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INTERVENCIONES ANTIOXIDANTES PARA EL TRATAMIENTO DEL ESTRÉS OXIDATIVO Y MUERTE CELULAR EN HEPATOCITOS HUMANOS EN CULTIVO

Raúl González Ojeda

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TITULO: *Intervenciones antioxidantes para el tratamiento del estrés oxidativo y muerte celular en hepatocitos humanos en cultivo*

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TÍTULO DE LA TESIS: INTERVENCIONES ANTIOXIDANTES PARA EL TRATAMIENTO DEL ESTRÉS OXIDATIVO Y MUERTE CELULAR EN HEPATOCITOS HUMANOS EN CULTIVO

DOCTORANDO/A: RAÚL GONZÁLEZ OJEDA

INFORME RAZONADO DEL DIRECTOR DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El proyecto de Tesis Doctoral de Raúl González profundiza en los mecanismos intracelulares de citoprotección de antioxidantes como N-acetilcisteína (NAC), coenzima Q (Co Q), análogo de superóxido dismutasa (MnTBAP) y α-tocoferol en el cultivo primario de hepatocitos humanos. En este sentido, el estudio demuestra que NAC, Co Q y MnTBAP ejercen su acción citoprotectora reduciendo el estrés oxidativo y preservando la función mitocondrial de los hepatocitos. De forma distinta, α-tocoferol regula la expresión de diversos genes implicados en la metabolización y transporte de drogas y tóxicos, así como promueve sus modificaciones postranslacionales mediadas principalmente por el óxido nítrico (NO).

Los resultados obtenidos han sido publicados en las revistas de más prestigio en el campo del estrés oxidativo y hepatología. Asimismo, el Sr. González ha demostrado por su actitud personal y su disciplina diaria, la gran importancia que representa en el actual organigrama del grupo siendo sin lugar a dudas de los investigadores más determinantes en el estado actual de excelencia de nuestro grupo de investigador.

Por todo lo dicho, es para mí un orgullo personal y científico el haber dirigido el actual trabajo de investigación que representa su Tesis Doctoral.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 11 de Diciembre de 2010

Firma del director

Fdo.: Jordi Muntané Relat

INTERVENCIONES ANTIOXIDANTES PARA EL TRATAMIENTO DEL ESTRÉS OXIDATIVO Y MUERTE CELULAR EN HEPATOCITOS HUMANOS EN CULTIVO

Memoria presentada por

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Doctor en Biología

Tesis Doctoral realizada bajo la dirección del Dr. Jordi Muntané Relat

En la Unidad de Investigación del Hospital Universitario Reina Sofía de Córdoba

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Fundación Hospital Reina Sofía-Cajasur

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"Lo que caracteriza al hombre de ciencia no es la posesión del conocimiento o de verdades irrefutables, sino la investigación desinteresada e incesante de la verdad".

Karl Popper

"Lo que sabemos es una gota de agua; lo que ignoramos es el océano".

Isaac Newton

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Antes de conocer a mi **esposa**, en mi vida solamente cabía el universo de la ciencia. Una vez que ella apareció, me hizo ver que había algo más y que se podía compatibilizar trabajo con vida personal. Por lo tanto, hago especial mención al apoyo incondicional, comprensión y paciencia de mi mujer, que me sirvieron de guía en el arduo camino de la investigación.

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Por último, evocar a "**la niña del ascensor**" que nos amenizaba las largas noches de trabajo abriendo el ascensor del fondo del pasillo.

A todos ellos, MUCHAS GRACIAS

ÍNDICE

Abreviaturas

Å: Amstrong

ABC: Proteínas transportadoras con dominio de unión a ATP

ADC: Adenomacarcinoma

ADH-5: Alcohol deshidrogenasa-5

ADN: Ácido dexosirribonucléico

ADP: Adenosina difosfato

Ahr: Receptor aril de hidrocarburo

Ala: Alanina

ALT: Alanina aminotransferasa

AP-1: Proteína activadora 1

ARE: Elemento de respuesta antioxidante

Arg: L-arginina

ARN: Ácido ribonucleico

ARNm: ARN mensajero

ARNr: ARN ribosómico

ARNt: ARN transferente

ASBT: Transportador apical de ácidos biliares dependiente de sodio

Asp: Aspartato

AST: Aspartato aminotransferasa

ATP: Adenosina trifosfato

A23187: Ionóforo del calcio

BAPTA-AM: 1,2-bis-(o-aminofenoxi)etano-N,N,N',N'-ácido tetraacético tetra-(acetoximetil) ester

BHT: 2,6-Di-tert-butyl-4-methyl-phenol

BH4: Tetrahidrobiopterina

BOP: N-nitroso bis(2-oxopropil)amina

BSEP: Bomba exportadora de sales biliares

CA: Ácido cólico

Ca^{2+}: Calcio

CAR: Receptor constitutivo de androstenona

CARD: Dominio de reclutamiento de activación de caspasas

CAT: Catalasa

CDCA: Ácido quenodeoxicólico

CDK: Quinasa dependiente de ciclina

C/EBP: Proteína estimuladora de unión a la secuencia CCAAT

CEHC: Carboxietil hidroxicromano

5'-CMBHC: 2,5,7,8-tetrametil-2-(4'-carboxi-4'metilbutil)-6-hidroxicromano

CMK: Kinasa II calcio-calmodulina

CO: Monóxido de carbono

CPT: Carnitina palmitoil transferasa

CSNO: S-nitroso-L-cisteína

CTF: Factor de transcripción unido a la caja CCAAT

CTLs: Linfocitos T citotóxicos

Cu: Cobre

Cys: Cisteína

CYP: Citocromo P 450

Da: Dalton

DC: dicarboxilato

DD: Dominio de muerte

DED: Dominio efector de muerte

DEM: Diethylmaleate

DEN: Dietilnitrosamina

D-GalN: D-galactosamina

DME: Enzimas metabolizadoras de drogas

DPQ: 3,4-dihidro-5-[4-(1-piperidinil)butoxil]-1(2H)-isoquinolinona

EDAR: Receptor de Ectodisplasina A

EGTA: Ácido etilenglicol-bis-amino etil éster)-N-N'- tetraacético

EGR: Respuesta temprana al crecimiento

EpRE: elemento de respuesta electrofílica

ERK: Proteína quinasa regulada por señales extracelulares

ERN: Especies reactivas de nitrógeno

ERO: Especies reactivas de oxígeno

ETC: Cadena de transporte de electrones mitocondrial

e^-: Electrón

FAD: Flavina adenina dinucleótido

FasL: Ligando de Fas

FasR: Receptor de Fas

Fe: Hierro

FMN: Flavina mononucleótido

FXR: Receptor X fernesoide

g: Gramo

GCA: Ácido glicocólico

GCDCA: Ácido glicoquenodeoxicólico

GCL: Glutamato cisteína ligasa

GCLC: Subunidad catalizadora de la glutamato cisteína ligasa

GCLM: Subunidad moduladora de la glutamato cisteína ligasa

GGT: γ-glutamil transpeptidasa

Gln: Glutamina

Glu: Glutamato

Gly: Glicina

GMPc: Guanosín monofosfato cíclico

GPx: Glutatión peroxidasa

GR: Glutatión reductasa

GRE: Elementos de respuesta a glucocorticoides

Grx: Glutarredoxina

GS: Glutatión sintetasa

GSH: Glutatión en forma reducida

GSH-EE: Glutatión-ethyl-ester

GSNO: S-nitrosoglutatión

GSSG: Glutatión en forma oxidada

GS^+: Radical glutatión

G6PDH: Glucosa-6-fosfato deshidrogenasa

h: Hora

HCC: Carcinoma hepatocelular

HDL: Lipoproteína de alta densidad

HepG2: Línea celular de hepatoma humano

HIF-1α: Factor inducible por hipoxia-1α

HIV: Virus de la inmunodeficiencia humana

HO-1: Hemoxigenasa-1

$HO_2^\cdot$: Radical hidroperoxilo

HSE: Elemento de choque térmico

HSF: Factor de choque térmico

Hsp: Proteína de choque térmico

H_2DCFDA: Diclorodihidrofluoresceina diacetato

H_2O: Agua

H_2O_2: Peróxido de hidrógeno

H^+: Protón

IBABP: Proteína de unión a ácidos biliares en el íleon

INF: Interferón

iNOS: Óxido nítrico sintasa inducible

IL: Interleuquina

IRF1: Factor 1 regulador de interferón

JAK: Quinasa de la proteína Jano

K: Kilo

kb: Kilobases

kg: Kilogramo

KLF2: Factor 2 Kruppel asociado a pulmón

L: Litro

LDH: Lactato deshidrogenasa

LDL: Lipoproteína de baja densidad

LDLR: Receptor de LDL

LH: Molécula lipídica

L-NAME: N-γ-nitroso-L-Arg metil ester

LOOH: Peróxido lipídico

LOO^+: Radical peróxido lipídico

LPL: Lipoproteinlipasa

LPO: Peroxidación lipídica

LPS: Lipopolisacárido

LSD: Test estadístico de mínimas diferencias significativas

LXR: Receptor X hepático

MAPK: Proteína quinasa activada por mitógeno

MDA: Malondialdehído

MDR: Proteína con resistencia a multidrogas

mg: Miligramo

MitoQ: Mitoquinona, análogo de Q_{10}

mM: Milimolar

Mn: Manganeso

MnTBAP: Mn(III)tetrakis(4-benzoic acid) porphyrin chloride

MRE: Elemento de respuesta a metales

MRPs: Proteínas asociadas a la resistencia a multidrogas

MTP: Transición permeabilidad mitocondrial

n: número

NAC: N-acetilcisteína

NAD^+: Nicotinamida adenina dinucleótido

NADH: Nicotinamida adenina dinucleótido en forma reducida

NADPH: Nicotinamida adenina dinucleótido fosfato en forma reducida

$NADP^+$: Nicotinamida adenina dinucleótido fosfato en forma oxidada

ND: No hay datos

NF-*k*B: Factor nuclear kappa B

NF-Y: Factor nuclear Y

NF1: Factor nuclear 1

NGFR: Receptor del factor de crecimiento nervioso

nM: Nanomolar

nm: Nanómetro

NO: Óxido nítrico

NOS: Óxido nítrico sintasa

NO_2: dióxido de nitrógeno

Nrf: Factor nuclear eritroide

NTCP: Proteína cotransportadora de ácido taurocólico dependiente de sodio

OATs: Transportadores de aniones inorgánicos

OATPs: Polipéptidos transportadores de aniones inorgánicos

OCTs: Transportadores de cationes orgánicos poliespecíficos

OGC: Oxo-glutarato

$^{\cdot}OH$: Radical hidroxilo

$ONOO^-$: Peroxinitrito

OST: Transportador de solutos orgánicos

O_2: Oxígeno

$O_2^{\cdot -}$: Anión superóxido

PARP-1: Poli (ADP-ribosa) polimerasa-1

pb: Pares de bases

PGE_1: Prostaglandina E1

Pi: Fósforo inorgánico

PI3K: Fosfatidilinositol 3-quinasa

PK: Proteína quinasa

PLTP: Proteína de transferencia de fosfolípidos

PPAR: Receptor activado por proliferadores de peroxisomas

PPRE: Elemento de respuesta a PPAR

Pro: Prolina

Prx: Peroxirredoxina

PTP: Poro de transición de permeabilidad

PXR: Receptor X de pregnano

P-450: Citocromo P-450

Q_{10}: Coenzima Q_{10}

RAR: Receptor de ácido retinoico

RARE: Elemento de respuesta a ácido retinoico

Redox: Reacciones de reducción-oxidación

RIF: Rifampicina

$RO^{\cdot}$: Radical alcoxilo

ROOH: Radical hidroperóxido

$RO_2^{\cdot}$: Radical peroxilo

RXR: Receptor X retinoico

S: Azufre

SAMe: S-adenosilmetionina

Se: Selenio

Ser: Serina

SH: Sulfidrilo

Smad: Proteínas homologas de las proteínas madre contra decapentaplegic de *Drosophila* y la proteína SMA de *C. elegans*

SNO: S-nitrosotiol

SOD: Superóxido dismutasa

Sp1: Proteína específica 1

SRE: Elemento de respuesta a esterol

SREBP: Proteína de unión a SER

STAT: Señales transductoras y activadoras transcripcionales

SULT: Sulfotransferasa

TAP: Proteína asociada a tocoferol

TCA: Ácido taurocólico

TCDCA: Ácido tauroquenodeoxicólico

TCF: Factor de células T

Tf: Transferrina

TGF-β1: Factor de crecimiento transformante β1

THC: Tetrahidrocannabinol

TNF-α: Factor de necrosis tumoral-α

TNF-R: Receptor de TNF

TRAIL-R: Receptor de TRAIL

Trx: Tiorredoxina

TrxR: Tiorredoxina reductasa

α-TTP: Proteína transportadora de α-tocoferol

Tyr: Tirosina

U: Unidades

UA: Unidades Arbitrarias

UCP: Proteína desacoplante

UDP: Uridina difosfato

UGT: Uridina difosfato glucuronosil transferasa

UTR: Región no traducida del gen

UV: Ultravioleta

Val: Valina

VCAM-1: Molécula 1 de adhesión celular vascular

VDAC: Canal iónico dependiente de voltaje

VDR: Receptor de vitamina D

VLDL: Lipoproteína de muy baja densidad

Zn: Zinc

Z-VAD-fmk: N-benziloxicarbonil-Val-Ala-Asp-fluorometilcetona

μm: micrómetro

μM: Micromolar

INTRODUCCIÓN

1. EL HÍGADO

El **hígado** es un órgano del cuerpo humano que en adulto presenta un peso de 1,5 Kg, y presenta una importante actividad metabólica y endocrina en el organismo.

1.1 Anatomía y circulación sanguínea en el hígado

El hígado se localiza en la región superior derecha de la cavidad abdominal, debajo del diafragma y por encima del estómago, el riñón derecho y de los intestinos (*Figura 1*). Su consistencia es blanda y depresible, y está recubierto por el peritoneo, **cápsula de Glisson ó cápsula fibrosa perivascular.**

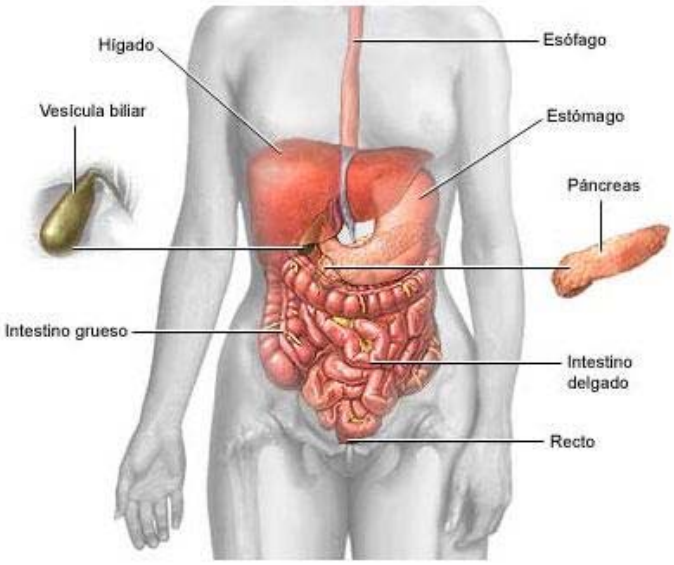

Figura 1: El hígado se localiza bajo el diafragma del cuerpo por encima del borde superior del estómago. La vesícula biliar y sus vías se encuentran exactamente debajo del lado derecho del hígado.

Está dividido en cuatro lóbulos (*Figura 2*):

• *Lóbulo derecho*, situado a la derecha del ligamento falciforme.

• *Lóbulo izquierdo*, extendido sobre el estómago y situado a la izquierda del ligamento falciforme.

• *Lóbulo cuadrado*. Se encuentra limitado por el surco umbilical a la izquierda, el lecho vesicular a la derecha y el hilio del hígado por detrás.

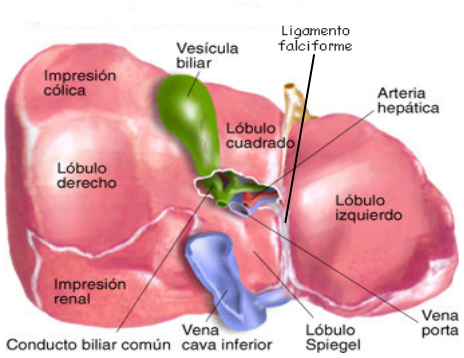

Figura 2: La anatomía morfológica del hígado, considera la división clásica del hígado, en un lóbulo derecho y otro izquierdo, separados

• *Lóbulo de Spiegel (lóbulo caudado)*, situado entre el borde posterior del hilio hepático por delante, la vena cava por detrás.

La **circulación hepática** es de naturaleza centrípeta y está formada por el sistema porta y la arteria hepática. El sistema con un flujo sanguíneo de 15 mL/min contiene sangre poco oxigenada y rica en nutrientes proveniente del tracto gastrointestinal y del bazo (**1**). La sangre oxigenada procede de la arteria hepática (irrigación nutricia) (*Figura 3*). La triada hepática, localizada en la confluencia de los lobulillos hepáticos que son formaciones hexagonales compuestas de células hepáticas, está compuesta por la vena porta, la arteria hepática y el conducto biliar que drenan a los hepatocitos en estructura radial a través de los sinusoides hepáticos en donde confluyen la sangre arterial y venosa portal. La sangre es recogida por la vena centrolobulillar que está localizada en el centro del propio lobulillo. La vena centrolobulillar confluye en la vena hepática, que finalmente transfiere la circulación venosa a la vena cava inferior. Por lo tanto, la sangre rica en nutrientes de la absorción intestinal (vena porta, proporciona **65-85%** de la sangre que llega al hígado) y en oxígeno (arteria hepática, proporciona 20-35% de la sangre hepática) se mezcla en los sinusoides hepáticos (espacios entre hepatocitos).

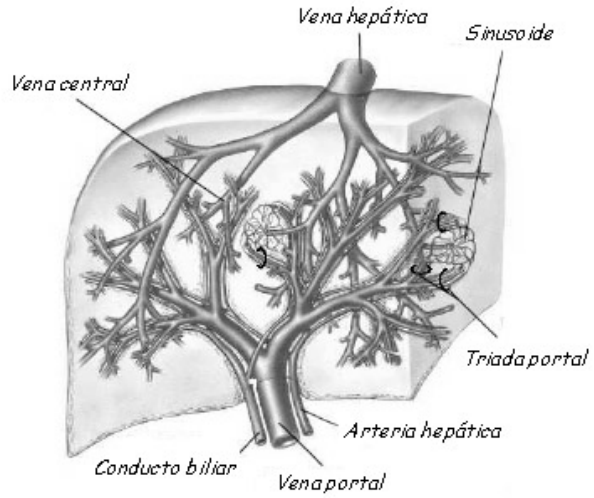

Figura 3: **Irrigación interna del hígado, con los vasos principales**. La irrigación y oxigenación del hígado se lleva a cabo por la vena porta y la arteria hepática. Estos grandes vasos penetran en el hígado por el hilio hepático, dividiéndose cada uno de ellos en 2 ramas, derecha e izquierda, que irrigan ambos lóbulos hepáticos, en cuyo interior se dicotomizan en ramas cada vez más pequeñas, terminando en una red vascular común, sinusoide hepático. También se forman las tríadas portales, constituidos por una arteria, una vena y un conducto biliar.

1.2 Fisiología del hígado

El hígado, en particular los hepatocitos, desempeña múltiples funciones en el *organismo* como son (**2**):

- *Producción de bilis:* el hígado sintetiza ácidos biliares que son excretados al duodeno a través de la vía biliar. La bilis es necesaria para la absorción de los compuestos lipídicos en el tracto intestinal.
- *Metabolismo de los carbohidratos: gluconeogénesis (formación de glucosa a partir de aminoácidos, lactato y glicerol), glucogenolísis (fragmentación de glucógeno para liberar glucosa en la sangre) y glucogenogénesis (síntesis de glucógeno a partir de glucosa).*
- *Eliminación de insulina y de otras hormonas.*
- *Metabolismo de los lípidos: síntesis de colesterol y triglicéridos.*
- *Síntesis de proteínas*: la albúmina y las lipoproteínas.
- *Síntesis de factores de coagulación*: el fibrinógeno (I), la protrombina (II), la globulina aceleradora (V), proconvertina (VII), el factor antihemofílico B (IX) y el factor Stuart-Prower (X).
- *Detoxificación de la sangre*: los productos tóxicos en el hígado sufren una serie de reacciones de transformación que los convierten en productos más hidrofílicos y por tanto más fácilmente excretables por vía urinaria.
- *Transformación del amonio en urea.*

- *Depósito de múltiples sustancias*, como: glucosa (en forma de glucógeno), vitamina B_{12}, hierro, cobre,...

El hígado cuenta con diversos complejos enzimáticos denominados de fase I y II destinados a la metabolización de drogas y productos tóxicos (DME, enzimas metabolizadoras de drogas) entre los cuales se encuentra el sistema del **citocromo p450** (P-450), flavin-monooxigenasas, peroxidasas, hidroxilasas, esterasas y amidasas. Otras enzimas también presentes son las glucuroniltransferasas, las sulfotransferasas, metilasas, acetiltransferasas, tioltransferasas.

1.3 Histología hepática

El parénquima hepático (*Figura 4*) está representado por dos estructuras funcionales distintas: lobulillo y acino hepático. La diferencia principal entre ambas estructuras radica en la dirección del flujo sanguíneo en relación con el centro de la unidad estructural. La vena centrolobulillar se localiza en el centro de la estructura en el lobulillo hepático (*Figura 4*). Sin embargo, la tríada hepática constituye el centro estructural en el acino hepático (*Figura 4*). Esta distribución diferencial del flujo sanguíneo conlleva la distinción de diversas áreas en el hígado en función de la presión parcial de oxígeno (*Figura 4*).

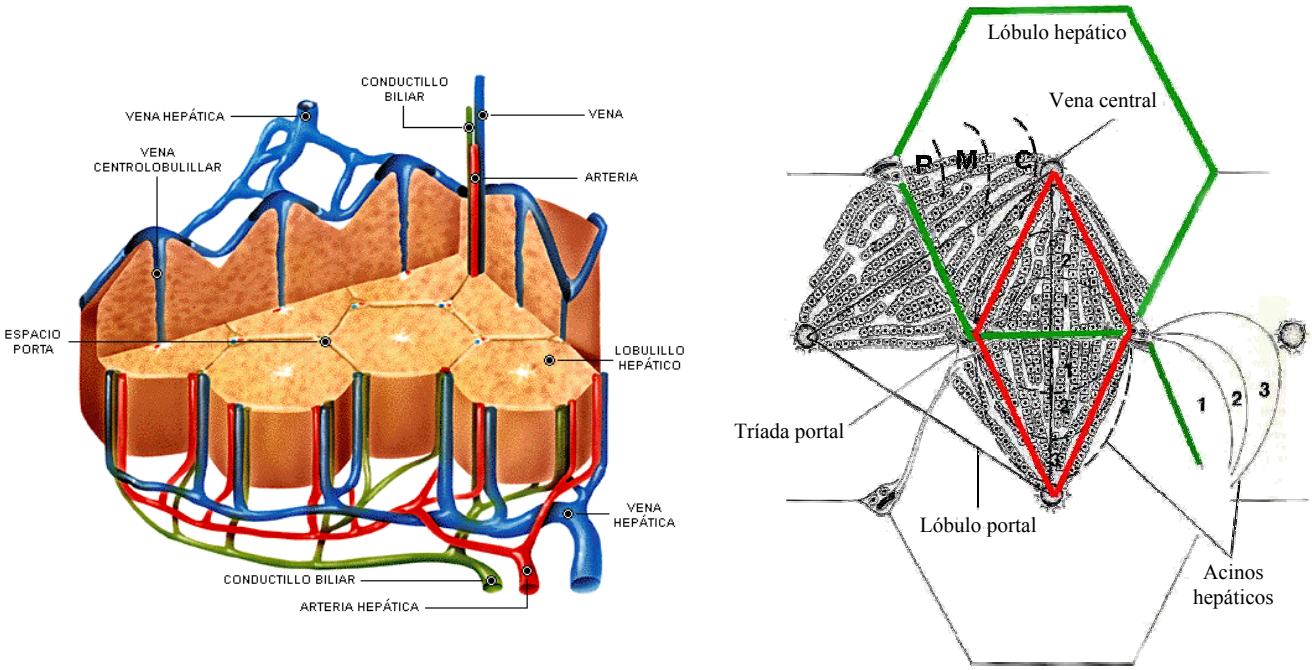

Figura 4: **Esquema de la unidad funcional hepática, lobulillo y acino hepático**. El hígado se divide en lobulillos hexagonales (representado por un hexágono de color verde) centrados por la vena central y en cuyas esquinas se sitúa el espacio ó tríada portal, por donde discurre una rama de la vena porta, una arteria hepática y un conducto biliar. El flujo sanguíneo se dirige desde la vena porta y arteria hepática hasta la vena central a través de los sinusoides, flanqueados por hileras de hepatocitos. Así, en el lobulillo hepático se distinguen 3 regiones: centrolubulillar (C), mediozonal (M) y periportal (P). También está el concepto de acino hepático (representado por un rombo de color rojo) para designar la unidad funcional hepática. La base del acino está formada por las ramas terminales de la vena porta y arteria hepática, equivaldría a cada uno de los lados del lobulillo. El acino se puede dividir también en 3 zonas: la zona 1 es la más cercana a la entrada de sangre, la zona 3 es la más próxima a la vena central y la zona 2 es intermedia. Los hepatocitos de la zona 1 están expuestos a una mayor presión parcial de oxígeno que los de la zona 3, la cuál, en comparación a otros tejidos del organismo, es hipóxica.

Los ***sinusoides hepáticos*** son capilares que se disponen entre las láminas de hepatocitos (*Figura 5*) y donde confluyen, desde la periferia de los lobulillos, las ramas de la arteria hepática y de la vena porta. La sangre fluye desde las tríadas hasta la vena central, circulando en forma centrípeta; la pared de los sinusoides está formada por una capa discontinua de células endoteliales con fenestraciones (poros de intercambio de nutrientes/desechos y gases) entre las células.

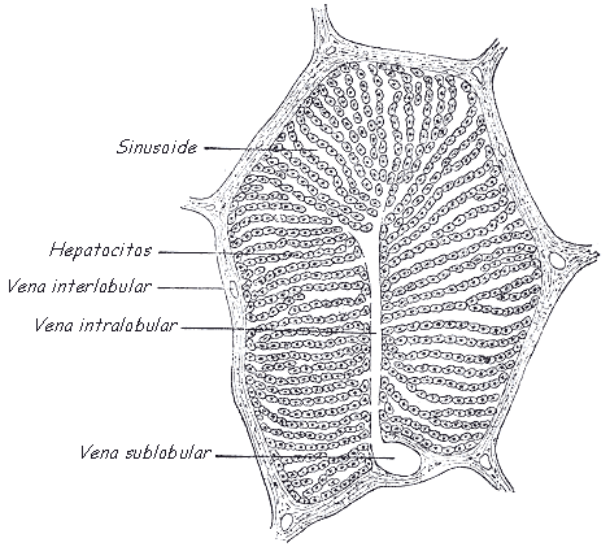

Figura 5: **Subdivisión del parénquima hepático**. En dicho parénquima se aprecian ramificaciones o sinusoides derivadas de los vasos sanguíneos con diámetro decreciente, como las venas interlobulillares, intralobulillares y sublobulillares donde drenan los hepatocitos de los lobulillos su sangre.

*El **espacio de Disse***: es un estrecho espacio perisinusoidal que se encuentra entre la pared de los sinusoides y las láminas de hepatocitos, ocupado por una red de fibras reticulares y plasma sanguíneo que baña libremente la superficie de los hepatocitos. En el espacio de Disse se produce el intercambio metabólico entre los hepatocitos y el plasma donde se forma la abundante linfa hepática.

1.4 Tipos celulares hepáticos

El hígado está constituido por las células parenquimales (hepatocitos) y células no parenquimales (células de Kupffer, células endoteliales y células estrelladas).

- ***Células parenquimales***: los **hepatocitos** constituyen alrededor del 80% de la población celular del tejido hepático. Son células poliédricas con 1 o 2 núcleos esféricos poliploides y un nucléolo prominente. Los hepatocitos presentan el citoplasma acidófilo con unos cuerpos basófilos, y con numerosos orgánulos como consecuencia de su elevada actividad metabólica (*Figura 6*). Además, en su citoplasma contienen inclusiones de glucógeno y grasa. La membrana plasmática de los hepatocitos presenta un dominio sinusoidal con microvellosidades que permite el intercambio de nutrientes/metabolitos y O_2/CO_2 con el espacio de Disse, y un dominio lateral orientado hacia el hepatocito vecino. En determinadas orientaciones de la membrana plasmática de dos hepatocitos contiguos se delimita un canalículo donde será secretada la bilis, **canalículo biliar**.

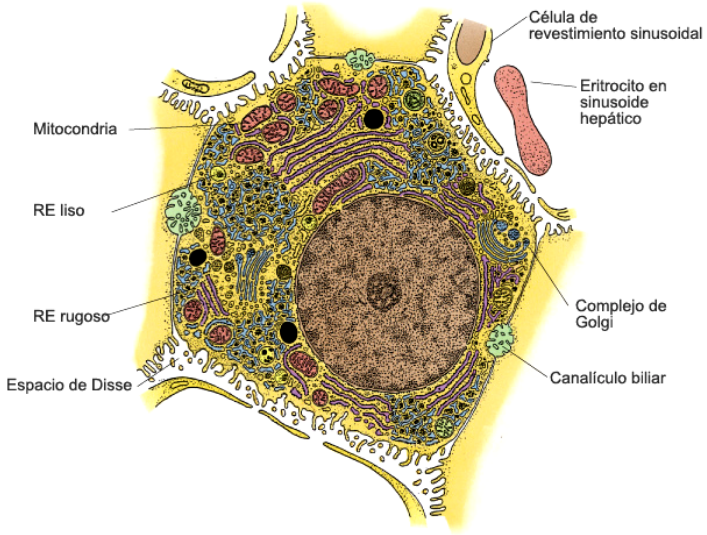

Figura 6: **Componentes intracelulares de la célula hepática**. Los hepatocitos presentan en su citoplasma numerosos orgánulos (ribosomas, retículo endoplásmico rugoso y liso, aparato de Golgi y mitocondrias) característico de células con elevada actividad metabólica. Los lípidos se depositan en gotas de VLDL. El glucógeno forma unas partículas de 20-30 nm de diámetro cerca del retículo endoplásmico liso.

- ***Células no parenquimales:***

- Células de Kupffer: son macrófagos residentes en el hígado pertenecientes al sistema fagocítico mononuclear que se encuentran en la parte interna de los sinusoides, y que emiten sus prolongaciones hacia el espacio de Disse. Su función es fagocitar las partículas exógenas extrañas y restos celulares que circulan por la sangre y que potencialmente pueden alterar la homeostasis del propio organismo actuando como células presentadoras de antígeno. A través de la liberación de citoquínas como el factor de necrosis tumoral-α (TNF-α), interleuquina-1 (IL-1β) e IL-6 permite la regulación de la respuesta de los hepatocitos en condiciones de inflamación. Liberan factores vasodilatadores como el óxido nítrico (NO).

- Células endoteliales: estas células poseen receptores que permiten la endocitosis de sustancias como el LDL y el ácido hialurónico. También son capaces de producir mediadores vasoconstrictores como la endotelina-1 y diversos mediadores inflamatorios.

- Células estrelladas: las células estrelladas se denominan de **Ito o lipocitos,** almacenan vitamina A en estado de reposo y están localizados en los espacios de Disse. Cuando se produce un daño hepático, estas células se activan a miofibroblastos sufriendo la pérdida de la vitamina A, e incrementan la síntesis de la matriz extracelular y de colágeno. Este proceso es esencial en los procesos de hipertensión portal y de fibrosis hepática.

2. MUERTE CELULAR

La muerte celular puede ser definida como una pérdida irreversible de la integridad de la membrana plasmática (**3**). Dicho proceso tiene lugar durante el desarrollo embrionario para la destrucción/generación de nuevos tejidos, así como durante el organismo adulto para el recambio celular como consecuencia de su senescencia o afectación patológica. La muerte celular es esencial en la homeostasis del organismo, pues elimina las células con lesiones importantes en su función celular (disfunción mitocondrial y/o permeabilidad de la membrana plasmática) y por la acumulación de mutaciones en el DNA celular.

Se han identificado tres tipos de muerte celular, autofagia, apoptosis y necrosis, atendiendo a criterios morfológicos y bioquímicos. El proceso de autofagia es consecuencia de los procesos de adaptación a cambios homeostáticos del entorno celular. Los procesos de apoptosis y necrosis son como consecuencia de alteraciones o daños importantes en la permeabilidad celular, función o estructuras celulares. Existe una transición entre ambas formas de muerte celular denominada necro-apoptosis en relación con el grado de alteración de la permeabilidad de la membrana externa mitocondrial y/o disfunción mitocondrial. Aún con ciertas variaciones, una célula muere por apoptosis o necrosis según la naturaleza, magnitud y tiempo de aplicación del estímulo inductor de la lesión, así como el tipo de célula afectada, la etapa de desarrollo del tejido y el estado fisiológico celular (**4, 5**).

2.1 Autofagia

La autofagia es un proceso catabólico altamente regulado y conservado en organismos eucariotas, que está caracterizado por el secuestro de material citoplásmico dentro de unas vesículas de doble membrana llamadas **autofagosomas** (**6**) que se fusionan con los lisosomas en donde son degradados por enzimas hidrolíticas ó **hidrolasas**. La activación de dichas enzimas requiere un pH más ácido que el del citosol, pH 5, que se logra por la acción de una bomba de protones en la membrana lisosomal (V-ATPasa). La membrana del lisosoma es impermeable a dichas enzimas y resistente a la acción de éstas. Una vez que los autofagosomas se han fusionado con los lisosomas, las vesículas resultantes se denominan **autolisosomas**, donde se lleva a cabo el reciclaje de las macromoléculas resultantes de la digestión (*Figura 7*).

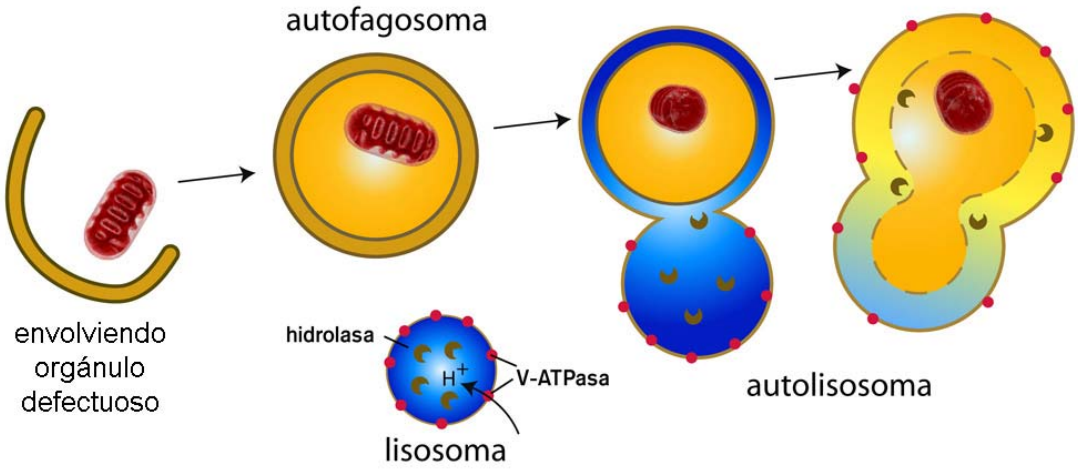

Figura 7: **Proceso básico de la autofagia celular**. La célula encapsula con una membrana doble las proteínas y orgánulos modificados o prescindibles dependiendo de las condiciones fisiológicas. Después el autofagosoma formado se funde con un lisosoma constituyéndose un autolisosoma, en el que se digiere enzimáticamente el contenido encerrado, reciclándose las macromoléculas resultantes.

Este proceso juega un papel esencial en la adaptación a las condiciones ambientales cambiantes, a la remodelación celular durante el desarrollo y eliminación de orgánulos alterados durante condiciones adversas o durante el envejecimiento (**7**). Los estudios más recientes muestran que la autofagia es clave para la producción de vacunas más eficaces (contra la tuberculosis) (**8**), en la comprensión del cáncer (gliomas) (**9**) y otros procesos de patogénesis.

2.2 Necrosis

La necrosis es un proceso de muerte celular que ocurre en condiciones extremas de lesión con reducción del contenido energético celular y drástica alteración de la permeabilidad celular que limitan la supervivencia celular (*Figura 8*) (**10, 11**). Este proceso conlleva la alteración de la función celular, alteración de los orgánulos celulares y ruptura de la membrana plasmática con liberación del contenido celular al medio extracelular promoviendo una respuesta inflamatoria (**4**, **5**, **10**).

El fenómeno de la necrosis incluye alteraciones mitocondriales con generación de especies reactivas de oxígeno (ERO) y permeabilización de la membrana mitocondrial (**12**), La alteración de la permeabilidad de la membrana plasmática incrementa la concentración de Ca^{2+} que activa la calpaína e induce liberación de enzimas lisosomales como la catepsina de tipo B, D, H y L (**13, 14**).

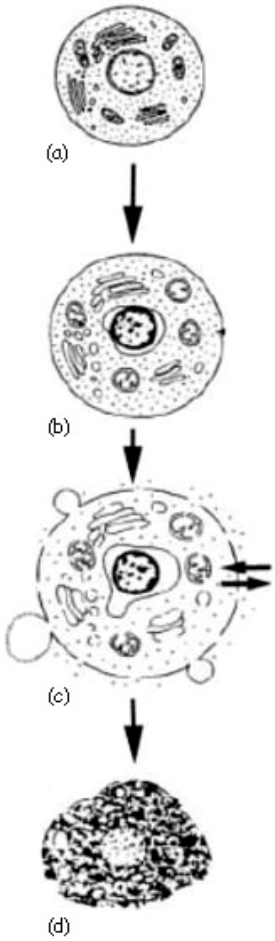

Figura 8: **Esquema de la ruta de muerte celular por necrosis**. En la cuál se distinguen varias fases:

(a) Célula con todos sus orgánulos intactos y totalmente viables.

(b) La célula sufre un hinchamiento celular (oncosis).

(c) En la membrana plasmática celular se forman poros.

(d) La célula sufre una lísis y liberación del contenido celular.

2.3 Apoptosis

La muerte celular por "apoptosis" se caracteriza por un cambio morfológico celular específico caracterizado por la reducción del volumen celular (picnosis) como consecuencia de la ruptura de las estructurales microtubulares de la célula y aparición de protuberancias en la membrana externa que incluye restos de orgánulos funcionales (cuerpos apoptóticos) que serán fagocitados por las células inflamatorias (*Figura 9*). Desde el punto de vista bioquímico se caracteriza por la condensación de la cromatina, fragmentación del núcleo (carorrexis) y el mantenimiento de la función de los orgánulos. El proceso de apoptosis ocurre durante el envejecimiento celular y como consecuencia de una lesión tóxica celular moderada. Se trata de un mecanismo homeostático que permite el mantenimiento de las poblaciones celulares funcionales de los tejidos (**10, 11**). Además, la apoptosis es responsable de la eliminación de células no deseadas durante el desarrollo, la maduración del sistema inmune y la supresión de tumores mediada por el sistema inmune (inmunovigilancia) (**15**).

El grupo de enzimas responsables de la señal de apoptosis celular son las caspasas que se encuentran en forma inactiva o zimógenos que son proteasas que presentan una *cisteína* en su centro activo y su secuencia diana presenta un residuo de aspartato de lo que deriva su nombre (**c**isteinil-**asp**artato prote**asas**). Las distintas caspasa se activan secuencialmente y conllevan todos los cambios morfológicos y bioquímicos asociados a la apoptosis. La activación de las caspasas es dependiente de energía (ATP) (**10**).

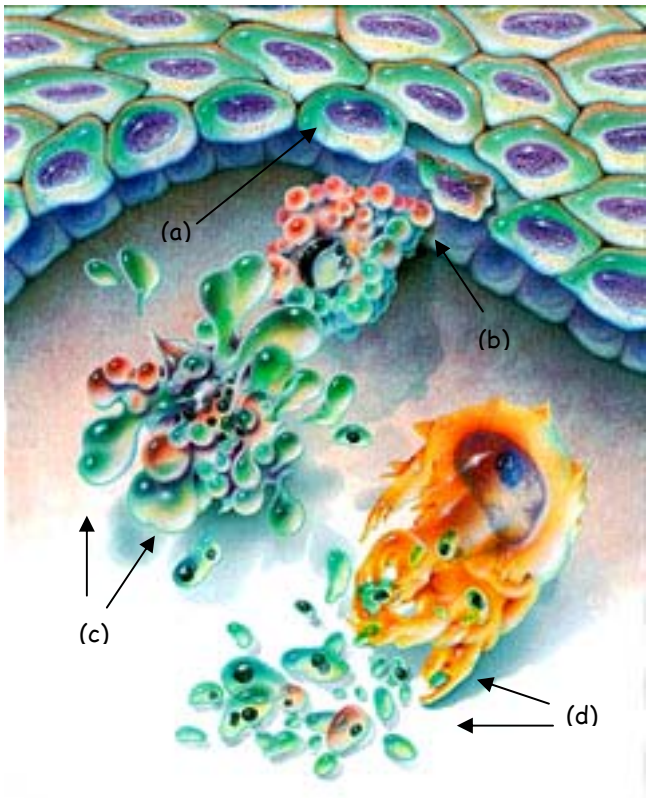

Figura 9: **Esquema de la ruta de muerte celular mediante apoptosis**.
En la cuál se distinguen las siguientes fases:

(a) Célula con todos sus orgánulos intactos y totalmente viables.

(b) La célula se encoge, se separa de las células vecinas y su cromatina se condensa.

(c) Se forman vesículas celulares

(d) La célula estalla, formando cuerpos apoptóticos que son fagocitados por células vecinas o macrófagos. No se produce inflamación celular.

La activación secuencial de las caspasas permite la amplificación rápida de la señal de muerte celular (**10**). Las caspasas implicadas en el proceso de inducción de muerte celular se pueden separa en **iniciadoras** (caspasa-2,-8,-9,-10) y **efectoras o ejecutoras** (caspasa-3,-6,-7) (15). Asimismo, existen una serie de caspasas (caspasa-1,-4,-5) que están implicadas en la proteólisis y liberación de las citoquinas en las células inflamatorias (**15, 16**).

Las investigaciones actuales indican que existen dos principales tipos de células atendiendo al tipo de muerte celular mayoritariamente involucrado (**10**):
-Células de tipo 1: cuya muerte está regulada por receptores localizados en la membrana celular (vía **extrínseca**).

-Células de tipo 2: en cuya muerte celular participa de forma mayoritaria la mitocondria como señal de expansión del proceso apoptótico (vía **intrínseca**).

Sin embargo, existen evidencias de que ambos tipos de muerte celular por apoptosis están conectados y que las moléculas de una ruta pueden influir en la otra (**17**).

2.3.1 Ruta de apoptosis extrínseca

La ruta de señalización extrínseca involucra a los receptores de muerte celular de la superfamilia de genes del receptor del TNF (**18**). La superfamilia de receptores al TNF incluye 8 receptores: TNF-R1 (p55, DR1), Fas/APO-1 (CD95, DR2), DR3, receptor al ligando inductor de apoptosis relacionados con TNF-α de tipo 1 (TRAIL-R1, DR4) y de tipo 2 (TRAIL-R2, DR5), DR6, ectodisplasina A (EDAR) y el receptor del factor de crecimiento nervioso (NGFR) (**19-23**). Los miembros de esta superfamilia de receptores presentan dominios extracelulares ricos en cisteína similares y poseen un dominio citoplásmico denominado "dominio muerte" (DD) (**24**). Los DD juegan un papel crucial en la transmisión de la señal de muerte desde la superficie celular hacia las rutas de señalización intracelular.

2.3.2 Ruta de apoptosis intrínseca

Diversos estímulos no mediados por receptores son canalizados por el tipo de muerte celular intrínseca que producen señales intracelulares que actúan sobre los procesos de muerte celular asociados a la mitocondria. La ruta de inducción de apoptosis intrínseca depende básicamente del tipo celular implicado. En este sentido, los hepatocitos tienen reducida la señal de muerte celular dependiente de la vía extrínseca principalmente dependiente de caspasa-8, con utilización de vías alternativas (Bid, etc) que dirigen la señal hacía la mitocondria. Existen también estímulos celulares independientes de la activación de receptores, como radiación, toxinas, hipoxia, hipertermia, infecciones virales y radicales libres, que pueden conllevar la activación de la vía extrínseca de muerte celular.

La liberación de factores pro-apoptóticos en mitocondria se inicia la apertura de un poro de transición relacionado con la permeabilidad mitocondrial (Poro de Transición de Permeabilidad o PTP), pérdida de potencial mitocondrial (Potencial Mitocondrial Transmembrana o MTP) y liberación de una serie de proteínas presentes en la membrana interna y espacio intermembrana mitocondrial como el citocromo c, Smac/DIABLO y la serina-proteasa HtrA2/Omi, AIF, endonucleasa G y CAD (**25-27**).

3. ESTRÉS OXIDATIVO

El estrés oxidativo se produce como consecuencia de una elevada producción de ERO que excede la capacidad antioxidantes de las células (**28**). Las ERO se producen como consecuencia de la actividad metabólica celular o tras una respuesta inflamatoria, y representa una constante amenaza en un entorno aeróbico en las células vivas que puede dañar severamente el ADN, proteínas y lípidos (**29**). Las ERO juegan un papel esencial durante la defensa frente a las infecciones como consecuencia de la actividad fagocítica de las células del sistema inmune (**30**). Sin embargo, las ERO son igulamente mediadores de señal intracelular en los procesos de supervivencia celular, participando en el balance redox celular y en la expresión de nuevos genes antioxidantes o metabolización de fármacos (**30-32**).

Las ERO es un término que incluye diversas especies como el anión superóxido ($O_2^{\cdot-}$), y los radicales hidroxilo ($^{\cdot}OH$), peroxilo ($RO_2^{\cdot}$), hidroperoxilo ($HO_2^{\cdot}$) y alcoxilo ($RO^{\cdot}$) que presentan un electrón (e^-) desapareado en último orbital de su estructura molecular. Asimismo, se incluyen en este grupo especies no radicales derivadas del O_2 como el peróxido de hidrógeno (H_2O_2) (**33**). La mayor fuente de radicales libres del oxígeno es la cadena respiratoria mitocondrial, aunque también se producen como consecuencia de la actividad del CYP450, xantina oxidasa, y metabolización del ácido araquidónico.

Los sistemas antioxidantes celulares pueden clasificarse en enzimáticos y no enzimáticos en función de la participación de una enzima que catalice la disminución de las ERO. Los antioxidantes no enzimáticos incluyen el glutatión reducido (GSH) el α-tocoferol (vitamina E), ácido ascórbico (vitamina C), flavonoides naturales, carotenoides, ácido lipoico, melatonina y otros componentes (**34**). Las defensas antioxidantes enzimáticas incluyen la superóxido dismutasa (SOD), catalasa (CAT), glutatión peroxidasa (GPx), hemoxigenasa (HO), tiorredoxina (Trx), peroxirredoxina (Prx) y glutarredoxina (Grx) (**35**) (*Figura 10*).

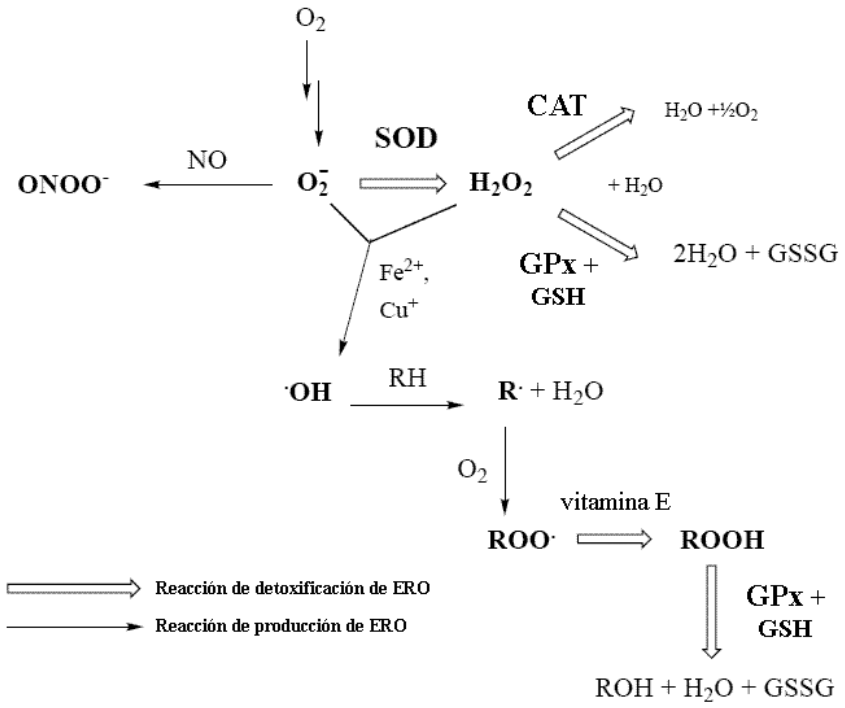

Figura 10: **Sistema de detoxificación de especies reactivas de oxígeno (ERO).** Las ERO son especies relacionadas con el oxígeno con elevada reactividad como consecuencia de la presencia de un electrón desapareado en su último orbital molecular. Las ERO más importantes son el anión superóxido (O2·-), y los radicales hidroxilo (·OH), peroxilo (RO2·), hidroperoxilo (HO2·) y alcoxilo (RO·), así como el peróxido de hidrógeno (H_2O_2) que no es un radical libre pero que participa en reacciones radicalarias. Las enzimas antioxidantes: superóxido dismutasa (SOD), glutatión peroxidasa (GPx) y catalasa (CAT). Si existe un desbalance entre producción de radicales libres y contenido de antioxidantes se genera estrés oxidativo.

3.1 Antioxidantes no enzimáticos

3.1.1 El glutatión

El glutatión reducido (GSH) es una molécula con grupos tiólicos con gran capacidad antioxidante y redox. La formula química es un tripéptido formado por L-cisteína (Cys), ácido L-glutámico (Glu) y glicina (Gly), con un enlace peptídico inusual entre el grupo amino de la cisteína y el grupo carboxilo de la cadena lateral del glutamato. El GSH es nucleofílico en azufre y ataca los aceptores conjugados electrofílicos tóxicos (**36**). El GSH es muy abundante en el citosol (2-10 mM) (**37**), núcleo (15 mM) (**37**), retículo endoplásmico (4-5 mM) (**38**) y mitocondria (5-10 mM) (**39**). El GSH es sintetizado en el citosol mediante la acción secuencial de la glutamato-cisteína ligasa (GCL) y glutamato sintetasa (GS). El GSH tiene la capacidad de reducir cualquier enlace disulfuro formado entre resíduos de cisteína dentro de proteínas

citoplasmáticas. En este proceso se genera la forma oxidada o GSSG (*Figura 11*). El GSSG se reduce a GSH mediante la glutatión reductasa (GR) que se expresa de forma constitutiva y que se incrementa su expresión durante el estrés oxidativo.

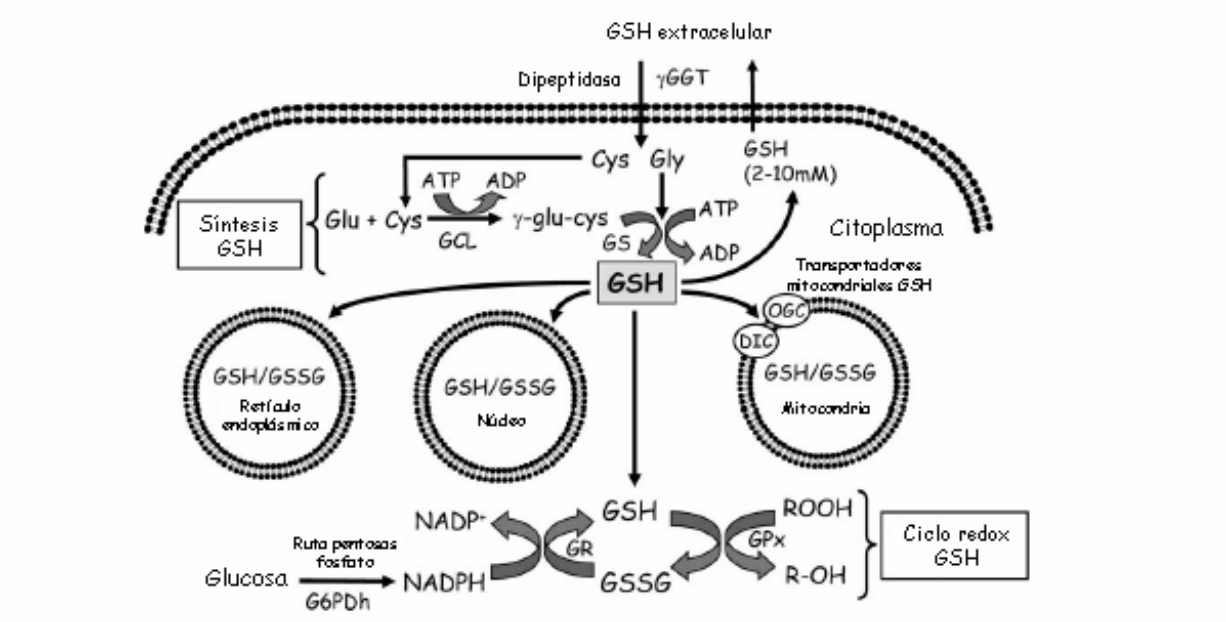

Figura 11: **Homeostasis del GSH: síntesis, ciclo redox y compartimentación**. El GSH se sintetiza mediante la acción de la glutamato-cisteína ligasa (GCL) y glutatión sintasa (GS). La hidrólisis de GSH extracelular es catalizada mediante γ-glutamil transpeptidasa (GGT) y dipeptidasa, y sus precursores (Cys y Cys) son transportados mediante transportadores de membrana para la síntesis intracelular de GSH. En el ciclo redox de GSH, la reducción de GSSG, generado por glutatión peroxidasa, es catalizada por la glutatión reductasa (GR). En la ruta de las pentosas fosfato, el NADPH es un donador de electrones para la reducción de GSSG. El GSH está localizado en distintos compartimentos celulares (mitocondria, núcleo y retículo endoplásmico). Los transportadores de tipo dicarboxilato (DC) y oxo-glutarato (OGC) permiten la internalización de GSH en la mitocondria. (Imagen procedente del articulo: Glutathione and apoptosis. Circu ML, Aw TY. *Free Radical Research*. 2008; 42: 689-706).

El GSH actúa como antioxidante mediante su interacción con ERO o actuando como cofactor de diversas enzimas antioxidantes, tales como GSH peroxidasas y GSH-S-transferasas (**40**). También participa en el transporte de aminoácidos a través de la membrana plasmática (**40**). Y además, es capaz de regenerar otros antioxidantes (vitaminas C y E) devolviéndoles a su forma activa (**40**).

3.1.2 La vitamina E

La vitamina E es una forma molecular que engloba a 8 isómeros: α-, β-, γ- y δ-tocoferol con un anillo aromático y una cadena monosaturada, y α-, β-, γ- y δ-tocotrienoles con una cadena monoinsaturada. El α-tocoferol es la forma principal de

vitamina E presente en plasma y tejidos humanos (**41**), que por su naturaleza lipídica se considera el antioxidante más importante para prevenir la lipoperoxidación de las membranas celulares (**42**). Asimismo, el α-tocoferol regula la expresión de genes involucrados en su propio metabolismo y fármacos, transporte lipídico, inflamación, adhesión celular, fibrosis, señalización celular y regulación del ciclo celular (**43**). Entre los primeros, cabe citar al citocromo CYP3A4 que participa en el catabolismo oxidativo de tocoferoles y que su expresión se ve incrementada por la administración de α-tocoferol en hepatocitos (**44, 45**). El α-tocoferol interacciona con el ácido ascórbico previniendo la oxidación de lipoproteínas de baja densidad (LDL) (**46**) (*Figura 12*).

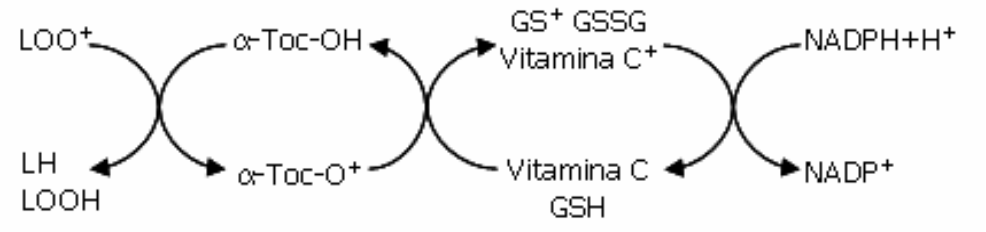

Figura 12: **Mecanismo antioxidante de tocoferoles**. El radical hidroxilo libre (OH) del anillo aromático del α-tocoferol es el responsable de las propiedades antioxidantes de dicho vitamero. El átomo de hidrógeno de dicho radical es donado al radical libre del peróxido lipídico (LOO^+), resultando una forma estable de vitamina E como radical libre (α-Toc-O^+: radical α-tocoferol) y una molécula lipídica (LH) estabilizada, liberándose un peróxido lipídico (LOOH). Todo esto lo puede realizar el α-tocoferol de forma conjunta con la vitamina C y el glutatión (GSH), los cuales pasan a estado oxidado (vitamina C^+: radical vitamina C, GS^+: radical glutatión, GSSG: glutatión en forma oxidada). Con la participación de NADPH (nicotinamida adenina dinucleótido difosfato reducido) y H^+ (protón), pasando a estado oxidado ($NADP^+$) (Imagen procedente del artículo: Alpha-tocopherol: looking beyond an antioxidant. Engin KN. *Molecular Vision*. 2009; 15: 855-60).

3.2 Antioxidantes enzimáticos

3.2.1 Superóxido Dismutasa (SOD)

Constituye una de las primeras barreras de defensa antioxidante que cataliza la reacción de eliminación de $O_2^{\cdot-}$ mediante su transformación en H_2O_2, el cual puede ser posteriormente eliminado por la acción de la catalasa o glutatión peroxidasa.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$$

Existen tres tipos de SOD (**47**): **Mn-SOD mitocondrial** (con un átomo de Mn en su sitio activo), **Cu/Zn SOD citosólica** (con un átomo de cobre y otro de zinc en su

sitio activo unidos por interacciones hidrofóbicas y electrostáticas) y **EC-SOD extracelular** (de naturaleza glicoproteica). La regulación de la expresión de SOD tiene lugar por acción de citoquinas y mediadores inflamatorios (**48**).

3.2.2 Catalasa (CAT)

La catalasa es una de las enzimas conocidas más eficientes, ya que no puede ser saturada por sustrato, que cataliza la reacción:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

La CAT es un tetrámero de 4 cadenas polipeptídicas con 4 grupos hemo porfirina que permiten a la catalasa reaccionar con el H_2O_2. La CAT se localiza normalmente en los peroxisomas. Aunque la catalasa no es esencial para algunos tipos de células en condiciones normales, tiene un importante papel en la adquisición de tolerancia al estrés oxidativo en la respuesta adaptativa de las células.

3.2.3 Glutatión peroxidasa (GPx)

La GPx es una proteína formada por cuatro subunidades idénticas, y cada una de ellas contiene un residuo de selenocisteína esencial para su actividad enzimática. Es capaz de catalizar la reacción llevada a cabo por la catalasa. Asimismo, puede catalizar la reducción de diferentes hidroperóxidos (ROOH y H_2O_2) usando el poder reductor del GSH según la reacción:

$$ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$$

En mamíferos se han identificado 7 isoenzimas de GPx presentes en diferentes tejidos y estructuras celulares: **GPx1** (localizada en el citoplasma celular), **GPx2** (localizada en citoplasma, y sintetizada en hígado y tracto gastrointestinal), **GPx3** (se secreta al plasma), **GPx4** (se encuentra en citosol, núcleo y mitocondria), **GPx5** (secretada en el epidídimo genital), **GPx6** (secretada y expresada en el epitelio olfativo) y **GPx7** (se secreta al medio extracelular). La GPx4 es la que presenta mayor capacidad antioxidante, ya que reduce directamente los fosfolípidos hidroperóxidos existentes en membranas y lipoproteínas (**49**).

3.2.4 Hemoxigenasa (HO)

Es una enzima que juega un papel fundamental en los mecanismos de defensa del organismo frente a los procesos oxidativos mediados por el radical hemo (**50**). Existen 3 isoformas de HO según el tipo celular en donde se expresa: **HO-1** (células de Kupffer) (**52**), **HO-2** (células parenquimales hepáticas) (**53**), **HO-3** (cerebelo, hipocampo y cortex cerebral) (**54**). La HO de tipo 1 degrada el grupo hemo con la participación de tres moléculas de oxígeno y de NADPH, originando una molécula de hierro (Fe^{2+}), monóxido de carbono (CO) y biliverdina IXα (*Figura 13*). La biliverdina es posteriormente reducida a bilirrubina por la enzima biliverdina reductasa utilizando como cofactor el NADPH. Se ha descrito que el CO generado tiene efectos antiinflamatorios, antioxidantes, antiapoptóticos, antiproliferativos y vasodilatadores. También se ha descrito que la biliverdina y bilirrubina tienen propiedades antioxidantes (**51**).

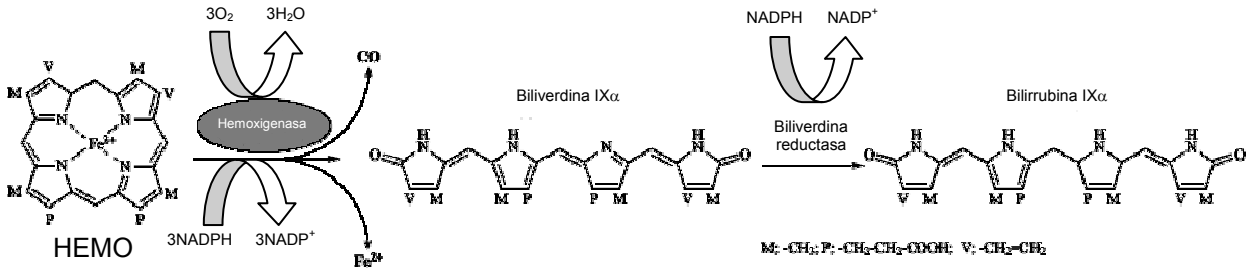

Figura 13: **Catálisis del grupo hemo por hemoxigenasa**. (Imagen modificada del artículo: Heme oxygenase-1: a fundamental guardian against oxidative tissue injuries in acute inflammation. Takahashi T, et al. *Mini-Reviews in Medicinal Chemistry*. 2007; 7: 745-53).

La expresión de HO-1 se induce durante los estados de estés oxidativo por radiación ultravioleta (UV), H_2O_2, metales pesados (cadmio, cobalto), hipoxia, lipopolisacárido (LPS) y NO (**55**).

3.2.5 Tiorredoxina (Trx)

La tiorredoxina (Trx) es uno de los principales sistemas de control redox celular. Existen dos isoformas: **Trx-1** (localizada en citoplasma) y **Trx-2** (situada en mitocondria). Ambas Trx son selenoproteínas mantenidas en estado reducida mediante la acción de la tiorredoxina reductasa-1 (TrxR1) y -2 (TrxR2) respectivamente, y NADPH (**56, 57**). A través de la reacción redox reversible de la Trx-1 se puede regular la actividad de diferentes sustratos proteicos en numerosas rutas, incluyendo miembros

de la familia de las peroxirredoxinas (Prx) que tienen como función principal la degradación de H_2O_2 (**58**). La Trx 1 y 2 comparten sistemas de acción similares (*Figura 14*).

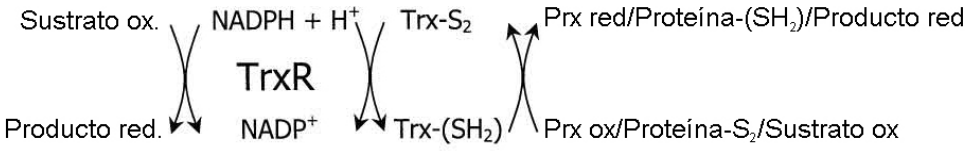

Figura 14: **Reacciones enzimáticas del sistema tiorredoxina**. La enzima tiorredoxina reductasa (TrxR) reduce directamente el centro activo disulfuro presente en la proteína tiorredoxina (Trx) y en otros diversos sustratos, bajo el consumo de NADPH. La Trx es altamente eficiente reduciendo disulfuros en proteínas y péptidos, incluyendo las peroxirredoxinas (Prx) y el glutatión oxidado (GSH). (Imagen modificada del artículo: Physiological functions of thioredoxin and thioredoxin reductase. Arner E, Holmgren A. *European Journal of Biochemistry*. 2000; 267: 6102-9).

Las Trx participan en el control redox asociado a diversas patologías en donde el estrés oxidativo tiene un papel relevante como en isquemia aguda tisular (**59**). Se han detectado niveles plasmáticos elevados de Trx en hepatocarcinoma (**60**) y SIDA (**61**).

3.2.6 Peroxirredoxina (Prx)

Las Prx son un grupo de peroxidasas que contribuyen al control redox celular gracias a su capacidad de eliminar hidroperóxidos orgánicos (LOOH) y H_2O_2 (**58**). Todas las Prx poseen un sitio catalítico, cisteína peroxidásica (cisteína reactiva, Cys-S_pH), que es oxidado a ácido sulfénico (Cys-SOH) por el peróxido (**62**). Dicho ácido rápidamente forma puentes disulfuro con otro residuo de cisteína en la subunidad C-terminal. La regeneración de la cisteína es catalizada por el sistema Trx/TrxR (**63**) (*Figura 15*).

Existen diferentes tipos de Prx según su localización intracelular: citoplasma (**Prx I** y **III**), mitocondria (**Prx II** y **V**), medio extracelular (**Prx IV**) y peroxisomas (**Prx V**).

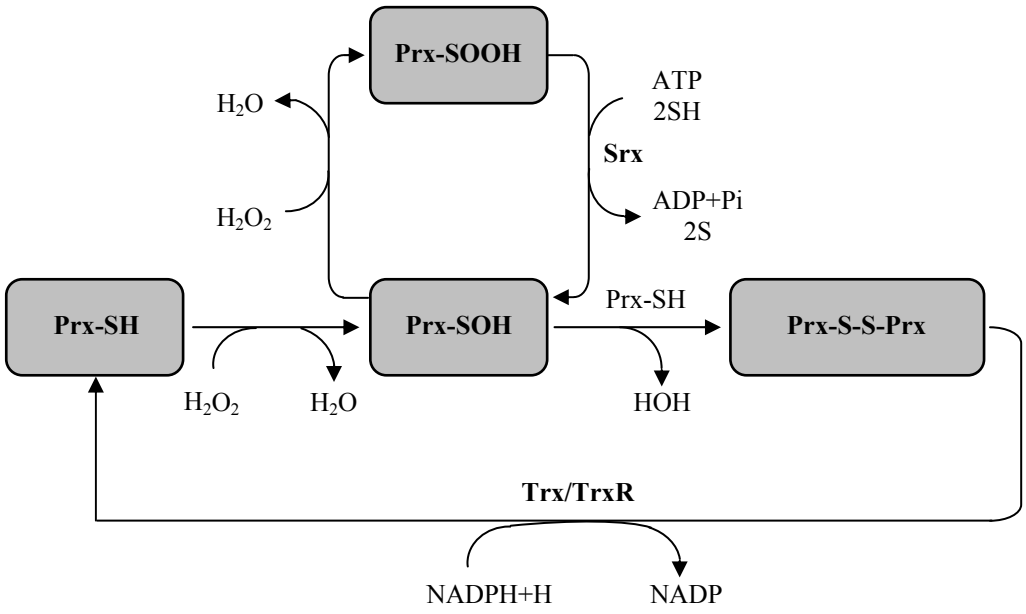

Figura 15: **Reacción catalítica cíclica de las peroxirredoxinas**. La cisteína peroxidásica de la peroxirredoxina (Prx) está representada como Prx-SH, la cuál sufre una reacción de peroxidación con la ayuda de H_2O_2. Posteriormente se forma un dímero de Prx unidas por puente disulfuro (Prx-S-S-Prx). El sistema tiorredoxina/tiorredoxina reductasa (Trx/TrxR) regenera los residuos de Cys de las Prx, con la ayuda de NADPH. La Prx puede ser inactivada por un exceso de oxidación del grupo tiol formándose Prx-SOOH, y revertiéndose esta reacción mediante sulfirredoxinas (Srx) con utilización de dos moléculas de sulfidrilo (SH) e hidrólisis de ATP.

3.2.7 Glutarredoxina (Grx)

La Grx es capaz de reducir los puentes disulfuro según el esquema de la *Figura 16* **(64).**

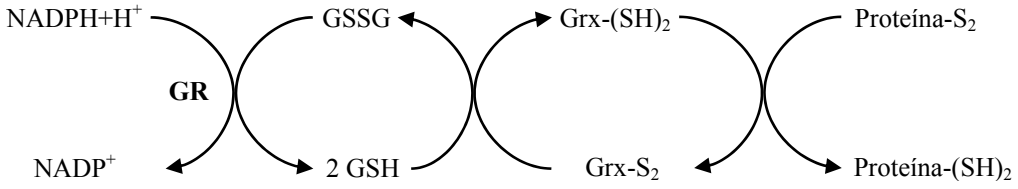

Figura 16: **Mecanismo general de acción del sistema de las glutarredoxinas**: en dicho sistema los electrones son transferidos desde el NADPH a la glutatión reductasa (GR), posteriormente a las dos moléculas de glutatión (2 GSH) y finalmente a la molécula de Grx. El puente disulfuro establecido en el sitio activo de la Grx ($Grx-S_2$) se rompe reduciéndose a dos moléculas de tiol (SH) unidas a la enzima. La reacción de formación del puente disulfuro a partir de las moléculas de SH permite la reducción del puente disulfuro de una proteína diana ($Proteína-S_2$). (Imagen procedente del articulo: Glutaredoxins: glutathione-dependent redox enzymes with functions far beyond a simple thioredoxin backup system. Fernandes AP, Holgren A. *Antioxidant and Redox Signaling*. 2004; 6: 63-74).

Las Grx son enzimas involucradas en diferentes procesos celulares. Se han propuesto dos mecanismos diferentes para explicar la actividad de las Grx, utilizando uno o los dos residuos de Cys presentes en su sitio activo (**65**):

- Mecanismo monotiólico: tan solo el residuo de Cys del extremo N-terminal de la Grx es requerido para la reducción de las moléculas diana.
- Mecanismo ditiólico: la Grx puede reducir moléculas de bajo peso molecular y proteínas disulfuro utilizando los dos residuos de Cys de su sitio activo.

En mamíferos se han identificado dos Grx ditiólicas (Grx 1 localizada en citoplasma y núcleo, y Grx 2 localizada en mitocondria y núcleo) (**66**) y una monotiólica (Grx 5 localizada en matriz mitocondrial) (**67**). La **Grx 1** está involucrada en múltiples procesos biológicos como la transferencia de poder redox a la ribonucleótido reductasa (**68**), regula la actividad de factores de transcripción (**69**), protege a las células frente a estrés oxidativo (**70**) y lleva a cabo la S-glutationilación reversible de proteínas (**71**). En cuanto a la **Grx2**, juega un papel fundamental en la respuesta mitocondrial al estrés oxidativo y en la señalización redox actuando como catalizador reversible de la S-glutationilación de las proteínas (**72**). La **Grx5** está involucrada en la biogénesis del grupo Fe-S en la matriz mitocondrial (**73**), los cuales se unen transitoriamente a Grx5 para la biosíntesis del grupo hemo (**67**). Además, esta Grx tiene la capacidad de reducir proteínas con puentes disulfuro (**74**).

3.3 Regulación génica del estrés oxidativo

3.3.1 Antioxidantes no enzimáticos

3.3.1.1 GSH

El factor nuclear eritroide de tipo 1 (Nrf1) y 2 (Nrf2) a través de los factores de transcripción NF-*k*B y AP-1 (**75, 76**), Jun y Maf (**77-79**) y c-Myc (**80**) regulan la expresión de la subunidad catalizadora (GCLC) y moduladora (GCLM) de GCL en condiciones de estrés. Además, la elevación brusca de la concentración citoplasmática de Ca^{2+} en condiciones patológicas inhibe la actividad de GCLC (**81**). La expresión de GS es regulada mediante los factores Nrf1 y 2 con la participación de los factores AP-1 y NF-*k*B (**82-84**). La expresión del enzima que proporciona un aporte continuado de císteina, γ-glutamil transpeptidasa (**GGT**), está regulada por el factor Nrf2 a través de NF-*k*B (**85**).

3.3.1.2 La vitamina E

La regulación de la vitamina E se puede entender a varios niveles:

a) Absorción y suministro de vitamina E a los tejidos:

La vitamina E se incorpora a lipoproteínas (HDL y LDL, lipoproteínas de alta y baja densidad respectivamente) y de proteínas transportadoras (**α-TTP**, proteína transportadora de α-tocoferol) (**86**) para su distribución a los distintos tejidos (**87, 88**) tras la ingesta. Las células diana presentan receptores a las lipoproteínas que facilitan la transferencia de la vitamina E al espacio intracelular en donde se une a la proteína asociada a tocoferol (**TAP)** para distribuirse a los diferentes orgánulos subcelulares (**89**). Los niveles de vitamina E regulan la expresión de α-TTP (**90**). Se ha visto que la proteína específica 1 (Sp1) (**91**) y proteínas de unión a elementos de respuesta a esterol (SREBP) (**91**) regulan la transcripción del gen LDLR con la participación de PI3K (fosfatidilinositol 3-quinasa) y ERK 1/2 (**91**).

b) Metabolismo de la vitamina E:

El α-tocoferol sufre una serie de oxidaciones secuenciales a través de diversas isoformas de citocromo P450 que conllevan la producción de α-carboxietil hidroxicromano (α-CEHC) (*Figura 17*) (**92**).

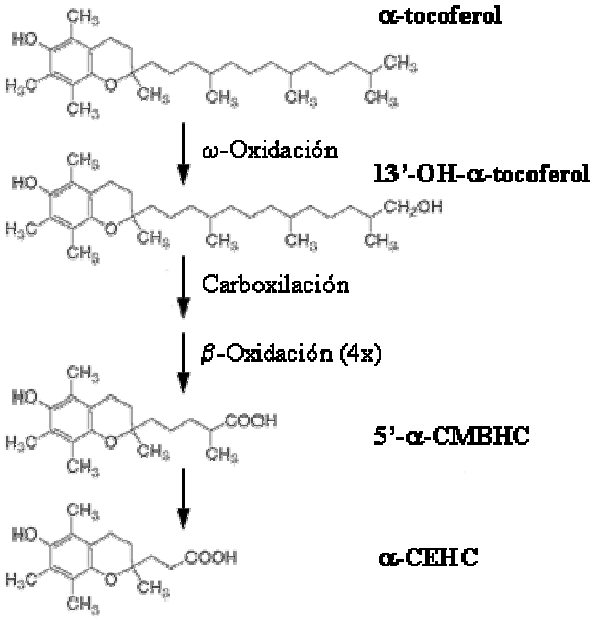

Figura 17: **Esquema del metabolismo del α-tocoferol** (Imagen procedente del articulo: Vitamin E. Mustacich DJ, et al. *Vitamins and Hormones*. 2007; 76: 1-21).

La mayor parte de los CEHC sufren conjugaciones enzimáticas posteriores catalizadas por sulfotransferasas (SULTs) ó uridina difosfato glucuronosil transferasas (UGTs) (ambas pertenecen al grupo de enzimas metabolizadoras de drogas de fase II), originando ésteres de sulfato y productos glucurónicos respectivamente (**93**).

3.3.2 Antioxidantes no enzimáticos

3.3.2.1 Superóxido Dismutasa

Se han determinado diversos factores de transcripción que regulan la expresión de la SOD. NF-*k*B mediante la ruta PI3K/PKB (**94**) y Sp1 junto con la proteína estimuladora de unión a la secuencia CCAAT (C/EBP) regulan la transcripción basal de SOD (**95**). De forma distinta, AP-1 (**96**) y AP-2 (**97**) (proteínas activadoras 1 y 2) regulan la transcripción de SOD Cu/Zn a nivel basal y en respuesta a diferentes estímulos. La regulación de la expresión basal e inducida de la SOD Mn está regulada por NF-*k*B (**98**), Sp1 (**99**, **100**), p53 (**101**), AP-1 (**95**), AP-2 (**102**) y C/EBP (**103**). La expresión de EC-SOD estña regulada por NF-*k*B (**104**), Sp1/Sp3 (**105**) y C/EBP (**106**). También a nivel postranscripcional existen unas regiones no traducidas de los genes de la SOD en posición 3' del gen, 3'UTR, que intervienen en la estabilización y traslación del ARNm (ARN mensajero) (**107**-**109**).

3.3.2.2 Catalasa

En el extremo 5' del promotor del gen de la CAT existe una secuencia CCAAT en la cual se localiza un sitio de unión al factor de transcripción unido a caja CCAAT (CTF) y factor nuclear 1 (NF1) que actúan como represores de su transcripción (**110**). Elementos de respuesta al receptor activado por proliferadores de peroxisomas (PPAR) a los que se une PPARγ actúan como inductor de la transcripción de CAT en respuesta a situaciones de estrés celular (**111**). Otras secuencias reguladoras relacionadas con Sp1, NF-Y, NF-kB, AP-1 y AP-2 han sido identificadas en el promotor del gen (**112**, **113**).

3.3.2.3 Glutatión peroxidasa

La enzima GPx1 es una selenoproteína regulada por estrógenos a través de NF-*k*B (**114**). Otros factores de transcripción, como el factor de células T (TCF) junto con β-catenina (**115**), y Nrf2 (**116**) regulan la actividad del promotor de GPx2. La expresión de GPx4 se ve incrementada como consecuencia de la acción de NF-*k*B, NF-Y, Sp1 y miembros de la familia de proteínas Smad (proteínas homologas de las proteínas madre contra decapentaplegico de *Drosophila* y la proteína SMA de *C. elegans*) (**117**,

118), así como se ve reducida por el factor de respuesta temprana al crecimiento de tipo 1 (EGR1) y SREBP1 (**119**). Sin embargo, la transcripción de GPx1 se ve estimulada por p53 (**120**), así como la de GPx3 lo es por AP-1 y Sp1/ factor inducible por hipoxia-1α (HIF-1α) (**121**, **122**) en condiciones de estrés oxidativo. Los niveles de selenio (Se) incorporados a través de la dieta tienen un efecto inductor en la expresión de ARNm y de su actividad (**123**).

3.3.2.4 Hemoxigenasa

La transcripción del gen de la HO está regulada por la quinasa regulada por señales extracelulares (ERK), quinasa del extremo N-terminal de c-Jun (JNK), p38, proteína quinasa C (PKC) y PI3K/PKB (proteína quinasa B) (**124-126**) a través de AP-1, NF-*k*B y Nrf2 (**127-129**) en condiciones de estrés oxidativo. Los niveles bajos de grupo hemo inducen una represión de la transcripción de HO-1 a través del factor de transcripción Bach1 (**130**).

3.3.2.5 Tiorredoxina

La inducción de estrés oxidativo o choque térmico incrementan los niveles del factor de transcripción de choque térmico de tipo 1 (HSF-1) y de tipo 2 (HSF-2) que se unen a elementos de choque térmico (HSE), así como de Nrf2 que se une a los elementos de respuesta antioxidante (ARE), ambas secuencias presentes en el promotor de Trx (131, 132). La exposición a ácido retinoico (vitamina A) y retinoides activan una serie de receptores de ácido retinoico (RAR) y receptores RXR (receptor X retinoico) que se unen a elementos de respuesta a ácido retinoico (RARE) y promueven la transcripción del promotor de Trx (**133**).

3.3.2.6 Peroxirredoxina

La inducción de estrés oxidativo incrementa los niveles de AP-1 y Nrf2 que induce la transcripción de Trx1 (**134-136**).

3.3.2.7 Glutarredoxina

La activación de AP-1 también es el responsable del aumento de la transcripción del promotor de Grx bajo condiciones de estrés oxidativo (**137**, **138**). La expresión de Grx2 también está regulada postranscripcionalmente mediante “splicing” (ensamblaje) alternativo (**139**).

4. ESTRÉS NITROSATIVO

El NO es un radical libre formado a partir de L-arginina (Arg) mediante una reacción catalizada por la óxido nítrico sintasa (NOS) de la cual existen 3 isoformas: neuronal (**nNOS** ó NOS-1), inducible (**iNOS** ó NOS-2) y endotelial (**eNOS** ó NOS-3) (**140**). La NOS-2 se expresa en numerosos tipos celulares y su expresión se incrementa de forma muy importante (inducible) por distintos mediadores inflamatorios. La NOS-1 y NOS-3 están presentes de forma basal (constitutiva) en diversos tipos celulares aunque su expresión se puede incrementar de forma moderada en diversas condiciones fisiopatológicas (**140**). El **estrés nitrosativo** se produce cuando existe una producción exacerbada de especies reactivas de nitrógeno (ERN) que excede la capacidad del organismo para neutralizarlas y eliminarlas (**141**).

4.1. Rutas de acción de NO dependientes de GMPc

La actividad biológica del NO está clasificada por rutas dependientes e independientes de GMPc (guanosín monofosfato cíclico) dependiendo de las condiciones fisiológicas y patológicas (**142**). Proteínas quinasas dependientes de GMPc, canales iónicos asociados a nucleótidos cíclicos y fosfodiesterasas reguladas por GMPc median diversos efectos celulares (**142**).

4.2. Rutas de acción de NO independientes de GMPc

En la célula se pueden distinguir dos tipos de reacciones producidos por el NO que se producen durante el estrés nitrosativo:

Los grupos tioles de los resíduos de Cys de las proteínas son dianas críticas para las ERN (en particular N_2O_3) (**143**). La reacción del NO más importante y reconocida es la llevada a cabo con los grupos tiólicos de los resíduos de Cys, denominándose a dicha reacción **S-nitrosilación** ó S-nitración, permitiendo la formación de nitrosotioles más estables (**142**). Dichos nitrosotioles pueden formarse por una reacción directa con NO ó mediante una reacción de nitrosilación de GSH generando nitrosotioles (S-nitrosoglutatión ó GSNO). Probablemente la presencia de secuencias diana específicas que permiten la accesibilidad de los grupos tioles al NO determine el patrón de proteínas nitrosiladas. La oxidación de Cys produce la modificación denominada S-nitrosilación que es reversible por las proteínas reguladoras del estado redox celular (Trx y Grx). El efecto de NO sobre la regulación génica es variable pudiendo actuar sobre factores de transcripción y proteínas, tales como NF-*k*B, AP-1, p53, HIF-1α, y

otros más, mediante modificaciones en resíduos de Cys permitiendo o impidiendo la unión al ADN para activar o inhibir la transcripción de diversos genes (**142**). Además el NO puede inducir la transcripción génica de forma indirecta mediante la activación/modulación de rutas de señalización en las que intervienen MAPK (proteína quinasa activada por mitógeno), proteínas G ó PI3K (**142**). S-nitrosilación de proteínas se ha detectado en condiciones de citotoxicidad hepatocelular (**144**), enfermedad neurodegenerativas (**145-147**), etc.

La segunda modificación postranslacional es la denominada **nitración** en residuos de tirosina (Tyr) que consiste en la transferencia del grupo nitro ($-NO_2$) del $ONOO^-$ al residuo de Tyr de las proteínas para formar 3-nitrotirosina (**148**). Esta modificación altera la conformación y actividad de la proteína (**149**). Esta modificación se ha detectado en hepatocitos sometidos a citotoxidad (**150**).

4.3 Regulación de la expresión y actividad de la óxido nítrico sintasa

La actividad de la NOS-1 y NOS-3 se encuentran reguladas por la concentración intracelular de Ca^{2+} (**151**). La transcripción de la NOS-3 está regulada por las quinasas Rho/rho (**152**), y por los factores de transcripción como el factor 2 de Kruppel asociado a pulmón (KLF2) (**153**), AP-1 y SP1 (**154, 155**). También se ha observado que la estabilidad del ARNm de NOS-3 está influenciada por los niveles de LDL oxidada y trombina, y por los procesos inflamatorios e hipoxia (**156**, **157**). Además, se ha demostrado una regulación postranscripcional de la NOS-1 por "splicing" alternativo (**158**, **159**).

La NOS-2 se encuentra regulada principalmente a nivel transcripcional tras estimulación de las células por citoquinas pro-inflamatorias (IFN-γ, IL-1β y TNF-α) que promueven la activación de una serie de quinasas y factores de transcripción como MAPK, NF-*k*B, quinasa de la proteína Jano (JAK), señales transductoras y activadoras de la transcripción (STAT) y el factor 1 regulador de interferón (IRF1) que estimulan la transcripción de la NOS-2 (**160-162**). La degradación proteolítica de NOS-2 es dependiente del sistema del proteosoma (**163**).

5. LA MITOCONDRIA

Son unos orgánulos celulares delimitados por una membrana mitocondrial externa y otra interna separados por un espacio intermembrana y que ambas limitan la matriz mitocondrial (**164**) (*Figura 18*). El tamaño mitocondrial varía entre 0,5-10 μm de diámetro y hasta 7 μm de longitud (**165**), y el número también según el tipo de organismo, tejido y las necesidades energéticas de la célula. Al conjunto de mitocondrias de la célula se denomina **condrioma celular**. La mitocondria tiene su propio genoma que codifica para 22 ARNt, ARNr 12S (Svedberg), ARNr 16S, 7 sub-unidades de la NADH deshidrogenasa (ND1-6, complejo I), citocromo b (complejo III), 3 sub-unidades de la citocromo c oxidasa (COX1-3, complejo IV) y 2 sub-unidades de la ATP sintasa (ATP6 y ATP8, complejo V). El resto de componentes de la cadena electrónica mitocondrial y sistemas enzimáticos están codificados por el genoma nuclear (**166**).

La membrana mitocondrial externa contiene proteínas que forman poros, llamadas porinas o **VDAC** (canal aniónico dependiente de voltaje) que permite el paso de proteínas de 5-6 kDa (**167**) y con secuencias específicas de señalización para ser transportadas a través de la membrana.

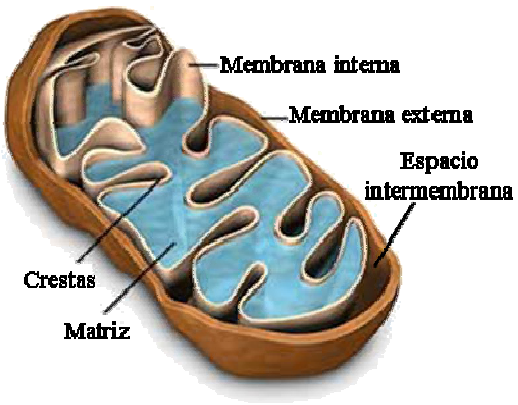

Figura 18: **Estructura de la mitocondria**.

La membrana mitocondrial interna es altamente impermeable a iones y solutos, y contiene canales iónicos específicos y sistemas de transporte. La membrana interna presenta invaginaciones denominadas **crestas** que incrementan la superficie funcional para la inserción de los complejos electrónicos encargados de generar el gradiente electrónico (potencial de membrana) y la fosforilación oxidativa celular. Las enzimas que componen la cadena respiratoria mitocondrial son:

1) El **complejo I** ó NADH deshidrogenasa está constituido por unos 40 componentes polipeptídicos, y contiene diversos grupos prostéticos (Fe^{2+}-S y flavina mononucleótido o FMN). El donador de electrones al complejo I es $NADH_2$ (nicotinamida adenina dinucleótido en forma reducida).
2) El **complejo II** ó succinato deshidrogenasa recibe los electrones del succinato a través de los grupos prostéticos flavina adenina dinucleótido (FAD) y Fe^{2+}-S.
3) El complejo I y II transfieren los electrones al **complejo III** a través del coenzima Q (ubiquinona). El complejo III ó citocromo bc_1 está compuesto por los citocromos b562, b566, c1 y c, y contiene diversos grupos prostéticos (Fe^{2+}-S y el grupo hemo). El complejo III cede los electrones al citocromo c.
4) El **complejo IV** ó citocromo c oxidasa contiene los citocromos a1 y a3, y contiene dos grupos hemo y dos átomos de Cu que están involucrados en los intercambios electrónicos. El complejo IV cede los electrones al O_2 para producir H_2O.
5) El gradiente de protones generado en el espacio intermembrana como consecuencia de la transferencia de electrones a lo largo de los complejos previamente descritos retorna a la matriz mitocondrial a través de un canal de H^+ denominado **ATP sintasa** (**complejo V**) que se acopla a la síntesis de ATP (fosforilación oxidativa).

En las crestas mitocondriales se encuentran proteínas transportadoras que permiten el paso de iones y moléculas (ácidos grasos, ácido pirúvico, ADP, ATP, O_2 y H_2O) entre las que se encuentran:

a) **Nucleótido de adenina translocasa**: encargada de transportar a la matriz mitocondrial el ADP citosólico formado durante las reacciones que consumen energía, translocando en paralelo hacia el espacio intermembrana el ATP recién sintetizado durante la fosforilación oxidativa.
b) **Fosfato translocasa**: transfiere el fosfato del espacio intermembrana junto con un H^+ (cotransporte) a la matriz. El fosfato es esencial para la fosforilación oxidativa.

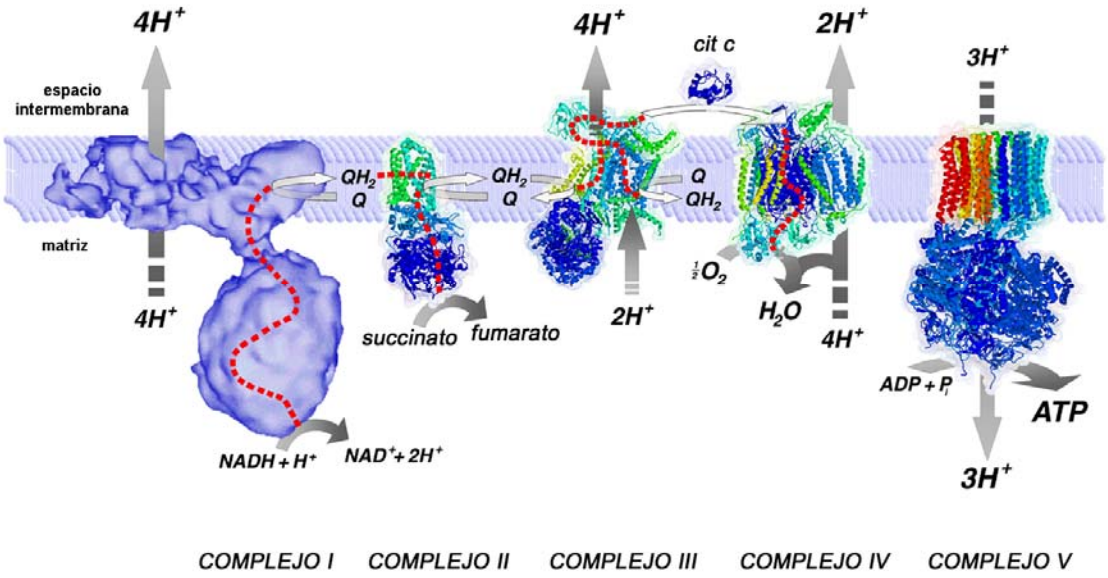

Figura 19: **Esquema de la cadena de transporte de electrones mitocondrial**.

En el espacio intermembrana se localizan diversos enzimas que intervienen en la transferencia del enlace de alto contenido energético del ATP (adenilato quinasa) y en el transporte de ácidos grasos desde el citosol hasta la matriz mitocondrial donde sufren una β-oxidación (carnitina). En este espacio se localizan diversos proteínas importantes que se liberan durante los procesos de muerte celular.

La matriz mitocondrial contiene iones, metabolitos a oxidar, ADN circular bicatenario, ribosomas tipo 70 Svedberg (que realizan la síntesis de algunas proteínas mitocondriales) y ARN mitocondrial. En dicha matriz tienen lugar diversas rutas metabólicas, como: ciclo de Krebs, β-oxidación de ácidos grasos, oxidación de aminoácidos, síntesis de la urea y grupos hemo.

La respiración mitocondrial depende de la propia actividad de la transferencia electrónica a través de la cadena de transporte electrónico, la cual está modulada por diversos factores entre los que se encuentra la disponibilidad de O_2, ADP, Pi, la magnitud del potencial mitocondrial, la actividad de las proteínas desacoplantes (UCP) y por la presencia del NO (**168**, **169**).

Papel de la mitocondria en el estrés oxidativo/nitrosativo y muerte celular

La producción de ERO es una consecuencia normal de la respiración mitocondrial aeróbica (5 %) como consecuencia de la salida de los e^- a nivel de complejo I y III y producción de $O_2^{\cdot-}$ (**170**, **171**). La presencia de Mn-SOD en la matriz mitocondrial cataliza la transformación del $O_2^{\cdot-}$ en H_2O_2. La producción masiva de ERO a nivel mitocondrial altera la estabilidad de los complejos mitocondriales y la fosforilación oxidativa (**172**), que puede comprometer la supervivencia de la célula (**173**).

Se ha descrito que el NO actúa como modulador de la respiración celular mediante su unión reversible al átomo de Cu del centro activo de la citocromo c oxidasa lo que inhibe parcialmente la transferencia de e^- al O_2 (**174**). Esto podría permitir limitar la producción de ERO en condiciones de hiperactividad mitocondrial. En estadios de elevada producción de ERO, se induciría la apertura del PTP y liberación del citocromo c que junto con la pro-caspasa-9 y ATP constituye el apoptosoma que es clave para la inducción de muerte celular por apoptosis en las células de tipo 2 (**175**).

6. SISTEMAS TRANSPORTADORES HEPÁTICOS

El hígado es el principal órgano involucrado en la metabolización de drogas y toxinas en el organismo (**176**). En este proceso participan una serie de enzimas denominados de fase I (citocromo P450), y de fase II (sulfotransferasas, quinona reductasas, UDP-glucuronosiltransferasas,…) que en conjunto incrementan la hidrofilicidad de los compuestos y facilitan su posterior excreción. Las drogas y compuestos lipofílicos atraviesan la membrana del sinusoide hepático por difusión pasiva. Mientras que los compuestos orgánicos polares requieren de transportadores específicos que pertenecen a la familia de transportadores con dominio de unión a ATP (ATP binding cassete, ABC). En el hígado, esas proteínas cumplen funciones de captación o excreción (**177**):

- **Transportadores con función de incorporación**: Se expresan en la membrana sinusoidal del hepatocito, y los distintos tipos se distinguen por las características físico-químicas de la molécula a transportar: proteína cotransportadora de ácido taurocólico dependiente de sodio (NTCP), polipéptidos transportadores de aniones inorgánicos (OATPs), transportadores de aniones inorgánicos (OATs) y transportadores de cationes orgánicos poliespecíficos (OCTs) (*Figura 20*). La proteína

NTCP reconoce drogas con estructura similar a ácidos biliares o unidas a taurocolato (**177**).

- **Transportadores con función de excreción**: estas proteínas de transporte son expresadas principalmente en la membrana lateral y canalicular del hepatocito, y por tanto eliminar compuestos hacia el sinusoide o al canalículo biliar, respectivamente. En la membrana lateral se encuentran proteínas asociadas a la resistencia a multidrogas (MRPs), cuya principal función es bombear drogas desde los hepatocitos hacia la sangre circulante, incluyen MRP1, 3, 4, 5 y 6 (**178**) (*Figura 20*). En la membrana canalicular, se encuentra: la glicoproteína P (proteína con resistencia a multidrogas 1, MDR1), MRP2, MDR3 y bomba exportadora de sales biliares (BSEP) (**179**) (*Figura 20*).

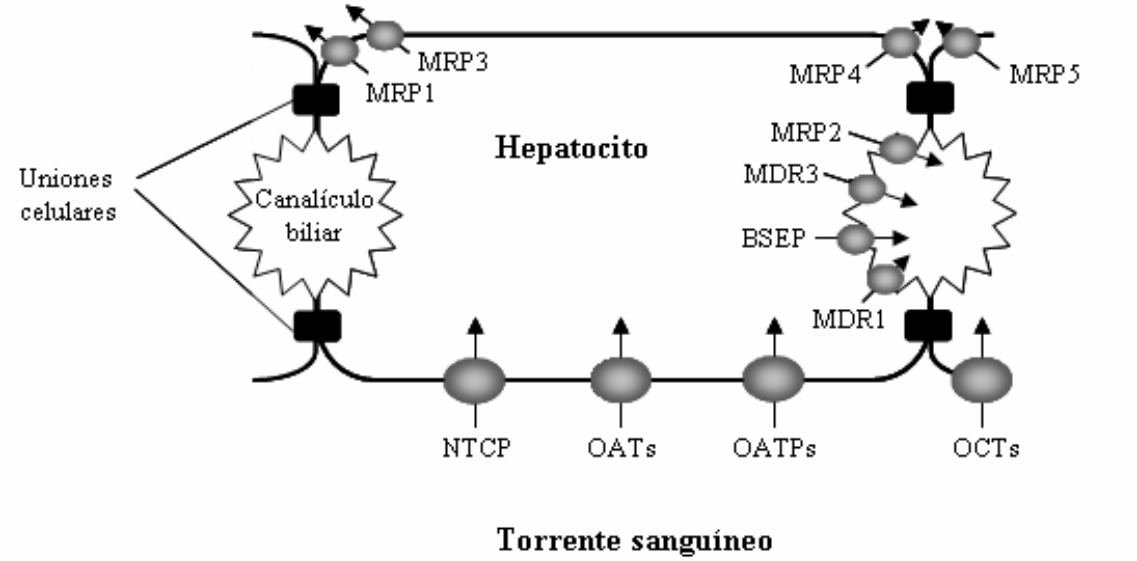

Figura 20: **Localización de los transportadores en el hepatocito**.

Las **interacciones droga-droga** inhiben la función de los transportadores hepáticos de drogas pudiendo en algunos casos modificar la disposición de los sustratos endógenos en el sistema hepatobiliar (ácidos biliares, bilirrubina) y hormona tiroidea (**180, 181**). Existen diferentes tipos de drogas que pueden inhibir el transporte de la bilirrubina (actuando sobre el transportador OATP1B1) o la excreción de ácidos biliares (reprimiendo a BSEP), provocando hiperbilirrubinemia o colestasis respectivamente (**181**).

Es especialmente importante para el presente proyecto de investigación los procesos de síntesis y modificaciones posteriores que conllevan la producción y/o eliminación de las sales biliares. En este sentido, estos compuestos se sintetizan en el hígado a partir del colesterol mediante una serie de reacciones en las que están involucrados el CYP7A1. Las sales biliares son excretadas en el duodeno para la absorción de grasas a través de la dieta, y posteriormente reabsorbidas a nivel de duodeno e ileon (95%) a través de un transportador apical de ácidos biliares dependiente de sodio (ASBT) localizado en el borde en cepillo de la membrana del enterocito (*Figura 21*). A nivel intestinal el ácido cólico (CA) y ácido quenodeoxicólico (CDCA) son modificados a ácido glicocólico (GCA) ó taurocólico (TCA), y ácido glicoquenodeoxicólico (GCDCA) ó tauroquenodeoxicólico (TCDCA) a través del CYP27A1 (*Figura 21*). Los ácidos biliares pasan a la circulación portal a través del transportador de solutos orgánicos (OST) y a través de NTCP se incorporan al hepatocitos y almacenados en la vesícula biliar para su posterior utilización (*Figura 21*).

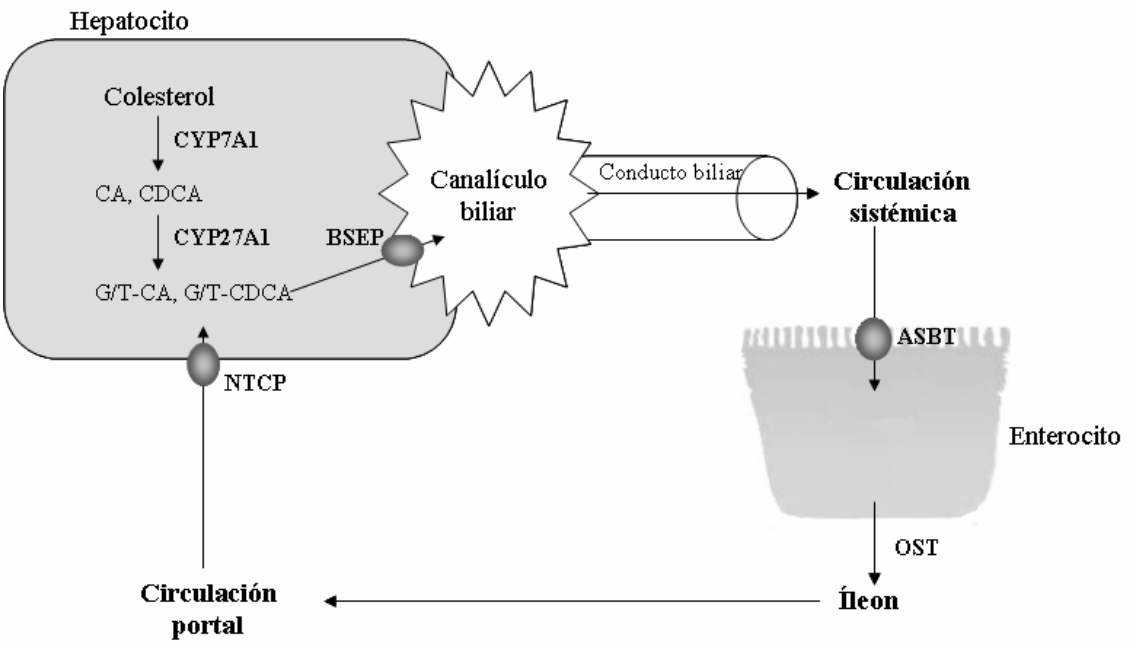

Figura 21: **Circulación enterohepática de los ácidos biliares**.

Regulación génica del transporte hepático

La expresión de los sistemas metabolizadores (CYP2B6, CYP2C9 y CYP3A4) y transportadores de fármacos (MRP2, MRP3, MRP4, OATP2) se encuentra regulada por diferentes **factores de transcripción** como NF-*k*B, AP-1 y C/EBP, y los **receptores nucleares** como el receptor aril de hidrocarburo (AhR), receptor de glucocorticoides (GR) y de la vitamina D (VDR), así como PXR, CAR y PPARα formando heterodímeros con RXR (**32**). Cabe mencionar al receptor X fernesoide (FXR), que juega un papel fundamental en la coordinación de la síntesis, secreción, reabsorción e incorporación de ácidos biliares hacia el hepatocito. FXR incrementa la expresión de BSEP (**182**), induce la expresión de la proteína de unión a ácidos biliares del íleon (IBABP) que reduce los niveles de ácidos biliares en el íleon, y OST (**183**) e inhibe la inducción vía RXR/RAR de la transcripción del transportador NTCP a través del receptor nuclear SHP (**184**).

ENFOQUE CIENTÍFICO DEL PROYECTO

Las enfermedades agudas y crónicas del hígado son de las patologías más prevalentes y con un gasto sanitario elevado en nuestro entorno (**185**). El origen de la disfunción hepática es multifactorial encontrándose componentes tanto de tipo ambiental (tóxico, viral, etc), dietéticos, y genético. El estudio del potencial uso terapéutico de los antioxidantes en el tratamiento de las enfermedades agudas y crónicas hepáticas es de interés. En este sentido, la aplicación experimental de diversos antioxidantes se ha investigado en el campo de las enfermedades alcohólicas, tóxicas, colestáticas identificando algunos de los mecanismos intracelulares de acción (**186-193**).

La citotoxicidad por D-galactosamina (D-GalN) es un buen modelo experimental de lesión hepatocelular con características comunes a la patología humana (**194-197**). La exposición de los hepatocitos a D-GalN altera la función celular, induce estrés oxidativo, modifica los procesos de transcripción/translación y el metabolismo energético con incremento de los procesos de muerte celular (**197**, **198**). Se ha demostrado que diferentes estrategias antioxidantes como S-adenosilmetionina (SAMe), vitamina E, NAC, ácido lipoico, melatonina y otros, reducen el estrés oxidativo y la muerte celular inducida por D-GalN en hepatocitos (**196**, **199-202**). Asimismo, la adición de sales biliares hidrofóbicas (como el GCDCA) es un buen modelo experimental para valorar los mecanismos de muerte celular en la colestasis, así como valorar posibles alternativas terapéuticas. Diferentes estrategias antioxidantes como el antioxidante Lazaroide U83836E, la melatonina, la vitamina E, el NAC, el SAMe, el ácido ursodesoxicólico, el óxido nítrico, entre otros, reducen el estrés oxidativo y la muerte celular inducida por sales biliares en hepatocitos (**189**, **203-208**). El presente proyecto de investigación evaluará el papel citoprotector de α-tocoferol, NAC, coenzima Q_{10} (Q_{10}) y un análogo de la SOD (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride, MnTBAP) en la lesión hepatocelular inducida por D-GalN y ácidos biliares. La administración de α-tocoferol ha demostrado ser eficiente para la prevención del estrés oxidativo y muerte celular en diversos modelos experimentales *in vivo* e *in vitro* de lesión hepatocelular (**189**, **190**, **192**, **199**, **209**). La inducción de estrés oxidativo por alteración de la función mitocondrial es muy relevante en los procesos de muerte celular de hepatocitos (**175**). En este sentido, el incremento de los niveles celulares, y en especial en la mitocondria, de antioxidantes es clave la prevención o disminución de la lesión hepatocelular. GSH es el tiol no proteico más abundante presente en las células de mamíferos, y es esencial en los sistemas de defensa antioxidante celular y

mitocondrial. El GSH interviene en la metabolización de moléculas orgánicas tóxicas y H_2O_2, con lo cual el mantenimiento del GSH mitocondrial es esencial para la función celular y prevención de la muerte hepatocelular. N-acetilcisteína (NAC) es una excelente fuente de cisteína para las células imprescindible para la síntesis de GSH. NAC ha demostrado ser beneficioso en los procesos de cáncer, infección por HIV (virus de la inmunodeficiencia humana) y en enfermedades de corazón, hígado y riñón (**210**). N-etilmaleato es una molécula que regula los niveles celulares de GSH en hepatocitos (**211**). El coenzima Q es un componente esencial del transporte mitocondrial con una importante actividad antioxidante en diversos modelos experimentales de lesión celular (**212**, **213**). Asimismo, la aplicación de un análogo de la SOD que reduce los niveles de $O_{2^{\cdot}}^{-}$ celulares y mitocondriales ha demostrado ejercen propiedades citoprotectoras en diversos modelos in vivo e in vitro de lesión celular (**214**, **215**).

El NO presenta un papel clave en el metabolismo y en la lesión hepática inducida por diferentes agentes. Se ha demostrado que el NO ejerce un efecto pro- o anti-apoptótico dependiendo del estado fisiopatológico celular, niveles y localización intracelular de su producción. Nuestro grupo de investigación ha demostrado el importante papel que el NO ejerce en la muerte celular por D-GalN (**216-220**). En el presente proyecto de Tesis Doctoral se valorará si el NO participa en los mecanismos de citoprotección por los antioxidantes en estudio en los modelos *in vivo* e *in vitro* de muerte celular inducida por sales biliares o D-GalN.

OBJETIVOS DEL ESTUDIO

Los objetivos del proyecto de Tesis Doctoral son:

1) Establecer las condiciones de muerte celular inducida por D-galactosamina (D-GalN) y ácido glicoquenodeoxicólico (GCDCA) en hepatocitos humanos en cultivo.

2) Determinar el efecto beneficioso de NAC, GSH, α-tocoferol, N-etilmaleato en la inducción de apoptosis y necrosis por D-GalN y GCDCA en el cultivo de hepatocitos humanos.

3) Valorar la capacidad de las moléculas en estudio para regular el estrés oxidativo y la función mitocondrial durante la muerte celular en los hepatocitos.

ARTÍCULOS

***Artículo 1*: Las propiedades citoprotectoras del α-tocoferol están relacionadas con la regulación génica en un cultivo de hepatocitos humanos tratados con D-galactosamina**

La vitamina E (α-tocoferol) presenta actividad antioxidante y regula la expresión de diversos genes (**43**). El NO media la muerte celular inducida por D-GalN en el cultivo primario de hepatocitos de rata (**219**). Sin embargo, los niveles o la pauta de producción de NO son claves para su efecto en la muerte celular en hepatocitos (**218**, **195**). Las propiedades beneficiosas del α-tocoferol y su relación con el estrés oxidativo y la regulación génica fueron determinadas durante la muerte celular por D-GalN. Las células hepáticas fueron aisladas por métodos enzimáticos con colagenasa de resecciones hepáticas humanas. El α-tocoferol (50 μM) fue administrado a las células en cultivo 10 horas después de la adición de D-GalN (40 mM). Se valoraron parámetros de muerte celular, estrés oxidativo, metabolismo del α-tocoferol y la expresión de genes bajo regulación de NF-*k*B, PXR y PPARα. Los resultados demuestran que aunque el α-tocoferol reduce de forma moderada el estrés oxidativo celular, es capaz de disminuir de forma importante los parámetros de apoptosis y necrosis inducidos por D-GalN. Utilizando inductores (rifampicina, RIF) o inhibidores (ketoconazole) se demostró que CYP3A4 potencia la muerte celular en los hepatocitos. La administración de α-tocoferol reduce la activación de NF-*k*B y la expresión de NOS-2, así como un aumento de la expresión de PPAR-α y CPT (carnitina palmitoil transferasa). En conclusión, las propiedades citoprotectoras de α-tocoferol están asociados con la regulación de la expresión de diversos genes (NOS-2, CPT) en lugar de con la actividad antioxidante en este modelo experimental de muerte celular en hepatocitos humanos.

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Original Contribution

Cytoprotective properties of α-tocopherol are related to gene regulation in cultured D-galactosamine-treated human hepatocytes

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Abstract

Vitamin E (α-tocopherol) has demonstrated antioxidant activity and gene-regulatory properties. D-Galactosamine (D-GalN)-induced cell death is mediated by nitric oxide in hepatocytes, and it is associated with hepatic steatosis. The beneficial properties of α-tocopherol and their relation to oxidative stress and gene regulation were assessed in D-GalN-induced cell death. Hepatocytes were isolated from human liver resections by a collagenase perfusion technique. α-Tocopherol (50 μM) was administered at the advanced stages (10 h) of D-GalN-induced cell death in cultured hepatocytes. Cell death, oxidative stress, α-tocopherol metabolism, and NF-κB-, pregnane X receptor (PXR)-, and peroxisome proliferator-activated receptor (PPAR-α)-associated gene regulation were estimated in the hepatocytes. D-GalN increased cell death and α-tocopherol metabolism. α-Tocopherol exerted a moderate beneficial effect against apoptosis and necrosis induced by D-GalN. Induction (rifampicin) or inhibition (ketoconazole) of α-tocopherol metabolism and overexpression of PXR showed that the increase in PXR-related CYP3A4 expression caused by α-tocopherol enhanced cell death in hepatocytes. Nevertheless, the reduction in NF-κB activation and inducible nitric oxide synthase expression and the enhancement of PPAR-α and carnitine palmitoyl transferase gene expression by α-tocopherol may be relevant for cell survival. In conclusion, the cytoprotective properties of α-tocopherol are mostly related to gene regulation rather than to antioxidant activity in toxin-induced cell death in hepatocytes.

Keywords: Tocopherol; Hepatocytes; Cell death; NF-κB; PXR; CYP3A4; PPAR-α; CPT1; NOS-2; Free radicals

D-Galactosamine (D-GalN) has been extensively used to induce experimental acute hepatotoxicity [1]. The toxicity of D-GalN is mainly related to the depletion of uridine pools that are associated with limited RNA and protein synthesis, thus altering hepatocellular function [2]. D-GalN induces extensive hepatocellular injury in rat livers [3–5]. Liver injury is mediated by TNF-α and is associated with a profound alteration of hepatic lipid metabolism leading to liver steatosis [6]. The induction of cell death by D-GalN is associated with the generation of reactive oxygen species (ROS) in cultured hepatocytes [7–11]. D-GalN-induced apoptosis is mediated by NF-κB-dependent inducible nitric oxide synthase (NOS-2) expression and nitric oxide production in cultured human and rat hepatocytes [12–14]. The presence of oxidative stress is caused by either

Abbreviations: CEHCs, carboxyethyl hydroxychromans; α-CPHC, alpha-carboxypropyl hydroxychroman; CPT1, carnitine palmitoyl transferase; D-GalN, D-galactosamine; DCFDA, 2′,7′-dichlorofluorescein diacetate; EMSA, electrophoretic mobility shift assay; NOS-2, inducible nitric oxide synthase; PPAR-α, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; ROS, reactive oxygen species; RXR, retinoid-X-receptor.

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an increase in ROS generation or a depletion of antioxidants. Different antioxidant strategies have shown to be useful to reduce oxidative stress and cell death in hepatocytes. α-Tocopherol has been successfully used to reduce hepatocellular damage induced by D-GalN. In addition, supplementation with α-tocopherol reduces the early fat and collagen accumulation in the liver after D-GalN administration in rats [15]. α-Tocopherol has been shown to exert antioxidant properties inhibiting lipid peroxidation and increasing enzymatic activities in different experimental models of liver injury [16–19]. Nevertheless, it has also been observed that the protection by α-tocopherol against D-GalN-induced liver injury is not related remarkably to prevention of lipid peroxidation [20].

Vitamin E is a general term that refers to eight different forms: α-, β-, γ-, and δ-tocopherol with a chromanol ring and saturated side chain and four compounds (α-, β-, γ-, and δ-tocotrienols) with an unsaturated side chain. α-Tocopherol is the main form of vitamin E present in human plasma and tissues [21]. α-Tocopherol is considered the most important lipophilic radical-quenching antioxidant in cell membranes [22]. In addition, α-tocopherol modulates two major signal transduction pathways centered on protein kinase C and phosphatidylinositol 3-kinase that are associated with changes in cell proliferation, platelet aggregation, and NADPH-oxidase activation [23]. α-Tocopherol also regulates the expression of genes related to its own metabolism, lipid uptake, inflammation, cell adhesion, and fibrosis, as well as cell signaling and cycle regulation [24]. The cellular concentration of tocopherols is influenced by both dietary intake and their rates of elimination. The degradation of vitamin E occurs via initial ω-oxidation followed by five cycles of β-oxidation resulting in the respective final products, the carboxyethyl hydroxychromans (CEHCs) [25,26]. CYP3A4 is involved in the oxidative catabolism of tocopherols to carboxychromans. α-Tocopherol regulates the expression of genes involved in its metabolism. In this sense, the vitamer enhances the expression of CYP3A4 in cultured HepG2 cells [27]. By contrast, CYP3A activity is significantly decreased in vitamin E-deficient rats [28]. Nevertheless, no studies have been undertaken to evaluate the ability of α-tocopherol to induce CYP3A4 in primary culture of human hepatocytes. It is relevant to evaluate if this effect might alter the potential beneficial effect of the α-tocopherol in hepatocytes.

Our work was undertaken to determine whether the antioxidant properties of α-tocopherol accounted for the cytoprotective properties of the vitamer against D-GalN-induced cell death in cultured human hepatocytes. We also assessed if the regulation of NF-κB-, pregnane X receptor (PXR)-, and peroxisome proliferator-activated receptor α (PPAR-α)-dependent genes by α-tocopherol was associated with the beneficial properties of the vitamer in toxin-treated human hepatocytes.

Materials and methods

Materials

All reagents were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. DME:Ham-F12 and William's E culture media were obtained from Sigma Chemical Co. and Applichem (Applichem GmbH, Darmstadt, Germany), respectively. Antibiotic–antimycotic solution and fetal bovine serum were from Life Technologies, Inc. (Paisley, UK). α-Tocopherol was obtained from Sigma Chemical Co. The peptide-based substrate for the measurement of caspase-3 was Ac-DEVD-AFC (Bachem AG, Bubendorf, Switzerland). The study protocol was approved by the ethical committee of the institution.

Preparation of primary human hepatocytes and cell culture

Liver resection was obtained from 22 patients (10 women, 12 men; 55±3.3 years old) who underwent surgical intervention for primary or secondary liver tumor resection, after written consent of the patient. The procedure of hepatocyte isolation was based on the two-step collagenase protocol described by Ferrini et al. [29]. Liver was first perfused with nonrecirculating chelating solution I (0.5 mM EGTA, 58 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 25 mM N-tris (hydroxymethyl)methylglycine, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), pH 7.2, at 37°C using a flow of 75 ml/min in order to remove remaining blood. Afterward, the liver was perfused with nonrecirculating washing solution II (20 mM Hepes, 120 mM NaCl, 5 mM KCl, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), pH 7.2, at 37°C using a flow of 75 ml/min. The liver was further perfused with recirculating dissociation solution III (0.05% collagenase, 20 mM Hepes, 120 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B), pH 7.2, at 37°C using a flow of 75 ml/min. The cell suspension was filtered through a nylon mesh (250 μm) and washed three times at 50*g* for 5 min at 4°C in supplemented culture medium. DEM:Ham-F12 and William's E media (1:1) were supplemented with 26 mM $NaHCO_3$, 15 mM Hepes, 0.29 g/L glutamine, 50 mg/L vitamin C, 0.04 mg/L dexamethasone, 2 mg/L insulin, 200 μg/L glucagon, 50 mg/L transferrin, and 4 ng/L ethanolamine. Cell viability was consistently >85%, as determined by trypan blue exclusion. Hepatocytes (8×10^6 cells; 150,000 cells/cm^2) were first seeded in type I collagen-coated dishes (Iwaki, Gyouda, Japan) and cultured in culture medium containing 5% fetal calf serum for 4 h. Afterward, the medium was removed and replaced by fresh culture medium without fetal bovine serum. The study was initiated 24 h after cell seeding. The culture medium was replaced again by fresh culture medium without fetal calf serum. An inducer of CYP3A4 expression (rifampicin, 10 μM) or an inhibitor of CYP3A4 activity (ketoconazole, 5 μM) was added in some experiments. The addition of solvent (0.1% dimethyl sulfoxide) has no effect on the studied parameters. Twenty-four hours later a kinetic study of cell death was induced by D-galactosamine (2-amino-2-deoxy-D-galactose; D-GalN) (40 mM) in cultured human hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN administration.

Plasmids

The ΔATG-hPXR expression vector was generated by PCR amplification of a cDNA encoding amino acids 1–434 of hPXR (kindly provided by Dr. S. Kliewer, Research Triangle Park, NC, USA) using the oligonucleotides sense, 5′-GGGTG-TGGGGAATTCACCACCATGGAGGTGAGACCCAAA-GAAAGC-3′, and antisense, 5′-GGGTGTGGGGGATCCT-CAGCTACCTGTGATGCCG-3′, and insertion into *Eco*RI/*Bam*HI-digested pSG5 (Promega). The CYP3A4 5′ flanking fragment (−262/+11) was generated by PCR from a previously isolated genomic clone [30] used as a template and from oligonucleotides which created artificial cloning sites 5′ (*Kpn*I) and 3′ (*Sma*I). These fragments were cloned into the pGL3-Basic reporter construct (Promega) to generate plasmid p(CYP3A4/−262/+11)-LUC. Plasmid p(CYP3A4/XREM −7800/−7200/−262/+11)-LUC was generated by inserting the PCR-amplified −7800/−7200 region of *CYP3A4* (XREM) [31] from human genomic DNA into plasmid p(CYP3A4/−262/+11)-LUC digested with *Kpn*I.

Cellular transfections

HepG2 cells (human hepatoma) were obtained from the NIH ATCC repository (Bethesda, MD, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA). Transfection of plasmid DNA was performed in single batches with FuGene-6 (Roche Diagnostics, Indianapolis, IN, USA) as instructed by the manufacturer. Transfections were performed using a cellular density of 10,000 cells/cm^2, 400 ng/ml reporter p(CYP3A4/−262/+11)-LUC plasmid, and 100 ng/ml expression ΔATG-hPXR vector. As an internal control plasmid, 40 ng/ml pSV-β-galactosidase (Promega, Madison, WI, USA) was added. pSG5 (Stratagene, La Jolla, CA, USA) empty vector was added to equalize the total concentration of transfected plasmid DNA. After 12–16 h of cell transfection, rifampicin (10 μM) or ketoconazole (5 μM) was added. The addition of solvent (0.1% dimethyl sulfoxide) has no effect on the studied parameters. Twenty-four hours later a kinetic study of cell death was induced by D-GalN (40 mM) in cultured human hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN administration. Cells were harvested in reporter lysis buffer (Promega) after 24 h of treatment, and cell extracts were analyzed for luciferase and β-galactosidase activities as described previously [32].

Analysis of tocopherols, tocotrienols, and α-CEHCs

Cells and culture media were collected for the measurement of α- and γ-tocopherol and α- and γ-tocotrienol, as well as α-CEHCs. Cells were washed two times with 0.5 ml PBS, scraped off from the plate in 1 ml PBS, homogenized in a tissue lyser (Qiagen) for 5 min at 30 Hz, and centrifuged at 9000*g* for 5 min. The cell lysate was mixed with 1 ml pyrogallole (6% in ethanol), 1 ml ethanol, and 1.0826 nmol δ-tocopherol as internal standard and vortexed for 1 min. Vitamin E was extracted two times with 2.5 ml *n*-hexane and the combined hexane phases were evaporated to dryness. Residues were dissolved in 200 μl 95% methanol. Vitamin E was analyzed by HPLC with electrochemical detection [33]. Tocopherols and tocotrienols were separated by HPLC with electrochemical detection as described [34]. Concentrations were calculated using the corresponding peak area and concentration of the internal standard δ-tocopherol. The response factors for α-tocopherol, γ-tocopherol, α-tocotrienol, and γ-tocotrienol were calculated to be 0.6, 0.61, 0.45, and 0.47, respectively.

For the estimation of CEHCs 8 ml of cell culture medium was mixed with 100 μl ascorbate (40 mg/ml H_2O), 1.2 nmol α-carboxypropyl hydroxychroman (α-CPHC) as internal standard [35]. For coulometric detection, the analytical cell was set to +0.55 V. α-CEHC content was calculated using peak area and concentration of the internal standard α-CPHC. CEHC standards were a kind gift from Dr. Abe, Eisai Co. Ltd., Japan. The response factor was calculated to be 1.22.

Measurement of lactate dehydrogenase (LDH) release

LDH in the culture medium was measured by modification of a colorimetric routine laboratory method. Briefly, a volume of medium ranging from 5 to 200 μl from cell lysate or culture medium was incubated with 0.2 mM β-NADH and 0.4 mM pyruvic acid diluted in PBS, pH 7.4. LDH concentration in the sample was proportional to the linear decrease in the absorbance at 334 nm. LDH concentration was calculated using a commercial standard. LDH release represents the percentage of LDH in the culture medium relative to the total LDH (culture medium and cytoplasm).

DNA fragmentation

The whole hepatocyte population, including the floating cells obtained from collected culture medium, was treated with 1 ml of lysis buffer (100 mM Tris–HCl, 5 mM EDTA, 150 mM NaCl, and 0.5% Sarkosyl), pH 8.0, for 10 min at 4°C. Samples were incubated with RNase (50 μg/ml) for 2 h at 37°C and proteinase K (100 μg/ml) for 45 min at 48°C. DNA was obtained by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Chemical Co.) extraction and precipitated with cold isopropanol (1:1) for 12 h at −20°C. DNA was recovered by centrifugation of the sample at 20,800*g* for 10 min at 4°C. Thereafter, the precipitate was washed with 70% ethanol, dried, and resuspended in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA) at pH 8.0. Samples (100 μg DNA) were analyzed on 1.5% agarose gels with ethidium bromide (0.5 μg/ml).

Determination of intracellular reactive oxygen species

The production of ROS was monitored using a fluorescent probe, such as 2′,7′-dichlorofluorescein diacetate (DCFDA) (Molecular Probes Europe BV, Leiden, The Netherlands), which oxidizes in the presence of hydrogen peroxide (H_2O_2). Hepatocytes were incubated with DCFDA (2 μM) for 30 min and, after the cells were washed, the production of H_2O_2 was

assessed in situ as the enhancement of the fluorescence at ex 500 and em 520 measured in a GENios Reader (TECAN, Salzburg, Austria).

Total RNA isolation and quantitative real-time RT-PCR procedure

Total RNA from the whole hepatocyte population was extracted using Trizol reagent according to the manufacturer's recommendations (Life Technologies, Inc.). RNA was precipitated using ice-cold isopropanol, washed with 75% ethanol, and resuspended in RNase-free water (Sigma). The RNA was treated with DNase I (Promega) (1 IU/μg RNA) at 37°C for 30 min. DNase I was degraded at 65°C for 10 min. The integrity of the RNA was verified after separation by electrophoresis on a 0.8% agarose gel containing 0.5% (v/v) ethidium bromide.

The expression of mRNA for NOS-2, CYP3A4, carnitine palmitoyl transferase (CPT1), PXR, and PPAR-α was examined by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics). RT-PCR was performed in one step, using the QuantiTect SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. First-strand cDNA synthesis was performed with 100 ng of total RNA diluted in a reaction mixture including Omniscript and Sensiscript reverse transcriptase, as well as the specific sets of primers for human NOS-2 (Biosource, Nivelles, Belgium) (Cat. No. GHO0144, cDNA reference GenBank U05810), CYP3A4 (sense, 5′-AGCAGAAACTGCAGGAGGAA-3′, and antisense, 5′-AGCGTTTCATTCACCACCAT-3′), CPT1 (sense, 5′-TCACATTCAGGCAGCAAGAG-3′, and antisense, 5′-GAATCGTGGATCCCAAAAGA-3′), PXR (sense, 5′-TCCGGAAAGATCTGTGCTCT-3′, and antisense, 5′-AGGGAGATCTGGTCCTCGAT-3′), PPAR-α (sense, 5′-ACGATTCGACTCAAGCTGGT-3′, and antisense, 5′-CGACAGAAAGGCACTTGTGA-3′), and 18S (MVG-Biotech AG, Getotek, Sabadell, Spain) (sense, 5′-GTAACCCGTTGAACCCCATT-3′, and antisense, 5′-CCATCCAATCGGTAGTAGCG-3′). The RT conditions were 20 min at 55°C and 15 min at 95°C. The amplification protocol consisted of 50 cycles of incubation after initial denaturation at 95°C for 15 s (20°C/s), 60°C for 30 s (20°C/s), and 72°C for 30 s (2°C/s). The melting conditions were fixed at 65°C (0.1°C/s). To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide, the PCR products were subjected to a melting curve analysis, and negative and positive controls containing either RNase-free water or control positive solution (Biosource) instead of sample were run to confirm that the samples were not cross-contaminated. Quantitation of relative expression was determined by the $2^{-\Delta(\Delta CT)}$ method [36].

Preparation of cytoplasmic and nuclear extracts

Nuclear extracts from hepatocytes were prepared according to Schreiber et al. [37]. Briefly, hepatocytes were recovered in 800 μl of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 0.6% Nonidet NP-40), allowing them to swell for 10 min on ice. Afterward, samples were homogenized and centrifuged at 15,000g for 1 min at 4°C. After removal of the supernatant (cytoplasmic fraction), the nuclear pellet was resuspended in 75 μl of nuclear extraction buffer (20 mM Hepes, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM DTT). The tube was incubated for 20 min on ice with continuous mixing and centrifuged at 15,000g for 5 min at 4°C. Aliquots of the supernatant (nuclear fraction) were stored at −80°C until use.

Western blot analysis for caspase-3 processing

The nuclear fractions obtained above were used to measure caspase-3 processing in cultured hepatocytes. Proteins (100 μg) were separated by 12% SDS–PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding commercial primary and secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), revealing protein content by ECL.

Assay for caspase-3-associated activity

The nuclear fractions obtained above were used to measure caspase-3 activity in hepatocytes. The enzymatic activity in the cell extract (25 μg) was measured using the corresponding peptide-based substrates (100 μM) in caspase-incubating buffer (50 mM Hepes, pH 7.5, 100 mM NaCl, 10% sucrose, 0.1% Chaps, 1 mM EDTA, and 5 mM DTT) up to 100 μl total volume. The increase in fluorescence of the sample from enzymatically released AFC was followed at ex 400 and em 505 using a GENios Reader (TECAN).

NF-κB activation

NF-κB expression in nuclear extract was evaluated by radioactive electrophoretic mobility shift assay (EMSA). NF-κB consensus oligonucleotides 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ (Promega) were used. Probes were labeled at the 5′ end with T4 polynucleotide kinase (Promega) and [γ-^{32}P] ATP (Amersham Biosciences, Uppsala, Sweden). Excess unreacted ATP was separated from the labeled probe using MicroSpin G25 columns (Amersham Biosciences). The binding reaction (25 μl) contained 15 μg nuclear protein, 5 μl incubation buffer (50 mM Tris–HCl, pH 7.5, 200 mM NaCl, 5 mM EDTA, 5 mM β-mercaptoethanol, and 20% glycerol), and 1 μg poly(dI–dC) (Promega). Samples were incubated for 15 min on ice and the corresponding amount of ^{32}P-labeled probe was added. Samples were incubated for 15 min at room temperature and loaded on a 6% native polyacrylamide gel for electrophoresis. Gels were fixed (30% methanol, 10% glacial acetic acid) and dried at 90°C under vacuum. Dried gels were visualized by autoradiography (Hyperfilm; Amersham Bio-

sciences). Antibody supershift assays were performed by incubation of the mixture with 1 μg/ml affinity-purified polyclonal antibodies against p50, p52, and p65 subunits (Santa Cruz Biotechnology) for 30 min on ice before addition of ^{32}P-labeled probe (Fig. 2A). The use of affinity-purified polyclonal antibodies against p50 and p65, but not p52, subunits induced a supershift with identification of the p65/p50 and p50/p50 complexes (Fig. 2A). A competitive assay was also carried out by incubating the mixture with cold probe (100-fold excess) for 30 min on ice before ^{32}P-labeled probe addition (Fig. 2A).

Statistical analysis

Results are expressed as means±SEM of five experiments. Data were compared using ANOVA with the least significant difference test as post hoc multiple comparison analysis. The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" in the figures were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

Results

Vitamin E attenuated apoptosis and necrosis, but not ROS generation, in D-GalN-treated hepatocytes

α-Tocopherol is the predominant form of vitamin E in primary cultures of human hepatocytes (Table 1). Interestingly, D-GalN enhanced the cellular concentration of α-tocopherol (110%), γ-tocopherol (125%), α-tocotrienol (176%), and γ-tocotrienol (128%), as well as that of α-CEHC (160%), in culture medium compared with control hepatocytes ($p \leq 0.05$). These data reflect an intense turnover of cellular tocopherols that could compromise the survival of hepatocytes subjected to an intense oxidative stress. In other experimental models, the preadministration of vitamin E markedly inhibited necrosis induced by acetaminophen in cultured mouse hepatocytes [38]. Also, the preadministration of α-tocopherol was able to reduce liver injury induced by D-GalN in rats [15]. The present study addressed also the beneficial properties of α-tocopherol supplementation during advanced stages of D-GalN-induced cell death in cultured human hepatocytes. D-GalN induced a rapid increase in ROS intracellular generation that reached a maximum 2–4 h after toxin administration (Fig. 1A). α-Tocopherol did not exert a significant antioxidant effect in D-GalN-treated hepatocytes (Fig. 1A). Nevertheless, the vitamer reduced H_2O_2 production in control hepatocytes (Fig. 1A). Although α-tocopherol was administered after D-GalN-induced ROS generation, shortly after its supplementation a reduction in DNA fragmentation (Fig. 1B) and caspase-3 activation (Figs. 1C and 1D) was observed in D-GalN-treated hepatocytes. This effect of α-tocopherol on cellular apoptosis was associated with a moderate but significant reduction in cell necrosis by the vitamer in D-GalN-treated hepatocytes (Fig. 1E).

Table 1
Concentration of tocopherols in hepatocytes and α-CEHC in culture medium from primary culture of human hepatocytes

	Control	D-GalN
α-Tocopherol	0.492±0.0101	0.549±0.012 *
γ-Tocopherol	0.054±0.0014	0.069±0.0015 *
α-Tocotrienol	0.0034±0.00014	0.0060±0.00096 *
γ-Tocotrienol	0.650±0.0017	0.834±0.1398 *
α-CEHC	0.048±0.0029	0.077±0.0094 *

D-Galactosamine (D-GalN) (40 mM) was administered to cultured human hepatocytes. Samples were collected at 24 h after D-GalN administration. α- and γ-tocopherol (nmol/mg protein) and α-(nmol/mg protein) and γ-tocotrienol (pmol/mg protein) were determined in cell lysates by HPLC. α-CEHC (nmol/mg protein) was determined in culture medium by HPLC. Each value represents the mean±SEM of four independent experiments. The statistical differences were set at $p \leq 0.05$.

* Significantly different vs the corresponding control group.

Effects of α-tocopherol on NF-κB activation and NOS-2 expression in human hepatocytes

The induction of apoptosis by D-GalN is mediated by the induction of NF-κB activation, NOS-2 expression, and nitric oxide production in human and rat hepatocytes [12–14,39]. The inhibition of NOS-2 activity prevented D-GalN-induced cell death in hepatocytes [13,14]. The degree of NF-κB activation was still high at 11–12 h, but declined at 24 h in D-GalN-treated hepatocytes. α-Tocopherol reduced NF-κB activation (Fig. 2B) and NOS-2 expression (Fig. 2C) in D-GalN-treated hepatocytes.

Role of CYP3A4 in D-GalN- and/or α-tocopherol-treated hepatocytes

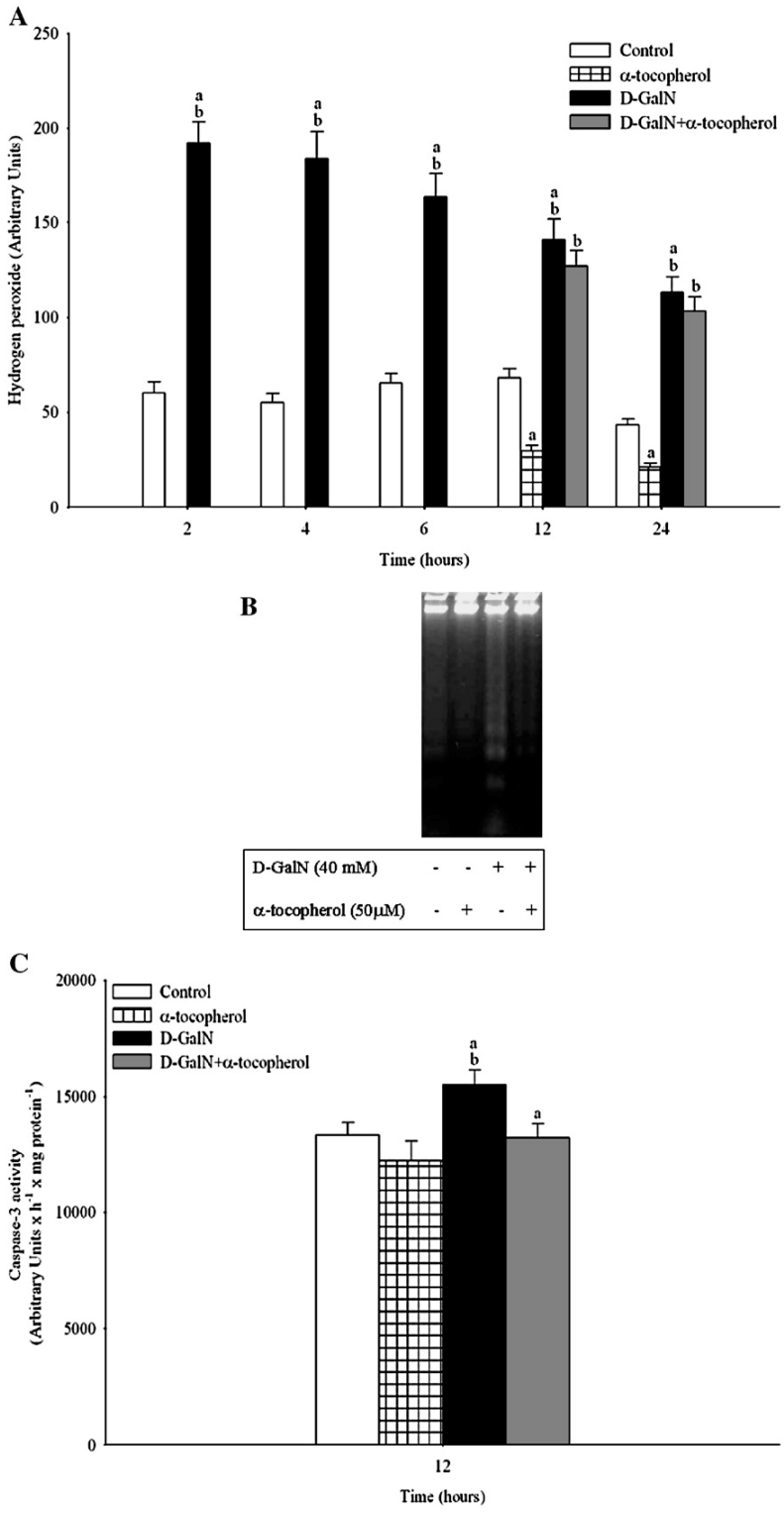
CYP3A4 is the one of the main mono-oxygenase systems involved in the metabolism of α-tocopherol [40,41]. The vitamer enhances the expression of CYP3A4 in HepG2 cells [27,28]. D-GalN reduced the expression of CYP3A4 in control and rifampicin-treated human hepatocytes (Fig. 3A), as well as luciferase activity in control and rifampicin p(CYP3A4/XREM −7800/−7200/−262/+11)-LUC-transfected HepG2 cells (Fig. 3B). No studies have been reported showing the ability of α-tocopherol to induce CYP3A4 in primary culture of human hepatocytes. The present study showed that the administration of α-tocopherol enhanced CYP3A4 expression in control and D-GalN-treated human hepatocytes (Fig. 3A). As expected, α-tocopherol enhanced luciferase activity in control and D-GalN-treated p(CYP3A4)-transfected HepG2 cells (Fig. 3B). The addition of vitamin E was not able to further increase luciferase activity in p(CYP3A4)-transfected HepG2 cells treated with rifampicin (Fig. 3B).

The induction of CYP3A4 expression by rifampicin drastically enhances the metabolism of vitamin E [40]. This effect of the inducer slightly enhanced DNA fragmentation (Fig. 4A) and caspase-3 activity (Fig. 4B) in D-GalN-treated hepatocytes. This effect was prevented by an inhibitor of CYP3A4 (ketoconazole) (Fig. 4). We wanted to assess further if the overexpression of PXR was able to mimic the effect of rifampicin-induced CYP3A4 overexpression in cell death.

Cellular transfection with the ΔATG-hPXR expression plasmid enhanced caspase-3 activity (12 h) in all experimental groups (Fig. 4C). Interestingly, PXR overexpression was not able to prevent the beneficial effects of α-tocopherol supplementation in D-GalN-treated hepatocytes (Fig. 4C).

Effects of α-tocopherol on PPAR-α-related cell signaling in D-GalN-treated hepatocytes

Disturbances of lipid metabolism are generally observed in liver injury. The expression of genes related to lipid metabolism



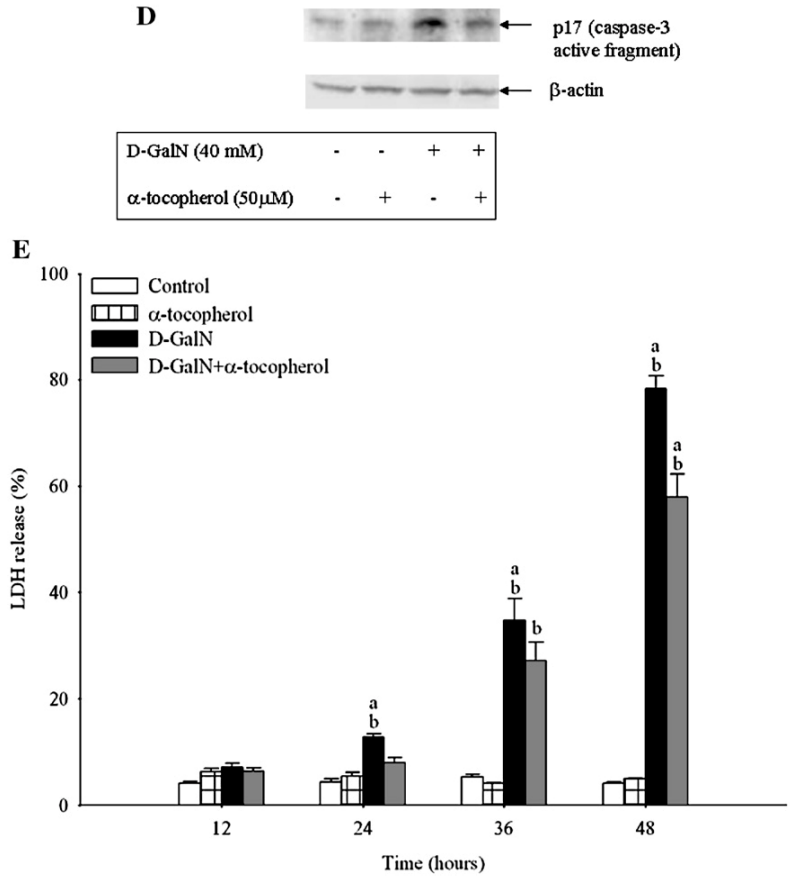


Fig. 1. Effects of α-tocopherol on (A) hydrogen peroxide production, (B) DNA fragmentation, (C) caspase-3 activity, (D) caspase-3 processing, and (E) cell necrosis induced by D-galactosamine (D-GalN) in primary culture of human hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN (40 mM) administration to cultured hepatocytes. Samples were collected at different time points after D-GalN administration. The production of hydrogen peroxide was monitored in situ using DCFDA. DNA fragmentation (12 h) was assessed by agarose gel electrophoresis of cell lysates. The gel is representative of five independent experiments. Caspase-3 activity and processing were assessed using peptide-based substrate and Western blot analysis of the nuclear fraction, respectively. LDH release, as a marker of cell necrosis, represents the percentage of LDH in culture medium relative to the total LDH. Each bar represents the mean ± SEM of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

is drastically altered during CCl4-induced liver injury [42]. The liver injury induced by D-GalN is associated with hepatic lipid steatosis in rats [6]. PPAR-α is a key regulator of lipid metabolism and transport, as well as fatty acid β-oxidation. CPT1 is under the genetic control of PPAR-α. D-GalN drastically reduced PPAR-α and CPT1 expression in cultured human hepatocytes (Fig. 5). The administration of α-tocopherol increased the expression (24 h) of PPAR-α and CPT1 in control hepatocytes. In addition, α-tocopherol enhanced PPAR-α and CPT1 expression in D-GalN-treated hepatocytes, but the values were still below those obtained in control cells (Fig. 5).

Discussion

Supplementation with α-tocopherol prevents cell death in different in vivo and in vitro experimental models of hepatocellular injury. α-Tocopherol is considered the most important lipophilic radical-quenching antioxidant in cell membranes [21]. Nevertheless, the vitamer also regulates different intracellular major signal transduction pathways and genes related to its metabolism, lipid uptake, inflammation, cell adhesion, fibrosis, and the cell cycle [23,24]. The present study was undertaken to assess if a reduction in free radical generation and/or gene regulation was relevant to the cytoprotective properties of α-tocopherol administered during the advanced stages of cytotoxicity in primary culture of human hepatocytes. α-Tocopherol exerted a moderate beneficial effect against apoptosis and necrosis induced by D-GalN in cultured human hepatocytes. The cytoprotective effect of the vitamer was not related to a significant antioxidant action, but reduced NF-κB-dependent NOS-2 expression and enhanced PPAR-α-associated CPT1 expression. The supplementation with α-tocopherol enhanced CYP3A4 expression in cultured human hepatocytes and PXR-driven CYP3A4 promoter activity in transfected HepG2 cells. The induction of CYP3A4 expression by rifampicin or PXR overexpression reduced cell survival in

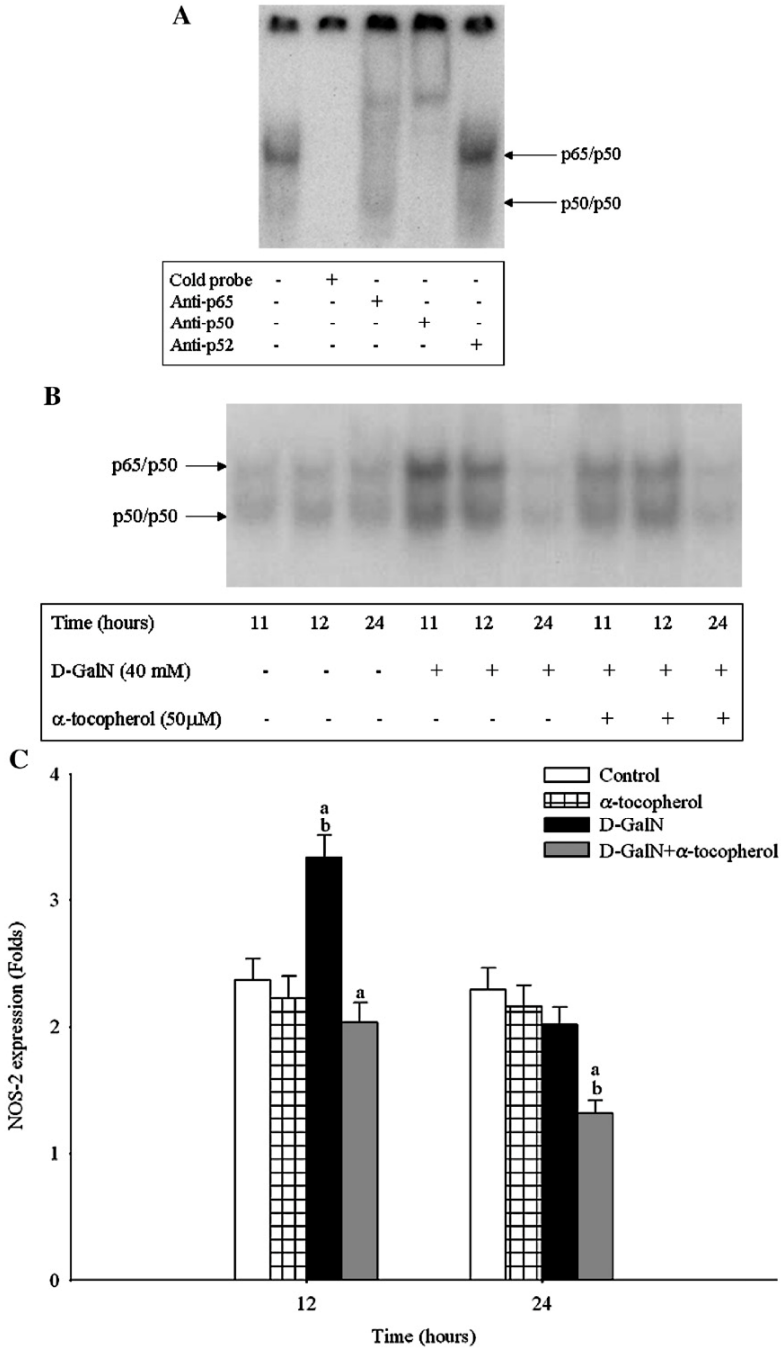


Fig. 2. Effects of α-tocopherol on (B) NF-κB activation and (C) NOS-2 expression in D-GalN-treated human hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN (40 mM) administration to cultured hepatocytes. Samples were collected at different time points after D-GalN administration. NF-κB activation was measured by radioactive EMSA in the nuclear fraction from D-GalN- and/or α-tocopherol-treated hepatocytes. (A) A competitive assay using cold probe and supershift assay using affinity-purified polyclonal antibodies against p50 and p65 subunits were used to validate the assay and identify p65/p50 and p50/p50 complexes. The images are representative of five independent experiments. NOS-2 expression was measured by real-time RT-PCR procedures. The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

control and D-GalN-treated hepatocytes. In consequence, the rise in PXR-driven CYP3A4 expression by α-tocopherol may not be included among the beneficial properties of the vitamer in cell survival in primary culture of human hepatocytes.

D-GalN has been used extensively to induce experimental acute hepatotoxicity [1]. D-GalN is actively incorporated into hepatocytes by carrier-mediated diffusion across the plasma membrane, phosphorylated, and transformed to UDP-galacto-

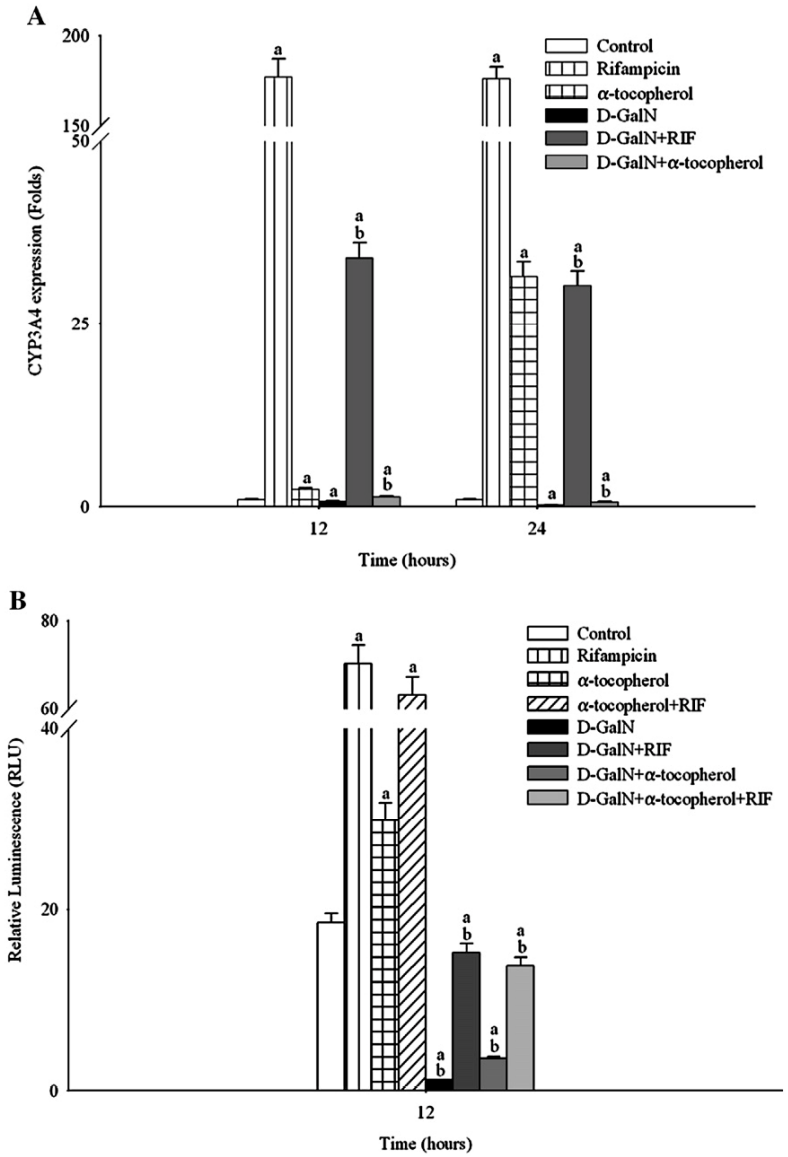


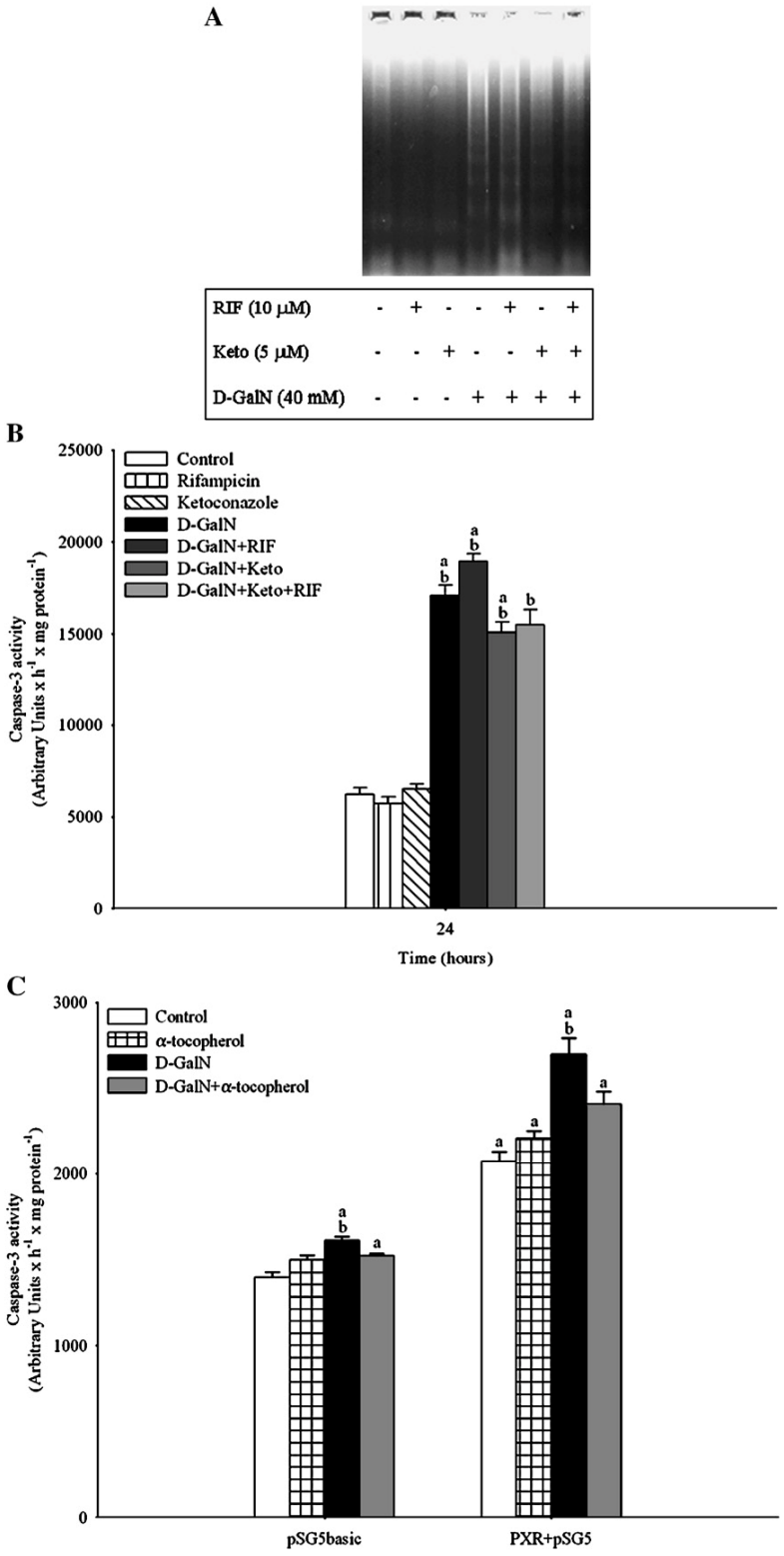
Fig. 3. Effects of α-tocopherol on (A) CYP3A4 expression in cultured human hepatocytes and (B) PXR-driven CYP3A4 promoter activity in HepG2-transfected cells. An inducer of CYP3A4 expression (rifampicin (RIF), 10 μM) was added 24 h before the initiation of a kinetic study of cell death induced by D-GalN (40 mM) in hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN administration. Samples were collected at different time points after D-GalN administration. CYP3A4 expression was measured by real-time RT-PCR in primary culture of human hepatocytes. The plasmid p(CYP3A4/XREM −7800/−7200/−262/+11)-LUC was transfected to HepG2 cells following the procedure described under Materials and methods. The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

samine by UDP-glucose:α-D-galactose-1-phosphate uridyltransferase. UDP-galactosamine is actively transformed to UDP-glucosamine by epimerization and incorporated to glycoprotein [43]; but nevertheless, the accumulation of UDP-galactosamine slows the regeneration of the UDP moiety for the synthesis of UDP-glucose and UDP-galactose levels and consequently depresses the synthesis of glycoproteins and glycolipids. The toxicity of D-GalN is mainly related to the depletion of uridine pools, which is associated with limited RNA and protein synthesis, thus altering hepatocellular function [2].

D-GalN induces apoptosis and necrosis in liver tissue and cultured rat hepatocytes [3,6,7,44–47]. Intracellular ROS generation is associated with cell death in cultured hepatocytes [7,8,48–50]. The induction of apoptosis by D-GalN was associated with a rapid rise in intracellular oxidative stress, caspase-3 processing, and DNA fragmentation in primary culture of human hepatocytes. Supplementation with α-tocopherol has been shown to reduce in vivo and in vitro oxidative stress and hepatocellular cell death in different experimental models [17,18,38,51]. α-Tocopherol did not

reduce significantly ROS production induced by D-GalN in hepatocytes, although a relevant antioxidant activity by α-tocopherol could be observed in control hepatocytes. Nevertheless, α-tocopherol was able to exert a moderate but significant beneficial effect on apoptosis and necrosis induced by D-GalN in cultured human hepatocytes.

Nitric oxide exerts a key role in hepatic metabolism and liver injury induced by various agents [52,53]. The induction of nitric



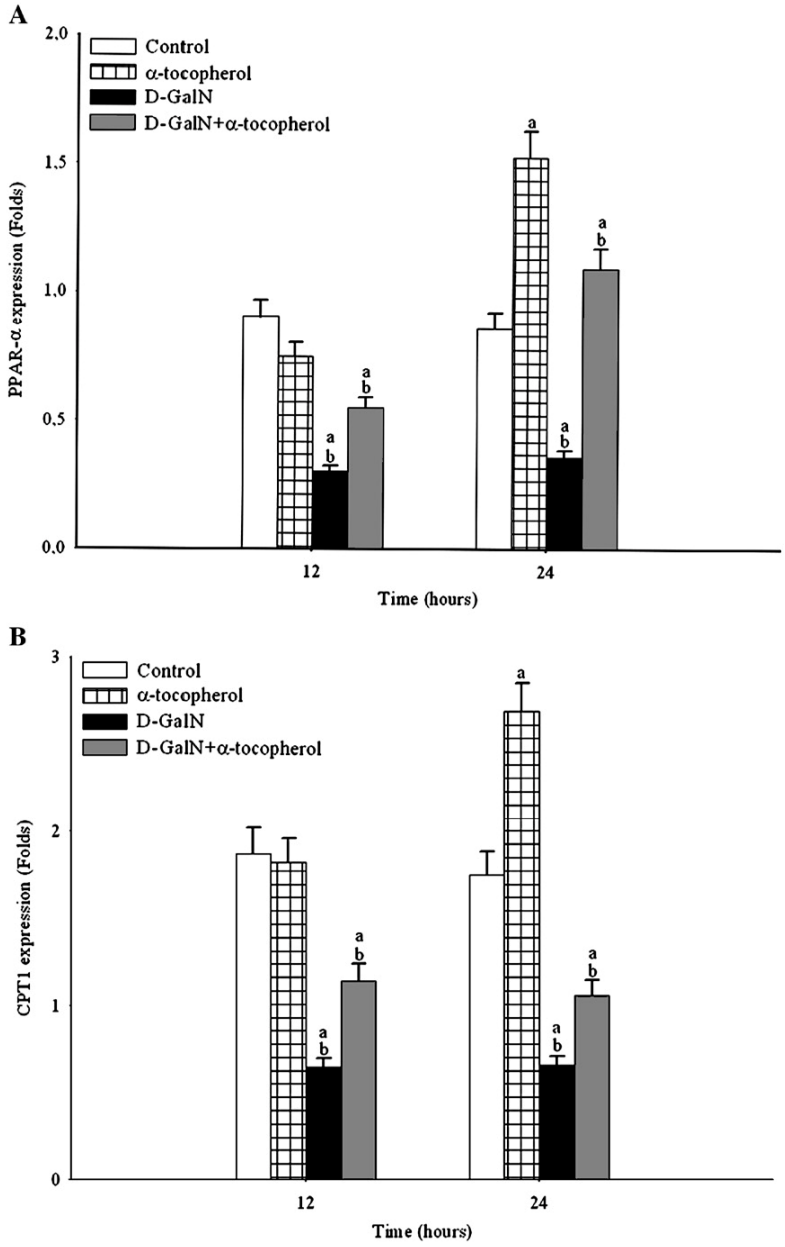


Fig. 5. Effects of α-tocopherol on (A) PPAR-α and (B) CPT1 expression in control and D-GalN-treated human hepatocytes. α-Tocopherol (50 μM) was administered 10 h after D-GalN (40 mM) administration to cultured hepatocytes. Samples were collected at 12 and 24 h after D-GalN administration. PPAR-α and CPT1 expression was measured by real-time RT-PCR procedures. The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

oxide production by NF-κB-dependent NOS-2 expression mediates apoptosis by D-GalN in cultured rat and human hepatocytes [12–14]. The inhibition of NOS-2 reduced D-GalN-induced apoptosis in primary culture of rat hepatocytes [13]. The beneficial properties of α-tocopherol were associated with a reduction in NF-κB activation and NOS-2 expression

Fig. 4. Effects of the inhibition of CYP3A4 expression (rifampicin or RIF) and activity (ketoconazole or Keto) on (A) DNA fragmentation and (B) caspase-3 activity in hepatocytes, as well as (C) the effects of PXR overexpression in HepG2 cells. RIF and/or Keto was added 24 h before D-GalN (40 mM). α-Tocopherol (50 μM) was administered 10 h after D-GalN administration. The presence of DNA fragmentation was assessed 24 h after D-GalN administration by agarose gel electrophoresis in cell lysate. The gel is representative of five independent experiments. Caspase-3 activity was assessed in the nuclear fraction using peptide-based substrate. HepG2 cells were transfected with an expression vector encoding hPXR (ΔATG-hPXR) or the corresponding control plasmid (pSG5). The statistical differences were set at $p \leq 0.05$. The groups labeled with "a" were significantly different vs the corresponding control group. The groups with "b" were significantly different vs the corresponding group without D-GalN.

induced by D-GalN in hepatocytes. The effect was sustained throughout the study, even at the latest time point when D-GalN-dependent NF-κB activation signal was already low. Several intracellular mechanisms may lead to NF-κB regulation in cells. The dephosphorylation of protein kinase C by α-tocopherol [54] is associated with inhibition of ROS generation systems such as NADPH oxidase [55]. This effect of the vitamer may account for a reduction in NF-κB activation. Nevertheless, the mild reduction in ROS by α-tocopherol did not support this mechanism in D-GalN-treated hepatocytes. The administration of PPAR-α agonist has been shown to prevent the induction by TNF-α of NF-κB activation and VCAM-1 expression in endothelial cells [56]. The induction of cell death by D-GalN was related to a reduction in PPAR-α and CPT1 gene expression, as well as NF-κB activation and a rise in NOS-2 expression in hepatocytes. The supplementation with α-tocopherol enhanced PPAR-α and CPT1 expression in D-GalN-treated hepatocytes twofold, but the values were still below those obtained in control cells. All these data may suggest that upregulation of PPAR-α by α-tocopherol precedes the reduction in NF-κB activation in cultured human hepatocytes. The inhibition of NF-κB activation by PPAR-α could result from a direct interaction with the NF-κB binding site in the NOS-2 promoter, as postulated for the interaction of NF-κB with the estrogen receptor [57]. Alternatively, the inhibitory effects might occur through competitive binding of transcriptional coactivators by PPAR-α or by PPAR-α-induced transcription factors. Such "negative cross talk" has also been suggested between other nuclear receptors and the transcription factor AP-1 [58]. Our data do not exclude a PPAR-α effect on IκB degradation or translocation of NF-κB to nucleus. The regulation of NOS-2 expression during D-GalN-induced cell death by α-tocopherol may be relevant for the cytoprotective properties of the vitamer in human hepatocytes.

PPAR-α is an orphan nuclear receptor that forms heterodimers with retinoid-X-receptor (RXR) to bind to DNA response elements and regulate the transcription of target genes [59]. Characteristic responses of hepatocytes to peroxisome proliferators include hepatomegaly, a marked proliferation of peroxisomes in parenchymal cells, and an increase in peroxisomal β-oxidation of fatty acids [60]. PPAR-α-associated peroxisome proliferator stimulation mediates the suppression of spontaneous and TGF-β-induced apoptosis in cultured hepatocytes [61,62]. A reduction in adiponectin and PPAR-α expression in liver was associated with insulin resistance and liver injury in rats maintained on a high-calorie solid diet [63]. The administration of PPAR-α ligand ameliorated the degree of liver injury [63]. D-GalN-induced hepatocellular injury is widely associated with hepatic lipid steatosis [3,6]. In the present study, D-GalN-induced hepatocellular injury was related to a reduction in PPAR-α and CPT1 gene expression. The rise in PPAR-α and CPT1 expression by α-tocopherol may have a beneficial impact on the disturbances in lipid metabolism and cell death induced by D-GalN in cultured hepatocytes.

Vitamin E has been shown to regulate CYP3A4 expression in vitro [27,64] and in vivo [28]. The transcription of CYP3A is mediated by PXR, which binds to its responsive element as a heterodimer with RXR [65]. The activation of a PXR-driven CAT reporter in HepG2 cells by tocopherols has recently been described [27]. The present study is the first report showing the alteration of α-tocopherol metabolism in control and toxin-treated cultured human hepatocytes. α-Tocopherol is the predominant form in primary culture of human hepatocytes. Interestingly, D-GalN enhanced the concentration of α- and γ-tocopherol and α- and γ-tocotrienol in hepatocytes, as well as that of α-CEHC in culture medium. These data reflect an intense turnover of cellular tocopherols that could compromise the survival of hepatocytes subjected to an intense oxidative stress. As tocopherols cannot be synthesized by mammals [66] and the commercial culture medium used in the present study includes only α-tocopherol, our data suggest that D-GalN induced the conversion between different forms of tocopherols to counteract the rise of α-CEHC in the culture medium. The stimulation of α-tocopherol catabolism to α-CEHC by D-GalN is associated with an inhibition of CYP3A4 expression by the toxin in control and rifampicin-treated hepatocytes. Supplementation with α-tocopherol enhanced the expression of CYP3A4 in control and D-GalN-treated hepatocytes. This effect of the vitamer was driven by the PXR-dependent induction of the CYP3A4 promoter in HepG2 cells. The studies involving PXR overexpression showed that PXR-cell signaling drives rifampicin-induced apoptosis. The study suggested that the induction of PXR-driven CYP3A4 overexpression by α-tocopherol may not be included among the beneficial properties of the vitamer in cell survival. The fact that α-tocopherol maintained the cytoprotection against D-GalN cytotoxicity in PXR-overexpressing HepG2 cells suggests that mechanisms unrelated to PXR are involved in the cytoprotective properties of the vitamer.

In conclusion, the induction of cell death by D-GalN was associated with the induction of oxidative stress and alterations in gene regulation in primary culture of human hepatocytes. The administration of α-tocopherol reduced D-GalN-induced apoptosis and necrosis, but without a remarkable reduction in ROS production in cultured hepatocytes. The induction of PXR-related CYP3A4 expression by α-tocopherol may not be included among the beneficial properties of the vitamer. By contrast, the opposite cross talk between enhanced PPAR-α and reduced NF-κB activation induced by α-tocopherol may have a beneficial impact on the survival of D-GalN-treated hepatocytes.

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Apéndice del artículo 1 (resultados preliminares o complementarios)

Selección de los antioxidantes en estudio en el modelo de toxicidad por D-GalN en hepatocitos humanos en cultivo

Se emplearon diversas moléculas relacionadas con el estrés oxidativo en este modelo experimental de citotoxicidad por D-GalN (40 mM) en hepatocitos humanos en cultivo (n=5 experimentos independientes):

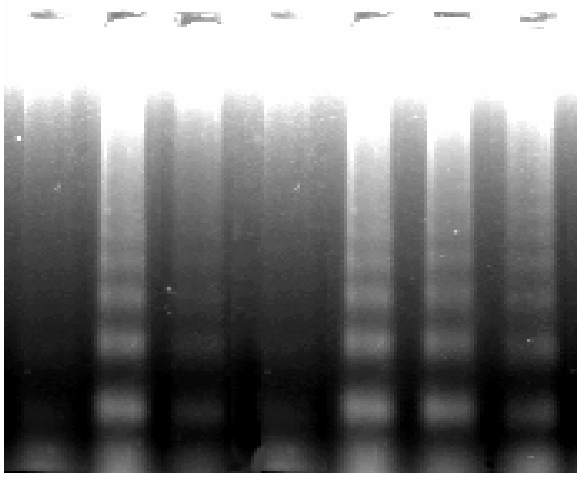
- α-tocoferol: concentración de 0,05 mM.
- N-acetilcisteína (NAC): concentración de 0,5 mM.
- Diethylmaleate (DEM) ó N-etilmaleate: concentración final de 2 mM.
- 2,6-Di-tert-butyl-4-methyl-phenol (BHT): concentración final de 0,02 mM.
- Glutatión-ethyl-ester (GSH-EE): concentración final de 5 mM.

En los estudios preliminares se realizó una co-administración de las moléculas con el tóxico, valorándose el estrés oxidativo y parámetros de muerte celular (apoptosis y necrosis). En los datos que a continuación se presentan (*Tabla 1* y *Figura 22*) se puede apreciar que:

- Medición de la producción de H_2O_2: La producción de H_2O_2 se midió con diclorodihidrofluoresceína diacetato (H_2DCFDA) a las 2 h (horas) después de administrar D-GalN. En este caso tan solo NAC reduce de forma significativa el estrés oxidativo inducido por D-GalN (*Tabla 1*) ($p \leq 0.05$).
- Apoptosis: Se valoró mediante la actividad asociada a caspasa-3 (*Tabla 1*) y la fragmentación del ADN celular en gel de agarosa (*Figura 22*) a 12 h de la administración de D-GalN. La administración de NAC y α-tocoferol disminuyeron de forma significativa la muerte celular inducida por D-GalN ($p \leq 0.05$).
- Necrosis: Se valoró mediante la medición del porcentaje de liberación de lactato deshidrogenasa (LDH) al medio de cultivo a las 24 h de la administración de D-GalN (*Tabla 1*). La administración de NAC disminuyó de forma significativa la necrosis celular inducida por D-GalN ($p \leq 0.05$).

Tratamientos	Peróxido de hidrógeno	Actividad asociada a caspasa-3	% LDH liberado
Control	60±5,6	13304±590,1	4±0,6
D-GalN	192±11,8*	15509±628,1*	13±0,6*
D-GalN+α-tocoferol	203±7,6*	14090±367,2*	11±1,7
D-GalN+NAC	164±7,5*	13028±1060,3*	9±0,3*
D-GalN+DEM	614±157,2*	15288±812,8	14±1,2
D-GalN+BHT	329±89,3*	15086±2250,0	13±1,0
D-GalN+GSH-EE	389±163,2*	16128±1710,1	14±1,7

Tabla 1: **Efecto de D-GalN y diferentes antioxidantes sobre estrés oxidativo y muerte celular en hepatocitos humanos en cultivo**. En cuanto al peróxido de hidrógeno se evaluó 2 h después de administrar el tóxico, y los valores están en Unidades Arbitrarias. La actividad asociada a la enzima caspasa-3 se analizó 12 h después de la adición de D-GalN sobre las células, y dichos valores están expresados en Unidades Arbitrarias (UA) x h^{-1} x mg proteína^{-1}. En último lugar la necrosis, se determinó 24 h después de la administración del hepatotóxico sobre las células. El estudio estadístico está realizado con el test de comparación múltiple LSD (mínimas diferencias significativas). Donde los grupos marcados con "*" presentan diferencias estadísticamente significativas frente a su correspondiente grupo control.



D-GalN	-	+	+	+	+	+	+
α-tocopherol	-	-	+	-	-	-	-
NAC	-	-	-	+	-	-	-
DEM	-	-	-	-	+	-	-
BHT	-	-	-	-	-	+	-
GSH-EE	-	-	-	-	-	-	+

Figura 22: **Efecto de D-GalN y diferentes antioxidantes sobre la apoptosis en hepatocitos humanos en cultivo**. En este gel de agarosa se analiza el efecto sobre la fragmentación del ADN que tienen las diferentes moléculas antioxidantes utilizadas en un cultivo de hepatocitos humanos 12 h después de la administración de D-GalN.

De estos estudios preliminares se seleccionó el α-tocoferol (artículo 1) y NAC (artículo 2) como antioxidante a profundizar en el modelo de lesión hepatocelular por D-GalN.

<u>Artículo 2</u>: N-acetilcisteína, coenzima Q_{10} y un mimético de la superóxido dismutasa previenen la disfunción celular mitocondrial y la muerte celular inducidas por D-galactosamina en el cultivo primario de hepatocitos humanos

D-GalN induce estrés oxidativo y muerte celular en hepatocitos en cultivo. En el presente estudio se evaluará el efecto citoprotector de NAC, Q_{10} y un análogo de la SOD (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride, MnTBAP) frente a la disfunción mitocondrial y la muerte celular en hepatocitos humanos en cultivo tratados con D-GalN. Los hepatocitos se aíslan de forma enzimática con colagenasa a partir de biopsias hepáticas humanas. NAC (0,5 mM), Q_{10} (30 μM) y MnTBAP (1 mg/mL) fueron co-administrados con D-GalN (40 mM) en el cultivo primario de hepatocitos. Se valoraron parámetros de estrés oxidativo, muerte celular, potencial mitocondrial (MTP), contenido energético (ATP), cocientes de GSSG/GSH y niveles de Q_{10} oxidado en mitocondria, actividad de la cadena de transporte electrónico (ETC), y la expresión de las subunidades codificadas en el núcleo y en la mitocondria del complejo I mitocondrial. Se observó que D-GalN indujo un incremento transitorio de la hiperpolarización mitocondrial y del estrés oxidativo, con incremento del cociente GSSG/GSH y del Q_{10} oxidado en mitocondria, y muerte celular en los hepatocitos humanos. La expresión de las subunidades en el complejo I se redujo con D-GalN. La administración de los antioxidantes en estudio (NAC, Q_{10} y MnTBAP) redujo la producción de ERO, recuperación del estado reducido de GSH y Q_{10}, así como de las actividades de los complejos mitocondriales I+III y II+III, y el contenido energético celular. Las propiedades citoprotectoras de Q_{10} y MnTBAP se relacionaron con un incremento en la expresión proteica de las subunidades nuclear y mitocondrial del complejo I. En resumen, la co-administración de NAC, Q_{10} y MnTBAP aumentó la expresión de las subunidades (nuclear y mitocondrial) del complejo I, y disminuyó la producción de ERO, el cociente GSSG/GSH y oxidación de Q_{10}, la disfunción mitocondrial y la muerte celular inducidas por D-GalN en el cultivo primario de hepatocitos humanos.

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N-acetylcysteine, coenzyme Q_{10} and superoxide dismutase mimetic prevent mitochondrial cell dysfunction and cell death induced by D-galactosamine in primary culture of human hepatocytes

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ABSTRACT

D-Galactosamine (D-GalN) induces reactive oxygen species (ROS) generation and cell death in cultured hepatocytes. The aim of the study was to evaluate the cytoprotective properties of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and the superoxide dismutase (SOD) mimetic against the mitochondrial dysfunction and cell death in D-GalN-treated hepatocytes. Hepatocytes were isolated from liver resections. NAC (0.5 mM), Q_{10} (30 μM) or MnTBAP (Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (1 mg/mL) were co-administered with D-GalN (40 mM) in hepatocytes. Cell death, oxidative stress, mitochondrial transmembrane potential (MTP), ATP, mitochondrial oxidized/reduced glutathione (GSH) and Q_{10} ratios, electronic transport chain (ETC) activity, and nuclear- and mitochondria-encoded expression of complex I subunits were determined in hepatocytes. D-GalN induced a transient increase of mitochondrial hyperpolarization and oxidative stress, followed by an increase of oxidized/reduced GSH and Q_{10} ratios, mitochondrial dysfunction and cell death in hepatocytes. The cytoprotective properties of NAC supplementation were related to a reduction of ROS generation and oxidized/reduced GSH and Q_{10} ratios, and a recovery of mitochondrial complexes I+III and II+III activities and cellular ATP content. The co-administration of Q_{10} or MnTBAP recovered oxidized/reduced GSH ratio, and reduced ROS generation, ETC dysfunction and cell death induced by D-GalN. The cytoprotective properties of studied antioxidants were related to an increase of the protein expression of nuclear- and mitochondrial-encoded subunits of complex I. In conclusion, the co-administration of NAC, Q_{10} and MnTBAP enhanced the expression of complex I subunits, and reduced ROS production, oxidized/reduced GSH ratio, mitochondrial dysfunction and cell death induced by D-GalN in cultured hepatocytes.

1. Introduction

Apoptosis is considered to be involved in the normal regulation of the organ size, as well as in the underlying mechanism of cell death in liver diseases. The induction of oxidative stress is a key event in the intracellular pathways leading to cellular apoptosis [1]. Several free radical generation sites, such as mitochondria or cytochrome P450-dependent metabolism, are involved in cell death [2,3]. The depletion of cellular reduced glutathione (GSH) content, as a consequence of intense intracellular oxidative stress, has been observed during cell death induced by different agents [4,5]. D-Galactosamine (D-GalN) is a suitable experimental model of human liver failure [6]. D-GalN induces oxidative stress and cell death in human and rat cultured hepatocytes [7,8]. The rapid generation of reactive oxygen species (ROS) was related to hyperpolarization of the mitochondrial membrane potential and apoptosis in D-GalN-treated human hepatocytes [9]. In addition, D-GalN-dependent cell

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necrosis was related to mitochondrial membrane depolarization [9].

Mitochondria provide most of the cell energy through the fatty acid β-oxidation, the tricarboxylic acid cycle, and oxidative phosphorylation. Endogenous compounds (such as cytokines or female sex hormones) or xenobiotics (including toxins, such as ethanol and drugs, such as aspirin, valproic acid, ibuprofen, or zidovudine) can inhibit β-oxidation directly or through a primary effect on the mitochondrial genome or the respiratory chain itself [10]. The mitochondrial ROS generation can be significantly enhanced by a rise of NADH supplementation or with the functional impairment of complexes I and III of electron transport chain (ETC) [11]. Although energy production is an important function of mitochondria, these organelles also participate in the initiation and execution of cell death, and maintaining calcium and iron homeostasis. The regulation of intracellular oxidative stress by antioxidants may determine cell fate and the mode of cell death [12]. Different antioxidant strategies have been shown to be useful to reduce oxidative stress and cell death in hepatocytes. GSH is the most abundant non-protein thiol present in mammalian cells, and acts as an essential component of the cellular antioxidant defense system. Due to its very reactive cysteine sulfhydryl moiety, GSH is enzymatically conjugated to toxic electrophiles by cellular GSH transferases. Additionally, GSH participates as a co-substrate in various GSH-dependent peroxidase reactions, protecting cells or tissues against oxidative damage by metabolizing toxic organic molecules and hydrogen peroxides. The maintenance of mitochondrial GSH content prevents cell damage in different *in vivo* [13] and *in vitro* [14] experimental models of hepatotoxicity. The administration of N-acetylcysteine (NAC), an excellent source of intracellular cysteine and free radical scavenger, has been shown to have clinical applications in HIV infection, cancer, heart disease, as well as in smoking, kidney and liver diseases [15]. The regulation of mitochondrial oxidative stress may be a useful strategy to prevent the depletion of GSH in the organelle. The aim of our study was the identification of the beneficial properties of NAC, coenzyme Q_{10} (Q_{10}) and superoxide dismutase (SOD) mimetic (MnTBAP) on oxidative stress, expression and/or activity of the mitochondrial electron transport chain (ETC) components and cell survival in D-GalN-treated hepatocytes. The study showed that the co-administration of NAC, Q_{10} and MnTBAP enhanced the expression of complex I subunits, and reduced ROS production, oxidized/reduced GSH ratio, mitochondrial dysfunction and cell death in D-GalN-treated hepatocytes.

2. Materials and methods

2.1. Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. DME:Ham-F12 and William's E culture mediums were obtained from Sigma Chemical Co. and Applichem (Applichem GmbH, Darmstadt, Germany), respectively. Antibiotics–antimycotic solution and fetal bovine serum were obtained from Life Technologies Inc. (Paisley, UK). NAC and Q_{10} were obtained from Sigma Chemical Co. MnTBAP (Mn (III)tetrakis(4-benzoic acid) porphyrin chloride was obtained from Calbiochem (Darmstadt, Germany). The peptide-based substrate for the measurement of caspase-3 activity was N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC, Bachem AG, Bubendorf, Switzerland). The study protocol has been approved by the Ethical Committee of the Institution.

2.2. Preparation of primary human hepatocytes and cell culture

The liver biopsy was obtained from 39 patients (17 women, 22 men; 58 ± 3.8 years old) submitted to surgical resection for liver tumor after written consent of the patient. The isolation of hepatocytes was based on the two-step collagenase procedure [16]. The liver was first perfused with a non-recirculating chelating solution I containing 0.5 mM ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetracetic acid (EGTA), 58 mM NaCl, 5 mM KCl, 0.44 mM KH_2PO_4, 0.34 mM $NaHPO_4$, 25 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (Trizma), 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min in order to remove the remaining blood. Afterwards, the liver was perfused with a non-recirculating washing solution II containing 20 mM N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)hemisodium (HEPES), 120 mM NaCl, 5 mM KCl, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min. The liver was later perfused with a recirculating dissociation solution III containing 0.05% type IV collagenase, 20 mM HEPES, 120 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 0.5% glucose, 100 μM sorbitol, 100 μM mannitol, 100 μM GSH, 100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B pH 7.2 at 37 °C using a flow of 75 mL/min. The cell suspension was filtered through nylon mesh (250 μm) and washed three times at 50 × *g* for 5 min at 4 °C in the supplemented culture medium. DME–Ham-F12 and William's E mediums (1:1) were supplemented with 26 mM NaHCO, 15 mM HEPES, 0.29 g/L glutamine, 50 mg/L vitamin C, 0.04 mg/L dexamethasone, 2 mg/L insulin, 200 μg/L glucagon, 50 mg/L transferrin and 4 ng/L ethanolamine. Cell viability was consistently >85%, as determined by trypan blue exclusion. Hepatocytes (8×10^6 cells; 150,000 cells/cm^2) were seeded at confluence on type I collagen-coated dishes (Iwaki, Gyouda, Japan), and maintained in a culture medium containing 5% fetal calf serum for 12 h. Afterwards, the medium was removed and replaced by a fresh culture medium without fetal bovine serum. The study was initiated 48 h after cell seeding to ensure cell stability. Cell death was induced by D-GalN (40 mM) that has been previously shown to induce oxidative stress and cell death in hepatocytes [7]. NAC (0.5 mM) [17,18], Q_{10} (30 μM) [19] or MnTBAP (1 mg/mL) [20] was co-administered with D-GalN.

2.3. Isolation of mitochondrial fraction

Mitochondria were obtained as previously described [21] with some modifications. Cells (16×10^6) were scraped and washed twice with ice-cold phosphate buffer solution (PBS) pH 7.4 at 500 × *g* for 5 min at 4 °C. Cells were disrupted with 9 volumes of buffer (HEPES 2 mM, pH 7.4, 0.15 mM $MgCl_2$, 10 mM KCl, 0.5 mM EGTA, 5 μg/mL aprotinin, 10 μg/mL leupeptin, and 0.5 mM phenylmethylsulfonyl fluoride) in a Dounce homogenizer. Afterwards, a concentrated sucrose solution was added to reach a final sucrose concentration of 0.32 M. Undisrupted cells and debris were removed by centrifugation at 1000 × *g* for 5 min. The pellet was recovered and named as F1 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The supernatant was considered as the post-nuclear cell extract, and it was used to obtain crude mitochondria by centrifugation at 10,000 × *g* during 10 min. The supernatant was recovered and named as F2 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The pellet was identified as the crude mitochondrial fraction, and layered onto a discontinuous sucrose gradient performed with 0.8, 1.0, 1.2, 1.4 and 1.6 M sucrose concentrations in 2 mM HEPES buffer pH 7.4. After centrifugation at 95,000 × *g* for 90 min, seven fractions were obtained. The first two fractions were recovered and named as F3 and F4 fractions for the characterization of mitochondrial isolation procedure (Fig. 1). The purified mitochondrial fraction located between 1.2 and 1.4 sucrose layers (correspond to the fraction 5

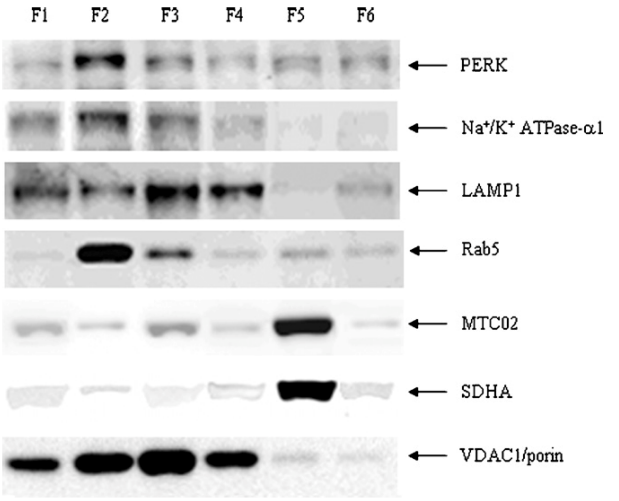


Fig. 1. Expression of mitochondria, endoplasmic reticulum, plasma membrane, lysosomes and endosomes markers in different fractions obtained during mitochondrial isolation procedure. Six fractions were isolated following the procedure described in Section 2. F1 fraction is obtained from the initial centrifugation of cell lysate at 1000 × *g*. The supernatant was submitted to centrifugation at 10,000 × *g*, and the resulting supernatant was identified as F2 fraction, and the pellet was layered onto a discontinuous sucrose gradient. After centrifugation at 95,000 × *g* for 90 min, seven fractions were obtained. The first two fractions were recovered and named as F3 and F4 fractions. The purified mitochondrial fraction located between 1.2 and 1.4 sucrose layers (correspond to the fraction 5 in the discontinuous gradient) was recovered and named as F5 fraction. The pellet obtained after ultracentrifugation was recovered and named as F6 fraction. The purity of the mitochondrial fraction was characterized in the F1–F6 fractions by the expression of voltage-dependent anion channel (VDAC1)/porin, succinate dehydrogenase (SDHA) and mitochondrial marker (MTC02). The expression of phospho-pancreatic endoplasmic reticulum kinase (PERK), Na^+/K^+ ATPase-α1, LAMP1 and Rab5A identify the expression of markers related to endoplasmic reticulum, plasma membrane, lysosomes and endosomes, respectively. The expression is assessed by SDS-PAGE followed by Western-blot analysis as described in Section 2. The images are representative of three different experiments.

in the discontinuous gradient) was recovered and named as F5 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The pellet was recovered and named as F6 fraction for the characterization of mitochondrial isolation procedure (Fig. 1). The expression of the markers in the isolated fractions (10 μg proteins) was assessed by 8–10% SDS-PAGE and Western-blot analysis. The purity of the mitochondrial fraction was characterized in the F1–F6 fractions by the expression of voltage-dependent anion channel (VDAC1)/porin (ab15895, Abcam, Cambridge, MA, USA) (1/1000), succinate dehydrogenase (SDHA) (2E3, ab14715, Abcam) (1/10,000) and mitochondrial marker (MTC02) (ab3298, Abcam) (1/200) (Fig. 1). The expression of SDHA and MTC02 was high, but very low that of VDAC1/porin, in the purified mitochondrial fraction (F5) (Fig. 1). The low expression of VDAC/porin in F5 may suggest that the outer mitochondrial has been separated from the mitochondrial inner membrane and matrix. The expression of phospho-pancreatic endoplasmic reticulum kinase (PERK) (ab65142, Abcam) (1/1000), Na^+/K^+ ATPase-α1 (ab2872, Abcam) (dilution 1/250), LAMP1 (ab25630) (1/10,000) and Rab5A (ab50523, Abcam) (1/1000) showed insignificant contamination with endoplasmic reticulum, plasma membrane, lysosomes and endosomes in the F5 fraction, respectively (Fig. 1). The endoplasmic reticulum, plasma membrane and endosomes were mostly present in F2 fraction, but lysosomes were mostly detected in the first two fractions of the discontinuous gradient (F3 and F4) (Fig. 1).

2.4. Preparation of cytoplasm and nuclear extracts

The cytoplasm and nuclear fractions from hepatocytes (3×10^6) were obtained by cell fractionation [22]. Briefly, hepatocytes were treated with lysis buffer (800 μL) containing 10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 0.1 mM ethylene glycol tetraacetic acid (EGTA), 5 μg/mL aprotinin, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol (DTT) and 0.6% Nonidet NP-40 for 10 min on ice. Afterwards, samples were homogenized and centrifuged at 15,000 × *g* for 3 min at 4 °C. Aliquots of the supernatant (cytoplasm fraction) were stored at −80 °C until use for the measurement of lactate dehydrogenase (LDH) release and caspase-3 activation. The pellet (nuclear faction) was discarded.

2.5. DNA fragmentation

The whole hepatocyte population (8×10^6 cells), including the floating cells obtained from the collected culture medium, was treated with lysis buffer (1 mL) containing 100 mM Trizma, 5 mM EDTA, 150 mM NaCl and 0.5% sarkosyl pH 8.0 for 10 min at 4 °C. Samples were incubated with RNAse (50 μg/mL) for 2 h at 37 °C, and with proteinase K (100 μg/mL) for 45 min at 48 °C. DNA was obtained by phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma Chemical Co.) extraction and precipitated with cold isopropanol (1:1) for 12 h at −20 °C. DNA was recovered by centrifugation of the sample at 20,800 × *g* during 10 min at 4 °C. Thereafter, the precipitate was washed with 70% ethanol, dried and re-suspended in Trizma–EDTA buffer (10 mM Trizma, 1 mM EDTA) at pH 8.0. Samples (100 μg DNA) were analyzed on 1.5% agarose gels with ethidium bromide (0.5 μg/mL).

2.6. Caspase-3 activation

Caspase-3 processing was assessed by Western-blot analysis in cytoplasm fraction. Proteins (50–100 μg) were separated by 14% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding primary rabbit polyclonal (1/1500) (Abcam) antibodies, and secondary goat anti-rabbit polyclonal (1/50,000) (sc-2301, Santa Cruz Biotechnology, Inc., California, USA) antibodies coupled to horseradish peroxidase, revealing the protein content by commercial enhanced chemiluminescence (ECL) assay (RPN2135, GE Healthcare, Buckinghamshire, UK). β-Actin, used as internal protein loading, was identified with commercial rabbit polyclonal antibodies (1/5000) (ab8227, Abcam). The enzymatic activities in the cytoplasm fraction (25 μg) were measured using Ac-DEVD-AFC (100 μM) in caspase-incubating buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10% sucrose, 0.1% CHAPS, 1 mM EDTA and 5 mM DTT) up to 100 μL total volume. The fluorescence of the sample (Ex 400, Em 505) was recorded using a GENios Microplate Reader (TECAN, Salzburg, Austria).

2.7. Measurement of lactate dehydrogenase release

LDH in the culture medium (130 μL) and cytoplasm fraction (5 μL) was assessed with 0.2 mM β-NADH and 0.4 mM pyruvic acid up to 200 μL PBS pH 7.4. LDH concentration in the sample was proportional to the rate of NADH oxidation measured by the absorbance at 334 nm (OD/min) using a GENios Microplate Reader (TECAN). LDH concentration in cytoplasm and culture medium was calculated using a commercial standard (GE Healthcare). The LDH release (%) was calculated as the ratio [(LDH culture medium)/(LDH cytoplasm + LDH culture medium)] × 100.

Table 1
Effect of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the mitochondrial complex activities in D-galactosamine (D-GalN)-treated hepatocytes.

Treatments	Complex I	Complex II	Complex III	Complexes I + III	Complexes II + III	Complex IV
Control	23.4 ± 3.4	23.0 ± 4.26	56 ± 6.3	116 ± 3.5	13.9 ± 0.51	29.2 ± 5.27
Q_{10}	63.9 ± 6.08*	36.7 ± 2.38*	150 ± 11.3*	194 ± 16.9*	22.1 ± 1.96*	48.3 ± 4.87*
NAC	45.1 ± 0.57*	19.1 ± 2.88	53 ± 5.7	124 ± 4.3	13.3 ± 0.89	30.2 ± 5.62
MnTBAP	23.5 ± 0.51	24.4 ± 1.12	67 ± 5.7	109 ± 2.0	13.1 ± 0.26	33.4 ± 3.53
D-GalN	69.5 ± 6.00*	25.7 ± 3.77	113 ± 1.5*	64 ± 3.4*	9.0 ± 0.41*	52.6 ± 2.00*
D-GalN + Q_{10}	52.8 ± 2.26*	22.0 ± 3.45	95 ± 6.5*	119 ± 11.0*	13.1 ± 0.50*	44.7 ± 1.33*
D-GalN + NAC	46.4 ± 2.60*	22.8 ± 3.01	79 ± 6.4*	85 ± 4.3*	11.8 ± 0.66*	45.2 ± 1.38*
D-GalN +MnTBAP	53.2 ± 2.50*	20.9 ± 2.31	76 ± 6.9*	87 ± 2.7*	11.9 ± 0.25*	46.0 ± 0.55*

NAC (0.5 mM), Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. The activity of complexes I, II, III, I + III, II + III and IV was measured 24 h after treatments in mitochondrial fraction following the procedure described in Section 2. All the values were expressed as a specific activity (nmol × min^{-1} × mg $protein^{-1}$). Each value represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

2.8. ATP measurement in hepatocytes

The hepatocyte population (3×10^6 cells), including the floating cells obtained from the collected culture medium, was treated with 1 mL of 85 mM perchloric acid, mixed for 20 s and centrifuged at 15,000 × *g* during 5 min at 4 °C. The samples were stored at −80 °C until use. The commercial luciferase solution (FL-AAM, Sigma) was diluted 1:25 with an ATP assay mix dilution buffer (FL-AAB, Sigma). The sample was also diluted 1:10 in 50 mM HEPES buffer solution pH 7.8. In the assay, 100 μL of luciferase was mixed with 100 μL of the sample in darkness, and the chemiluminescence of the mixture was measured using a GENios Microplate Reader (TECAN). The ATP concentration in the sample was proportional to the emitted chemiluminescence. The ATP concentration was calculated using a commercial standard (FL-AAS, Sigma).

2.9. GSH measurement in mitochondrial fraction

GSH and its oxidized form (GSSG) were measured using a commercial o-phthalaldehyde based assay (BioVision Research, Mountain View, CA, USA). The DTNB-enzyme cycling or the monochlorobimane GSH based assays can hardly detect differences between GSH + GSSG and GSH measurements. Briefly, the mitochondrial fraction (16×10^6 cells) was treated with 6N perchloric acid solution (3:1, v/v). The o-phthalaldehyde reacts with GSH, but not with GSSG, generating a fluorescent product. The specific measurement of GSSG, requires the addition of GSH quencher to the sample preventing its reaction with o-phthalaldehyde. The later addition of a reducing agent allows the removal of the excess of GSH quencher and the conversion of GSSG to GSH that will be measured by reaction with o-phthalaldehyde. The assay provides a specific procedure to eliminate protein thiol interference and to stabilize GSH and GSSG in solution. The fluorescence of the sample (Ex 340, Em 420) was recorded using a GENios Microplate Reader (TECAN). GSH concentration was obtained using commercial standards included in the assay.

2.10. Coenzyme Q_{10} determination

The content of oxidized and reduced Q_{10} in mitochondria was determined by high performance liquid chromatography (HPLC) after extraction with hexane [23]. Briefly, the mitochondrial fraction obtained from 16×10^6 cells were re-suspended in 500 μL PBS and 500 μL 10 mM butylated hydroxytoluene, and incubated for 10 min on ice. An aliquot of the sample (50 μL) was diluted with 50 μL 2% SDS and mixed by vortex during 1 min, 2 mL of ethanol/isopropanol (95:5) HPLC grade was added and mixed again for 1 min. Finally, 500 μL of hexane was added, mixed and centrifuged at 19,000 × *g* for 5 min at 4 °C. The extraction of Q_{10} with hexane was repeated twice, and the different upper organic phases were collected, pooled and dried with nitrogen. The dried residue was reconstituted with 50 μL mobile phase composed of *n*-propanol/methanol (40:60) containing 19.9 mM lithium perchlorate, and then subjected immediately to reversed phase chromatography at a flow-rate of 1 mL/min in a C18 kromasil 100 column (5-μm particle, 25 cm × 0.45 cm) (Scharlab, S.L., Sentmenat, Spain) installed in a HPLC device (Beckman-Coulter, Inc., Fullerton, USA). Monitoring was carried out with a Coulochem II electrochemical detector (ESA, Chelmsford, MA, USA) fitted with a Model 5010 analytical cell with the electrodes set at potentials of −500 mV and +300 mV. Both reduced and oxidized Q_{10} forms were detected from the electrochemical signal obtained at the second electrode. Using this protocol, reduced Q_{10} eluted at 13.5 min, whereas oxidized Q_{10} eluted at 21 min. Concentrations were calculated by integration of peak areas and comparison with external standards (oxidized or sodium borohydride-reduced Q_{10}). Pure Q_{10} was obtained from Sigma Chemicals.

2.11. Mitochondrial ETC activities and ROS production

The mitochondrial fraction obtained from 16×10^6 cells was used to measure the activity of complexes I, II, III, I + III, II + III, and IV [24]. NADH dehydrogenase (complex I) activity was determined as the rate of NADH-dependent consumption at 340 nm with coefficient of extinction or $\varepsilon = 6.81\ mM^{-1}\ cm^{-1}$ using 200 μM NADH, 1 mM NaN_3 in the presence or the absence of 10 μM rotenone. The activity of complex II (succinate dehydrogenase) was measured spectrophotometrically in the presence of 100 μM 2,6-dicholophenolindophenol, 32 mM succinate, and 50 μM Q_1. The decrease of dicholorophenolindophenol was followed at 600 nm using $\varepsilon = 20.5\ mM^{-1}\ cm^{-1}$. The complex III (ubiquinol–cytochrome *c* reductase) activity was assayed using 50 μM decilubiquinol and 50 μM cytochrome *c* as substrates. The complex III activity was measured by cytochrome *c* reduction in the presence or absence of 18.22 μM antimycin A. The reduction of cytochrome *c* was followed at 550 nm using $\varepsilon = 18.5\ mM^{-1}\ cm^{-1}$. The decilubiquinol was obtained by the chemical reduction of decilubiquinone with sodium borohydride. Cytochrome *c* oxidase (complex IV) activity was determined as the rate of oxidation of 80 μM reduced cytochrome *c* at 550 nm ($\varepsilon = 20.5\ mM^{-1}\ cm^{-1}$). Cytochrome *c* was reduced by sodium borohydride. The activities of complexes I + III and II + III consisted, respectively in the NADH- and succinate-dependent cytochrome *c* reduction assessed at 550 nm.

ROS were detected using 2,7-dichlorofluorescein diacetate dye (DCFDA; Molecular Probes Europe BV, Leiden, The Netherlands) in hepatocytes. Anion superoxide ($O_2^{\bullet -}$) production was monitored using dihydroethidium (DHE; Molecular Probes) probe in hepatocytes. The contribution of hydrogen peroxide (H_2O_2) to the oxidation of DCFDA was determined with the pre-incubation

(30 min) of the cells with catalase (500 U/mL). Hepatocytes were incubated with DHE (10 μM) or DCFDA (2 μM) for 20 and 30 min, respectively. Cells were treated with digitonin (10 μM) for 5 min in order to eliminate probe not retained in mitochondria. The culture medium was removed and replaced with PBS in order to avoid any interference with the measurement of cell fluorescence. The fluorescence emitted by DHE (Ex 510, Em 590) and DCFDA (Ex 500, Em 520) was assessed *in situ* using a GENios Microplate Reader (TECAN).

The mitochondrial transmembrane potential (MTP) was monitored using a membrane potential-sensitive fluorescent probe, such as tetramethylrhodamine methyl ester (TMR) (Molecular Probes) in hepatocytes. Cells were incubated with TMR (2 μM) for 30 min. Cells were treated with digitonin (10 μM) for 5 min in order to eliminate any probe not retained in mitochondria. The culture medium was removed and replaced with PBS in order to avoid any interference with the measurement of cell fluorescence. The fluorescence emitted by TMR (Ex 550, Em 570) was assessed *in situ* using a GENios Microplate Reader (TECAN).

2.12. Measurement of nuclear- and mitochondrial-DNA ratio

Hepatocytes were collected, washed twice with cold PBS pH7.4, re-suspended in 250 μL TE (10 mM Trizma, pH 7.5; 1 mM EDTA), 1.25 μL proteinase K (20 mg/mL), 5 μL 25% SDS and 5 μL ribonuclease A (10 mg/mL), and incubated overnight at 37 °C. DNA was extracted once with 1.5 volumes of phenol:chloroform:isoamyl alcohol (25:24:1, saturated with a solution of 10 mM Trizma pH 8, 1 mM EDTA), and twice more with chloroform:isoamyl alcohol (24:1). DNA was precipitated by the addition of 50 μL 7.5 M ammonium acetate and 2 volumes of cold pure ethanol for 3 h at −20 °C. DNA was recovered by centrifugation at 18,000 × *g* for 30 min, and the dry pellet was re-suspended in 10 mM Trizma buffer pH 8.0 or DNase free water (Sigma).

DNA was quantified by real-time polymerase chain reaction (RT-PCR) using the LightCycler Thermal Cycler System (Roche Diagnostics, Indianapolis, USA) and QuantiTect SYBR Green RT-PCR Kit (Qiagen GmbH, Hilden, Germany). Total cellular DNA (27 ng) was used as a template and was amplified with specific oligodeoxynucleotides for mitochondrial-encoded cytochrome oxidase subunit 2 gene (mt-Co2) (sense 5′-CGATCCCTCCCTTACCATCA-3′, and antisense 5′-CCGTAGTCGGTGTACTCGTAGGT-3′) and nuclear-encoded β-globin gene (sense 5′-GTGCATCTGACTCCTGAGGAGA-3′, and antisense 5′-CCTTGATACCAACCTGCCCAG-3′).

RT-PCR amplification was carried out in accordance with the manufacturer's recommendations in a final volume of 18 μL. The final concentrations of primers were 0.25 μM. The amplification protocol consisted of 55 cycles of incubation after the initial denaturing at 95 °C during 10 s (20 °C/s), 58 °C for 10 s (20 °C/s) and 72 °C for 10 s (2 °C/s). The melting conditions were fixed at 65 °C (0.1 °C/s). We calculated the mtDNA copy number per cell using a β-globin amplification as a reference for nuclear DNA content. The values (%) are calculated in relation to the control values of DNA copy number calculated using the formula $2 \times 2^{(\Delta Ct\ \text{nuclear-mitochondrial})}$.

2.13. Quantitative mRNA expression of nuclear-encoded complex I subunit by real-time PCR

The expression of the nuclear-encoded p17 subunit (NDUFB6 gene) was assessed with specific oligodeoxynucleotides (sense 5′-TGCTGCCCCCACAGAAGA-3′, and antisense 5′-TACCCCATGGACCATTTTCC-3′) and using the expression of the Ribosomal Protein L13A gene (sense, 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, and antisense 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′) as a reference.

RT-PCR amplification was carried out in a final volume of 18 μL. The final concentrations of primers and total RNA were 0.55 μM

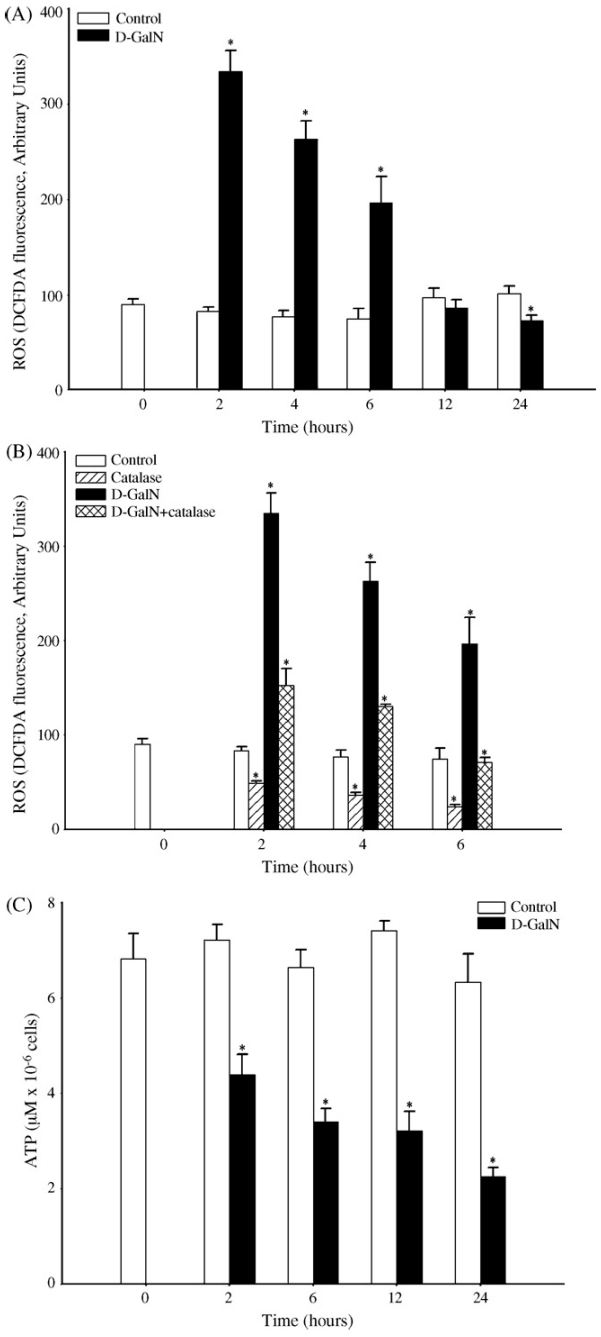


Fig. 2. Reactive oxygen species (ROS) (A) and ATP (C) in D-galactosamine (D-GalN)-treated hepatocytes. A kinetic study of D-GalN (40 mM)-induced cell death was carried out in cultured human hepatocytes. ROS were *in situ* detected using 2,7-dichlorofluorescein diacetate dye (DCFDA) in digitonin-treated cells. The contribution of H_2O_2 to the oxidation of the DCFDA was evaluated by the pre-administration (30 min) of catalase (500 U/mL) in control and D-GalN-treated hepatocytes (B). ATP was measured in acid cell extracts using luciferase reaction. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

and 5.5 ng/μL, respectively. The amplification protocol consisted of 35 cycles of denaturing at 95 °C during 15 s (20 °C/s), annealing at 60 °C for 15 s (20 °C/s) and synthesis at 72 °C for 25 s (2 °C/s). The melting conditions were fixed at 65 °C (0.1 °C/s). The used primers were tested to be optimum for quantitative analysis. To confirm amplification specificity, the amplified fragments were analyzed by 1.5% agarose gel electrophoresis containing ethidium bromide. The melting curve analysis with the PCR products showed that there

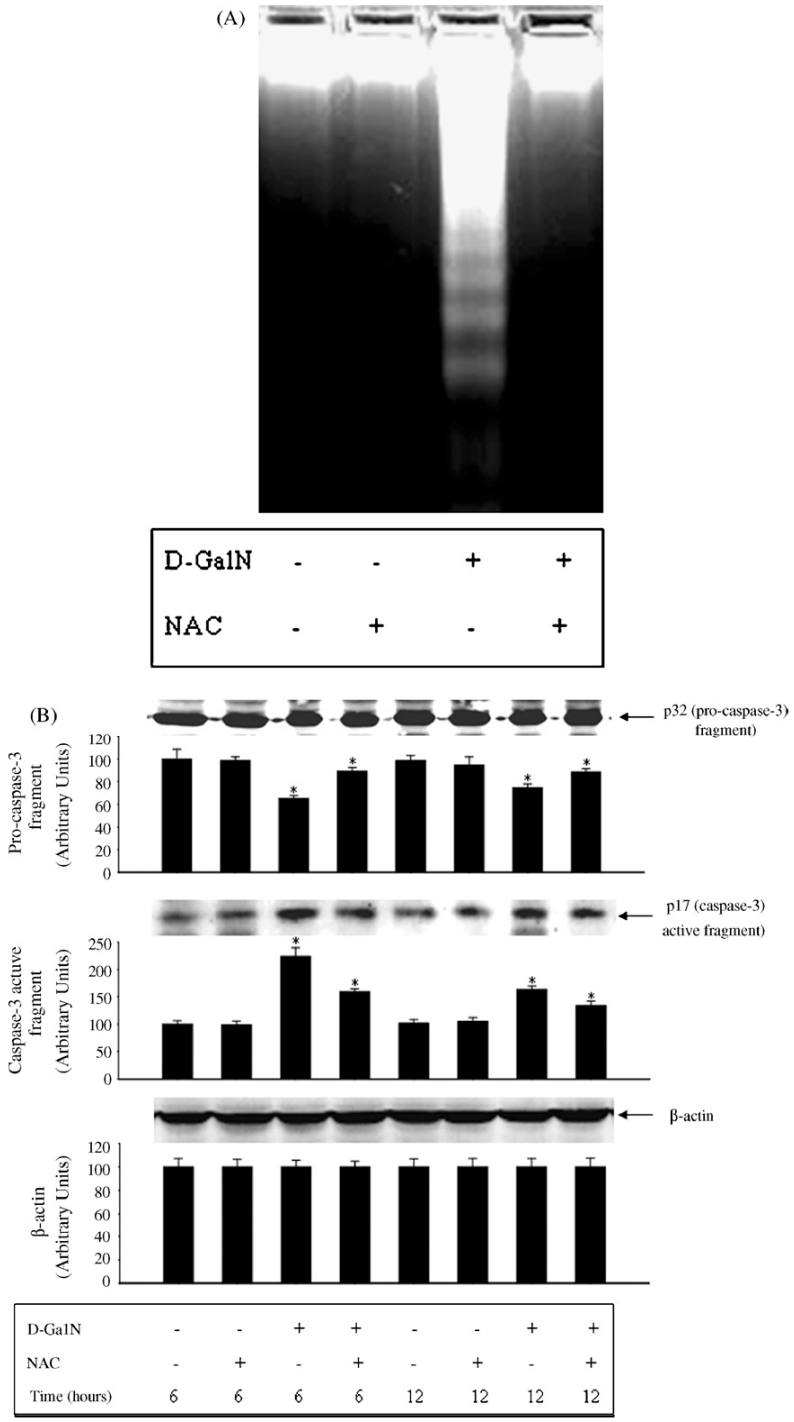


Fig. 3. Effect of N-acetylcysteine (NAC) treatment on DNA fragmentation (A), caspase-3 processing (B), caspase-3 activity (C) and lactate dehydrogenase (LDH) release (D) in D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM) was co-administered with D-GalN (40 mM) in cultured human hepatocytes. DNA fragmentation (6 h) was assessed by agarose gel electrophoresis in cell lysate including hepatocytes and floating cells present in the culture medium. The expression of pro-caspase-3 (p32) and caspase-3 active fragment (p17) was assessed by SDS-PAGE and Western-blot analysis. The statistical analysis of densitometric values of the spots is shown below each blot. β-Actin was used as internal protein loading. Caspase-3-associated activity was assessed using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) as the corresponding peptide-based substrate. LDH release represents the percentage of LDH in the culture medium in relation to total LDH. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

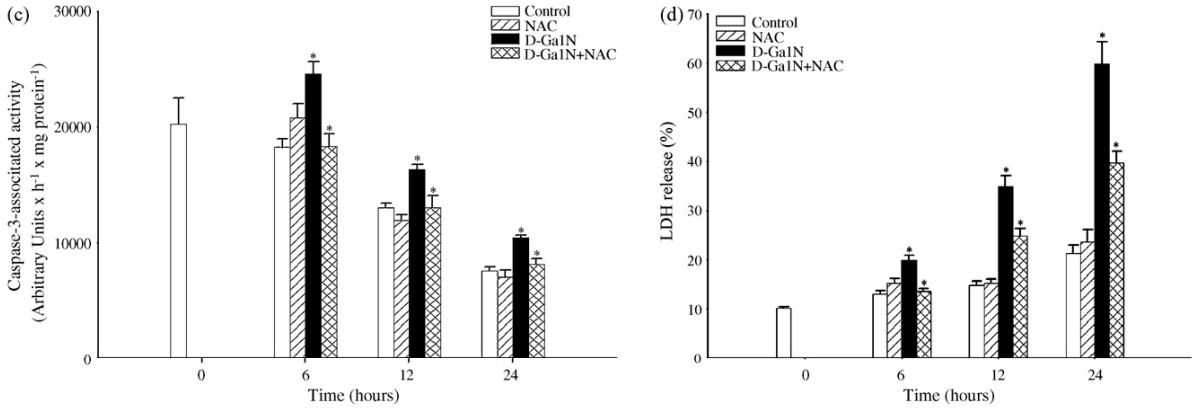


Fig. 3. (*Continued*).

was no primer dimer formation. Quantitation of relative expression was determined by the $2^{(\Delta Ct)}$.

2.14. Measurement of nuclear- and mitochondrial-encoded complex I protein expression

The protein expression of nuclear-encoded 17 kDa and mitochondrial-encoded 20 kDa subunits of mitochondrial complex I was assessed by Western-blot analysis in the mitochondrial fraction. Proteins (5 μg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose. The membranes were incubated with the corresponding commercial primary antibodies against p17 (1/10,000) (A21359, Molecular Probes Europe BV) and p20 (1/5000) (A31857, Molecular Probes Europe BV) subunits. Secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology, Inc.) were used, revealing protein content by ECL.

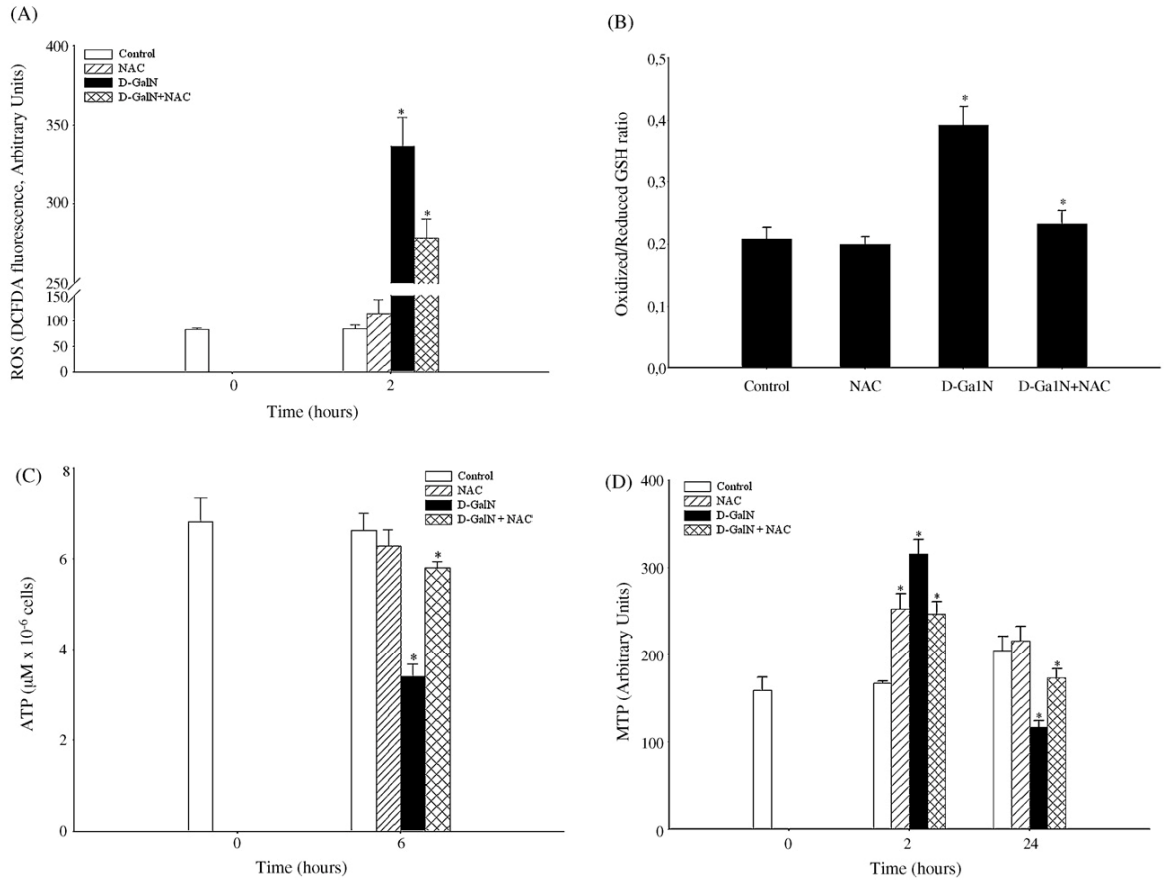


Fig. 4. Effect of N-acetylcysteine (NAC) treatment on reactive oxygen species (ROS) (A), mitochondrial oxidized/reduced glutathione (GSH) ratio (B), ATP (C) and mitochondrial transmembrane potential (MTP) (D) in D-GalN-treated human hepatocytes. NAC (0.5 mM) was co-administered with D-galactosamine (D-GalN) (40 mM) in cultured human hepatocytes. ROS were monitored *in situ* using 2,7-dichlorofluorescein diacetate dye (DCFDA) in digitonin-treated cells. GSH and its oxidized form (GSSG) were measured by the o-phthalaldehyde based assay. ATP was measured in acid cell extracts using a luciferase reaction. MTP was monitored *in situ* using a membrane potential-sensitive fluorescent probe, such as tetramethylrhodamine methyl ester (TMR), in hepatocytes. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

MTC02, used as internal protein loading, was identified with commercial primary rabbit polyclonal antibodies (1/200) (Abcam).

2.15. Statistical analysis

Results are expressed as mean ± SE of five experiments. Data were compared using the analysis of variance with the Least Significant Difference's test as post hoc multiple comparison analysis. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

3. Results

3.1. NAC reduced cell death in D-GalN-induced cytotoxicity

D-GalN induces oxidative stress and cell death in primary culture of rat and human hepatocytes [7,9]. D-GalN induced a rapid and transient induction of mitochondrial ROS generation measured by DCFDA (Fig. 2A) ($p \leq 0.05$). The contribution of hydrogen peroxide (H_2O_2) to the oxidation of the DCFDA was determined with the pre-incubation (30 min) of the cells with catalase (500 U/mL). Catalase showed that 50% of DCFDA oxidation was due to H_2O_2 (Fig. 2B). The profile of ROS generation mimicked the progressive reduction of cellular ATP concentration in D-GalN-treated hepatocytes (Fig. 2C). NAC administration has been shown to reduce oxidative stress, cellular GSH depletion and cell death induced by different agents in cultured rat hepatocytes [25,26]. The co-administration of NAC (0.5 mM) reduced DNA fragmentation (Fig. 3A), the expression of active caspase-3 fragment (Fig. 3B), caspase-3 activity (Fig. 3C) and cell necrosis (Fig. 3D) induced by D-GalN in hepatocytes ($p \leq 0.05$). The prevention of D-GalN-induced cell death by NAC was related to a partial reduction of ROS generation (Fig. 4A), as well as a recovery of mitochondrial oxidized/reduced GSH ratio (Fig. 4B), cellular ATP (Fig. 4C), and MTP (Fig. 4D) ($p \leq 0.05$).

3.2. Cytoprotective properties of NAC on mitochondrial function in D-GalN-treated hepatocytes

The intense oxidative stress by D-GalN did not affect the ratio of mitochondrial/nuclear DNA copy number (data not shown). Nevertheless, the alteration of ROS production (Fig. 2A), MTP (Fig. 4D) and ATP (Fig. 2C) content induced by D-GalN suggests a profound mitochondrial dysfunction. The complex I (NADH coenzyme Q reductase) and the complex III (ubiquinol cytochrome *c* reductase) are sites in which ROS production may take place [11]. In addition to the bioenergetic role of Q, as a component of the mitochondrial respiratory chain, its redox properties suggest that it exerts relevant antioxidant properties [11]. Interestingly, D-GalN increased complexes I, III and IV activities, but reduced the coupled complexes I + III and II + III activities (Table 1) ($p \leq 0.05$). The last data suggest that the toxin induced a drastic alteration of Q status in the mitochondrial membrane. In concordance, D-GalN reduced Q_{10} content (Fig. 5A) and enhanced the ratio of oxidized/reduced Q_{10} (Fig. 5B) in mitochondria ($p \leq 0.05$). Interestingly, the increase of reduced Q_{10} (Fig. 5A) content and reduction of the oxidized/reduced Q_{10} ratio (Fig. 5B) in mitochondria by NAC co-administration was related to a reduction of complexes I, II and IV activities, as well as a restoration of coupled complexes I + III and II + III activities (Table 1) from D-GalN-treated hepatocytes ($p \leq 0.05$).

3.3. The supplementation with Q_{10} or MnTBAP restored mitochondrial activities and reduced cell death in D-GalN-treated hepatocytes

The data described above showed that the induction of cell death by D-GalN was related to an increase of mitochondrial oxidative stress, depletion of mitochondrial reduced Q_{10} content and mitochondrial dysfunction in hepatocytes. The co-administration of Q_{10} could enhance reduced Q_{10} (Fig. 5A) content, but it did not recover oxidized/reduced Q_{10} ratio (Fig. 5B) in mitochondria from D-GalN-treated hepatocytes ($p \leq 0.05$). This effect of Q_{10} was related to a reduction of ROS (Fig. 6A) production measured by DCFDA fluorescence in D-GalN-treated hepatocytes ($p \leq 0.05$). The co-administration of a SOD mimetic, MnTBAP, reduced $O_2^{\bullet -}$ production measured by DHE in D-GalN-treated hepatocytes (Fig. 6A) ($p \leq 0.05$). Both experimental strategies (Q_{10} and MnTBAP) reduced mitochondrial oxidized/reduced GSH content (Fig. 6B) and complexes I, III and IV activities (Table 1), as well as increased the coupled complexes I + III and II + III activities (Table 1) in D-GalN-treated hepatocytes ($p \leq 0.05$). Q_{10} and MnTBAP reduced DNA fragmentation (Fig. 6C), caspase-3 activity (Fig. 6D), and cell necrosis (Fig. 6E) in hepatocytes treated with D-GalN ($p \leq 0.05$). The reduction of oxidative stress and the recovery of functional ETC activities by NAC, Q_{10} and MnTBAP were related to an increase of the protein expression of the p17 nuclear- and p20 mitochondria-encoded complex I subunits (Fig. 7A), as well as the mRNA expression of the p17 nuclear-encoded complex I subunit (Fig. 7B) in D-GalN-treated hepatocytes ($p \leq 0.05$).

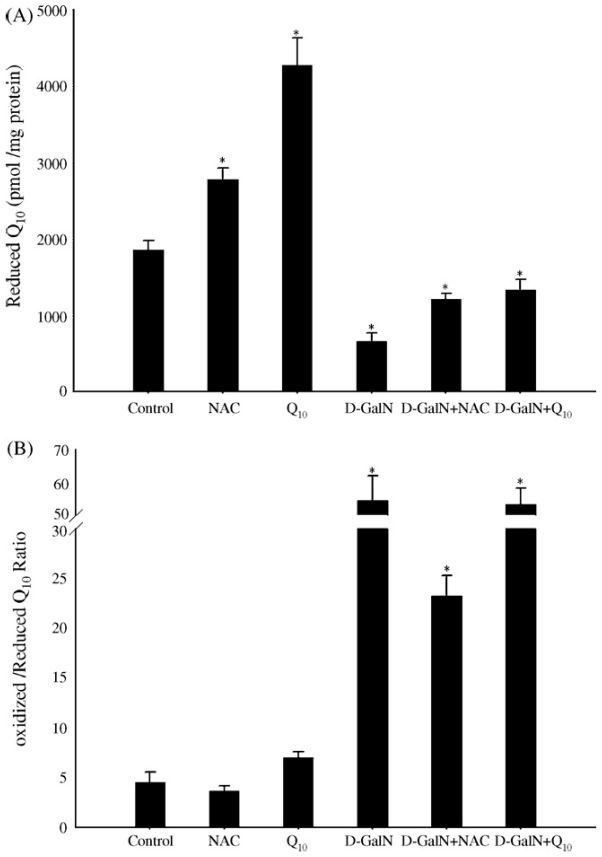


Fig. 5. Effect of N-acetylcysteine (NAC) and coenzyme Q_{10} (Q_{10}) treatments on the reduced Q_{10} content (A) and the oxidized/reduced Q_{10} ratio (B) in mitochondria from D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM) or Q_{10} (30 μM) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. Oxidized and reduced Q_{10} content (24 h) were determined by high performance liquid chromatography in mitochondria following the procedure described in Section 2. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group.

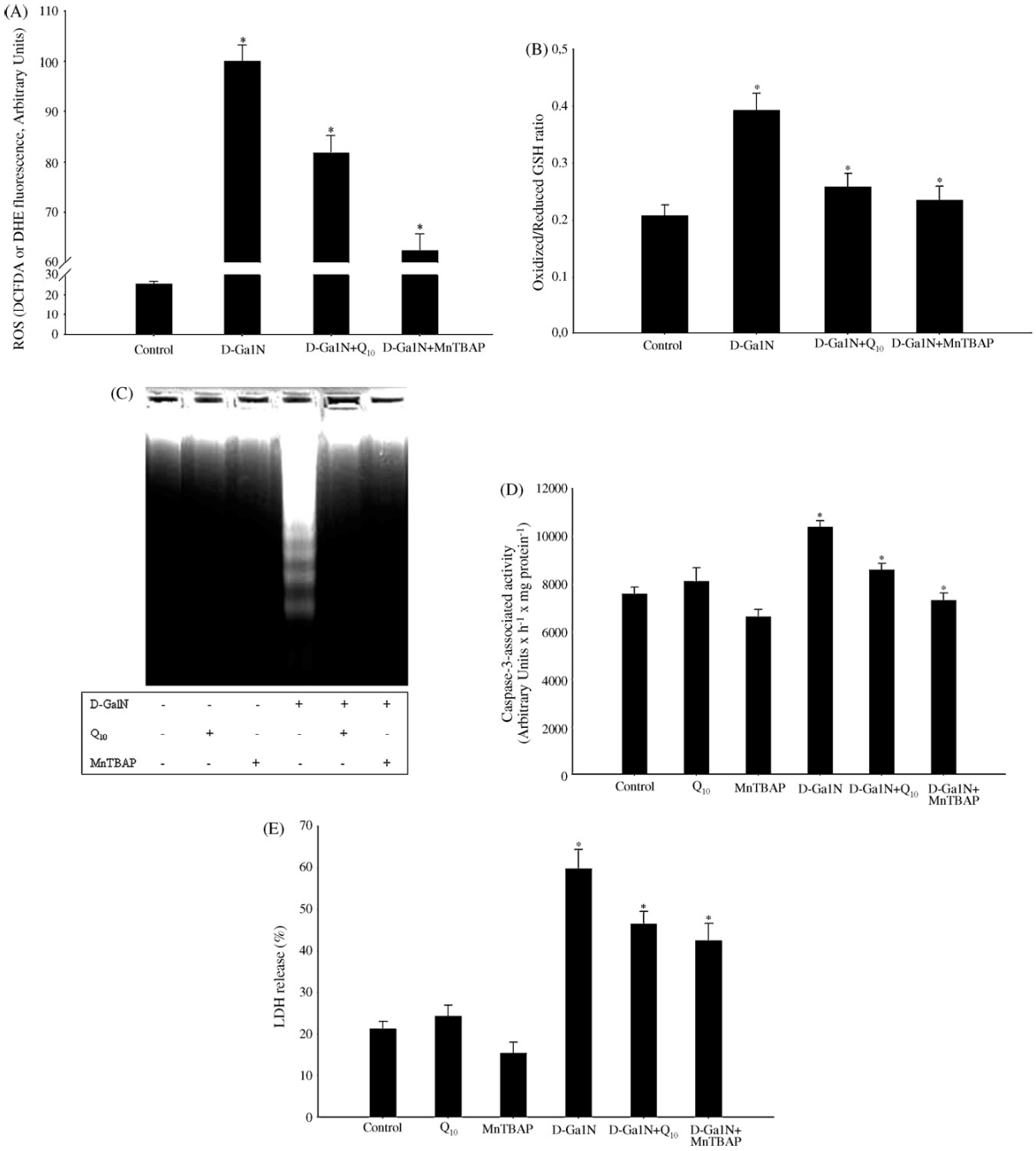
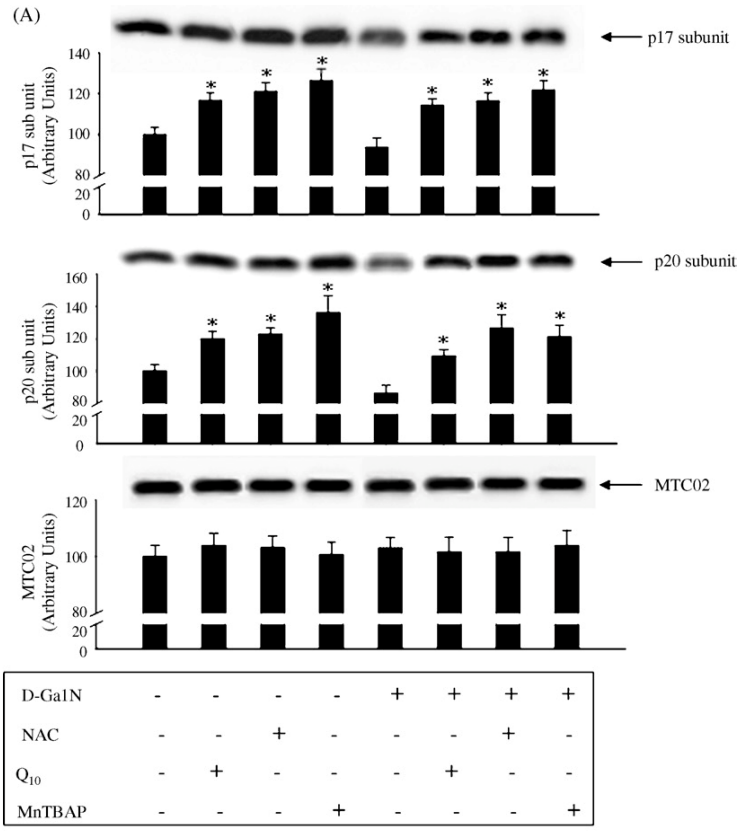


Fig. 6. Effect of coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the reactive oxygen species (ROS) (A) and the oxidized/reduced Q_{10} ratio (B) in mitochondria, DNA fragmentation (C), caspase-3 activity (D) and lactate dehydrogenase (LDH) release (E) in D-galactosamine (D-GalN)-treated human hepatocytes. Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. ROS production (2 h) was monitored *in situ* using 2,7-dichlorofluorescein diacetate dye (DCFDA) in control, D-GalN and D-GalN + Q_{10}-treated hepatocytes. Anion superoxide ($O_2^{\bullet-}$) production (2 h) was monitored *in situ* using dihydroethidium (DHE) in D-GalN and D-GalN + MnTBAP-treated hepatocytes. Oxidized and reduced Q_{10} content (24 h) were determined by high performance liquid chromatography in mitochondria following the procedure described in Section 2. DNA fragmentation (6 h) was assessed by agarose gel electrophoresis in cell lysate including hepatocytes and floating cells present in the culture medium. Caspase-3-associated activity (24 h) was assessed using N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC) as the corresponding peptide-based substrates. LDH release (24 h) represents the percentage of LDH in the culture medium in relation to total LDH. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

4. Discussion

Oxidative stress plays a relevant role in the induction of cell death in hepatocytes. NAC has been shown to exert cytoprotection in different thiol-depleting experimental models of cell death in hepatocytes [25,27]. D-GalN-induced ROS production, mitochondrial dysfunction and cell death in hepatocytes. The study showed that the reduction of oxidative stress, recovery of mitochondrial



Treatments	Ct mRNA RPL13A (%)
Control	100±1.6
D-GalN	106±1.9
D-GalN+Q_{10}	107±2.3
D-GalN+NAC	108±1.6
D-GalN+MnTBAP	110±3.1

Fig. 7. Effect of N-acetylcysteine (NAC), coenzyme Q_{10} (Q_{10}) and Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) treatments on the protein expression of nuclear- and mitochondria-encoded complex I subunits (A), and the mRNA expression of nuclear-encoded complex I subunit (B) in D-galactosamine (D-GalN)-treated human hepatocytes. NAC (0.5 mM), Q_{10} (30 μM) and MnTBAP (1 mg/mL) were co-administered with D-GalN (40 mM) in cultured human hepatocytes. The samples were obtained 24 h after D-GalN administration. The protein expression of nuclear- and mitochondrial-encoded complex I subunits (p17 and p20, respectively) was assessed by SDS-PAGE and Western-blot analysis. The statistical analysis of densitometric values of the spots is shown below each blot. Mitochondrial marker (MTC02) was used as internal protein loading. The mRNA expression of complex I subunit (p17) was assessed by RT-PCR analysis, and referred to the expression of the constitutive gene RPL13A. The statistical analysis of Ct values of all samples is shown below the mRNA expression graph. Each bar represents the mean ± SE of five independent experiments. The statistical differences were set at $p \leq 0.05$. The groups with "*" were significantly different from the corresponding control group. The images are representative of five independent experiments.

oxidized/reduced GSH and reduced Q_{10} content, and the restoration of ETC by antioxidants are key factors for cell survival in D-GalN-treated hepatocytes. The study also suggests that improved mitochondrial function by NAC, Q_{10} and MnTBAP was related to an increase of the expression of nuclear- and mitochondrial-encoded subunits of complex I in D-GalN-treated hepatocytes.

D-GalN has been used to induce experimental acute hepatotoxicity [6]. D-GalN is actively incorporated to hepatocytes by

carrier-mediated diffusion across the plasma membrane, phosphorylated and transformed to UDP-galactosamine by UDP-glucose:α-D-galactose-1-phosphate uridyltransferase. Consequently, the toxicity of D-GalN is related to the depletion of uridine pools and alteration of RNA and protein synthesis [6]. This experimental model is particularly interesting for the assessment of mitochondrial protective agents. The UTP synthesis also affects the activity of a mitochondrial enzyme, dihydroorotate dehydrogenase, which catalyzes the oxidation of dihydroorotate to orotate with the reduction of Q. In this sense, the activity of dihydroorotate dehydrogenase requires a functional mitochondrial chain activity [23].

D-GalN induces apoptosis and necrosis in liver tissue and cultured hepatocytes [7,9,28]. The intracellular ROS generation is associated with cell death in cultured hepatocytes [2,3,29]. The complexes I and III are major sites of mitochondrial ROS production [11]. The rapid hyperpolarization of mitochondrial membrane by D-GalN was related to an increase of complexes I, III and IV activities, as well as a rapid and transient ROS production. The generation of ROS reduces antioxidant content and may damage mitochondrial components through lipid peroxidation products, protein oxidation and nitrosylation, as well as mtDNA mutations in the liver [11,30–32]. The induction of oxidative stress by D-GalN was associated with a sharp increase of mitochondrial oxidized/reduced GSH and Q_{10} ratios, and reduction of coupled complexes I + III and II + III activities, expression complex I subunits and cellular ATP content. Oxidative stress [7,12] and ATP depletion [33] are related to a shift from an apoptotic to a necrotic cell death pathway. The rapid induction of ROS production (4.3-folds at 2 h) may be responsible for the early presence of apoptotic and necrotic cell death markers in D-GalN-treated hepatocytes. The great reduction of ATP content at advanced stages of the study (24 h) was associated with more prominent hepatocellular necrosis.

The maintenance of mitochondrial GSH content prevents cell damage in different *in vivo* [13] and *in vitro* [14] experimental models of hepatotoxicity. The depletion of intracellular GSH by diethylmaleate exacerbates D-GalN-induced cell death in hepatocytes [34]. The administration of NAC, as a precursor of GSH and free radical scavenger, has beneficial clinical implications in HIV infection and cancer, as well as in heart, kidney and liver diseases [15]. In particular, NAC has been shown to improve the hemodynamic and metabolic parameters in patients with fulminant hepatic failure [35], as well as reduces cellular GSH depletion during the induction of cell death in cultured rat hepatocytes [25,26]. The alteration in the mitochondrial Q_{10} content has also been related to severe dysfunction in muscle and nervous system [36,37]. The supplementation with Q_{10} or analogues, such as idebenone, improves the neurodegenerative disorders [38–40], and reduces ROS generation and apoptosis induced by bile acid in hepatocytes [41]. In our study, the reduction of ROS generation and cell death by NAC or Q_{10} administration was related to a recovery of the oxidized/reduced GSH ratio, reduced Q_{10} content, and the coupled complexes I + III and II + III activities in mitochondria in D-GalN-treated hepatocytes.

The direct involvement of ROS production in mitochondrial function and cell death was addressed using a SOD mimetic. The administration of MnTBAP reduces liver injury during acute liver failure induced by Fas in mice [20]. The administration of MnTBAP reduced mitochondrial $O_2^{\bullet-}$ production, recovered mitochondrial oxidized/reduced GSH ratio and coupled complexes I + III and II + III activities, and reduced cell death induced by D-GalN in hepatocytes.

The administration of NAC, Q_{10} and MnTBAP enhanced the protein expression of nuclear- and mitochondria-encoded complex I subunits in control and D-GalN-treated hepatocytes. It appears that this effect was related to an early increase of MTP (NAC) and complex I-associated activity (NAC and Q_{10}) in control cells. Nevertheless, the increase of complex I subunit expression by antioxidants was related to a reduction of complex I activity, restoration of complexes I + III and II + III activities and reduction of cell death in D-GalN-treated hepatocytes. These results suggest a relevant role of antioxidants in the fine regulation of complex I expression and activity that may reduce the potential deleterious effect of ETC-derived ROS production and cell death induced by D-GalN. The improvement of the mitochondrial function with up-regulation of protein components of ETC complex has been observed in a comparative study using different neuronal cell types [42], as well as during the aging studies in which the compensatory mechanism is sustained until advanced stages of the process [43].

In conclusion, D-GalN-induced cell death was related to a rise of oxidative stress, depletion of mitochondrial reduced GSH and Q_{10} contents and mitochondrial dysfunction. The reduction of mitochondrial oxidative stress by NAC, Q_{10} and MnTBAP enhanced mitochondrial GSH and reduced Q_{10} content, improved mitochondrial function and prevented cell death. In addition, it was particularly interesting the beneficial properties of antioxidants on the expression of nuclear- and mitochondrial-encoded complex I subunits that may affect the stability and/or efficiency of ETC in hepatocytes submitted to toxic agents.

Conflict of interest

None declared.

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Apéndice del artículo 2 (resultados preliminares o complementarios):

Evaluación de los efectos de la administración de diferentes moléculas sobre un modelo de lesión hepática *in vivo*

Tras valorar el efecto citoprotector de NAC, Q_{10} y MnTBAP sobre la lesión hepatocelular inducida por D-GalN en un cultivo de hepatocitos humanos, se determinó su papel beneficioso en un modelo experimental *in vivo* de lesión hepática por D-GalN. Se utilizaron ratas Wistar (210-230 g de peso) que se distribuyeron en 5 grupos (n= 5 animales): Control, D-GalN, Q_{10}+D-GalN, MnTBAP+D-GalN y NAC+D-GalN. Se indujo una lesión hepática por D-GalN (1 g/kg peso, vía intraperitoneal) durante 24 h. La administración de Q_{10} (10 mg/kg), MnTBAP (10 mg/kg) y NAC (150 mg/kg peso) se realizó por vía intraperitoneal cada 12 h iniciándose el tratamiento 24 h antes de la administración de D-GalN hasta 12 h antes del sacrificio. Los animales se sacrificaron con extracción de sangre por punción cardíaca y extracción del hígado. Se valoraron parámetros de:

- Lesión hepática: Se determinaron los niveles de alanina aminotransferasa (ALT), aspartato aminotransferasa (AST) y LDH en el suero, y actividad asociada a caspasa-3 y la fragmentación del ADN en el homogenado hepático. Se realizó la tinción de hematoxilina-eosina para valorar la alteración de la arquitectura hepática.
- El contenido energético: El contenido en ATP se valoró en homogenado hepático.
- Los niveles de GSH y GSSG se determinaron en la fracción mitocondrial procedente del homogenado de hígado.
- Enzimas antioxidantes: Se analizó la actividad enzimática de CAT, SOD y GPx en fracción mitocondrial y en el lisado tisular.

Los datos mostraron que las tres moléculas empleadas redujeron la concentración de ALT, AST y LDH en suero, así como el incremento de actividad caspasa-3 inducido por D-GalN (*Tabla 2*). Los antioxidantes reducen la fragmentación del ADN y mejoraron la arquitectura hepática en los animales tratados con D-GalN (*Figura 23*).

	ALT (U/L)	**AST (U/L)**	**LDH (U/L)**	**Actividad asociada a caspasa-3 (UA x h^{-1} x mg $proteína^{-1}$)**
Control	28±3,1	66±6,7	96±5,7	10228±258,1
D-GalN	159±6,3*	208±13,6*	447±24,9*	29310±603,8*
MnTBAP+D-GalN	58±0,8*	130±4,7*	272±13,1*	20100±603,8*
Q_{10}+D-GalN	64±3,7*	133±3,4*	296±9,5*	23328±700,6*
NAC+D-GalN	75±4,7*	148±5,1*	290±10,3*	22893±1278,0*

Tabla 2: **Efecto de MnTBAP, Q_{10} y NAC en las transaminasas, LDH y actividad caspasa-3 en ratas tratadas con D-GalN.** Las transaminasas y la LDH se midieron por procedimientos rutinarios de laboratorio. La actividad caspasa-3 se midió mediante sustratos específicos según el Material y Métodos del artículo 2. El estudio estadístico está realizado con el test de comparación múltiple LSD (mínimas diferencias significativas). Donde los grupos marcados con "*" presentan diferencias estadísticamente significativas frente a su correspondiente grupo control.

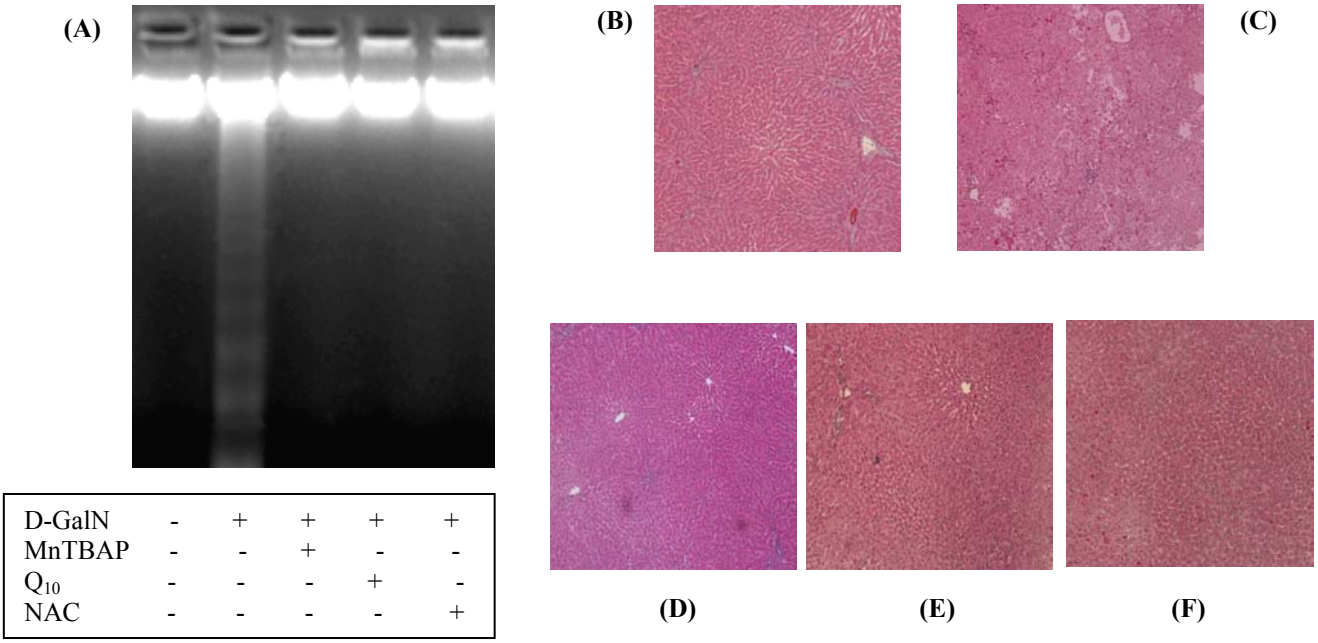

Figura 23: **Reducción de la fragmentación del DNA (A) y lesión tisular histológica (B-F) por MnTBAP, Q_{10} y NAC en ratas tratadas con D-GalN**. La fragmentación del DNA se valoró en un gel de agarosa según el Material y Métodos del artículo 2. La lesión tisular se valoró en secciones hepáticas fijadas y teñidas con hematoxilina-eosina de animales control (B), D-GalN (C), MnTBAP+D-GalN (D), Q_{10}+D-GalN (E) y NAC+D-GalN (F). Las imágenes son representativas de 5 experimentos diferentes.

Los tratamientos antioxidantes recuperaron el contenido de ATP celular e incrementaron la relación de GSH/GSSG en la fracción mitocondrial de los lisados hepáticos tratados con D-GalN (*Tabla 3*).

	ATP (μM/mg proteína^{-1})	**GSH/GSSG**
Control	703±23,3	4±0,4
D-GalN	458±21,3*	1±0,1*
D-GalN+MnTBAP	584±26,0*	3±0,2*
D-GalN+Q_{10}	614±17,3*	2±0,2*
D-GalN+NAC	597±14,9*	3±0,1*

Tabla 3: **Efecto de MnTBAP, Q_{10} y NAC sobre el contenido energético celular y en el cociente de GSSG/GSH mitocondrial en el hígado de ratas tratadas con D-GalN.** La medición de ATP y GSSG/GSH se valoró según lo descrito en el Material y Métodos del artículo 2. El estudio estadístico se realizó con el test de comparación múltiple LSD (mínimas diferencias significativas). Donde los grupos marcados con "*" presentan diferencias estadísticamente significativas frente a su correspondiente grupo control.

La administración de los antioxidantes en estudio recuperaba la actividad de tres antioxidantes enzimáticos como SOD, CAT y GPX en el homogenado y en la fracción mitocondrial de hígados procedentes de animales tratados con D-GalN (*Tabla 4*).

	SOD (homogenado) (U/mg proteína)	**SOD (mitocondria)** (U/mg proteína).	**CAT (homogenado)** (U/mg proteína)	**CAT (mitocondria)** (U/mg proteína)	**GPX (homogenado)** (U/mg proteína)	**GPX (mitocondria)** (U/mg proteína)
Control	0,23±0,025	2,91±0,182	549±33,0	68,8±6,56	254±14,4	185±31,0
D-GalN	0,06±0,007*	0,92±0,040*	201±22,0*	41,5±1,61*	192±9,9*	98±4,2*
MnTBAP+D-GalN	0,21±0,014*	1,81±0,050*	371±30,9*	58,8±3,42*	245±20,1*	112±5,7*
Q_{10}+D-GalN	0,24±0,025*	1,19±0,047*	257±21,3*	48,1±4,44*	208±15,7*	105±3,0*
NAC+D-GalN	0,26±0,019*	1,83±0,155*	360±66,3*	55,1±4,68*	235±19,6*	119±7,0*

Tabla 4: **Efecto de MnTBAP, Q_{10} y NAC sobre la actividad de antioxidantes enzimáticos en homogenado o fracción mitocondrial procedente de hígado de rata con lesión hepática por D-GalN.** La obtención del lisado tisular y la fracción mitocondrial se realizaron según lo descrito en el Material y Métodos del artículo 2. El estudio estadístico está realizado con el test de comparación múltiple LSD (mínimas diferencias significativas). Donde los grupos marcados con "*" presentan diferencias estadísticamente significativas frente a su correspondiente grupo control.

El estudio demuestra que la administración de los antioxidantes en estudio es capaz de prevenir el estrés oxidativo y lesión hepatocelular inducida por D-GalN en ratas.

<u>Artículo 3</u>: El óxido nítrico mimetiza la regulación transcripcional y postranslacional durante la citoprotección frente a la muerte celular inducida por el ácido glicoquenodeoxicólico en hepatocitos

Las ERO y el NO ejercen un papel clave en el desarrollo de citotoxicidad hepática inducido por ácidos biliares. Se plantearon experimentos *in vivo* e *in vitro* para evaluar si el α-tocoferol regula el estrés oxidativo y nitrosativo, la expresión de transportadores de ácidos biliares y sus modificaciones postranslacionales, y la muerte celular en hepatocitos. Los experimentos se realizaron en hepatocitos humanos en cultivo aislados de forma enzimática a partir de biopsias hepáticas tratados con GCDCA, y en ratas con obstrucción biliar del colédoco. Se valoró el efecto de α-tocoferol y/o donadores de NO, como DETA-NONOate ó CSNO (S-nitroso-L-cisteína) (estudio *in vitro*) y V-PYRRO/NO (estudio *in vivo*). Se valoraron parámetros de lesión hepática, producción de anión superóxido, expresión de NOS-2, CYP7A1, HO-1 y transportadores de ácidos biliares, S-nitrosilación y nitración de NTCP, y la incorporación de ácido taurocólico (TCA) en hepatocitos. Los resultados demostraron que la muerte celular por GCDCA se relacionó con un incremento de la producción de $O_2^{\cdot-}$, expresión de NTCP y HO-1, y una disminución de la expresión de CYP7A1 y NOS-2 en hepatocitos. El tratamiento con α-tocoferol disminuyó la muerte celular, la producción de $O_2^{\cdot-}$ y la expresión de CYP7A1, aunque incrementó la expresión de NOS-2 y la producción de NO en los hepatocitos tratados con GCDCA. El incremento de la S-nitrosilación y nitración de NTCP por α-tocoferol y el donador de NO redujo la incorporación de TCA en los hepatocitos en cultivo. La administración de α-tocoferol y el donador de NO redujeron la lesión hepática y la expresión de NTCP en hígado de ratas con obstrucción biliar. En conclusión, la regulación de la expresión de CYP7A1, NTCP y HO-1 es importante para el efecto citoprotector de α-tocoferol y NO frente a la disfunción mitocondrial, el estrés oxidativo y la muerte celular en hepatocitos tratados con GCDCA. La regulación de las modificaciones postranslacionales de NTCP dependientes de NO llevada a cabo por el α-tocoferol y el donador de NO reducen la incorporación de ácidos biliares hidrofóbicos en los hepatocitos.

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Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferease; ABC, ATP binding cassette; BSEP, bile acid export pump to bile duct; Biotin-HPDP, N-[6-(biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide; CSNO, S-nitroso-L-cysteine; CYP, cytochrome P450; DHE, dihydroethidium; GCDCA, glycochenodeoxycholic acid; HO-1, Heme oxygenase-1; LDH, lactate dehydrogenase; MTP, mitochondrial transmembrane potential; Mrp4, multidrug resistance-associated protein 4; NO, nitric oxide; NOS, nitric oxide synthase; NONOate, 2,2'-(hydroxynitrosohydrazino)*bis*-ethanamide; NTCP, Na^+-taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; ROS, reactive oxygen species; $O_2^{\bullet -}$, superoxide anion; SOD, superoxide dismutase; TMR, tetramethylrhodamine methyl ester; TC, taurocholic acid; V-PYRRO/NO, 1-[(ethenyloxy)-NNO-azoxy]-pyrrolidine.

Keywords: Apoptosis, Bile acids, Cysteine S-nitrosylation, Free Radicals, Mitochondria, Necrosis, Transporters, Tyrosine nitration

ABSTRACT

Background/Aims: Reactive oxygen species (ROS) and nitric oxide (NO) exert a relevant role during bile acid-induced hepatotoxicity. Whether α-Tocopherol regulates oxidative and nitrosative stress, bile acid transporter expression and their NO-dependent post-translational modifications, and cell death were assessed *in vitro* and *in vivo*. *Methods:* α-Tocopherol and/or NO donors (DETA-NONOate or CSNO, and V-PYRRO/NO) were administered to glycochenodeoxycholic acid (GCDCA)-treated cultured human hepatocytes or to bile duct obstructed rats. Cell injury, superoxide anion ($O_2^{\bullet -}$) production, as well as inducible nitric oxide synthase (NOS-2), cytochrome P4507A1 (CYP7A1), heme oxygenase-1 (HO-1) and bile acid transporter expression were determined. Cysteine S-nitrosylation and tyrosine nitration of Na^+-taurocholate co-transporting polypeptide (NTCP), as well as taurocholic acid (TC) uptake were also evaluated. *Results:* GCDCA-induced cell death was associated with increased $O_2^{\bullet -}$ production, NTCP and HO-1 expression, and with a reduction of CYP7A1 and NOS-2 expression. α-Tocopherol reduced cell death, $O_2^{\bullet -}$ production, CYP7A1, NTCP and HO-1 expression, as well as increased NOS-2 expression and NO production in GCDCA-treated hepatocytes. α-Tocopherol and NO donors increased NTCP cysteine S-nitrosylation and tyrosine nitration, and reduced TC uptake in hepatocytes. α-Tocopherol and V-PYRRO/NO reduced liver injury and NTCP expression in obstructed rats. *Conclusions:* The regulation of CYP7A1, NTCP and HO-1 expression may be relevant for the cytoprotective properties of α-Tocopherol and NO against mitochondrial dysfunction, oxidative stress and cell death in GCDCA-treated hepatocytes. The regulation of NO-dependent post-translational modifications of NTCP by α-Tocopherol and NO donors reduces the uptake of toxic bile acids by hepatocytes.

Word counts: 243

INTRODUCTION

Cholestatic liver disorders are an heterogeneous group of diseases with impaired bile flow that are implicated in different relevant liver dysfunctions such as biliary atresia, primary biliary cirrhosis and primary sclerosing cholangitis [1]. Cholic acid (CA) and chenodeoxycholic acid (CDCA) are the two major primary bile acids in humans [2]. Bile acids are secreted into bile canaliculi after glycine or taurine conjugation. More than 95% of the bile acid pool is re-absorbed from the intestine and transported to the liver where bile acids play a key role in the inhibitory feedback control of their own synthesis. The retention of certain di-hydroxy-hydrophobic bile acids, such as the glycine conjugates of chenodeoxycholic acid (glycochenodeoxycholic acid, GCDCA) in the liver is believed to play an important role in the pathogenesis of cholestatic liver injury [3, 4]. Several markers of cellular apoptosis have been also identified in bile acid-induced cell injury in cultured hepatocytes [5, 6]. The involvement of mitochondria- and endoplasmic reticulum oxidative stress has been shown during bile acid-induced apoptosis and necrosis in hepatocytes [7-8].

Vitamin E is a general term that refers to the eight different forms: α-, β-, γ-, and δ-Tocopherol with a chromanol ring and saturated side chain, and four compounds (α-, β-, γ-, and δ-Tocotrienols) with an unsaturated side chain. α-Tocopherol is the main form of vitamin E present in human plasma and tissues [9]. α-Tocopherol is considered the most important lipophilic radical-quenching antioxidant in cell membranes [10] but this function *in vivo* has been questioned [11]. In addition, α-Tocopherol modulates cell proliferation, platelet aggregation, and NADPH-oxidase activation [12], as well as regulates the expression of genes related to its own metabolism, lipid uptake, inflammation, cell adhesion and fibrosis [13]. The supplementation with α-Tocopherol

has been shown to prevent the loss of cell viability [14] and reduce the release of mitochondrial pro-apoptotic factors induced by toxic hydrophobic bile acids in hepatocytes [15].

Nitric oxide (NO) exerts a key role on the hepatic metabolism and liver injury induced by different agents [16, 17]. The three described nitric oxide synthase (NOS) isoforms are present in the liver, being the inducible (iNOS, NOS-2) and endothelial constitutive (eNOS, NOS-3) forms the most abundant ones [17]. NO signaling activity may be mediated by its direct effects on certain proteins, e.g. activation of soluble guanylate cyclase and inhibition of cytochrome c oxidase, or by producing covalent protein post-translational modifications, such as cysteine S-nitrosylation and tyrosine nitration, that can alter protein function [18]. NO has been shown to exert pro-apoptotic and anti-apoptotic effect in cultured hepatocytes [19-21]. GCDCA prevented NOS-2 expression and NO production in cytokine-treated rat hepatocytes [22]. The supplementation with a NO donor prevented caspase-3 activity and apoptosis induced by bile acids in cultured rat hepatocytes [22, 23].

The present study showed that the induction of cell death by GCDCA was related to oxidative stress, increased expression of proteins involved in bile acid cellular accumulation such as Na^+-taurocholate co-transporting polypeptide (NTCP) and heme oxygenase (HO-1), and reduction of NOS-2 expression and NO production. These effects were counteracted by α-Tocopherol administration in GCDCA-treated hepatocytes. In addition, the induction of protein cysteine S-nitrosylation and tyrosine nitration by α-Tocopherol and NO donors reduced the incorporation of toxic bile acids to hepatocytes. The data obtained *in vitro* were confirmed in an experimental model cholestasis. The administration of α-Tocopherol or NO donors reduced NTCP

expression and liver injury in obstructed rats.

MATERIAL AND METHODS

Materials

All reagents were from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA) unless otherwise stated. 2,2'-(hydroxynitrosohydrazino)*bis*-ethanamide (NONOate or NOC-18) was purchased from Calbiochem (Darmstadt, Germany). 1-[(ethenyloxy)-NNO-azoxy]-pyrrolidine (V-PYRRO/NO) was purchased from Cayman (Ann Arbor, MI, USA). N-[6-(biotinamido)hexyl]-3´-(2´-pyridyldithio)propionamide (Biotin-HPDP) was from Pierce (Rockford, IL, USA). ^{3}H-taurocholic acid (^{3}H-TC) was from Perkin-Elmer (Boston, MA). S-nitrosocysteine was synthesized as described elsewhere [24] by incubation of L-cysteine with acidified sodium nitrite and quantification by absorbance at 334 nm using a molar absorption coefficient of 0.74 mM^{-1} cm^{-1}. The study protocol has been approved by the Ethical Committee of the Institution.

Preparation of primary human hepatocytes and cell culture

Liver resection was obtained from 47 patients (18 women, 29 men; 53 ± 12.5 years old) submitted to surgical intervention for primary or secondary liver tumor resection after written consent of the patient. The procedure of hepatocyte isolation was based on the two-step collagenase procedure described by Ferrini et al [25]. Hepatocytes (8 x 10^{6} cells; 150000 cells/cm^{2}) were seeded in type I collagen-coated dishes (Iwaki, Gyouda, Japan) and cultured in supplemented DEM-Ham-F12 and William's E mediums (1:1). NO donors such as NONOate (0.2 mM) and CSNO (0.2 mM) were administered 2 h

and 30 min before GCDCA (0.5 mM), respectively. α-Tocopherol (50 μM) was added 10 h after GCDCA.

Experimental in vivo model cholestasis

Animals (n=24, 6 in each group) were distributed in each of the following groups: sham operated (SO), obstructive jaundice (OJ), OJ+α-tocopherol, and OJ+V-PYRRO/NO. All the surgical procedures were carried out in anesthetized animals with ketamine (60 mg/kg i.p.) and midazolam (4 mg/kg i.p.). Cephazoline (17 mg/kg i.m.) was used as antibiotic prophylaxis. SO animals were submitted to laparatomy and abdominal closure without bile duct intervention. The procedure for OJ was started by a midline ventral incision with exposure of the extra-hepatic bile duct. A double ligature with silk suture was done and the bile duct was sectioned. A two-layer running suture was used for abdominal closure with polyglycolic acid and silk. α-tocopherol (50 mg/kg/day in ethanol) and V-PYRRO/NO (5 mg/kg/day in ethanol) were subcutaneously administered daily since the day of surgical intervention until the day before the sacrifice of animals. The administration of the solvent did not change the parameters in control and OJ rats (data not shown). The animals were sacrificed under anesthesia 7 days after OJ. Blood was collected by cardiac puncture and serum was frozen at -80 ºC until the measurement of alanine aminotransferase (ALT), aspartate aminotransferease (AST) and total bilirubin by routine laboratory methods. Liver was obtained and some pieces were immediately frozen at -80 ºC for biochemical analysis, and others were fixed for confocal microscopy analysis.

Measurement of cell death, mitochondrial transmembrane potential, ROS production and SOD activity

Cell death was determined by DNA fragmentation, caspase-3 activation and lactate dehydrogenase (LDH) release following the methods described previously [26]. The mitochondrial transmembrane potential (MTP) and superoxide anion ($O_2^{\cdot-}$) production were monitored using tetramethylrhodamine methyl ester (TMR, 2 μM) and dihydroethidium (DHE, 10 μM) (Molecular Probes Europe BV, Leiden, The Netherlands) in hepatocytes. Cells were incubated with TMR for 30 min or DHE for 20 min, washed with PBS, and treated with digitonin (10 μM) for 5 min in order to eliminate any probe not retained in mitochondria. The fluorescence emitted by TMR (Ex 550, Em 570) or DHE (Ex 510, Em 590) was assessed *in situ* using a GENios Microplate Reader (TECAN).

Superoxide dismutase (SOD) activity in mitochondrial fraction was determined using a commercial assay (19160 SOD determination kit, Sigma-Aldrich Chemical Co.). The method is based in the Dojindo's highly water-soluble tetrazolium salt that produces a water-soluble formazan dye upon reduction with $O_2^{\cdot-}$. The rate of the reduction with $O_2^{\cdot-}$ are linearly related to the xanthine oxidase activity exogenously added to the assay, and is inhibited by SOD from the sample.

Measurement of nitric oxide

NO production was followed by the measurement of nitrite+nitrate+nitrosothiols concentration in culture medium using NOA (GE Healthcare, Inc) analyzer. A volume (300 μL) was deproteinized in 4% $ZnSO_4$ and 0.2 N NaOH for 15 min at room

temperature and centrifuged at 19400 *g* 5 min 4ºC. The supernatant was injected to the analyzer chamber containing 50 mM $VaCl_3$ in 2.8 % HCl, and measuring the resulting chemiluminiscence. A solution of 10 mM $NaNO_3$ was used as standard.

Detection of NTCP cysteine S-nitrosylation by the biotin switch assay

The procedure was performed as previously described [27] with recently described modifications [28]. Hepatocytes and liver were treated with lysis solution (50 mM Tris-HCl pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1 mM neocuproine, 1 % Triton X-100, 5 µg/mL aprotinin and 10 µg/mL leupeptin), and after centrifugation at 10000 g the supernatants (0.5 µg/µL) were incubated with 4 volumes of blocking buffer (225 mM HEPES, pH 7.7, 0.9 mM EDTA, 90 µM neocuproine, 2.5% SDS and 20 mM methyl methane thiosulfonate or MMTS) for 20 min at 50ºC, and precipitated with 3-4 volumes of cold acetone. The dried pellet was resuspended in 500 µL HENS (HEN and 1% SDS) with 100 mM sodium ascorbate and 167 µL 4 mM Biotin-HPDP for 1 h, precipitated with cold acetone, and dried pellet resuspended in 200 µL HENS buffer and 800 µL of neutralization buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100). Samples were treated with 45 µL Neutravidin Plus UltralinK resin (Pierce) for 1 hour in agitation, washed (20 mM HEPES pH 7.7, 600 mM NaCl, 1 mM EDTA and 0.5% Triton X-100), and incubated in 20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM 2-mercaptoethanol for 20 min at 37ºC. After centrifugation, supernatants were loaded into 10% SDS-PAGE electrophoresis in reducing conditions. NTCP was detected by Western Blot analysis using either anti-human NTCP (sc-107030, Santa Cruz Biotechnology Inc., CA, USA) (1/1000) or anti-rat NTCP (sc-107029, Santa Cruz Biotechnology Inc.) (1/1000) as primary antibodies,

and anti-goat (sc-2056, Santa Cruz Biotechnology Inc.,) (1/50000) as secondary antibodies.

Detection of NTCP nitration

A volume of the cell lysate obtained above (500 µg protein) was mixed with immunoprecipitation buffer (20 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, 1 mM PMSF, and commercial inhibitors) and either 2 µg anti-human NTCP (sc-107030, Santa Cruz Biotechnology Inc.) or anti-rat NTCP (sc-107029, Santa Cruz Biotechnology Inc.) antibodies at 4ºC overnight. Afterwards, protein G Sepharose (35 µL) (GE Healthcare) was added, agitated for 3 hours, and the washed resin was loaded into 10% SDS-PAGE electrophoresis. Nitrated NTCP was assessed by Western Blot analysis using a anti-3-nitrotyrosine (N5538, Sigma-Chemical) (1/10000) as primary antibodies, and anti-mouse (sc-2031, Santa Cruz Biotechnology Inc.,) (1/50000) as secondary antibodies.

NOS-2, CYP7A1, HO-1 and bile acid transporter protein and mRNA expression

Total RNA from whole hepatocyte population was extracted using Trizol reagent according to the manufacturer's recommendations (Life Technologies Inc.). The expression of NOS-2, cytochrome P4507A1 (CYP7A1), bile acid export pump to bile duct (BSEP), multidrug resistance-associated protein 4 (MRP4), NTCP and HO-1 mRNA was examined by quantitative real-time RT-PCR using the LightCycler thermal cycler system (Roche Diagnostics, Indianapolis, USA). RT-PCR was performed in one step, using the QuantiTect SYBR Green RT-PCR kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. The following primers were used:

NOS-2 (BIOSOURCE, Nivelles, Belgium) (Catalog number GHO0144, cDNA reference GenBank U05810), CYP7A1 (sense, 5'-CTGCAGAACACCCTCACCACACA-3', and antisense, 5'-CACCCGAAGAACCCCACCATC-3'), BSEP (sense, 5'-GTGAAGGAATGGTGACCGTGGA-3', and antisense, 5'-TCCTTGGCAGCTTGGACTATGTCT-3'), MRP4 (sense, 5'-TGCAAGGGTTCTGGGATAAAGA-3', and antisense, 5'-CTTTGGCACTTTCCTCAATTAACG-3'), NTCP (sense,5'-AATGGACGGTGCAGACGCA-3', and antisense, 5'-GGTGGAAAGGCCACATTGAGGA-3'), HO-1 (sense, 5'-ATGACACCAAGGACCAGAGC-3', and antisense, 5'-GTGTAAGGACCCATCGGAGA-3') and 18S (MVG-Biotech AG, Getotek, Sabadell, Spain) (sense, 5'-GTAAC CCGTT GAACC CCATT-3', and antisense, 5'-CCATC CAATC GGTAG TAGCG-3'). The RT conditions were adapted for each specific conditions of measurement.

The protein expression was determined by SDS-PAGE electrophoresis at 4% (MRP4 and BSEP), 6% (NOS-2), 8% (CYP7A1 and NTCP) and 12% (HO-1), and Western-blot analysis using anti-MRP4 (dilution 1/600) (ab32550, Abcam), BSEP (1/1000) (sc-17292, Santa Cruz Biotechnology Inc.), NOS-2 (1/4000) (PA1-036, Affinity Bioreageants, Golden, USA), CYP7A1 (1/200) (ab79847, Abcam), human NTCP (1/1000) (sc-107030, Santa Cruz Biotechonology Inc.), rat NTCP (1/1000) (sc-107029, Santa Cruz Biotechnology Inc.) and HO-1 (1/500) (OSA-110, Stressgen) antibodies, and the corresponding secondary antibodies. NOS-2 antigen (ref 360862, Cayman Chemical, Ann Arbor, USA) was used as standard, as well as Na^+/K^+ ATPase-α1 (1/250) (ab2872, Abcam) and β-actin (1/5000) (ab8227, Abcam) as cell protein

loading controls.

NTCP expression in liver OCT fixed section (5 μm) mounted on glass slides coated with polylysine was also assessed by immunefluorescence. Samples were incubated overnight at 4ºC with goat anti-rat NTCP (1/50) (sc-107029, Santa Cruz Biotechnology Inc.) antibodies diluted in 100 mM PBS pH 7.4 containing 2% normal goat serum and 0.05% Triton X-100. Sections were washed three times with PBS and incubated for 1 hour at room temperature with anti-goat Alexa Fluor 488 (1/200) (A11055, Invitrogen, Molecular Probes, Inc, Willow Creek Road Eugene, OR, USA) labeled antibodies. Finally, sections were washed several times with PBS, embedded in 70% glycerol and mounted with cover slide. The specificity of the immunoreactivity was verified by incubating sections with primary antibodies in the absence of secondary antibodies, and secondary antibodies in the absence of primary antibodies. Six replicated immunostained sections were analyzed by LSM 5 Exciter confocal microscope (Carl Zeiss, Jena, Germany) using confocal imaging system (ZEN 2008, Microimaging Carl Zeiss, Germany).

Taurocholate incorporation

Cells were washed twice with 0.5 mL HEPES buffer, and incubated with 20 μM ^{3}H-taurocholate (TC) (100 dpm/pmol) for 2 min. After washing, cells were treated with 20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 500 nM okadaic acid, and 1 mM orthovanadate at pH 7.5. After centrifugation, the incorporated radioactive was determined in the supernatant fraction (cpm/mg protein).

Liver injury in obstructed rats

Liver injury was assessed in deparaffinised fixed hepatic sections (5 μm) stained with hematoxylin-eosin. Liver (1 g) was homogenized by Ultraturrax in 20 mL of 50 mM HEPES pH 7.5, 2 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1% Nonidet NP40, 5 μg/mL aprotinin and 10 μg/mL leupeptin for the measurement of caspase-3 activity. Caspase-3-associated activity was determined using Ac-DEVD-AFC (100 μM) (Bachem AG, Bubendorf, Switzerland) substrate following a procedure described previously [26]. The caspase-3-derived fluorescence (Ex 400, Em 505) was recorded using a GENios Microplate Reader (TECAN, Salzburg, Austria).

Statistical analysis

Results are expressed as mean ± SE (n=5-6). Data were compared using the analysis of variance with the Least Significant Difference's test as post-hoc multiple comparison analysis. The statistical differences were set at $p \leq 0.05$. The groups with "a" were significantly different vs. the corresponding control group. The groups with "b" were significantly different vs. the corresponding group without GCDCA.

RESULTS

NO donor mimics the cytoprotective properties of α-Tocopherol in cultured hepatocytes

The induction of mitochondrial-dependent oxidative stress has been related to hydrophobic bile acid-induced cytotoxicity in cultured hepatocytes [7]. NO exerts cytoprotective properties on GCDCA-induced cell death in hepatocytes [22, 23]. GCDCA reduced NOS-2 mRNA (Fig. 1A, 12h) and protein (Fig. 1B, 24h) expression, and nitrite+nitrate+nitrosothiols (Fig. 1C, 24h) concentration in culture medium from

hepatocytes ($p \leq 0.05$). This effect was slightly but significantly counteracted by α-Tocopherol (Fig. 1) ($p \leq 0.05$). Interestingly, α-tocopherol increases NOS-2 protein (Figure 1B) expression and nitrite+nitrate+nitrosothiols (Figure 1C) concentration in culture medium, but not NOS-2 mRNA (Figure 1A) expression in control hepatocytes. The increase of NO production by α-Tocopherol was associated with the reduction of caspase-3-associated activity (Fig. 2A, 24h), caspase-3 processing (Fig. 2B, 24h), DNA fragmentation (Fig. 2C, 24h) and LDH release (Fig. 2D, 24h) induced by GCDCA in hepatocytes (Fig. 2) ($p \leq 0.05$). The administration of NO donor (NONOate) mimicked the cytoprotective properties of α-Tocopherol against GCDCA-induced cell death in hepatocytes ($p \leq 0.05$).

Regulation of mitochondrial potential and oxidative stress

The mitochondrial dysfunction and oxidative stress have been related to the induction of cell injury [29]. GCDCA induced hyperpolarization of mitochondrial membrane (Fig. 3A, 12h) and $O_2^{\cdot -}$ production (Fig. 3B, 12h) ($p \leq 0.05$). GCDCA-induced oxidative stress was associated with an increase of SOD activity in mitochondrial fraction (Fig. 3C, 24) ($p \leq 0.05$). α-Tocopherol or NONOate reduced MTP (Fig. 3A), oxidative stress (Fig. 3B) and SOD activity (Fig. 3C) ($p \leq 0.05$).

α-Tocopherol and NO donor regulate CYP7A1 and bile acid transporter expression in GCDCA-treated hepatocytes

CYP7A1 is involved in cholesterol and bile acid metabolism. In concordance with other studies [30], GCDCA reduced CYP7A1 mRNA (Fig. 4A, 12h) and protein

(Fig 4B, 24h) expression ($p \leq 0.05$). α-Tocopherol, but not NONOate, reduced CYP7A1 protein, but not mRNA, expression (Fig. 4) ($p \leq 0.05$). The ATP binding cassette (ABC) transporters, such as BSEP and MRP4, are involved in the export of bile acids across the canalicular and basolateral membrane, respectively. The basolateral NTCP together, with a much lower contribution by several members of the family of organic anion transporting polypeptide (OATPs), is the major mechanism accounting for bile acid uptake by hepatocytes [31]. GCDCA increased BSEP (Fig. 5A and 5B), MRP4 (Fig. 5C and 5D) and NTCP (Fig. 5E and 5F) mRNA (12h) and protein (24h) expression, respectively ($p \leq 0.05$). α-Tocopherol or NO donor reduced mRNA BSEP, but not that of MRP4 and NTCP, induced by GCDCA in hepatocytes (Fig. 5A, 5C and 5E) ($p \leq 0.05$). α-Tocopherol or NO donor reduced BSEP, MRP4 and NTCP protein expression in GCDCA-treated hepatocytes (Fig. 5B, 5D and 5F) ($p \leq 0.05$).

Bilirubin content and HO-1 expression

The intracellular concentration of bilirubin is also influenced by HO-1 activity. GCDCA, α-Tocopherol and NONOate significantly increased the concentration of total bilirubin in control hepatocytes (Fig. 6A, 24h) ($p \leq 0.05$). α-Tocopherol and NONOate reduced the levels of bilirubin in GCDCA-treated hepatocytes (Fig. 6A) ($p \leq 0.05$). The pattern of bilirubin concentration was closely correlated to HO-1 mRNA (Fig. 6B, 12h) and protein (Fig. 6C, 24h) expression in control and GCDCA-treated hepatocytes ($p \leq 0.05$).

Cysteine S-nitrosylation and tyrosine nitration of NTCP in GCDCA-treated hepatocytes

NO donor reduces the uptake of different molecules by NTCP in hepatocytes [32]. GCDCA, α-Tocopherol, NONOate and CSNO increased cysteine S-nitrosylation (Fig. 7A) and tyrosine nitration (Fig. 7B) of NTCP which correlated to significant reduction of TC uptake (Fig. 7C) in control hepatocytes. However, α-Tocopherol, NONOate and CSNO reduced both post-translational modifications in NTCP, but without altering TC uptake, in GCDCA-treated hepatocytes (Fig. 7C).

NO donor mimics the cytoprotective properties of α-Tocopherol in bile duct obstructed rats

Bile duct obstruction increased ALT, AST and total bilirubin concentration in blood (Table 1). The increase of transaminases was related to the presence of features of hepatocellular necrosis and bile duct cellular proliferation in tissue hepatic sections stained with hematoxylin-eosin (Fig. 8), and caspase-3-associated activity in liver homogenate (Fig. 9) obtained from obstructed rats ($p \leq 0.05$). The administration of α-Tocopherol and V-PYRRO/NO exerted an improvement of all parameters of liver injury in obstructed animals (Fig. 8-9, Table 1).

Cysteine S-nitrosylation and tyrosine nitration of NTCP in liver from obstructed animals

The obstruction of bile duct flow for seven days increased NTCP protein expression and its degree of cysteine S-nitrosylation and tyrosine nitration in liver (Fig. 10). The administration of α-Tocopherol and V-PYRRO/NO drastically reduced NTCP expression and its posttranslational modifications in liver from obstructed animals (Fig. 10).

DISCUSSION

Liver injury induced by cholestasis is characterized by the induction of oxidative and nitrosative stress, as well as activation of cell death which leads to biliary fibrosis and cirrhosis [33]. The study showed that α-Tocopherol increases NO production, and reduces oxidative stress and cell death in GCDCA-treated hepatocytes. The reduction of CYP7A1, NTCP and HO-1 protein expression by α-Tocopherol may have a clear positive impact in the survival of GCDCA-treated hepatocytes. The supplementation with α-Tocopherol, NONOate and CSNO increased S-nitrosylation and tyrosine nitration of NTCP, and reduced TC uptake in control hepatocytes.

The increase of MTP and ROS production was related to induction of cell injury in GCDCA-treated hepatocytes [7, 8]. The cytoprotective properties of different agents were related to the reduction of oxidative stress and cell death in bile acid-induced citotoxicity [7, 34-37]. α-Tocopherol reduces oxidant injury induced by bile acids in cultured hepatocytes [14, 15] and in experimental models of cholestasis [38, 39]. α-Tocopherol reduced the hyperpolarization of MTP, ROS production and cell death induced by GCDCA in hepatocytes. The beneficial effect of the vitamin was mimicked by NO donor administration. GCDCA reduced NOS-2-associated NO production in hepatocytes [22], and NO donor prevented GCDCA-induced apoptosis in rat hepatocytes [22, 23]. α-Tocopherol increased NOS-2 protein expression and nitrite+nitrate+nitrosothiols concentration in culture medium, but not NOS-2 mRNA expression, in control hepatocytes. This data may suggest a potential inhibitory effect of α-Tocopherol in NOS-2 degradation in hepatocytes. In fact, α-Tocopherol completely attenuated the induction of protein degradation in murine myotubes [40]. However, α-Tocopherol increases NOS-2 mRNA and protein expression and NO production in

GCDCA-treated hepatocytes. The transcriptional regulation of NOS-2 gen is under the control of different transcriptional factors and nuclear receptors [21, 41]. The administration of α-Tocopherol counteracts the effect of GCDCA on the expression/activation of different transcription factors (NF-*k*B) and nuclear receptors (PXR, RXR, FXR and PPAR-α) in hepatocytes (data not shown). The administration of α-Tocopherol and NO donor reduced mitochondrial-associated oxidative stress and cell death in GCDCA-treated hepatocytes.

The human body has evolved versatile inducible metabolizing enzymes and efflux transporters to facilitate the metabolism and elimination of potentially harmful drugs, toxins and/or xenobiotics that are introduced from the environment or produced during physiopathological mechanisms. Different transcriptional factors and nuclear receptors participate in an integrated complex regulatory network that regulate the expression of a broad number of proteins such as members of CYP superfamily (phase I drug metabolizing enzymes), detoxification system (phase II drug metabolizing enzymes) and import/export transporters [42]. The study evaluates the effect of experimental interventions on the expression of proteins involved on bile acid synthesis (CYP7A1), antioxidants (SOD), redox-active (HO-1) and import/export (NTCP, MRP4 and BSEP) proteins.

Cholestatic liver damage is counteracted by repression of *de novo* bile acid synthesis and the hepatic bile acid uptake. α-Tocopherol, but not NONOate, potently reduced protein CYP7A1 expression in GCDCA-treated hepatocytes. In addition, α-Tocopherol and NONOate reduced HO-1 protein expression in GCDCA-treated hepatocytes. Froh et al. [43] have suggested that the overexpression of HO-1 may exert antioxidant properties during bile duct ligation in rats. HO-1 expression followed a similar pattern than observed of $O^{\cdot}$ production and SOD activity in mitochondria

suggesting that intracellular oxidative stress may regulate both parameters. However, the intracellular concentration of bilirubin was also closely correlated to the expression of HO-1 in control and GCDCA-treated hepatocytes. Under cholestatic conditions the regulation of import/export of bile acids into hepatocytes is relevant to limit hepatocellular injury. NTCP is responsible for the 80% of hepatocellular bile acid incorporation [31]. NTCP is downregulated in various cholestatic liver diseases [44]. The repression of NTCP has been suggested to be cytokine mediated during endotoxemia [45], and cytokine-independent in obstructive cholestasis [46]. However, we have observed an increase of NTCP protein expression in cultured GCDCA-treated hepatocytes, as well as in hepatic tissue sections from bile duct obstructed animals. The different results may be related to the short period of cholestasis used in the present study, or as a consequence of animal specificity. α-Tocopherol and NO donor reduced NTCP protein expression in both *in vitro* and *in vivo* models of experimental cholestasis. The inhibition of NO production by aminoguanidine prevented the reduction of NTCP expression by LPS in hepatocytes [47].

The export of bile acid is mainly mediated by MRP4 and BSEP at the basolateral and canalicular membranes, respectively. GCDCA increased MRP4 expression in hepatocytes. In concordance with previous studies [48], GCDCA also induced the expression of BSEP in hepatocytes. α-Tocopherol and NONOate reduced MRP4 and BSEP protein expression in GCDCA-treated hepatocytes. However, the functional repercussions of these effects were limited. α-Tocopherol and NONOate reduced total bilirubin intracellular concentration in GCDCA-treated hepatocytes.

The transcriptional regulation of CYP7A1, HO-1 and import/export transporter expression is a consequence of the extensive crosstalk between PXR, RXR, FXR, CAR and PPAR-α transactivation activity which shared response elements present in their

promoter regions [42]. As described above, α-Tocopherol and GCDCA alter the expression of different transcription factors and nuclear receptors in hepatocytes (data not shown). The study showed that NO mimicks transcriptional and translational regulation related to the cytoprotective properties of α-Tocopherol against GCDCA-induced cell death. NO may exert its cellular action by guanylate cyclase-dependent, as well as by guanylate cyclase-independent pathways including postranslational modifications in cysteine (S-nitrosylation or S-nitrosation) and tyrosine (nitration) residues, mixed disulfide formation (S-nitrosoglutathione or GSNO), or promoting further oxidation protein stages which have been related to altered protein function and gene transcription regulation. In particular, the prolonged exposure to NO shifts the cellular redox potential to a more oxidized state which regulates gene expression through alteration of MAPK activation and/or redox state of transcriptional factors containing zinc finger motifs or cysteines within the DNA-binding domain [42, 49].

We have studied the effect of NO-dependent posttranslational modifications of NTCP by α-Tocopherol, GCDCA and NO donors on TC uptake in hepatocytes. NO donor (sodium nitroprusside) reduced the NTCP-mediated TC uptake, but not the OCT-mediated tributylmethylammonium and triethylmethylammonium uptake in hepatocytes [32]. The potential noxious effect of GCDCA-induced increase of NTCP expression may be partially counteracted by its cysteine S-nitrosylation and tyrosine nitration which reduced TC uptake in hepatocytes. The increase of both translational modifications of NTCP by α-Tocopherol, NONOate and CSNO was also associated with a reduction of TC uptake in control hepatocytes. The drastic reduction of NTCP protein expression by α-Tocopherol and NO donors seems to be relevant for the reduction of its cysteine S-nitrosylation and tyrosine nitration during *in vitro* and *in vivo* experimental models of cholestasis.

In conclusion, the study suggests that α-Tocopherol-derived NO was able to reduce NTCP expression, and increase its cysteine S-nitrosylation and tyrosine nitration which overall have a clear beneficial impact in order to reduce GCDCA-induced mitochondrial dysfunction, oxidative stress and cell death in hepatocytes. The reduction of CYP7A1, HO-1 and NTCP expression by α-Tocopherol and NO might be the consequence of the alteration of transcription factor and nuclear receptor activation/expression and their redox state, which regulate the transcription of numerous genes such as CYPs, antioxidants, redox-active proteins and import/export transporters.

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FIGURE LEGENDS

Figure 1. Effect of α-Tocopherol on mRNA (A) and protein (B) nitric oxide synthase (NOS)-2 expression, and nitrite+nitrate+nitrosothiols (C) concentration in culture medium in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of five independent experiments.

Figure 2. Effect of α-Tocopherol and nitric oxide donor (NONOate) on caspase-3-associated activity (A), caspase-3 processing (B), DNA fragmentation (C) and cell necrosis (D) in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of five independent experiments.

Figure 3. Effect of α-Tocopherol and nitric oxide donor (NONOate) on mitochondrial transmembrane potential (MTP) (A), anion superoxide ($O_2^{\bullet -}$) production (B) and superoxide dismutase (SOD) activity (C) in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively.

Figure 4. Effect of α-Tocopherol and nitric oxide donor (NONOate) on CYP7A1 mRNA (A) and protein (B) expression in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of five independent experiments.

Figure 5. Effect of α-Tocopherol and nitric oxide donor (NONOate) on bile acid export pump to bile duct (BSEP) (A and B), multidrug resistance-associated protein 4 (MRP4) (C and D), and Na^+-taurocholate co-transporting polypeptide (NTCP) (E and F) mRNA and protein respectively expression in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of five independent experiments.

Figure 6. Effect of α-Tocopherol and nitric oxide donor (NONOate) on the total bilirubin content (A), heme oxygenase-1 (HO-1) mRNA (B) and protein (C) expression in glycochenodeoxycholate (GCDCA)-treated hepatocytes. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of five independent experiments.

Figure 7. Effect of α-Tocopherol and nitric oxide donors (NONOate and CSNO) on cysteine S-nitrosylation (A) and tyrosine nitration (B) of Na^+-taurocholate co-

transporting polypeptide (NTCP), as well as taurocholic acid (TC) uptake in glycochenodeoxycholate (GCDCA)-treated hepatocytes. The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively. The images are representative of three independent experiments.

Figure 8. Liver histology was evaluated in bile duct obstructed rats. Liver injury was evaluated in tissue section stained with hematoxylin-eosin from sham operated (A) and obstructive jaundice (7 days) (B). The effect of subcutaneously daily administration of α-Tocopherol (C) and NO donor (V-PYRRO/NO) (D) was determined. The images are representative of six independent experiments. Magnification 50x.

Figure 9. Effect of α-Tocopherol and NO donor (V-PYRRO/NO) administration on caspase-3-associated activity in livers from bile duct obstructed rats. Data is expressed as mean ± SEM ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without GCDCA, respectively.

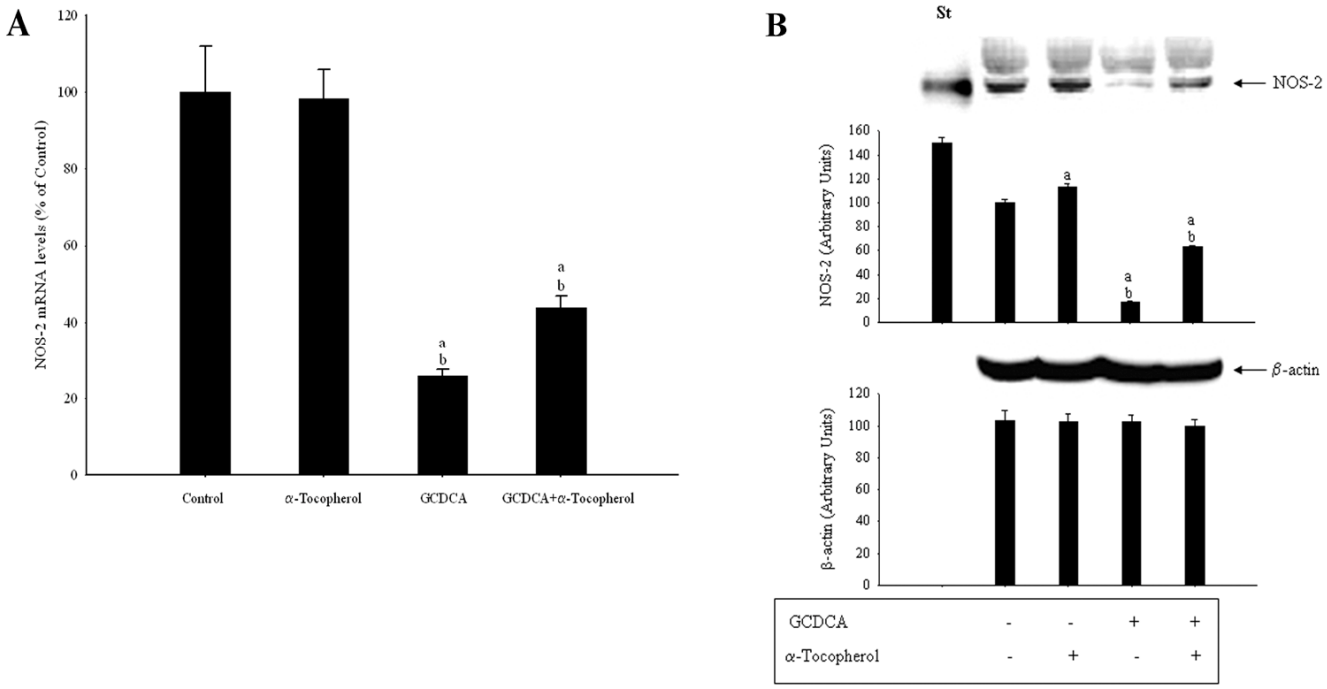
Figure 10. Effect of α-Tocopherol and NO donor (V-PYRRO/NO) administration on NTCP expression and its cysteine S-nitrosylation and tyrosine nitration (A) in liver homogenate from bile duct obstructed rats. The expression of NTCP was also evaluated by confocal microscopy in liver fixed sections obtained from sham operated (B), obstructive jaundice (OJ) (7 days) (C), OJ+α-Tocopherol (D) and OJ+V-PYRRO/NO (E) animals. The images are representative of six independent experiments. Magnification 400x.

Table 1

TABLE 1. Measurement of parameters of liver injury and cholestasis in blood from obstructed animals.

	ALT (IU/L)	AST (IU/L)	Total Bilirubin (mg/dL)
Sham operated	30±2.9	31±1.5	0.9±0.03
Bile duct obstruction	89±9.4[a,b]	160±13.5[a,b]	14.1±1.35[a,b]
Bile duct obstruction+ α-Tocopherol	58±4.0[a]	82±6.0[a]	8.1±0.81[a]
Bile duct obstruction+ V-PYRRO/NO	70±5.0[a,b]	118±9.3[a]	10.3±0.45[a]

α-Tocopherol (50 mg/kg) and nitric oxide donor (V-PYRRO/NO) (5 mg/kg) were injected subcutaneously daily from the day of the bile duct ligature until the day preceding the sacrifice (7 days). Data is expressed as mean ± SEM of six independent experiments ($p \leq 0.05$). The groups with "a" and/or "b" were significantly different vs. the corresponding control group with/without obstructive jaundice, respectively.


A
NOS-2 mRNA levels (% of Control)
120
100
80
60
40
20
0
a
b
a
b
Control
α-Tocopherol
GCDCA
GCDCA+α-Tocopherol
B
St
NOS-2
NOS-2 (Arbitrary Units)
160
140
120
100
80
60
40
20
0
a
b
a
b
β-actin
β-actin (Arbitrary Units)
120
100
80
60
40
20
0
GCDCA - - + +
α-Tocopherol - + - +

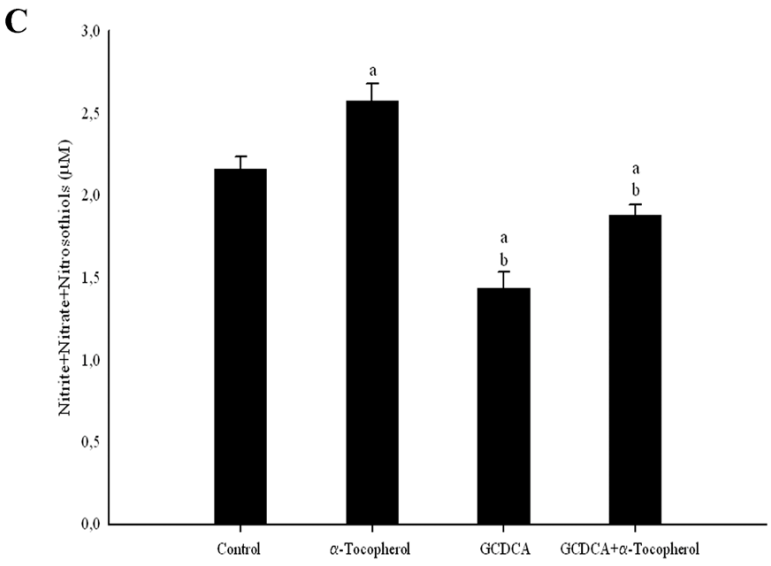

C
Nitrite+Nitrate+Nitrosothiols (μM)
3,0
2,5
2,0
1,5
1,0
0,5
0,0
a
a
b
a
b
Control
α-Tocopherol
GCDCA
GCDCA+α-Tocopherol

Figure 1

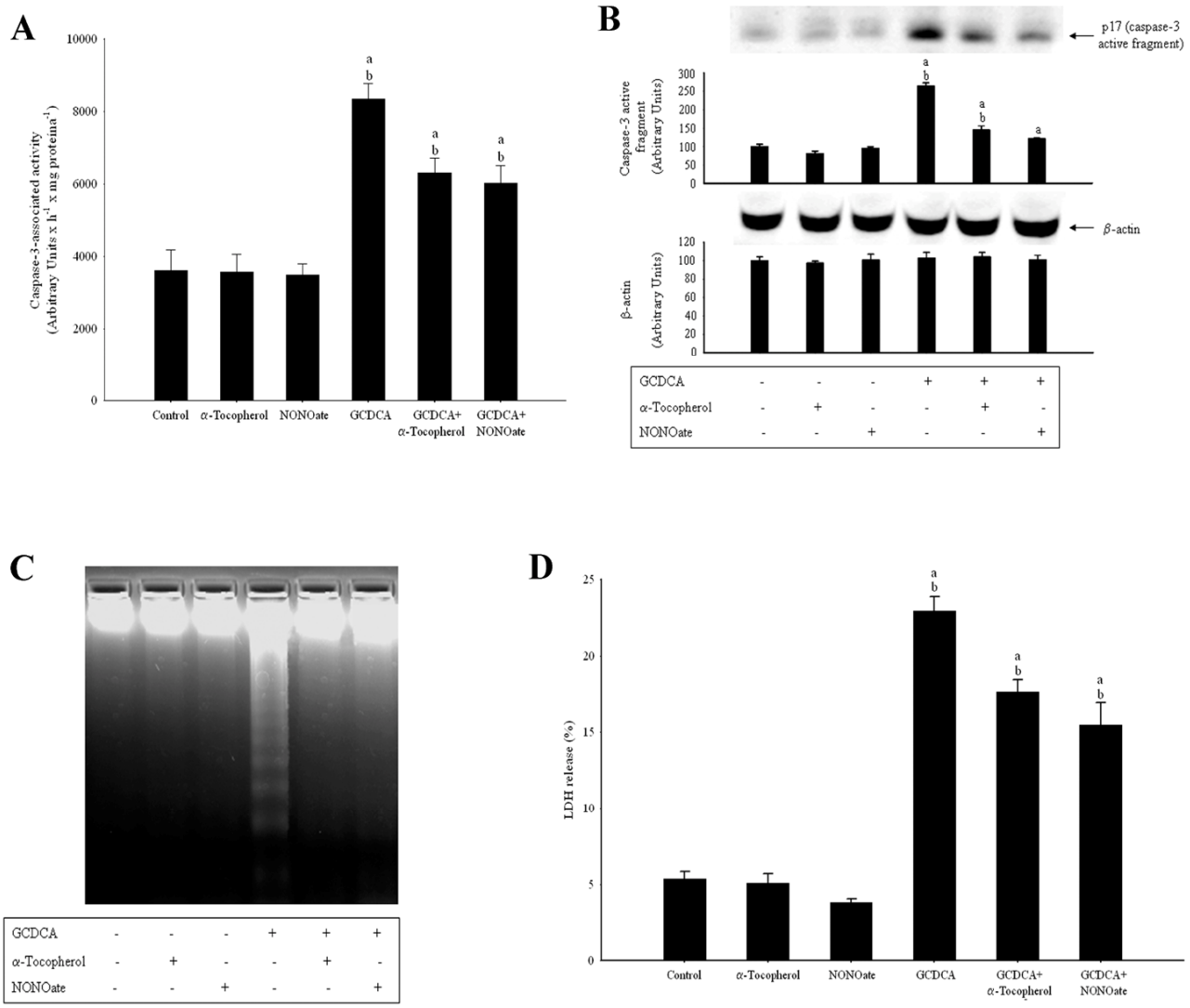

A
Caspase-3-associated activity (Arbitrary Units x h-1 x mg proteina-1)
10000
8000
6000
4000
2000
0
a
b
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
B
p17 (caspase-3 active fragment)
Caspase-3 active fragment (Arbitrary Units)
300
250
200
150
100
50
0
β-actin
β-actin (Arbitrary Units)
120
100
80
60
40
20
0
GCDCA - - - + + +
α-Tocopherol - + - - + -
NONOate - - + - - +
C
GCDCA - - - + + +
α-Tocopherol - + - - + -
NONOate - - + - - +
D
LDH release (%)
25
20
15
10
5
0
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate

Figure 2

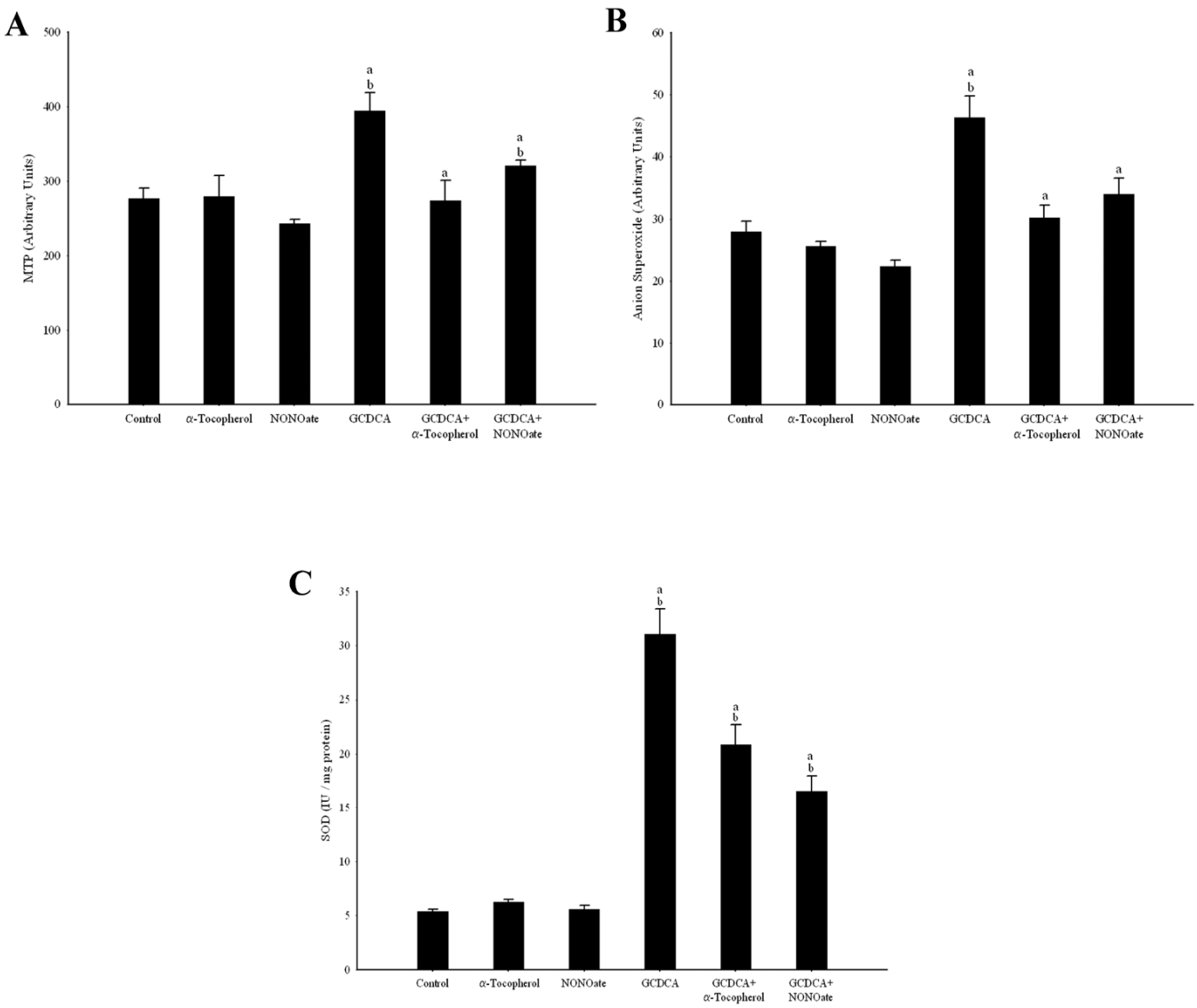

A
MTP (Arbitrary Units)
500
400
300
200
100
0
a
b
a
a
b
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
B
Anion Superoxide (Arbitrary Units)
60
50
40
30
20
10
0
a
b
a
a
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
C
SOD (IU / mg protein)
35
30
25
20
15
10
5
0
a
b
a
b
a
b
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate

Figure 3

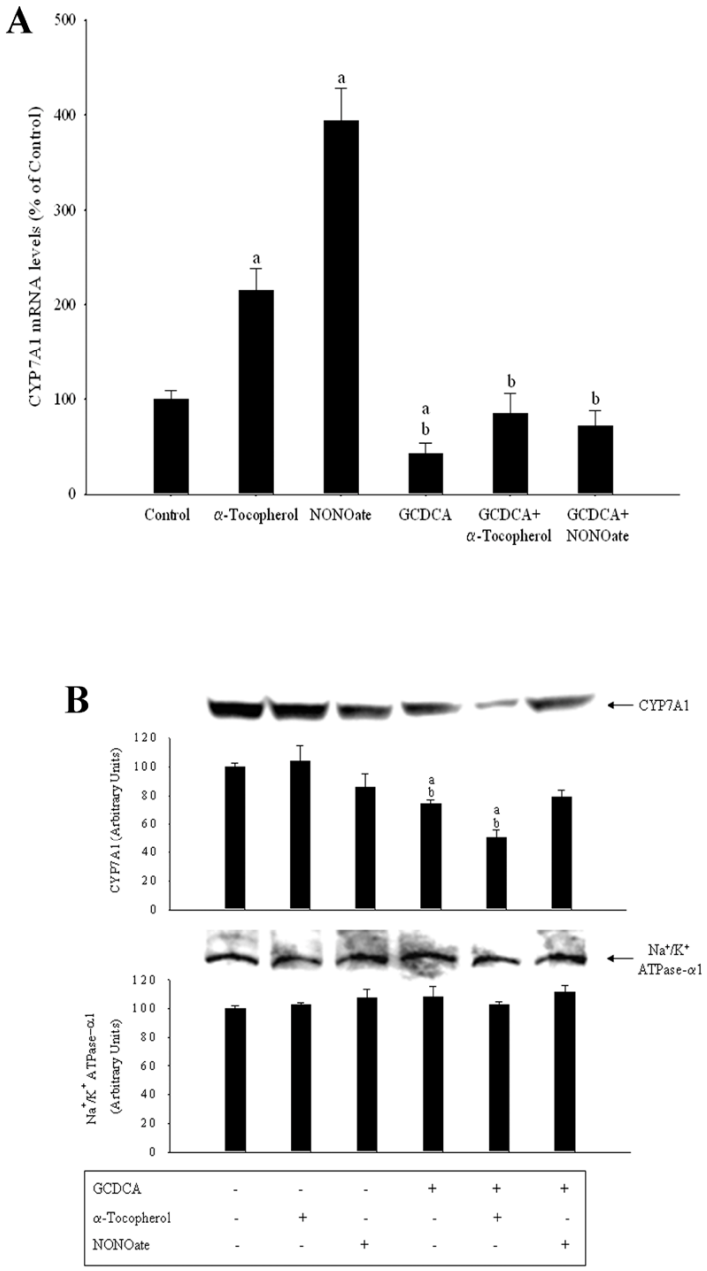

A
CYP7A1 mRNA levels (% of Control)
500
400
300
200
100
0
a
a
a
b
b
b
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
B
CYP7A1
CYP7A1 (Arbitrary Units)
120
100
80
60
40
20
0
Na+/K+ ATPase-α1
Na+/K+ ATPase-α1 (Arbitrary Units)
GCDCA - - - + + +
α-Tocopherol - + - - + -
NONOate - - + - - +

Figure 4

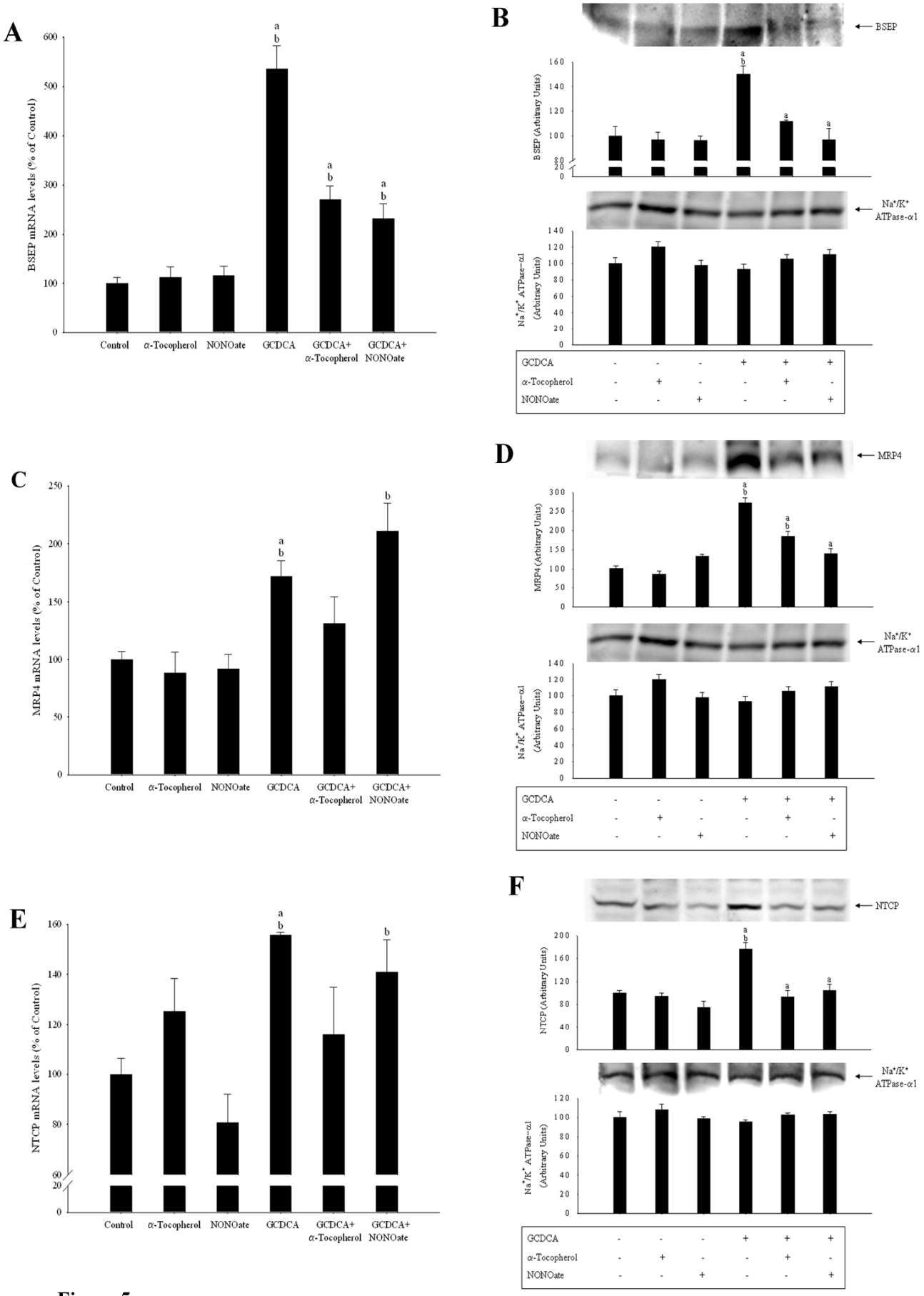

A
BSEP mRNA levels (% of Control)
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
B
BSEP
BSEP (Arbitrary Units)
Na+/K+ ATPase-α1
Na+/K+ ATPase-α1 (Arbitrary Units)
GCDCA
α-Tocopherol
NONOate
C
MRP4 mRNA levels (% of Control)
D
MRP4
MRP4 (Arbitrary Units)
E
NTCP mRNA levels (% of Control)
F
NTCP
NTCP (Arbitrary Units)

Figure 5

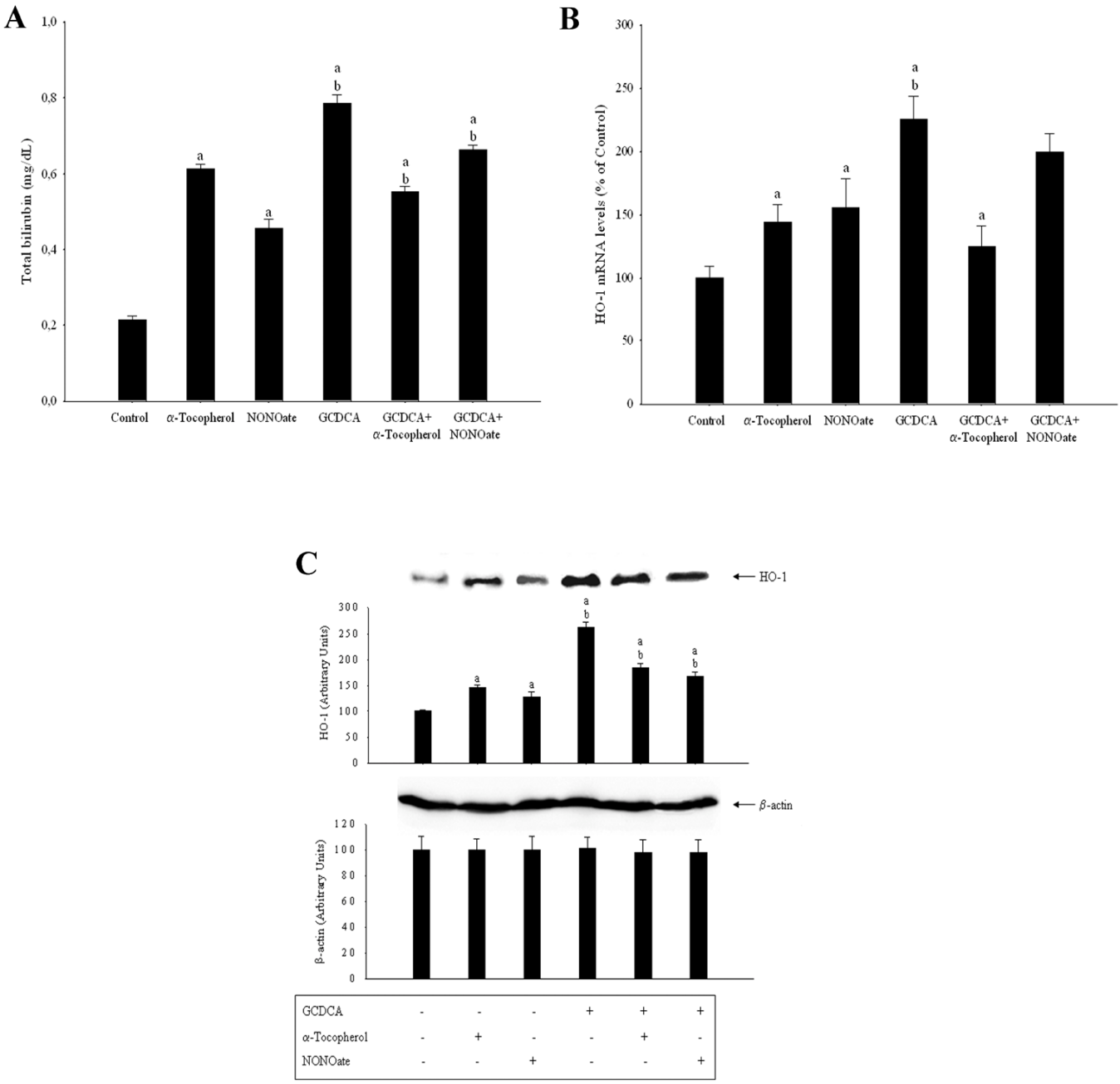

A
Total bilirubin (mg/dL)
1,0
0,8
0,6
0,4
0,2
0,0
Control
α-Tocopherol
NONOate
GCDCA
GCDCA+ α-Tocopherol
GCDCA+ NONOate
B
HO-1 mRNA levels (% of Control)
300
250
200
150
100
50
0
C
HO-1
β-actin
HO-1 (Arbitrary Units)
β-actin (Arbitrary Units)
GCDCA
α-Tocopherol
NONOate

Figure 6

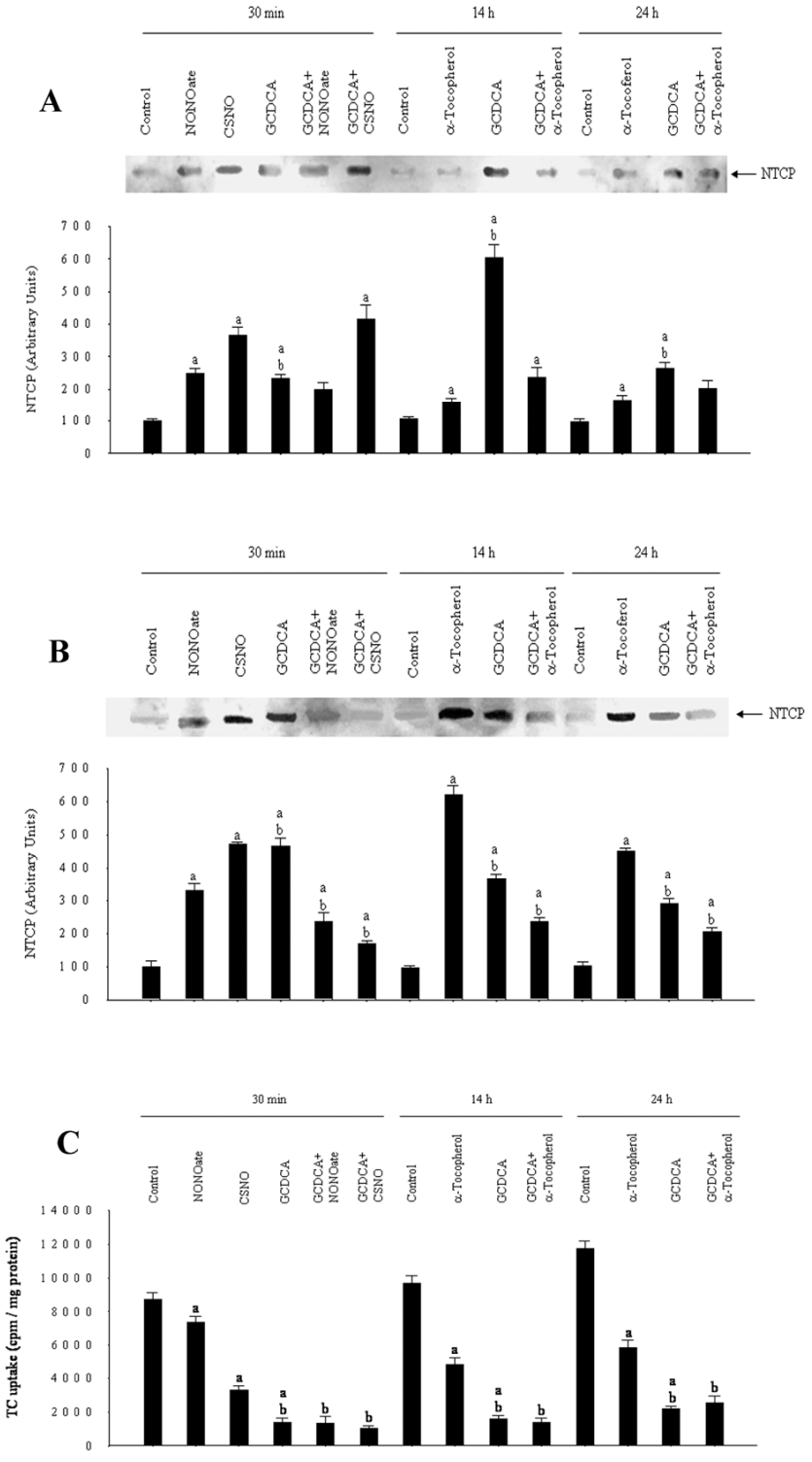

A
30 min
14 h
24 h
Control
NONOate
CSNO
GCDCA
GCDCA+ NONOate
GCDCA+ CSNO
Control
α-Tocopherol
GCDCA
GCDCA+ α-Tocopherol
Control
α-Tocoferol
GCDCA
GCDCA+ α-Tocopherol
NTCP
NTCP (Arbitrary Units)
B
30 min
14 h
24 h
Control
NONOate
CSNO
GCDCA
GCDCA+ NONOate
GCDCA+ CSNO
Control
α-Tocopherol
GCDCA
GCDCA+ α-Tocopherol
Control
α-Tocoferol
GCDCA
GCDCA+ α-Tocopherol
NTCP
NTCP (Arbitrary Units)
C
30 min
14 h
24 h
Control
NONOate
CSNO
GCDCA
GCDCA+ NONOate
GCDCA+ CSNO
Control
α-Tocopherol
GCDCA
GCDCA+ α-Tocopherol
Control
α-Tocopherol
GCDCA
GCDCA+ α-Tocopherol
TC uptake (cpm / mg protein)

Figure 7

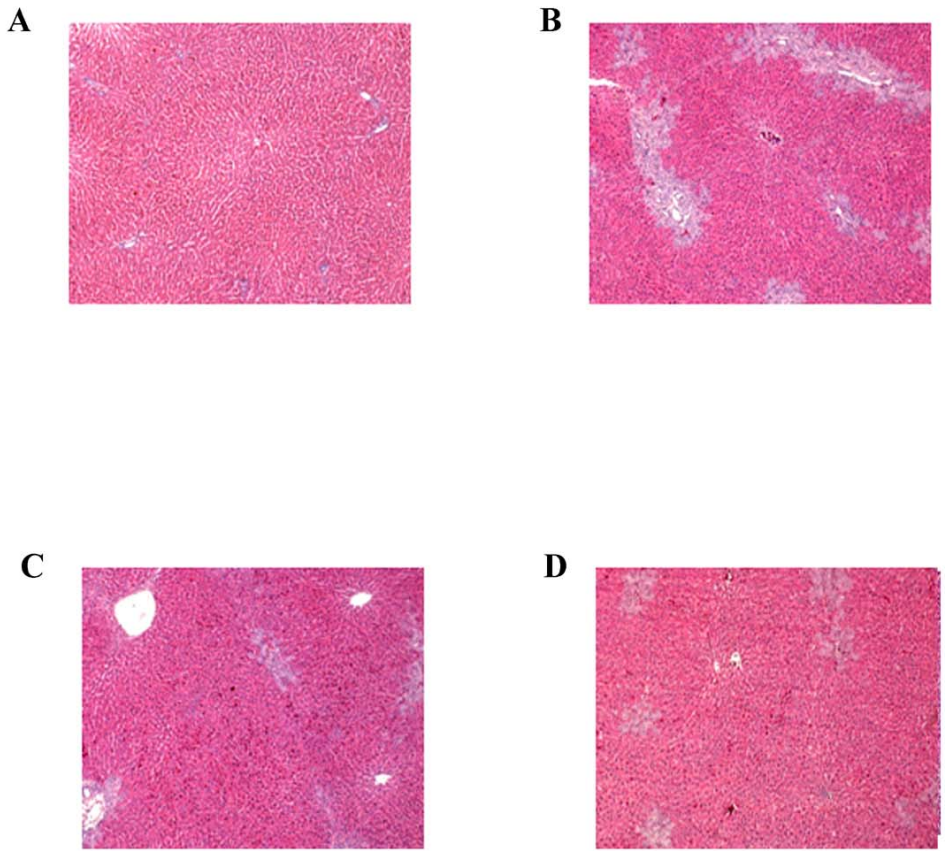


Figure 8

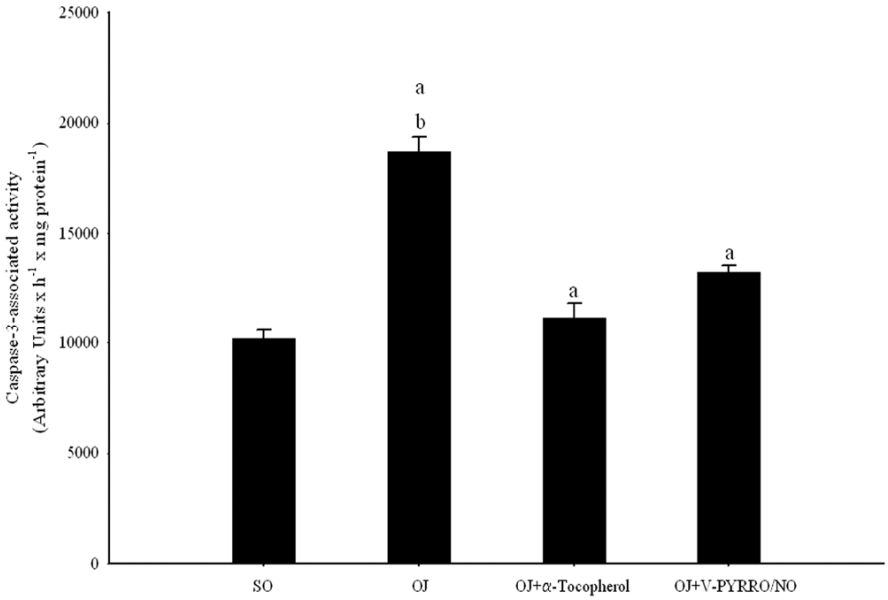

25000
20000
15000
10000
5000
0
Caspase-3-associated activity
(Arbitrary Units x h-1 x mg protein-1)
a
b
a
a
SO
OJ
OJ+α-Tocopherol
OJ+V-PYRRO/NO

Figure 9

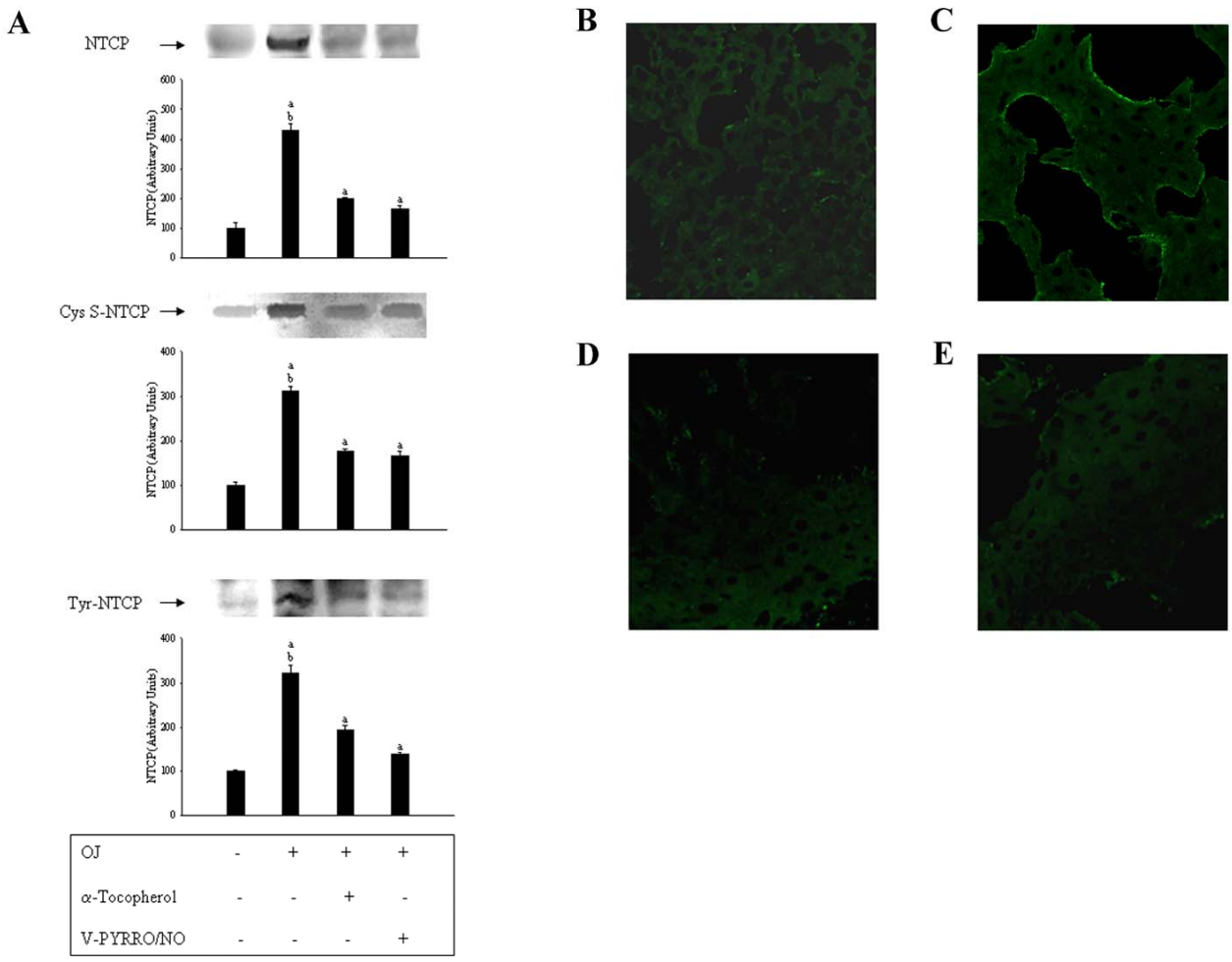

A
NTCP
NTCP(Arbitrary Units)
Cys S-NTCP
NTCP(Arbitrary Units)
Tyr-NTCP
NTCP(Arbitrary Units)
OJ - + + +
α-Tocopherol - - + -
V-PYRRO/NO - - - +
B
C
D
E

Figure 10

RESULTADOS GLOBALES Y DISCUSIÓN

En este proyecto de Tesis Doctoral se han estudiado diversas estrategias antioxidantes frente a la lesión hepatocelular inducida *in vivo* e *in vitro* por un hepatotóxico específico (D-GalN) y por sales biliares hidrófobas (GCDCA). En ambos modelos experimentales de lesión hepática se han ensayado diversas estrategias citoprotectoras, tales como prostanoides (PGE1) (**195**, **216-220**), antioxidantes (SAMe, α-tocoferol, NAC, ácido lipoico, melatonina, antioxidante Lazaroide U8383E, análogo de la SOD) (**196**, **199-206**, **208**, **209**, **214**, **215**, **221-226**), óxido nítrico (**208**, **227-229**), quinonas (mitoquinona ó mitoQ, Q_{10}) (**212**, **213, 230**) y tratamientos que alteran el metabolismo de los ácidos biliares (ácido ursodeoxicólico) (**207**). La muerte celular inducida por D-GalN y colestasis se caracteriza por estar asociada a la disfunción mitocondrial con hiperpolarización de la membrana mitocondrial (MTP) y estrés oxidativo (**195**, **203-208**, **217-220, 231**). Las propiedades citoprotectoras de numerosos agentes se relacionan con la reducción del estrés oxidativo y muerte celular en ambos modelos experimentales de lesión hepatocelular (**193-210**, **212**, **213**, **216-226, 230**). En el presente estudio se ha profundizado en los mecanismos intracelulares de citoprotección por α-tocoferol, NAC, Q_{10} ó MnTBAP en el caso de la lesión hepatocelular por D-GalN, así como de α-tocoferol y un donador de NO en los estudios de citotoxicidad por GCDCA. A pesar de que todos los tratamientos han reducido el estrés oxidativo y la muerte celular, existen claras diferencias en cuanto a la extensión de su actividad antioxidante, así como la identificación de nuevas vías de señal intracelular mediante la cual ejercen su acción citoprotectora. En el trabajo de investigación contemplado en la presente Tesis Doctoral, NAC, Q_{10} ó MnTBAP reducen la muerte celular en el modelo de toxicidad con D-GalN atenuando el estrés oxidativo mitocondrial, recuperando el contenido de GSH y Q_{10} reducido, e incrementando la actividad de los complejos mitocondriales y síntesis de ATP. La administración de NAC (**193**, **200, 221-224, 226**), Q_{10} ó mito Q (**210, 230**) y el análogo de la SOD (MnTBAP) (**225**) es capaz de reducir el estrés oxidativo y muerte celular en diversos modelos experimentales de lesión hepatocelular. La administración de α-tocoferol ha demostrado reducir la citoxicidad por sales biliares hidrófobas a través de la regulación de la disfunción mitocondrial y estrés oxidativo (**209**). Diversos estudios han demostrado la capacidad de α-tocoferol para regular la actividad transactivadora de diversos receptores nucleares (PPAR-γ) y factores de transcripción (NF-*k*B) (**232-237**). Nuestros estudios sugieren que la activación o reducción de la activación de NF-*k*B dependen del entorno fisiopatológico celular. En este sentido, α-tocoferol reduce o disminuye la activación de NF-*k*B en los

hepatocitos tratados con D-GalN o GCDCA, respectivamente. La lesión hepatocelular inducida por D-GalN está asociada a una esteatosis hepática (**216**). El incremento de la expresión de genes como CPT-1 por α-tocoferol tiene un impacto positivo en la supervivencia del hepatocito. CPT-1 incrementa la entrada de ácido grasos en los hepatocitos, facilitando su metabolismo lo que se ha relacionado con supervivencia celular (**238**). Los datos presentados son los primeros estudios demostrando la capacidad de α-tocoferol para regular la expresión de los transportadores de sales biliares en hepatocitos. Nuestros estudios sugieren que este efecto está mediado por el NO. Otros autores han demostrado previamente que el NO regula la expresión de diversos genes en hepatocitos (**239-242**).

El NO ya ha demostrado ejercer un papel clave en el metabolismo hepático y en la lesión hepática inducida por diversos agentes (**243**, **244**). El efecto beneficioso de la administración de α-tocoferol se ha relacionado con una disminución de la activación de NF-*k*B y de la expresión de NOS-2 en hepatocitos humanos en cultivo (artículo 1). Diversos estudios previos de nuestro grupo han demostrado que la inducción de la expresión de NOS-2 y producción de NO induce muerte celular por apoptosis en hepatocitos humanos y de rata en cultivo (**195**, **218**, **219**). La inhibición competitiva por L-NAME de NOS-2 reduce la apoptosis inducida por D-GalN en hepatocitos de rata (**219**). La regulación transcripcional del gen NOS-2 se encuentra bajo el control de diferentes receptores nucleares y factores de transcripción (**219**, **245**). La administración de α-tocoferol altera de forma drástica el patrón de expresión y activación de diferentes factores de transcripción (NF-*k*B) y receptores nucleares (PXR, RXR, FXR, PPAR-α) en hepatocitos tratados con D-GalN y GCDCA. La administración de agonistas de PPAR-α previene la activación de NF-*k*B y la expresión de VCAM-1 (molécula 1 de adhesión celular vascular) por TNF-α en células endoteliales (**246**). El incremento de PPAR-α por α-tocoferol podría estar relacionado con la reducción de la activación de NF-*k*B y expresión de NOS-2 en los hepatocitos tratados con D-GalN. De hecho, se ha demostrado la existencia de interacción entre NF-*k*B y PPAR-α para la unión de NF-*k*B a su secuencia de unión en la región promotora del gen del receptor de estrógenos (**247**).

La expresión de CYP3A, enzima implicado en el metabolismo de α-tocoferol, está regulado por PXR, RXR, VDR y GR (**248-250**). Nuestros, así como otros (**43**), demuestran que α-tocoferol incrementa la expresión de CYP3A4, lo que sugiere una importante alteración del patrón de regulación transcripcional al resultar PXR clave en la expresión de numerosos genes de DME y transporte de moléculas. El estudio descrito en el artículo 1 muestra que D-GalN reduce la expresión de CYP3A4 e incrementa la concentración de las diferentes isoformas del tocoferol y su catabolismo a

α-CEHC en los hepatocitos en cultivo. En este sentido, la inducción de CYP3A4 a través de PXR por α-tocoferol no se relacionaría con su efecto beneficioso general. Nuestros datos sugieren que el efecto citoprotector de α-tocoferol se produce a través de un mecanismo independiente de PXR.

El mantenimiento del contenido de GSH mitocondrial es importante para prevenir la lesión hepatocelular (**191**, **193**). Los resultados de nuestros estudios están de acuerdo con investigaciones anteriores en las que se observó que la reducción en el GSH intracelular se relaciona con la muerte celular por D-GalN (**196**). NAC es un precursor importante en la ruta sintética de GSH, demostrando efectos beneficiosos en otros modelos de lesión celular (**210**). Los complejos mitocondriales I y III son los mayores productores de ERO mitocondrial (**251**). El incremento de la actividad de los complejos I, III y IV se asocia con hiperpolarización de la MTP y mayor producción de ERO en las células tratadas con D-GalN. La inducción de estrés oxidativo altera física y funcionalmente diversos componentes de la membrana plasmática y mitocondrial, que es clave en los procesos de envejecimiento y muerte celular (**251**). La disfunción mitocondrial y reducción de la fosforilación oxidativa son procesos importantes durante la inducción de la muerte celular por necrosis y apoptosis (**217**, **252**). El estrés oxidativo por D-GalN se asocia con la oxidación de componentes clave del funcionamiento mitocondrial como el Q_{10} y GSH, que conlleva en última instancia la depleción del ATP celular y compromete la supervivencia celular. La administración de NAC, Q_{10} (**212** y **213**) ó MnTBAP restaura el estado reducido del GSH y del Q_{10}, ATP celular y previene la muerte celular por D-GalN en hepatocitos humanos. Los resultados sugieren que los antioxidantes permiten la restauración de la expresión y actividad del complejo I en los hepatocitos tratados con D-GalN.

Los sistemas metabolizadores y de transporte de los compuestos exógenos y endógenos de origen tóxico, xenobiótico o farmacológico han ido evolucionando para incrementar la hidrofilicidad de los compuestos y facilitar su excreción a nivel renal o biliar (**253**). Diferentes factores de transcripción y receptores nucleares participan en la regulación de la expresión de los sistemas enzimáticos metabolizadores de drogas (DME) constituidos por los CYP, así como de los transportadores (**32**, **182**-**184**). En el estudio expuesto en el artículo 3 se evaluó el efecto del α-tocoferol y un donador de NO (NONOate) sobre la expresión de proteínas involucradas en la síntesis de ácidos biliares (CYP7A1), antioxidantes (SOD), reacciones redox (HO-1) y transporte de incorporación/excreción de ácidos biliares (NTCP, MRP4 y BSEP). Se observó que α-tocoferol y NONOate atenuaron la inducción de la expresión de HO-1 y SOD en los hepatocitos tratados con GCDCA. En estudios previos se ha demostrado que la presencia de estrés oxidativo puede ser regulado por HO-1 en ratas colestáticas (**254**).

En nuestro estudio, observamos que la producción de $O_2^{\cdot-}$ incrementa la expresión de HO-1 y la actividad SOD en hepatocitos tratados con GCDCA. La regulación de la incorporación y excreción de los ácidos biliares tiene un impacto importante en la lesión hepatocelular por sales biliares. NTCP es el principal mecanismo de incorporación de ácidos biliares hacia el hepatocito (**177**). La administración de α-tocoferol y el donador de NO (NONOate ó V-PYRRO/NO) redujeron *in vivo* e *in vitro* la expresión de NTCP. La expresión de MRP4 (membrana basolateral) y BSEP (canalículo biliar) son los principales transportadores de excreción de ácidos biliares del hepatocito. En el estudio *in vitro* se observó que tanto α-tocoferol como el donador de NO (NONOate) atenuaron el incremento de la expresión de ambos transportadores por GCDCA en hepatocitos. Estos datos sugieren que el efecto citoprotector de α-tocoferol y NO se relacionan con la disminución de la expresión de los transportadores de sales biliares al interior del hepatocito.

Diferentes receptores nucleares como PXR, RXR, FXR, CAR y PPAR-α están involucrados en la regulación de la transcripción de CYP7A1, HO-1 y de los transportadores de ácidos biliares (**32, 182, 184**). En el presente estudio se ha observado que el NO mimetiza la alteración de la expresión de los transportadores de ácidos biliares (NTCP) y modificaciones postranslacionales observadas con la administración de α-tocoferol frente a la muerte celular inducida por GCDCA.

NO ejerce su acción celular mediante una ruta dependiente de GMPc y también a través de una ruta independiente de GMPc que incluye las modificaciones en resíduos de Cys (S-nitrosilación) y Tyr (nitración), que en circunstancias de elevado estrés oxidativo puede promover la oxidación llegando a alterar la función proteica y/o alterar la transcripción génica (**142**). La administración de GCDCA induce una disminución de la expresión de NOS-2 y de la producción de nitrito+nitrato en el medio de cultivo de los hepatocitos. La administración de donadores de NO ha demostrado ejercer un efecto beneficioso frente a la citotoxicidad por sales biliares (**208**, **227**-**229**). El efecto citoprotector por α-tocoferol se relaciona con el incremento de la expresión de NOS-2 en los hepatocitos tratados con GCDCA. Se ha demostrado que la administración de donadores de NO reduce la captación de moléculas dependientes de NTCP en hepatocitos (**227**). En el modelo experimental *in vitro* e *in vivo* investigado en el artículo 3 se ha analizado el efecto de las modificaciones postranslacionales de NTCP dependientes de NO debidas al α-tocoferol y a donares de NO (NONOate y CSNO). Se observa que la modificación por S-nitrosilación y nitración de NTCP por α-tocopherol y el donador de NO previene la incorporación de TC al hepatocito.

En resumen, el presente proyecto de Tesis Doctoral avanza en el conocimiento de los mecanismos intracelulares de los antioxidantes utilizados (NAC, Q_{10} y MnTBAP) sobre

la restauración de la función mitocondrial y prevención de la muerte celular inducida por D-GalN y por GCDCA en hepatocitos. La Tesis abre nuevas vías de investigación sobre el importante papel de α-tocoferol en la regulación de la expresión de numerosos genes implicados en la metabolización e incorporación de moléculas con actividad hepatotóxica.

CONCLUSIONES

Los resultados de dicho trabajo de investigación permiten extraer una serie de conclusiones:

1) La muerte celular inducida por D-GalN se asocia con un incremento del estrés oxidativo, y de la oxidación del GSH y del Q_{10} mitocondrial en los hepatocitos humanos en cultivo.

2) El efecto beneficioso de NAC, Q_{10} ó MnTBAP, pero no de α-tocoferol, está relacionado con la disminución del estrés oxidativo mitocondrial, y recuperación del contenido de GSH y Q_{10} reducido durante la citotoxicidad por D-GalN en hepatocitos humanos en cultivo.

3) El efecto citoprotector de α-tocoferol se asocia con el incremento de la expresión de PPAR-α y CPT-1, y disminución de la activación de NF-*k*B y expresión de NOS-2 durante la citotoxicidad por D-GalN en hepatocitos humanos en cultivo.

4) El NO mimetiza el efecto de α-Tocoferol sobre la reducción de la expresión de NTCP, así como en el incremento de sus modificaciones postranslacionales (S-nitrosilación y nitración) en hepatocitos. Este efecto tiene un claro impacto en la prevención del estrés oxidativo y muerte celular inducida por GCDCA en los hepatocitos humanos en cultivo.

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