyl contents (Fig. 1). Besides, EC alone did not modify any enzymatic activity tested, and CPE alone increased GPx and GR activities when compared to control cells (Fig. 2).

Under high-glucose conditions, pre-treatment of HepG2 cells with EC and CPE prevented GSH depletion and reversed the 30 mM glucose-induced ROS production and protein carbonyl content to pre-stress values (Fig. 1). In line with this, pre-treatment with EC recovered GPx activity although not GR activity, and CPE restored GPx and GR activities to control levels (Fig. 2). CAT activity remained unchanged after all incubations and the 30 mM glucose-decreased GST activity was unaffected by EC or CPE pre-treatment. These results indicate that EC, and more clearly CPE, protect HepG2 cells against the redox imbalance caused by a high-glucose concentration.

3.4 EC and CPE modulate high-glucose-induced up-regulation of Nrf2 and MAPKs

To continue the study of the protective effect of EC and CPE against a high-glucose challenge, cells were pre-treated with both natural substances, and levels of Nrf2 and MAPKs were evaluated. As shown in Fig. 3, EC and CPE alone increased nuclear content (100 and 57kDa) and total levels of Nrf2 in comparison to control unchallenged HepG2 cells (Fig. 3). Thus, cells incubated with EC alone showed higher levels for nuclear (100kDa) and total Nrf2 than those of high-glucose treated cells. Interestingly, increased nuclear and total Nrf2

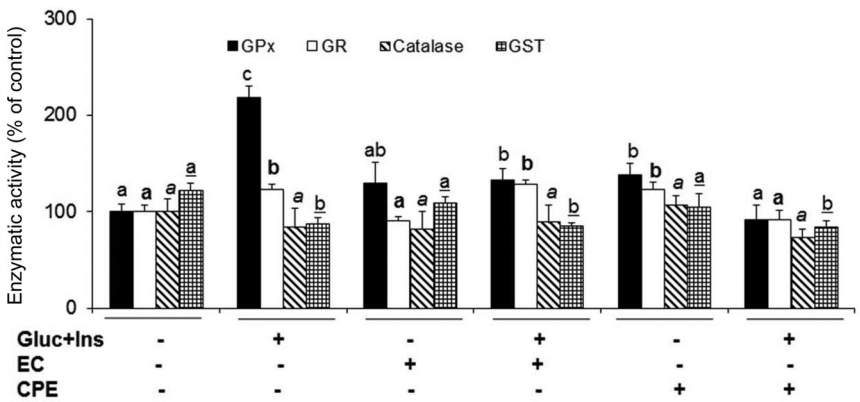

Figure 2. Protective effect of EC and CPE on the activity of antioxidant/detoxifying enzymes GPx, GR, CAT and GST. HepG2 cells treated with 10 μM EC or 1 μg/mL CPE for 24 h were later incubated with 30 mM glucose (Gluc) for additional 24 h and further exposed to 100 nM (Ins) for 10 min. Values as percentage relative to control condition are means ± SD ($n = 7$–9). Different letters over bars indicate statistically significant differences ($p < 0.05$). Different styles of letters (normal, bold, italics and underlined) have been used for each parameter depicted within the same graph.

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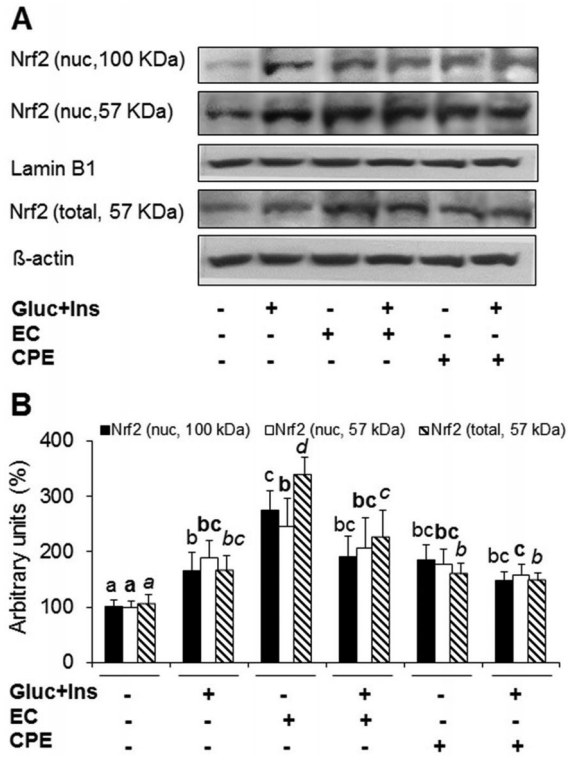

Figure 3. Effect of EC and CPE on Nrf2 in HepG2. Cells were incubated with 10 μM EC or 1 μg/mL CPE for 24 h prior to 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (A) Western blot bands of representative experiments. (B) Densitometric quantification of nuclear levels of phosphorylated Nrf2 (100 kDa), (C) nuclear levels of Nrf2 (57kDa) and (D) total levels of Nrf2 (57kDa). Values are expressed as percentage relative to the control condition (means ± SD, $n = 6$–8). Equal loading of Western blots was ensured by β-actin or lamin B1 for nuclear protein extracts. Means without a common letter differ significantly ($p < 0.05$). Different styles of letters (normal, bold and italics) have been used for each parameter depicted within the same graph.

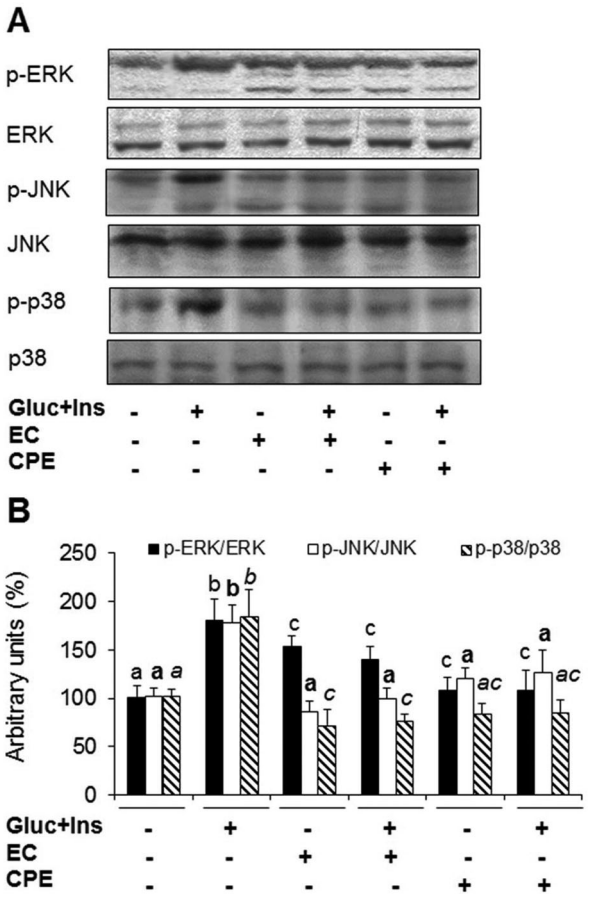

Figure 4. Effect of EC and CPE on phosphorylated and total levels of ERK, JNK, p-38 in HepG2. Cells treated with 10 μM EC or 1 μg/mL CPE for 24 h were later incubated with 30 mM glucose (Gluc) for 24 h and further exposed to 100 nM (Ins) for 10 min. (A) Western blot bands of representative experiments. Percentage data of (B) p-ERK/ERK, (C) p-JNK/JNK and (D) p-p38/p38 ratios relative to control condition (means±SD, $n = 6$–9). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly ($p < 0.05$). Different styles of letters (normal, bold and italics) have been used for each parameter depicted within the same graph.

levels were unaltered when HepG2 cells were pre-treated with both natural substances as compared to glucose-challenged cells (Fig. 3).

Additionally, EC and CPE alone increased p-ERK values, but did not modify p-JNK levels, whereas EC decreased p-p38 levels in comparison to control cells (Fig. 4). It is worth noting that EC- and CPE-induced increase in p-ERK levels was significantly lower than that evoked by 30 mM glucose. In addition, pre-treatment with EC and CPE diminished the enhanced phosphorylated levels of all three MAPKs induced by 30 mM glucose (Fig. 4). Total levels of ERK, JNK and p38 were not modified by any treatment.

Since CPE demonstrates a better protective effect on high-glucose-induced stress (Fig. 2), shows Nrf2 stimulation similar to EC (Fig. 3) and remarkably improves glucose uptake better than EC [20], CPE was selected over EC to perform further experiments.

3.5 MAPKs regulate CPE-induced changes of Nrf2 in high-glucose-challenged hepatic cells

To further clarify the involvement of MAPKs in the modulation of Nrf2 and GPx and GR activities induced by CPE in cells incubated with high glucose, the effect of MAPKs selective inhibitors on these processes was assayed. First, HepG2 cells were pre-treated for 1 h with ERK or p38 inhibitors (PD98059 or SB203580, respectively), as both kinases have been demonstrated to be main regulators of Nrf2 [10], and then treated with 1 μg/mL CPE for 24 h. Supporting Information Fig. 4 shows that the inhibition of the two kinases reduced nuclear (100 and 57 kDa) and total (57 kDa) levels of Nrf2 induced by CPE to values that were lower than those of controls.

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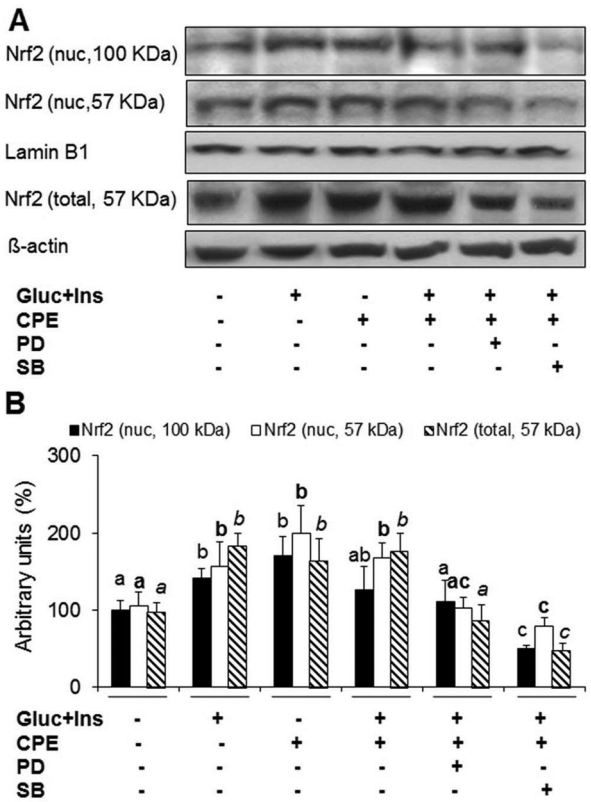

Figure 5. Effect of CPE and selective inhibitors of ERK (PD, PD98059) and p38 (SB, SB203580) on Nrf2. HepG2 cells incubated with 1 μg/mL CPE for 24 h were later treated with 50 μM PD or 10 μM SB for 1 h prior to the 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (A) Western blot bands of representative experiments. Percentage data of (B) of nuclear levels of phosphorylated Nrf2 (100 kDa), (C) nuclear levels of Nrf2 (57 kDa) and (D) total levels of Nrf2 (57 kDa) relative to the control condition (means ± SD, $n = 6–8$). Equal loading of Western blots was ensured by β-actin or lamin B1 for nuclear protein extracts. Means without a common letter differ significantly ($p < 0.05$). Different styles of letters (normal, bold and italics) have been used for each parameter depicted within the same graph.

Additionally, blockage of ERK and p38 decreased the nuclear and total levels of Nrf2 enhanced by the glucose challenge, showing intermediate values between those of untreated and challenged cells. This fact shows that CPE-induced Nrf2 stimulation is mediated by ERK and p38 in a non-pathological situation, and that the blockage of both MAPKs seems to act in a similar manner of that of CPE to modulate Nrf2.

Next, it was examined whether ERK and p38 inhibition would also abolish the increase in the expression of Nrf2 provoked by CPE on high-glucose-challenged cells. As shown in Fig. 5, inhibition of ERK and p38 evoked a decrease of nuclear (100 and 57 kDa) and total (57 kDa) levels of Nrf2 in comparison to CPE- and high-glucose-treated cells. These results suggest the involvement of both pathways in CPE-induced Nrf2 stimulation and that MAPKs inhibitors improved the protection induced by CPE.

3.6 MAPKs regulate CPE-induced changes of GPx and GR activities and ROS production in high-glucose-exposed hepatic cells

Following, the role of MAPKs on the activity of GPx and GR and ROS generation was analysed. Inhibition of ERK and JNK diminished GPx activity in cells pre-treated with CPE, whereas p38 blockade did not alter this enzymatic activity (Fig. 6). In addition, ERK and p38 inhibitors increased GR activity of CPE pre-incubated cells, but the blockage of JNK did not affect this enzymatic activity (Fig. 6). Therefore, ERK and JNK inhibitors abrogated the effect of the high-glucose challenge and CPE in GPx activity, whereas blockage of ERK and p38 enhanced the effect of the high-glucose challenge and CPE in GR activity.

As shown in Fig. 6, overproduction of ROS induced by 30 mM glucose was avoided by CPE, showing comparable levels to unchallenged cells. Similarly, the presence of MAPKs inhibitors abolished the effect of high glucose in ROS production, which could suggest that CPE-induced protective effect was enhanced by blocking MAPKs.

3.7 MAPKs regulate CPE-induced changes of p-(Ser307)-, p-(Ser(636/639))-IRS-1/IRS-1 ratio and glucose uptake in high-glucose-challenged hepatic cells

Since high-glucose provokes oxidative stress and insulin resistance, and MAPKs are involved in both processes, the effect of CPE and specific MAPKs inhibitors in p-(Ser307)-IRS-1, p-(Ser(636/639))-IRS-1 and total IRS-1 levels and glucose uptake under a high-glucose concentration were analysed.

CPE pre-treatment prevented the increase of p-(Ser307)-IRS-1 and p-Ser(636/639)-IRS-1/IRS-1 ratio induced by 30 mM glucose, achieving levels similar to those of CPE-treated cells (Fig. 7). In high-glucose-challenged cells, the blockage of JNK and ERK suppressed the increase of p-(Ser307)- and p-(Ser636/639)-IRS-1/IRS-1 ratios induced by the high-glucose concentration, and displayed levels comparable to those of unchallenged cells. Additionally, the inhibition of JNK or ERK in CPE-pre-treated cells diminished the ratios for the phosphorylated versus total IRS-1 to values lower than those of CPE pre-treated cells or cells incubated with CPE alone. Similarly, p38 inhibition prevented the enhancement in p-(Ser636/639)-IRS-1/IRS-1 ratio induced by 30 mM glucose, showing levels intermediate to those of unchallenged and high-glucose-treated cells, whereas the blockage of p38 in CPE pre-treated cells exhibited values comparable to those of CPE pre-treated cells (Fig. 7C and D). These results show the involvement of JNK and ERK, and a possible redundant role for p38 regulation by CPE, in the stimulation of IRS-1 induced by CPE during insulin-resistance in HepG2 cells.

As presented in Fig. 7E, 30 mM glucose reduced the glucose uptake, whereas CPE alone increased the glucose uptake and in pre-treated cells prevented the high-glucose-induced

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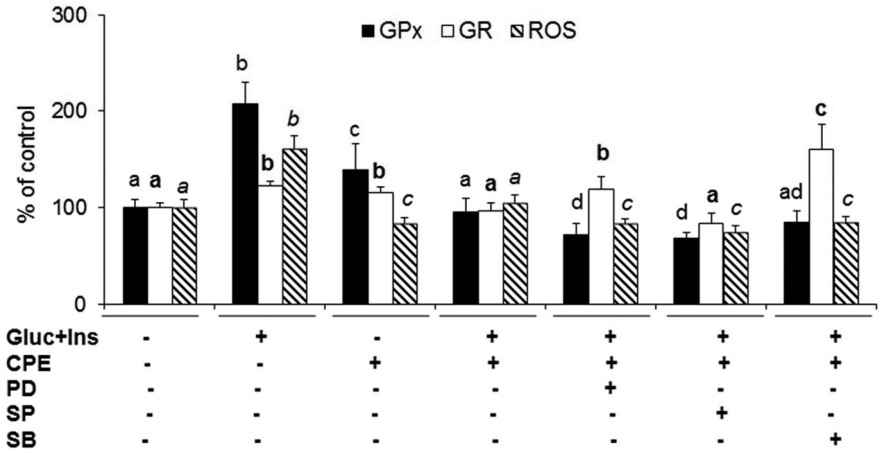

Figure 6. Effect of CPE and selective inhibitors of ERK (PD, PD98059), JNK (SP, SP600125) and p38 (SB, SB203580) on GPx and GR activities, and on intracellular ROS production. HepG2 cells incubated with 1 μg/mL CPE for 24 h were later treated with 50 μM PD, 40 μM SP, or 10 μM SB for 1 h prior to 24-h glucose (Gluc) challenge, and further exposed to 100 nM (Ins) for 10 min. Activity of GPx and GR and intra-cellular ROS production are expressed as percentage of control as means ± SD (n = 6–9). Different letters over bars indicate statistically significant differences ($p < 0.05$). Different styles of letters (plain, bolt and italics) have been used for each parameter depicted within the same graph.

diminished glucose uptake, as previously reported [20,21]. Inhibition of all three MAPKs partly blocked the high-glucose-induced reduction of glucose uptake in insulin-resistant cells, displaying values equal to those of control and CPE treated cells. Similarly, the blockage of MAPKs in CPE-pre-treated cells completely prevented the diminished glucose uptake of the insulin resistant cells and evoked a similar or higher beneficial effect than that of CPE alone (Fig. 7E). All these points to the relevance of MAPKs on the improved glucose uptake induced by CPE after a high-glucose challenge in hepatic cells.

4 Discussion

Flavonoids have been suggested as anti-diabetic agents and have drawn attention because of their safety [5, 6]. T2DM is associated with insulin resistance and reduction in antioxidants, indicating the causal role of oxidative stress in its pathogenesis [2, 3], and pointing to natural antioxidants, such as flavonoids as a promising therapy [5, 6]. However, insufficient investigation at molecular level, especially in the liver, has been performed to support these observations. In this study, for the first time it has been shown that cocoa flavanols proved to efficiently prevent the unbalance of cellular redox status provoked by a high dose of glucose by reducing ROS overproduction, recovering altered antioxidant defences, restoring the impairment in glucose uptake and first steps of the insulin transduction route (p-(Ser)-IRS-1/ total IRS-1), and restraining signalling pathways related to stress namely MAPKs.

Cocoa is a rich source of flavonoids such as (−)-EC, (+)-catechin and procyanidins, being EC the most abundant flavanol in the CPE employed in this work [17]. It is worth mentioning that concentrations used in the study are not far from realistic and they are within the range recommended for *in vitro* studies [29]. In this regard, an array of concentrations between 35 and 50 μM of EC have been reported in rat serum 1 h after oral administration of 172 μM of EC per kg of body weight [30], and serum levels of 6 μM EC and 41 nM procyanidin B2 have been observed in humans after ingestion of 26g cocoa [31]. Besides, it should be taken into account the possible synergic effect of phenolic compounds in CPE, the potential contribution of EC and flavanol metabolites to the biological activity and the possible degradation of EC and phenolic compounds in CPE at a physiological pH [32]. All these aspects, which are unclear at present, should not be underestimated, and require further studies.

In T2DM, oxidative stress has been proved as a result of long-term high glucose and mediates diabetic complications [33]. Consistent with this, our results revealed that high doses of glucose lead to an overproduction of ROS, and as a direct consequence oxidative damage to proteins increased and GSH was depleted, as previously reported in the liver of diabetic animals [3, 34] and in human serum [35]. Furthermore, antioxidant enzymes, such as GR, GPx, GST and CAT, which play a key role in defence mechanisms against free radicals, were altered. CAT, GST and GPx are involved in eliminating peroxides providing an important cellular defence mechanism against oxidative damage, and GR is responsible for the regeneration of oxidized glutathione [8, 9]. Consequently, enhanced enzymatic activities contribute to prevent the damage induced by ROS overproduction [8, 17, 34, 36] and may reflect an adaptive mechanism in response to elevated oxidative stress during hyper-glycaemia [34], in agreement with the present results. However, in these conditions a decreased GST activity was observed in HepG2 treated with high concentrations of glucose. This fact has been related to a diminished hepatic enzymatic expression because of lower insulin stimulation during insulin resistance in T2DM [37].

In the present insulin-resistant model, the glucose-induced increase in ROS generation and carbonyl content and decrease of GSH concentration were prevented in cultured cells pre-treated with EC or CPE. These results are in line with those reporting a protective effect of EC and cocoa flavonoids in cells or animals against an induced oxidative insult, which has been related to a more efficient ROS quenching in flavanol pre-treated cells or animals [13, 14, 17, 19, 28, 38]. In

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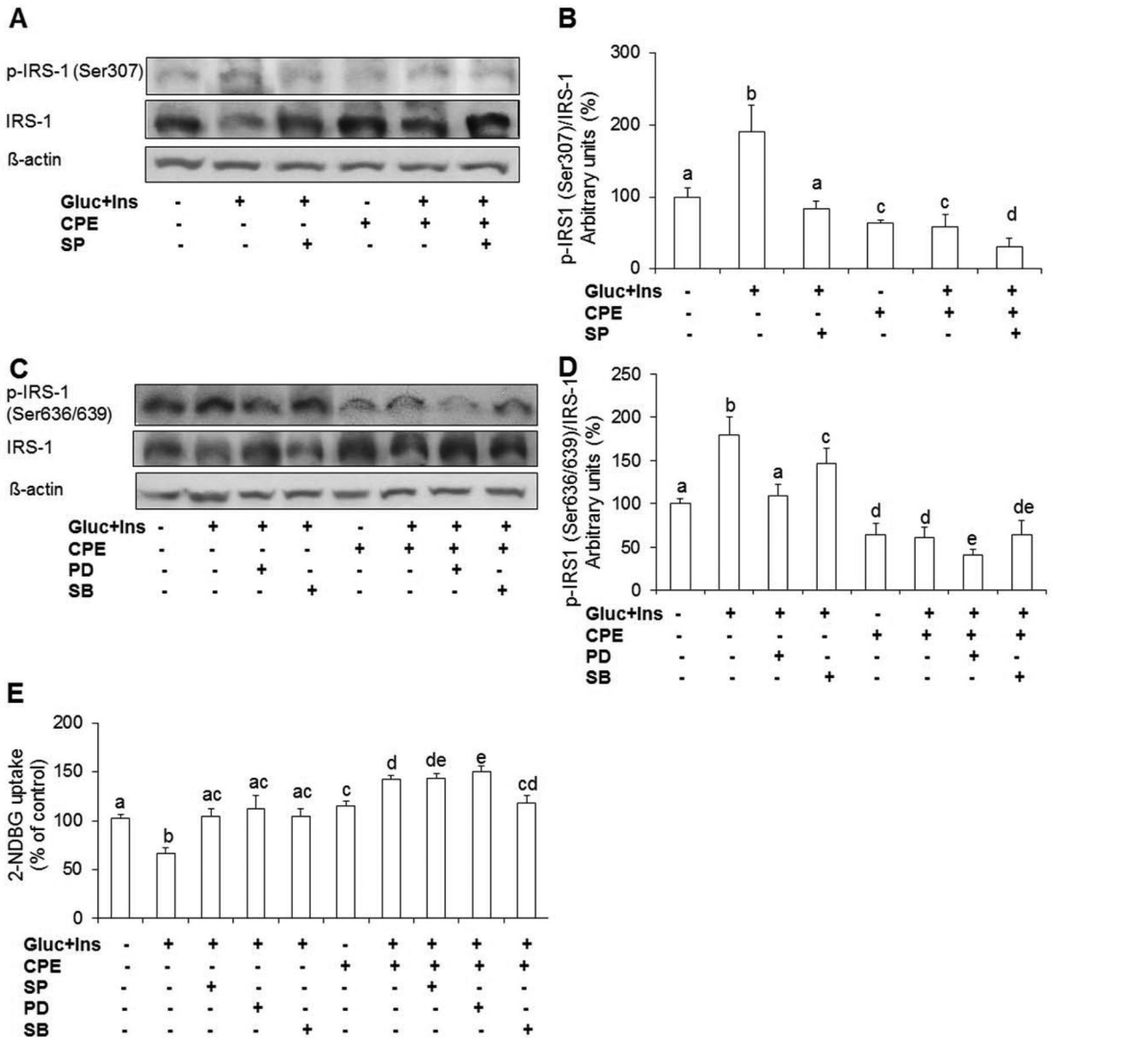

Figure 7. Effect of CPE and selective inhibitors of JNK (SP, SP600125), ERK (PD, PD98059) and p38 (SB, SB203580) on levels of p-(Ser)-IRS-1. HepG2 cells incubated with 1 μg/mL CPE for 24-h were later treated with 40 μM SP, 50 μM PD or 10 μM SB for 1 h prior to 24-h glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. (A) Western blot bands of representative experiments. (B) Densitometric quantification p-IRS1(Ser307) as a percentage to the control condition (means ± SD, $n = 5$–7). (C) Western blot bands of representative experiments. (D) Percentage values of p-IRS1(Ser636/639) relative to the control condition (means ± SD, $n = 6$–9). Equal loading of Western blots was ensured by β-actin. (E) Glucose uptake expressed as percentage of control as means ± SD ($n = 8$–10). Means without a common letter differ significantly ($p < 0.05$).

addition, plant phenolics, and therefore CPE, seem to provide a parallel protection by increasing the activity of antioxidant enzymes, which prepares cells to deal with ROS generation in the presence of stressors and thus to suppress or minimize the oxidative stress induced damage [17, 34, 36, 39]. Consistent with this, our results revealed that pre-treatment of HepG2 cells with CPE, although not EC, increased GPx and GR activities, as previously shown in non-insulin-resistant hepatic cells with higher concentrations [14, 18]. Moreover, EC and CPE effectively inhibited the alterations on antioxidant enzymatic activities exerted by a high dose of glucose, in agreement with other studies performed with phenolic compounds, such as curcumin and grape proanthocyanidins, in insulin-resistant models [34, 39].

Nrf2 transcription factor is activated by stress, and plays a central role in the induction of phase II enzymes through its binding to the antioxidant response element after its nuclear translocation and phosphorylation [10]. In agreement

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with our results, it has been reported that Nrf2 levels are increased in diabetic patients, indicating an adaptive response to counteract oxidative stress in the disease [40]. We have shown that EC stimulates Nrf2 [11] and that Nrf2 induction by phenolic compounds activates protein and gene expressions and activities of antioxidant enzymes [24, 25, 41]. In the present study, we have found that CPE evokes an increase in GPx and GR activities in HepG2 cells, which correlates with Nrf2 activation. However, EC stimulated Nrf2 without enhancing GPx and GR activities. In line with this, it has been described that other natural compounds evoked increases in Nrf2 and antioxidant enzyme protein levels, but this effect was not observed on the enzymatic activities in the liver of insulin-resistant animals [7]. Although this finding deserves further investigations, the results above indicate that EC- and CPE-treated HepG2 cells are in favourable conditions to face the increasing generation of ROS induced by the high dose of glucose. Additionally, pre-treatment with EC and CPE retained the increase of nuclear (100 and 57 kDa) and total Nrf2 levels in hepatic cells. Consistent with these findings, it has been shown that grape polyphenol resveratrol induces Nrf2 translocation and activation during insulin resistance, which was associated with a protective effect [42, 43].

Oxidative stress induced by hyper-glycaemia alters signal transduction pathways, resulting in the activation of key stress mediators involved in insulin resistance, such as MAPKs [3, 16, 44], which are upstream signals involved in Nrf2 activation [10]. In full agreement with this, increased phosphorylated levels of all three MAPKs were found in insulin-resistant HepG2 cells. Additionally, pre-treatment with EC and CPE prevented the activation of MAPKs in hepatic cells treated with a high dose of glucose. In this line, it has been shown that EC and CPE restrained the enhanced levels of p-JNK induced by oxidative stress in different cultured cells [14, 19, 36]. Moreover, other flavonoids have also demonstrated to exert anti-diabetic effects through the down-regulation of proteins close-related to stress, such as MAPKs [45–47]. Nevertheless, we have already reported enhanced levels of p-ERK induced by EC and CPE in HepG2 cells, which have been associated to the promotion of cell protection and survival against oxidative stress [11, 14].

MAPKs are also implicated in the up-regulation of antioxidant/detoxificant enzymatic activities [10, 14, 37], which together with Nrf2 induction constitutes an important pathway to protect cells against oxidative damage [9, 10]. Supporting this, we found that selective inhibitors of ERK and p38 prevented the nuclear phosphorylation and accumulation of Nrf2 induced by CPE in HepG2 cells, also in response to high-glucose stress. Consequently, GPx and GR activities restored by CPE in high-glucose challenged cells were modulated by selective inhibitors of ERK and JNK, and ERK and p-38 MAPK pathway, respectively. These results could indicate that the regulation of MAPK pathways by CPE could be implicated in Nrf2, and GPx and GR stimulation, as kinase inhibitors seemed to improve the protection induced by CPE. In this regard, flavanols and other phenolic compounds increase the cellular antioxidant defence capacity in hepatic cells by inducing cytoprotective enzymes via the activation of PI3K/AKT and/or the MAPKs pathways [11, 24, 48, 49]. Indeed, we have demonstrated that the major phenolic component in CPE, EC, up-regulates Nrf2 through PI3K/AKT and ERK-MAPK [11], and that a cocoa procyanidin induces Nrf2 through p38 and ERK [41]. Moreover, the involvement of ERK in the modulation of GPx and GR activities induced by CPE in hepatic cells has been shown [14]. Altogether suggests that CPE could induce the activity of GPx and GR through a mechanism involving the stimulation of Nrf2 and the modulation of MAPK pathways.

Overproduction of ROS activates signalling cascades involving MAPKs [44]. In the present work, CPE-mediated cytoprotection against a high dose of glucose was improved in the presence of specific inhibitors of all three MAPKs. In this regard, it has been reported that attenuation of ROS release by natural antioxidant compounds is accompanied by activation of Nrf2 [9–12], and that a blocked ROS production in HepG2 cells inhibited Nrf2-induced expression and nuclear translocation [11, 12, 50]. These results could suggest that phosphorylation of all three MAPKs via ROS and the subsequent modulation of glutathione-related enzymes and Nrf2, which probably decrease the oxidative stress, are involved in the protective mechanism exerted by CPE against the oxidative stress induced by the hyper-glycaemia in HepG2 cells.

Chronic activation of MAPKs may induce insulin resistance by affecting glucose transporter expression and insulin signalling via modulation of total IRS-1 values and phosphorylation of IRS serine residues [2, 51]. In this regard, serine phosphorylation of IRS-1 is involved in the desensitization of insulin by chronic high-glucose treatment [20, 22, 23], and activation of ERK and JNK induces negative regulators of insulin sensitivity, such as p-(Ser636/639)-IRS-1 and p-(Ser307)-IRS-1, respectively [22, 52]. In this study, the blockage of ERK and JNK decreased the ratio of serine phosphorylated levels of IRS-1 versus total IRS-1 and improved the glucose uptake in insulin-resistant CPE-treated cells, whereas p38 seemed to play a redundant role with CPE in these processes. In agreement with these results, suppression of ERK by grape powder extract attenuated TNFα-induced insulin resistance [47], and JNK blockage could also mediate the inhibition of IRS-1 serine phosphorylation [22], contributing to the stimulation of the hepatic insulin sensitivity, whereas p38 seemed to moderately affect p-(Ser)-IRS-1 levels [51]. Moreover, oxidative stress inhibited insulin-stimulated glucose transport in adipocytes, which was reversed by pre-incubation with an antioxidant [53], and a role for ERK on the glucose uptake has been reported in hepatic cells [54]. Interestingly, impaired glucose utilization could be provoked because of ROS interfering with insulin signalling cascade [55]. Thus, inhibition of all three MAPKs was accompanied by a reduction in ROS generation and an improvement in glucose uptake. All this suggests that CPE improves the cellular redox status and insulin resistance in high-glucose-exposed HepG2 cells

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through the modulation of MAPKs, since kinase inhibitors enhanced the protection induced by CPE.

GLUT2 maintains the intra-cellular and extracellular glucose in equilibrium in the liver, although this balance could be altered during insulin resistance [2, 23]. We have demonstrated that GLUT-2 levels and glucose uptake decreased in HepG2 cells exposed to a high dose of glucose, and this was reverted when cells were previously treated with EC or CPE without varying GLUT-2 content [20]. However, higher doses of CPE were able to increase GLUT-2 levels in HepG2 cells. Therefore, it could be hypothesized that glucose uptake and GLUT-2 content could also be related and stimulated by CPE [21]. In fact, the anti-diabetic drugs telmisartan, sitagliptin and metformin recovered the diminished levels of hepatic GLUT-2 on insulin-resistant mice, suggesting a normalization of post-receptor insulin signalling and a restoration of the hepatic insulin sensitivity [56]. These studies deserve further investigations.

In summary, we provide evidence for the first time that EC and CPE may be bioactive natural substances with anti-diabetic effect as they preserve biochemistry of hepatic cells against high-glucose-induced oxidative stress by modulating antioxidant defences. Furthermore, CPE beneficial effects on redox status and insulin resistance seem to be mediated at least by its ability to target MAPKs. Although these findings are encouraging to consider cocoa flavanols as potential candidates for preventing and/or treating diabetes, further investigations to trace out the intimate mechanism of action are required.

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Supporting information.

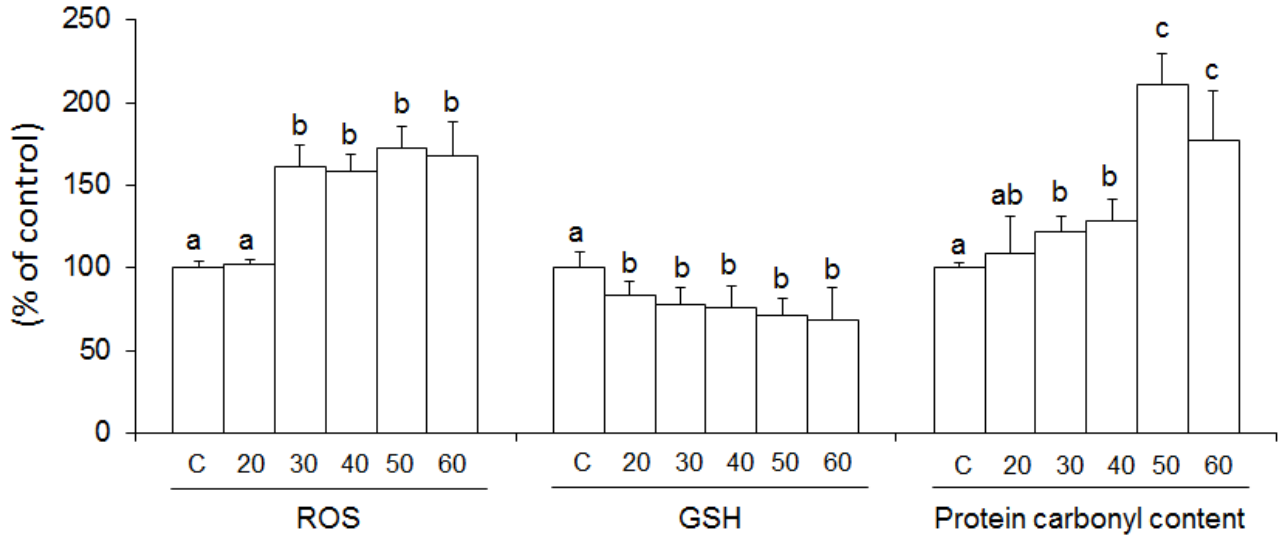

Figure 1. Effect of glucose concentrations on intracellular ROS production, GSH content and protein carbonyl levels. HepG2 cells were treated with glucose concentrations (20-60 mM) for 24 h. Data are expressed as percentage relative to the control condition. Values are means ± SD of 8-10 different samples per condition. Different letters over bars indicate statistically significant differences (P<0.05).

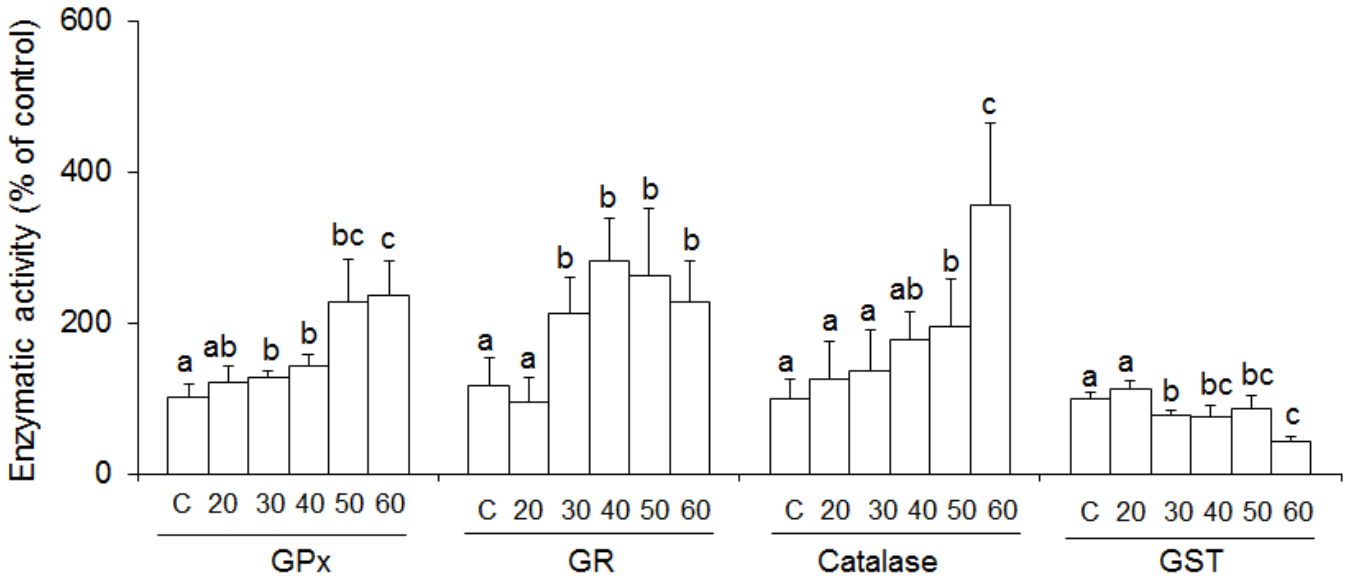

Figure 2. Effect of glucose concentrations on the activity of GPx, GR, CAT and GST. HepG2 cells were treated with glucose concentrations (20-60 mM) for 24 h. Data are expressed as percentage relative to the control condition and are means ± SD, n=6-9. Different letters over bars indicate significant differences ($P<0.05$).

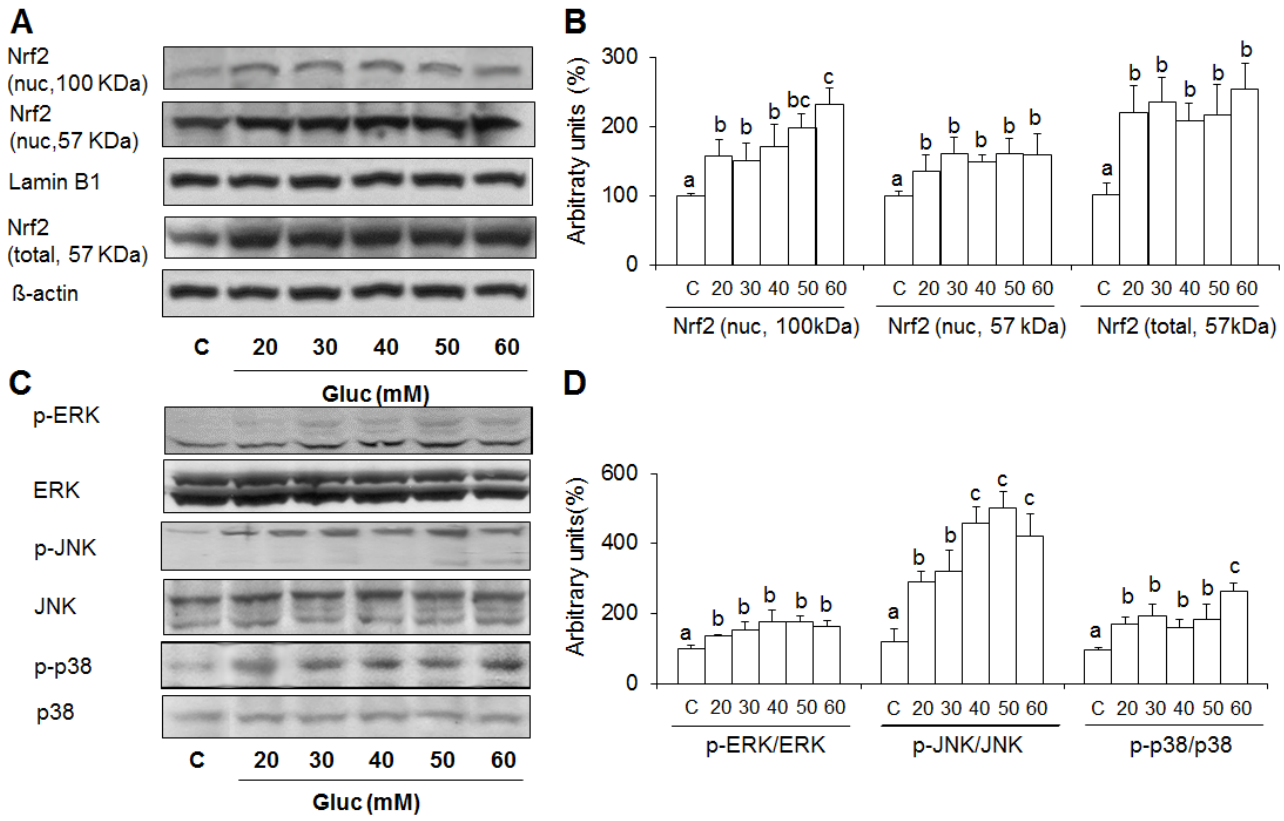

Figure 3. Effect of glucose concentrations on Nrf2 and phosphorylated and total levels of MAPKs (ERK, JNK, p38). HepG2 cells were incubated with glucose (20-60mM) for 24h. (A) Bands of representative experiments for Nrf2. (B) Densitometric quantification of nuclear levels of phosphorylated (100kDa) and total (57kDa) Nrf2 and total values of Nrf2 (57kDa). Values are expressed as a percentage relative to the control condition (means±SD, n=6-8). (C) Bands of representative experiments of MAPKs. (D) Percentage data of p-ERK/ERK, p-JNK/JNK and p-p38/p38 ratios relative to control condition (means±SD, n=5-7). Equal loading of Western blots was ensured by β-actin or lamin B1 for nuclear extracts. Different letters over bars indicate statistically significant differences ($P<0.05$).

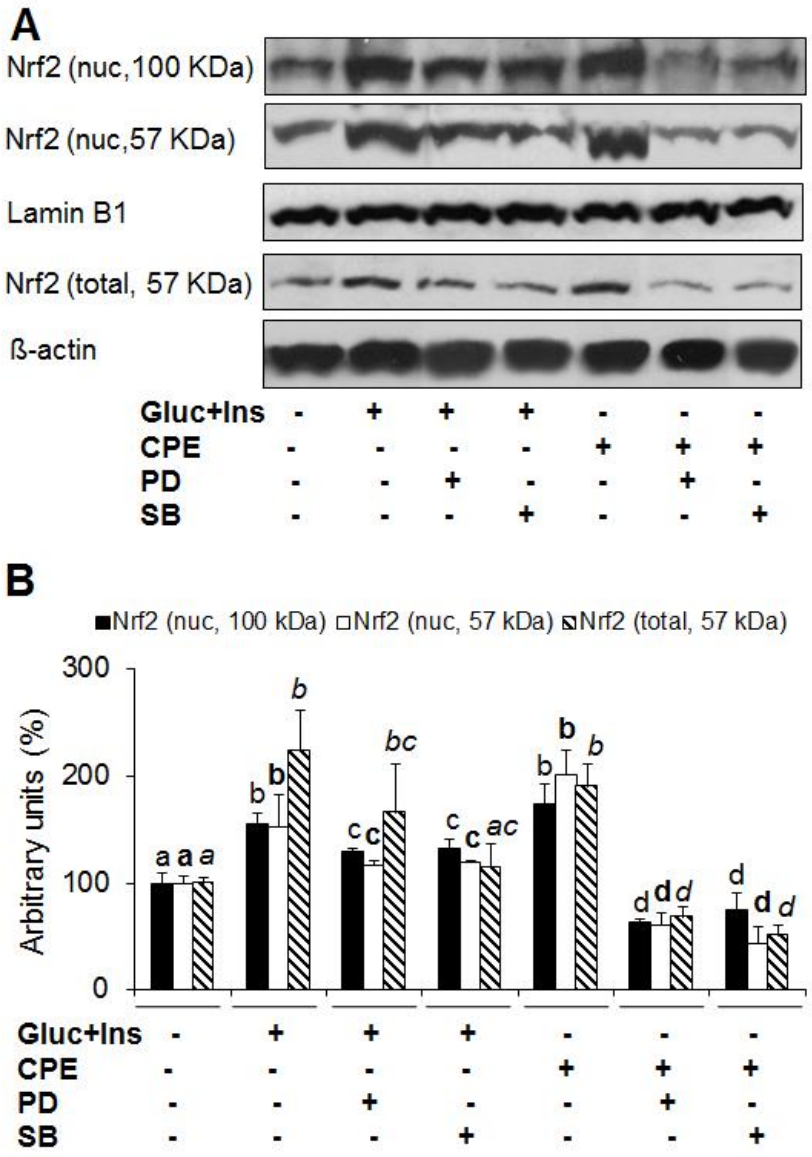

Figure 4. Effect of CPE and selective inhibitors of ERK (PD, PD98059) and p38 (SB, SB203580) on Nrf2. HepG2 cells incubated with 50µM PD, or 10µM SB for 1h prior to the 24h-CPE (1µg/mL) incubation or to the 24h-glucose (Gluc) challenge and further exposed to 100nM (Ins) for 10min. (A) Bands of representative experiments. (B) Percentage data of nuclear levels of phosphorylated Nrf2 (100kDa), nuclear levels of Nrf2 (57kDa) and total levels of Nrf2 (57kDa) relative to the control condition (means±SD, n=5-7). Equal loading of Western blots was ensured by lamin B1 for nuclear fractions or β-actin. Means without a common letter on different bars differ

significantly ($P<0.05$). Different styles of letters (plain, bolt and italics) have been used for each parameter depicted within the same graph.

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Cocoa intake ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats

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ABSTRACT

Chronic hyperglycemia in diabetes is associated with oxidative stress-mediated tissue damage. The present study is aimed to explore the role of a cocoa-enriched diet in ameliorating the oxidative stress-induced damage in the liver of young type 2 diabetic Zucker diabetic fatty (ZDF) rats. Male ZDF rats were fed a control or cocoa-rich diet (10%), and Zucker Lean (ZL) animals received the control diet. ZDF rats fed with cocoa (ZDF-Ca) decreased body weight gain, glucose and insulin levels, and improved glucose tolerance and insulin resistance. Cocoa diet further reduced reactive oxygen species (ROS) levels and carbonyl content in the liver of ZDF animals. The diminished activity of superoxide dismutase (SOD) and the enhanced activity of heme oxygenase (HO-1) in ZDF-C were returned to ZL values upon cocoa administration. Cocoa did not restore the decreased glutathione-S-transferase (GST) activity in both ZDF groups in comparison to ZL rats. Glutathione (GSH) content and activities of glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) remained unaltered among all animal groups. Moreover, cocoa-rich diet suppressed total and phosphorylated nuclear factor erythroid-derived 2-like 2 (Nrf2), as well as p65-nuclear factor-kappaB (NF- B) enhanced levels observed in ZDF rats. The results indicate that cocoa protects the hepatocytes by improving the antioxidant competence in the liver of young type 2 diabetic ZDF rats.

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1. Introduction

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by hyperglycaemia resulting from peripheral insulin resistance and β-cell pancreatic failure (Evans, Goldfine, Maddux, & Grodsky, 2002; Rochette, Zeller, Cottin, & Vergely, 2014). There is growing evidence that alterations in this metabolic disorder are in part a consequence of the oxidative stress that appears in the pre-diabetic milieu, and also plays a critical role in the development and progression of diabetes (Evans et al., 2002; Rochette et al., 2014). Indeed, an oxidative environment has been linked to the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, mitochondrial dysfunction and diabetes complications (Evans et al., 2002; Rochette et al., 2014).

The primary defence against oxidative stress is regulated by antioxidant/detoxifying enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), heme oxygenase (HO-1), and catalase (CAT), as well as by non-enzymatic defences such as glutathione (GSH) (Masella, Benedetto, Varì, Filesi, & Giovanninni, 2005; Valko et al., 2007). Secondly, increased reactive oxygen species (ROS) activates stress-sensitive transcription factors such as nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and nuclear factor-kappaB (NF- B) (Gloire, Legrand-Poels, & Piette, 2006). In this line, it has been reported that diabetes in experimental animals and humans is associated with reductions in antioxidants such as ascorbic acid and GSH, suggesting the critical role of oxidative stress in its pathogenesis (Evans et al., 2002).

Liver is the crucial organ responsible for oxidative and detoxifying processes, and it actively responds to the oxidative stress by modulating different signals such as Nrf2 and NF- B, which lead to the regulation of critical genes of enzymatic defences (Eggler, Gay, & Mesecar, 2008; Masella et al., 2005), and to the increased expression of pro-inflammatory genes (Gloire et al., 2006), respectively.

Dietary antioxidants play an important role in the prevention of T2DM (Dey & Lakshmanan, 2013). Accordingly, recent attention has focused on the identification of natural antioxidants with anti-diabetic

Abbreviations: AUC, area under curve; CAT, catalase; DCFH, dichlorofluorescein; DNPH, dinitrophenylhydrazine; GSH, glutathione; GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-S-transferase; GTT, glucose tolerance test; HO-1, heme oxygenase; HOMA-IR, homeostasis model assessment of insulin resistance; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NF- B, nuclear factor-kappaB; OPT, o-phthalaldehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; T2DM, type 2 diabetes mellitus; *t*-BOOH, *tert*-butylhydroperoxide; ZDF, Zucker diabetic rat; ZL, Zucker Lean.

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properties, and this constitutes a promising approach for the prevention and/or treatment of T2DM. Apart from the conventional anti-diabetic treatment, antioxidants may benefit in having fewer side-effects than anti-diabetic drugs currently used (Babu, Liu, & Gilbert, 2013), and it should also be considered that those natural antioxidant compounds are abundant in nature and inexpensive to produce.

Cocoa has been recognized as a rich source of phenolic compounds, mainly flavanols such as epicatechin and oligomeric procyanidins (Martin, Cordero-Herrera, Bravo, Ramos, & Goya, 2014; Martin et al., 2008). It has been showed that cocoa possesses a powerful antioxidant activity (Vinson et al., 2006), decreases blood glucose levels (Dorenkott et al., 2014; Jalil, Ismail, Pei, Hamid, & Kamaruddin, 2008; Ruzaidi, Amin, Nawalyah, Hamid, & Faizul, 2005), improves glucose tolerance (Dorenkott et al., 2014; Jalil et al., 2008; Ruzaidi et al., 2005), and diminishes metabolic syndrome-related inflammation (Gu & Lambert, 2013). More importantly, human clinical studies in diabetic patients have demonstrated that cocoa and dark chocolate improve insulin sensitivity (Grassi et al., 2008) and modulate oxidative stress markers in skeletal muscle (Ramirez-Sanchez et al., 2013). Despite this, the precise antioxidant defence mechanism for the preventive activities of cocoa during diabetes in the liver is not fully understood. Thus, there is scarce data regarding the effect of cocoa on hepatocytes *in vivo* and data related to the systemic antioxidant defence response is contradictory, as enhanced, decreased and unchanged enzymatic activities have been reported in serum of T2DM patients (Ahmed, Naqvi, & Shafiq, 2006; Ramakrishna & JailKhani, 2010).

The aim of this study was to evaluate the potential effect of cocoa-rich diet on the redox status in the liver of male Zucker diabetic fatty [ZDF; ZDF/*crl-lepr* (fa/fa)] rats, during the pre-diabetic stage (6–15 weeks of life). Here, we report that cocoa reduces hyperglycaemia and hyperinsulinemia, ameliorates glucose intolerance, and alleviates oxidative stress by modulating key proteins of the antioxidant defence in the liver of young ZDF rats.

2. Materials and methods

2.1. Materials and chemicals

Glutathione reductase (GR), reduced and oxidized glutathione (GSH and GSSG, respectively), nicotine adenine dinucleotide reduced salt (NADH), nicotine adenine dinucleotide phosphate reduced salt (NADPH), *tert*-butylhydroperoxide (*t*-BOOH), o-phthalaldehyde (OPT), dichlorofluorescein (DCFH), dinitrophenylhydrazine (DNPH) hydrogen peroxide and SOD determination kit were purchased from Sigma Chemicals (Madrid, Spain). GST fluorometric activity kit and rat HO-1 ELISA kit were acquired from BioVision (Deltaclon, Madrid, Spain). Rat insulin ELISA kit was obtained from Mercodia (AD Bioinstruments, Barcelona, Spain). Anti-Nrf2 (C-20, sc-722), anti-Nrf2 (H-300, sc-13032) and anti-NF-κB p65 (sc-7151) were purchased from Santa Cruz (Qimigen, Madrid, Spain). Anti-β-actin was obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Materials and chemicals for electrophoresis and Bradford reagent were from BioRad Laboratories S.A. (Madrid, Spain).

2.2. Cocoa

Natural Forastero cocoa powder (a kind gift from Nutrexpa S.L., Barcelona, Spain) was used for this study. It contains epicatechin (383.5 mg/100 g), catechin (116 mg/100 g), procyanidins (254.5 mg/100 g) and non-flavonoids compounds such as theobromine. A detailed description of this cocoa is given elsewhere (Martin et al., 2008). Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain) and provide all nutrients required by adult rats. The 10% cocoa diet was produced by adding 100 g/kg cocoa to AIN-93G. The total phenolic content of cocoa, as determined with the Folin–Ciocalteu method, was 2 g/100 g (Martin et al., 2008). The composition of the diets is given in Table 1.

Table 1
Composition of the experimental control and cocoa-rich diets.

Components (g/kg dry weight)	Control	Cocoa
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
tert-Butylhydroquinone	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Choline bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa powder	–	100

2.3. Animals and diets

Five week-old male Zucker Lean (ZL, n = 8) and Zucker diabetic ZDF/*crl-lepr fa* (n = 16) rats were purchased from Charles River Laboratories (L'Arbresle, France). These animals possess a mutation in the leptin receptor and spontaneously develop severe obesity, hyperglycaemia, hyperlipidaemia, and insulin resistance (Leonard, Watson, Loomes, Phillips, & Cooper, 2005). Rats were housed per groups in cages in a controlled environment (19–23 °C, 50–60% humidity and 12 h light-dark cycles). After one week of acclimatisation, ZDF rats were randomly assorted into two different experimental groups (8 animals per group): one group received a standard diet (ZDF-C) and the other one was fed with the cocoa-supplemented diet (ZDF-Ca). ZL animals (8 rats) were fed with the standard control diet. All experimental groups were provided with food and water *ad libitum* and treated according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas, CSIC).

At 15 weeks of age, rats were fasted overnight and sacrificed. Blood was harvested from the trunk after decapitation, and the serum was separated by centrifugation at 1000 *g* for 10 min at 4 °C for further biochemical analysis. Livers were collected, weighted and frozen in liquid N_2 and stored at −80 °C.

2.4. Biochemical analysis

Blood glucose was determined using an Accounted Glucose Analyzer (LifeScan, Madrid, Spain). Serum insulin was analysed with a rat insulin ELISA kit with a detection limit lower than 0.15 ng/mL (Mercodia,AD Bioinstruments, Barcelona, Spain).

Insulin sensitivity from the final fasting insulin and glucose values was estimated by the Homeostasis model assessment of insulin resistance (HOMA-IR) according to the following formula: [fasting glucose (mM) × fasting insulin (mUI/L)] / 22.5.

2.5. Glucose tolerance test (GTT)

One week before ending the study, GTT was performed in overnight fasted rats. Animals were administered with 35% glucose solution (1 g/kg of body weight) *via* saphenous vein and blood samples were taken from the tail vein before the glucose load (t = 0) and at 15, 30, 60, 90 and 120 min after glucose administration. Blood glucose was immediately determined using an Accounted Glucose Analyzer (LifeScan). Blood samples were centrifuged (1000 *g* at 4 °C for 10 min) and stored at −80 °C until insulin determination. Overall changes in glucose and insulin during GTT were calculated as the area under the curve (AUC) above the basal level (ΔGlucose and ΔInsulin areas respectively). The ratio of ΔGlucose area to ΔInsulin area was used as an index of whole body insulin sensitivity (Levy, Davenport, Clore, & Stevens, 2002).

2.6. Preparation of liver homogenates

Samples of frozen liver were homogenized 1:5 (w:v) in extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na_3VO_4, 5 μg/mL leupeptin, 20 μg/mL aprotinin, 2 mM benzamidine and 2 mM phenylmethylsulphonyl fluoride]. Homogenates were centrifuged at 14,000 *g* for 1 h and the supernatants were collected, assayed for protein concentration by using the Bradford reagent (Bradford, 1976) and stored at −80 °C until use.

2.7. Determination of ROS production

ROS were quantified as described based on the oxidation of DCFH assay to dichlorofluorescein (DCF) that emits fluorescence (Martin et al., 2008). Briefly, liver homogenates were diluted 1:20 (v/v) with ice-cold Locke's buffer [154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2 mM $CaCl_2$, 10 mM D-glucose and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfocnic acid, pH 7.4] and incubated with 5 μM DCFH for 30 min at 37 °C. Fluorescence was measured in a microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT).

2.8. Determination of carbonyl groups

Protein oxidation of liver homogenates was measured as carbonyl group content, as previously described (Granado-Serrano, Martín, Bravo, Goya, & Ramos, 2009). Liver tissues were homogenized (1:5 w/v) in 0.25 M Tris (pH 7.4), 0.2 M sucrose and 5 mM 1,4-dithiothreitol (DTT) buffer. Absorbance was measured at 360 nm and carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22,000 nmol/L/cm. Protein was measured by the Bradford reagent (Bradford, 1976).

2.9. Determination of GSH

GSH content was quantified by the fluorometric assay of Hissin & Hilf (1976). The method is based on the reaction of GSH with OPT at pH 8.0, which generates fluorescence. Livers were homogenized (1:20 w/v) in 50 mM phosphate buffer pH 7.0, proteins were precipitated with 5% trichloroacetic acid and then centrifuged for 30 min at 10,000 *g* (Granado-Serrano et al., 2009). Fluorescence was measured at an excitation wavelength of 340 nm and emission wavelength of 460 nm. The results were interpolated in a GSH standard curve (5 ng–1 μg) and expressed as nmol GSH/mg protein, which was determined by the Bradford reagent (Bradford, 1976).

2.10. Determination of GPx, GR, GST, CAT, SOD and HO-1 activities

The activity of antioxidant enzymes (GPx, GR, GST, CAT, SOD and HO-1) was determined in liver homogenates. Livers were homogenized (1:5 w/v) in 0.25 M Tris, 0.2 M sucrose and 5 mM DTT buffer pH 7.4 and centrifuged at 3000 *g* for 15 min. Determination of GPx activity is based on the oxidation of GSH by GPx, using *t*-BOOH as a substrate, coupled to the disappearance of NADPH by GR at 340 nm (Granado-Serrano et al., 2009), expressing the results as mU/mg protein. GR activity was assayed by following the decrease in absorbance at 340 nm due to the oxidation of NADPH utilized in the reduction of oxidized glutathione. GR activity was expressed as mU/mg protein. GST activity was analysed by a commercial fluorometric activity assay kit. The assay utilizes monochlorobimane, a dye that reacts with GSH catalysed by GST to produce a fluorescent dye. GST activity was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm, and expressed as U/mg protein (Granado-Serrano et al., 2009). CAT activity was determined by the decomposition of H_2O_2 as a decrease in absorbance at 240 nm, and expressed as UI/mg protein. SOD activity was assayed by using a commercial kit following the manufacturer's instructions (Sigma). The assay is based on the capacity of SOD to reduce superoxide anion, coupled with the disappearance of Dojindo's highly water-soluble tetrazolium salt (WST-1) to yield a dye. SOD activity was quantified by measuring the decrease in the absorbance at 440 nm and was expressed as U/mg protein. Analysis of HO-1 activity was quantified by a colorimetric immunoassay measuring at 450 nm (ELISA; BlueGene Biotech, Deltaclon) and expressed as ng/mg protein. Protein concentration was measured by the Bradford reagent (Bradford, 1976).

2.11. Protein determination by Western blot analysis

To detect Nrf2 (57 and 100 kDa) and NF-κB (p65), equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Millipore, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare, Madrid, Spain) immunoglobulin. Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was ensured by β-actin and band quantification was carried out with a scanner (HP Scanjet G2710, Hewlett-Packard, Spain), and accompanying software (Scion Image, Scion Corporation based on NIH Image, National Institutes of Health, USA).

2.12. Statistics

Prior to statistical analysis, it was verified that all the variables analysed follow a normal distribution using the Shapiro–Wilk test. Then, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way ANOVA was followed by the Tukey test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P < 0.05$ was considered significant. A SPSS version 21.0 program has been used.

3. Results

3.1. Characteristics of the animals

Final body weight and total food intake were significantly increased in both groups of ZDF rats in comparison to ZL animals (Table 2). Interestingly, the final body weight of animals fed with cocoa diet (ZDF-Ca) was reduced as compared to ZDF-C group. The body weight gain was higher in ZDF-C rats than in ZDF-Ca animals, which showed a body weight gain value similar to that of ZL group (Table 2).

ZDF groups reported higher values of fasting glycaemia and insulinemia than ZL rats, although ZDF-Ca animals exhibited significantly lower values than ZDF-C rats (Table 2). Likewise, cocoa-enriched diet reduced the glycaemia during GTT, and maintained lower levels of insulin as compared to ZDF rats. In concert, insulin resistance indexes

Table 2
Body weight, food intake, glucose and insulin levels of rats fed with standard (ZL and ZDF-C) and cocoa-enriched (ZDF-Ca) diets.*

	ZL	ZDF-C	ZDF-Ca
Final body weight (g)	327.00 ± 11.83^{a}	447.00 ± 11.60^{c}	369.71 ± 12.86^{b}
Body weight gain (g in 9 weeks)	201.25 ± 8.99^{a}	299.00 ± 5.09^{b}	222.4 ± 20.14^{a}
Total food Intake (g in 9 weeks)	983.97 ± 54.58^{a}	1353.54 ± 96.01^{b}	1279.10 ± 58.49^{b}
Glucose (mg/dL)	86.50 ± 5.35^{a}	202 ± 26.60^{c}	160.5 ± 16.52^{b}
Insulin (ng/mL)	0.48 ± 0.19^{a}	5.50 ± 0.46^{c}	3.08 ± 0.52^{b}
HOMA-IR	1.52 ± 0.56^{a}	30.99 ± 3.96^{c}	13.70 ± 1.02^{b}
Insulin sensitivity index (AUC insulin/ AUC glucose)	10.43 ± 2.64^{a}	60.22 ± 3.12^{c}	39.25 ± 4.15^{b}

* Data represent the means ± SD. Means in a row without a common letter differ, $P < 0.05$.

(HOMA-IR and AUC insulin/AUC glucose ratio) increased in ZDF rats, and a significant decrease was observed in ZDF-Ca animals in comparison with ZDF-C group (Table 2). However, ZDF-Ca values were still higher than those of ZL rats. All these results could suggest that the cocoa-rich diet ameliorates hyperglycaemia and hyperinsulinemia, and alleviates glucose intolerance and insulin resistance.

3.2. Cocoa-rich diet prevents ROS production and protein oxidation in ZDF rats

Oxidative stress and ROS production are elevated during insulin resistance and diabetes, which could lead to an enhanced macromolecule oxidation (Evans et al., 2002). In order to study whether cocoa could prevent these processes, ROS production and the protein carbonyl content, as a protein oxidation marker, were measured in the liver of all rat groups.

ROS production was increased in ZDF-C animals when compared to ZL and ZDF-Ca groups, whereas ROS generation levels in ZDF-Ca rat's liver were similar to those of ZL group (Fig. 1A). In concert, a significant increase in protein carbonyl content in ZDF-C animals was observed in comparison to ZL group, whereas carbonyl content was similar in ZL and ZDF-Ca rats (Fig. 1B). Altogether, it suggests that cocoa ameliorates the hepatic oxidative stress by reducing ROS production and protein oxidation in the liver of young ZDF rats.

3.3. GSH levels and antioxidant/detoxifying enzymatic activities in ZDF rat's liver

In order to continue studying the redox status in the liver, GSH levels and key antioxidant/detoxifying enzymes activity were analysed.

As shown in Fig. 2A–C, GSH levels, and GPx and GR activities were not modified in any group of rats. However, a decreased GST activity was observed in both ZDF groups in comparison to ZL rats (Fig. 2D). Additionally, no differences were found for CAT activity among all animal groups (Fig. 3A), and SOD activity diminished in ZDF-C rats when compared to their lean counterparts, although this enzymatic activity was restored to ZL values by feeding the animals with the cocoa-rich diet (Fig. 3B). Interestingly, ZDF-C rats showed the highest levels of HO-1 activity of the three groups of animals, whereas ZDF-Ca showed values similar to those of ZL rats (Fig. 3C). All these results indicate that cocoa-enriched diet modulates relevant antioxidant defences, that is, SOD and HO-1, in the liver of ZDF rats.

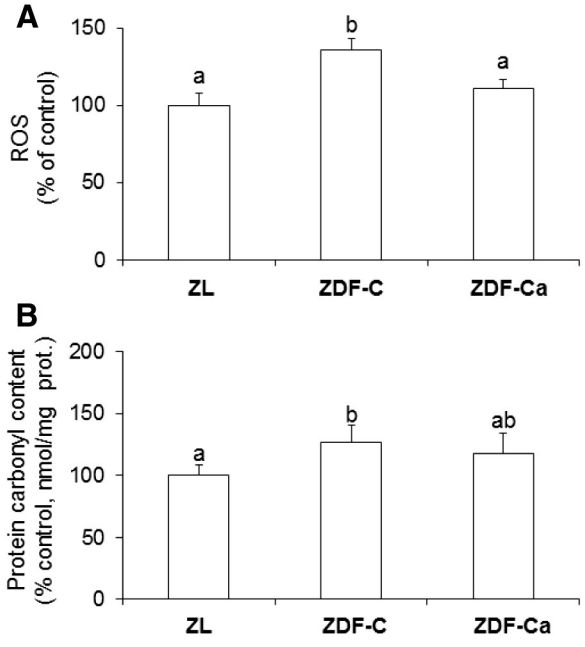

Fig. 1. Effect of the cocoa-enriched diet on (A) ROS production and (B) protein carbonyl content in the liver of young ZDF rats. Data are expressed as percentage of controls (means ± SD, n = 6–8). Means without a common letter differ ($P < 0.05$).

3.4. Cocoa-enriched diet modulates Nrf2 and NF-κB expression in ZDF rat's liver

Nrf2 and NF-κB are well-known sensors of the cellular redox status (Gloire et al., 2006), which are regulated during T2DM (Rochette et al., 2014). Then, the modulation of both transcription factors by the cocoa-rich diet was studied, and total and phosphorylated levels of Nrf2, as well as total levels of p65-NF-κB were analysed by Western blot.

Hepatic levels of total (57 kDa) and phosphorylated (100 kDa) Nrf2 were significantly increased in ZDF-C rats when compared to ZL and ZDF-Ca animals (Fig. 4A–C). Cocoa-rich diet prevented the enhanced levels of Nrf2 (100 and 57 kDa), and ZDF-Ca group presented similar levels of both proteins to those of ZL rats, although total Nrf2 (57 kDa) in ZDF-Ca group also showed similar values to those of ZDF-C rats. Additionally, p65-NF-κB levels increased in ZDF-C rats in comparison to ZL group, whereas this effect was totally repressed in animals receiving the cocoa rich-diet (Fig. 4A and D). All these results suggest that cocoa might contribute to protection of ZDF liver against the oxidative insult through the modulation of relevant closed-related redox transcription factors.

4. Discussion

T2DM is a major concern in the society due to its high prevalence, and there have been persistent efforts to identify potential compounds that can prevent or delay the onset of the disease. Rising evidence proposes flavonoids as potential anti-diabetic agents (Ali, Ismail, & Kersten, 2014; Babu et al., 2013; Brand-Miller, Holt, de Jong, & Petocz, 2003; Grassi et al., 2008), although insufficient investigation *in vivo* has been performed to support these observations.

The present study demonstrates that a cocoa-enriched diet improves the hepatic redox status, the glycaemic control and insulin sensitivity in young ZDF rats. Moreover, the cocoa-supplemented diet ameliorates the hepatic dysfunction evoked by the oxidative stress that takes place in T2DM by restraining the increase of ROS generation, protein carbonyl content, and the enhanced levels of Nrf2 and NF- B. Furthermore, cocoa recovers the hepatic activities of SOD and HO-1 to control values, although GST activity remains decreased.

Cocoa is a rich source of bioactive compounds, mainly flavanols such as epicatechin, catechin and oligomeric procyanidins (Martin et al., 2008) that seem to exert different biological activities contributing to prevent oxidative-stress related diseases such as T2DM as well as other metabolic disorders (Ali et al., 2014; Babu et al., 2013). Young ZDF rats presented an increased food intake and body weight, which corroborate the hyperphagia of ZDF animals (Leonard et al., 2005). However, despite no differences in food intake between ZDF groups were observed, body weight of ZDF-Ca rats was similar to their lean counterparts, which confirms the potential effect of the cocoa-enriched diet on obesity related diseases (Ali et al., 2014). In this line, it has been shown that the cocoa polyphenolic fraction possesses the ability to reduce fat adipose tissue (Ramiro-Puig et al., 2008; Rodríguez-Ramiro et al., 2011), modulates the lipid metabolism, prevents fat deposition and activates energy expenditure (Ali et al., 2014; de Oliveira & Genovese, 2013). In fact, cocoa polyphenols activate 5′-AMP-activated protein kinase, which leads to the stimulation of downstream events that suppress hyperglycaemia and fat deposition, and to the prevention of hyperglycaemia, hyperinsulinemia and insulin resistance (Ali et al., 2014).

The pre-diabetic stage in ZDF animals is characterized by insulin resistance, which is compensated by hyperinsulinemia to maintain a normoglycaemia (Etgen & Oldham, 2000), as rats develop hyperglycaemia between 7 and 12 weeks (Leonard et al., 2005).

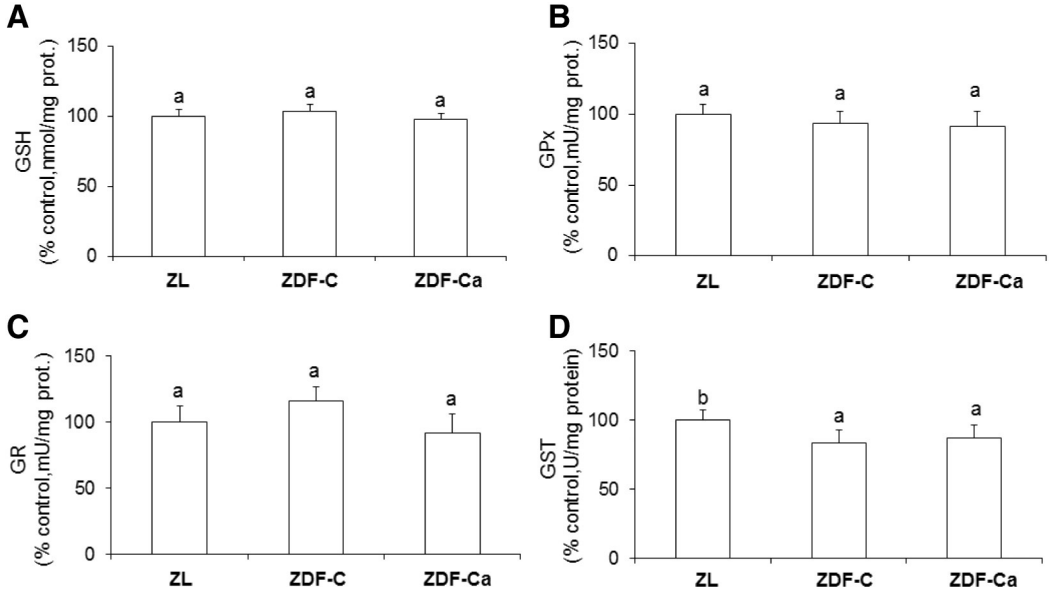

Fig. 2. Effect of the cocoa-rich diet on (A) GSH content and the activity of antioxidant/detoxifying enzymes (B) GPx, (C) GR and (D) GST. Values are expressed as a percentage relative to ZL rats (means ± SD, n = 6–8). Means without a common letter differ ($P < 0.05$).

Accordingly, glycaemia and insulinemia were higher in both ZDF groups when compared to ZL rats. However, cocoa supplementation reduced fasting blood glucose and insulin levels, and AUC values for glucose and insulin (Table 2). Indeed, ZDF-Ca rats as compared to ZDF-C group presented a reduced AUC insulin/AUC glucose ratio and HOMA-IR as compared to ZDF-C group, indicative of alleviated insulin resistance. Moreover, reduced body weight of ZDF-Ca animals was consistent with the increased insulin sensitivity of this group, as previously shown for high-fat fed mice supplemented with oligomeric cocoa procyanidins (Dorenkott et al., 2014). In fact, the diminution in body weight has been related to reduced circulating glucose blood levels and improved lipid profiles in cocoa-fed diabetic rats (Ruzaidi et al., 2005). Also in line with the present results, different cocoa extracts, oligomeric cocoa procyanidins, and epicatechins have shown to reduce glucose levels in diabetic rodents (Dorenkott et al., 2014; Fu, Yuskavage, & Liu, 2013; Jalil et al., 2008; Ruzaidi et al., 2005), and procyanidins from cocoa or grapes have been reported to diminish insulin values in insulin-resistant rodents (Dorenkott et al., 2014; Montagut et al., 2010). All together, it indicates that cocoa could attenuate hyperglycaemia and improve postprandial glycaemic control in young ZDF rats. Indeed, these beneficial effects of cocoa on glucose tolerance and insulin resistance, among other factors, have been related to the ability of cocoa phenolic compounds to inhibit the activity of key digestive enzymes that reduce macronutrient absorption/digestion, (Gu & Lambert, 2013), as well as to a more efficacious stimulation of insulin secretion and/or production (Dorenkott et al., 2014; Fu et al., 2013; Gu & Lambert, 2013), activation

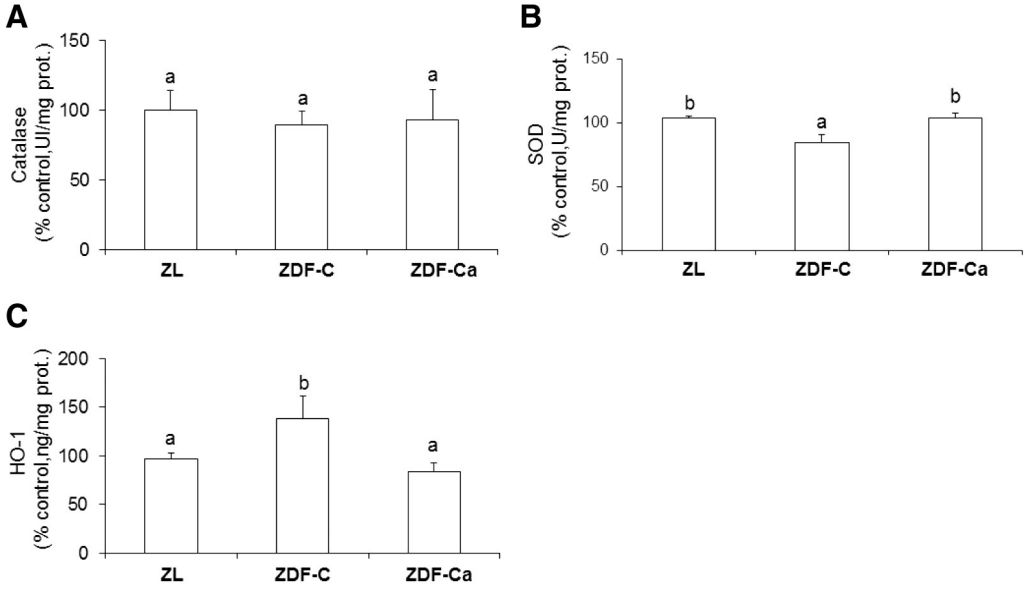

Fig. 3. Effect of the cocoa-supplemented diet on the activity of (A) CAT, (B) SOD and (C) HO-1. Values are expressed as a percentage relative to ZL rats and are means ± SD, n = 6–8. Means without a common letter differ ($P < 0.05$).

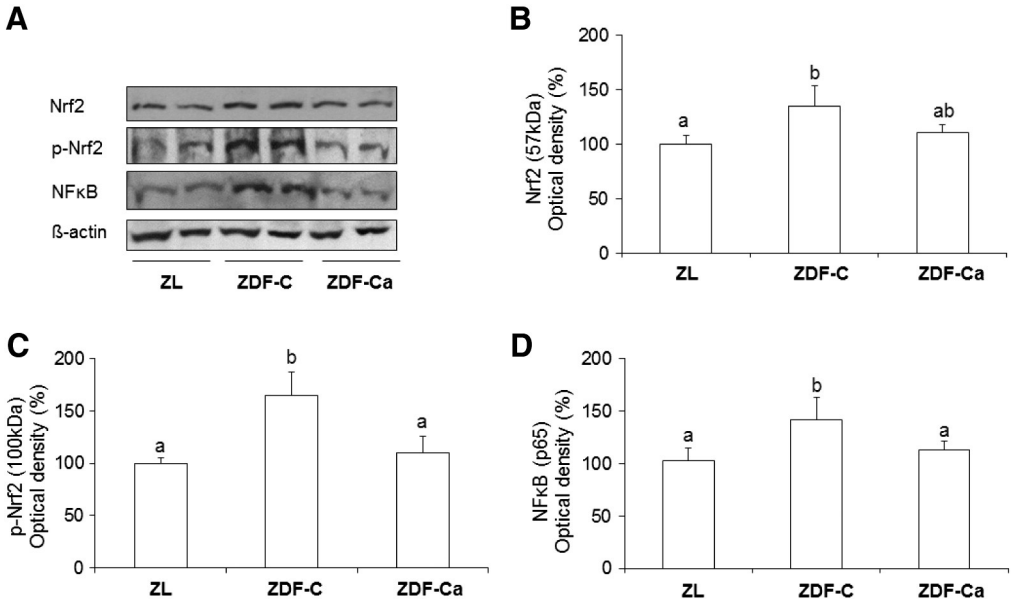

Fig. 4. Effect of the cocoa-enriched diet on total and phosphorylated levels of Nrf2 and total values of p65-NF- B in the liver of young ZDF rats. (A) Bands of representative experiments. Densitometric quantification of (B) levels of phosphorylated Nrf2 (100 kDa) and (C) total levels of Nrf2 (57 kDa) and (D) total levels of p65-NF- B. Values are expressed as a percentage relative to ZL animal group. Equal loading of Western blots was ensured by β-actin (n = 6–8). Means without a common letter differ ($P < 0.05$).

of insulin signalling and glucose uptake (Cordero-Herrera, Martin, Bravo, Goya, & Ramos, 2013; Cordero-Herrera, Martín, Goya, & Ramos, 2014; Martin et al., 2014).

Recent evidences suggest that chronic hyperglycaemia-mediated oxidative stress is a major risk factor for liver dysfunction in diabetes (Evans et al., 2002; Rochette et al., 2014). Accordingly, the levels of ROS and the marker of protein oxidation (carbonyl groups) increased in ZDF-C rats, whereas these effects were prevented by the cocoa-supplemented diet. In this line, anthocyanins derived from purple sweet potato (Zhang et al., 2013) and a combination of flavonoids (Parveen, Khan, Mujeeb, & Siddiqui, 2010) have shown to attenuate ROS generation and protein carbonyls in different diabetic models.

Oxidative stress is counteracted by non-enzymatic antioxidant defences, as GSH, and antioxidant/detoxifying enzymes (Masella et al., 2005). In this line, it has been reported that the liver is the largest GSH reservoir and that the modulation of intracellular GSH content is a possible disease-preventing effect of phenolic compounds (Masella et al., 2005). In the present work, hepatic GSH content was equal among all animal groups, as well as CAT, GPx and GR activities. However, SOD and GST activities decreased, and HO-1 activity increased in ZDF-C rats as compared to their lean counterpart. Cocoa-rich diet returned the activities of SOD and HO-1 to control values. Similarly, unmodified GSH hepatic content and CAT activity, although diminished SOD activity, have been reported in insulin-resistant rats (Bagul et al., 2012). This situation was reverted upon treatment of the insulin-resistant animals with the grape polyphenol resveratrol, and it was associated with a reduction of oxidative stress in the liver (Bagul et al., 2012). Therefore, it could be suggested that the antioxidant defence system is reinforced to face oxidative stress, as previously shown in diabetic and high-fat fed rats (Aksoy, Vural, Sabuncu, & Aksoy, 2003; Alezandro, Granato, & Genovese, 2013; Shrivastava, Chaturvedi, Singh, Saxena, & Bhatia, 2013). Interestingly, the detoxifying enzyme GST has been proved to decrease in diabetic mice (Barnett, Abbott, Bailey, Flatt, & Ioannides, 1992), which has been related to a diminished hepatic enzymatic expression and activity because of lower insulin stimulation during hepatic resistance (Kim, Woodcroft, & Novak, 2003). Indeed, insulin resistance was partly prevented in ZDF-Ca rats when compared to ZDF-C animals, according to insulin sensitivity indexes AUC glucose/AUC insulin ratio and HOMA-IR (Table 2), but this seems not to be enough to restore the hepatic GST activity. Additionally, overexpression of HO-1 in the liver of diabetic rats can be down-regulated by antioxidant supplementation (Patriarca et al., 2005). Concurrently, in the present work ZDF-C rats showed increased hepatic HO-1 activity, whereas the cocoa-enriched diet diminished its activity, suggesting a reduced oxidative stress. In fact, it has been proposed that the modulation of antioxidant defences is connected to the severity, duration and treatment of diabetes (de Oliveira & Genovese, 2013). Thus, if the damaging effect of oxidative stress is maintained, accumulated impairment would decrease the antioxidant defences in the tissue, as previously suggested (Aksoy et al., 2003). Altogether it suggests that the modulation of antioxidant/detoxifying enzymes is a complex mechanism that could be a consequence of an attempt to regulate body's redox balance.

Several signalling pathways are activated in response to ROS overproduction, such as Nrf2 and NF- B (Gloire et al., 2006). Nrf2 is a key player in the antioxidant/detoxifying response, and its primary control of function lies on its subcellular distribution and/or phosphorylation (Eggler et al., 2008; Masella et al., 2005). Moreover, Nrf2 has been shown to be essential in the upregulation of certain cytoprotective enzymes in response to oxidative stress, such as HO-1 (Eggler et al., 2008). Thus, ZDF-C rats showed increased phosphorylated and total Nrf2 hepatic levels in comparison to ZL animals, as previously shown in another diabetic model (Jiang et al., 2010). These effects were abolished by feeding the animals with the cocoa-supplemented diet. Interestingly, in the liver of insulin-resistant rats, resveratrol did not alter total Nrf2 levels and increased phosphorylated Nrf2 values, whereas the anti-diabetic drug metformin diminished the total Nrf2 levels and unaltered phosphorylated Nrf2 values, showing in both cases a protective effect against the hepatic oxidative stress (Bagul et al., 2012). Moreover, changes observed in HO-1 activity among all groups of rats were parallel to those showed by Nrf2, which could indicate the regulation of the enzymatic activity by the transcription factor, but these studies deserve further investigations.

NF- B also plays a central role in oxidative stress and inflammation (Gloire et al., 2006). In the current study, ZDF-C rats showed increased

levels of p65-NF- B that were returned to control values in cocoa-fed animals. Accordingly, other phenolic compounds and antioxidants such as anthocyanins derived from purple sweet potato, proanthocyanidins and L-cysteine reversed the high levels of NF- B detected in diabetic rats to control values (Jain, Velusamy, Croad, Rains, & Bull, 2009; Noh, Kim, Park, Fujii, & Yokozawa, 2010; Zhang et al., 2013), which was associated to a diminished oxidative stress. In this regard, it should be mentioned that a potential anti-inflammatory effect of cocoa, which merits further studies, could not be ruled out. A low-grade inflammation impairs insulin sensitivity and it has been demonstrated that cocoa inhibits the metabolic endotoxemia and reduced systemic inflammation through the modulation of NF- B, leading to a diminished cytokine production and eicosanoid metabolism, among others (Dorenkott et al., 2014; Gu & Lambert, 2013). Therefore, cocoa might also contribute to alleviate glucose intolerance by this mechanism. All these results suggest that increased levels of these transcription factors provide an adaptive response to counteract oxidative stress in T2DM, and that cocoa might improve the oxidative stress condition *via* the restoration of Nrf2 and NF- B in young ZDF rats.

5. Conclusions

Cocoa-enriched diet alleviates glycaemic homeostasis and oxidative stress in young ZDF rats. Cocoa ameliorates the redox imbalance, as shown by the diminished ROS generation and protein oxidation, through its ability to modulate the antioxidant/detoxifying defence system, as it decreases hepatic HO-1 and re-establishes SOD activity, as well as levels of Nrf2 and NF- B. This study provides *in vivo* evidence about the preventive effects of cocoa in T2DM and potential therapeutic targets of antioxidant treatment for the prevention and therapy of T2DM.

Acknowledgments

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Capítulo 3: Efecto de los flavanoles del cacao sobre el metabolismo lipídico hepático durante la resistencia a la insulina y la diabetes.

Resumen

Antecedentes: Durante la resistencia a la insulina se produce la alteración del metabolismo lipídico, lo que está directamente relacionado con la desregulación de la señalización de la insulina y el desarrollo de la DMT2. Distintos estudios muestran que el cacao y sus metabolitos parecen modular el metabolismo lipídico, sin embargo no se conoce completamente el mecanismo molecular por el cual ejercen su efecto beneficioso.

Objetivo: Analizar el efecto de una dieta enriquecida en cacao en las ratas ZDF, y de la EC, flavanol mayoritario en el cacao, en las células HepG2 expuestas a una concentración alta de glucosa sobre el metabolismo lipídico hepático y los mecanismos moleculares subyacentes.

Metodología: Los estudios se llevaron a cabo en ratas ZDF y ZL alimentadas con dieta estándar (ZDF-C y ZL) o enriquecida con un 10% en cacao (ZDF-Co), y con las células HepG2 preincubadas con EC (10 μM) durante 24 horas y posteriormente tratadas con 30 mM de glucosa (24 horas). Se analizaron los niveles séricos y hepáticos de los lípidos en los animales y las células HepG2. Además, se evaluaron los niveles de proteínas clave del metabolismo lipídico como SREBP1-c, FAS, PPARα, AKT, AMPK y PKCζ. Además, en las células HepG2 se estudió el papel de la PKCζ en el efecto protector inducido por la EC durante la resistencia a la insulina.

Resultados: La dieta enriquecida en cacao redujo el peso final de las ratas ZDF-Co respecto a las ZDF-C sin modificar la ingesta. Además, mejoró los perfiles lipídicos sérico y hepático: se produjo un descenso en los niveles de TG, colesterol total (T-Cho), LDL-Cho, NEFA y la acumulación de gotas lipídicas, y un aumento en los niveles de HDL-Cho. De manera similar, la EC restauró los valores lipídicos alterados (TG, T-Cho y NEFA) por la alta concentración de glucosa en las células HepG2. El cacao y la EC también previnieron el descenso en los niveles fosforilados de AKT, AMPK y PPARα, así como el aumento de p-PKCζ, SREBP1-c y FAS, lo que se asoció con la estimulación de la lipolisis y la inhibición de la lipogénesis. La regulación de la PKCζ por la EC contribuyó a mejorar el metabolismo lipídico a través de la AKT y la AMPK.

Conclusión: Los flavanoles del cacao alivian la hiperlipidemia y la esteatosis hepática al atenuar la lipogénesis y estimular la β-oxidación de los ácidos grasos a través de la

modulación de la AKT, AMPK y PKCζ, que juega un papel crucial en este proceso. Este estudio aporta nuevos conocimientos sobre el mecanismo antidiabético del cacao, y sugieren que este alimento podría ser útil para prevenir o retrasar el desarrollo y el progreso de la DMT2 y sus complicaciones, y enfermedades asociadas, como la esteatosis hepática.

Cocoa flavanols improve hepatic lipid metabolism in Zucker Diabetic Fatty rats and human hepatic cells. Role of PKCζ

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A B S T R A C T

Diabetes alters lipid metabolism that could lead to hepatic steatosis. The present study aimed to investigate the effect of a cocoa-enriched diet in type 2 diabetic Zucker Diabetic Fatty (ZDF) rats and that of cocoa-flavanol epicatechin (EC) in high-glucose-exposed HepG2 cells on hepatic lipid metabolism. Male ZDF cocoa-fed-rats decreased body weight gain and improved circulating and hepatic lipid levels. Similarly, EC alleviated altered lipid values induced in high-glucose-challenged HepG2 cells. The lipid-lowering effect was related to diminished fatty-acid synthesis (sterol-regulatory-element-binding protein-1-c and fatty-acid synthase down-regulation), and increased fatty-acid oxidation (proliferator-activated-receptorα up-regulation). These effects depended on 5´-AMP-activated-protein-kinase (AMPK), protein kinase B (AKT) and protein kinase C (PKC)-ζ, which phosphorylated levels returned to control values upon cocoa or EC administration. Moreover, PKCζ played a role on AKT and AMPK regulation. These findings suggest that cocoa and EC protect hepatocytes *in vivo* and *in vitro* by improving lipid metabolism through multiple signaling pathways.

Keywords:

Cocoa
Epicatechin
Hepatic HepG2 cells
Hepatic lipid deposition
Lipid metabolism
Type 2 diabetic ZDF rats

1. Introduction

Type 2 diabetes (T2D) is an increasing health global problem in both developed and developing countries (American Diabetes Association, 2005). This chronic disease is characterized by peripheral insulin resistance and insulin secretion defects (Babu, Liu & Gilbert, 2013; Klover & Mooney, 2004). At early stages, the impairment of glucose metabolism leads to hyperglycemia and hyperinsulinemia, as well as to the activation of lipogenic and pro-inflammatory pathways (Klover & Mooney, 2004). T2D treatment has been much focused on the control of glycaemia, but it has been long documented that lipid abnormalities are also involved in the dysregulation of insulin signaling. Accordingly, the interest for the identification of natural compounds that improve glucose and/or lipid metabolism has started to grow rapidly and is considered an interesting strategy to prevent or treat T2D (Babu et al., 2013; Im, Issac, Ninan, Kuttan & Maliakel, 2014; Mokiran, Ismail, Azlan, Hamid & Hassan, 2014).

Liver plays a critical role regulating the whole body metabolism, and during T2D insulin control of hepatic metabolism is impaired (Babu et al., 2013; Klover & Mooney, 2004). As a consequence, there is an inefficient glucose uptake and suppression of glucose production, which leads to hyperglycemia (Klover & Mooney, 2004), as well as a reduced lipid oxidation and increased lipid synthesis, which leads to an augmented lipid accumulation, resulting in hepatic steatosis (Farese & Sajan, 2010). At molecular level key proteins involved in glucose and lipid metabolism namely 5´-AMP-activated protein kinase (AMPK) and protein kinase B (AKT) are inhibited (Klover & Mooney, 2004), and protein kinase C (PKC) is activated (Farese & Sajan, 2010; Guillou, Martin & Pineau, 2008; Viollet et al., 2006). AMPK constitutes a central regulator of the cellular metabolism, able to repress hepatic glycolytic and lipogenic genes (Viollet et al., 2006), and similarly, both AKT and atypical protein kinase C (aPKC) mediate insulin effects on hepatic glucose utilization and lipogenesis (Farese & Sajan, 2010; Guillou et al., 2008). Indeed, PKCζ, which detailed role is still unclear, is an aPKC isoform abundant in the liver particularly important in diabetes, as it is involved in lipid alterations and has been suggested as an initiating factor for the development of insulin resistance (Farese & Sajan, 2010). Moreover, transcription factors sterol regulatory element-binding protein 1-c (SREBP1-c) and proliferator activated receptor α (PPARα) play a pivotal role in hepatic lipid metabolism as they control synthesis and degradation of fatty acids, respectively (Guillou et al., 2008).

Cocoa and its derived products are widely consumed (Vinson et al., 2006), and together with its phenolic compounds have increasingly attracted attention because of their potential ability to act as highly effective chemo-preventive agents in diabetes and metabolic-related diseases (Ali, Ismail & Kersten, 2014; Babu et al., 2013). Accordingly, we have previously demonstrated that (-)-epicatechin (EC), the main cocoa flavanol, and a cocoa phenolic extract improve insulin sensitivity as well as both glucose uptake and production in insulin-resistant hepatic HepG2 cells (Cordero-Herrera, Martín, Goya & Ramos, 2014). Additionally, a cocoa phenolic extract and EC alone strengthen insulin signaling by activating key proteins such as AKT and AMPK in HepG2 cells (Cordero-Herrera, Martín, Bravo, Goya &

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Ramos, 2013). Moreover, it has been reported that metabolites derived from a proanthocyanidin-rich extract of cocoa decreased lipid synthesis and excretion in HepG2 cells (Guerrero et al., 2013). Different studies have also demonstrated that cocoa or its main components, such as EC, exert hypoglycemic and hypolipidemic effects on several animal models of diabetes and insulin resistance (Cordero-Herrera, Martín, Goya & Ramos, 2015a; Matsuia et al., 2005; Mokiran et al., 2014; Ramos et al., 2008; Ruzaidi, Amin, Nawalyah, Hamid & Faizul, 2005; Si et al., 2011). Moreover, cocoa and dark chocolate have been reported to improve insulin sensitivity and lipid profile in healthy humans and T2D patients, alleviating the atherosclerotic cholesterol profile (Baba et al., 2007; Grassi et al., 2008; Mellor, Sathyapalan, Kilpatrick, Beckett & Atkin, 2010). However, to the best of our knowledge, limited studies have evaluated the effects of cocoa and EC on lipid metabolism during diabetes by using animal and cellular models. Furthermore, the molecular mechanism for the potential anti-diabetic effect of cocoa related to lipid metabolism during diabetes in the liver is not fully understood.

The aim of this study was to analyze the effect of cocoa on lipid metabolism in the liver of a widely used model that mimics human T2D, Zucker diabetic fatty rats, during the pre-diabetic stage (6-15 weeks of life). Furthermore, molecular mechanisms involved in the potential beneficial effects of the main cocoa flavanol, EC, on lipid metabolism, as well as the relevance of the scarcely studied although crucial aPKC in T2D, PKCζ, were analyzed in high-glucose-challenged HepG2 cells. Here, it is reported that cocoa and EC improve lipid metabolism by attenuating lipogenesis and stimulating lypolisis in the liver of ZDF rats and cultured hepatic cells through the modulation of key proteins such as AKT, AMPK and PKCζ, playing PKCζ a main role in all these processes.

2. Materials and methods

2.1. Materials and chemicals

*(–)-EC (>95% of purity), D-gl*ucose, gentamicin, penicillin G, streptomycin, compound C (6-[4-(2-Piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine), LY294002 (2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride), and oil red-O were purchased in Sigma Chemicals (Madrid, Spain). Anti-AKT, anti-phospho-Ser473-AKT, anti-AMPK, anti-phospho-Thr172-AMPK, FAS and anti-β-actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-PKCζ, anti-phospho-Thr410-PKCζ, SREBP1 and PPARα were purchased from Santa Cruz (sc-216, sc-271962, sc-366 and sc-9000, respectively, Qimigen, Madrid, Spain). NEFA kit was obtained from Wako Chemicals GmbH (Neuss, Germany). Triglycerides and cholesterol reagent kits were acquired from BioSystems (Atom, Madrid, Spain). PKCζ pseudosubstrate (myristoylated) was purchased from Enzo Life Sciences (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Lonza (Madrid, Spain), respectively. Materials and chemicals for electrophoresis were obtained from BioRad Laboratories S.A. (Madrid, Spain).

2.2. Cocoa

Natural Forastero cocoa powder (a kind gift from Nutrexpa S.L., Barcelona, Spain) was used for this study. It contains epicatechin (383.5 mg/100 g), catechin (116 mg/100 g) and procyanidins (254.5 mg/100 g) and non-flavonoid compounds such as theobromine. A detailed composition of this cocoa is given elsewhere (Martin et al., 2008). Diets were prepared from an AIN-93G formulation (Panlab S.L., Barcelona, Spain) providing all nutrients required by adult rats. The 10% cocoa diet was produced by adding 100 g/Kg cocoa to AIN-93G, and the composition of the diets is given in Table 1.

Table 1. Composition of the experimental control (C) and cocoa-rich (Co) diets (g/Kg dry weight).

	C	Co
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ[1]	0.008	0.008
Mineral mix.	35	35
Vitamin mix.	10	10
L-Cys	1.8	1.8
Cholin bitartrate	2.5	2.5
Cellulose	100	66
Starch	415.7	349.7
Cocoa powder	-	100

[1]*tert*-butylhydroquinone

2.3. Animals and diets

Five-week-old male Zucker Diabetic Fatty (ZDF, n=16) rats and littermate Zucker lean (ZL, n=8) rats were purchased from Charles River Laboratories (L'arbresle, France) and were placed under controlled conditions (19-23ºC, 50-60% humidity and 12 h light/dark cycles). ZDF [ZDF/*crl-lepr* (fa/fa)] animals possess a mutation in the leptin receptor and spontaneously develop severe obesity, hyperglycaemia, hyperlipidaemia, and insulin resistance (Leonard, Watson, Loomes, Phillips & Cooper, 2005; Sparks et al., 1998). After one week of acclimation (at 6 weeks of age), ZDF rats were randomly assorted into two different experimental groups: one group received the standard diet (ZDF-C) and the other group was fed with the cocoa rich diet (ZDF-Co). ZL animals remained as a unique group and were fed with the standard control diet. Both diets were isocaloric and all experimental groups were provided with food and water *ad libitum*.

At 15 weeks of age, animals were fasted overnight and killed by decapitation. Trunk blood was collected in the absence of anesthesia, and serum was separated by centrifugation at 1000g, 10 min, 4ºC for further biochemical analysis. Liver was removed and divided into two samples: one was fixed by immersion in paraformaldehyde (PFA) for histological analysis and the other one was frozen in liquid N_2 and stored at -80°C. Animals were treated according to Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Cientificas, CSIC).

2.4. Determinations of serum and hepatic lipid levels in rats

In serum, triglycerides (TG), total cholesterol (T-Cho) and HDL-Cho were determined as described elsewhere (Ramos et al., 2008). LDL-Cho was analyzed following the method by Wieland and Seidel (Wieland & Seidel, 1983). Non-esterified fatty acids (NEFA) were measured using a colorimetric enzyme assay (Wako Chemicals, BioAnalitica, Madrid, Spain) according to manufacturer's instructions.

Liver lipids were extracted from 100 mg frozen-liver using chloroform-methanol (1:1, v/v) solution, as described by Bligh and Dyer (Bligh & Dyer, 1959). The extraction solution was used to determine levels of TG, T-Cho and NEFA with kits employed for serum lipid determinations.

2.5. Histological analysis

Livers were removed, fixed overnight in 4% PFA in 0.1 M phosphate buffer pH 7.4 and routinely paraffin embedded. Serial sections (5 µm) were mounted on glass slides, hydrated and stained with Hematoxylin and Eosin (H&E). Images of stained sections were acquired using a digital camera (XCD-U100CR; Sony, Tokyo, Japan) attached to a light microscope (Eclipse 80i; Nikon, Tokyo, Japan).

2.6. Preparation of liver lysates for Western blotting

Samples of frozen liver were homogenized 1:5 (w:v) in extraction buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% glycerol, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2 mM EDTA, 1% Nonidet P-40, 2 mM Na_3VO_4, 5 µg/mL leupeptin, 20 µg/mL aprotinin, 2 mM benzamidin and 2 mM phenylmethylsulphonyl fluoride (PMSF)]. Homogenates were centrifuged at 14000g for 1 h and supernatants were collected, assayed for protein concentration by using the Bradford reagent and stored at -80ºC until use for Western blot analyses.

2.7. Western blot analysis

To detect p-AKT, AKT, p-AMPK, AMPK, p-PKCζ, PKCζ, FAS, SREBP1 and PPARα, equal amounts of protein were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF)

filters (Millipore, Madrid, Spain). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit (GE Healthcare, Madrid, Spain) or anti-mouse (Sigma, Madrid, Spain) immunoglobulin. Blots were developed with the ECL system (GE Healthcare, Madrid, Spain). Normalization of Western blot was ensured by β-actin and band quantification was carried out with a scanner and accompanying software.

2.8. Cell culture and treatments

Human HepG2 cells were grown in DMEM-F12 medium supplemented with 2.5% fetal bovine serum (FBS) and 50 mg/L antibiotics (gentamicin, penicillin and streptomycin). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2. One day after plating, the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and FBS, and the culture was continued. Subsequently, to evaluate the protective effect of EC against a high glucose challenge, cells were preincubated for 24 h with 10 µM EC; then, the medium was discarded and fresh medium containing 30 mM glucose was added for additional 24 h. At the end of the treatment, cells were incubated with 100 nM insulin for 10 min to test the response to insulin and then, cells were harvested. Cells exposed to high-glucose constitute a reliable hepatic model to study insulin resistance and steatosis (Cordero-Herrera et al., 2014; Cordero-Herrera et al., 2015a; Nakajima et al., 2000; Shang et al., 2008; Zang et al., 2004).

In the experiments with the inhibitors, cells were pre-incubated with 25 µM LY294002 (specific AKT inhibitor, LY) 40 µM compound C (specific AMPK inhibitor, CC), 30 µM pseudosubstrate (specific PKCζ inhibitor, P) for 1 h prior to the high glucose challenge.

2.9. Oil red-O staining in cells

To measure cellular neutral lipid droplet accumulation, HepG2 cells were stained by the oil red-O method (Lin, Huang & Lin, 2007). HepG2 cells were fixed with 3.7 % formaldehyde for 20 min at room temperature. Then, cells were washed with PBS and stained with oil red-O solution (0.2 mg/mL) for 2 h. Later, HepG2 cells were washed with PBS to remove unincorporated dye, isopropanol added and absorbance measured at 500 nm.

2.10. Analysis of lipids in cultured cells

Cellular lipids were extracted from HepG2 cells using hexane:isopropanol (2:1, v/v) solution, as described previously (Hozumi, Kawano & Jordan, 2000). TG, T-Cho and NEFA levels were determined by using commercial kits (see section 2.4.). Protein content was measured using the Bradford reagent.

2.11. Preparation of total cell lysates for Western blotting

To detect p-AKT, AKT, p-AMPK, AMPK, p-PKCζ, PKCζ, FAS, SREBP1 and PPARα, cells were lysed at 4ºC in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 µg/mL leupeptin and 1 mM PMSF. Supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at -80ºC until used for Western blotting (see section 2.7.).

2.12. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene. For multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. $P<0.05$ was considered significant. A SPSS version 22.0 program has been used.

3. Results

3.1. Cocoa-rich diet decreases body weight gain and improves serum and hepatic lipid profile in ZDF rats

Initial body weight was significantly enhanced in ZDF rats in comparison to ZL animals (ZL: 124.50 ± 5.21 g; ZDF: 146.75 ± 12.98 g; ZDF-Co: 145.87 ± 14.46 g). However, the final body weight of ZDF-Co animals (363.00 ± 22.08 g) was reduced as compared with ZDF-C animals (444.13 ± 13.47 g), showing intermediate values to those of ZDF-C and ZL rats (328.67 ± 18.46 g). There was a significant increase of total food intake in ZDF animals in comparison to ZL rats, but interestingly, there was no difference between ZDF groups (ZL: 983.97 ± 54.58 g; ZDF-C: 1353.54 ± 96.01 g; ZDF-Co: 1279.10 ± 58.49 g).

Serum TG, LDL-Cho, HDL-Cho and NEFA levels were significantly elevated in ZDF rats in comparison to ZL group, and those values were greater in ZDF-C animals than in ZDF-Co rats (Table 2). ZDF groups showed significantly higher levels of T-Cho than ZL rats. Interestingly, despite ZDF groups presented equal T-Cho levels, cocoa-rich diet significantly increased HDL-Cho levels and decreased LDL-Cho levels in comparison to ZDF-C animals (Table 2).

Hepatic TG and T-Cho content were elevated in ZDF groups in comparison to ZL rats, and those values were smaller in ZDF-Co animals than in ZDF-C rats (Table 2). Additionally, ZDF-C rats displayed the highest values of hepatic NEFA, whereas in ZL and ZDF-Co levels were comparable. In concert, morphological analysis of liver indicated that lipid droplet accumulation was more pronounced in ZDF rats as compared to ZL group. However, cocoa-rich diet resulted in a reduced number and smaller size of lipid droplets in comparison to ZDF-C animals, although still more pronounced than in ZL rats (Figure 1). All these results could suggest that cocoa-rich diet ameliorates the lipid profiles in serum and liver of ZDF rats.

3.2. Cocoa-rich diet increases phosphorylated levels of AKT and AMPK and decreases PKCζ phosphorylated values in ZDF rat's liver

During a pre-diabetic situation hepatic insulin signaling is impaired, whereas insulin promotion of lipogenesis is maintained (Farese & Sajan, 2010). Thus, in order to study the potential protective effect of cocoa-rich diet on lipid alterations, phosphorylated and total levels of key proteins that mediate insulin effects on lipid metabolism, such as AMPK, AKT and PKCζ were assayed by Western blot in rat's liver of different animal groups.

Phosphorylated levels of AKT were decreased in ZDF-C rats as compared with ZDF-Co animals, which presented similar levels to those of ZL rats (Figures 2A and 2B). Similarly, ZDF-C rats

Table 2. Lipid profile in serum and in liver of rats fed with standard (ZL and ZDF-C) and cocoa-rich (ZDF-Co) diets for 9 weeks*.

	ZL	ZDF-C	ZDF-Co
Serum TG (mmol/L)	0.96 ± 0.13^{a}	6.35 ± 0.40^{c}	4.42 ± 0.32^{b}
Serum T-Cho (mmol/L)	2.38 ± 0.16^{a}	5.85 ± 0.38^{b}	6.46 ± 0.25^{b}
Serum HDL-Cho (mmol/L)	1.94 ± 0.20^{a}	3.78 ± 0.29^{b}	5.43 ± 0.21^{c}
Serum LDL-Cho (mmol/L)	0.79 ± 0.07^{a}	2.44 ± 0.30^{c}	1.33 ± 0.32^{b}
Serum NEFA (mmol/L)	0.46 ± 0.03^{a}	0.86 ± 0.06^{c}	0.67 ± 0.05^{b}
Liver TG (µmol/g liver)	73.32 ± 4.93^{a}	139.66 ± 6.36^{c}	117.45 ± 8.15^{b}
Liver T-Cho (µmol/g liver)	4.77 ± 0.24^{a}	8.12 ± 0.62^{c}	6.58 ± 0.23^{b}
Liver NEFA (µmol/g liver)	1.04 ± 0.12^{a}	1.56 ± 0.07^{b}	1.37 ± 0.07^{ab}

* Data represent the means ± SD. Means in a row without a common letter differ, $P<0.05$.

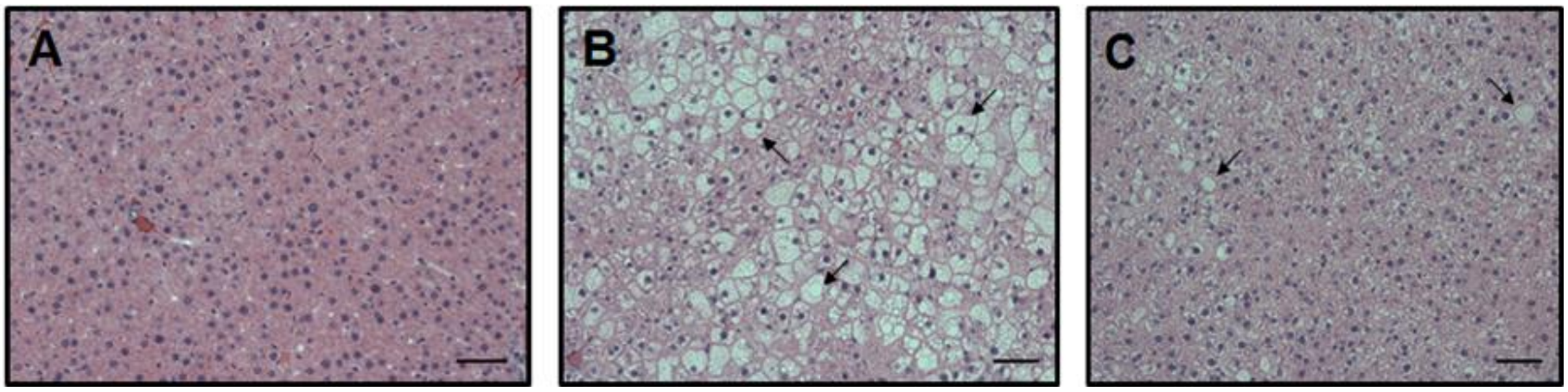

Figure 1. Representative images of histological sections of livers stained with H&E from (A) ZL, (B) ZDF-C and (C) ZDF-Co rats. Arrows indicate lipid droplets (scale bar: 50µm).

showed diminished p-AMPK values that were increased to achieve similar levels to those of ZL group by feeding the animals with the cocoa-rich diet (Figures 2A and 2C). In addition, phosphorylated levels of PKCζ were elevated in ZDF-C rats, whereas ZDF-Co maintained similar values to those of their lean littermates (Figures 2A and 2D). There was no difference in total levels of AKT, AMPK and PKC among all groups. Altogether suggests that the cocoa-rich diet modulates main proteins related to insulin response and lipid metabolism, which could contribute to attenuate the alterations of the lipid metabolism in the liver of ZDF animals.

3.3. Cocoa-rich diet regulates key proteins of lipid metabolism in the liver of ZDF rats

To continue with the study of the hepatic lipid metabolism, crucial proteins involved in hepatic lipid synthesis and degradation, such as SREBP1 and its precursor (pre-SREBP1), FAS and PPARα, were evaluated by Western blot in all animal groups. Levels of hepatic SREBP1-c and its precursor were significantly elevated in ZDF-C group when compared with ZL rats (Figures 3A-3C), and this effect was totally repressed in animals receiving the cocoa rich-diet. Indeed, ZDF-Co rats showed comparable (pre-SREBP1) or even lower (SREBP1-c) levels to those of ZL group (Figures 3A and 3C). In this line, ZDF-C rats presented increased FAS levels that were diminished by feeding the animals with cocoa to achieve intermediate values to those of ZL and ZDF-C groups (Figures 3A and 3D). Additionally, levels of PPARα were decreased in ZDF-C rats, whereas ZDF-Co animals maintained similar values to those of ZL group (Figures 3A and 3E). All these results suggest that cocoa-rich diet might contribute to alleviate the impaired hepatic lipid metabolism by regulating proteins related to lipolysis and lipogenesis in ZDF rats.

3.4. EC modulates phosphorylated levels of PKCζ in high glucose-exposed hepatic cells

In order to confirm that the positive effects of the cocoa-rich diet on lipids could be related to its polyphenol content and to gain further insight into the mechanism involved, the beneficial activity of EC, the most abundant flavanol in the cocoa used (Martin et al., 2008) in the *in vivo* experiment, was next investigated. To this end, the effect of EC (10 µM) on PKCζ, was analyzed in high-glucose challenged HepG2 cells. EC dose was selected based on our previous works, since this realistic concentration alleviated insulin resistance in hepatic HepG2 cells by activating key proteins of the insulin signalling pathway (Cordero-Herrera et al., 2013), attenuating high glucose-induced insulin signalling blockade and oxidative stress (Cordero-Herrera et al., 2014; Cordero-Herrera et al., 2015a), and improving glucose uptake and production (Cordero-Herrera et al., 2013; Cordero-Herrera et al., 2014).

EC treatment did not affect phosphorylated levels of PKCζ, whereas EC pre-treatment prevented the increase of p-PKCζ induced by 30 mM glucose, achieving even lower levels to those of control and EC-treated cells (Figure 4). This result indicates the potential protective effect of the flavanol by preventing PKCζ enhancement. Total levels of PKCζ were not modified under any experimental condition.

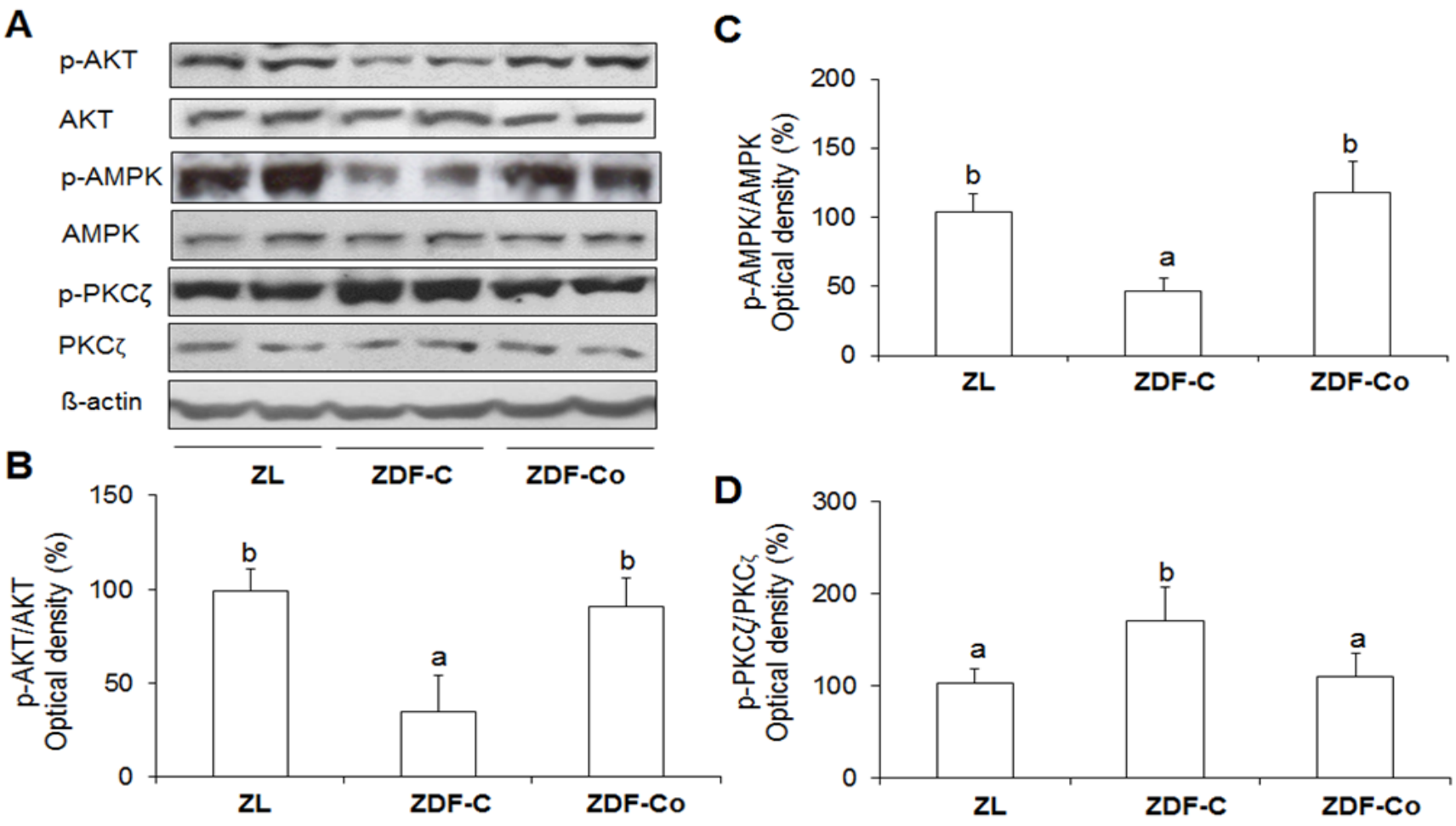

Figure 2. Effect of the cocoa-rich diet on phosphorylated and total levels of AKT, AMPK, and PKCζ in liver of ZL and ZDF rats. (A) Bands of representative blots. Percentage data of (B) p-AKT/AKT, (C) p-AMPK/AMPK and (D) p-PKCζ/PKCζ ratios relative to ZL group. Values are expressed as means ± SD, n=6-8. Equal loading of Western blots was ensured by β-actin. Means without a common letter differ (P<0.05).

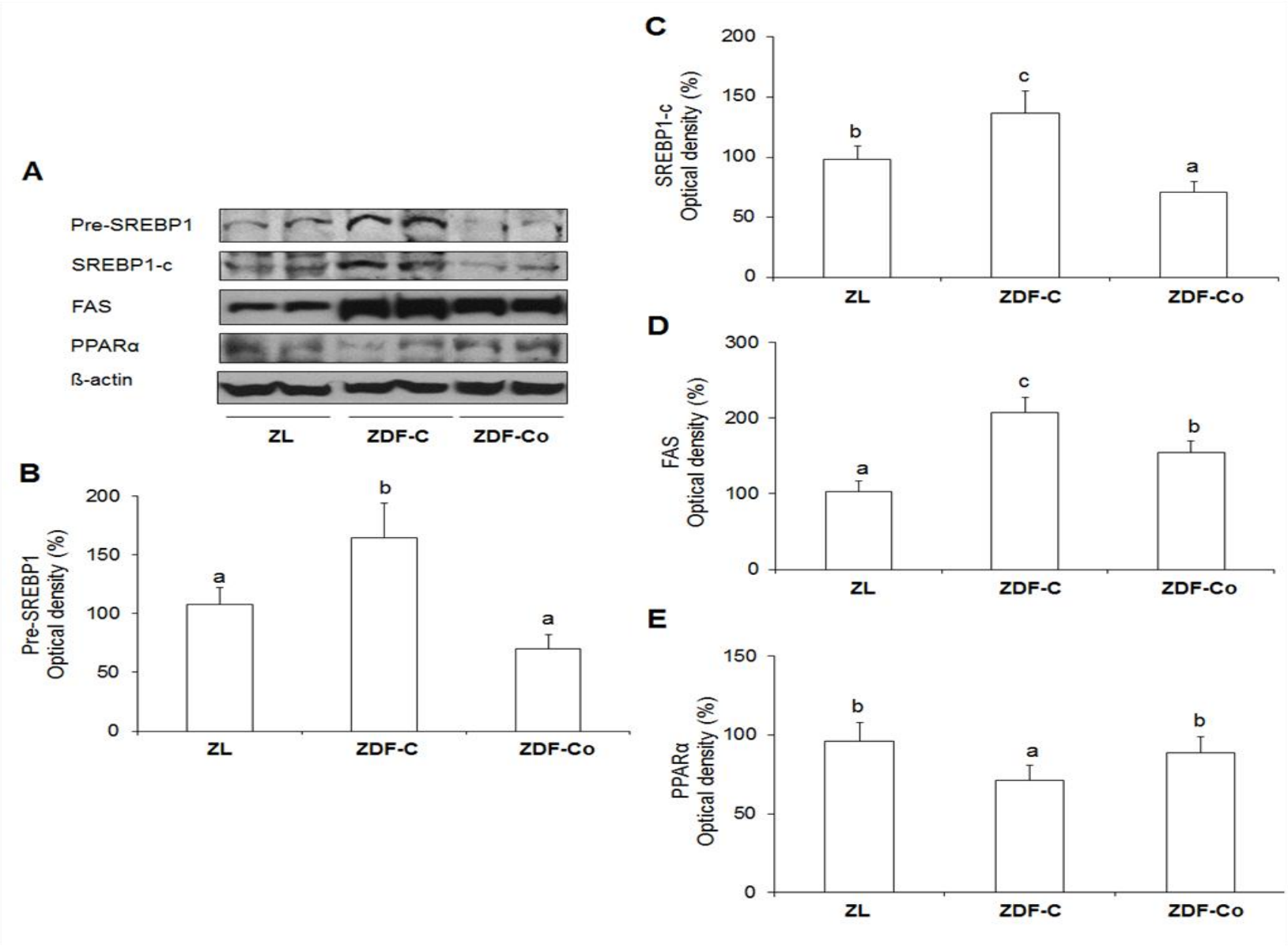

Figure 3. Effect of the cocoa-rich diet on total levels of SREBP1-c and its precursor (pre-SREBP1), FAS and PPARα in liver of ZL and ZDF rats. (A) Bands of representative experiments. Densitometric quantification of (B) pre-SREBP1, (C) SREBP1-c, (D) FAS and (E) PPARα. Values are expressed as a percentage relative to ZL animal group. Equal loading of Western blots was ensured by β-actin (n=6-8). Means without a common letter differ ($P<0.05$).

3.5. EC regulates key proteins of lipid metabolism via PKCζ in high glucose-challenged hepatic cells

To further elucidate the potential beneficial effect of EC and the role of PKCζ on hepatic lipid metabolism, key proteins such as SREBP1 and its precursor, FAS and PPARα were analyzed in high-glucose challenged HepG2 cells, as well as the effect of a PKCζ specific inhibitor, pseudosubstrate (P).

As shown in Figures 5A-5C, EC alone did not modify hepatic SREBP1-c and its precursor levels in comparison to unchallenged HepG2 cells. Pre-treatment with EC reverted the enhanced levels of SREBP1-c and its precursor induced by 30 mM glucose. In addition, the blockage of PKCζ did not affect SREBP1 precursor levels, but decreased SREBP1-c levels in high-glucose treated cells, showing intermediate values to those of untreated and challenged cells. Enhanced levels of SREBP1-c were completely repressed after inhibiting PKCζ in cells that were previously treated with EC, whereas its precursor values were not modified. Similarly, EC pre-treatment prevented the increase of FAS levels induced by 30 mM glucose, achieving levels similar to those of EC-treated cells and slightly lower than those of controls (Figures 5A and 5D). In high glucose-challenged cells, the blockage of PKCζ suppressed the enhanced levels of FAS induced by 30 mM glucose, and displayed values comparable to those of control cells. Additionally, the inhibition of PKCζ in EC-pre-treated cells diminished FAS levels, reaching similar values to those of EC-pre-treated cells and cells incubated with EC alone.

PPARα levels were reduced in high-glucose exposed cells, whereas EC alone increased those values, showing higher levels than those of untreated cells. Besides, EC pre-incubation prevented the high glucose-induced reduction of PPARα values, and displayed comparable levels to unchallenged cells. Inhibition of PKCζ abolished the effect of high glucose on PPARα in both non-pretreated and pre-incubated-EC cells, and exhibited similar values to those of EC-treated cells. All these results point to the relevance of PKCζ for the hepatic lipid metabolism and suggests a possible role for PKCζ repressed by EC in the stimulation of key proteins related to lipid metabolism in high glucose-challenged HepG2 cells.

3.6. EC decreases TG, T-Cho and NEFA levels via PKCζ in high glucose-challenged hepatic cells

In order to confirm the positive response of EC and the role of PKCζ on lipid regulation, levels of TG, T-Cho and NEFA were investigated in high-glucose exposed HepG2 cells, as well as in the presence of a specific inhibitor for PKCζ (pseudosubstrate, P).

In concert with previous results of the present work, increased TG, T-Cho and NEFA levels induced by 30 mM glucose were totally prevented by EC pre-treatment, showing similar levels to those of unchallenged and EC-treated cells (Table 3). Moreover, in high glucose-challenged cells the blockage of PKCζ partly reduced TG, T-Cho and NEFA levels, whereas TG, T-Cho and NEFA values were restored to control levels when cells were previously treated with EC. All these results suggest that EC improves the lipid profile in high-glucose challenged HepG2 cells and that PKCζ plays a role in the diminution of lipid content induced by EC in high glucose-exposed HepG2 cells.

3.7. EC reduces total lipid content in high glucose-exposed hepatic cells via AKT, AMPK and PKCζ

AKT, AMPK and PKCζ are key proteins that mediate insulin effects on lipid metabolism (Ali et al., 2014; Farese & Sajan, 2010). To elucidate the role of these proteins, the effect of EC in the presence of selective inhibitors of AKT (LY294002, LY), AMPK (Compound C, CC) and PKCζ (pseudosubstrate, P) in high-glucose challenged HepG2 cells was analyzed.

As shown in Figure 6 and in agreement with results presented in Table 3, total cellular lipid content increased in high glucose-challenged cells, whereas EC pre-treatment prevented this enhancement, achieving levels similar to those of control and EC-treated cells. In high glucose-challenged cells, the blockage of AKT or AMPK increased the intracellular lipid accumulation, although this effect was partly prevented when cells were previously incubated with EC. Similarly, the inhibition of PKCζ

partly abolished the detrimental effect on lipid content induced by 30 mM glucose, but values just returned to control levels when cells were previously treated with EC.

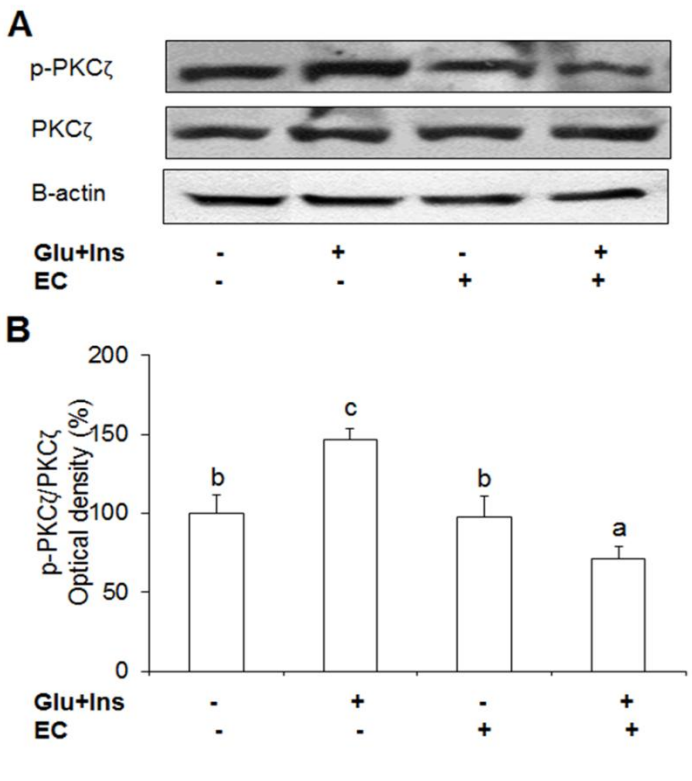

Figure 4. Effect of EC on PKCζ in HepG2. Cells were incubated with 10 µM EC for 24 h prior to 24 h-glucose (Glu) challenge and further exposed to 100 nM (Ins) for 10 min. (A) Bands of representative experiments. (B) Percentage data of p-PKCζ/PKCζ ratio relative to control condition (means±SD, n=6-8). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly (*P*<0.05).

3.8. EC modulates high glucose-induced down-regulation of AKT and AMPK via PKCζ in HepG2 cells

As mentioned, the role of PKCζ on hepatic lipid metabolism seems to be crucial, although still remains largely unknown (Farese & Sajan, 2010). Therefore, the effect of EC in the presence of the PKCζ selective inhibitor (pseudosubstrate, P) on AKT and AMPK in high-glucose challenged HepG2 cells was studied.

Figures 7A-7C showed that 30 mM glucose reduced phosphorylated levels of AKT and AMPK, whereas EC alone increased these values and EC pre-treatment prevented high glucose-induced diminished phosphorylated levels, as previously reported (Cordero-Herrera et al., 2013; Cordero-Herrera et al., 2014). Moreover, inhibition of PKCζ abolished the reduction of AKT and AMPK phosphorylated levels in high glucose-exposed cells, displaying values equal to those of EC-treated- and control-cells, respectively. Similarly, the blockage of PKCζ in EC-pre-treated cells completely prevented the diminished p-AKT and p-AMPK values of the high-glucose challenged cells, and evoked a higher beneficial effect than that of EC alone (Figure 7). All these data point to the relevance of PKCζ on the improved phosphorylated levels of AKT and AMPK induced by EC after a high-glucose challenge in hepatic cells and that EC improved the protection induced by PKCζ inhibition.

Discussion

T2D is a growing health problem and is currently one of the most common chronic diseases worldwide. Rising evidence proposes flavonoids as potential anti-diabetic agents (Ali et al., 2014; Babu et al., 2013), although insufficient investigations *in vivo* and at molecular level to unravel their mechanism of action have been performed to support these observations.

The present study highlights the potential benefits of cocoa, a source of dietary flavanols such as EC, in alleviating hepatic lipid metabolism in ZDF rats and high glucose-exposed HepG2 cells. Cocoa and EC administration improves hepatic lipid levels, alleviates miss-regulation of key protein of the insulin pathway involved in lipid metabolism (AKT, AMPK and PKCζ), and thus ameliorates alterations of crucial proteins involved in fatty acid oxidation (PPARα) and synthesis (FAS and SREBP1-c). In this context, PKCζ plays a main role in regulating AKT and AMPK as well as in hepatic lipid metabolism.

Cocoa is a rich source of flavonoids such as (−)-EC, (+)-catechin, and procyanidins, being EC the most abundant flavanol in the cocoa employed in this work (Martin et al., 2008). Cocoa and EC seem to exert diverse biological activities contributing to prevent alterations related to lipid metabolism as occurs in T2D and other metabolic disorders (Ali et al., 2014; Babu et al., 2013; Vinson et al., 2006). We have recently demonstrated that EC, at the same concentration used in this study, and a cocoa phenolic extract improve insulin sensitivity as well as glucose uptake and production in high glucose-challenged hepatic HepG2 cells (Cordero-Herrera et al., 2014). Similarly, we have shown that the same cocoa-enriched diet used in the present work alleviates hepatic insulin resistance and oxidative stress in ZDF rats (Cordero-Herrera, Martín, Goya & Ramos, 2015b). It is worth mentioning that EC concentrations used in the study are not far from realistic and they are within the range recommended for *in vitro* studies (Kroon et al., 2004). Thus, serum levels of 6 µM EC have been detected in humans after ingestion of 26 g cocoa (Holt et al., 2002).

ZDF rats presented an increased food intake and body weight, which corroborates the hyperphagia of ZDF animals (Leonard et al., 2005). However, cocoa-rich diet decreased body weight gain without influencing total food intake in comparison to ZDF-C group, which indicates a potential beneficial effect for cocoa on obesity related diseases (Ali et al., 2014). Indeed, it has been shown that cocoa phenolics modulate lipid metabolism, prevent fat deposition and activate energy expenditure (Ali et al., 2014), although the detailed molecular mechanism remains elusive.

The pre-diabetic stage in ZDF animals is characterized by insulin resistance (Etgen & Oldham, 2000) together with elevated levels of serum lipids (TG, NEFA, T-Cho, HDL-Cho and LDL-Cho) (Leonard et al., 2005; Sparks et al., 1998). Accordingly, circulating TG, NEFA, T-Cho, HDL-Cho and LDL-Cho were higher in both ZDF groups when compared to ZL rats. However, cocoa supplementation reduced serum TG, NEFA and LDL-Cho values. Indeed, ZDF-Co rats presented reduced hepatic lipid droplets and levels of TG, NEFA and T-Cho as compared to ZDF-C group. Similarly, EC restored high TG, NEFA and T-Cho levels in high glucose-challenged HepG2 cells to control values. In line with these results, different cocoa extracts and EC reduced serum

Table 3. Effect of treatment with EC (10 µM), pre-treatment with EC plus pseudosubstrate (EC+P) on percentage modifications of triglycerides (TG), total cholesterol (T-Cho) and non-esterified fatty acids (NEFA) induced by high glucose-exposure (Glu+Ins). Percentages were related to untreated HepG2 cells.*

	TG (%)	T-Cho (%)	NEFA (%)
C	100.00 ± 3.87^{a}	100.00 ± 5.76^{a}	100.00 ± 4.16^{a}
C+Glu+Ins	139.63 ± 10.81^{c}	159.93 ± 8.99^{c}	199.83 ± 14.34^{c}
EC	100.26 ± 10.31^{a}	105.96 ± 9.80^{a}	84.04 ± 10.38^{a}
EC+Glu+Ins	88.08 ± 11.04^{a}	105.70 ± 4.38^{a}	102.88 ± 11.57^{a}
(C+P)+Glu+Ins	114.64 ± 5.13^{b}	125.80 ± 8.62^{b}	129.12 ± 7.66^{b}
(EC+P)+Glu+Ins	110.80 ± 6.52^{ab}	112.74 ± 6.87^{ab}	84.16 ± 8.24^{a}

* HepG2 cells incubated with 10 µM EC for 24 h were later treated with 30 µM P for 1 h prior to 24 h-glucose (Gluc) challenge and further exposed to 100 nM (Ins) for 10 min. Data represent the means ± SD. Means in a row without a common letter differ, $P< 0.05$.

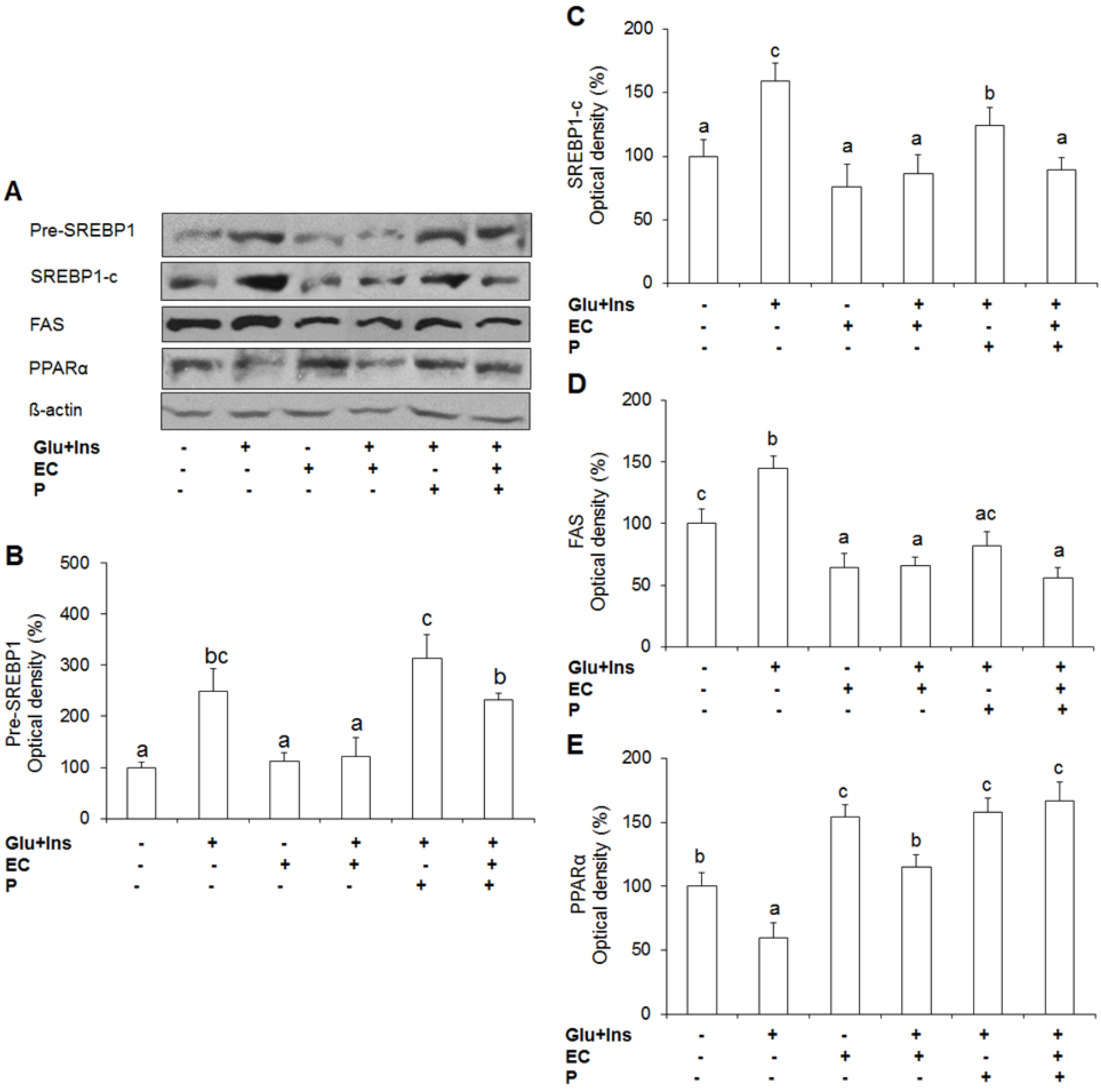

Figure 5. Effect of EC and the selective inhibitor of PKCζ (pseudosubstrate, P) on levels of SREBP1-c and its precursor (pre-SREBP1), FAS and PPARα. HepG2 cells incubated with 10 µM EC for 24 h were later treated with 30 µM P for 1 h prior to 24 h-glucose (Glu) challenge and further exposure to 100 nM (Ins) for 10 min. (A) Bands of representative experiments. Densitometric quantification of (B) pre-SREBP1, (C) SREBP1-c, (D) FAS and (E) PPARα. Values are expressed as a percentage relative to control cells. Equal loading of Western blots was ensured by β-actin (n=7-9). Means without a common letter differ significantly (*P*<0.05)

and hepatic lipid levels in diabetic rodents (Jalil, Ismail, Chong, Hamidc & Syed Kamaruddina, 2009; Jalil, Ismail, Pei, Hamid & Kamaruddin, 2008; Ruzaidi et al., 2005; Si et al., 2011), and grapes procyanidins diminished lipid values in HepG2 cells (Guerrero et al., 2013). Moreover, it has also been reported that resveratrol and extracts rich in phenolic compounds from fruits and plants reversed the impaired lipid profile in the liver of diabetic rodents (Im et al., 2014; Kopec, Piatkowska, Leszczynska & Koronowicz, 2013; Kamaruddin, 2008; Ruzaidi et al., 2005; Si et al., 2011), and grapes procyanidins diminished lipid values in HepG2 cells (Guerrero et al., 2013). Moreover, it has also been reported that resveratrol and extracts rich in phenolic compounds from fruits and plants reversed the impaired lipid profile in the liver of diabetic rodents (Im et al., 2014; Kopec, Piatkowska, Leszczynska & Koronowicz, 2013; Mokiran et al., 2014), as well as in lipid-overloaded hepatic cells (Wu et al., 2013). Interestingly, ZDF-Co group showed the highest HDL-Cho levels, which might be indicative of some potential protective effects against hypercholesterolemia risks in T2D (Ramos et al., 2008; Ruzaidi et al., 2005). All together indicates that cocoa and EC could alleviate the altered lipid levels in T2D.

Increased circulating lipids play a critical role in exacerbating insulin resistance and therefore in the pathogenesis of T2D (Farese & Sajan, 2010; Guillou et al., 2008; Klover & Mooney, 2004). In fact, hepatic insulin resistance can simultaneously result in an excessive output of glucose, and in a reduced lipid oxidation and augmented lipid accumulation, which could lead to hepatic steatosis (Farese & Sajan, 2010; Klover & Mooney, 2004). Although the underlying pathogenesis of hepatic steatosis remains undefined, it is clearly associated with states of hepatic insulin resistance, playing AKT, AMPK and PKCζ a main role (Farese & Sajan, 2010; Klover & Mooney, 2004). Indeed, AKT and AMPK phosphorylation is decreased in hepatic insulin resistance and steatosis in T2D (Klover & Mooney, 2004; Pu et al., 2012 ; Viollet et al., 2006; Zhang et al., 2013), whereas p-PKCζ levels are enhanced (Farese & Sajan, 2010), as we have shown in the present study. However, the cocoa-rich diet and EC treatment prevented all these critical aspects for the development of insulin resistance and hepatic steatosis in ZDF rats and HepG2 cells. In agreement with this, we have recently reported that a cocoa phenolic extract and EC attenuated insulin resistance by enhancing both AKT and

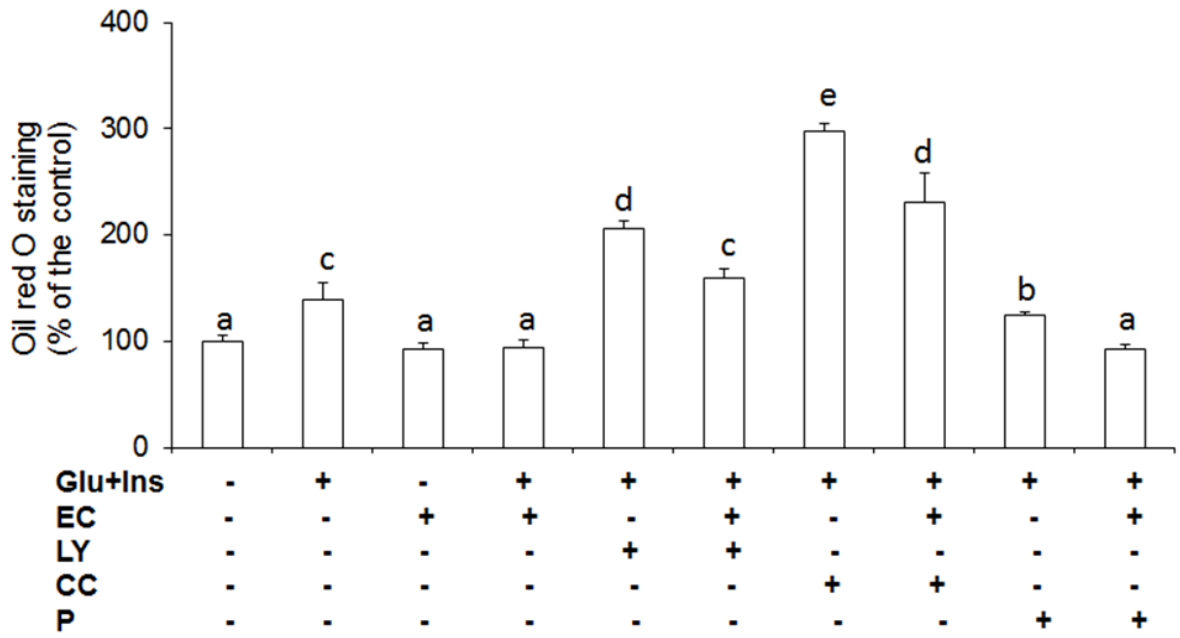

Figure 6. Effect of EC and the selective inhibitor of AKT (LY294002, LY), AMPK (compound C, CC) and PKCζ (pseudosubstrate, P) on intracellular lipid accumulation. HepG2 cells incubated with 10 µM EC for 24h were later treated with 25 µM LY, 40 µM CC or 30 µM P for 1h prior to 24h-glucose (Glu) challenge and further exposure to 100 nM (Ins) for 10 min. Values are expressed percentage relative to control cells as means ± SD, n=6-8. Means without a common letter differ significantly (*P*<0.05).

phosphorylation in high-glucose-exposed HepG2 cells (Cordero-Herrera et al., 2014). Similarly, improved hepatic insulin sensitivity and lipid metabolism through the enhancement of AMPK phosphorylated levels has shown by EC in the liver of obese diabetic mice (Si et al., 2011) and by resveratrol in high glucose-challenged HepG2 cells and high-fat fed rats (Shang et al.,2008), respectively. Additionally, stimulation of AMPK and inhibition of PKC activation by epigallocatechin gallate alleviated insulin resistance and suppressed lipid accumulation in insulin-resistant myoblast cells (Deng, Chang, Lee & Lin, 2012).

Hepatic lipid accumulation and increased production of TG in T2D are associated with alterations in lipogenesis and lipid oxidation (Farese & Sajan, 2010; Viollet et al., 2006). It has been demonstrated that SREBPs regulate enzymes of TG and fatty acid synthesis, such as FAS, and PPARα overexpression in the liver leads to fat accumulation, as lipid oxidation results inhibited (Guillou et al., 2008). In the current study, cocoa and EC ameliorated hepatic lipid accumulation, as determined by diminished number of lipid droplets in ZDF rats and decreased hepatic NEFA, TG and Cho contents in ZDF animals and high glucose-challenged cells. These results were accompanied by decreased levels of lipogenic proteins, including SREBP1-c and FAS, as well as enhanced hepatic fatty acid oxidation, namely PPARα, in both experimental models. In this line, improved lipid metabolism as reduced TG, T-Cho and fatty acid levels through decreased FAS and SREBP1-c and increased PPARα have been observed in rats fed with high-fat-fructose diet with FAS, as well as enhanced hepatic fatty acid oxidation, namely PPARα, in both experimental models. In this line, improved lipid metabolism as reduced TG, T-Cho and fatty acid levels through decreased FAS and SREBP1-c and increased PPARα have been observed in rats fed with high-fat-fructose diet with proanthocyanins from grape seed (Yogalakshmi, Sreeja, Geetha, Radika & Anuradha, 2013) and in fatty-acid-treated HepG2 cells submitted to polyphenolic extracts from *Sechium edule* shoots (Wu et al., 2014) and mulberry leaf (Wu et al., 2013). Also in agreement with the present results, pomegranate flower extract rich in polyphenols reduced hepatic TG and droplets through the restoration of PPARα values in ZDF rats and HepG2 cells (Xu, Zhu, Kim, Yamahara & Li, 2009).

AMPK and AKT are known to down-regulate SREBP1-c

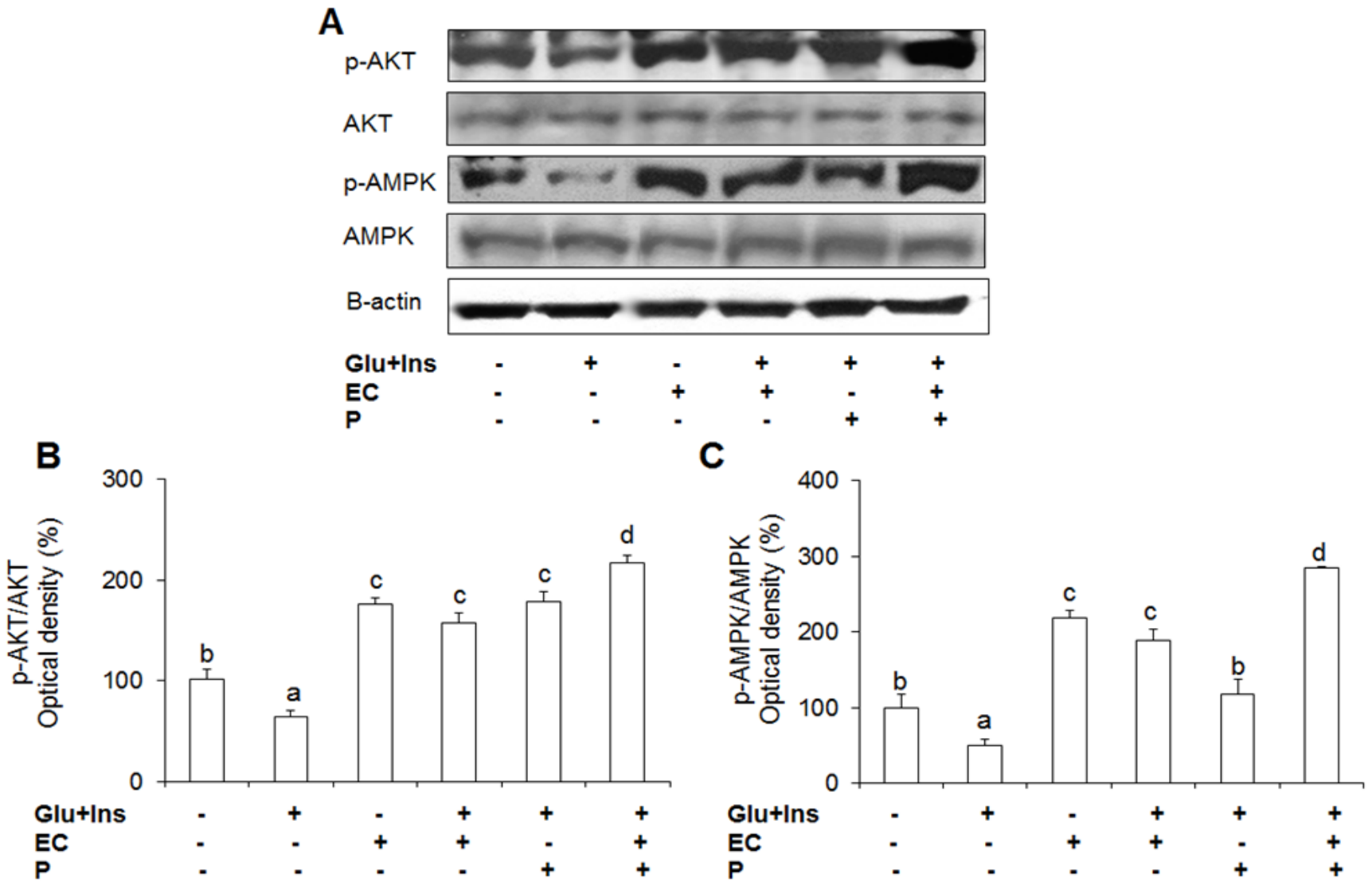

Figure 7. Effect of EC and the selective inhibitor of PKCζ (pseudosubstrate, P) on levels of AMPK and AKT. HepG2 cells incubated with 10 µM EC for 24 h were later treated with 30 µM P for 1 h prior to 24 h-glucose (Glu) challenge and further exposure to 100 nM (Ins) for 10 min. (A) Bands of representative experiments. Percentage values of (B) p-AKT/AKT and (C) p-AMPK/AMPK ratios relative to the control condition (means±SD, n=6-9). Equal loading of Western blots was ensured by β-actin. Means without a common letter differ significantly (*P*<0.05).

(Guillou et al., 2008; Viollet et al., 2006), which is a factor regulating *de novo* lipogenesis in the liver and is predominantly responsible for the regulation of genes involved in fatty acid synthesis, such as FAS (Guillou et al., 2008). Thus, in T2D phosphorylated values of AMPK and AKT are attenuated, leading to increased SREBP-1c levels and its target genes (i.e. FAS) (Guillou et al., 2008; Viollet et al., 2006), and suppressed PPARα (Guillou et al., 2008). Indeed, the blockage of AKT and AMPK in HepG2 cells exposed to a high dose of glucose worsen the situation by enhancing cellular total lipid content and NEFA values (Figure 6), although these effects were prevented by EC treatment in HepG2 cells. Therefore, it could be suggested that hepatic lipid accumulation could be attenuated by EC and cocoa through decreasing *de novo* lipogenesis (down-regulation of SREBP1-c and FAS) and increasing fatty acid oxidation (up-regulation of PPAR-α), playing AKT and AMPK a main role in these processes. In full agreement, in mice fed with a high-fat diet the flavonoid baicalein induced lipid-lowering effects as activated hepatic AMPK, which repressed lipogenesis by decreasing SREBP1-c and FAS and up-regulating PPARα (Pu et al., 2012). Similarly, anthocyanins derived from purple sweet potato color attenuated insulin resistance and hepatic lipid deposition in high-fat-diet-treated mice through the activation of AKT and AMPK pathways (Zhang et al., 2013).

Hepatic aPKC, which mediates insulin effects on lipid synthesis, is activated in T2D and increases gluconeogenesis, lipogenesis and inflammation, which trigger insulin resistance, obesity, hepatosteatosis and hyperlipidemia (Farese & Sajan, 2010). Accordingly, in EC pre-treated cells and later exposed to high glucose, the blockage of PKCζ ameliorated hepatic steatosis, as diminished lipid and NEFA contents, which is in concert with decreased levels of SREBP1-c and FAS, and enhanced PPARα values. Furthermore, inhibition of PKCζ in high glucose-exposed cells increased the phosphorylated levels of AKT and AMPK. Interestingly, these protective effects were more remarkable when cells were previously treated with EC. Therefore, EC-induced regulation of PKCζ contributes to improve the hepatic lipid metabolism through AKT and AMPK. These findings are supported by a previous study which demonstrated that inhibition of PKCζ diminished SREBP1-c and FAS expressions, and this was accompanied by an improvement in hepatic TG, Cho and NEFA levels and insulin signaling via AKT (Sajan et al., 2009). Similarly, a relation between AMPK and hepatic aPKC has been suggested, as the anti-diabetic agent metformin, which activates AMPK, modulates hepatic aPKC activity in human hepatocytes (Sajan, Ivey III & Farese, 2013). Therefore, despite further studies are needed to elucidate the mechanism of action for PKCζ on AKT and AMPK regulations, it could be suggested that cocoa and EC reduce phosphorylated levels of PKCζ to control values in both ZDF rats and high glucose-challenged cells, and contribute to decrease lipid accumulation through diminished lipogenesis, increased lipid oxidation and improved phosphorylation of AKT and AMPK.

In conclusion, cocoa and its main flavanol epicatechin can alleviate hyperlipidemia and hepatic steatosis through inhibition of *de novo* key lipogenic proteins (SREBP1-c and FAS) and the activation of fatty acid oxidation (PPARα). Likewise, cocoa- and EC-induced changes in hepatic lipid content were regulated by AMPK, AKT and PKCζ. Indeed, EC-induced AKT and AMPK regulation seemed to be mediated by PKCζ. This study provides novel mechanistic insight into the anti-diabetic effects of cocoa, which may be helpful in preventing or delaying the development and/or progression of T2D or metabolic diseases and its complications such as hepatic steatosis.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Discusión integradora

1. Efecto de los flavanoles del cacao sobre la señalización de la insulina y el metabolismo glucídico hepático en condiciones no patológicas y durante la resistencia a la insulina y la diabetes.

Diversos estudios han mostrado que el consumo de polifenoles tiene efectos beneficiosos para la salud, pudiendo ejercer un efecto protector frente a la diabetes, enfermedad cardiovascular y cáncer, entre otras enfermedades [2, 24]. En concreto, en los últimos años se ha despertado el interés por el cacao debido a su elevado consumo en Europa y EEUU [78] y por su alto contenido en flavanoles [102]. Diversos estudios han puesto de manifiesto los efectos beneficiosos de los flavanoles del cacao, ya que son buenos antioxidantes y son capaces de neutralizar directamente los radicales libres [79, 86], aumentar la capacidad antioxidante plasmática en animales y humanos [78, 103, 104], activar las defensas antioxidantes celulares [90], y disminuir el estrés para proteger frente al daño oxidativo al hígado [99, 107] y el colon [100, 122]. Además, los flavanoles del cacao modulan diferentes vías de señales celulares, lo que se puede llegar a traducir en una reducción del riesgo de padecer enfermedad cardiovascular [79, 123], o una disminución de la inflamación [94]. En este sentido, distintos estudios llevados a cabo en células, animales y humanos han puesto de manifiesto el efecto antidiabético del cacao y sus flavanoles [24, 25]; sin embargo, no se conocen bien los mecanismos moleculares que están implicados en dicho efecto beneficioso, y son muy escasos los estudios que han investigado las propiedades beneficiosas de los flavanoles del cacao en el hígado. Por tanto, en este trabajo se planteó el estudio de los mecanismos de acción molecular implicados en el efecto antidiabético de los flavanoles del cacao sobre la señalización de la insulina, el metabolismo glucídico y lipídico y el estado redox hepáticos. Para ello se utilizaron un modelo *in vitro*, las células HepG2, y un modelo animal, las ratas ZDF, en los que está presente la resistencia a la insulina hepática que aparece en la diabetes. Además, para llevar a cabo estos estudios se han empleado concentraciones de EC y CPE realistas y alcanzables a través de la dieta; en este sentido, cabe mencionar que se han detectado niveles de 6 µM de EC en sangre en humanos tras el consumo de 26 g de cacao [124].

En primer lugar se analizó el posible efecto de los flavanoles de cacao en condiciones fisiológicas sobre la señalización de la insulina en las células hepáticas HepG2. La incubación de las células con EC (1, 5, 10 µM) y CPE (1, 5, 10 µg/mL)

reforzó la ruta de señalización de la insulina desde los primeros eslabones de la vía. EC y CPE incrementaron los niveles totales y fosforilados en tirosina de IR e IRS-1 y-2, lo que resultó en el reclutamiento y activación de las proteínas que están por debajo en la ruta de señalización de la hormona [125]. En consonancia con estos resultados, se ha descrito que los polifenoles del té verde aumentan los niveles de ácido ribonucleico mensajero (ARNm) de IRS-2 en el hígado [126], y que el EGCG del té verde y las prociandinas oligoméricas de las semillas de uva incrementan los niveles fosforilados en tirosina y totales de IR y los totales de IRS [127, 128] Además, se ha descrito que los polifenoles del cacao pueden modular la actividad quinasa de IR a través de su unión al receptor [129].

La activación de IRS-1/-2 estimula la ruta PI3K/AKT, necesaria para poner en marcha las acciones metabólicas de la insulina en el hígado, y es responsable de la inhibición de GSK3 y la activación de GS [40]. En este sentido, ambas sustancias EC y CPE activaron la vía PI3K/AKT al aumentar los niveles fosforilados de AKT y GSK3 y disminuir los de GS. Estos resultados están de acuerdo con otros estudios previos en los que se ha mostrado que EC y CPE son capaces de estimular a AKT en las células HepG2 [110, 130]. De manera similar, la naringenina activó la ruta de AKT en las células HepG2 [131] y los polifenoles del té verde incrementaron los niveles de ARN de PI3K/AKT y GSK3 en el hígado de ratas insulino-resistentes [126].

La AMPK es un importante sensor energético intracelular y se considera una importante diana terapéutica para la diabetes [132]. De hecho, la metformina, el fármaco hipoglucemiante más empleado en el tratamiento de la diabetes tipo 2, activa a la AMPK [71]. EC y CPE aumentaron los niveles fosforilados de la AMPK, al igual que EGCG, las teflavinas del té negro y el ginsenósido Rg2 del ginseng en hepatocitos primarios y células HepG2 [133-135].

PEPCK es una de las enzimas principales implicadas en la regulación de la gluconeogénesis [40]. EC y CPE parecen capaces de modular el metabolismo glucídico hepático, ya que disminuyeron los niveles de PEPCK y redujeron la producción de glucosa. En este sentido, otros compuestos fenólicos como EGCG, naringina y las antocianinas disminuyeron los niveles de PEPCK y la producción de glucosa en células hepáticas y en el hígado de ratones resistentes a la insulina [133, 136-138].

AKT y AMPK suprimen la gluconeogénesis en el hígado [40, 71, 125, 132]. En este estudio se ha demostrado que AKT y AMPK están implicadas en la modulación de PEPCK y en la producción de glucosa en las células HepG2 tratadas con EC y CPE; así, al inhibir AKT o AMPK se pierde parcialmente el efecto inhibitorio de EC y CPE sobre la gluconeogénesis, lo que sugiere que el extracto de cacao y la EC podrían mejorar la hiperglucemia al modular los niveles de PEPCK y la producción de glucosa a través de AKT y AMPK. De acuerdo con esto, algunos compuestos naturales como EGCG, narigina y naringenina son capaces de modular la gluconeogénesis mediante la activación de AKT [139] o AMPK [133, 136].

El trasportador de glucosa GLUT2 es el responsable del flujo de glucosa a través de la membrana plasmática en los hepatocitos [40]. En este estudio, la concentración más alta de CPE usada (10 µg/mL) incrementó los niveles de GLUT2, mientras que EC no alteró los niveles de este transportador. Aunque la influencia de los compuestos fenólicos sobre los GLUTs se ha estudiado poco, especialmente en el hígado, la administración de polifenoles del té verde no modificó los niveles de ARNm de GLUT2 en el hígado de las ratas insulino-resistentes [126], aunque el resveratrol y algunos fármacos antidiabéticos (pioglitazona y rosiglitazona) fueron capaces de aumentar la expresión de este transportador en las células beta pancreáticas [140, 141]. Estos resultados podrían indicar que el cacao contribuye de una manera más eficaz que la EC a la captación de glucosa.

En conjunto, todos estos resultados muestran que EC y CPE poseen una actividad similar a la de la insulina, y son capaces de activar la vía de señalización de la hormona desde los primeros eslabones a concentraciones que no son tóxicas y resultan fácilmente alcanzables con la dieta. Además, estos estudios han puesto de manifiesto un nuevo mecanismo por el cual EC y CPE modulan en las células hepáticas a PEPCK y la producción de glucosa a través de AKT y AMPK.

A la vista de estos resultados, se estudió el posible efecto protector de los polifenoles del cacao sobre las células hepáticas durante una situación de resistencia a la insulina y diabetes. Para llevar a cabo estos trabajos se utilizaron un modelo animal de diabetes tipo 2 ampliamente aceptado, las ratas ZDF, y se puso a punto un modelo celular en el que la resistencia a la insulina se inducía mediante la exposición de las células hepáticas HepG2 a altas dosis de glucosa.

En las ratas ZDF, la hiperglucemia aparece entre las semanas 7 y 12 [142]. De acuerdo con esto, los animales utilizados en este estudio eran ratas jóvenes (6-15 semanas de vida) que se encontraban en un periodo de prediabetes, caracterizado por producirse una resistencia a la insulina e intolerancia a la glucosa, pero sin presentar una hiperglucemia muy severa [142], como muestran los valores de glucemia e insulinemia de las ratas ZDF alimentadas con la dieta control (ZDF-C) frente a los animales control (ZL). La administración de la dieta rica en cacao (grupo ZDF-Co) mejoró la tolerancia a la glucosa, lo que se tradujo en una reducción de la hiperglucemia a corto y largo plazo. Este efecto a corto plazo en roedores parece estar relacionado con la supresión de la absorción intestinal de la glucosa por la inhibición de enzimas digestivas por parte de los flavanoles del cacao [115], así como con un aumento de la secreción postprandial de insulina [115, 127]. Por su parte, el efecto a largo plazo se ha encontrado también en ratas insulino-resistentes alimentadas con extractos de procianidinas de semillas de uva [127]. De acuerdo con esta mejora de la tolerancia a la glucosa, la dieta rica en cacao provocó un descenso de los niveles de insulina plasmática a corto y largo plazo, lo que pone de manifiesto la mejora de la sensibilidad a la hormona, como evidencia el descenso en el índice HOMA-IR. Además, la administración de cacao redujo el peso corporal sin variar la ingesta de comida, lo que también contribuyó a mejorar la sensibilidad de la insulina, al igual que ocurre en ratones alimentados con una dieta rica en grasa y suplementada con procianidinas [115]. Este último aspecto será discutido en el apartado 3 de esta sección.

El aumento de la relación peso del hígado/peso corporal se utiliza para investigar posibles cambios en el peso del hígado que puede estar asociado con efectos adversos para la salud, como la aparición de esteatosis y/o el incremento de los niveles de glucógeno hepático [142]. Las ratas ZDF mostraron valores mayores en la relación mencionada que los animales ZL, sin embargo no se observaron diferencias entre los grupos ZDF-C y ZDF-Co, por lo que la dieta rica en cacao no parece ejercer ningún efecto sobre este parámetro, al igual que se ha observado en otros estudios realizados con ratas ZDF que recibían dieta ricas en diferentes compuestos naturales [143].

Durante la resistencia a la insulina, se produce un fallo en la estimulación del IR y en la fosforilación en tirosina de los IRS, y se favorece la fosforilación en residuos de serina de IRS-1 [40]; estos cambios del IR e IRS conducen a una menor asociación entre los IRS y PI3K [40, 144], y como consecuencia, disminuyen los niveles

fosforilados de AKT y de GSK3 y aumentan los niveles fosforilados de GS [40]. Estas alteraciones características de la resistencia a la insulina se observaron en el hígado de las ratas ZDF-C, así como en las células HepG2 incubadas con altas concentraciones de glucosa. No obstante, el pretratamiento con EC y CPE en las células, y la administración de la dieta rica en cacao a las ratas ZDF, impidieron el bloqueo de la ruta de señalización de insulina. Así, en las células HepG2, EC y CPE previnieron el descenso de los niveles totales y fosforilados en tirosina de IR, IRS-1 y -2, así como el aumento de los valores fosforilados en Ser(636/639) de IRS-1. Del mismo modo, la dieta rica en cacao impidió la fosforilación en Ser(307) y Ser(636/639) de IRS-1 en las ratas ZDF. Además, EC, CPE y la dieta rica en cacao restauraron los niveles fosforilados de AKT, GSK3 y GS a valores similares a los de los control, o incluso, mejoraron la situación. En conjunto, estos resultados sugieren que los flavanoles del cacao incrementan la sensibilidad a la insulina en una situación de resistencia a la hormona y diabetes. En consonancia con esto, se ha demostrado que en modelos de células en cultivo y animales, otros compuestos fenólicos como EGCG [65, 128, 145], berberina [146], las antocianinas de la patata morada dulce [147], así como los extractos polifenólicos del té [126] y de procianidinas de la semilla de la uva [127] son capaces de reactivar la vía de señalización de la insulina; estos compuestos reducen los niveles de fosforilación en serina de IRS-1, previenen el descenso de la fosforilación en tirosina y/o aumentan los niveles fosforilados de AKT y GSK3 en diferentes modelos de resistencia a la insulina y en la diabetes.

El estado de fosforilación de IR e IRS resulta crucial para el desarrollo de la resistencia a la insulina y la activación de proteínas clave de la vía que se sitúan por debajo de los elementos mencionados [40, 144]. No obstante, se ha de considerar también que la AMPK juega un papel muy relevante, y su fosforilación está inhibida en el hígado en situaciones de resistencia a la insulina [132]. En este sentido, EC y CPE mostraron un efecto protector sobre la AMPK, dado que impidieron su inactivación por las altas concentraciones de glucosa. Estos resultados están de acuerdo con otros estudios en los que EC y un extracto de procianidinas del licor de cacao restauraron los valores de p-AMPK en el hígado de ratas diabéticas y adipocitos de ratones insulino-resistentes [148, 149]. Además, otros compuestos fenólicos como EGCG y el resveratrol activaron a la AMPK durante una situación de resistencia a la insulina en las células HepG2 [65, 150].

En la diabetes, también se altera el metabolismo glucídico en el hígado: se reducen la captación de glucosa a través de GLUT2, la glicolisis y la síntesis de glucógeno, y permanece activa la gluconeogénesis [40, 144]. En este contexto, las proteínas PEPCK y GK juegan un papel crucial, ya que son enzimas limitantes de la gluconeogénesis y de la glucolisis y de la captación de glucosa, respectivamente [40, 151]. Durante la resistencia a la insulina y la diabetes PEPCK se activa, lo que estimula la producción de glucosa hepática y contribuye a agravar la hiperglucemia [40]. GK se estimula por la insulina, por lo que al contrario que PEPKC, los niveles de GK disminuyen a medida que se agrava la resistencia a la insulina en las ratas ZDF y en las células HepG2 [151]. EC y CPE fueron capaces de proteger a las células HepG2 durante una situación de resistencia a la insulina, previnieron la activación de PEPCK y, consecuentemente, redujeron la producción de glucosa. De manera similar, la dieta rica en cacao redujo los niveles de PEPCK en el hígado de las ratas ZDF a valores similares a los de los animales ZL y aumentó los niveles de GK, lo que se podría relacionar con un incremento de la utilización de glucosa sanguínea para producir energía [152]. De acuerdo con nuestros resultados, se ha mostrado que otros compuestos fenólicos también son capaces de modular a PEPCK y/o GK en células hepáticas en cultivo y en el hígado de roedores mediante la estimulación de la glicolisis y la síntesis de glucógeno [133, 136, 151]. Durante la resistencia a la insulina se produce un descenso en los niveles de glucógeno en el hígado [40], lo que está de acuerdo con lo observado en las células HepG2 incubadas con una dosis alta de glucosa. Además, el pretratamiento con EC y CPE restauró los niveles de glucógeno a valores similares a los del control, como se ha descrito para otros polifenoles en células hepáticas [65, 146, 153]. Por su parte, en las ratas ZDF-C se encontró un aumento de los niveles de glucógeno hepático en comparación con los animales ZL; este hecho es una característica de este modelo animal, y se ha relacionado con la ausencia de un verdadero estado postprandial en las ratas ZDF que active la glucogenolisis debido a la hiperfagia que padecen [142]. La dieta enriquecida en cacao restauró los niveles de glucógeno a valores similares al grupo ZL.

EC, CPE y la dieta rica en cacao incrementaron los niveles de GLUT2 en las células HepG2 y en las ratas ZDF-Co, respectivamente, lo que puede contribuir a aumentar la sensibilidad a la insulina en el hepatocito y al efecto hipoglucemiante observado en los animales. Además, EC y CPE inhibieron el descenso en la captación

de glucosa en las células HepG2. De acuerdo con estos resultados, se ha descrito que algunos fármacos antidiabéticos como la metformina, telmisartan y sitagliptina aumentan los niveles del GLUT2 en el hígado de ratones resistentes a la insulina [154], y también algunos compuestos polifenólicos como EGCG y un extracto de procianidinas de la semilla de la uva son capaces de estimular la captación de glucosa en las células adiposas y hepáticas resistentes a la insulina [65, 127].

Las proteínas MAPKs están implicadas en la resistencia la insulina, ya que favorecen la fosforilación inhibitoria de IRS-1 en Ser307 y Ser636/639 [65, 155, 156]. En el hígado de las ratas ZDF-C los niveles de p-ERK, p-p38 y p-JNK aumentaron respecto a los de las ratas ZL. Sin embargo, la dieta rica en cacao disminuyó los valores de p-p38 y p-JNK, mientras que los niveles de p-ERK permanecieron incrementados. En este sentido, se ha descrito que el bloqueo de las JNK media la inhibición de la fosforilación en serina del IRS-1 en el hígado de las ratas ZDF y en células hepáticas humanas [65]. Además, la supresión de JNK y p38 en las ratas ZDF se ha asociado con una actividad antidiabética [156]. Todas estas acciones se han relacionado con una mejora de la resistencia a la insulina, y la activación de las ERK por el cacao se ha asociado con un mecanismo protector frente al estrés oxidativo que implica el aumento de las defensas antioxidantes [130].

En resumen, los polifenoles del cacao mejoran la tolerancia a la glucosa e incrementan la sensibilidad a la insulina en los hepatocitos. Estos efectos parecen producirse porque los flavanoles de cacao restauran la señalización de la insulina en las células hepáticas al reactivar al IR, IRS-1, IRS-2, la vía de la AKT y AMPK e inhibir la fosforilación en serina de IRS-1 (Ser307 y Ser636/639). Además, los flavanoles del cacao alivian el daño sobre la funcionalidad hepática, dado que contribuyen a preservar los niveles de GLUT2, la captación de glucosa y a modular a las enzimas gluconeogénicas y glicolíticas. Estos resultados muestran nuevos datos sobre los mecanismos moleculares de acción por los cuales los flavanoles del cacao ejercen su efecto preventivo en la resistencia a la insulina hepática y la DMT2, así como su potencial utilidad para prevenir o retrasar el desarrollo de la DMT2.

2. Efecto de los flavanoles del cacao sobre el estrés oxidativo hepático durante la resistencia a la insulina y la diabetes.

Distintos estudios reflejan que las alteraciones que se producen en la diabetes, como la resistencia a la insulina, están causadas al menos en parte por el estrés oxidativo, y que dicho estrés oxidativo también está implicado en el progreso y en la aparición de las complicaciones de esta enfermedad [47, 64]. No obstante, a pesar de que se han determinado algunos de los mecanismos de protección celular en relación con el estado redox en la diabetes, todavía no se conocen con exactitud cómo los flavanoles del cacao podrían llevar a cabo la activación y mejora de dichos mecanismos. Así pues, dado el efecto beneficioso de los flavanoles del cacao frente a la resistencia a la insulina y las alteraciones del metabolismo glúcido hepático y por su carácter antioxidante, se estudió el posible efecto protector de los flavanoles del cacao sobre el estado redox hepático en una situación de resistencia a la insulina y diabetes.

La hiperglucemia, que constituye un factor de riesgo principal para la disfunción hepática en la diabetes, induce estrés oxidativo [47, 64]. De acuerdo con esto, la producción de ROS y los niveles de un marcador de daño oxidativo a proteínas (grupos carbonilo) se incrementaron en las células HepG2 incubadas con altas concentraciones de glucosa y en el hígado de las ratas ZFD-C. Sin embargo, el pretratamiento con EC o CPE y la administración de la dieta suplementada con cacao previnieron estas alteraciones del estado redox hepático. En este sentido, otros estudios llevados a cabo en células hepáticas en cultivo y animales sanos y diabéticos han mostrado que la EC y los flavanoles del cacao, al igual que otros polifenoles, son capaces de proteger a los hepatocitos frente al estrés oxidativo mediante la atenuación de la generación de ROS y la disminución de la oxidación de las macromoléculas, como las proteínas (grupos carbonilo) [99, 107, 147, 157].

Las defensas antioxidantes enzimáticas y no enzimáticas constituyen los principales mecanismos celulares para mantener el equilibrio redox [52]. Dichas defensas se alteraron en las células hepáticas en cultivo y en el hígado de las ratas ZDF-C, lo que resultó indicativo de una perturbación del estado redox celular provocada por la hiperglucemia, como se ha descrito en otros estudios con animales resistentes a la insulina [158, 159]. La incubación con CPE, pero no con EC, incrementó la actividad de las enzimas GPx y GR, aunque el pretratamiento con ambas sustancias previno el

aumento en la actividad de estas enzimas provocado por la dosis alta de glucosa, al igual que el descenso en los niveles de GSH. Por su parte, en las ratas ZDF-C, la actividad de GPx, GR, CAT y los niveles de GSH no se vieron modificados por ningún tratamiento. Sin embargo, en estos animales, disminuyó la actividad de SOD y se incrementó la de HO-1, aunque la administración de la dieta rica en cacao previno estas alteraciones. Estos resultados están de acuerdo con el efecto protector de EC y los polifenoles del cacao demostrado frente al daño oxidativo causado por un pro-oxidante [107, 108]. Además, el tratamiento con CPE (en ausencia de estrés) aumentó las defensas antioxidantes, lo que parece preparar a las células para afrontar en mejores condiciones una situación de estrés y, por tanto, para minimizar el potencial daño oxidativo [107, 108]. Este incremento en la actividad de algunas enzimas antioxidantes en ambos modelos se podría considerar como un mecanismo adaptativo para contrarrestar el estrés causado por la hiperglucemia [159]. En este sentido, también se ha visto que otros polifenoles de la dieta fueron capaces de restaurar el estado redox en distintos modelos de resistencia a la insulina [158-160]. No obstante, se ha de tener en cuenta que la modulación de las defensas antioxidantes está relacionada con la severidad, la duración y el tratamiento de la diabetes [161]; así pues, el mantenimiento del efecto deletéreo del estrés oxidativo en tiempo y/o intensidad, conduciría al aumento del daño acumulado y, por tanto, al descenso de las defensas antioxidantes del tejido afectado [162].

La actividad de la enzima detoxificante GST se redujo en el hígado de las ratas ZDF-C y en las células HepG2 expuestas a una dosis alta de glucosa, y su actividad permaneció disminuida tras la administración de los distintos tratamientos (EC, CPE y dieta rica en cacao). Este descenso en la actividad de la GST se relaciona con una disminución en la expresión de la enzima causada por una menor estimulación de la insulina durante la resistencia a la hormona en la diabetes [59]. Así, aunque en las células HepG2 pretratadas con EC y CPE, y en las ratas ZDF-Co, se observó una mejora de la situación de resistencia a la insulina, esta no pareció ser suficiente para restaurar los niveles de la actividad GST hepática. Todos estos resultados sugieren que la modulación de las defensas antioxidantes/detoxificantes es compleja y parecen ser la consecuencia de un intento para mantener el equilibrio redox.

El exceso en la generación de ROS además de producir daños oxidativos sobre las macromoléculas (ADN, lípidos y proteínas), también puede modular vías de señalización sensibles a cambios redox como los factores de transcripción Nrf2 y NF-

κB, y las MAPKs [64, 66, 68, 163]. El Nrf2 se activa por el estrés y estimula la expresión de ciertas enzimas antioxidantes [66]. En las células HepG2 resistentes a la insulina y las ratas ZDF-C se encontró una activación del Nrf2 como consecuencia del estrés oxidativo. Este aumento en los niveles del factor de transcripción se ha descrito en pacientes diabéticos, y resulta indicativo de la aparición de una repuesta adaptativa para contrarrestar el estrés oxidativo en esta enfermedad [164]. Además, en las células HepG2, el tratamiento con EC y CPE estimuló al Nrf2, y el pretratamiento con ambas sustancias mantuvo elevados los niveles de este factor de transcripción, sugiriendo que las células tratadas con EC y CPE estarían mejor preparadas para enfrentarse al aumento de ROS provocado por la hiperglucemia. Dicho incremento en los niveles de Nrf2 se podría correlacionar, en el caso del CPE y la dieta rica en cacao, con un aumento de la actividad de GPx y GR, y de la actividad HO-1, respectivamente, mientras que dicha relación no se encontró para EC. Estos resultados están de acuerdo con otros estudios en los que se ha descrito que algunos compuestos naturales ejercen un efecto protector frente al estrés oxidativo mediante el aumento de los niveles de Nrf2 y de las enzimas antioxidantes, aunque dicha actividad enzimática puede verse o no incrementada [165, 166].

El NF-κB juega un papel crucial en el estrés oxidativo y la inflamación [68]. Los niveles de p65-NF-κB aumentaron en las ratas ZDF-C, y los animales que recibieron la dieta rica en cacao recuperaron los valores de las ratas control (ZL). Este resultado se ha relacionado con un efecto protector asociado a la disminución del estrés oxidativo y la inflamación. En este sentido, se ha observado un descenso del estrés en otros modelos de animales diabéticos alimentados con dietas suplementadas con compuestos fenólicos [147, 167]. Además, el cacao ejerce un efecto antiinflamatorio que también podría contribuir a aliviar la intolerancia a la glucosa [115].

La hiperglucemia activa diferentes proteínas mediadoras del estrés como las MAPKs [64], que a su vez están reguladas por el Nrf2 [66]. Así pues, las altas concentraciones de glucosa incrementaron los niveles fosforilados de las ERK, JNK y p38-MAPK en las células HepG2. Sin embargo, el pre-tratamiento con EC y CPE previno el aumento en los niveles de las p-JNK y p-p38, aunque los valores de p-ERK se mantuvieron elevados, como se ha descrito para las ratas ZDF-Co (ver apartado 1 de esta sección). Esta disminución de los niveles de p-JNK y p-p38, y paradójicamente también el aumento de los valores de p-ERK, se ha relacionado con un efecto positivo asociado a la protección y supervivencia en las células HepG2 frente al estrés oxidativo

[98, 130], y con un efecto antidiabético [168]. En este sentido, en este trabajo se ha observado que la regulación de las MAPKs por CPE está implicada en la estimulación del factor de transcripción Nrf2 y de las actividades de GPx y GR, ya que los inhibidores de las quinasas mejoraron la protección ejercida por el extracto de cacao en las células HepG2 expuestas a la alta dosis glucosa. Así pues, se podría sugerir que CPE indujo la actividad de las enzimas GPx y GR a través de un mecanismo que implica la modulación de las MAPKs y la estimulación de Nrf2, lo que parece estar asociado a una disminución del estrés oxidativo. De acuerdo con estos resultados, otros estudios han mostrado que los flavanoles y otros polifenoles son capaces de aumentar la capacidad de defensa antioxidante celular mediante la inducción de enzimas citoprotectoras a través de la activación de vías como las MAPKs y/o PI3K/AKT [110, 130, 169, 170].

La activación crónica de las MAPKs puede inducir resistencia a la insulina al afectar a la modulación de los niveles totales y fosforilados en serina de IRS-1 [40, 171]. En este estudio, la inhibición de las JNK y ERK disminuyó la relación p-IRS-1/IRS-1 y mejoró la captación de glucosa en las células expuestas a la dosis alta de glucosa previamente tratadas con CPE, mientras que p38 parecía jugar un papel redundante con el CPE en este proceso, dado que mantuvo ambos parámetros (relación p-IRS-1/IRS-1 y captación de glucosa) en niveles similares a los mostrados en las células incubadas sólo con CPE. En consonancia con nuestros resultados, se ha descrito que la supresión de las ERK por un extracto de uva atenuó la resistencia a la insulina [155], y el bloqueo de JNK medió la inhibición de la fosforilación en serina de IRS-1 [65], lo que contribuyó a estimular la sensibilidad a la hormona. Por su parte, p38 parece afectar de forma moderada a los niveles de p-(Ser)-IRS-1 [171].

En resumen, los flavanoles del cacao parecen ejercer un efecto antidiabético, ya que mantienen la bioquímica de las células hepáticas frente a un estrés oxidativo inducido por los altos niveles de glucosa, y previenen el desequilibrio redox mediante la modulación de enzimas antioxidantes y señales celulares como Nrf2 y NF-κB. Además, el efecto beneficioso de CPE en el estado redox y la resistencia a la insulina parece estar mediado, al menos en parte, por su habilidad para modular las MAPKs en las células hepáticas en cultivo. Estos estudios muestran el potencial efecto preventivo y/o terapéutico del cacao frente a la DMT2.

3. Efecto de los flavanoles del cacao sobre el metabolismo lipídico hepático durante la resistencia a la insulina y la diabetes.

Durante mucho tiempo el tratamiento de la DMT2 se ha centrado en el control glucémico, sin embargo, las alteraciones del metabolismo lipídico también están implicadas en la desregulación de la señalización a la insulina. En consecuencia, en los últimos años ha aumentado el interés por compuestos que puedan modular el metabolismo glucídico y lipídico durante esta situación de resistencia y diabetes como nueva estrategia de tratamiento.

El cacao y la EC parecen modular el metabolismo lipídico, lo que contribuye a disminuir las alteraciones lipídicas que ocurren durante la diabetes y otros desórdenes metabólicos [27, 78], aunque no se conocen con exactitud los mecanismos moleculares por lo que son capaces de ejercer este efecto. Por tanto, en este estudio se investigó el efecto de una dieta rica en cacao en las ratas ZDF y el efecto de la EC en las células HepG2 resistentes a la insulina sobre el metabolismo lipídico hepático.

Los grupos de ratas ZDF (ZDF-C y ZDF-Co) mostraron un peso corporal y una ingesta de comida mayor que los de los animales ZL, lo que corrobora el estado de hiperfagia de estos animales ZDF [142]. Sin embargo, la dieta enriquecida en cacao disminuyó el aumento del peso corporal sin modificar la ingesta en comparación con las ratas ZDF-C, lo que podría ser indicativo de un potencial efecto beneficioso del cacao frente la obesidad y enfermedades relacionadas. Estos resultados están en consonancia con estudios que muestran un efecto antilipogénico del cacao, ya que disminuye el acúmulo de grasa y aumenta el gasto de energía [27]. De hecho, los polifenoles del cacao activan a la AMPK, lo que conduce a la estimulación de otros eventos que inhiben la producción de glucosa y el depósito de grasa, y ayudan en la prevención de la hiperglucemia, hiperinsulinemia y la resistencia a la insulina [27]. De manera adicional, y como se discutirá más adelante, los flavanoles del cacao también modulan la síntesis y degradación de lípidos y promueven la termogénesis, lo que también puede contribuir a reducir el depósito de grasa intra-abdominal y a mejorar la hiperlipidemia y la esteatosis hepática [27].

Durante la resistencia a la insulina se produce un aumento de los niveles lipídicos séricos y la acumulación de grasa ectópica [63, 142]. De acuerdo con esto, las

ratas ZDF mostraron unos niveles de TG, NEFA, T-Cho, HDL-Cho y LDL-Cho séricos más elevados que los de las ratas ZL, pero la suplementación de la dieta con cacao mejoró el perfil lipídico de estos animales, ya que redujo los niveles de TG, NEFA, T-Cho y LDL-Cho séricos, aunque no se llegaron a alcanzar los valores control. Además, las ratas ZFD-Co mostraron una reducción en la acumulación de grasa, ya que en el hígado de estos animales disminuyeron los depósitos de lípidos, en concreto, descendieron los niveles de TG, NEFA y T-Cho. De manera similar, en las células HepG2 resistentes a la insulina, la EC restauró los niveles de TG, NEFA y T-Cho a valores similares a los de los controles. En este sentido, estudios realizados con distintos extractos de cacao y EC han mostrado una reducción de los lípidos hepáticos y séricos en roedores diabéticos [105, 113, 148], y las procianidinas de la uva redujeron los niveles lipídicos en las células HepG2 [119]. Además, los animales ZDF-Co mostraron el valor más alto de HDL-Cho de los tres grupos, lo que podría sugerir un potencial efecto beneficioso frente a los riesgos de la hipercolesterolemia de la DMT2 [172].

La excesiva producción de glucosa junto con la reducción de la lipolisis y el aumento de la acumulación lipídica, presentes en la resistencia a la insulina y la DMT2, pueden llegar a producir esteatosis hepática [40, 44]. Aunque no se conoce bien la patogénesis de la esteatosis, parece que la AKT, AMPK y PKCζ tendrían un papel relevante, ya que en esta situación se produce un descenso en los niveles fosforilados de AKT y AMPK [40, 147, 173, 174], y un aumento de los niveles de p-PKCζ [44]. Así, en consonancia con esto, en las ratas ZDF-C y en las células HepG2 insulino-resistentes se observó un descenso en los niveles de p-AKT y p-AMPK, y un aumento en los de p-PKCζ. Sin embargo, la administración de la dieta rica en cacao y el pretratamiento con EC previnieron la alteración de los niveles fosforilados de las tres proteínas. De manera similar, distintos estudios han mostrado una mejora de la resistencia a la insulina y el metabolismo lipídico en el hígado por el aumento de los niveles de p-AMPK inducido por EC y por el resveratrol en ratones obesos y células HepG2 resistentes a la insulina [148, 175]. Así mismo, la inhibición de PKCζ y la activación de AMPK observada en mioblastos insulino-resistentes incubados con EGCG se relacionaron con una mejora de la sensibilidad a la insulina y del metabolismo lipídico [176].

Los factores de transcripción SREBP1-c y PPARα regulan el metabolismo lipidíco: estimulan la lipogénesis y la lipolisis, respectivamente [70]. SREBP1-c regula la síntesis de TG y de ácidos grasos mediante la estimulación de enzimas lipolíticas

como FAS, y PPARα activa el catabolismo de ácidos grasos. En este estudio, en ambos modelos experimentales, se observó un descenso de las proteínas lipolíticas SREBP1-c y FAS, a la vez que se incrementaron los niveles de PPARα, lo que está de acuerdo con el descenso de la acumulación lipídica hepática descrito previamente. En consonancia con estos resultados, se ha observado una mejora del metabolismo lipídico en ratas alimentadas con una dieta rica en fructosa y procianidinas de la uva [177], así como en las células HepG2 tratadas con ácidos grasos y un extracto polifenólico de *Sechium edule* [178] y de hojas de mora [179], ya que se detectó una reducción de los niveles de T-Cho, TG, ácidos grasos, SREBP1-c y FAS y un aumento en los de PPARα. Igualmente, la administración de un extracto polifenólico de la flor de la granada redujo los depósitos lipídicos del hígado y los TG, y restauró los niveles de PPARα en las ratas ZDF y en células HepG2 [180].

Durante la diabetes disminuyen los niveles fosforilados y, por tanto, la actividad de AKT y AMPK. Ambas proteínas modulan a SREBP1-c, por lo que durante la DMT2 el descenso en los niveles de p-AKT y p-AMPK está relacionado con el aumento de SREBP1-c y la inhibición de PPARα. De hecho, el bloqueo de AKT y AMPK con inhibidores específicos agravó la situación en las células HepG2 sometidas a altas concentraciones de glucosa, y se produjo un aumento de los niveles lipídicos totales y de los NEFA. Sin embargo, este incremento de los depósitos lipídicos se previno en parte al pretratar las células con EC. Estos resultados sugieren que EC y el cacao podrían reducir la acumulación lipídica en el hígado al disminuir la lipogénesis *de novo* (inhibición de SREBP1-c y FAS) y al aumentar la lipolisis (incremento de PPARα), jugando AKT y AMPK un papel clave en dicho proceso. De acuerdo con esto, en ratones alimentados con dieta grasa, el flavonoide baicaleína redujo los niveles lipídicos mediante la activación de la AMPK, lo que reprimió la lipogénesis a través del descenso de SREBP1-c y FAS y el aumento de PPARα [173]. En este sentido, también los antocianos de la patata morada dulce atenuaron la resistencia a la insulina y el depósito lipídico hepático mediante la activación de AKT y AMPK [147].

La PKCζ hepática media los efectos lipolíticos de la insulina y se activa durante la DMT2, favoreciendo la gluconeogénesis, la lipogenésis y la inflamación que promueven la resistencia a la insulina, la obesidad, la hepatoesteatosis y la hiperlipidemia [44]. El pretratamiento con EC en las células HepG2 incubadas con la dosis alta de glucosa, y la dieta rica en cacao en las ratas ZDF disminuyeron los niveles

de p-PKCζ y los de los NEFA, lo que está en consonancia con el descenso en los niveles de lípidos en el hígado, de SREBP1-c y FAS, y el aumento de los de PPARα. Además, el bloqueo de PKCζ inducido mediante el uso de un inhibidor específico en las células HepG2 insulino-resistentes mostró unos efectos similares a los ejercidos por EC sobre el metabolismo lipídico hepático (descenso de los niveles de NEFA, SREBP1-c y FAS, e incremento de los de PPARα) y aumentó los niveles fosforilados de AKT y AMPK, lo que se asoció con una mejoría de la situación. Este efecto fue todavía mayor en las células HepG2 resistentes a la insulina pretratadas con EC, lo que sugiere que la regulación de PKCζ inducida por EC contribuye a mejorar el metabolismo lipídico hepático a través del aumento de los niveles de p-AKT y p-AMPK. Estos resultados están apoyados por un estudio previo en el que se ha demostrado que la inhibición de PKCζ disminuía la expresión de SREBP1-c y FAS, a la vez que mejoraban los niveles hepáticos de NEFA, TG y Cho, así como la señalización de la insulina mediada por la AKT [181]. Además, se ha descrito la existencia de una relación entre las PKCa hepáticas y AMPK, ya que la metformina activa a la AMPK y modula la actividad de PKCa en los hepatocitos humanos [181]. Así, aunque es necesario realizar más estudios para determinar con detalle el mecanismo de acción de la PKCζ sobre AMPK y AKT, se podría sugerir que la EC y el cacao son capaces de modular dichas proteínas para regular el metabolismo lipídico y mejorar la resistencia a la insulina hepática.

En conclusión, los flavanoles del cacao pueden aliviar la hiperlipidemia y la esteatosis hepática mediante la inhibición de proteínas clave para la lipogénesis *de novo* (SREBP1-c y FAS) y la activación de la oxidación de los ácidos grasos (PPARα). Estos cambios provocados por EC y el cacao en el metabolismo lipídico están regulados por AMPK, AKT y PKCζ; de hecho, la activación de AKT y AMPK inducida por la EC parece estar mediada por PKCζ. Estos resultados aportan nuevos conocimientos sobre el mecanismo molecular implicado en el efecto antidiabético del cacao, el cual podría ser útil para prevenir o retrasar el desarrollo y/o progreso de la DMT2 y las complicaciones relacionadas como la esteatosis hepática.

En resumen, los flavanoles del cacao podrían considerarse como una potencial herramienta frente a la resistencia a la insulina y la diabetes tipo 2, dado que son capaces de incrementar la sensibilidad a la insulina y mejorar la tolerancia a la glucosa,

el estado redox y el metabolismo lipídico en los hepatocitos. Estos efectos parecen producirse a través de distintos mecanismos: i) restaurando la señalización de la insulina en los hepatocitos mediante la reactivación de la vía de la hormona; ii) aliviando el daño sobre la funcionalidad hepática mediante la preservación de los niveles de GLUT2, la captación de glucosa y la modulación de enzimas gluconeogénicas y glicolíticas; iii) previniendo el desequilibrio redox mediante la regulación de enzimas antioxidantes y señales celulares como Nrf2, NF-κB y MAPKs; y iv) inhibiendo la lipogénesis y estimulando la oxidación de los ácidos grasos a través de la modulación de señales clave para el metabolismo lipídico hepático (AKT, AMPK y PKCζ). Aunque todas estas evidencias podrían sugerir que el consumo moderado de cacao, como fuente importante de polifenoles, y al igual que las frutas y las verduras, puede contribuir a mejorar la salud hepática y a prevenir algunas de las patologías asociadas con la resistencia a la insulina, como la diabetes tipo 2, son necesarios más estudios para determinar de manera inequívoca el mecanismo de acción de estos compuestos fenólicos.

Conclusiones

En relación con los objetivos planteados al comienzo de esta tesis y los resultados obtenidos, ha sido posible extraer las siguientes conclusiones:

1. El tratamiento de las células hepáticas HepG2 con dosis realistas del flavanol epicatequina (1-10 µM) y del extracto polifenólico de cacao (1-10 µg/mL) ejerce un efecto similar al de la insulina que contribuye a fortalecer la señalización de la hormona. Este efecto se relacionó con la activación del receptor de la insulina, los sustratos de dicho receptor (IRS-1 y -2), la vía PI3K/AKT y AMPK, y la inhibición de la producción de glucosa.

   El pretratamiento de las células HepG2 insulino-resistentes con epicatequina (10 µM) o el extracto polifenólico de cacao (1 µg/mL) y la administración de una dieta rica en cacao a las ratas ZDF mejoraron la homeostasis glucídica y la sensibilidad hepática a la hormona. Este retraso o prevención de la potencial disfunción hepática se asoció con la atenuación del bloqueo de la ruta de la insulina que tiene lugar durante la resistencia a la insulina y la diabetes, dado que se previno la inhibición de IR, IRS-1 y -2, la ruta PI3K/AKT y de la AMPK. Todos los tratamientos administrados también contribuyeron a mantener la funcionalidad de las células hepáticas, puesto que impidieron el incremento de los niveles de PEPCK y restauraron el contenido de glucógeno y los niveles de GLUT2. Además, en las células HepG2, la epicatequina y el extracto polifenólico de cacao previnieron el descenso en la captación de glucosa y el aumento en la producción de glucosa causado por la alta dosis de glucosa, mientras que la dieta rica en cacao incrementó los niveles de GK y suprimió la activación de las JNK y p38-MAPK en el hígado de los animales ZDF.

2. El pretratamiento de las células HepG2 insulino-resistentes incubadas con epicatequina (10 µM) o el extracto polifenólico de cacao (1 µg/mL) y la administración de una dieta enriquecida en cacao a las ratas ZDF protegieron frente al estrés oxidativo. Este efecto protector se relacionó con el refuerzo de los niveles de las defensas antioxidantes celulares (GPx y GR en las células HepG2 insulino-resistentes, y SOD y HO-1 en las ratas ZDF), así como con la modulación de factores de trascripción clave relacionados con el

estado redox (Nrf2 y NF-κB). Además, en las células HepG2 insulino-resistentes, la epicatequina y el extracto polifenólico de cacao previnieron la activación de p38 y JNK inducida por la dosis alta de glucosa, sugiriendo que los efectos beneficiosos del extracto de cacao se deben en parte a su acción moduladora sobre las MAPKs.

3. El pretratamiento de las células HepG2 insulino-resistentes con epicatequina (10 µM) y la administración de una dieta enriquecida en cacao a las ratas ZDF aliviaron la hiperlipidemia y la esteatosis hepática mediante la regulación de múltiples vías de señalización. El descenso de los niveles lipídicos se relacionó con la inhibición de proteínas clave para la lipogénesis *de novo* (SREBP1-c y FAS) y la activación de la oxidación de los ácidos grasos (PPARα); estos efectos parecen depender de AMPK, AKT y PKCζ. Además, en las células HepG2, la activación de AKT y AMPK inducida por la epicatequina parece estar mediada por PKCζ.

Conclusión general.

En resumen, los resultados obtenidos en cultivos celulares y en animales de experimentación han puesto de manifiesto que los flavanoles del cacao en las células hepáticas parecen capaces de modular el metabolismo glucídico y lipídico, lo que se traduce en una mejora de la sensibilidad a la insulina y de la tolerancia a la glucosa en una situación de resistencia a la insulina y diabetes. Además, los flavanoles del cacao son capaces de proteger el potencial antioxidante del hepatocito para mantener el equilibrio redox durante el estrés oxidativo que tiene lugar durante la resistencia a la insulina y diabetes.

Conclusions

According to the aims proposed and the results obtained, the following conclusions may be drawn:

1. Treatment of HepG2 cells with realistic concentrations of epicatechin (1-10 µM) and a cocoa polyphenolic extract (1-10 µg/mL) exerts an insulin-like activity that contributes to strengthen the hormone pathway. This effect was associated to the activation of insulin receptor, insulin substrates (IRS-1 and -2), PI3K/AKT and AMPK pathways and the inhibition of glucose production.

   Pretreatment of insulin-resistant HepG2 cells with epicatechin (10 µM) or cocoa polyphenolic extract (1 µg/mL), as well as administration of a cocoa-rich diet to ZDF rats improve glucose homeostasis and hepatic insulin sensitivity. Cocoa and EC could prevent or ameliorate the hepatic dysfunction associated with the insulin signalling blockage that occur during insulin resistance and diabetes by preventing inhibition of IR, IRS-1 y -2, PI3K/AKT and AMPK pathways. All treatments contribute to maintain hepatic functionality as they avoided the increase of hepatic PEPCK values and restored glycogen content and GLUT2 levels. Moreover, epicatechin and the cocoa polyphenolic extract prevented the diminution of glucose uptake and the enhanced glucose production induced by the high glucose concentration in HepG2 cells. In addition, cocoa- rich diet increased GK levels and suppressed hepatic JNK and p38-MAPK activation in the liver of ZDF rats.

2. Pretreatment of insulin-resistant HepG2 cells with epicatechin (10 µM) or cocoa polyphenolic extract (1 µg/mL), as well as the administration of a cocoa-rich diet in ZDF rats protected against the oxidative stress. This protective effect was related to a reinforcement of the antioxidant defences (GPx y GR activities in insulin-resistant HepG2 cells and SOD y HO-1 activities in ZDF rats), and to the modulation of relevant close-related redox transcriptional factors (Nrf2 and NF-κB). Besides, epicatechin and the cocoa polyphenolic extract prevented p38 and JNK activation in insulin resistance

HepG2 cells, suggesting that the cocoa extract beneficial effects seem to be mediated at least in part by its ability to target MAPKs.

3. Epicatechin (10 μM) pretreatment of insulin resistant HepG2 cells and cocoa-rich diet administration to ZDF rats alleviated the hyperlipidemia and hepatic steatosis through the modulation of multiple signalling pathways. Cocoa- and epicatechin-treatment decreased hepatic lipid levels, which was associated to the inhibition of *de novo* key lipogenic proteins (SREBP1-c and FAS) and the activation of fatty acid oxidation (PPARα). Likewise, cocoa- and EC-induced changes in hepatic lipid content were regulated by AMPK, AKT and PKCζ. Indeed, EC-induced AKT and AMPK regulation seemed to be mediated by PKCζ in HepG2 cells.

General conclusion.

In summary, the results obtained in cell culture and experimental animals demonstrate that cocoa flavanols might modulate glucose and lipid metabolism in hepatic cells, which leads to an improved insulin sensitivity and glucose tolerance during insulin resistance and diabetes. Moreover, cocoa flavanols seem to be able to protect the hepatocyte antioxidant capacity to maintain the redox homeostasis against the oxidative stress that occurs during insulin resistance and diabetes.

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via activation of AMPK signals in HepG2 cells. *Journal of Agricultural and Food Chemistry*. 2014. **62**(3): 705-759.

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Anexo

Isabel Cordero Herrera

PERSONAL DATA

Place and date of birth:	Madrid, SPAIN, 4th January 1986
Address:	Calle Sangenjo 37 6ºF 28034 Madrid, Spain
Telephone:	+34 630837493
E-mail:	cordero.isa@gmail.com

EDUCATION

SEPTEMBER 2011	**Ph.D. Student**. Ph.D dissertation, July 2015.
SEPTEMBER 2015	**Institute of Food Science, Technology and Nutrition, Spanish National Research Council (ICTAN-CSIC)**. Doctorate Program in Biochemistry, Molecular Biology and Biomedicine at Universidad Complutense de Madrid (UCM). Topic: Mechanism of action of cocoa flavanols in hepatic cells during insulin resistance and diabetes. Study in cultured cells and experimental animals. Supervisor: Dr. Sonia Ramos Rivero
2010-2011	**Master** in Biochemistry Molecular Biology and Biomedicine at **Universidad Complutense de Madrid (UCM)**. Master Thesis: Study of the redox status and the insulin pathway in HepG2 cells after to high glucose concentrations exposure. Supervisor: Dra. Sonia Ramos Rivero.
2004-2009	**Graduate** in Biochemistry at **UCM** (5 year program).

SKILLS

- Animal and cellular models to study diseases related to oxidative stress, metabolism and nutrition (diabetes and insulin resistance).
- Techniques: Cell culture, animal handling, western-blot, redox status and antioxidant activity, glucose tolerance test, gene silencing with small interfering RNA (siRNA), quantitative real time PCR, epigenetics, behavioral tests (radial arm water maze, inhibitory avoidance task, open field), microdialysis *in vivo*.

LANGUAGES

SPANISH:	Fluent (mother tongue).
ENGLISH:	Fluent: reading, writing and speaking.
GERMAN:	Basic level (A1.1). Goethe Institut, Madrid (2015).

RESEARCH EXPERIENCE

PARTICIPATION IN RESEARCH PROJECTS

2011-2014	Project title: Effect of diet flavanols on type 2 diabetes development and prevention. Studies in cultured cells and experimental animals. Funded by Spanish Ministry of Science and Innovation (MINECO), Ref: AGL2010-17579. Institutions involved: ICTAN (CSIC). Principal Investigator: Luis Goya Suárez.

RESEARCH STAYS IN FOREIGN CENTERS

AUGUST 2014- NOVEMBER 2014	Laboratory of Dr. Sridevi Devaraj, **Texas Children's Hospital and Baylor College of Medicine, HOUSTON, Texas (USA).** Project: Study the potential preventive role of (-) epicatechin under high glucose condition by epigenetic modifications of the histones in the monocyte THP-1 cell line.
SEPTEMBER 2013- DECEMBER 2013	Laboratory of Prof. Dr. Ulf Smith, **Sahlgrenska Academy at Gothenburg University. Lundberg Laboratory for Diabetes Research, GOTHENBURG, Sweden.** Project: Study the role of wisp2 and Wnt signaling on myogenesis in the C2C12 myoblast cell line.

OTHER COLLABORATIONS

SEPTEMBER 2009- MARCH 2010	Laboratory of Prof. Dr. Francisco Mora Teruel, **Department of Physiology at Medicine School, UCM.** Project: Effect of environmental enrichment on working memory.
SEPTEMBER 2007- JUNE 2009	Laboratory of Prof. Dr. Inmaculada Fernández Fernández. **Department of Biochemistry Molecular Biology I at Chemistry School, UCM.** Project: Antiapoptotic mechanisms induced by sphingolipids in human neuroblastoma: role of mitochondria.
SEPTEMBER 2006- JUNE 2007	Laboratory of Dr. Rosario Linacero de La Fuente. **Department of Genetics at Biology School, UCM.** Project: Study of the changes in methylation patterns in regenerated plants *in vitro* of *Secale cereal L*.

HONORS AND SCHOLARSCHIPS

2015	**Young Investigator Fellowship** for attending the 83rd European Atherosclerosis Annual Society (EAS) Congress.
2014	**FPI grant for international mobility awarded by Spanish Ministry of Economy and Competitiveness** (MINECO).
2013	**FPI grant for international mobility awarded by** MINECO.
SEPTEMBER 2011- AUGUST 2015	**Research Fellowship** FPI program awarded by **Spanish Ministry of Science and Innovation** (MICINN).
SEPTEMBER 2008 JUNE 2009	**Scholarship to collaborate** with the Department of Biochemistry and Molecular Biology I, **Chemistry School, UCM**.

PUBLICATIONS

- I. Cordero-Herrera, M.A. Martín, Fernando Escrivá, Carmen Álvarez, L. Goya and S. Ramos."Cocoa-rich diet ameliorates hepatic insulin resistance by modulating insulin signaling and glucose homeostasis in Zucker diabetic fatty rats." *J Nutr Biochem.* (2015). In press. (Doi:10.1016/j.jnutbio.2015.01.009).
- I. Cordero-Herrera, M.A. Martín, L. Goya and S. Ramos. "Cocoa intake ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats". *Food Res. Int.* 69:194-201 (2015).
- I. Cordero-Herrera, M.A. Martín, L. Goya and S. Ramos. "Cocoa flavonoids protect hepatic cells against high-glucose-induced oxidative stress: Relevance of MAPKs." *Mol Nutr Food Res. 59*: 597-609 (2015).
- E. Fernandez-Millán, I. Cordero-Herrera, S. Ramos, Fernando Escrivá, Carmen Álvarez, L. Goya and M.A. Martín. "Cocoa-rich diet attenuates beta cell mass loss and function in young Zucker diabetic fatty rats by preventing oxidative stress and beta cell apoptosis." *Mol Nutr Food Res. 59 (4): 820-824* (2015).

- I. Cordero-Herrera, M.A. Martín, L. Goya and S. Ramos. "Cocoa flavonoids attenuate high glucose-induced insulin signaling blockade and modulate glucose uptake and production in human HepG2 cells." *Food Chem Toxicol.* 64:10-19 (2014).
- M. A. Martín, I. Cordero-Herrera, L. Bravo, S. Ramos, L. Goya."Cocoa flavanols show beneficial effects in cultured pancreatic beta cells and liver cells to prevent the onset of type 2 diabetes." *Food Res. Int.* 63:400-408 (2014).
- S. Fernández-Tomé, S. Ramos, I. Cordero-Herrera, I. Recio, L. Goya, B. Hernández-Ledesma. "*In vitro* chemo-protective effect of bioactive peptide lunasin against oxidative stress in human HepG2 cells." *Food Res. Int.* 62:793-800 (2014).
- I. Cordero-Herrera, M.A. Martín, L. Bravo, L. Goya and S. Ramos. "Cocoa flavonoids improve insulin signalling and modulate glucose production via AKT and AMPK in HepG2 cells." *Mol Nutr Food Res. 57*: 974-985 (2013).
- M.A. Martín, S. Ramos, I. Cordero-Herrera, L. Bravo and L. Goya. "Cocoa phenolic extract protects pancreatic beta cells against oxidative stress". *Nutrients.* 5: 2955-68 (2013)
- I. Cordero-Herrera, M. A. Martín, L. Bravo, L. Goya and S Ramos. "Epicatechin gallate induces cell death via p53 activation and stimulation of p38 and JNK in human colon cancer SW480 cell." *Nutr Cancer*. 65(5): 719-728 (2013).
- I. Cordero-Herrera, S. Cuello, L. Goya, Y. Madrid, L. Bravo, C. Camara and S. Ramos. "Molecular mechanisms involved in the protective effect of selenocystine against methylmercury-induced cell death in human HepG2 cells." *Food Chem Toxicol.* 59: 554-63 (2013).
- P. Garrido, M. De Blas, G. Ronzoni, I. Cordero, M. Antón, E. Giné, A. Santos, A. Del Arco, G. Segovia, F. Mora. "Differential effects of environmental enrichment and isolation housing on the hormonal and neurochemical responses to stress in the prefrontal cortex of the adult rat: relationship to working and emotional memories." *J Neural Transm. 20 (5): 829-843* (2012).

INTERNATIONAL CONFERENCES CONTRIBUTION

2015 83rd European Atherosclerosis Annual Society Congress, Glasgow (Moderated Poster).
2014 3rd International Conference on Food Digestion, Wageningen (Poster).
2014 8th World Congress on Polyphenols Applications, Lisbon (Poster).
2013 49th Annual Meeting of the European Association for the Study of Diabetes, Barcelona (Oral Poster).
2013 2nd CoCoTea Meeting, Naples (Poster)
2011 5th international conference on polyphenols and health (ICPH), Sitges (Poster).
2010 7th Forum of European Neuroscience (FENS), Amsterdam (Poster).

NATIONAL CONFERENCES CONTRIBUTION

2014 XXXVII Congreso de la Sociedad Española de Bioquímica y Biología Molecular, Granada, (Oral presentation and Poster).
2013 XXXVI Congreso de la Sociedad Española de Bioquímica y Biología Molecular, Madrid, (Oral Poster).
2007 XXXVI Congreso de la Sociedad Española de Genética, León. (Poster).

ATTENDANCE TO COURSES

2015 21st "**European Nutrition Leadership Platform Seminar**" Luxembourg (1 week).
2013 VI Conference-Workshop "**Fermented Beverages and Health**" Pharmacy School, UCM (10h).
2011 20th Scientific Symposium "**Obesity Today**" San Lorenzo de El Escorial (20h).
2010 Course "**Security and good practices in the laboratory**" CSIC (10h).
2009 Conference "**Progress in neuroscience: neurotransmitters and nervous pathologies**" Real Academia de Farmacia (30h).
2007 Course "**Chemical forensic Analysis**" Summer School of UCM (100h).

2006 Course "**Stem cell biology: know more to heal better**." Summer courses of UCM in San Lorenzo de El Escorial (30h).

2005 **Paleontologic excavations** in Cerro de los Batallones (Madrid), organized by the Geology School at UCM and the Natural Sciences Museum, (CSIC) (150 h).

VOLUNTEERING

2011 Participation in the divulgation activity "**Semana de la Ciencia 2011.**"

2007-Present Member of **Kin-ball Association of Madrid** (Asociación de Kinball de Madrid). Designed projects (including proposal preparation, budgeting and workplan formulation), work as trainer and referee and organize national and international Championships.

JULY 2013 **Instructor** at **Diabetic Association of Madrid** (Asociación de Diabéticos de Madrid). Working for a non-profit educational organization to educate children and teenagers on diabetes.

2004-2006 **Instructor at Guides Association of Madrid** (Asociación Guías de Madrid). Working for a non-profit civic and educational organization for children and youth, designed educational projects (including proposal preparation, budgeting and workplan formulation), conducted educational programs, and carried out project and program evaluations.

INTERESTS

Sports: Hiking and kin-ball (member of the Kin-ball Spanish National Team 2011-2013).
Hobbies: Reading, travelling, baking.

REFERENCES

Available on request