Investigación de nuevas estrategias terapéuticas para la inflamación y la fibrosis hepática

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INVESTIGACIÓN DE NUEVAS ESTRATEGIAS TERAPÉUTICAS PARA LA INFLAMACIÓN Y LA FIBROSIS HEPÁTICA

Memoria presentada por

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ABREVIATURAS

Acteil-CoA	Acetil coenzima A.
AgRP	Péptido relacionado con agouti.
AMP	Adenosín monofosfato.
Ang II	Angiotensina II.
ATP	Adenosín trifosfato.
bFGF	Factor básico de crecimiento de fibroblastos.
cAMP	Adenosín monofosfato cíclico.
CB	Receptor de cannabinoides.
CBP	Cirrosis biliar primaria.
CCL21	Quimiocina 21.
CCl_4	Tetracloruro de carbono.
CTGF	Factor de crecimiento del tejido conectivo.
DDR	Receptores con dominio discoidina.
EGF	Factor de crecimiento epitelial.
ERK	Quinasas reguladas por señales extracelulares.
FADD	Proteína asociada a Fas con dominio de muerte.
FAK/PI3K/Akt	Quinasa de adhesión focal/fosfatidilinositol-3-quinasa/Akt.
FGF	Factor de crecimiento de fibroblastos.
GH	Hormona del crecimiento.
GHRH	Hormona liberadora de la hormona del crecimiento.
GHS-R	Receptor de los secretagogos de la hormona del crecimiento.
HA	Hepatitis alcohólica.
HGF	Factor de crecimiento de hepatocitos.
HMG-CoA	3-hidroxi-3-metilglutaril-coenzima A.
HSC	Células estrelladas hepáticas.
HVC	Hepatitis por virus de la hepatitis C.
ICAM	Molécula de adhesión intercelular.
IFN	Interferón.
IGF	Factor de crecimiento similar a la insulina.
IL	Interleucina.
IP-10	Proteína tipo 10 inducible por interferón-γ.
I-TAC	Citocina quimioatrayente de células T inducida por interferón.
JNK	Quinasas N-terminales de c-Jun.
LBP	Proteína de unión a lipopolisacárido.
LPS	Lipopolisacárido.
MAPK	Proteínas quinasas activadas por mitógenos.

MCP-1	Proteína quimioatrayente de macrófagos 1.
M-CSF	Factor estimulador de colonias de macrófagos.
MEC	Matriz extracelular.
MEK	Quinasa de proteínas quinasas activadas por mitógenos.
MHC	Complejo mayor de histocompatibilidad.
Mig	Monocina inducida por interferón-γ.
MIP-2	Proteína inflamatoria de macrófagos 2.
MKK	Quinasa de quinasas de proteínas quinasas activadas por mitógenos.
MMP	Metaloproteasa.
mTOR	Diana de mamíferos para la rapamicina.
NASH	Esteatohepatitis no alcohólica.
NCAM	Molécula de adhesión neural.
NF-κB	Factor nuclear κB.
NS	Proteína no estructural.
PAF	Factor activador de plaquetas.
PDGF	Factor de crecimiento derivado de plaquetas.
PDGF-R	Receptor del factor de crecimiento derivado de plaquetas.
PGE2	Prostaglandina E2.
PKA	Proteína quinasa A.
PPAR	Receptor activado por proliferación peroxisomal.
RIP	Proteína que interacciona con receptor.
RNAi	Ácido ribonucleico de interferencia.
ROS	Especies reactivas de oxígeno.
RXR	Receptor X de retinoides.
SCF	Factor de células madre.
SRA	Sistema renina-angiotensina.
STAT	Proteína transductora de señales y activadora de transcripción.
TGF-β	Factor de crecimiento transformante β.
Th	T helper.
TIMP	Inhibidor de metaloproteasa.
TLR	Receptor *Toll-like*.
TNF	Factor de necrosis tumoral.
TNF-R	Receptor del factor de necrosis tumoral.
TRADD	Proteína con dominio de muerte asociada al receptor 1 del factor de necrosis tumoral.
TRAF	Factor asociado al receptor del factor de necrosis tumoral.
VCAM	Molécula de adhesión vascular celular.

VEGF	Factor de crecimiento del endotelio vascular celular.
VHC	Virus de la hepatitis C.
VIH	Virus de la inmunodeficiencia humana.

ÍNDICE

ÍNDICE DE FIGURAS

ÍNDICE DE TABLAS

"Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad."

Albert Einstein

1. INTRODUCCIÓN

1.1. LA INFLAMACIÓN HEPÁTICA.

1.1.1. Concepto.

La inflamación es un mecanismo homeostático que se desencadena ante una agresión en un tejido vascularizado. Tiene como finalidad la reparación de la lesión tisular y la eliminación de las células y tejidos necróticos (1). La inflamación es pues un proceso clave en la progresión de diversas enfermedades como el cáncer, el asma, la enfermedad inflamatoria intestinal o las enfermedades hepáticas crónicas.

Debido a su función y situación anatómica, el hígado está constantemente expuesto a agresiones que pueden inducir inflamación. Es por ello que ha desarrollado numerosos mecanismos de defensa como la presencia de un elevado número de macrófagos residentes (células de Kupffer) (2), una elevada concentración de moléculas antioxidantes como el glutatión (3;4) y una alta capacidad de regeneración (5). Ante ciertas agresiones, estos mecanismos de defensa pueden ser insuficientes, lo que conduciría a un aumento de la muerte de los hepatocitos y a la consiguiente respuesta inflamatoria (6).

La inflamación es un hallazgo común en la mayoría de enfermedades crónicas del hígado. Las causas más frecuentes de inflamación hepática en España son la infección por virus de la hepatitis C (VHC) y el consumo excesivo de alcohol (7), aunque existen otras causas (Tabla 1).

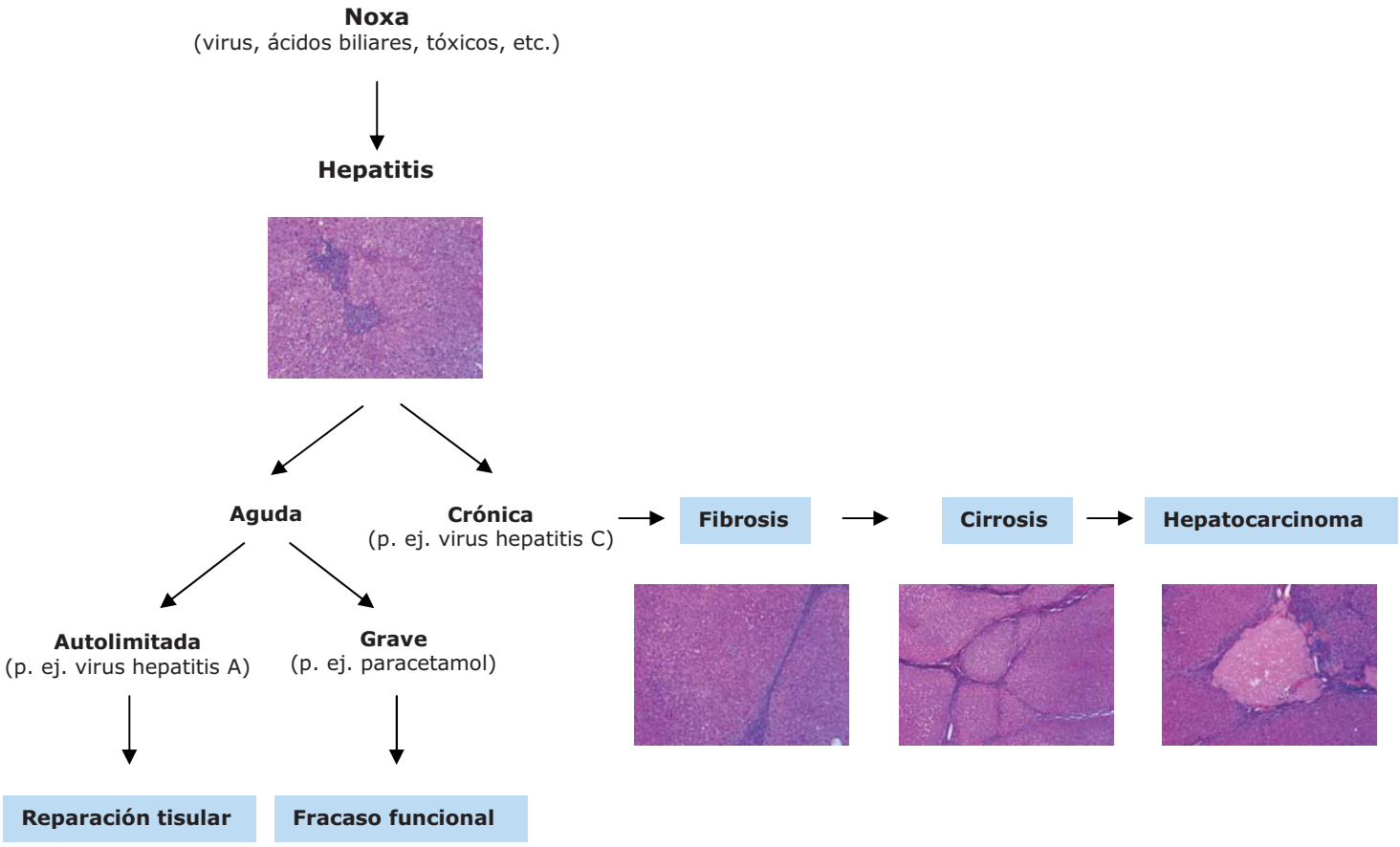
El pronóstico de la inflamación hepática es muy diferente según la etiología. La inflamación se denomina aguda si se resuelve en un corto periodo de tiempo y crónica si se mantiene durante años. Dentro de la hepatitis aguda, ésta se puede clasificar en autolimitada y grave. La infección por virus de la hepatitis A es un ejemplo de hepatitis aguda autolimitada, ya que generalmente se resuelve rápidamente y no suele presentar complicaciones clínicas. Sin embargo, la inflamación aguda, producida por algunos tóxicos (como el paracetamol) o por el virus de la hepatitis B, puede dar lugar a una hepatitis grave y asociarse a insuficiencia hepática (8).

Tabla 1. Causas más frecuentes de inflamación hepática.

Etiología	Ejemplos
Vírica	Virus de la hepatitis: A, B, C, D y E Otros virus hepatotropos (Epstein-Barr, varicela-zóster, citomegalovirus, etcétera)
Tóxica	Consumo abusivo de alcohol Fármacos (paracetamol, metotrexato, anestésicos, etcétera) Drogas de abuso
Inmunitaria	Hepatitis autoinmune Rechazo de hígado trasplantado Enfermedad del injerto contra el huésped
Genética	Enfermedad de Wilson (depósito de cobre) Hemocromatosis (depósito de hierro) Enfermedad de Gaucher (depósito de lípidos) Glucogenosis (depósito de glucógeno) Déficit de alfa1-antitripsina
Alteraciones biliares	Obstrucción del conducto biliar Cirrosis biliar primaria Colangitis esclerosante
Bacteriana	Fiebre tifoidea Tuberculosis Sífilis
Parasitaria	Esquistosomiasis
Alteraciones circulatorias	Hepatitis isquémica Síndrome de Budd-Chiari
Nutricional	Obesidad (esteatohepatitis no alcohólica, NASH) Kwashiorkor

Cuando la hepatitis se cronifica suele desarrollarse fibrosis, que consiste en el acúmulo de matriz extracelular (MEC), principalmente colágeno. Ésta puede evolucionar hasta cirrosis, en algunos pacientes. La cirrosis implica un mayor riesgo de desarrollar cáncer hepatocelular (Figura 1).

Figura 1. Historia natural de las enfermedades hepáticas.



1.1.2. Bases moleculares.

La inflamación está dirigida por mediadores bioquímicos que pueden circular en el plasma (mediadores sistémicos o plasmáticos) o pueden ser producidos en el propio tejido afectado (mediadores locales) (1). En las enfermedades hepáticas, los primeros son sintetizados por el hígado y necesitan ser activados para ejercer su función y los segundos pueden estar preformados y almacenados en gránulos dentro de células (especialmente mastocitos, basófilos o neutrófilos) o sintetizados *de novo*. Un resumen de los mediadores químicos más importantes que intervienen en la inflamación está representado en la Tabla 2.

Tabla 2. Mediadores bioquímicos de la inflamación.

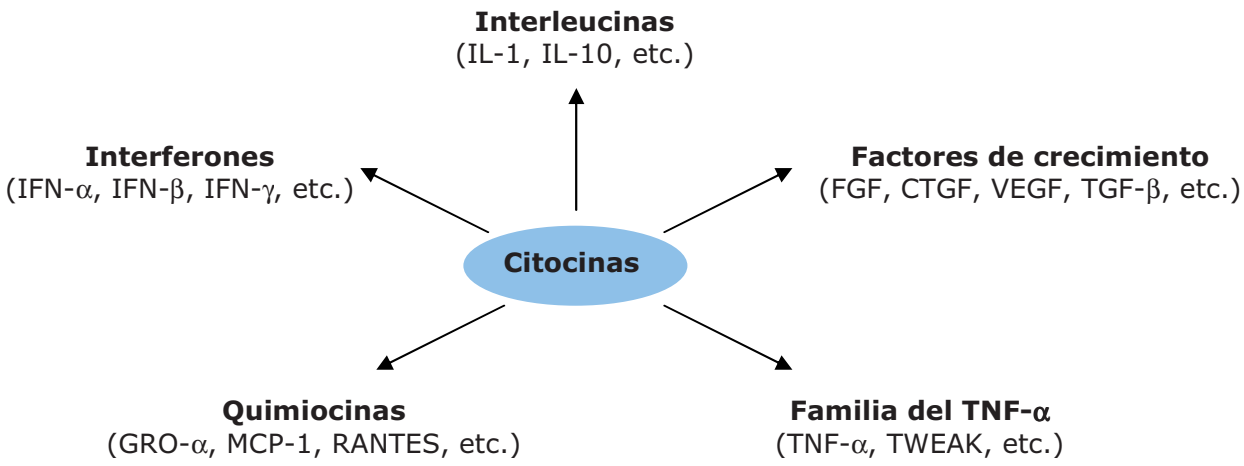
MEDIADORES LOCALES		
	Sustancias	**Acciones biológicas**
Preformados	Histamina	Vasodilatación y permeabilidad
	Serotonina	Vasodilatación y permeabilidad
	Enzimas lisosomales	Iniciación daño
Sintetizados *de novo*	Prostaglandinas	Vadodilatación, dolor, permeabilidad
	Leucotrienos	Permeabilidad, quimiotaxis
	PAF	Permeabilidad, transmigración
	ROS	Iniciación daño
	Óxido nítrico	Vasodilatación
	Citocinas	Quimiotaxis, propagación
	Selectinas	Transmigración
MEDIADORES SISTÉMICOS (plasmáticos)		
	Sustancias	**Acciones biológicas**
Sistema de las cininas	Bradicinina	Vasodilatación, dolor
	Calicreína	Quimiotaxis
Sistema de la coagulación/	Trombina	Coagulación, quimiotaxis
fibrinolisis	Fibrina	Coagulación
Sistema del complemento	C3a, C5a, C3b y C5b-9	Permeabilidad, opsonización y quimiotaxis

En su conjunto, los mediadores de la inflamación son responsables de los cambios locales que se producen en el tejido inflamado. Su función es la de facilitar la entrada de células inflamatorias al foco de la lesión y la entrada de proteínas plasmáticas al tejido dañado. Dichos mediadores promueven mecanismos específicos como la vasodilatación, el aumento de la permeabilidad vascular, el incremento de la viscosidad de la sangre, la quimiotaxis y la transmigración de células inflamatorias.

Las citocinas son unos de los mediadores clave de la inflamación. Además de modular la respuesta inflamatoria, tienen la capacidad de regular otros procesos biológicos como la muerte celular, la angiogénesis, la fibrosis y la regeneración tisular. Las citocinas son un extenso grupo de proteínas formado por varias familias: interleucinas (IL), interferones (IFN), miembros de la familia del factor de necrosis tumoral (TNF), quimiocinas y factores de crecimiento (9) (Figura 2). Las citocinas

pueden ser proinflamatorias (como la IL-1, la GRO-α o el TNF-α) o antiinflamatorias (como la IL-4 o la IL-10). Las citocinas modulan la respuesta inflamatoria local a la vez que pueden ejercer efectos sistémicos como la inhibición del apetito o la fiebre (IL-1, IL-6 y TNF-α) (1;10).

Figura 2. Clasificación de las citocinas.



Además de los mediadores inflamatorios clásicos y las citocinas, existen otras moléculas implicadas en el proceso de inflamación hepática como la angiotensina II (Ang II), los endocannabinoides (11) o las adipocinas (leptina, adiponectina, resistina y visfatina) (12;13). La Ang II es una de las moléculas más importantes en la inflamación hepática. Este péptido, que constituye el principal componente activo del sistema renina-angiotensina (SRA), ejerce diversas funciones proinflamatorias y profibrogénicas en el hígado. Se sabe que la simple infusión de Ang II en ratas sanas, es capaz de inducir fenómenos inflamatorios en el hígado. Por otro lado, la Ang II promueve acciones profibrogénicas en las células estrelladas hepáticas (HSC) (14). Por su parte, los endocannabinoides tienen un efecto proinflamatorio si actúan mediante el receptor de cannabionoides (CB) tipo 1 y antiinflamatorio si actúan mediante el CB2 (11). Entre las adipocitocinas, se ha descrito que la resistina es capaz de producir efectos proinflamatorios sobre las HSC mientras que la

adiponectina ejerce una función antiinflamatoria en modelos animales de inflamación hepática (15).

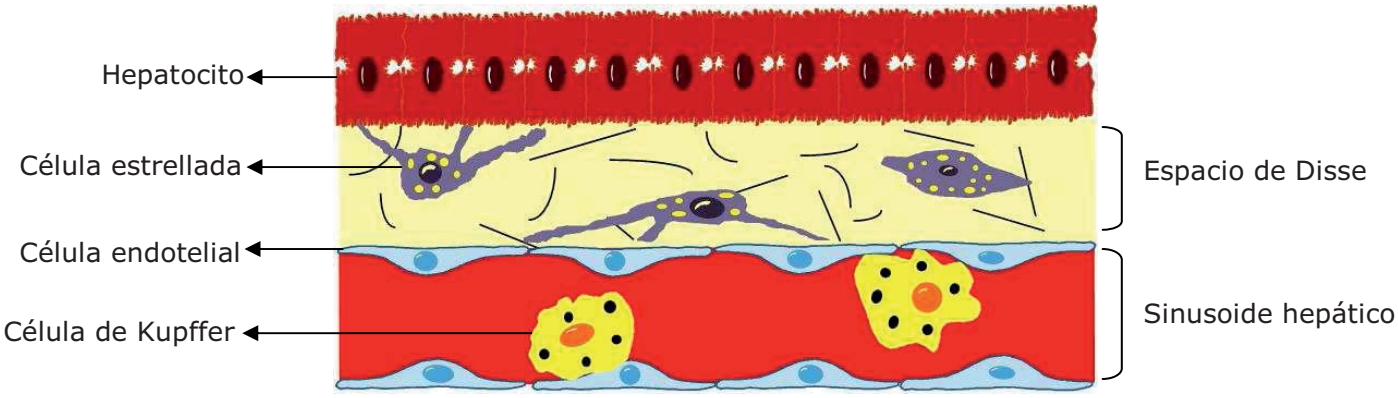
En cada tipo de enfermedad hepática predomina un tipo de mediador inflamatorio. Por ejemplo, las quimiocinas son especialmente importantes en la inflamación hepática aguda. Concretamente, en la hepatitis alcohólica (HA), hay un incremento en la expresión de las quimiocinas de la familia CXC (especialmente IL-8 y GRO-α) en el hígado, lo cual favorece el infiltrado inflamatorio, especialmente de neutrófilos (16). En la hepatitis C, son más importantes otras quimiocinas responsables de la atracción de linfocitos T, como la RANTES, la proteína inflamatoria de macrófagos (MIP)-1α, la MIP-1β, la proteína tipo 10 inducible por interferón-γ (IP-10), la monocina inducida por interferón-γ (Mig) y la quimioatrayente de células T inducida por interferón (I-TAC). Se ha demostrado que la expresión hepática de estas citocinas se halla incrementada en pacientes con hepatitis por virus C (HVC) (17). En el NASH inducido por obesidad, pueden ser más importantes las adipocitocinas, especialmente la leptina y la resistina que promueven la inflamación. La adiponectina, en cambio, es conocida por sus efectos antiinflamatorios y se ha observado que los pacientes con NASH tienen una concentración baja de adiponectina en suero (18). El TNF-α también influye en el progreso del NASH tal y como sugiere un estudio experimental en el que se demuestra que ratones deficientes en los receptores 1 y 2 del TNF-α muestran una respuesta inflamatoria disminuida en un modelo animal de NASH (dieta deficiente en colina y metionina) (19).

1.1.3. Bases celulares.

Las células involucradas en la respuesta inflamatoria del hígado se pueden clasificar en dos grupos: células residentes en el hígado (principalmente hepatocitos, HSC, células endoteliales y células de Kupffer) y células circulantes del sistema inmunitario que infiltran el hígado.

Las células residentes en el hígado se pueden describir según su posición respecto al sinusoide hepático. La sangre procedente de la vena porta entra en el hígado a través del sinusoide hepático. Éste está constituido por células endoteliales fenestradas dispuestas en línea y sin membrana basal y está separado de los hepatocitos por el espacio de Disse donde se encuentran las HSC. Las células residentes del sistema inmunitario (como las células de Kupffer o las células pit) se encuentran en el lumen del sinusoide hepático. La Figura 3 muestra un esquema de la distribución de las células alrededor del sinusoide hepático.

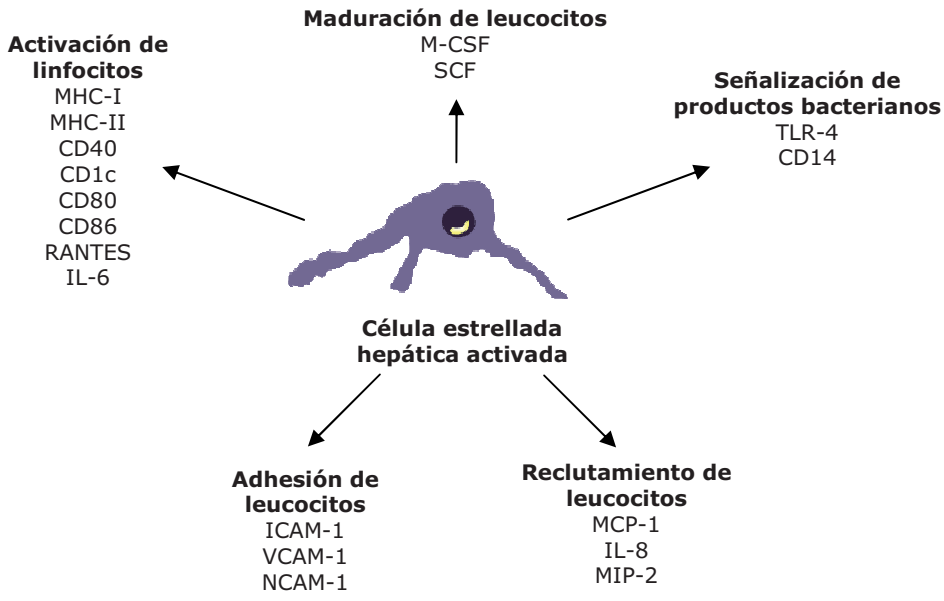
Figura 3. Disposición de las células alrededor del sinusoide hepático.



Los hepatocitos son las células que forman el parénquima hepático. Sus funciones son variadas pero cabe mencionar su importancia en la producción de bilis, en el metabolismo de productos tóxicos así como de carbohidratos y de lípidos, y en la síntesis de proteínas y factores de coagulación. Las células endoteliales forman la estructura de los vasos sanguíneos y regulan la circulación. Las células estrelladas hepáticas son un almacén de vitamina A (constituyen el reservorio el más importante de esta vitamina en el organismo), participan en la homeostasis del espacio de Disse y en la regulación del flujo sanguíneo del sinusoide hepático. Por último, las células de Kupffer son macrófagos especializados y por tanto, su función es la de eliminar patógenos o células transformadas.

La respuesta inflamatoria del hígado suele iniciarse cuando los hepatocitos son dañados. Éstos participan en la inflamación liberando citocinas y especies reactivas de oxígeno. Estos primeros mediadores inflamatorios, la muerte de los hepatocitos y las propias moléculas causantes del daño inducen la activación del resto de células hepáticas y el reclutamiento de leucocitos del torrente sanguíneo (20). El papel de las células endoteliales en la inflamación hepática consiste en incrementar la expresión de quimiocinas como la proteína quimioatrayente de macrófagos 1 (MCP-1) o la IL-8 y de otras moléculas que promueven la atracción y migración de leucocitos del torrente sanguíneo (20). Las moléculas que participan en la activación de las HSC derivan principalmente de las células de Kupffer y del infiltrado inflamatorio. La activación de las HSC consiste en un cambio fenotípico que se asocia a la adquisición de funciones profibrogénicas y proinflamatorias. Aunque estas células son especialmente importantes en el proceso de fibrogénesis hepática, como se explica en el apartado 1.2, también juegan un papel importante en el inicio del daño hepático, ya que secretan diversos mediadores inflamatorios y expresan moléculas de adhesión (20) (Figura 4). De manera similar, las células de Kupffer activadas secretan mediadores inflamatorios como especies reactivas de oxígeno (ROS), citocinas y eicosanoides.

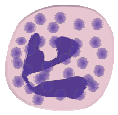
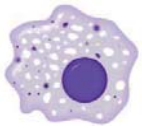
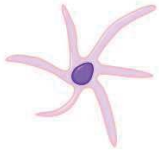
Figura 4. Actividades proinflamatorias de las células estrelladas hepáticas.



Como se ha explicado anteriormente, las células del sistema inmunitario también juegan un papel importante en la inflamación hepática. El sistema inmunitario se divide en dos: innato y adaptativo. La inmunidad innata es la respuesta rápida, inicial y no específica a estímulos dañinos (patógenos, estrés oxidativo, células malignas). El sistema inmunitario adaptativo es la respuesta tardía (necesita días para su activación) y específica contra un antígeno determinado (10). Las células más importantes del sistema inmunitario se detallan en la Tabla 3.

Ante una lesión en el hígado, algunas células circulantes del sistema inmunitario son atraídas hacia el foco de la inflamación mediante los mediadores quimioatrayentes. De manera simplificada, las células del sistema inmunitario participan en la inflamación mediante la secreción de citocinas (linfocitos, células NKT y macrófagos), la producción de sustancias citotóxicas (neutrófilos), la presentación de antígenos (macrófagos y células dendríticas), la inducción de apoptosis en células infectadas (células natural killer o NK y linfocitos T), la fagocitosis (neutrófilos, macrófagos y células dendríticas) y la producción de anticuerpos (linfocitos B). La etiología de la enfermedad hepática y el tipo de inflamación (aguda o crónica) determina el tipo celular más importante en el infiltrado inflamatorio, por ejemplo en las hepatitis agudas como la HA es muy importante el infiltrado de células polimorfonucleares (básicamente neutrófilos) mientras que los linfocitos son mucho más abundantes en las hepatitis virales.

Tabla 3. Células implicadas en el sistema inmunitario.

SISTEMA INMUNITARIO INNATO		
	**Neutrófilos**	Es el primer tipo celular en migrar al foco de inflamación. Son fagocitos. Almacenan sustancias citotóxicas en gránulos que liberan ante una reacción inflamatoria. Su participación es fundamental en la HA.
	**Macrófagos**	Proceden de los monocitos circulantes. Funcionan como fagocitos y células presentadoras de antígeno. Secretan citoquinas proinflamatorias. Los residentes en el hígado se denominan células de Kupffer.
	**Células NK (natural killer)**	Inducen apoptosis en células infectadas (virus, bacterias intracelulares, parásitos) y células transformadas. Las residentes en el hígado se denominan células pit. Efecto antiinflamatorio y antifibrogénico en el hígado.
	**Células dendríticas**	Proceden de los monocitos circulantes. Son fagocitos y presentadoras de antígeno. Inducen la activación de los linfocitos T.
SISTEMA INMUNITARIO ADAPTATIVO		
	**Linfocitos T**	Participan en la respuesta inmune celular. Existen diferentes tipos de linfocitos T con diferentes funciones (ayudar a la maduración de otras células del sistema inmunitario como los linfoctios B o los macrófagos, destruir células infectadas por virus o células no recocidas como propias, etcétera).
	**Linfocitos B**	Producen anticuerpos contra antígenos específicos (respuesta inmune humoral).
	**Células NKT**	Participan en la prevención del desarrollo de tumores y metástasis y en la regulación de enfermedades autoinmunes.

1.2. LA FIBROSIS HEPÁTICA.

1.2.1. Concepto.

La inflamación crónica del hígado se asocia con el depósito de colágeno y otras moléculas de la matriz extracelular (MEC) en el parénquima hepático, un proceso conocido como fibrosis hepática. Si el agente causante de la inflamación persiste, la fibrosis altera lentamente la arquitectura hepática formando puentes fibrosos y nódulos de regeneración, lo que constituye la cirrosis hepática. Por tanto, la fibrosis hepática es un paso previo y necesario para el desarrollo de cirrosis. En la cirrosis existe una clara disfunción hepatocelular y resistencia intrahepática al flujo sanguíneo, lo cual tiene como consecuencia hipertensión portal. El hígado cirrótico causa una serie de alteraciones en el organismo que desencadenan en síntomas como el sangrado por varices esofágicas, la ascitis o la encefalopatía hepática. Es entonces cuando se habla de cirrosis descompensada. Cuando la cirrosis se descompensa, el pronóstico del paciente es malo. Además, la cirrosis es un factor de riesgo para desarrollar carcinoma hepatocelular (21).

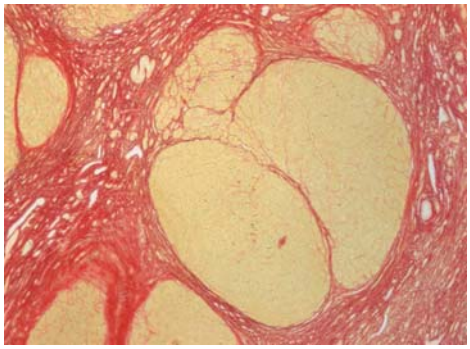
La evolución de la fibrosis hepática es un proceso muy lento en la mayoría de los pacientes. Como ejemplo, un paciente con HVC puede tardar 30 años en desarrollar cirrosis. Sin embargo, la progresión de la fibrosis es variable en cada paciente y está determinada por diversos factores tanto genéticos como no genéticos (21). La Tabla 4 muestra algunos de estos factores.

La evaluación del grado de fibrosis hepática es importante para decidir el tratamiento y establecer el pronóstico de los pacientes con hepatopatías crónicas. La biopsia hepática es todavía el método de elección, aunque los métodos no invasivos son cada vez más precisos. El examen histológico mediante tinciones para proteínas de la matriz extracelular (tricrómico de Masson o Sirius Red) permite evaluar el grado de fibrosis (Figura 5). Sin embargo, este método conlleva serios inconvenientes que promueven la investigación de métodos alternativos.

Tabla 4. Factores genéticos y no genéticos que influyen en la progresión de la fibrosis hepática.

Etiología	Factores genéticos	Factores no genéticos
HVC	Gen de la hemocromatosis hereditaria Angiotensinógeno TGF-β1 TNF-α Apolipoproteína E Hidrolasa epóxido microsomal MCP-1 MCP-2 Factor de la coagulación V	Edad Sexo Consumo de alcohol Coinfección con VIH o virus de la hepatitis B Trasplante hepático Diabetes mellitus No respuesta al tratamiento Obesidad Consumo de cannabis
Enfermedad hepática alcohólica	IL-10 IL-1β Alcohol deshidrogenasa Aldehído deshidrogenasa Citocromo P450 2E1 TNF-α Proteína citotóxica 4 asociada a linfocitos T Transportador 2 Manganeso superóxido dismutasa	Edad Sexo Consumo de alcohol Episodios de HA
NASH	Gen de la hemocromatosis hereditaria Angiotensinógeno TGF-β1	Edad Obesidad Diabetes mellitus
Cirrosis biliar primaria	IL-1β TNF-α Apolipoproteína E	

Figura 5. Tinción con Sirius Red en una biopsia hepática de un paciente con cirrosis secundaria a enfermedad hepática alcohólica. La tinción por Sirius Red tiñe de rojo las fibras de colágeno.



Entre los probemas asociados al diagnóstico del grado de fibrosis mediante biopsia hepática estan: la obtención de la biopsia (que se realiza mediante una técnica invasiva y puede causar dolor y/o complicaciones clínicas), la variabilidad intra e inter-observador a la hora de establecer el diagnóstico (lo cual dificulta la objetividad del resultado) y el error de muestreo, que se refiere a que la biopsia puede no ser representativa del resto del hígado (22). Se han propuesto diversos métodos no invasivos para determinar el estadio de fibrosis como tests formulados a partir de análisis séricos rutinarios (tiempo de protrombina, aminotransferasas, plaquetas) (23;24) o el análisis de la concentración en sangre de proteínas implicadas en el proceso de fibrosis como el inhibidor de metaloproteasas (TIMP) 1 o el ácido hialurónico (25). Sin embargo, aunque estos métodos son capaces de discernir entre una fibrosis leve y una avanzada son poco precisos para el caso de estadios intermedios. También se pueden utilizar técnicas de imagen como la elastografía de transición (Fibroscan®), la ultrasonografía, la tomografía computerizada o la resonancia magnética (26;27).

El patrón de acumulación de MEC depende de la etiología. Así, en la enfermedad hepática alcohólica existe más fibrosis en la zona del sinusoide hepático; en la esteatohepatitis no alcohólica en la zona de la vena central y en la cirrosis biliar primaria o en HVC en la zona periportal (28;29).

1.2.2. Bases moleculares.

A nivel molecular, la fibrosis está regulada tanto por los propios componentes de la MEC como por mediadores solubles. Se sabe que la MEC no es inerte y que componentes de la MEC como el colágeno tipo IV y el fibrinógeno estimulan la actividad de los miofibroblastos activando citocinas latentes como el TGF-β (30). Además, los colágenos fibrilares pueden estimular la activación de las HSC mediante su unión a integrinas de membrana o a receptores con dominio discoidina. Por otro lado, la MEC sirve como reservorio de mediadores involucrados en fibrosis como el TGF-β, el factor de crecimiento derivado de plaquetas (PDGF) y las metaloproteasas (MMP). Con respecto a los mediadores solubles, se ha identificado una larga lista de moléculas que influyen en la fibrosis hepática, algunos de ellos se muestran en la Tabla 5. Entre estos mediadores son especialmente importantes el TGF-β y el PDGF. El TGF-β, producido sobre todo por los macrófagos, es la citocina profibrogénica por excelencia debido a su habilidad para estimular la síntesis de proteínas de la MEC. En el hígado, se ha demostrado que los ratones transgénicos que sobreexpresan TGF-β tienen una mayor respuesta fibrogénica que los ratones salvajes (31). Por otro lado, la inhibición de la ruta de señalización del TGF-β, causa una reducción de la respuesta fibrogénica en modelos animales de fibrosis hepática (32). El PDGF, por su parte, está producido principalmente por las células de Kupffer y es un potente mitógeno para las HSC, lo que constituye su mayor contribución al desarrollo de fibrosis hepática (33). En un estudio reciente, se ha observado que los ratones transgénicos con una expresión aumentada de PDGF-A presentan fibrosis hepática espontánea (34).

Tabla 5. Principales mediadores implicados en la fibrosis hepática.

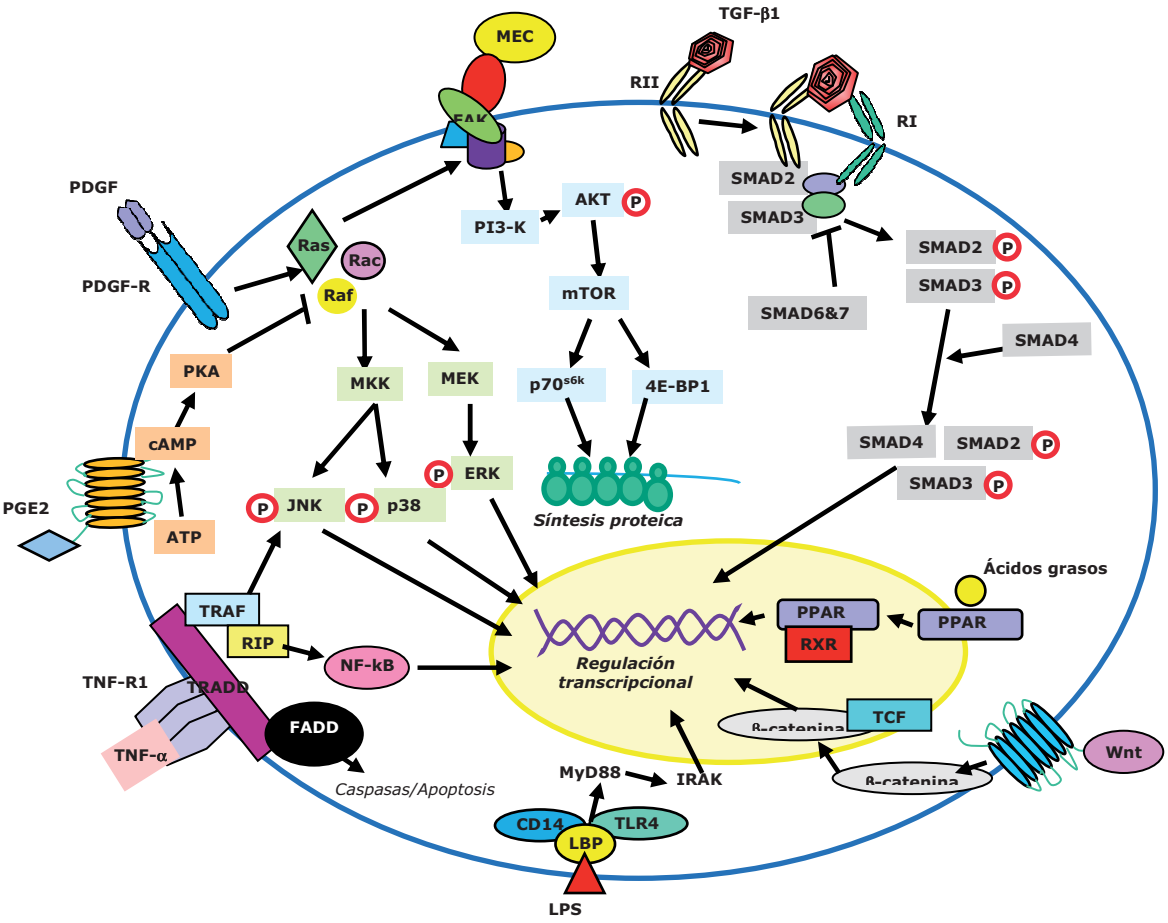
Grupo	Mediador	Efecto (cita)
Citocinas	MCP-1	Profibrogénico (35)
	RANTES	Profibrogénico (36)
	IL-1	Profibrogénico (37)
	IL-10	Antifibrogénico (38)
	IFN-γ	Antifibrogénico (39)
Factores de crecimiento	TGF-β	Profibrogénico (40)
	PDGF	Profibrogénico (41)
	CTGF	Profibrogénico (42)
	VEGF	Profibrogénico (43)
	FGF 1 y 2	Profibrogénico (44)
	HGF	Antifibrogénico (45)
	IGF-1	Antifibrogénico (46)
Sustancias vasoactivas	Ang II (receptor 1)	Profibrogénico (47)
	Ang II (receptor 2)	Antifirogénico (48)
	ET-1 (receptor A)	Profibrogénico (49)
	Norepinefrina	Profibrogénico (50)
	Relaxina	Antifibrogénico (51)
	Prostaglandina E2	Antifibrogénico (52)
	Adrenomedulina	Antifibrogénico (53)
	Óxido nítrico	Antifirogénico (54)
Adipocitocinas	Leptina	Profibrogénico (55)
	Adiponectina	Antifibrogénico (56)
Otros	Serotonina	Profibrogénico (57)
	Endocannabinoides (CB2)	Antifibrogénico (58)
	Endocannabinoides (CB1)	Profibrogénico (59)

1.2.3. Vías de señalización intracelular.

Las moléculas implicadas en la fibrosis hepática activan diferentes vías de señalización intracelular en los diferentes tipos celulares. La quinasa de adhesión focal/fosfatidilinositol-3-quinasa/Akt (FAK/PI3K/Akt) media diversas acciones fibrogénicas en las HSC incluyendo proliferación, quimiotaxis y la transcripción de genes profibrogénicos (60;61). Esta vía se activa mediante factores de crecimiento con receptores tirosina quinasa (como el PDGF), quimiocinas como la MCP-1, integrinas, ligandos de receptores acoplados a proteína G (como la Ang II) y adipocinas como la leptina. La activación de Akt mediante fosforilación induce la actividad de la diana de mamíferos para la rapamicina (mTOR) y esto finalmente lleva a la síntesis de proteínas y a la estimulación de crecimiento celular. La vía de las proteínas quinasas activadas por mitógenos (MAPKs) incluye las quinasas reguladas por señales extracelulares (ERKs), las quinasas N-terminales de c-Jun (JNKs) y la p38. La activación de las MAPK se produce por diversos mediadores de la fibrosis como factores de crecimiento o péptidos vasoactivos. En HSC, regulan los procesos de proliferación, secreción de quimiocinas, migración y síntesis de colágeno (62). La vía de Smad consiste en moléculas efectoras y sus inhibidores. Es la principal vía de señalización del TGF-β y promueve acciones profibrogénicas en las HSC (63). El factor nuclear κB (NF-κB) es un factor de transcripción que modula las acciones proinflamatorias del TNF-α y otras moléculas inflamatorias como la Ang II (64). Las HSC activadas tienen esta vía permanentemente activada y por tanto, expresan de manera constitutiva genes como la IL-6 o la molécula de adhesión intercelular (ICAM)-1. Otra vía de señalización es la de los receptores nucleares. Son receptores de sustancias liposolubles como los ácidos grasos o los eicosanoides, que se encuentran directamente en el núcleo celular. Cuando se une su ligando funcionan como un factor de transcripción. En el hígado son importantes los receptores activados por proliferación peroxisomal (PPARs). La activación del PPAR-γ reduce las actividades proinflamatorias y profibrogénicas de las HSC (65). La vía de La Wnt/β-

catenina también señaliza para citocinas y promueve La inflamación y fibrosis en el hígado (66). Los receptores *Toll-like* (TLR) reconocen patrones moleculares asociados a patógenos como el lipopolisacárido (LPS). Su activación induce inflamación y fibrosis (67). La vía de JAK/STAT se refiere a las quinasas Janus que se unen a receptores acoplados a protreína G o receptores tirosina quinasa. Cuando se unen forman sitios de unión para las proteínas transductoras de señales y activadoras de transcripción (STATs). Una vez activadas, las STATs dimerizan y entran al núcleo donde regulan la transcripción génica. Esta vía participa en el daño, la inflamación y la inhibición de la regeneración hepática (68). Otra vía es la activada por el TNF-α y otros miembros de la superfamilia del TNF. Cuando el TNF-α se une a su receptor se activa una cascada de vías intracelulares que regulan la transcripción de genes implicados en inflamación (JNK y NF-κB). La vía de la quinasa activada por adenosín monofosfato (AMP), en cambio, produce la activación de vias catabólicas (productoras de adenosín trifosfato, ATP) y la inactivación de vias que consumen ATP. De esa manera, inhibe la proliferación, migración, secreción de quimiocinas y producción de colágeno por parte de las HSC (69). La Figura 6 muestra un esquema representativo de las vías intracelulares de las HSC más importantes implicadas en fibrosis hepática.

Figura 6. Vías intracelulares implicadas en las acciones fibrogénicas de las las células estrelladas hepáticas (HSC). La mayoría de moléculas que influyen en la respuesta fibrogénica del hígado se unen a sus receptores en las HSC, lo que conlleva la activación de diferentes vías de señalización. Las HSC procesan estas señales y generan una respuesta a partir de la modificación de la expresión génica o proteica de moléculas implicadas en fibrosis como el colágeno.



Adaptado de Moreno M (62)

1.2.4. Bases celulares.

Tras un daño agudo en el hígado se desencadena una respuesta inflamatoria con la finalidad de reparar el tejido dañado. Durante este proceso, se produce un pequeño depósito transitorio de MEC de manera simultánea a la regeneración hepatocelular. Si el daño persiste, los mecanismos reparadores del hígado no son suficientes y la capacidad de regeneración de los hepatocitos disminuye a la vez que se acumula un esceso de MEC. En el proceso de fibrosis no sólo ocurre un aumento en la cantidad de MEC (hasta 6 veces más de lo normal) sino también un cambio en su composición (se incrementa la proporción de colágenos I, III, IV y V, fibronectina, undulina, elastina, laminina, hialuronano y proteoglicanos) (70). Este acúmulo de MEC se produce debido tanto a un aumento en la síntesis de proteínas de MEC como a una disminución de su degradación por una menor actividad de las MMPs (71). Eventualmente, si el agente causante del daño se elimina, la fibrosis puede revertir. La figura 7 muestra un esquema detallado de la patogenia del proceso de fibrosis desde el inicio del daño hasta la resolución y los tipos celulares implicados más importantes.

De manera similar a lo que ocurre en la inflamación hepática, durante la fibrogénesis tiene lugar una compleja relación entre los diferentes tipos celulares. Cuando se dañan los hepatocitos, liberan especies reactivas de oxígeno a la vez que mediadores proinflamatorios y profibrogénicos. De este modo, reclutan células inflamatorias procedentes del torrente sanguíneo y activan a las células de Kupffer. Además, estimulan la activación de las HSC, las principales células productoras de MEC en el hígado (72). Por otro lado, los leucocitos reclutados contribuyen a esta activación (73). A su vez, las HSC activadas secretan quimiocinas y moléculas de adhesión e inducen la activación de las células inflamatorias (74). Así, se establece un ciclo en el que las células fibrogénicas y las inflamatorias se estimulan las unas a las otras.

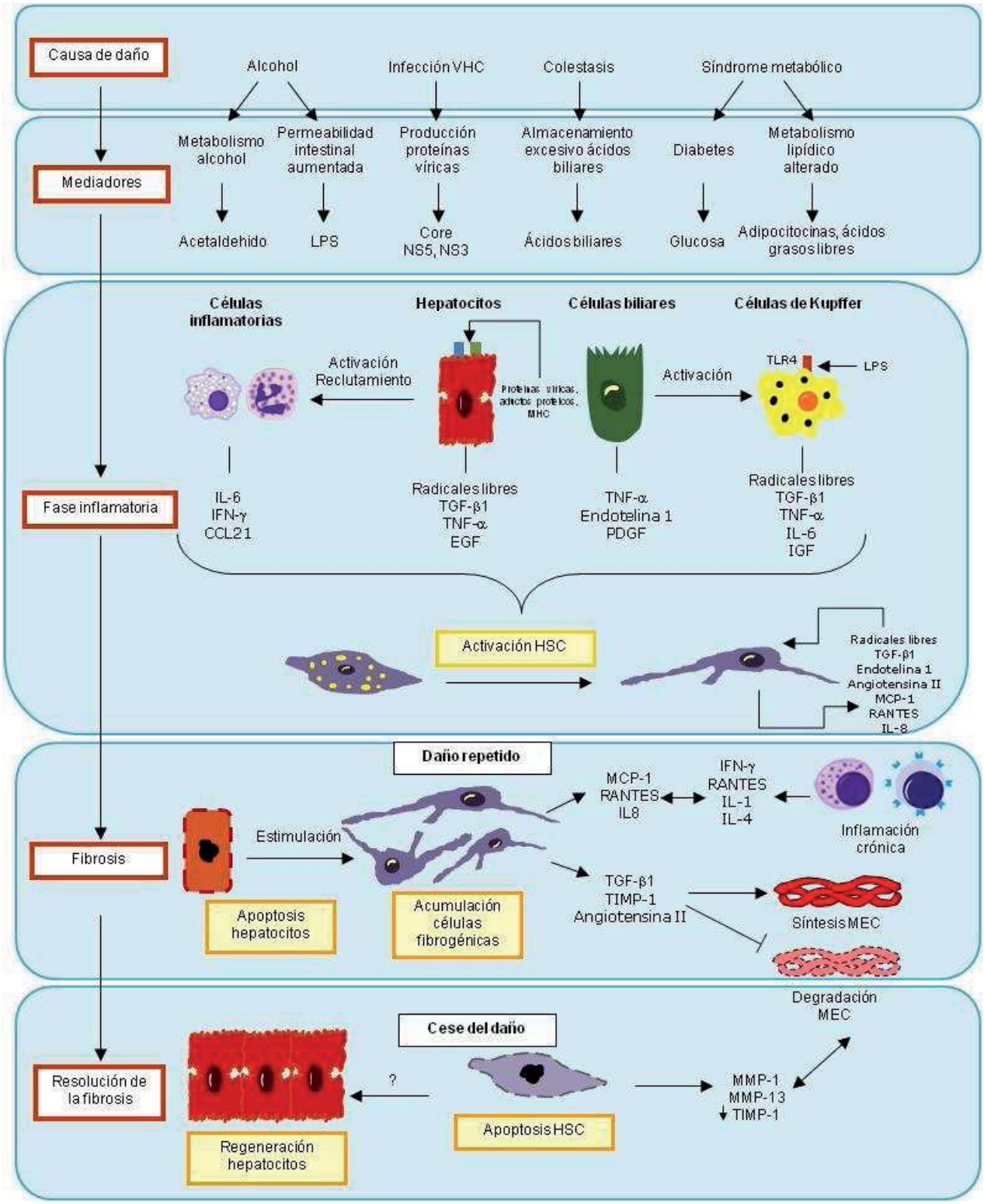
La etiología de la enfermedad determinará los mecanismos concretos que medien la fibrosis en cada caso. Por ejemplo, en la fibrosis por colestasis hay modestos signos de inflamación (75) mientras que en la fibrosis producida por tóxicos

(p. ej. alcohol) el componente inflamatorio es muy importante, sobre todo por parte de los neutrófilos. Además, en la enfermedad hepática alcohólica se produce una alteración en la flora bacteriana y una inhibición de la motilidad intestinal, resultando en un sobrecrecimiento de bacterias gram negativas. Por otro lado, se produce un incremento en la permeabilidad intestinal. Como consecuencia, la concentración de LPS (componente de la pared de bacterias gram negativas) aumenta en la circulación portal. Ésto conduce a la activación de las células de Kupffer. Por su parte, el acetaldehído, el principal producto del metabolismo del alcohol, induce un marcado estrés oxidativo en los hepatocitos y estimula la activación de las células de Kupffer y las HSC (76). Además, los radicales libres y las citocinas producidas inducen la activación y proliferación de las HSC.

En la patogenia de la fibrosis inducida por el VHC participan más activamente los linfocitos. El VHC infecta a los hepatocitos causando estrés oxidativo y el reclutamiento de células inflamatorias. Ambos factores inducen la activación de las HSC y al depósito de colágeno. Además, se ha demostrado que varias proteínas del VHC estimulan directamente las acciones inflamatorias y fibrogénicas de las HSC (77).

Para explicar la fibrosis inducida por esteatohepatitis no alcohólica se ha propuesto el modelo de los dos golpes (*two hits*) que consiste en un primer golpe en el que se produce esteatosis hepática, que en un principio es benigna pero sensibiliza al órgano a otras agresiones y un segundo golpe en el que hay un aumento de estrés oxidativo y citocinas proinflamatorias que promueven la apoptosis de hepatocitos y el reclutamiento de células inflamatorias.

Figura 7. Patogenia de la fibrosis hepática. Principales procesos, tipos celulares y mediadores implicados en el proceso de la fibrosis hepática.



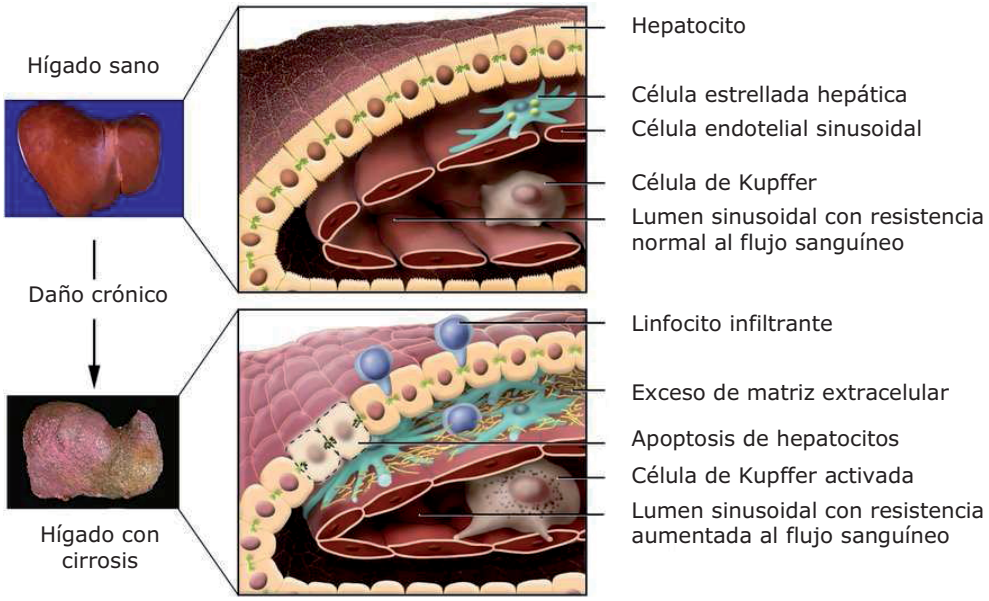
Adaptado de Bataller R (21)

Además de las relaciones intercelulares y los mecanismos patogénicos expuestos, durante el proceso de fibrosis ocurren una serie de cambios fenotípicos en todas las células del sinusoide hepático, debido a las relaciones paracrinas entre ellas, a cambios en la composición de la MEC o a los agentes causantes del daño hepático (alcohol, acetaldehído, LPS, proteínas virales, ácidos grasos, ácidos biliares, etcétera). Las HSC se activan, lo que conduce a su proliferación y al acúmulo excesivo de MEC. Esto contribuye a la pérdida de microvilli de los hepatocitos y la pérdida de fenestras en las células endoteliales. Todo ello resulta en un deterioro de la función hepática. Por otro lado se activan las células de Kupffer. La activación de las células de Kupffer se refiere a un aumento de la secreción de citocinas (factores de crecimiento, quimiocinas) así como un incremento en la producción de ROS, lo cual contribuye de manera paracrina a la activación de las HSC (21) (Figura 8).

Las células productoras de MEC son los miofibroblastos. Éstos provienen mayoritariamente de las HSC. Las HSC fueron descubiertas en el año 1876 por el anatomista alemán Karl Wilhelm von Kupffer. También son conocidas como lipocitos o células de Ito. Se han podido estudiar en detalle debido a la existencia de un método fácil de aislamiento a partir de hígado sano por métodos de gradiente de densidad aprovechando que su contenido lipídico les confiere una baja densidad. Una vez cultivadas en plástico y en presencia de suero, se activan de manera muy similar a lo que ocurre en el hígado durante el proceso de fibrosis adquiriendo propiedades de miofibroblasto. En el hígado sano, las HSC residen en el espacio de Disse y son el mayor almacén de vitamina A del organismo. Tras un daño en el hígado, en respuesta a citocinas como el TGF-β, el PDGF, el factor de crecimiento del tejido conectivo (CTGF) o el TNF-α procedentes de otras células residentes en el hígado o de células inflamatorias se activan adquiriendo propiedades contráctiles, proinflamatorias, proliferativas, migratorias y fibrogénicas (Figura 9). Pierden sus gránulos de vitamina A y migran hacia el lugar de inflamación donde secretaran grandes cantidades de proteínas de MEC (78). El proceso de activación de las HSC, es por tanto, un evento

clave en la patogenia de la fibrosis hepática. La expresión de α-actina, desmina y miosina son marcadores de activación de estas células.

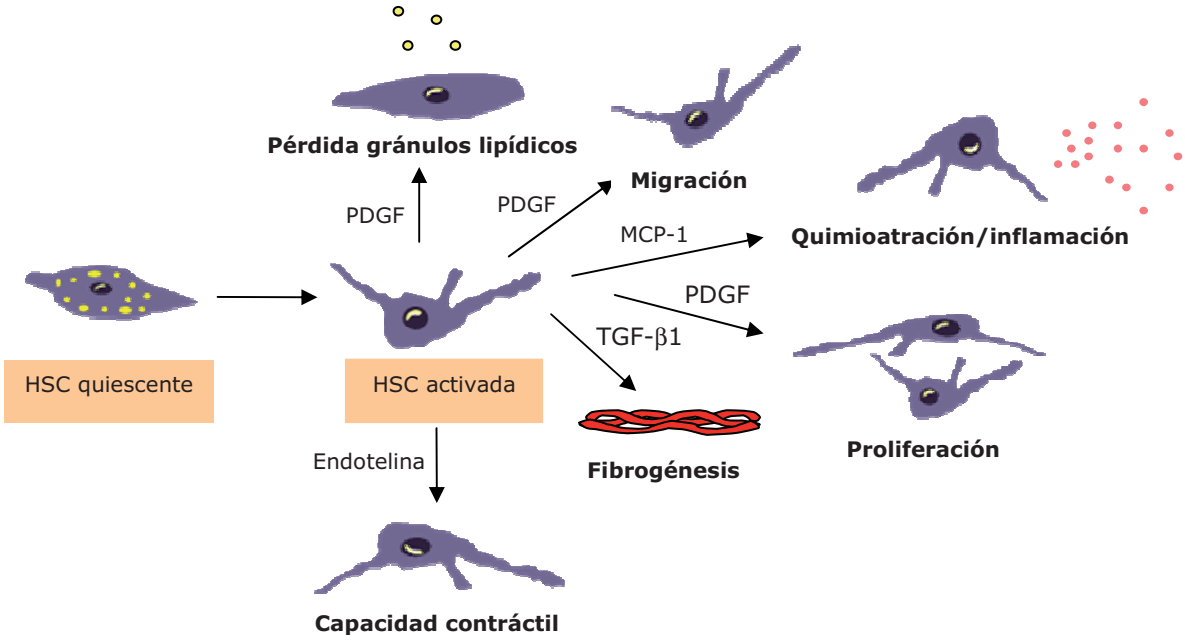
Figura 8. Cambios fenotípicos que ocurren en el sinusoide hepático durante la fibrosis. Se oberva muerte de los hepatocitos, infiltrado de leucocitos, activación de HSC células de Kupffer y una disminución de las fenestras del endotelio y del volumen del lumen sinusoidal. Además, un incremento en la deposición de matriz extracelular en el espacio de Disse.



Adaptado de Bataller R (21)

Hay otras fuentes de células profibrogénicas o miofibroblastos que participan en la fibrogénesis hepática como son los miofibroblastos portales, los miofibroblastos derivados de médula ósea (79;80) y los producidos a partir de la transición epitelio-mesenquimal de hepatocitos y células epiteliales biliares (81;82). La α-actina y el colágeno son los dos marcadores que reconocen toda la gama de miofibroblastos de cualquier origen.

Figura 9. Cambios fenotípicos derivados de la activación de las células estrelladas hepáticas. Tras su activación, las HSC pierden los gránulos lipídicos y adquieren propiedades migratorias, inflamatorias, proliferativas, fibrogénicas y contráctiles.



Las células del sistema inmunitario también participan en la respuesta fibrogénica. Con respecto a los neutrófilos, aunque se ha observado que son rápidamente reclutados a las zonas donde hay daño tisular, parece ser que no tienen ningún efecto sobre la fibrosis hepática según los resultados obtenidos a partir de modelos animales (83;84). Es posible que su papel sea más importante en la inflamación aguda que en la fibrosis. El papel de los macrófagos en la fibrosis es complejo. A través de estudios con animales, se sabe que los macrófagos juegan un papel profibrogénico o antifibrogénico dependiendo del estado de la fibrosis (en las fases inicales es profibrogénico y en la fase de reversión es antifibrogénico) (85-87). Por otro lado, las células NK inducen la muerte de las HSC activadas, y por tanto, ejercen un papel antifibrogénico en el hígado (88). Los linfocitos T están divididos en varios tipos. Los linfocitos T $CD8^+$ promueven la fibrosis mientras que los linfocitos T $CD4^+$ o T helper (Th), que colaboran en la respuesta inmunitaria adaptativa mediante la producción de citocinas, pueden ejercer un efecto profibogénico o antifibrogénico.

Según el patrón de citocinas que presentan los linfocitos $CD4^+$ se diferencian dos subtipos (Th1 y Th2). En general, las citocinas producidas por linfocitos Th1 promueven la inmunidad mediada por células e incluyen IFN-γ, TNF y IL-2. Estas citocinas inhiben la progresión de la fibrosis. Sin embargo, las producidas por células Th2 (IL-4, IL-5, IL-6 e IL-13) promueven la respuesta humoral y favorecen la progresión de la fibrosis. El papel de los linfocitos B en la fibrosis no está claro. En estudios experimentales se ha observado que ratones deficientes en células B muestran una fibrosis atenuada con respecto a ratones salvajes en un modelo tóxico de fibrosis (administración de tetracloruro de carbono) (89) mientras que muestran una fibrosis mayor en un modelo de esquistosomiasis (90).

1.2.5. Reversibilidad de la fibrosis.

La fibrosis hepática ha sido considerada tradicionalmente como un proceso pasivo e irreversible, consecuencia de la sustitución del parénquima hepático por tejido fibroso. Sin embargo, ahora se sabe que es un proceso potencialmente reversible, al menos en las primeras fases. Se ha observado una disminución de la fibrosis en varios tipos de enfermedad hepática tras la eliminación el agente causal, por ejemplo tras el drenaje biliar en el caso de obstrucción del conducto biliar (91) o tras el tratamiento antiviral en el caso de HVC (92). También se ha demostrado que la fibrosis es un proceso reversible en modelos animales, lo que permite investigar este fenómeno biológico. En la mayoría de modelos animales, la reversión de la fibrosis es fácil y rápida; sin embargo, hay que tener en cuenta que en humanos puede durar varios meses, dependiendo de la etiología y de otros factores. Este hallazgo ha estimulado a los investigadores a buscar maneras de revertir la fibrosis.

Los mecanismos involcucrasos en la resolución de la fibrosis son básicamente dos: la apoptosis de las células estrelladas hepáticas y la modificación del balance entre MMPs y TIMPs. En el hígado con fibrosis o cirrótico hay un aumento de la expresión de MMPs que degradan la matriz extracelular. Sin embargo, su actividad se

ve inhibida por el aumento desproporcionado de la actividad de sus inhibidores. Durante la resolución de la fibrosis hay una disminución de la expresión TIMPs de manera que las MMPs (sobre todo la 1, la 8 y la 13) pueden degradar el exceso de MEC. Por otro lado, las HSC reciben señales de supervivencia mediante el contacto con componentes de la MEC a través de integrinas de membrana (93) o de receptores con dominio discoidina (DDR) (94). Cuando empieza a degradarse la MEC por el efecto de las metaloproteasas las HSC pierden estas señales de supervivencia, lo que contribuye a la regresión de la fibrosis. El retroceso de la inflamación, también contribuye a la muerte de las HSC activadas (95).

1.3. MODELOS ANIMALES DE INFLAMACIÓN Y FIBROSIS HEPÁTICA.

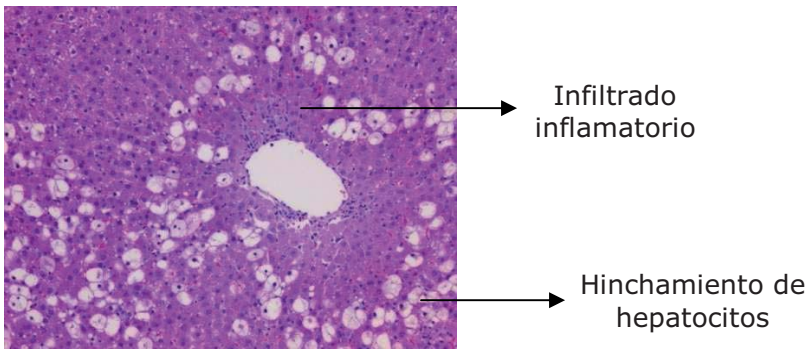
El objetivo de los modelos animales de inflamación o fibrosis hepática es el de entender mejor la fisiopatología de las enfermedades del hígado y determinar la relevancia de nuevas moléculas que puedan estar implicadas en el daño hepático o la efectividad de nuevos posibles tratamientos. Debido a la amplia variedad de enfermedades hepáticas que producen inflamación o fibrosis y ya que no hay ningún modelo animal que reproduzca exactamente lo que ocurre en humanos, existen numerosos modelos animales complementarios. La mayoría de ellos se desarrollan en especies de roedores (sobre todo rata y ratón) aunque también se realizan estudios en otras especies como pez zebra, chimpancé, perro o cerdo. Por otro lado, para determinar la importancia de ciertas moléculas en el proceso de inflamación hepática (o en el proceso de fibrosis) se recurre cada vez más a animales modificados genéticamente y tecnologías como la del ácido ribonucleico interferencia (RNAi) o los adenovirus que permiten la manipulación de la expresión de un gen determinado. Los modelos de inflamación y fibrosis hepática más utilizados, son la administración de tetracloruro de carbono (CCl_4) y la ligadura de conducto biliar.

1.3.1. Administración de tetracloruro de carbono.

El CCl_4 es uno de los hepatotóxicos más utilizados en hepatología experimental. Es un compuesto químico que se utilizó durante años como extintor de fuego, refrigerador y solvente de pinturas entre otros usos pero debido a su toxicidad se dejó de utilizar hace aproximadamente 40 años. Su toxicidad ha sido demostrada en hígado, riñones y sistema nervioso (96), siendo el hígado el órgano más afectado. El CCl_4 se metaboliza en el hígado mediante el citocromo P450 dando lugar al radical libre triclorometil y a especies reactivas de oxígeno que inician la peroxidación de lípidos y proteínas de la membrana plasmática de los hepatocitos, lo que causa su muerte. Se sabe, además, que la administración de CCl_4 disminuye la actividad de moléculas

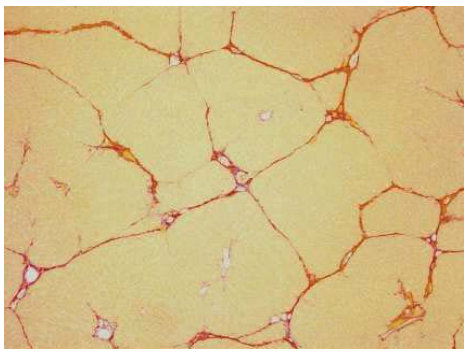
antioxidantes en el hígado como el glutatión, la superóxido dismutasa y la catalasa (97). Administrado de manera puntual causa un daño agudo en el hígado en sólo unas horas, caracterizado por un aumento en el infiltrado inflamatorio, muerte de hepatocitos (evidenciada por una elevada concentración de transaminasas en sangre), hinchamiento de hepatocitos y estrés oxidativo (Figura 10). Se suele administrar por vía intraperitoneal, aunque también puede administrarse por vía subcutánea u oral.

Figura 10. Tinción de hematoxilina-eosina que muestra los daños histológicos causados por la administración aguda de tetracloruro de carbono en rata (24 horas).



El CCl_4 administrado de manera crónica es probablemente el modelo de fibrosis hepática más utilizado y mejor caracterizado. Es un modelo muy reproducible y predecible. La administración suele ser dos veces por semana y puede ser por vía subcutánea, intraperitoneal o inhalatoria. Se puede acompañar de tratamiento con fenobarbital (en el agua de bebida) para inducir la activación del citocromo P450 y acelerar así la metabolización del CCl_4, y por tanto, el proceso de fibrosis. El desarrollo de fibrosis en puentes aparece entre 4 y 6 semanas tras el inicio del tratamiento y a las 12 semanas ya se puede observar una cirrosis avanzada micronodular (98). Características de este modelo son el estrés oxidativo, la necrosis y la fibrosis centrolobulillar (Figura 11).

Figura 11. Tinción con Sirius Red en una muestra de tejido de rata sometida a administración crónica de tetracloruro de carbono (8 semanas).



1.3.2. Ligadura del conducto biliar.

La ligadura del conducto biliar, se utiliza como modelo de fibrosis biliar secundaria. Este modelo consiste en una maniobra quirúrgica en la que se expone el conducto biliar y se realizan una serie de ligaduras (2 o 3) a lo largo del conducto biliar con el objetivo de bloquear el paso de la bilis (Figura 12). Se puede realizar un corte entre las ligaduras para asegurar que toda la bilis producida se quedará retenida en el hígado. El resultado es que los ácidos biliares se acumulan en el hígado produciendo inflamación y fibrosis. Histológicamente se caracteriza por fibrosis portal y proliferación de nuevos conductos biliares. Se observa fibrosis en puentes aproximadamente a las 2 semanas tras la ligadura y cirrosis en unas 5 o 6 semanas (Figura 13).

Figura 12. Representación esquemática de la ligadura del conducto biliar en la rata.

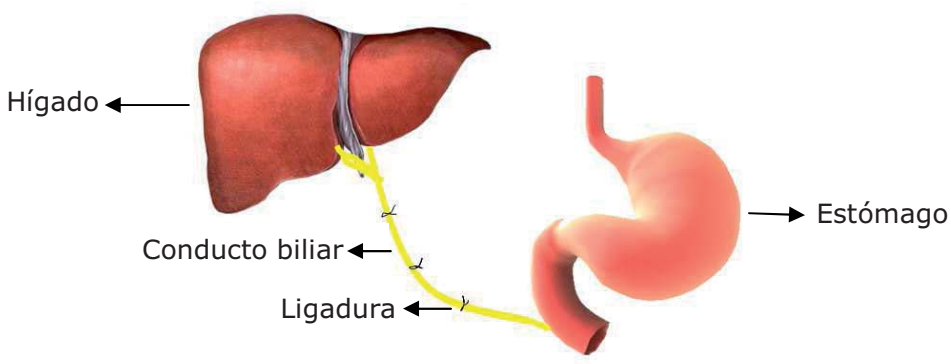
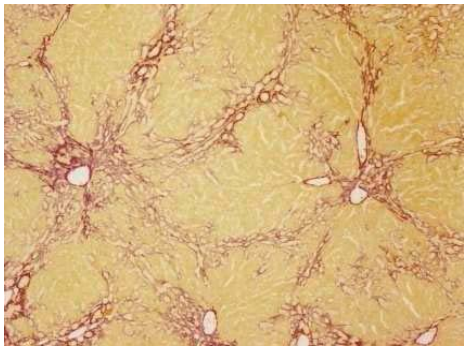


Figura 13. Tinción con Sirius Red en una muestra de tejido de rata sometida a ligadura del conducto biliar (2 semanas).



1.3.3. Otros modelos de daño hepático.

Existen otros modelos utilizados para estudiar la inflamación o la fibrosis hepática. Los modelos más importantes con los que se trabaja son modelos en los que se administran tóxicos (paracetamol o acetaminofén, tioacetamida, etanol), modelos de activación del sistema inmunitario (concanavalina A), modelos en los que se induce apoptosis hepatocitaria (D-galactosamina/LPS, Fas), modelos de enfermedad del hígado graso no alcohólica (dieta cafetería, rica en grasas, dieta deficiente en colina y metionina) y modelos quirúrgicos (de resección hepática, de isquemia-reperfusión, de transplante hepático).

Además, existen otros modelos menos frecuentes y más específicos como por ejemplo modelos animales para la hepatitis C como el de chimpancé, en el que se infectan los animales con el VHC (99) o ratones transgénicos que expresan proteínas del VHC (100). Otros modelos específicos para una enfermedad son los de esquistosomiasis en ratones o ratas. Consisten en una inyección endovenosa de huevos de alguna de las especies de Schistosoma o inyección percutánea de cercaria (larvas) (101).

1.4. TRATAMIENTO DE LA INFLAMACIÓN Y LA FIBROSIS HEPÁTICA.

1.4.1. Generalidades.

Actualmente no hay ningún tratamiento bien establecido para la inflamación o la fibrosis hepática y por tanto, la mejor estrategia terapéutica para los pacientes con enfermedades hepáticas es eliminar el agente causante del daño (ingesta de alcohol, virus, etcétera). Sin embargo esto no siempre es posible, como por ejemplo en los pacientes que no responden al tratamiento antiviral. En los pacientes con cirrosis avanzada, el tratamiento de elección es el trasplante hepático, pero es una solución muy limitada debido al bajo número de donantes. Además, en pacientes con cirrosis por virus C, el hígado trasplantado se reinfecta rápidamente y vuelve a aparecer fibrosis y cirrosis en cuestión de poco tiempo (102).

Existen numerosos estudios experimentales en los que se demuestra la efectividad de diferentes tratamientos tanto para el daño hepático agudo como para la fibrosis. Sin embargo, hay dificultad para realizar ensayos clínicos en pacientes, especialmente en el ámbito de la fibrosis hepática, debido a una serie de limitaciones. Primero, se necesita realizar biopsias seriadas para determinar los cambios histológicos que se producen con el tratamiento, esto es éticamente incorrecto por los perjuicios que conlleva la obtención de la biopsia hepática. Segundo, la necesidad de realizar estudios muy largos (años), ya que la fibrosis es un proceso crónico y no se puede esperar que su reversión sea un proceso rápido. Finalmente, muchos posibles tratamientos que se investigan a nivel experimental no se pueden utilizar en la práctica clínica debido a la falta de estudios de seguridad o a posibles efectos secundarios.

Una consideración importante a tener en cuenta es que frecuentemente los tratamientos que se prueban en animales son preventivos, puesto que se administran desde el inicio del daño hepático o incluso antes. Sin embargo, los pacientes que

necesitan un tratamiento ya tienen una fibrosis establecida y necesitan un tratamiento curativo. Es por ello que los tratamientos que funcionan en animales en modo preventivo se deben probar en modo curativo antes de pasar a estudiarlos en ensayos clínicos.

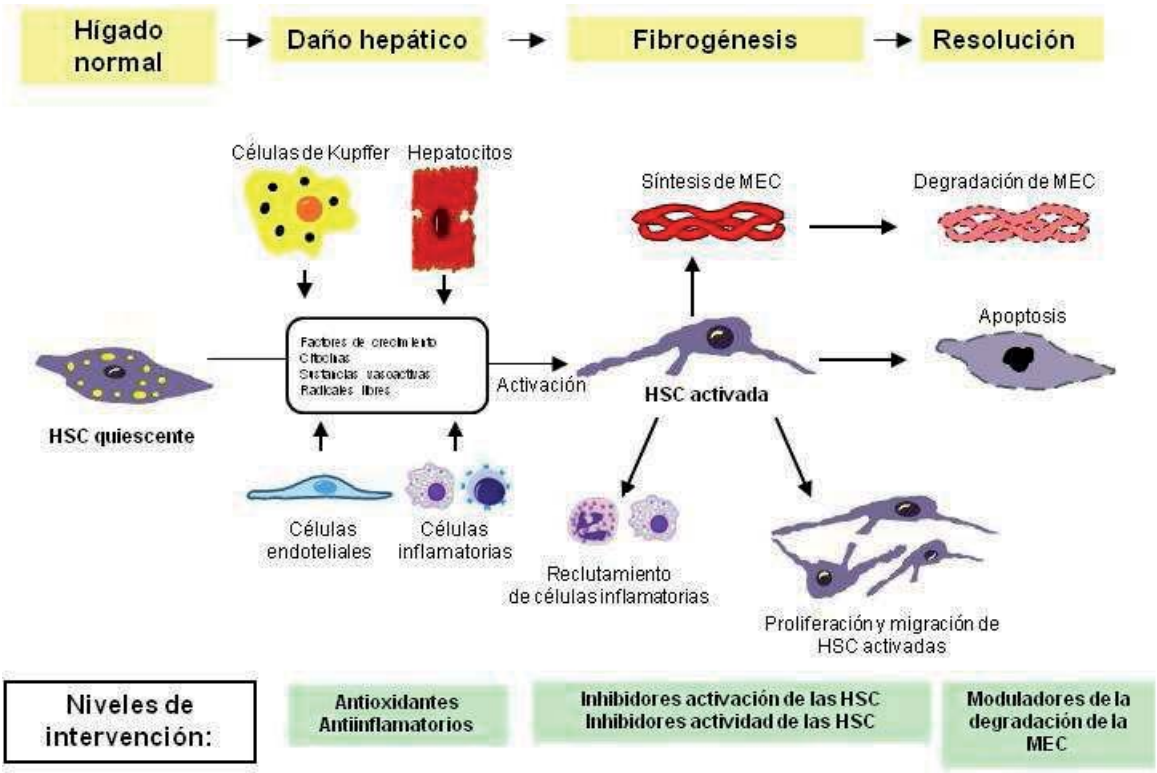
Actualmente se están investigando algunos tratamientos dirigidos a un tipo celular concreto mediante moléculas transportadoras específicas, que pueden ser una gran ventaja para los fármacos que puedan causar efectos secundarios por sus acciones a nivel sistémico. Por ejemplo, en un estudio reciente de nuestro grupo se administró losartan unido a un transportador que lo llevó hasta las HSC y se demostró un efecto antifibrogénico a una dosis de losartan mucho menor de lo que hubiera sido necesario con una administración de losartan convencional (103). Sin embargo este tipo de estudios está todavía en fase de experimentación.

Las características ideales de un fármaco antifibrogénico son un bajo coste, un buen perfil de seguridad para tratamientos prolongados y un escaso potencial hepatotóxico.

1.4.2. Niveles de intervención.

Dado que la inflamación o el daño hepático y la fibrosis son dos procesos estrechamente relacionados, muchos de los tratamientos que se han propuesto para reducir la fibrosis hepática, son también hepatoprotectores. Aunque la mayoría de los tratamientos propuestos pueden disminuir la fibrosis mediante más de un mecanismo, las diferentes estrategias para inhibir la fibrosis se podrían resumir de la siguiente manera: tratamientos antiinflamatorios, tratamientos antioxidantes, inhibidores de la activación de las HSC, inhibidores de la actividad de las HSC activadas y tratamientos que promueven la degradación de la MEC (Figura 14).

Figura 14. Niveles de intervención en el tratamiento de la fibrosis hepática.



1.4.3. Potenciales tratamientos farmacológicos.

• **Antiinflamatorios.** Debido a que la inflamación hepática precede a la fibrosis, varios tratamientos antiinflamatorios se han propuesto para reducir la fibrosis hepática. De entre ellos, los corticosteroides están indicados para la hepatitis aguda alcohólica (no hay evidencias de que mejore la fibrosis pero sí la supervivencia) (104) y se ha demostrado que disminuyen la fibrosis en pacientes con hepatitis autoinmune (105). Por otro lado, la administración de la citocina inmunomoduladora IL-10 a pacientes con HVC no respondedores a la terapia antiviral también disminuye la fibrosis (106).

• **Antioxidantes.** Debido a que en la mayoría de enfermedades del hígado se observa un aumento de estrés oxidativo, y éste promueve la inflamación y la fibrosis, la

administración de antioxidantes se ha propuesto como tratamiento para las enfermedades hepáticas. Así, antioxidantes como la vitamina E (107), la S-adenosil metionina (108) o la silimarina (109) (derivada del *Silybum marianum*) han mostrado un efecto antifibrogénico en modelos animales. Aunque se han realizado algunos ensayos clínicos que muestran un efecto beneficioso en pacientes con hepatopatías crónicas, son necesarios más estudios para determinar su efecto antifibrogénico (110). En este grupo de tratamientos se encuentra el Sho-saiko-to, que se utiliza en la medicina tradicional oriental para el tratamiento de la hepatitis crónica. En modelos animales de fibrosis hepática, se ha demostrado que tiene propiedades antifibrogénicas y antioxidantes (111). De la misma manera, el extracto de la planta *Salvia miltiorrhiza* tiene propiedades antioxidantes y se ha demostrado que tiene efectos beneficiosos en modelos animales de fibrosis hepática (112). Además, inhibe la proliferación y promueve la apoptosis de las HSC en cultivo (113). Finalmente, la curcumina es un polifenol aislado de la cúrcuma, especia obtenida de la planta *Curcuma longa* y tiene efectos antiinflamatorios y antioxidantes. En HSC inhibe la síntesis de α-actina y colágeno (114;115) y en modelos animales de fibrosis hepática muestra un efecto antifibrogénico (116). Es una opción prometedora, ya que actualmente se está estudiando en fase clínica II para otra indicación (pacientes con cáncer) y se ha observado que es bien tolerada (117).

• **Inhibidores de la activación de las HSC.** Como se ha explicado anteriormente, la activación de las HSC es un evento clave para la fibrosis hepática. Por ese motivo, se han investigado moléculas capaces de inhibir este paso. En este grupo se incluyen los ligandos del PPAR-γ como la rosiglitazona. Se ha visto que ejercen efectos beneficiosos tanto en modelos animales de fibrosis hepática (118) como en pacientes con esteatohepatitis no alcohólica (119). El IFN-γ, también inhibe la activación de las HSC en cultivo (120) y es antifibrogénico en modelos animales (121). Además, varios estudios sugieren un papel antifibrogénico del IFN-γ en pacientes con HVC independientemente de su eficacia antiviral (122).

• **Inhibidores de la actividad fibrogénica de las HSC activadas.** Debido a que la actividad de las HSC es fundamental en la fibrosis hepática, su modulación es otra estrategia a seguir en el tratamiento de la fibrosis hepática. El PDGF-BB, a través de su receptor tirosina-quinasa, es un potente estimulador de la proliferación de las HSC activadas. La inhibición de los receptores tirosina-quinasa es una de las estrategias que ha resultado eficiente en la reducción de la fibrosis hepática experimental (123). Además, existe un inhibidor tirosina-quinasa disponible (Imanitib) que se utiliza de manera segura en pacientes oncológicos (124). En este grupo de tratamientos se incluyen también inhibidores del TGF-β. Como se ha explicado anteriormente, el TGF-β es la citocina más importante que promueve la producción de colágeno por parte de las HSC y se ha observado que el bloqueo de la activación del TGF-β atenúa la fibrosis hepática experimental (125). Sin embargo, en humanos no se ha hecho ningún estudio y podría tener efectos secundarios indeseables. El SRA es un estimulador de las propiedades de las HSC activadas, como por ejemplo la contractilidad. La inhibición del SRA es una de las estrategias más prometedoras para el tratamiento de la fibrosis hepática. Los fármacos que interfieren en la síntesis y acción de la Ang II (inhibidores del enzima convertidor de Ang II y/o antagonistas de los receptores AT1 de la Ang II) tienen un efecto antifibrogénico en pacientes con enfermedades crónicas renales o cardíacas (126;127). A pesar de la evidencia antifibrogénica de estos fármacos, muy estudiada en modelos animales (128;129), existen pocos estudios en pacientes con hepatopatías. Estudios preliminares sugieren una mejora en la progresión de la fibrosis en pacientes con NASH y HVC (130-132). Actualmente se están realizando estudios clínicos para determinar si estos tratamientos son recomendables en la práctica clínica rutinaria. Por otro lado, el factor de crecimiento de hepatocitos (HGF), además de inhibir la muerte de los hepatocitos, inhibe la expresión de TGF-β y la fibrosis hepática experimental (133) aunque como tratamiento a largo plazo en humanos, podría conducir a carcinoma hepatocelular. Otro de los fármacos que se podría englobar en este grupo es la pentoxifilina, que se ha utilizado en el tratamiento de la HA (134). Se ha visto que disminuye la

proliferación y producción de colágeno por parte de fibroblastos *in vitro* y que disminuye el depósito de colágeno un en ratas sometidas a fibrosis hepática experimental (135).

• **Degradación de la MEC.** El uso de compuestos que inhiben de forma directa la síntesis de colágeno o favorecen su degradación es otra estrategia a considerar en la fibrosis hepática. Dentro de este grupo, se encuentra la halofuginona. Es un alcaloide inicialmente aislado de la planta *Dichroa febrifuga*. Se ha observado que disminuye específicamente la síntesis de colágeno α1(I) en fibroblastos de la piel. En modelos animales se ha visto que previene y cura la fibrosis hepática experimental. Probablemente, al inhibir la síntesis de colágeno α1(I) afecta a la viabilidad de las HSC y acelera la degradación de la MEC. Por otro lado, se ha observado que la metaloproteasa 8 y el activador de plasminógeno tipo uroquinasa estimulan la degradación de colágeno *in vivo*. Por su parte, la relaxina, una hormona estudiada inicialmente por su efecto durante el embarazo y la preparación del parto (inhibe la síntesis de colágeno y estimula la degradación de colágenos y proteoglicanos), tiene un efecto antifibrogénico tanto *in vitro* como en fibrosis hepática experimental (136). La eficacia de estos compuestos en humanos es desconocida y pueden tener efectos secundarios importantes (137).

• **Otros.** Hay otras moléculas que tienen efectos beneficiosos sobre la fibrosis hepática y no se pueden englobar en ninguna de las categorías anteriores. El ácido ursodeoxicólico es un ácido biliar citoprotector en los hepatocitos que reduce la inflamación aunque no se ha determinado que disminuya la fibrosis (138). Se ha estudiado principalmente en la cirrosis biliar primaria pero los resultados no son consistentes (139). Otra de las moléculas de este grupo es la gliotoxina, que es una toxina fúngica. Induce apoptosis en células del sistema inmunitario y en las HSC. Así, se ha visto que disminuye la fibrosis hepática en modelos animales (140;141). Finalmente, la colchicina es un alcaloide que inhibe la polimerización de microtúbulos, un proceso necesario para la secreción de colágeno. Aunque en modelos animales

reduce la fibrosis hepática, se han hecho varios estudios clínicos en los que no se observa que mejore la fibrosis. Sin embargo, sí mejora la supervivencia y marcadores de daño hepático.

La Tabla 6 muestra un resumen de los tratamientos, efectivos en modelos animales, que más se han estudiado.

Tabla 6. Resumen de posibles tratamientos farmacológicos para la fibrosis hepática.

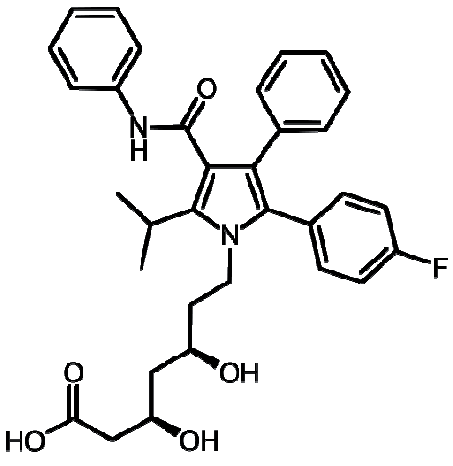
Grupo	Tratamiento	Estudios en pacientes
Antiinflamatorios	Corticosteroides	Estudios favorables
	IL-10	Estudio favorable (HVC)
Antioxidantes	Vitamina E	Pocos estudios
	S-adenosil metionina	Pocos estudios
	Silimarina	Pocos estudios
	Sho-saiko-to	Pocos estudios
	Curcumina	No hay estudios
Inhibición activación HSC	Ligandos PPAR-γ	Estudios favorables (NASH)
	IFN-γ 1b	Estudios favorables
Inhibición actividad HSC	Inhibición TGF-β	No hay estudios
	Inhibición SRA	Estudios favorables
	HGF	No hay estudios
Degradación MEC	Halofuginona	No hay estudios
	MMP-8	No hay estudios
	Relaxina	No hay estudios
Otros	Ácido ursodeoxicólico	No consistentes (CBP)
	Gliotoxina	No hay estudios
	Colchicina	Estudios contradictorios

1.5. ESTATINAS: LA ATORVASTATINA.

1.5.1. Introducción.

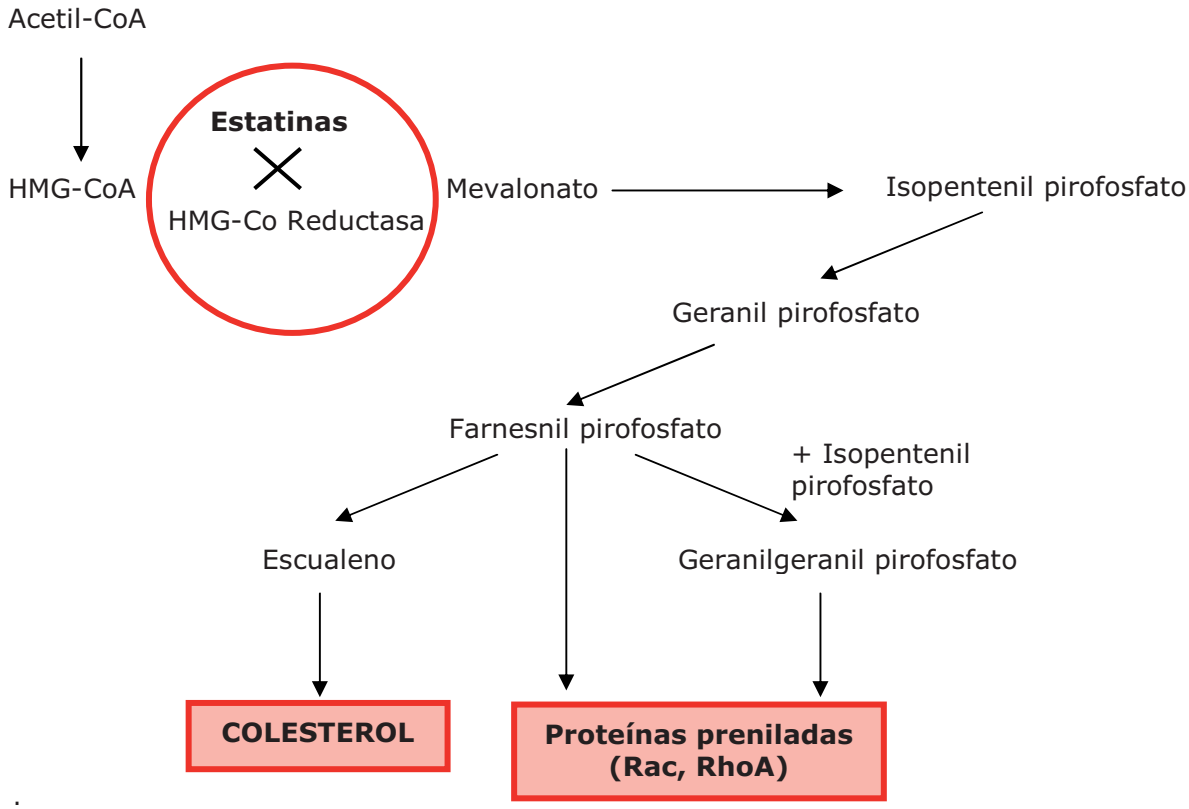
La atorvastatina es un fármaco de la familia de las estatinas. Las estatinas son el grupo de medicamentos más prescrito a nivel mundial (142). Estan fundamentalmente indicadas para el tratamiento de la hipercolesterolemia y se ha demostrado que son efectivas en la prevención de la enfermedad coronaria. Su principal mecanismo de acción es la inhibición de la síntesis de colesterol mediante el bloqueo de la 3-hidroxi-3-metilglutaril-coenzima A reductasa (HMG-CoA reductasa). Esta enzima cataliza la conversión del HMG-CoA a mevalonato, que es la reacción limitante en la ruta de biosíntesis de colesterol (143). Existen varias estatinas, algunas de ellas derivadas de fermentos fúngicos (lovastatina, simvastatina y pravastatina). Los hongos las utilizan para defenderse de microorganismos que necesitan derivados del mevalonato para mantener sus paredes celulares en buen estado. Otras son totalmente sintéticas (atorvastatina, fluvastatina, rosuvastatina). La estructura química de la atorvastatina está representada en la Figura 15. Las estatinas son fármacos generalmente bien tolerados y rara vez producen efectos secundarios.

Figura 15. Estructura química de la atorvastatina.



Diversos estudios clínicos han puesto de manifiesto que el efecto beneficioso de las estatinas no se debe sólo a la disminución en los niveles de colesterol, ya que otros fármacos que reducen los niveles de colesterol no llegan a tener la efectividad de las estatinas a la hora de prevenir la enfermedad coronaria (144). La explicación a este hallazgo es que la inhibición de la HMG-CoA reductasa lleva también a una reducción en la producción de isoprenoides como el farnesilpirofosfato y el geranilgeranil pirofosfato (Figura 16). Éstos están implicados en la modificación post-traduccional (isoprenilación) de varias proteínas como la subunidad γ de proteínas G heterotriméricas, lamininas nucleares o proteínas pequeñas de unión a guanosín trifosfato como Ras, Rho, Rac, Rab, Ral y Rap.

Figura 16. Mecanismo de acción de las estatinas.



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1.5.2. Efectos terapéuticos.

Las estatinas son absorbidas por el tracto gastrointestinal y principalmente se dirigen al hígado. Así, la mayoría de efectos que producen son resultado de su acción sobre el hígado. Sólo una pequeña parte se une a proteínas plasmáticas y viaja al resto del organismo. Además de la disminución de la colesterolemia las estatinas tienen otros efectos beneficiosos que incluyen efectos antiinflamatorios, antioxidantes y mejora de la función endotelial.

1.5.2.1. Efecto sobre el colesterol.

La mayor parte del colesterol circulante se sintetiza en el hígado mediante la vía de la HMG-CoA reductasa (Figura 16). El colesterol también se obtiene directamente de la dieta. Tanto el procedente de la síntesis hepática (y secretado mediante la bilis) como el ingerido con los alimentos llega al intestino. De éste, aproximadamente la mitad es absorbido. Para su transporte en sangre, el colesterol se une a lipoproteínas. Los quilomicrones llevan el colesterol desde el intestino delgado, donde es absorbido, hacia el hígado. Las lipoproteínas de baja densidad y las de muy baja densidad llevan el colesterol desde el hígado hacia el resto del organismo. Una concentración sérica elevada de colesterol asociado a lipoproteínas de baja densidad se asocia con un mayor riesgo de enfermedad coronaria. Las proteínas de alta densidad, por el contrario, llevan el colesterol sobrante de nuevo al hígado para su eliminación a través de la bilis y una concentración de éstas elevada en sangre se asocia con protección frente al daño cardiovascular. Las estatinas inhiben la síntesis de colesterol en el hígado mediante la inhibición de la HMG-CoA reductasa. La menor producción de colesterol induce un aumento en la expresión de receptores de lipoproteínas de baja densidad, lo cual lleva a una disminución de éstas en sangre. Otros efectos derivados son una disminución de los niveles séricos de triglicéridos y lipoproteínas de muy baja densidad y un incremento de los niveles de lipoproteínas de alta densidad (145).

1.5.2.2. Otros efectos de las estatinas.

Varios estudios preclínicos muestran que las estatinas ejercen un efecto protector en modelos animales de daño en corazón (146), cerebro (147), intestino (148), pulmón (149) y riñón (150;151). También podrían ejercer un papel beneficioso en enfermedades autoinmunes gracias a su efecto antiinflamatorio, como se ha demostrado en un modelo de esclerosis múltiple en ratón (152). En humanos se ha demostrado que protege el corazón y el riñón en enfermos con dolencias en estos órganos (153), disminuye la gravedad de accidentes cerebrovasculares y ejerce efectos beneficiosos en la enfermedad de Alzheimer y en cardiopatías entre otras patologías (154).

Estos efectos beneficiosos no están relacionados con la síntesis de colesterol sino con la inhibición de la isoprenilación de diferentes proteínas (principalmente de la familia de Ras: RhoA, Rac) debido al bloqueo en la producción de geranilgeranil pirofofato y farnesilpirofosfato, dos isoprenoides necesarios para que se lleven a cabo estas modificaciones post-traduccionales. La isoprenilación permite la activación de estas proteínas. Las proteínas de la familia Ras juegan un papel esencial en procesos como la polarización celular, la adhesión célula-célula o célula-matriz, el tráfico intracelular de vesículas, la regulación del citoesqueleto, la proliferación celular o la formación de lamelipodios. Rac1 es responsable de la activación del complejo NADPH oxidasa que promueve la formación de superóxido y así, de estrés oxidativo (155). La inhibición de la isoprenilación de estas moléculas produce, en diferentes tipos celulares, los efectos que se resumen en la Tabla 7.

Tabla 7. Efectos biológicos de las estatinas independientes de la inhibición de la síntesis de colesterol.

Células endoteliales
Incremento de la expresión y actividad de la sintasa endotelial de óxido nítrico.
Disminución de la síntesis de endotelina.
Disminución de la síntesis de especies reactivas de oxígeno.
Disminución de la producción de citocinas proinflamatorias.
Disminución de la expresión de moléculas de adhesión celular.
Células de músculo liso
Inhibición de la proliferación y la migración.
Disminución de la síntesis de especies reactivas de oxígeno.
Disminución de la actividad de la NADPH oxidasa.
Disminución de la expresión del receptor 1 de la Ang II.
Inducción de apoptosis.
Macrófagos
Inhibición de la proliferación.
Disminución de la expresión y secreción de metaloproteasas.

1.6. LA GHRELINA.

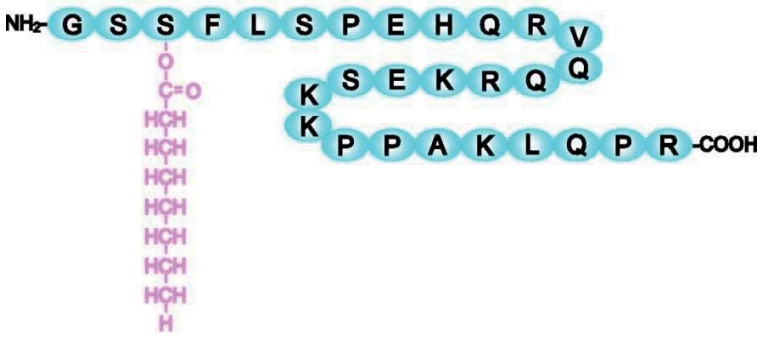
1.6.1. Introducción.

La ghrelina es una hormona peptídica producida principalmente por el estómago. Es especialmente estudiada por sus efectos sobre la secreción de la hormona del crecimiento (GH) y sobre la inducción del apetito, aunque también por otras acciones que se explican más adelante. El descubrimiento de la ghrelina es un ejemplo de farmacología reversa. Su historia comienza en 1976, cuando se sintetizaron los primeros secretagogos de la GH (156). Eran derivados sintéticos de la Met-encefalina y se desarrollaron como una herramienta para el diagnostico la deficiencia de GH. Más tarde, en 1996, se identificó el receptor al que se unían estas moléculas, el receptor de los secretagogos de la hormona del crecimiento (GHS-R). Se hicieron entonces experimentos en los que se exponía una línea celular que expresaba este receptor a extractos de varios tejidos, con el fin de encontrar el ligando endógeno de este receptor. Fue en 1999 cuando dos grupos independientes identificaron esta molécula, a la que se denominó ghrelina (157;158). La palabra ghrelina deriva de "ghre" que significa crecimiento en lengua proto-indoeuropea, haciendo referencia a su habilidad para estimular la secreción de GH.

Su estructura consta de 28 aminoácidos y para su actividad sobre el GHS-R es imprescindible una modificación en el aminoácido 3 de su secuencia (serina) que consiste en la unión de un ácido graso, principalmente ácido n-octanoico (Figura 17). No está claro el procedimiento por el cual ocurre esta modificación, pero se ha observado que la ghrelina se asocia con un tipo de lipoproteína de alta densidad, que podría ser responsable de su esterificación (159). Además, el tipo de ácidos grasos ingeridos con la dieta parecen estar relacionados con esta modificación post-traduccional (160). Sin embargo, en sangre la mayor parte de la ghrelina se encuentra en su forma no acilada (70-80%) (161) y algunos investigadores postulan que la ghrelina no acilada podría unirse a otro receptor y tener otras funciones (162).

La ghrelina es, de momento, la única hormona conocida que tiene esta modificación post-traduccional.

Figura 17. Secuencia aminoacídica de la ghrelina humana.



Adaptado de Kojima M et al. (162)

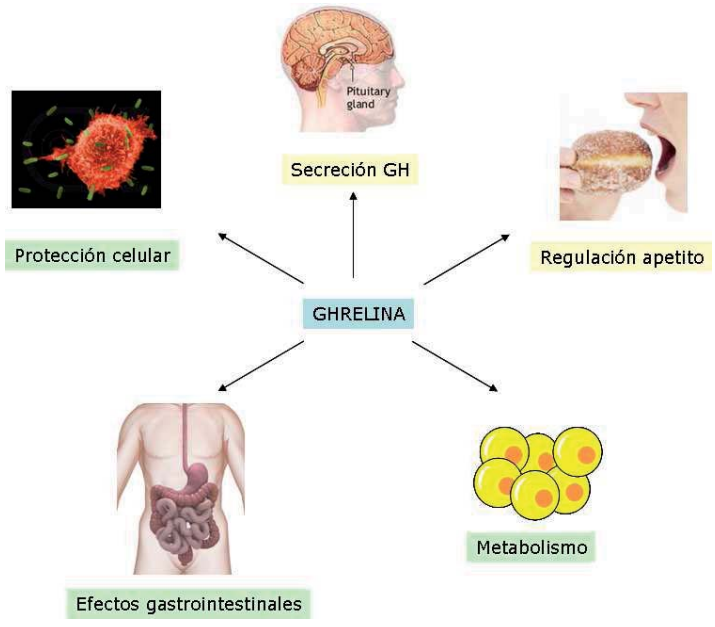
El sistema ghrelina/GHS-R está muy conservado entre especies. Se ha visto que la ghrelina estimula la secreción de GH incluso en peces (163). Además, los 10 primeros aminoácidos de la ghrelina son idénticos en todas las especies de mamíferos estudiadas (162). De hecho los 4 primeros aminoácidos de la estructura son suficientes para activar el receptor GHS-R *in vitro* (164) aunque no *in vivo* (165).

El GHS-R tiene dos variantes procedentes del mismo gen, la 1a y la 1b. La variante 1a corresponde a la proteína entera y es un receptor acoplado a proteína G con 7 dominios transmembrana (166). Es un receptor biológicamente activo. Se ha observado que la adenosina es otro ligando de este receptor aunque se une a otro "bolsillo" (167). La variante 1b es derivada de *splicing* alternativo del gen del GHS-R y se corresponde con una proteína truncada, en la que hipotéticamente sólo habría 5 dominios transmembrana. No se activa con la ghrelina ni con otros secretagogos de la GH y se desconoce su función (163). Por otro lado, se sabe que la ghrelina debe activar otro(s) receptor(es) además del GHS-R1a, ya que es biológicamente activa sobre células que carecen de dicho receptor (168).

La ghrelina se produce principalmente en el estómago y se secreta a la circulación. Prueba de ello es que tras una gastrectomía los niveles de ghrelina disminuyen marcadamente (entre un 40 y un 60%), aunque existen mecanismos compensatorios que modulan la concentración sérica de ghrelina (169;170). Además de producirse en el estómago, se ha detectado expresión de ghrelina (aunque en menor medida) a nivel proteico o de ARN mensajero en la mayoría de órganos del cuerpo como intestino, páncreas, riñón, placenta, tejido linfático, gónadas ovario, glándula tiroides, pulmón, cerebro o corteza adrenal (171-177) A su vez, su receptor, aunque mayoritariamente expresado en hipocampo, hipófisis e hipotálamo, también se expresa en otras zonas del cerebro y en varios órganos periféricos como corazón, pulmón, riñón, páncreas, estómago, intestino, tejido adiposo y células del sistema inmunitario (162).

Aunque las principales acciones de la ghrelina son a nivel central (secreción de GH y estimulación del apetito), se han descrito diversos efectos periféricos como son la protección de la muerte celular, efectos gastrointestinales (aumenta la secreción gástrica y la motilidad intestinal) y efectos en el metabolismo energético (Figura 18).

Figura 18. Principales acciones de la ghrelina.



1.6.2. Niveles séricos de ghrelina en diferentes situaciones patológicas.

En personas sanas, los niveles de ghrelina varían a lo largo del día en relación con las comidas. La concentración plasmática de ghrelina aumenta hasta el doble de su concentración basal durante el ayuno y una hora después de haber ingerido alimentos, vuelve a su estado basal (178). Se sabe que para la disminución postpandrial de los niveles de ghrelina no basta una distensión física del estómago, por ejemplo con agua, sino que es necesario un aporte energético (179). Por otro lado, los niveles de ghrelina en sangre se ven modificados en diferentes patologías (Tabla 8). En el caso de individuos obesos, además de tener niveles disminuidos de ghrelina (y de GH) no se producen esas variaciones en relación con la ingesta de comida (180). De la misma manera ocurre en el síndrome de Prader-Willi, aunque en este caso, a pesar de la obesidad, los niveles plasmáticos de ghrelina están aumentados en relación con individuos control pareados por índice de masa corporal. Probablemente, ésta es la causa de la hiperfagia que se observa en los individuos con este síndrome (181). El sexo y la edad parecen no influir en la concentración plasmática de ghrelina aunque hay cierta controversia en este campo (182;183).

Tabla 8. Niveles séricos de ghrelina en diferentes situaciones patológicas.

Aumentados	**Disminuidos**
Síndrome de Prader-Willi (181)	Obesidad (184)
Caquexia (185;186)	Infección por *Helicobacter pylori* (187)
Bulimia (188)	Síndrome de intestino corto (189)
Cirrosis hepática (190)	Síndrome del ovario poliquístico (191)
Fallo renal (192)	Síndrome metabólico (193)
	Esteatohepatitis no alcohólica (194)

1.6.3. Efectos centrales.

La ghrelina tiene dos principales acciones a nivel central: la estimulación de la secreción de la GH y la estimulación del apetito, que se explicaran más en detalle más adelante, ya que son los más estudiados. Además, tiene otros efectos centrales como la estimulación de la secreción de prolactina o la estimulación del eje hipotálamo-pituitario adrenal (195), que podría contribuir a la ganancia de peso inducida por la ghrelina (196). Por otro lado, se ha observado que aumenta la ansiedad y la retención de memoria en roedores (197).

1.6.3.1. Papel sobre la secreción de la hormona del crecimiento.

Clásicamente, se sabe que la liberación de hormona del crecimiento (GH) por parte de las células somatotropas de la glándula pituitaria está regulada por la hormona liberadora de la hormona del crecimiento (GHRH) y la somatostatina (198). La primera estimula la secreción de la GH y la segunda la inhibe. Tras el descubrimiento de la ghrelina, se añadió un nuevo factor que promueve la liberación de la GH. Este efecto ocurre por diversos mecanismos. Por una parte, la ghrelina estimula directamente las células productoras de GH en la pituitaria (162). Por otra parte, a nivel hipotalámico, aumenta la producción de GHRH (199). Por otro lado, recientemente se ha demostrado que la GHRH es un ligando activo del GHS-R que potencia el efecto de la ghrelina (200), lo que hace pensar que estas dos hormonas se potencian mutuamente. Finalmente, en un estudio en ratas se demostró que el efecto de la ghrelina sobre la secreción de GH es mediado por el nervio vago, ya que en ratas con el nervio vago bloqueado no se produce el aumento esperado de los niveles sanguíneos de GH tras un estímulo con ghrelina (201). Sin embargo esto no ha podido ser confirmado en el caso de pacientes vagotomizados (202).

1.6.3.2. Papel sobre la regulación del apetito.

El papel biológicamente más importante de la ghrelina podría ser su función en el control del apetito y el control energético y metabólico del organismo, ya que los niveles de ghrelina se correlacionan negativamente con el índice de masa corporal (203) y las oscilaciones en la concentración de ghrelina plasmática a lo largo del día están en relación con la ingesta de alimentos (178).

Aunque ya había indicios de que los secretagogos sintéticos de la GH estimulaban el apetito (204;205), no se conoció este efecto de la ghrelina hasta el año 2000 gracias a un estudio en el que se demostró que el tratamiento con ghrelina incrementa el peso corporal en roedores mediante una reducción de la utilización del tejido adiposo y un incremento de la ingesta de alimentos (179). El efecto de la ghrelina sobre el apetito está mediado por la estimulación de las neuronas que secretan los péptidos orexigénicos *agouti related peptide* (AgRP), neuropéptido Y (206) y las orexinas (207). Por otro lado, este efecto de la ghrelina también viene mediado por el nervio vago, ya que el bloqueo del nervio vago en ratas y en humanos inhibe la estimulación del apetito inducida por la ghrelina (201;208).

1.6.4. Efectos periféricos.

Además de los bien conocidos efectos centrales, el receptor de la ghrelina se expresa en muchos otros órganos y tipos celulares y se han descubierto otras acciones que se detallan a continuación.

1.6.4.1. Papel en el metabolismo energético.

Como se ha explicado, el incremento de peso inducido por la ghrelina es producto tanto de un aumento de la ingesta de alimento como de un cambio en el metabolismo lipídico (179). La ghrelina tiene efectos pro-adipogénicos, ya que estimula la diferenciación de los pre-adipocitos e inhibe la lipólisis (209). Por tanto,

reduce la utilización de grasa, la temperatura corporal y el consumo de oxígeno (179). Por otro lado, estimula la utilización de carbohidratos y produce hiperglicemia (210;211).

1.6.4.2. Efecto citoprotector.

Estudios recientes sugieren que la ghrelina ejerce un papel protector en el parénquima de varios órganos. Se ha demostrado que protege frente a lesiones gástricas inducidas por alcohol en ratas y que este efecto podría estar mediado por el óxido nítrico (212). En pacientes con infección crónica del sistema respiratorio, la ghrelina reduce la inflamación y mejora la calidad de vida (213). En el corazón, reduce la sobrecarga cardiaca y el gasto cardíaco sin aumentar la frecuencia cardiaca (214). Esto puede explicarse porque la ghrelina tiene un importante efecto vasodilatador tanto por efecto directo sobre las arterias (215) como por efecto a nivel central sobre el núcleo del tracto solitario (216). Estos efectos beneficiosos sobre el corazón, se han demostrado en pacientes con enfermedades cardiacas (217;218) y parece que es independiente de la GH, ya que también se produce en ratas deficientes en GH (219). En riñón e hígado también hay algún trabajo que indica efectos beneficiosos de la ghrelina (220;221). Algunos de los efectos beneficiosos de la ghrelina podrían deberse a su efecto orexigénico y lipogénico, ya que previene la caquexia asociada a enfermedades crónicas, aunque también podrían deberse a un efecto anti-apoptótico en las células parenquimatosas (222) y/o a su efecto antiinflamatorio (223).

Además, la ghrelina ejerce efectos sobre la proliferación celular, como se ha demostrado *in vitro* y dependen del tipo celular. Así, inhibe la proliferación en algunas líneas celulares (carcinoma folicular de tiroides y carcinoma de mama) (224;225) mientras que tiene efectos proliferativos en otras (células somatotropas, endoteliales, adipocitos y cardiomiocitos) (226-229).

1.6.4.3. Efectos gastrointestinales.

La ghrelina tiene, además, importantes efectos a nivel del tracto gastrointestinal. Se ha observado que en el estómago, estimula la secreción ácida y que promueve la motilidad y el vaciado del tubo digestivo (230). En ratas, se ha demostrado que es la molécula más potente para revertir el íleo gástrico posoperatorio (231). Este efecto está mediado por diversos mecanismos. A nivel central, se ha demostrado que la ghrelina estimula el centro de coordinación de motilidad visceral (núcleo de la oliva inferior) (232). Por otro lado, el nervio vago también parece jugar un papel en este efecto, ya que la vagotomía inhibe tanto la motilidad intestinal como la secreción ácida inducida por ghrelina. Además, es un efecto mediado por el sistema colinérgico, ya que la atropina (antagonista del receptor muscarínico de la acetilcolina) inhibe estas acciones (230). Otro de los efectos gastrointestinales de la ghrelina es que inhibe la secreción pancreática tanto *in vivo* como *in vitro* (233).

2. OBJETIVOS

El propósito de esta tesis ha sido el de investigar nuevas vías terapéuticas para el tratamiento de la inflamación y la fibrosis hepática. De manera general, esta tesis doctoral tiene dos objetivos:

1. Estudiar el efecto de la atorvastatina sobre la inflamación hepática.

1.1. Investigar el efecto del tratamiento con atorvastatina en un modelo animal de inflamación hepática inducida mediante la infusión sistémica de Ang II.

1.2. Estudiar el efecto de la atorvastatina y el losartan en un modelo de daño agudo en el hígado (CCl_4).

1.3. Estudiar los mecanismos mediante los cuales la atorvastatina ejerce sus efectos beneficiosos en el hígado.

1.4. Investigar los efectos de la atorvastatina sobre las células estrelladas hepáticas en cultivo.

2. Estudiar el papel de la ghrelina en las enfermedades hepáticas.

2.1. Investigar el efecto de la administración de ghrelina en un modelo animal de fibrosis hepática en rata (ligadura de conducto biliar).

2.2. Investigar la respuesta fibrogénica de ratones deficientes en ghrelina ante un estímulo fibrogénico (CCl_4).

2.3. Explorar los mecanismos mediante los cuales la ghrelina ejerce su efecto antifibrogénico en los modelos animales.

2.4. Investigar el efecto de la administración de ghrelina en un modelo de daño agudo en hígado (CCl_4).

2.5. Evaluar los mecanismos responsables de los efectos hepatoprotectores de la ghrelina.

2.6. Investigar los efectos de la ghrelina sobre las células estrelladas hepáticas en cultivo.

2.7. Analizar los niveles plasmáticos de ghrelina en pacientes con diferentes enfermedades del hígado.

2.8. Determinar la expresión hepática de ghrelina en pacientes con diferentes enfermedades hepáticas.

2.9. Analizar seis polimorfismos del gen de la ghrelina y su relación con el desarrollo de fibrosis en enfermos con hepatitis crónica C.

3. RESULTADOS

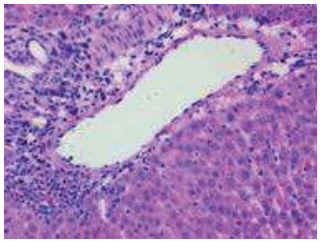
ESTUDIO 1. Efecto de la atorvastatina sobre la inflamación y el daño hepático

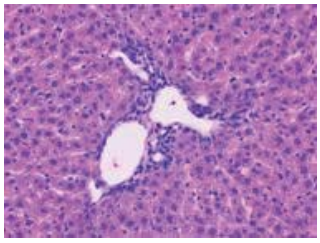
En este primer estudio, se investigan los efectos de la atorvastatina sobre las acciones proinflamatorias y profibrogénicas de la Ang II. El SRA juega un papel importante en la fibrosis hepática ya que promueve la inflamación y el depósito de colágeno en el hígado, siendo la Ang II la principal molécula efectora del sistema (62). La inhibición del sistema renina-angiotensina es una de las estrategias terapéuticas más prometedoras para el tratamiento de la fibrosis hepática (234;235). Se sabe que las estatinas inhiben las acciones proinflamatorias de la Ang II en riñón (236) y en células de músculo liso (237). Por otro lado, se ha observado que las estatinas inhiben la proliferación de HSC en cultivo (238). Sin embargo, se desconocía si la atorvastatina también ejerce acciones beneficiosas en el hígado.

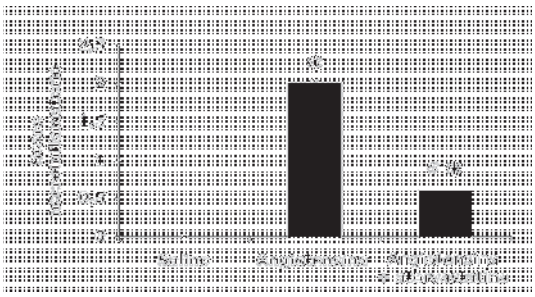
En este trabajo se demuestra un efecto hepatoprotector de la atorvastatina sobre el daño inducido por la infusión de Ang II y se corrobora el efecto hepatoprotector con otro modelo de inflamación hepática (inyección de CCl_4). Además, se analizan los efectos biológicos de la atorvastatina sobre las células estrelladas hepáticas en cultivo. Los principales resultados obtenidos son:

- **La administración de atorvastatina previene la inflamación hepática inducida por la infusión de Ang II.** Se infundió Ang II a ratas Wistar durante 4 semanas mediante minibombas osmóticas y se les administró atorvatatina o vehículo por vía oral a diario. Se determinó el daño histológico mediante un score de necroinflamación y se cuantificó el número de células inflamatorias ($CD43^+$) en cortes histológicos. Las ratas tratadas con atorvastatina tuvieron una respuesta inflamatoria menor que las ratas tratadas con vehículo y mostraron una histología más preservada. Este resultado se muestra en la Figura 1 del primer artículo:

Ang II **Atorvastatina + Ang II**

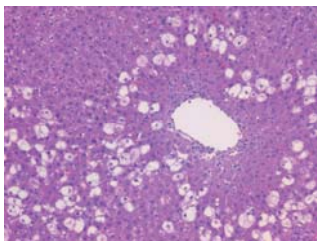


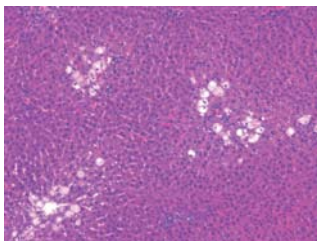


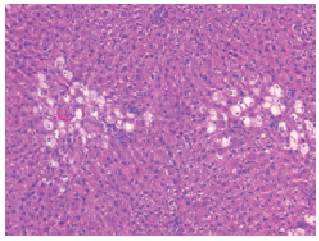


- **La administración de losartan y de atorvastatina previene el daño inducido por CCl_4**. Se utilizó un modelo de daño hepático agudo (administración de CCl_4, 24 horas) para confirmar el efecto hepatoprotector de la atorvastatina y para determinar si el tratamiento con un antagonista del receptor 1 de la Ang II (losartán) también posee un papel hepatoprotector. Se administró atorvastatina y/o losartan por vía oral en el agua de bebida un día antes de la administración de CCl_4 y se mantuvieron los tratamientos hasta el momento del sacrificio. Las ratas tratadas con losartán o atorvastatina mostraron hígados mejor preservados a nivel histológico. Sin embargo, el tratamiento simultáneo de atorvastatina y losartán no produjo ningún efecto beneficioso en este modelo. Estos resultados se muestran en la Figura 1 del primera artículo:

CCl_4 **Losartan + CCl_4** **Atorvastatina + CCl_4**



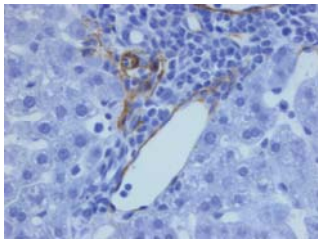
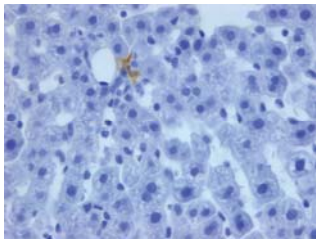
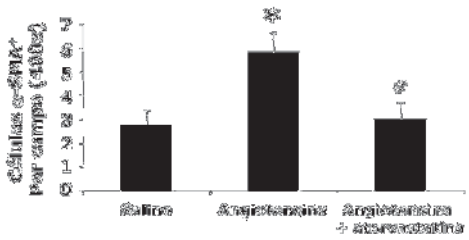




- **La administración de atorvastatina atenúa el acúmulo de células fibogénicas.** En el modelo de hepatitis inducida por Ang II, se observó un incremento en el número de células profibrogénicas (positivas para α-actina de músculo liso). Las

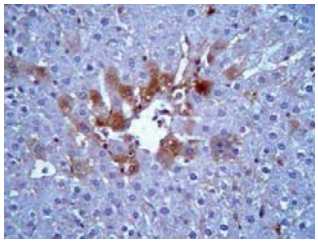
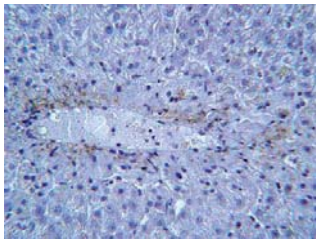
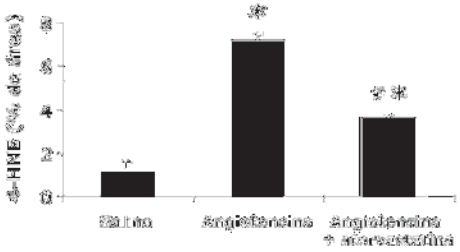
ratas tratadas con atorvastatina mostraron un menor número de éstas. Este resultado se muestra en la Figura 2 del primer artículo:

Ang II **Atorvastatina + Ang II**

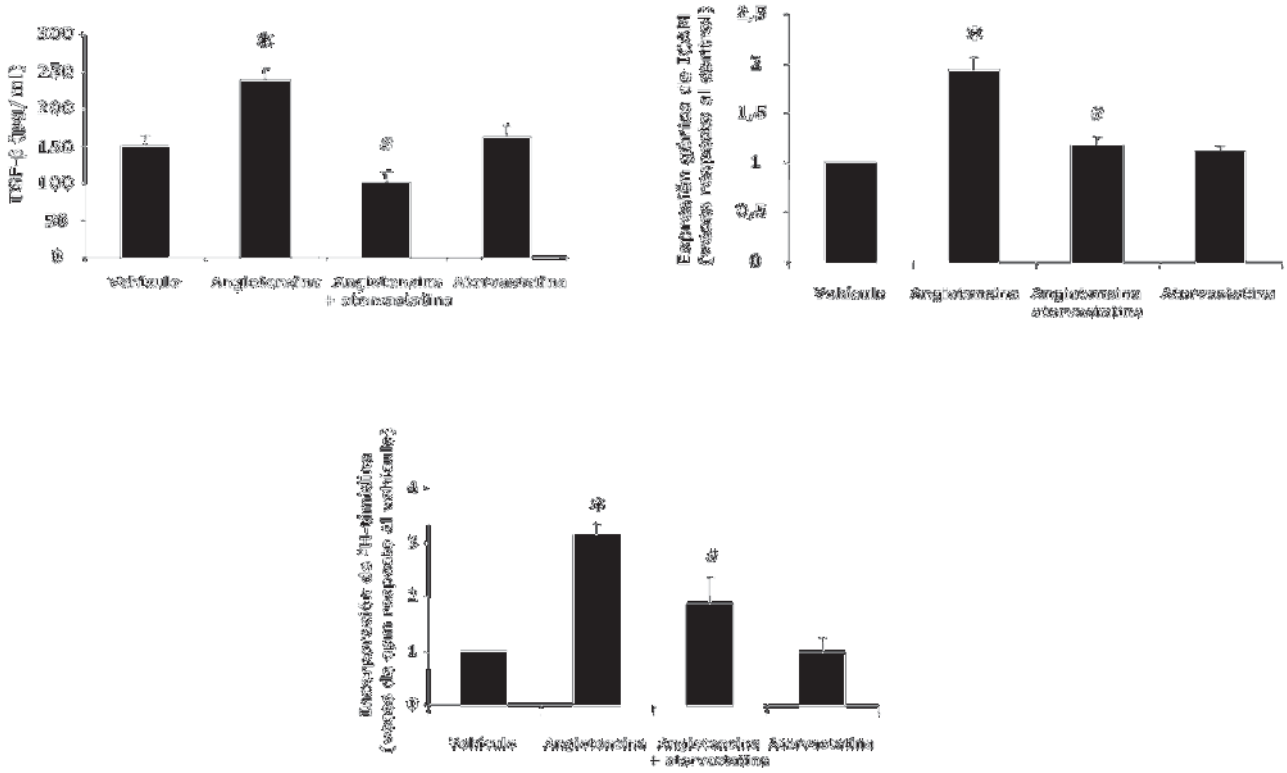
• **La administración de atorvastatina modula el estrés oxidativo en el modelo de inflamación hepática inducida por la infusión de Ang II.** En el modelo de hepatitis inducida por Ang II, se observó estrés oxidativo en el hígado (evaluado por inmunohistoquímica de 4-hidroxinonenal). Las ratas tratadas con atorvastatina mostraron un menor grado de éste. Este resultado se muestra en la Figura 2 del primer artículo:

Ang II **Atorvastatina + Ang II**

• **La atorvastatina modula la expresión de genes profibrogénicos y proinflamatorios en el modelo de inflamación hepática inducida por la infusión de Ang II.** El tratamiento con atorvastatina previno el aumento de expresión de algunos genes proinflamatorios y profibrogénicos como TGF-β1 e IL-6 inducido por Ang II. También previno la sobreexpresión de Rac1, un miembro de la familia de proteínas de unión a guanosin trifosfato.

• **La atorvastatina inhibe acciones proinflamatorias y profibrogénicas en las HSC**. Se estimularon HSC con Ang II en presencia o ausencia de atorvastatrina y se analizó la proliferación, la secreción de IL-8 y de TGF-β y la translocación de la subunidad p65 del factor de transcripción NFκB (que promueve acciones proinflamatorias en las HSC). La atorvastatina inhibió todas estas acciones inducidas por la Ang II. Además, inhibió el aumento en la expresión de los genes ICAM-1, procolágeno α1(I) y TGF-β inducido por Ang II. Sin embargo, no moduló la actividad de Rac1, la expresión de α-actina de músculo liso ni la fosforilación de ERK. Estos resultados se muestran en la Figuras 4 y 5 del primer artículo:



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Atorvastatin attenuates angiotensin II-induced inflammatory actions in the liver

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Moreno M, Ramalho LN, Sancho-Bru P, Ruiz-Ortega M, Ramalho F, Abraldes JG, Colmenero J, Dominguez M, Egido J, Arroyo V, Ginès P, Bataller R. Atorvastatin attenuates angiotensin II-induced inflammatory actions in the liver. *Am J Physiol Gastrointest Liver Physiol* 296: G147–G156, 2009. First published December 4, 2008; doi:10.1152/ajpgi.00462.2007.—Statins exert beneficial effects in chronically damaged tissues. Angiotensin II (ANG II) participates in liver fibrogenesis by inducing oxidative stress, inflammation, and transforming growth factor-β1 (TGF-β1) expression. We investigate whether atorvastatin modulates ANG II-induced pathogenic effects in the liver. Male Wistar rats were infused with saline or ANG II (100 ng·kg^{-1}·min^{-1}) for 4 wk through a subcutaneous osmotic pump. Rats received either vehicle or atorvastatin (5 mg·kg^{-1}·day^{-1}) by gavage. ANG II infusion resulted in infiltration of inflammatory cells (CD43 immunostaining), oxidative stress (4-hydroxynonenal), hepatic stellate cells (HSC) activation (smooth muscle α-actin), increased intercellular adhesion molecule (ICAM-1), and interleukin-6 hepatic gene expression (quantitative PCR). These effects were markedly blunted in rats receiving atorvastatin. The beneficial effects of atorvastatin were confirmed in an additional model of acute liver injury (carbon tetrachloride administration). We next explored whether the beneficial effects of atorvastatin on ANG II-induced actions are also reproduced at the cellular level. We studied HSC, a cell type with inflammatory and fibrogenic properties. ANG II (10^{-8}M) stimulated cell proliferation, proinflammatory actions (NF-κB activation, ICAM-1 expression, interleukin-8 secretion) as well as expression of procollagen-$\alpha_{1(I)}$ and TGF-β1. All of these effects were reduced in the presence of atorvastatin (10^{-7}M). These results indicate that atorvastatin attenuates the pathogenic events induced by ANG II in the liver both in vivo and in vitro. Therefore, statins could have beneficial effects in conditions characterized by hepatic inflammation.

statins; fibrosis; renin-angiotensin system; hepatic stellate cells

CHRONIC INFLAMMATION OF THE hepatic parenchyma eventually leads to fibrosis. Liver fibrosis is the excessive accumulation of extracellular matrix proteins, including collagen, which occurs in most types of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation (2). Drugs capable of attenuating inflammation and/or fibrosis progression in patients with chronic liver diseases are currently under investigation.

Experimental and clinical data strongly indicate that the renin-angiotensin system may play a major role in liver fibrosis by promoting inflammation and collagen synthesis (2, 7). Angiotensin II (ANG II), the main effector of this system, exerts an array of inflammatory and fibrogenic actions in hepatic stellate cells (HSC), the major fibrogenic cell type in the injured liver (5). Moreover, we previously demonstrated that ANG II infusion into normal rats induces HSC activation and proinflammatory events in the liver (3, 4). Most importantly, pharmacological inhibition of the renin-angiotensin system attenuates liver fibrosis in rodents (13, 18, 22, 31, 33, 35, 42, 46, 47, 49).

A large body of evidence indicates that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, so-called statins, have beneficial properties in atherosclerosis (38, 43). Moreover, statins modulate the deleterious effects of the renin-angiotensin system in several organs (34). Atorvastatin, a widely used statin, reduces NF-κB activation and chemokine expression induced by ANG II in vascular smooth muscle cells (32), as well as reduces free radical production (44). Statins also exert anti-inflammatory and antifibrogenic activity in the kidney in vitro and in vivo (10, 29). These effects are due to a decrease in serum lipid levels and to its lipid-independent, pleiotropic effects.

Recent reports suggest that atorvastatin may have beneficial effects in patients with nonalcoholic steatohepatitis associated with the metabolic syndrome, suggesting a potential usefulness for this drug in the treatment of chronic liver diseases (20). In addition, lovastatin and simvastatin inhibit cell growth of cultured HSC (36). The combinatory use of pitavastatin and candesartan, an ANG II receptor, type 1 (AT1) blocker, inhibits liver fibrogenesis in carbon tetrachloride (CCl_4)-treated rats (28). Nevertheless, simvastatin, used without an ANG II type 1-receptor blocker, does not seem to affect liver fibrogenesis in vivo (30).

To provide novel insights on the potential effects of statins on liver inflammation, the current study investigates whether atorvastatin modulates the pathogenic effects of ANG II on the liver both in vitro and in vivo as well as its effect on a model of acute liver injury (CCl_4-induced liver damage). Here, we provide evidence that atorvastatin markedly reduces the deleterious effects induced by ANG II and CCl_4. These results reinforce the hypothesis that statins may have beneficial effects in patients with chronic liver diseases.

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Experimental Procedures

Experimental protocol. For the ANG II experimental model, male 200 g Wistar rats were infused with either saline or ANG II (Sigma-Aldrich, St. Louis, MO) at a dose of 100 $ng \cdot kg^{-1} \cdot min^{-1}$, dissolved in saline through an osmotic minipump (Alza, Palo Alto, CA) for 4 wk as described previously (4). Minipumps were placed subcutaneously and replaced after 2 wk. Rats were treated daily with either vehicle or atorvastatin (Pfizer, Madrid, Spain) (at 5 $mg \cdot kg^{-1} \cdot day^{-1}$) by gavage. Before death, systolic arterial pressure was measured by a tail-cuff pletysmograph (Narco Bio-Systems, Houston, TX), as previously described (11). Ten rats were included in each group. At the end of the infusion period, rats were weighed and killed and liver and blood samples were harvested. For the CCl_4 experimental model, rats were administered vehicle, atorvastatin (at 5 $mg \cdot kg^{-1} \cdot day^{-1}$), losartan (Pfizer, Madrid, Spain) (at 10 $mg \cdot kg^{-1} \cdot day^{-1}$), or atorvastatin plus losartan by gavage at *day 0* and at *day 1*. At *day 1* rats were also administered vehicle (olive oil) or 30% CCl_4 (Sigma, Madrid, Spain) (at 1 ml/kg) by a single intraperitoneal injection. Twenty-four hours later, rats were killed and liver and blood samples were harvested. Eight rats were included in each group. Animal protocols were reviewed and approved by the local committee according to the guidelines for ethical care of experimental animals of the European Community.

Biochemical analysis. Serum alanine aminotransferase (ALT) was measured with an automatic biochemical analyzer.

Histological studies. Livers were fixed in 10% phosphate-buffered formalin for 24 h at room temperature and then embedded in paraffin. Liver inflammation and fibrosis were assessed in 5-μm sections, which were stained with hematoxylin and eosin and Sirius red, respectively. Samples were blindly scored by an expert pathologist (L. N. Ramalho). For the ANG II infusion model, the scoring system used was: inflammation (0 = absence, 1 = mild, 2 = moderate, 3 = severe) and fibrosis (0 = absence, 1 = portal fibrosis, 2 = portal fibrosis and few septa, 3 = evident septal fibrosis without cirrhosis, 4 = cirrhosis) (9). For the CCl_4 model, hepatic necroinflammation was estimated by quantifying the presence of necrosis, hepatocytes ballooning, and/or swelling, inflammatory cell infiltration, and lipid droplets. The degree of necroinflammatory changes was assessed as the percentage of hepatic parenchyma with any of the above-described changes: 0 = lower than 20%; 1 = 20–40%; 2 = 40–60%; 3 = 60–75%; 4 = >75%. For immunohistochemical analysis, sections were deparaffinized, rehydrated, and stained by using the Dako Envision system (Dako, Carpinteria, CA). Sections were incubated with anti-CD43 (1:1,000; Serotec, Raleigh, NC), anti-(E)-4-hydroxynonenal (4-HNE) (1:500, AG Scientific, San Diego, CA), anti-smooth muscle α-actin (α-SMA) (1:1,000, Dako, Carpinteria, CA) and anti-transforming growth factor-β (TGF-β) (1:500, Chemicon, Temecula, CA) for 30 min at room temperature. As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. Morphometric assessments were performed using an optic microscope (Eclipse E600; Nikon, Kanagawa, Japan) connected to a high-resolution camera (model CC12; Soft-Imaging System, Münster, Germany) as described previously (12).

Analysis of gene expression. RNA was isolated from either frozen liver samples and cultured cells using RNeasy mini kit (Hilden, Germany) and Trizol (Life Technologies, Rockville, MD), respectively. Retrotranscription was performed to obtain cDNA. Quantitative PCR was performed with predesigned TaqMan Gene Expression Assay probes and primer pairs for collagen-$\alpha_{1(I)}$, transforming growth factor-β-1 (TGF-β1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), Rac1, AT1, and ribosome subunit 18S, as described previously (40). Information on these assays is available at: http://www.appliedbiosystems.com. TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 Machine (Applied Biosystems, Foster City, CA). Results were normalized to 18S expression. Results are expressed as fold respect to saline.

Isolation and culture of primary human HSC. HSC were isolated from fragments of normal human livers obtained from resections of liver metastasis, as described previously (5). Briefly, liver tissues were digested by two enzymatic solutions. First, digestion was performed in Gey's balanced salt solution containing 0.33% pronase, 0.035% collagenase, and 0.001% DNase for 30 min at 37°C (all from Roche Diagnostics, Mannheim, Germany). Second, digestion was performed in Gey's balanced salt solution containing 0.06% pronase, 0.035% collagenase, and 0.001% DNase for 30 min at 37°C. The resulting cell pellet was centrifuged over a gradient of 10% Nycodenz (Sigma-Aldrich). Average yield per isolation was 5×10^5 cells/g liver. A subset of immunocytochemistry studies was performed in HSC freshly isolated from normal human livers (quiescent phenotype). In all cell cultures, no staining was found for CD45, factor VIII-related antigens, and CAM 5.2 (Dako), indicating the absence of mono/macrophagic, endothelial, and epithelial cells. HSC were studied after the second serial passage (culture-activated phenotype). Cells were cultured in standard conditions in DMEM (BioWhittaker, Verviers, Belgium) containing 15% fetal bovine serum, glutamine, sodium piruvate, nonessential amino acids and insulin. Cells were serum starved for at least 12 h before the experiments. The protocol was approved by the Ethical Committee of the Hospital Clínic of Barcelona.

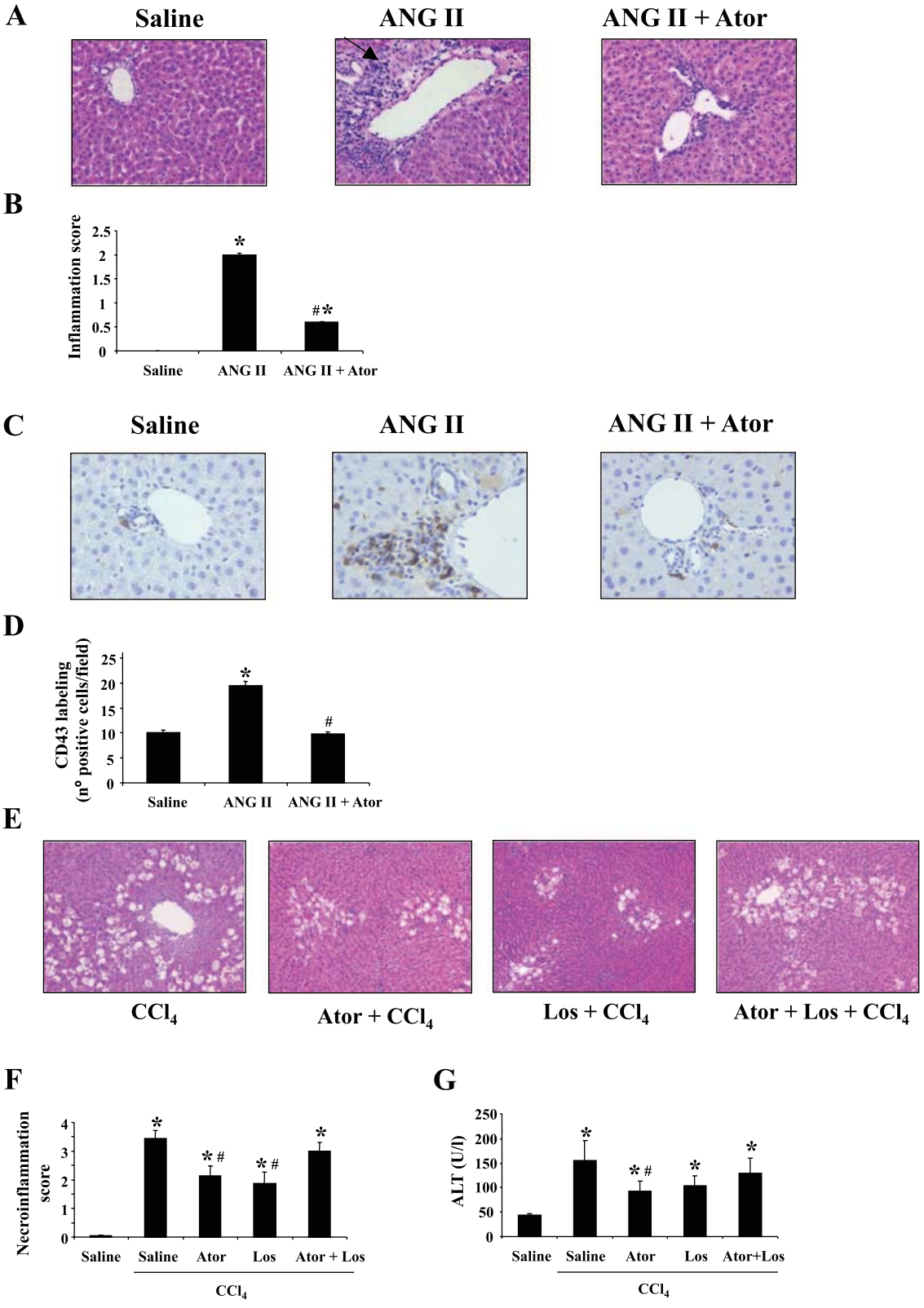
Immunocytochemistry studies. HSC were stimulated for 12 h with agonists in the presence or absence of atorvastatin (10^{-7} M). Cells were then fixed in methanol at −20°C for 10 min, blocked in PBS containing 0.1% BSA for 30 min, and incubated with anti-p65 for 1 h (Santa Cruz Biotechnology, Santa Cruz, CA). Cells were incubated with fluorescein-labeled secondary antibody for 1 h. An isotope-matched antibody was used as a negative control. The p65 nuclear translocation was estimated as the media of the index of nuclear/cytoplasmatic staining in 10 fields at ×400 magnification.

Cell proliferation assay. DNA synthesis was estimated by [methyl-^{3}H]-labeled thymidine (Amersham Biosciences, Buckinghamshire, UK) incorporation, as described in detail previously (6). Cells were serum starved for 24 h, stimulated for 18 h with agonists in the presence or absence of atorvastatin (10^{-7} M), and then pulsed for 6 h with 1 μCi/ml [methyl-^{3}H]-labeled thymidine. Results are expressed as fold stimulation compared with cells incubated with buffer.

IL-8 and TGF-β1 secretion. HSC were cultured in six-well plates at a density of 4×10^5 cells/well for 24 h. Medium was removed, and cells were incubated in serum-free medium for 24 h in the presence of agonists. Supernatants were collected, and a sandwich ELISA for human IL-8 (BLK Diagnostics, Barcelona, Spain) (39), or TGF-β1 (R&D Systems, Minneapolis, MN) was performed.

Fig. 1. Atorvastatin (Ator) reduces liver inflammation. *A*: rats infused with saline showed a normal hepatic histology. An inflammatory infiltrate of mononuclear cells and thickening of the limiting membrane was observed in portal tracts from ANG II-treated rats (arrow). Treatment with Ator markedly attenuated these effects (hematoxylin and eosin staining; original magnification, ×200). *B*: grading of inflammatory changes in liver specimens (see scoring scale in *Histological studies*) ($^*P < 0.05$ vs. saline; $^\#P < 0.05$ vs. ANG II) from rats infused with saline or ANG II. *C*: detection of inflammatory cells by CD43 immunostaining. ANG II infusion induced an infiltration of CD43-positive cells in the rat livers, which was attenuated in the presence of Ator (CD43 immunohistochemistry; original magnification, ×200). *D*: morphometric quantification of the number of CD43-positive cells per high-power field in saline or ANG II-infused rats ($^*P < 0.05$ vs. saline; $^\#P < 0.05$ vs. ANG II). *E*: representative pictures of liver specimens from rats receiving CCl_4 (hematoxylin and eosin staining; original magnification, ×100). Treatment with Ator and/or losartan (Los) significantly reduced CCl_4-induced hepatotoxic effects. *F*: grading of the necroinflammatory score in CCl_4-damaged rats (see scoring scale in *Histological studies*). *G*: alanine aminotransferase (ALT) serum levels in CCl_4-injured rats. Treatment with Ator markedly reduced ALT serum levels ($^*P < 0.05$ vs. saline; $^\#P < 0.05$ vs. CCl_4).

Western blot analysis. Whole cell extracts were obtained in lysis buffer containing protease and phosphatase inhibitors. Fifty micrograms were loaded onto 12% SDS acrylamide gels, electrophoresis was carried out, and proteins were blotted onto nitrocellulose membranes. Membranes were blocked for 2 h with nonfat milk and incubated with antibodies against AT1 (1:200; Santa Cruz Biotechnology), or phospho-extracellular-regulated kinase (1:1,000; Cell Signaling, Beverly, MA) overnight at 4°C. After extensive washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody. Proteins were



detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Rac1 pull-down assay. Rac1 activity was determined by a pull-down assay kit (Assay Designs, Ann Arbor, MI). Briefly, after incubation with agonists, cell extracts were obtained in lysis buffer containing protease inhibitors. Part of the lysates was used to analyze total Rac1, and the rest was incubated with GST-human Pak1-PBD to pull down active Rac1 in the presence of a glutathione disc at 4°C for 1 h. After incubation, the mixture was centrifuged at 7200 *g* for 30 s to remove unbound proteins. The resins were rinsed with washing buffer, and the samples were eluted by adding 50 μl of SDS sample buffer. Half of the sample volume was loaded onto 12% SDS acrylamide gel, electophoresis was carried out, and proteins were transferred onto a nitrocellulose membrane. Active Rac1 was detected by using a specific mouse monoclonal anti-Rac1 antibody diluted 1:1,000. Goat anti-mouse antibody conjugated with horseradish peroxidase (1:3,000; Cell Signaling, Beverly, MA) was used as the secondary antibody. Proteins were detected by enhanced chemiluminiscence (Amersham, Buckinghamshire, UK).

Data analysis. Data presented herein are expressed as means ± SE. Histology data, liver serum enzymes, and gene expression are means of at least eight animals per group. In vitro assays are representative of five independent experiments. Statistical analysis was performed by Student's *t*-test for pairwise comparisons and analysis of variance with a post hoc test of Tukey for multiple comparisons. The Kruskall-Wallis test with a post hoc Dunn's test was used for multiple nonparametric analyses.

RESULTS

Atorvastatin reduces ANG II-induced inflammation and oxidative stress in the liver. ANG II infusion, either with saline or atorvastatin, was well tolerated in all rats. No rats showed noticeable side effects. ANG II infusion induced a marked increase in arterial pressure (157 ± 5 and 118 ± 7 mmHg in rats receiving ANG II and saline, respectively, $P < 0.01$). Concomitant administration by atorvastatin did not decrease ANG II-induced arterial hypertension (147 ± 18 mmHg, $P =$ not significant vs. ANG II plus saline). ANG II induced a slight increase in ALT serum levels (42 ± 5.5 U/l vs. 28 ± 4.6 U/l in ANG II and saline-treated rats, respectively; $P < 0.05$). Histological examination of ANG II-infused livers showed preserved hepatic parenchyma with no apparent hepatocyte damage. Infiltration of mononuclear cells and thickening of the limiting membrane were observed in most portal tracts (Fig. 1*A*). The median inflammatory score in ANG II-treated rats was 2, while no inflammation was seen in saline-infused rats (Fig. 1*B*). To further demonstrate that ANG II infusion results in hepatic inflammation, infiltrating inflammatory cells were stained with CD43, a pan-leukocyte antibody. CD43 is typically expressed by infiltrating mononuclear cells and lymphocytes. Quantification of CD43-positive cells showed that ANG II infusion increased the amount of inflammatory cells infiltrating the hepatic parenchyma (Fig. 1, *C* and *D*). Concomitant treatment with atorvastatin, but not saline, resulted in reduced inflammatory changes, both the inflammatory degree (median inflammatory scores 1 and 2, respectively, $P < 0.05$) and the amount of CD43 positive infiltrating cells. As previously shown, ANG II infusion into normal rats did not cause parenchymal fibrosis (4). The degree of liver fibrosis and the amount of Sirius red staining as assessed using a computer-based morphometric method did not differ from rats receiving saline and ANG II (data not shown). HSC activation, as indicated by α-SMA immunostaining, was slightly increased in ANG II-treated rats. Atorvastatin treatment blunted this effect (Fig. 2, *A* and *B*). ANG II infusion also induced an increase in TGF-β1 hepatic expression as assessed by immunohistochemistry. This effect was not modified by atorvastatin treatment (data not shown). We next explored whether atorvastatin modulates oxidative stress, a major pathogenic event induced by ANG II in the liver (4, 8). For this purpose, we assessed lipid peroxidation by staining liver specimens with anti-4-HNE antibody, a well-known lipid peroxidation product (15). As shown in Fig. 2*C*, there was a marked increase in 4-HNE immunostaining in ANG II-infused rats compared with rats infused with saline. Oxidative stress was mainly located in pericentral areas. This finding was confirmed by morphometric quantification of the 4-HNE positive area (Fig. 2*D*). Importantly, atorvastatin administration markedly reduced ANG II-induced oxidative stress, as indicated by decreased 4-HNE staining (Fig. 2, *C* and *D*). These results indicate that ANG II basically induces hepatic inflammation, HSC activation, and oxidative stress, which are attenuated by the concomitant administration of atorvastatin.

Atorvastatin modulates ANG II-induced expression of inflammatory and fibrogenic genes in the liver. We previously demonstrated that ANG II stimulates the expression of genes involved in hepatic inflammation and fibrogenesis both in vivo and in vitro (3, 4, 8). We next studied whether atorvastatin modulates these effects. We assessed key genes involved in hepatic fibrogenesis [procollagen-$\alpha_{1(I)}$ and TGF-β1], inflammation (ICAM-1, and IL-6), a chief component of the nonphagocytic NADPH oxidase (Rac1), and a key component of the renin-angiotensin system (AT1). ANG II infusion induced an upregulation of genes involved in fibrogenesis, inflammation, and Rac1, as described in Fig. 3. Atorvastatin treatment significantly reduced the expression of TGF-β1, IL-6, and Rac1. AT1 expression was not modified by any treatment. These results suggest that infusion of ANG II to normal rats stimulates inflammatory and fibrogenic gene expression, which is largely attenuated in the presence of atorvastatin.

Atorvastatin reduces CCl_4-induced acute liver damage. We next investigated whether the protective effects induced by atorvastatin are reproduced in a different experimental model. For this purpose, rats were exposed to a single intraperitoneal injection of CCl_4 in the presence or absence of atorvastatin. As expected, CCl_4 administration caused profound hepatic histological changes, including inflammatory infiltrate, necrosis, hepatocytes ballooning, and steatosis. Importantly, atorvastatin administration resulted in a reduction of the necroinflammatory score (Fig. 1, *E* and *F*) and ALT serum levels (Fig. 1*G*). This result reinforces our hypothesis that atorvastatin exerts protective effects against liver injury. To explore whether endogenous ANG II participates in the pathogenesis of acute liver injury, a group of rats were exposed to losartan before CCl_4 administration. We found that losartan also reduced the extent of liver damage induced by CCl_4 (Fig. 1, *E* and *F*). Interestingly, concomitant treatment with atorvastatin and losartan did not induce any synergistic effect.

Atorvastatin reduces the proinflammatory effects of ANG II in HSC. We finally investigated whether the beneficial effects of atorvastatin in rats are reproduced at the in vitro level. For this purpose, we studied cultured HSC, a key fibrogenic cell type in the injured liver that also displays powerful inflammatory properties. We first studied HSC proliferation by measur-

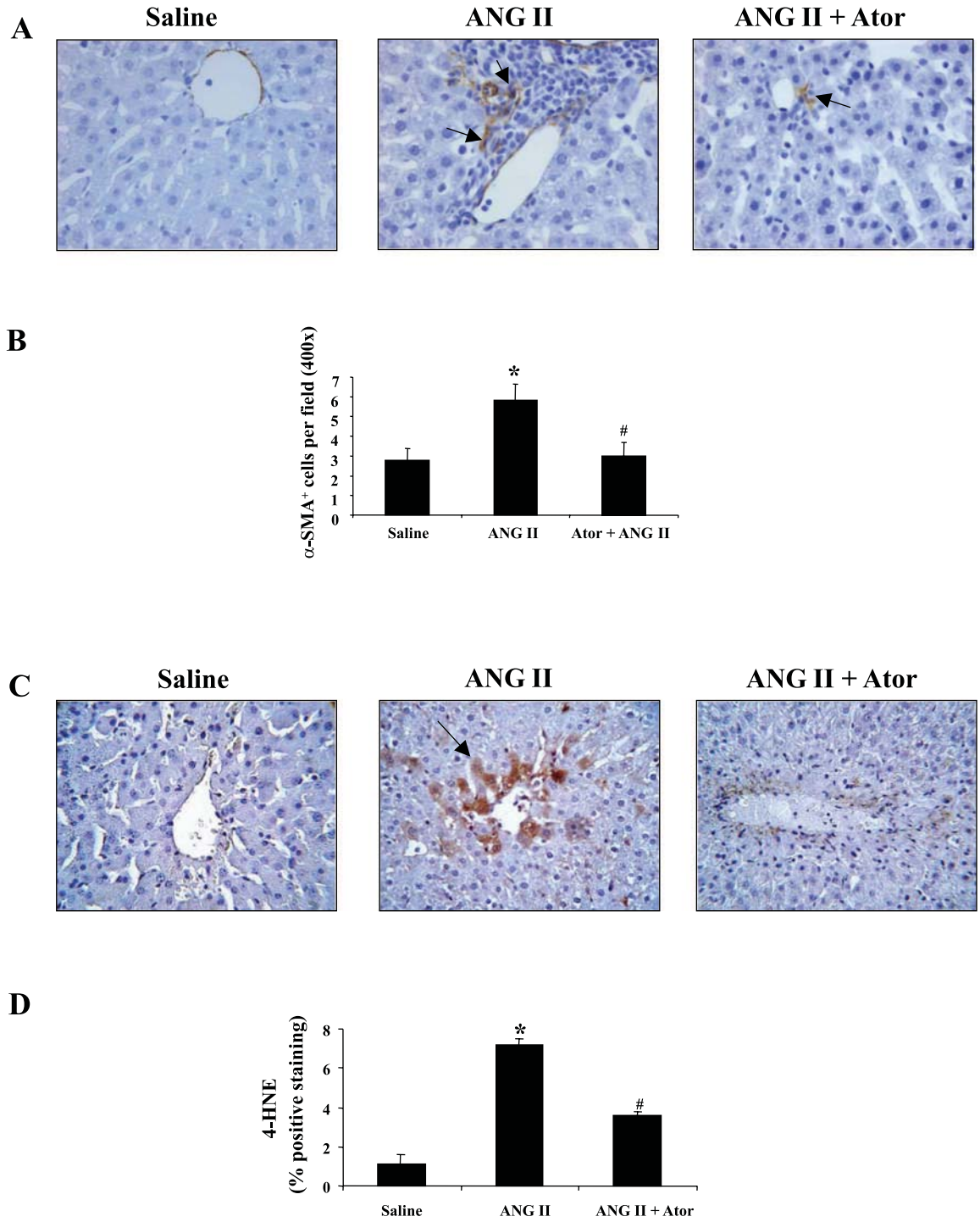


Fig. 2. Effect of Ator on ANG II-induced effects in fibrosis and oxidative stress. *A*: smooth muscle α-actin (α-SMA) immunostaining of liver specimens from rats treated with saline and ANG II with or without Ator. ANG II infusion resulted in a mild increase in α-SMA immunostaining (arrows) that was reduced by Ator. Original magnification, ×400. *B*: quantification of the amount of α-SMA positive cells ($^{*}P < 0.05$ vs. saline; $^{\#}P < 0.05$ vs. ANG II). *C*: ANG II infusion induced oxidative stress in the liver as assessed by increased detection of 4-hydroxynonenal (4-HNE) protein adducts in pericentral areas (arrow). Concomitant treatment with Ator reduced signs of oxidative stress (4-HNE immunohistochemistry; original magnification, ×200); *D*: morphometric quantification of %area stained with 4-HNE in all groups ($^{*}P < 0.05$ vs. saline; $^{\#}P < 0.05$ vs. ANG II).

ing [^{3}H]-labeled thymidine incorporation. ANG II stimulated HSC growth, as shown in Fig. 4*A*. The effect induced by ANG II was significantly attenuated in the presence of atorvastatin. This result corroborates the antiproliferative effects of statins in HSC (27, 36). We next explored whether atorvastatin attenuates the inflammatory effects induced by ANG II in HSC. We studied the activation of the transcription factor NF-κB, a signaling pathway that participates in the inflammatory actions in HSC (24). ANG II and TNF-α, a powerful inflammatory cytokine, stimulated NF-κB activation, as indicated

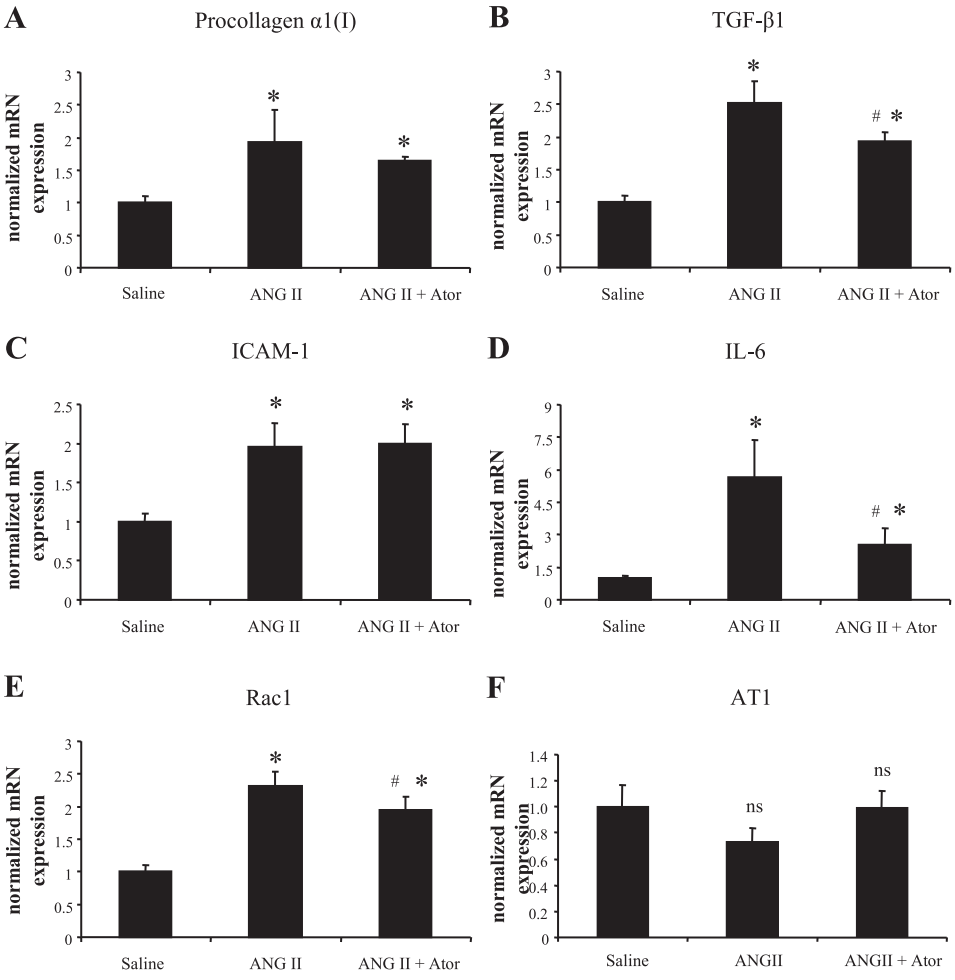


Fig. 3. Effect of Ator on ANG II-induced effects in hepatic gene expression as assessed by quantitative PCR (see *Analysis of gene expression*). Expression of procollagen-$\alpha_{1(I)}$ (*A*), transforming growth factor-β1 (TGF-β1) (*B*), intercellular adhesion olecule-1 (ICAM-1) (*C*), IL-6 (*D*), and Rac1 (*E*) were significantly upregulated in ANG II-infused rats compared with control animals (*$P < 0.05$ vs. saline). Concomitant treatment with Ator reduced the expression of TGF-β1, IL-6, and Rac1 (#$P < 0.05$ vs. ANG II) but not the expression of procollagen-$\alpha_{1(I)}$ and ICAM-1. ANG II receptor, type 1 (AT1) (*F*) expression was not modulated by any treatment. ns, not significant.

by increased nuclear translocation of the subunit p65 (Fig. 4, *B* and *C*). NF-κB activation by ANG II and TNF-α was markedly reduced by cell preincubation with atorvastatin. We next explored the secretion of IL-8, an inflammatory chemokine, involved in liver fibrogenesis (12). As we previously reported, ANG II stimulated the secretion of IL-8 by cultured HSC (8). Preincubation with atorvastatin markedly reduced IL-8 secretion to basal levels (Fig. 4*D*). Similarly, ANG II stimulated the expression of ICAM-1, a membrane protein involved in the interaction between HSC and lymphocytes. Again, this effect was blunted in the presence of atorvastatin (Fig. 5*A*). We next investigated whether atorvastatin regulates the expression of genes involved in liver fibrogenesis, such as procollagen-$\alpha_{1(I)}$ and TGF-β1. As previously described, ANG II induced an upregulation of both genes. This effect was attenuated in the presence of atorvastatin (Fig. 5*B*). Moreover, ANG II induced TGF-β1 release by HSC. As shown in Fig. 4*E*, this effect was attenuated by atorvastatin. However, atorvastatin did not modify α-SMA expression, a marker of HSC activation, (Fig. 4*F*). Next, we assessed whether atorvastatin modifies ANG II receptor activation by analyzing ERK phosphorylation, an important intracellular pathway stimulated by ANG II. Atorvastatin did not modify ERK phosphorylation (Fig. 4*G*). Overall, these results indicate that atorvastatin attenuates the inflammatory effects of ANG II in HSC. We then analyzed whether atorvastatin treatment reduces Rac1 activity. Atorvastatin did not modify Rac1 activation in cells treated with ANG II (Fig. 4*H*). Finally, we studied whether atorvastatin modulates AT1 expression. We found that atorvastatin did not modify AT1 expression in cultured HSC (Fig. 4*I*).

DISCUSSION

The present study investigates the effects of a statin (atorvastatin) on the inflammatory actions of ANG II in the liver. We provide evidence that atorvastatin attenuates the pathogenic events induced by ANG II in the liver, including oxidative stress, inflammatory events, and expression of profibrogenic genes. These results confirm previous observations that statins attenuate the atherogenic effects of ANG II (17). Moreover, our results indicate that atorvastatin exerts protective effects in a model of acute liver injury. Losartan (an AT1 antagonist) treatment also reduced the extent of liver damage, suggesting that endogenous ANG II plays a role in the pathogenesis of hepatic inflammation. Because ANG II is believed to play a role in liver inflammation both in rodents and in humans, the beneficial effects of atorvastatin suggest that this family of drugs could exert beneficial effects in the liver. Further studies should evaluate this hypothesis.

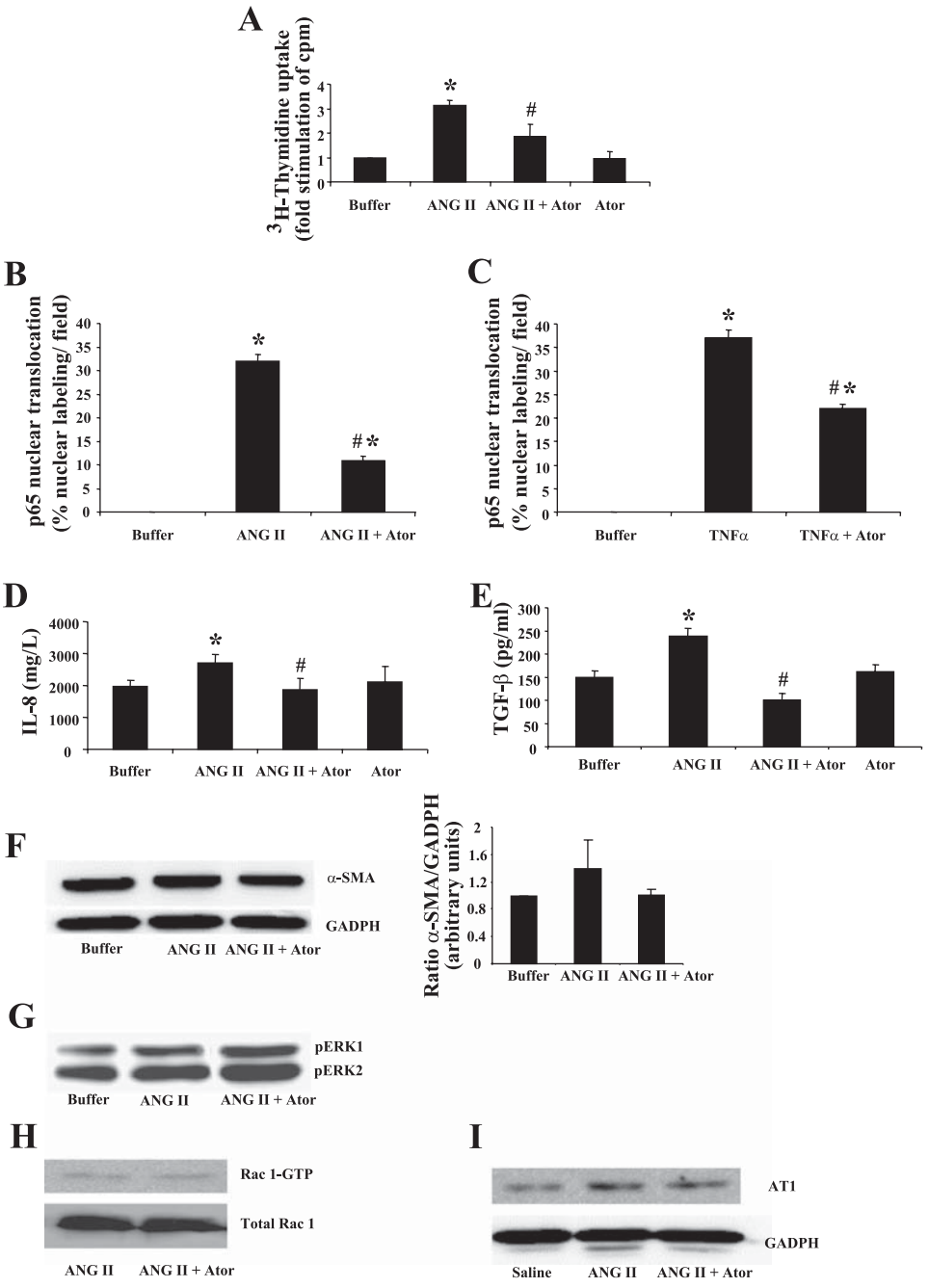


Fig. 4. Effect of Ator on ANG II-induced biological effects in cultured primary hepatic stellate cells (HSC). *A*: effects of different treatments on HSC growth as assessed by [^{3}H]-labeled thymidine incorporation. ANG II (10^{-8}M) stimulated cell proliferation, which was attenuated in the presence of Ator (10^{-7}M) (*$P < 0.05$ vs. buffer; $^{\#}P < 0.05$ vs. ANG II). *B* and *C*: effects of different treatments on NF-κB activation as assessed by p65 nuclear translocation (see *Immunocytochemistry studies*). Results are expressed as %cells with positive nuclear labeling per field. Both ANG II (10^{-8}M) and TNF-α (10 ng/ml) markedly increased p65 nuclear translocation (*$P < 0.05$ vs. buffer). Pretreatment with Ator (10^{-7}M) blunted agonist-induced p65 nuclear translocation ($^{\#}P < 0.05$ vs. ANG II and TNF-α); *D*: secretion of IL-8 by cultured HSC. ANG II (10^{-8}M) stimulated the secretion of IL-8 to the culture media (*$P < 0.05$ vs. buffer) as assessed by ELISA. Incubation with Ator (10^{-7}M, 20 min) prevented ANG II-induced IL-8 secretion ($^{\#}P < 0.05$ vs. ANG II). *E*: secretion of TGF-β1 by cultured HSC as assessed by ELISA. ANG II (10^{-8}M, 24 h) stimulated the secretion of TGF-β1 to the culture media (*$P < 0.05$ vs. buffer) as assessed by ELISA. Preincubation with Ator (10^{-7}M, 20 min) prevented ANG II-induced release of TGF-β1 ($^{\#}P < 0.05$ vs. ANG II). Results are means of 5 independent experiments. *F*: α-SMA expression as assessed by Western blotting was not modified by ANG II treatment (10^{-8}M, 15 h) nor by Ator (10^{-7}M, 1 h before ANG II and throughout the experiment). *G*: ANG II (10^{-8}M, 30 min) induced ERK phosphorylation. Ator treatment (10^{-7}M, 20 min) did not modify this effect. *H*: ANG II (10^{-8}M, 10 min) induced Rac1 activity as assessed by pull-down assay (see *Rac1 pull-down assay*). Ator treatment (10^{-7}M, 20 min) did not modulate Rac1 activation. *I*: expression of AT1 in primary cultured HSC was assessed by Western blotting. Incubation with ANG II (10^{-8}M, 15 h) increased AT1 expression. Treatment with Ator (10^{-7}M, throughout the experiment) did not reduce the effect induced by ANG II. Pictures are representative of three independent experiments.

To test the effects of atorvastatin in the liver, we have used a well-characterized model of continuous infusion of ANG II into rats (4). This model was chosen since ANG II is a powerful proinflammatory substance that plays a role in the pathogenesis of liver inflammation. Moreover, drugs inhibiting ANG II generation and/or binding to its receptors (such as losartan) are considered the most promising approach to treat liver fibrosis in humans (7). The model of continuous infusion of ANG II has been widely used in other organs, such as the kidney and the heart (14, 37). Also, it was previously demonstrated that this model is associated with hepatic inflammation, oxidative stress, and activation of profibrogenic mediators, such as TGF-β1 (3, 16). To determine whether the effect of atorvastatin was specific for the ANG II infusion model of inflammation, we confirmed the beneficial effects of atorvastatin in a well-characterized model of liver injury (CCl_4 administration). Besides, we tested the effects of atorvastatin in cultured HSC. This cell type plays a pivotal role in the hepatic wound healing response to injury (5). Moreover, HSC are an active source of free radicals during liver fibrogenesis and amplify the inflammatory response to injury (4, 8). Finally, there is strong evidence that ANG II is a powerful inflammatory and fibrogenic agonist for these cells (5, 19). Atorvastatin attenuated most of the pathogenic effects of ANG II in these cells. Further studies should investigate whether atorvastatin or other statins blunt the effects of ANG II on other nonparenchymal cell types such as Kupffer cells or sinusoidal endothelial cells.

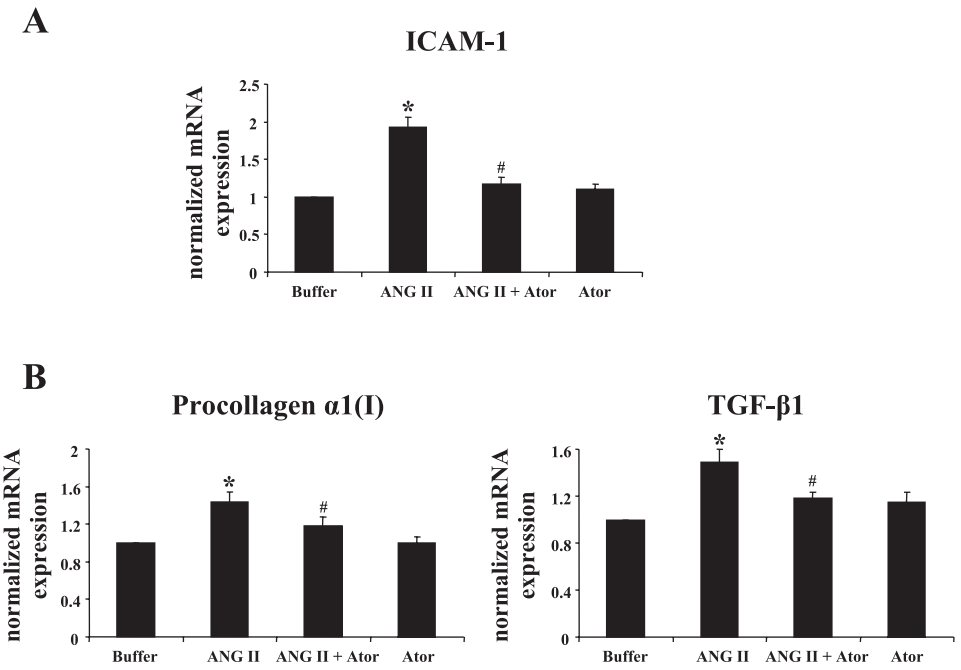


Fig. 5. Effect of Ator on ANG II-induced changes in gene expression in cultured HSC. Stimulation of HSC with ANG II (10^{-8}M, 24 h) resulted in a significant increase in ICAM-1 (*A*), procollagen-$\alpha_{1(I)}$, and TGF-β1 gene expression (*B*). Pretreatment with Ator (10^{-7}M, 20 min before ANG II and throughout the experiment) reduced the increase in ICAM-1, procollagen-$\alpha_{1(I)}$, and TGF-β1 expression ($^{*}P < 0.05$ vs. buffer, $^{\#}P < 0.05$ vs. ANG II). Results are means of 5 independent experiments.

Our results strongly suggest that statins exert anti-inflammatory effects in the liver. This effect was demonstrated in vivo and in cultured HSC. The mechanisms involved in this effect are largely unknown. Statins reduced the expression of proinflammatory cytokines, which promote recruitment of inflammatory cells (26). Moreover, atorvastatin reduced oxidative stress in the liver, which is an important event leading to hepatic inflammation (8). Finally, we recently demonstrated that statins decrease endothelial dysfuntion in rats with experimental cirrhosis, which is a pathogenic event linked to local inflammation and fibrogenesis (1). Besides this effect, we showed that atorvastatin attenuates the expression of procollagen-$\alpha_{1(I)}$ and reduced the accumulation of activated HSC. Further studies should investigate whether statins attenuate liver fibrogenesis.

A relevant result of this study is that atorvastatin reduces the prooxidant effects of ANG II in the liver. There is extensive evidence demonstrating that ANG II is a powerful prooxidant agent on the liver (4, 8). ANG II stimulates NADPH oxidase-derived reactive oxygen species generation in cultured HSC (8), and ANG II infusion induces hepatic oxidative stress in vivo (4). Importantly, mice lacking AT1 receptors do not develop oxidative stress following chronic liver injury, suggesting that local ANG II plays a key role in reactive oxygen species generation in chronically damaged livers (48). The antioxidant effects of statins have been previously suggested in different organs (25, 41). Inhibition of the small GTP-binding proteins, including Rac1, plays an important role in mediating the antioxidant effects of statins (25, 41). Membrane translocation of Rac1, which is required for the activation of NAD(P)H oxidase, is inhibited by atorvastatin in other organs (45). However, in our study, atorvastatin does not modulate ANG II-induced Rac1 activation in HSC. Further studies should investigate the molecular mechanisms involved in the antioxidant effect of atorvastatin.

At the cellular level, we explored the effects of atorvastatin on ANG II-induced biological effects in human primary HSC. Activated HSC proliferate and accumulate at the areas of active inflammation. This cell type plays a major role in the hepatic wound healing response to injury by promoting inflammation and fibrosis (2). ANG II is a powerful agonist for these cells, inducing cell growth and inflammatory and profibrogenic effects (5). We confirmed previous data that statins reduce proliferation of HSC (27, 36). Moreover, we provide evidence that atorvastatin reduce the inflammatory actions (IL-8 secretion and ICAM-1 expression) stimulated by ANG II (17, 23). This effect was associated with a reduction in ANG II-induced NF-κB activation (32). This biological effect of atorvastatin has been reported in hepatocytes (21). Importantly, we demonstrate that atorvastatin blunted the effect of ANG II on fibrogenic gene expression, including procollagen-$\alpha_{1(I)}$ and TGF-β1. Moreover, atorvastatin reduced the effects of ANG II in TGF-β1 cell release in HSC. These results are relevant, since HSC are the major source of collagen in the injured liver and play a pivotal role in liver fibrogenesis (2). Further studies should evaluate whether statins attenuate fibrosis in experimental models of chronic liver injury.

Taken together, our results demonstrated that ANG II exerts inflammatory properties in the liver, both in vivo and in vitro. Administration of atorvastatin reduced mainly the inflammatory effects of ANG II in vivo, as well as inflammatory and profibrogenic events in vitro. These results suggest that statins, besides their lipid-lowering properties, may exert beneficial effects in patients with chronic liver injury.

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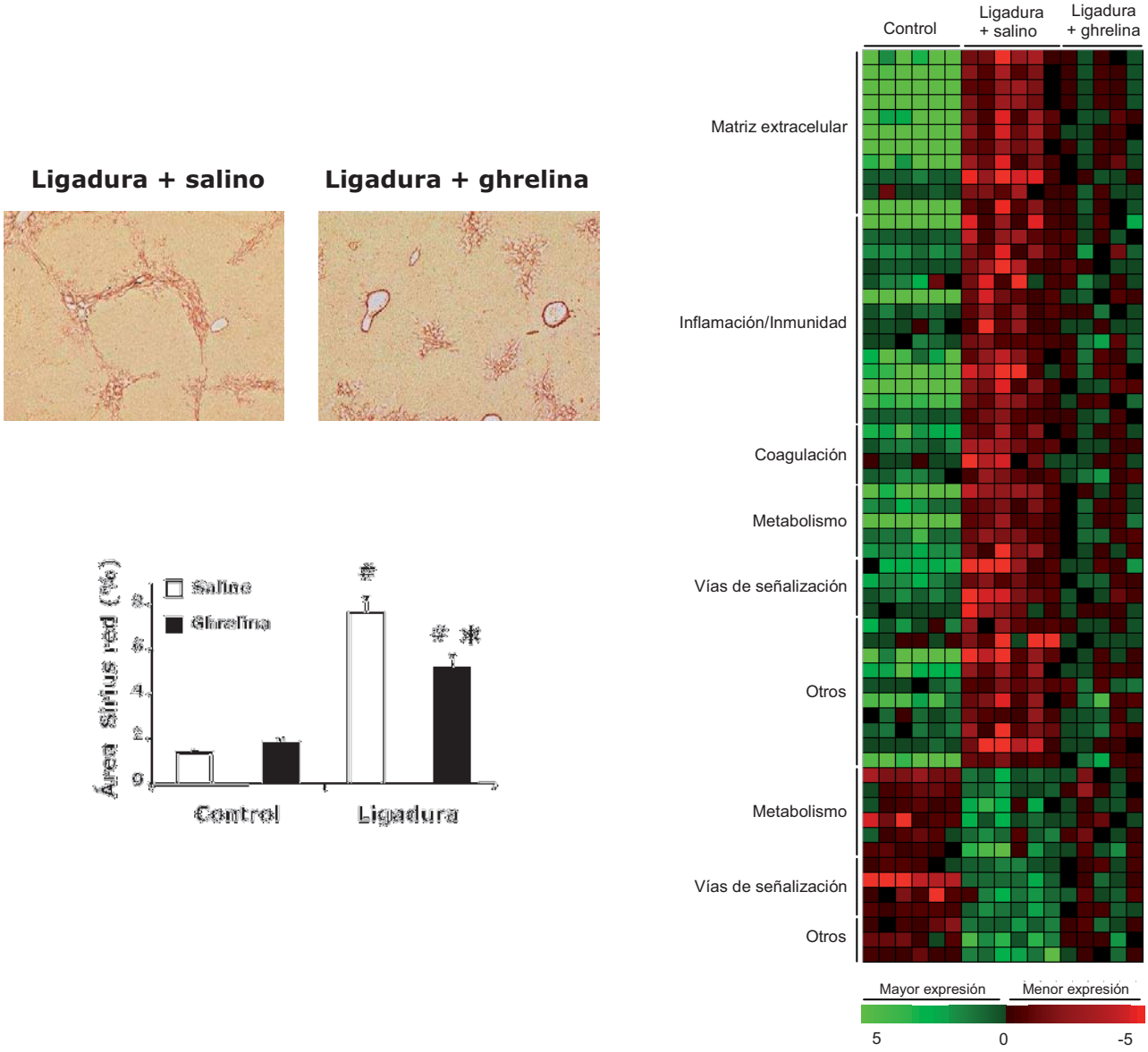
ESTUDIO 2. Efectos de la ghrelina sobre la inflamación y la fibrosis hepática

Este segundo trabajo es un estudio traslacional sobre el papel de la ghrelina en la inflamación y la fibrosis hepática que incluye análisis de muestras humanas, estudios en modelos animales y estudios con células estrelladas hepáticas. La ghrelina, que inicialmente se describió por sus efectos sobre la estimulación de la secreción de la GH y el apetito, se considera una diana terapéutica en diversas patologías que cursan con anorexia o caquexia (239;240). Además, por sus propiedades antiinflamatorias y protectoras de la muerte celular en células parenquimatosas, también se ha sugerido que podría ejercer efectos beneficiosos en pacientes con enfermedad pulmonar crónica o enfermedad cardíaca (213;218).

En este estudio se demuestra un efecto beneficioso de la ghrelina sobre la fibrosis inducida por la ligadura del conducto biliar. Por otro lado, se demuestra la importancia de la ghrelina producida por el propio organismo a la hora de modular la fibrosis inducida por el daño hepático (estudio de fibrosis en ratones deficientes en ghrelina). Además, se demuestra que la ghrelina también ejerce efectos beneficiosos sobre el daño hepático agudo inducido por la administración de CCl_4. Por otro lado, también se muestran algunos efectos antifibrogénicos de la ghrelina sobre células estrelladas hepáticas en cultivo y algunos datos que muestran su posible implicación en las enfermedades hepáticas (análisis de muestras procedentes de pacientes). Los principales resultados obtenidos son:

- **La administración de ghrelina recombinante atenúa el desarrollo de fibrosis hepática.** Se indujo fibrosis hepática mediante la ligadura del conducto biliar durante dos semanas en ratas Wistar. Durante ese periodo de tiempo se administró ghrelina o vehículo mediante minibombas osmóticas. La fibrosis hepática se determinó mediante diferentes técnicas. A partir de muestras hepáticas, se cuantificó el depósito de colágeno en el hígado (tinción con Sirius Red) así como la

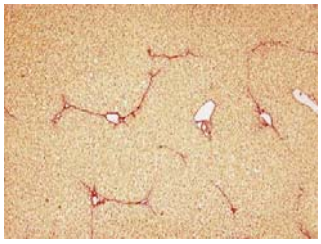
expresión de α-actina de músculo liso (inmunohistoquímica y *western blot*), y el contenido hepático de hidroxiprolina. Se observó que las ratas tratadas con ghrelina tenían un menor grado de fibrosis. Además, el tratamiento con ghrelina previno muchos de los cambios de expresión génica inducidos por la ligadura del conducto biliar como demostró el estudio de microarrays (principalmente genes implicados en inflamación, fibrosis y metabolismo). Estos resultados se muestran en las Figuras 1 y 2 y en la Tabla suplementaria 1 del segundo artículo:

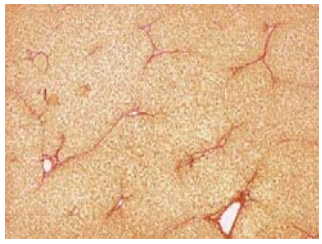


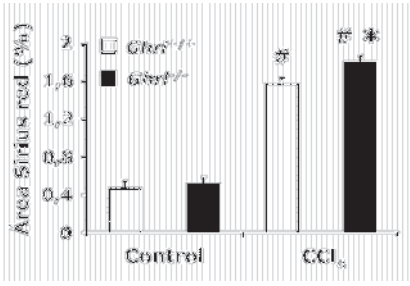
• **La administración de un agonista del receptor de la ghrelina previene la fibrosis hepática.** Se repitió el procedimiento de ligadura del conducto biliar administrando un agonista del receptor de la ghrelina, (Des-Ala^3)-GHRP-2, o su vehículo. El resultado fue que las ratas tratadas con el agonista mostraron un grado de fibrosis menor que las tratadas con vehículo (menor depósito de colágeno).

• **Los ratones deficientes en ghrelina son más susceptibles a desarrollar fibrosis hepática**. Se utilizó un modelo de fibrosis (administración de CCl_4 durante 4 semanas) en ratones deficientes en ghrelina (knockout) y salvajes. Los ratones deficientes en ghrelina fueron más susceptibles al daño inducido por el CCl_4 que los ratones salvajes (mayor depósito de colágeno, analizado por cuantificación de la tinción Sirius Red; mayor daño hepatocelular y mayor expresión de genes involucrados en fibrosis). Este resultado se muestran en la Figura 3 del segundo artículo:

Ratones salvajes **Ratones knockout**

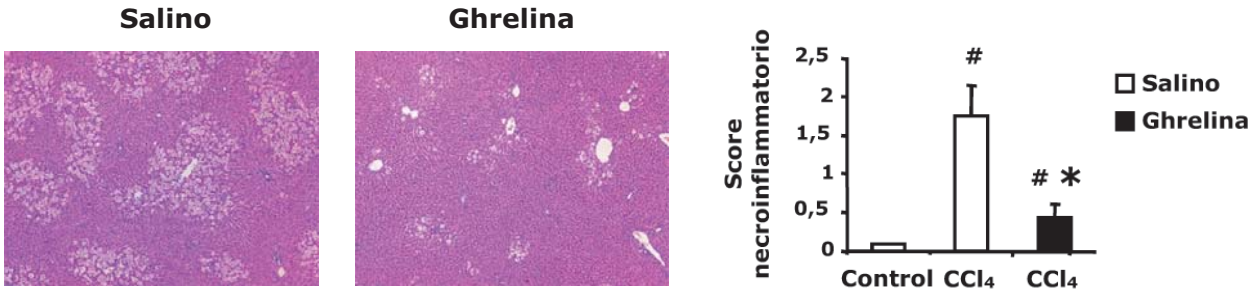




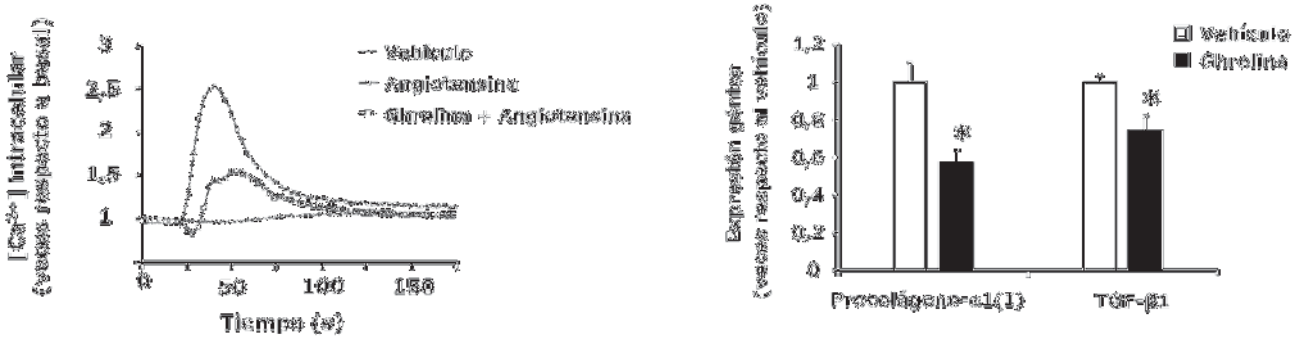


• **La administración de ghrelina previene el daño hepático agudo inducido por el CCl_4.** Se administró ghrelina (vía endovenosa) de manera preventiva una hora antes del tratamiento con CCl_4 (vía intraperitoneal) a ratas Wistar. Después de 24 horas de la administración del CCl_4, se sacrificaron los animales. Se analizó el daño necroinflamatorio (tinción con hematoxilina-eosina), la inflamación (número de células positivas para CD43), el estrés oxidativo (cuantificación de 4-hidroxinonenal), la apoptosis (TUNEL) y la expresión de p65. Las ratas que habían recibido ghrelina mostraron una disminución en todos estos parámetros de daño

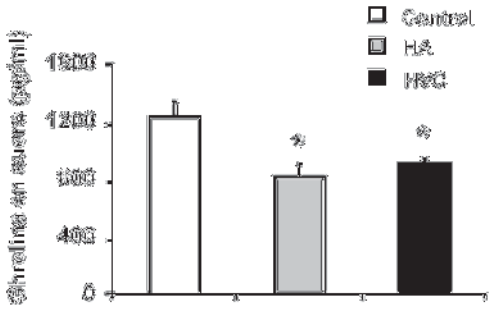
hepático. Además, también se observó un incremento en la actividad de Akt y ERK, dos vías intracelulares implicadas en la supervivencia de hepatocitos. Este resultado se muestra en la Figura 5 del segundo artículo:



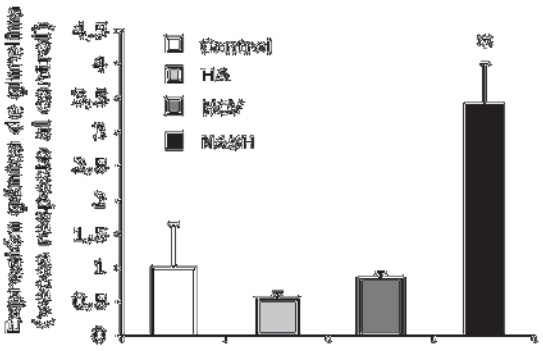
• **La ghrelina reduce las propiedades fibrogénicas de las HSC.** Se estimularon HSC con Ang II en presencia o ausencia de ghrelina y se analizó la concentración de calcio intracelular. Como era de esperar, la Ang II indujo un incremento en la concentración de calcio intracelular. La ghrelina inhibió este efecto. Por otro lado, la estimulación de las HSC con ghrelina redujo la expresión de genes involucrados en fibrosis (TGF-β y procolágeno-α1(I). Sin embargo, no modificó la actividad del factor de transcripción NF-κB (involucrado en inflamación) ni la secreción de IL-8. Estos resultados se muestran en la Figura 6 del segundo artículo:



• **Los niveles séricos de ghrelina se encuentran disminuidos en pacientes con hepatopatías.** Se analizaron muestras de pacientes con HA, HVC y NASH, así como de individuos sanos (control). Los niveles de ghrelina sérica en ayunas fueron menores en los tres grupos de pacientes comparado con los individuos control. Además, los niveles fueron menores en los casos de fibrosis avanzada en comparación con los pacientes con fibrosis leve. Este resultado se muestra en la Figura 7 del segundo artículo:



• **La expresión hepática de ghrelina está aumentada en NASH.** Se analizó la expresión hepática de ghrelina mediante PCR cuantitativa en pacientes con HA, HVC y NASH así como en muestras de hígado sano (control). Los pacientes con NASH mostraron una expresión de ghrelina mayor que las muestras control. Por otro lado, la expresión de ghrelina se correlacionó positivamente con la expresión de genes implicados en inflamación y fibrosis. Este resultado se muestra en la Figura 7 y la Tabla suplementaria 2 del segundo artículo:



• **Dos polimorfismos del gen de la ghrelina se asocian a fibrosis avanzada en pacientes con HVC.** Se analizaron seis polimorfismos del gen de la ghrelina en 284 pacientes con HVC. Se compararon los diferentes genotipos entre los pacientes con fibrosis leve y los pacientes con fibrosis avanzada. Se encontró que un genotipo y un haplotipo de dos loci se asociaban con fibrosis avanzada, especialmente en mujeres. Estos resultados se muestran en la Tabla 1 del segundo artículo:

Polimorfismo -994 CT				
Sexo	**Genotipo**	**n (%)**		**Odd ratio**	**p**
		F0-F2	**F3-F4**	**(95% IC)**	
Todos	CC	134 (84.3)	93 (74.4)	1.00	--
	TT + CT	25 (15.7)	32 (25.6)	1.79 (0.96-3.37)	.068
Mujeres	CC	55 (90.2)	29 (69)	1.00	--
	TT+ CT	6 (9.8)	13 (31)	**9.75 (1.34-71.05)**	**.010**
Hombres	CC	79 (80.6)	64 (77.1)	1.00	--
	TT+ CT	19 (19.4)	19 (22.9)	1.01 (0.47-2.19)	.981

Haplotipo					
Sexo	**-994 CT**	**-604 GA**	**n (%)**		**Odd ratio**	**p**
			F0-F2	**F3-F4**	**(95% IC)**	
Todos	C	A	82 (51.64)	63 (50.4)	1.00	--
	C	G	64 (40.5)	44 (35.6)	0.97 (0.66-1.41)	.850
	T	A	11 (7.17)	17 (14.0)	**2.06 (1.08-3.91)**	**.028**
	T	G	1 (0.7)	0 (0.0)	0.00	1.00
Mujeres	C	A	32 (51.64)	21 (51.19)	1.00	--
	C	G	26 (43.44)	13 (30.95)	0.96 (0.35-2.66)	.943
	T	A	3 (4.92)	8 (17.86)	**8.47 (1.31-54.84)**	**.029**
	T	G	0 (0.00)	0 (0.00)	0.00	1.00
Hombres	C	A	51 (51.82)	42 (50.00)	1.00	--
	C	G	38 (38.49)	31 (37.95)	1.12 (0.61-2.05)	.712
	T	A	8 (8.39)	10 (12.05)	1.40 (0.48-4.05)	.54
	T	G	1 (1.31)	0 (0.00)	1.08 (0.00-1088)	.982

Ghrelin Attenuates Hepatocellular Injury and Liver Fibrogenesis in Rodents and Influences Fibrosis Progression in Humans

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There are no effective antifibrotic therapies for patients with liver diseases. We performed an experimental and translational study to investigate whether ghrelin, an orexigenic hormone with pleiotropic properties, modulates liver fibrogenesis. Recombinant ghrelin was administered to rats with chronic (bile duct ligation) and acute (carbon tetrachloride) liver injury. Hepatic gene expression was analyzed by way of microarray analysis and quantitative polymerase chain reaction. The hepatic response to chronic injury was also evaluated in wild-type and ghrelin-deficient mice. Primary human hepatic stellate cells were used to study the effects of ghrelin *in vitro*. Ghrelin hepatic gene expression and serum levels were assessed in patients with chronic liver diseases. Ghrelin gene polymorphisms were analyzed in patients with chronic hepatitis C. Recombinant ghrelin treatment reduced the fibrogenic response, decreased liver injury and myofibroblast accumulation, and attenuated the altered gene expression profile in bile duct–ligated rats. Moreover, ghrelin reduced the fibrogenic properties of hepatic stellate cells. Ghrelin also protected rats from acute liver injury and reduced the extent of oxidative stress and inflammation. Ghrelin-deficient mice developed exacerbated hepatic fibrosis and liver damage after chronic injury. In patients with chronic liver diseases, ghrelin serum levels decreased in those with advanced fibrosis, and ghrelin gene hepatic expression correlated with expression of fibrogenic genes. In patients with chronic hepatitis C, polymorphisms of the ghrelin gene (−994CT and −604GA) influenced the progression of liver fibrosis. *Conclusion:* Ghrelin exerts antifibrotic effects in the liver and may represent a novel antifibrotic therapy. (HEPATOLOGY **2010;51:974-985.**)

Hepatic fibrosis is the progressive accumulation of extracellular matrix that occurs in most types of chronic liver diseases. In patients with advanced fibrosis, liver cirrhosis ultimately develops. Currently, the only effective therapy to treat liver fibrosis is to eliminate the causative agent (e.g. successful antiviral therapy in patients with chronic hepatitis C). For those patients in whom the underlying cause cannot be removed, there are no effective antifibrotic therapies. During recent years, research has focused on molecular and cellular mechanisms involved in liver fibrosis, and many pharmacological interventions have been successfully tested in experimental models of liver fibrosis.[1] However, most of the information derives from the experimental setting,

Abbreviations: α-SMA, α-smooth muscle actin; BDL, bile duct ligation; CCl_4, carbon tetrachloride; $Ghrl^{-/-}$, ghrelin knockout; $Ghrl^{+/+}$, ghrelin wild-type; GHS-R, growth hormone secretagogue receptor; HSC, hepatic stellate cell; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α; TUNEL, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling.

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while translational studies with human samples and clinical trials are scarce. In the current study, we used both experimental and translational approaches to characterize a new potential antifibrotic substance for patients with chronic liver diseases.

Ghrelin is a gut hormone (28 amino acids) firstly discovered as a potent growth hormone secretagogue. Moreover, it plays a major role in the regulation of food intake.[2] Recently, peripheral effects such as cytoprotection, vasodilatation, regulation of energy balance, and gastrokinesis have been also attributed to ghrelin.[3] The primary site of ghrelin synthesis is the stomach, but ghrelin transcripts have been detected in many other organs, including the liver, bowel, pancreas, kidneys, and lungs.[4] Most ghrelin actions are mediated by growth hormone secretagogue receptor (GHS-R),[2] which is mainly expressed in the pituitary gland but also in other organs, including the pancreas, spleen, and adrenal gland.[4] However, ghrelin probably binds to another yet unknown receptor, because cells not expressing GHS-R respond to ghrelin stimulus.[5]

Recent data indicate that ghrelin has protective effects in different organs and cell types including the pancreas, heart, and gastrointestinal tract.[6-8] Recombinant ghrelin has been successfully administered to patients with a variety of disorders such as anorexia,[9] caquexia,[10] and gastroparesis.[11] Moreover, ghrelin reduces muscle wasting and improves functional capacity in elderly patients with congestive heart failure and chronic obstructive pulmonary disease.[12,13] We hypothesize that ghrelin regulates hepatic injury and fibrogenesis. To prove this hypothesis, we investigated the effect of recombinant ghrelin in different models of acute and chronic liver injury. Moreover, we evaluated whether changes in endogenous ghrelin regulate hepatic fibrosis in mice and in patients with chronic liver diseases due to hepatitis C virus infection. We provide evidence that recombinant ghrelin exerts protective and antifibrotic effects in the injured liver. Our results also suggest that endogenous ghrelin plays a role in hepatic fibrogenesis, because ghrelin knockout ($Ghrl^{-/-}$) mice are more susceptible to carbon tetrachloride (CCl_4)-induced liver injury than ghrelin wild-type ($Ghrl^{+/+}$) mice. Moreover, we demonstrate that ghrelin is locally produced in the human liver.

Materials and Methods

Chronic Liver Injury Models in Rodents. Male Wistar rats (250 g) were induced to chronic liver injury and hepatic fibrosis by prolonged bile duct ligation (BDL) as described.[14] Either saline, rat recombinant ghrelin (Phoenix Pharmaceuticals; Burlingame, CA), or ghrelin receptor agonist (Des-Ala^3-GHRP-2) (Bachem; Bubendorf, Switzerland) were administered to rats through a subcutaneous osmotic minipump (Alza Corporation; Palo Alto, CA) at a rate of 200 μL/hour^{-1} throughout the experiment. Doses were chosen from existing data in the literature. Preliminary studies in rats with advanced fibrosis (CCl_4 for 8 weeks) were performed to assess the tolerability of both ghrelin and (Des-Ala^3-GHRP-2). The selected doses for the peptides (10 $\mu g \cdot kg^{-1} \cdot day^{-1}$ for recombinant ghrelin and 30 $\mu g/kg^{-1}/d^{-1}$ for Des-Ala^3-GHRP-2) were well tolerated and did not cause arterial hypotension. Experimental groups were as follows (n = 12 per group): rats with BDL or sham-operated rats infused with saline, recombinant rat ghrelin, or the ghrelin receptor agonist (Des-Ala^3-GHRP-2). $Ghrl^{-/-}$ mice (C57BL/6 background) were obtained from Regeneron Pharmaceuticals (Tarrytown, NY). The generation and characterization of these mice has been described extensively.[15] We used mice aged 8 to 10 weeks. Because C57BL/6 mice develop biliary infarcts early and have a high rate of mortality following BDL,[16] we used a different experimental model to induce chronic liver injury and hepatic fibrosis. CCl_4 (Sigma-Aldrich; St. Louis, MO) was administered intraperitoneally at a dose of 1 mL/kg^{-1}, 12.5% diluted in olive oil (Sigma-Aldrich) twice a week for 4 weeks. Control mice were given olive oil at the same dose. Each group included at least 12 mice. Rats and mice were housed in temperature and humidity-controlled rooms and kept on a 12-hour light/dark cycle. Animal procedures were approved by the Ethics Committee of Animal Experimentation of the University of Barcelona and were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Assessment of Hepatic Necroinflammatory Injury and Fibrosis. Paraffin-embedded liver sections were stained with hematoxylin-eosin. Hepatic necroinflamma-

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Additional Supporting Information may be found in the online version of this article.

tion was estimated by quantifying the presence of necrosis, hepatocyte ballooning and/or swelling, inflammatory cell infiltration, and lipid droplets. The degree of necroinflammatory changes was assessed as the percentage of hepatic parenchyma with any of the aforementioned changes: 1, <30%; 2, 30-60%; 3, >60%. Analyses were blindly performed by an expert pathologist (L. N. R.). To assess liver fibrosis, liver specimens were stained with picrosirius red (Gurr-BDH Lab Supplies; Poole, England). The positive area stained with picrosirius red was quantified using a morphometric method. Briefly, six images per specimen were obtained with an optic microscope (Nikon Corporation; Tokyo, Japan) at a magnification of ×40. Images were imported to an image analysis software (AnalySIS, Olympus; Münster, Germany) and automatically merged.

Acute Liver Injury Model in Rats. Acute liver injury was induced in male Wistar rats (250 g) through a single intraperitoneal injection of CCl_4 (Sigma-Aldrich; 1 mL/kg^{-1} body weight, 30% diluted in olive oil). Control rats received the same amount of olive oil. Animals were treated with either saline or 20 $\mu g/kg^{-1}$ rat recombinant ghrelin (Phoenix Pharmaceuticals) intravenously 1 hour before CCl_4 administration. Rats were divided into three experimental groups (n = 8 per group): rats receiving saline and olive oil, rats receiving saline and CCl_4, and rats receiving ghrelin and CCl_4. Twenty-four hours after intraperitoneal injection, animals were anesthetized and sacrificed for blood and tissue sample collection. Rats were housed in temperature- and humidity-controlled rooms and kept on a 12-hour light/dark cycle. Animal procedures were conducted in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Human Samples. For analysis of ghrelin serum levels, blood samples from patients with chronic hepatitis C (n = 67) and alcoholic hepatitis (n = 24) were obtained. Moreover, samples from healthy controls (n = 24) matched for age, sex, and body mass index with patients were collected. Blood samples were obtained after an overnight fasting. Hepatic gene expression was assessed in liver specimens obtained by a transjugular approach from patients with alcoholic hepatitis (n = 37) and by a percutaneous approach in patients with chronic hepatitis C (n = 45) and in patients with nonalcoholic steatohepatitis (n = 23). Normal liver specimens (n = 5) were obtained from fragments of resections of colon metastases before the vascular clamping as described.[17] For the analysis of the role of variations of the ghrelin gene on the progression of liver fibrosis, DNA from patients with chronic hepatitis C (n = 284) was obtained from peripheral blood. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of the Hospital Clínic of Barcelona. All patients gave informed consent.

Data Analysis. Data are representative of at least three independent experiments. Results are expressed as the mean ± standard error of the mean (SEM). The normality of the data was assessed by the Kolmogorov-Smirnov test. Comparisons between groups were performed using the Student *t* test or nonparametric Mann-Whitney test depending on the normality of data. Statistical analysis of correlations was performed by Spearman rho. P values < 0.05 were considered significant. For multiple comparisons, Bonferroni correction was applied to P values, with significance set at $P < 0.001$.

Other methods are shown in Supporting Materials and Methods.

Results

Liver Fibrosis is Reduced in Rats Treated with Recombinant Ghrelin. To investigate whether recombinant ghrelin regulates hepatic fibrogenesis following chronic liver injury, a model of secondary biliary fibrosis was induced in rats through prolonged ligation of the common bile duct. Both BDL or sham-operated rats were continuously infused with either saline or recombinant ghrelin through a subcutaneous osmotic pump for 2 weeks. BDL rats infused with saline showed severe septal hepatic fibrosis with a marked disruption of the hepatic architecture (Fig. 1A). Hepatic collagen content was increased over seven-fold compared with control rats. In contrast, BDL rats infused with ghrelin had only mild collagen deposition without formation of bridging fibrosis. Morphometric analysis revealed that ghrelin decreased collagen deposition by about 40%. To uncover the mechanisms underlying this beneficial effect, we first investigated whether ghrelin modulates the accumulation of myofibroblastic fibrogenic cells (α-smooth muscle actin [α-SMA]–positive cells). Myofibroblastic cells accumulated markedly throughout the hepatic parenchyma in BDL rats. Ghrelin treatment reduced the amount of fibrogenic cells by 25% (Fig. 1B). Moreover, ghrelin treatment decreased α-SMA protein expression, as assessed by western blotting (Fig. 1C) and hepatic content of hydroxyproline (Fig. 1D). In addition, ghrelin infusion reduced the elevation of serum aspartate aminotransferase levels, a parameter indicative of hepatocellular damage, induced by BDL (Fig. 1E). Because ghrelin stimulates guanosin 3',5'-cyclic monophosphate production in other tissues,[18] we next studied whether the beneficial effect of ghrelin is associated with increased guanosin

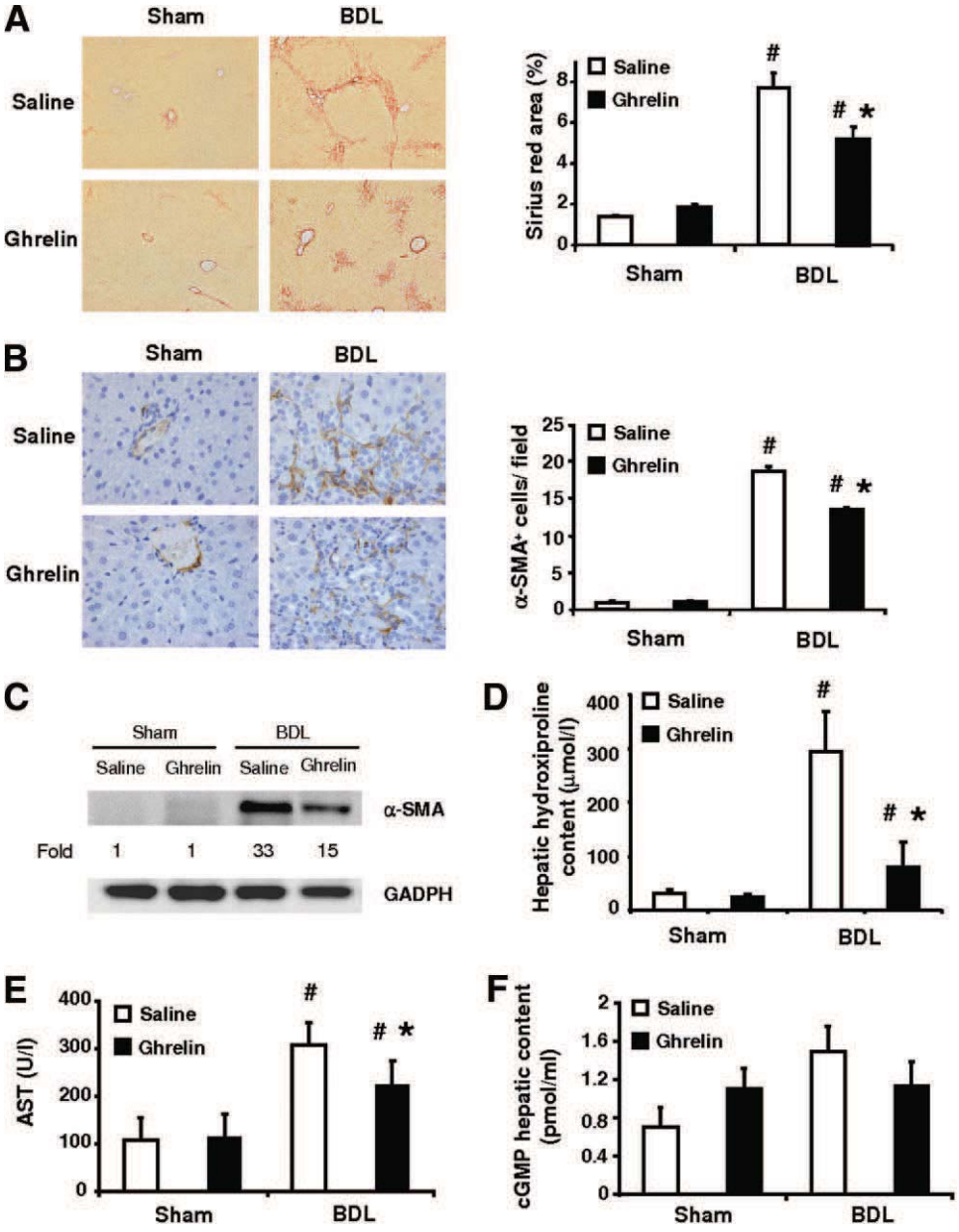

Fig. 1. Ghrelin reduces hepatic fibrosis induced by BDL in rats. (A) Representative pictures of Sirius red staining (magnification ×40) from rats submitted to BDL or sham-operated rats treated with saline or ghrelin. Graph shows quantification of Sirius red–stained area. (B) Representative pictures and quantification of α-SMA–positive cells (magnification ×400). (C) Representative western blotting for α-SMA in liver samples. (D) Hydroxyproline content in liver samples from sham-operated rats or rats submitted to BDL treated or not with ghrelin. (E) Aspartate aminostransferase serum levels from all groups of rats. (F) Guanosin 3',5'-cyclic monophosphate hepatic content in liver extracts from all groups of rats. Data shown are the means from at least 10 animals per group; error bars show SEM. $^{\#}P < 0.05$ (sham-operated rats). $^{*}P < 0.05$ (saline-BDL rats).

3',5'-cyclic monophosphate hepatic content. We did not find differences between any of the groups (Fig. 1F).

Recombinant Ghrelin Prevents Changes in Hepatic Gene Expression During Liver Fibrogenesis. To explore the effects induced by ghrelin in the fibrotic liver, we analyzed changes in hepatic gene expression by way of complementary DNA microarray analysis. BDL stimulated the hepatic expression of 1,543 genes and repressed the expression of 997 genes compared with sham-operated rats. Ghrelin treatment attenuated changes in the expression of 231 genes including collagen-α1(II), plasminogen activator-urokinase receptor, matrix metallopeptidase 2 and chemokine receptor 5 (Fig. 2A). A list of all the genes modified by ghrelin treatment is shown in Supporting Table 1. The complete dataset is available at the National Center for Biotechnology Information's Gene Expression Omnibus public database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE13747. Quantitative polymerase chain reaction confirmed the changes found in microarray analysis in some selected genes (Fig. 2B). Rat liver samples were clusterized depending on gene expression profile. Rats were perfectly classified in the different experimental groups. A heatmap of the clustering can be seen in Supporting Fig. 1.

Increased Liver Injury and Fibrogenesis in Ghrl$^{-/-}$ Mice. To investigate the role of endogenous ghrelin in liver fibrogenesis, we next analyzed the fibrogenic response in Ghrl$^{-/-}$ and *Ghrl*$^{+/+}$ mice. Chronic liver injury was induced by intraperitoneal injections of CCl_4 twice a week for 4 weeks. The extent of liver fibrosis was assessed

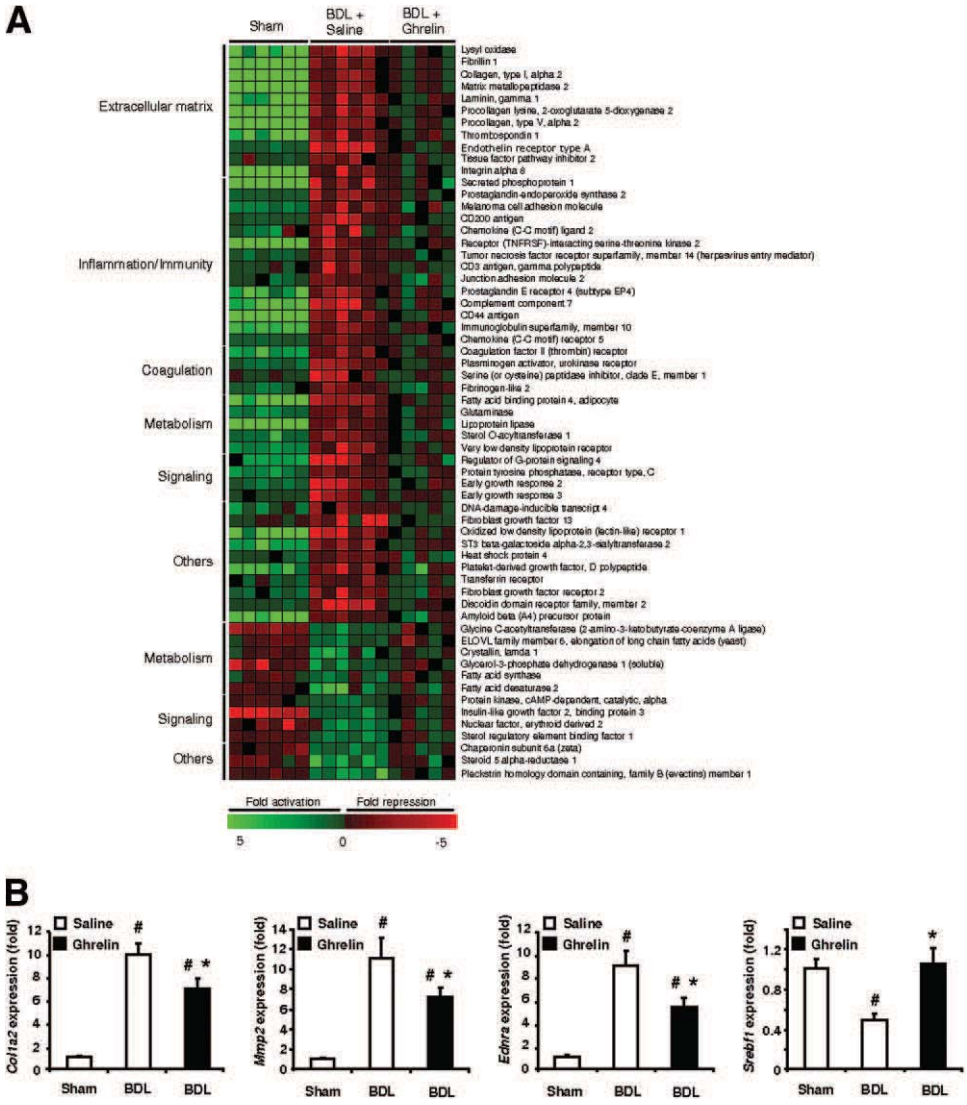

Fig. 2. Hepatic gene expression in rats submitted to sham operation or BDL. (A) Microarray data from hepatic complementary DNA. Expression of key genes was modified by BDL. Ghrelin treatment attenuated changes in gene expression profile. All genes have a false discovery rate <0.2 and are deviated from the control by at least ± 1.8-fold. (B) Quantitative polymerase chain reaction confirmed the results obtained in the microarray analysis in procollagen-α1(II) (*Col1a2*), matrix metallopeptidase 2 (*Mmp2*), endothelin receptor type A (*Ednra*), and sterol regulatory element-binding factor 1 (*Srebf1*). Data shown are expressed as the mean from at least 5 animals per group; error bars show SEM. $^{\#}P < 0.05$ (sham-operated rats). $^{*}P < 0.05$ (saline-BDL rats).

in both groups of mice. We found that *Ghrl*$^{-/-}$ mice were more susceptible to CCl_4-induced liver fibrosis and liver injury than *Ghrl*$^{+/+}$ mice, as indicated by increased collagen deposition (Fig. 3A,B) and increased necroinflammatory score (Fig. 3C). Moreover, *Ghrl*$^{-/-}$ mice treated with CCl_4 showed a reduced weight gain compared with *Ghrl*$^{+/+}$ mice (Fig. 3D). In addition, procollagen-α2(I) and TIMP1 expression were overexpressed in *Ghrl*$^{-/-}$ mice treated with CCl_4 compared with *Ghrl*$^{+/+}$ littermates (Fig. 3E,F).

A GHS-R Agonist Attenuates Liver Fibrosis. We first analyzed the expression of GHS-R in human and rat liver samples by way of polymerase chain reaction. We found transcripts of GHS-R in both human and rat livers (Fig. 4A,B). Specifically, we detected GHS-R expression in human hepatocyes and activated hepatic stellate cells (HSCs) but not in quiescent HSCs (Fig. 4B). To investigate whether stimulation of GHS-R attenuates liver fibrosis new groups of rats were submitted to BDL or sham operation in the presence or absence of a GHS-R agonist (Des-Ala^3-GHRP-2) for 2 weeks. We found that the degree of liver fibrosis was reduced in rats treated with the GHS-R agonist, as indicated by decreased collagen deposition (Fig. 4C,D).

Recombinant Ghrelin Reduces Hepatocellular Injury in a Model of Acute Liver Injury in Rats. The results in BDL rats suggest that ghrelin may attenuate fibrosis by exerting a hepatoprotective effect. To prove this hypothesis, we analyzed the effects of ghrelin in a model of acute liver injury in rats (single intraperitoneal administration of CCl_4). Ghrelin or vehicle were administered to rats intravenously 1 hour before CCl_4. Pretreatment with ghrelin, but not saline, strongly reduced the hepatocellular injury induced by CCl_4, as indicated by decreased necroinflammatory score (Fig. 5A) and aspartate aminotransferase serum levels (170 and 90 IU/L in CCl_4-damaged rats in the absence and the presence of ghrelin, respectively, $P < 0.05$). This beneficial effect was

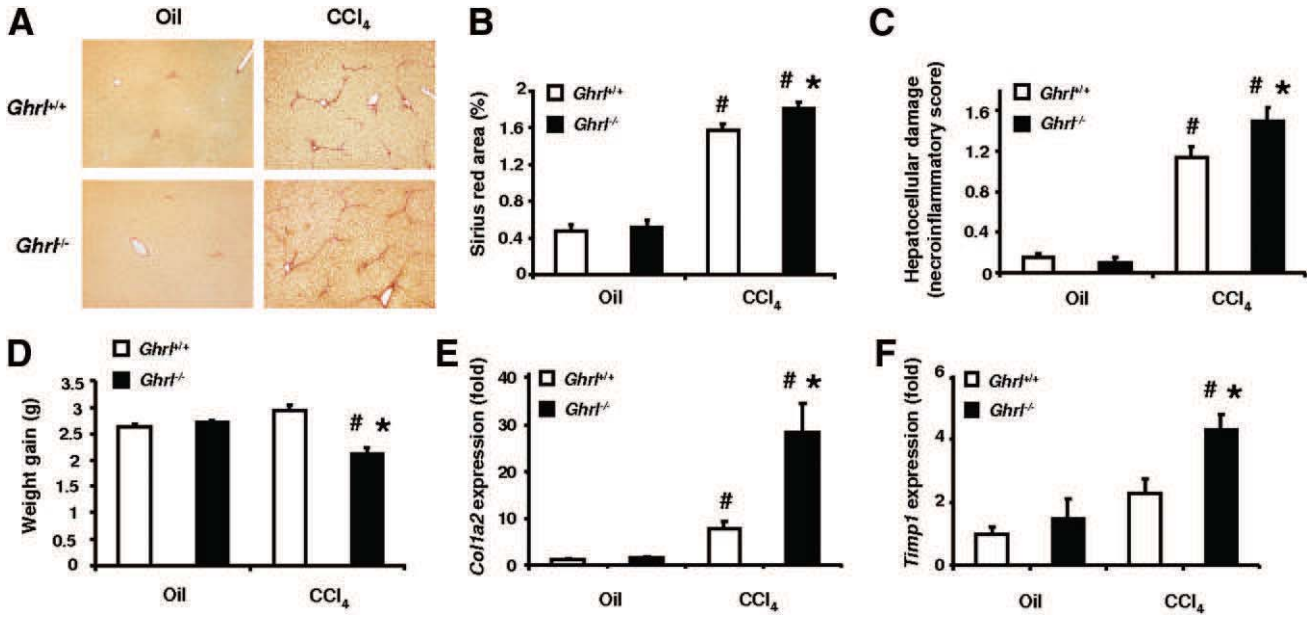

Fig. 3. Role of endogenous ghrelin in liver fibrosis in mice. $Ghrl^{+/+}$ and $Ghrl^{-/-}$ mice were induced to liver fibrosis by administration of CCl_4 for 4 weeks. $Ghrl^{-/-}$ mice show a modest increase in the extent of liver fibrosis and increased liver damage after chronic liver injury induced by CCl_4 compared with $Ghrl^{+/+}$. (A) Representative pictures and (B) quantification of Sirius red staining (magnification ×40) from $Ghrl^{+/+}$ and $Ghrl^{-/-}$ mice treated chronically with oil or CCl_4. (C) Necroinflammatory score of liver samples from $Ghrl^{+/+}$ and $Ghrl^{-/-}$ mice chronically treated with oil or CCl_4. (D) Weight increase during the 4 weeks of CCl_4 treatment in all groups of mice. (E,F) Gene expression of genes involved in fibrogenesis. Procollagen-α2(I) and tissue inhibitor of metalloproteases (TIMP-1) were overexpressed in $Ghrl^{-/-}$ mice induced to liver fibrosis when compared with $Ghrl^{+/+}$ mice. Data shown are expressed as the mean from at least 10 animals per group; error bars show SEM. $^{\#}P < 0.05$ (oil-treated mice). $^{*}P < 0.05$ (CCl_4–wild-type mice).

associated with decreased infiltration of inflammatory cells, as assessed by quantification of infiltrating leukocytes (CD43-positive cells) in liver sections ($P < 0.05$, Fig. 5B). Because oxidative stress mediates CCl_4-induced hepatocellular injury, we also explored whether ghrelin reduces this pathogenic event by quantifiying 4-hydroxynonenal protein adducts. As shown in Fig. 5C, ghrelin attenuated the accumulation of 4-hydroxynonenal in hepatocytes. We next explored the effects on hepatocyte cell death by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) analysis. Ghrelin diminished the number of TUNEL-positive hepatocytes, indicating that it reduces cell apoptosis (Fig. 5D). This effect was associated with decreased activation of nuclear factor κB, as assessed by p65 nuclear translocation (Fig. 5E). Moreover, ghrelin treatment at-

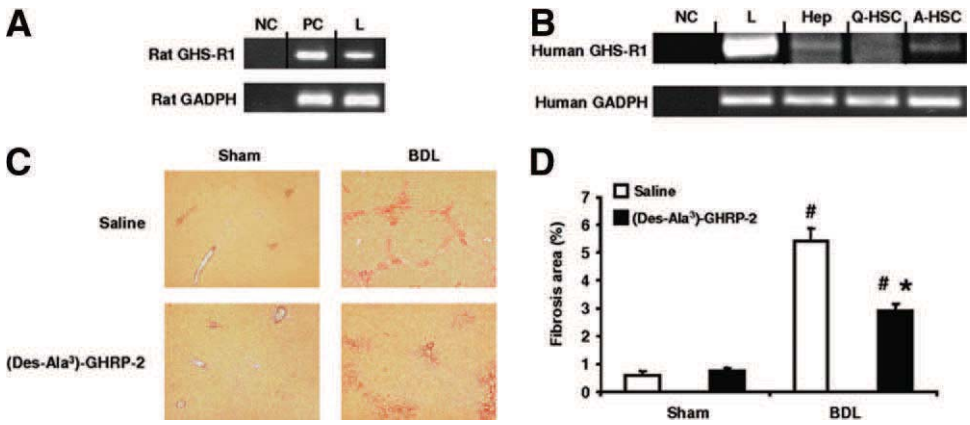

Fig. 4. Effects of a GHS-R agonist, (Des-Ala3)-GHRP-2, on experimental liver fibrosis. Expression of GHS-R was detected in (A) rat and (B) human livers. NC, negative control; PC, positive control; L, liver; Hep, hepatocytes; Q-HSC, quiescent HSCs; A-HSC, activated HSCs. A GHS-R agonist, (Des-Ala3)-GHRP-2, was infused in sham-operated rats and rats with BDL during the 2 weeks of the experiment. (C) Representative pictures and (D) quantification of the area stained by Sirius red (magnification ×40). Data shown are expressed as the mean ± SEM from 8 rats per group. $^{\#}P < 0.05$ (sham-operated rats). $^{*}P < 0.05$ (saline-BDL rats).

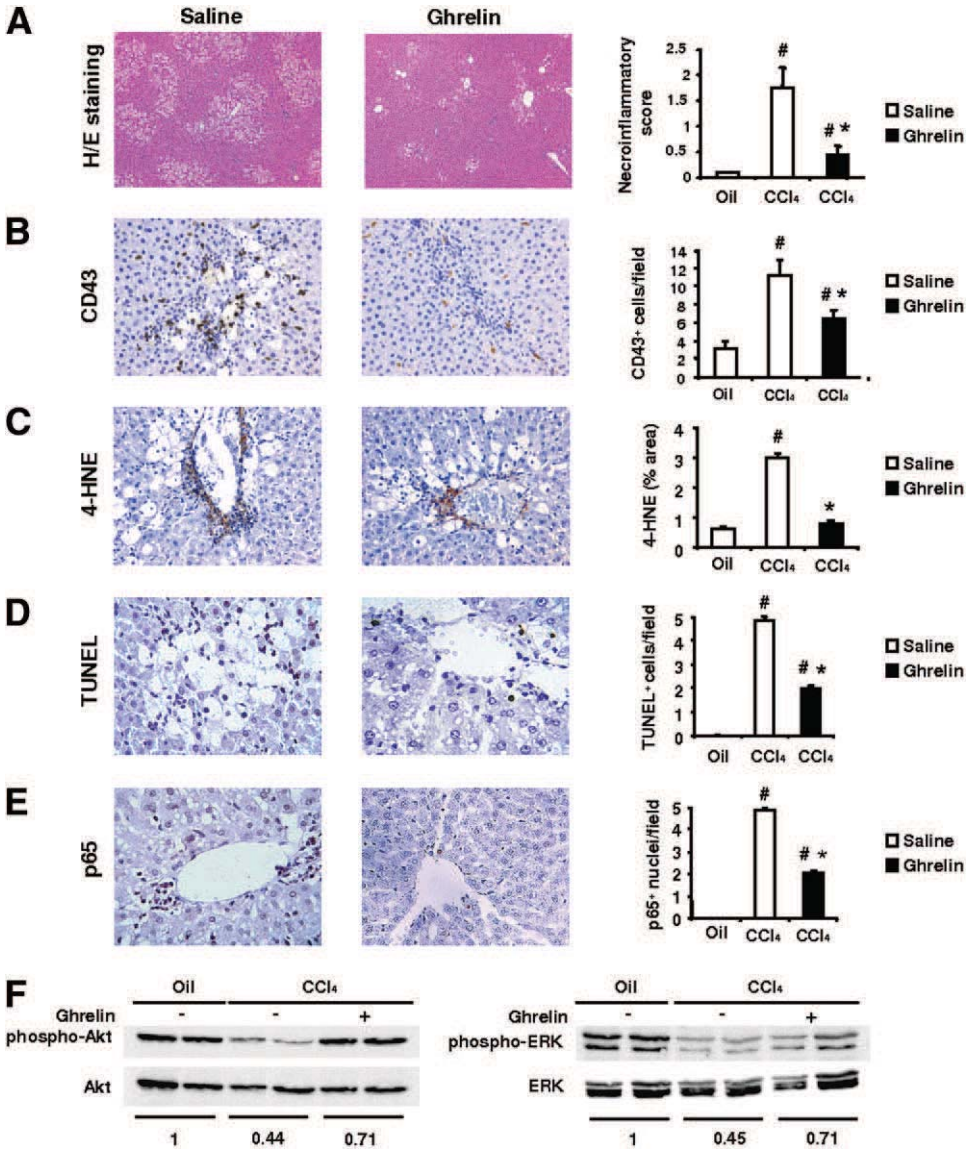

Fig. 5. Ghrelin exerts hepatoprotective effects in rats with acute liver injury induced by CCl_4. Rats received ghrelin (20 $\mu g/kg^{-1}$) intravenously 1 hour before CCl_4 administration. (A) Representative pictures of hematoxylin-eosin staining in livers from CCl_4-injured rats treated with saline or ghrelin (magnification $\times$200). CCl_4 induced hepatocyte ballooning, parenchymal necrosis, and inflammatory infiltrate. Graph shows evaluation of the necroinflammatory score. (B) Representative pictures of CD43 immunostaining in CCl_4-treated rats (magnification $\times$400). Graph shows quantification of CD43-positive cells per field (magnification $\times$200). (C) Representative pictures of 4-hydroxynonenal immunostaining in CCl_4-treated rats (magnification $\times$400). Quantification of the area stained is shown in the graph. (D) Representative pictures of TUNEL immunostaining in CCl_4-treated rats (magnification $\times$400). Graph shows quantification of TUNEL-positive cells per field (magnification $\times$400). (E) Representative pictures of p65 immunostaining (magnification $\times$400). Graph shows quantification of p65-positive nuclei per field (magnification $\times$400). $^{\#}P < 0.05$ (control). $^{*}P < 0.05$ (rats receiving saline-CCl_4). (F) Intracellular pathways involved in CCl_4-induced liver damage and ghrelin hepatoprotection. Western blot analyses showing Akt and extracellular signal-regulated kinase phosphorylation in extracts from rat livers. Numbers underneath represent fold expression compared with oil-treated rats. Data are expressed as the mean $\pm$ SEM from 8 animals per group.

tenuated the effects of CCl_4 on Akt and extracellular signal-regulated kinase phosphorylation, two intracellular pathways involved in hepatocyte survival and proliferation (Fig. 5F). All together, these results indicate that ghrelin exerts hepatoprotective effects.

Ghrelin Modulates Fibrogenic, But Not Proinflammatory, Properties of Hepatic Stellate Cells. To further elucidate possible mechanisms of the protective effects of ghrelin in the liver, we next investigated whether ghrelin modulates the fibrogenic actions of HSCs, the main fibrogenic cell type in the injured liver.[1] Stimulation of primary cultured HSCs with angiotensin II (0.1 μM), a well-known fibrogenic agonist, resulted in a marked increase in intracellular calcium concentration ($[Ca^{2+}]_i$). Preincubation with ghrelin (0.1 μM) for 10 minutes attenuated angiotensin-II–induced $[Ca^{2+}]_i$ increase (Fig. 6A). Ghrelin (0.1 μM) also reduced by 40% the expression of collagen-α1(I) and transforming growth factor-β1 in unstimulated HSCs (Fig. 6B). We then investigated whether ghrelin inhibits the proinflammatory actions of HSCs. Ghrelin did not modulate the activation of nuclear factor κB or the release of interleukin-8 (Fig. 6C and 6D, respectively). These results indicate that ghrelin reduces the fibrogenic but not the inflammatory properties of cultured HSCs.

Serum Ghrelin Levels and Hepatic Ghrelin Expression in Patients with Chronic Liver Diseases. To analyze the potential role of ghrelin in chronic human liver diseases, serum ghrelin concentration was measured in control subjects (n = 24) and in patients with liver fibrosis including alcoholic hepatitis (n = 24) and chronic hepatitis C (n = 67). Serum ghrelin levels were significantly lower in both patients with alcoholic hepatitis and chronic hepatitis C compared with control subjects, after adjusting for age, sex, and body mass index (Fig. 7A).

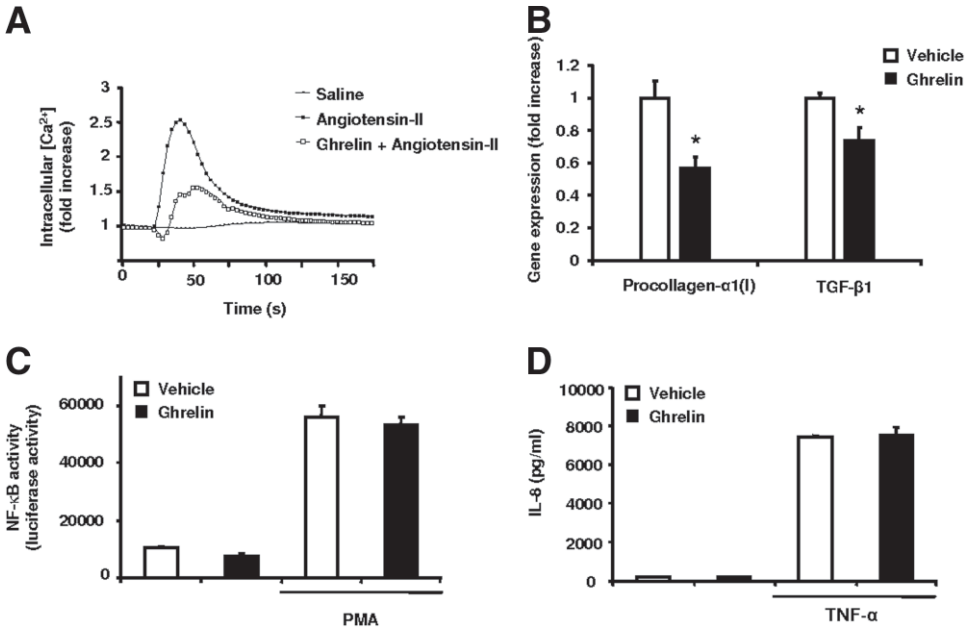

Fig. 6. Effects of ghrelin on profibrogenic and proinflammatory properties in primary human HSCs. (A) Intracellular calcium concentration ([Ca^{2+}]) as evidenced by Fura 2 intensity in HSCs. Cells were preincubated for 10 minutes with ghrelin (0.1 μmol/L) and then challenged with angiotensin II (0.1 μmol/L). (B) Expression of procollagen-α1(I) and transforming growth factor-β1 messenger RNA in HSCs exposed to ghrelin (0.1 μmol/L) for 24 hours. *$P < 0.05$ (vehicle). (C) Activity of nuclear factor κB assessed by luciferease reporter gene assay. Cells were infected with an adenovirus containing luciferase gene with the promoter region for nuclear factor κB transcription factor and incubated overnight with vehicle, ghrelin, or phorbol 12-myristate 13-acetate. Ghrelin (0.1 μmol/L) did not modulate nuclear factor κB activity in HSCs. 12-myristate 13-acetate (1 mg/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of 12-myristate 13-acetate. (D) Cells were incubated with vehicle, ghrelin, or tumor necrosis factor-α (TNF-α) for 24 hours. Medium was collected to analyze interleukin 8 concentration. Ghrelin (0.1 μmol/L) did not modulate interleukin 8 release by HSCs to the culture medium. TNF-α (1 ng/mL) was used as a positive control. Preincubation of cells with ghrelin for 10 minutes did not modulate the effect of TNF-α. Data are expressed as the mean $\pm$ SEM from 3 independent experiments.

Interestingly, ghrelin serum levels were lower in patients with advanced fibrosis (Metavir score 3-4) than in those with mild fibrosis (Metavir score 0-2) (Fig. 7B). Next, we assessed ghrelin gene (*GHRL*) expression in normal (n = 5) and diseased human livers (37 patients with alcoholic hepatitis, 45 patients with chronic hepatitis C, and 23 patients with nonalcoholic steatohepatitis). Ghrelin transcripts were found in both normal and diseased livers. Interestingly, *GHRL* was clearly overexpressed in livers with nonalcoholic steatohepatitis compared with the rest of the groups (Fig. 7C). Moreover, in the whole series of patients with chronic liver diseases, *GHRL* hepatic expression positively correlated with the expression of genes involved in fibrogenesis (Supporting Table 2) as well with body mass index ($r = 0.675$, $P < 0.0001$). At the cellular level, *GHRL* transcripts were found in both hepatocytes and HSCs freshly isolated from human livers as well as in culture-activated human HSCs (Fig. 7D).

Polymorphisms in the Ghrelin Gene Are Associated with the Degree of Fibrosis in Patients with Chronic Hepatitis C. Finally, we investigated whether ghrelin gene polymorphisms are associated with the progression of liver fibrosis in patients with chronic liver diseases. For this purpose, we analyzed six single nucleotide polymorphisms on the ghrelin gene (Supporting Fig. 2A): −994CT, −604GA, −501AC, Arg51Gln, Met72Leu, and Leu90Gln (GeneBank numbers can be found in Supporting Materials and Methods) in 284 patients with HCV-induced liver disease. One single nucleotide polymorphism in the promoter (−994CT) was differently represented between women with advanced fibrosis (F3-F4) and those with mild fibrosis (F0-F2). Moreover, we found that patients with the haplotype −994T and −604A are more susceptible to severe liver fibrosis after adjusting by age and sex (Table 1). These results suggest that variations in *GHRL* modulate the progression of chronic hepatitis C. To investigate the functionality of these polymorphisms, we constructed plasmids containing the promoter of ghrelin with different haplotypes (wild-type and −994CT −604GA) bound to the luciferase gene. Plasmids were transfected to Huh7 hepatocytes. The plasmid with the promoter containing the haplotype associated with an increased risk to develop advanced fibrosis was found to be more active than the plasmid containing the wild-type promoter (Supporting Fig. 2B).

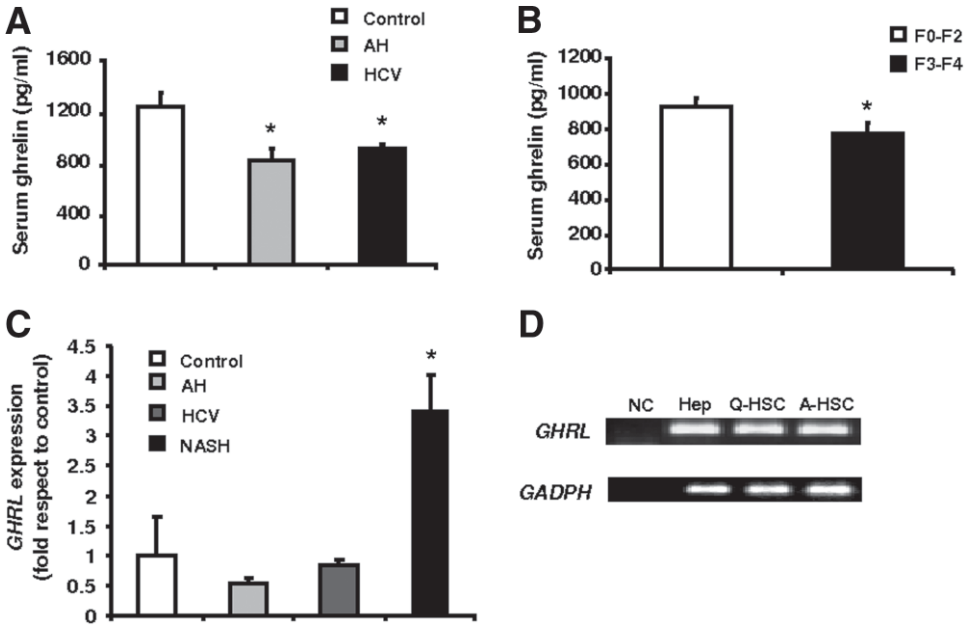

Fig. 7. Ghrelin serum levels and hepatic ghrelin expression in control subjects and in patients with chronic liver diseases. (A) Fasting ghrelin serum levels were analyzed in blood samples from patients with chronic hepatitis C virus infection, alcoholic hepatitis, and healthy controls. Serum ghrelin levels were decreased in all groups of patients. (B) Ghrelin levels were lower in patients with advanced fibrosis compared with those with mild fibrosis. *$P < 0.05$ (control or F0-F2). (C) *GHRL* hepatic expression was analyzed in samples from controls, chronic hepatitis C, alcoholic hepatitis, and nonalcoholic steatohepatitis patients. *$P < 0.05$ (all groups). (D) Ghrelin expression was analyzed in different hepatic cell types. AH, alcoholic hepatitis; A-HSC, human in culture-activated HSCs; HCV, hepatitis C virus; Hep, primary human hepatocytes; NASH, nonalcoholic steatohepatitis; NC, negative control; Q-HSC, quiescent human HSCs.

Discussion

Gut hormones play a major role in food intake and energy homeostasis at different levels, from central regulation of appetite to motility of the gastrointestinal tract. They also regulate inflammatory and fibrogenic processes in a variety of tissues. Ghrelin is a gut hormone that is also produced by extraintestinal tissues and exerts a variety of pleiotropic effects in parenchymal cells.[3] We provide extensive evidence that ghrelin exerts antifibrotic and hepatoprotective effects in the injured liver in rodents. We demonstrate that recombinant ghrelin regulates the fibrogenic response of the liver to acute and chronic injury. Moreover, endogenously produced ghrelin also regulates fibrogenesis in mice and humans. The hepatoprotective effects of ghrelin confirm previous studies indicating that ghrelin exerts protective effects in parenchymal cells and in damaged tissues such as the heart and the colon.[6,19] In the liver, a single study[20] suggests protective effects of ghrelin in a model of chronic liver injury. Our study extensively expands this notion by demonstrating a role for ghrelin in liver fibrosis. This new effect of ghrelin has potential therapeutic implications, as discussed later.

The main finding of our study is that ghrelin regulates hepatic fibrosis. Although a number of studies have suggested that ghrelin has protective effects against cell death,[5,21] the current study expands this effect by demonstrating that ghrelin also prevents scar tissue formation in chronically injured tissues. Most importantly, we demonstrate for the first time that endogenously produced ghrelin regulates fibrogenesis in the liver. In addition to the effects in experimental models of liver injury (BDL and CCl_4), we used a translational approach to study the potential role of ghrelin in samples from patients with chronic liver injury. First, we analyzed ghrelin hepatic expression in patients with different liver diseases. We found ghrelin expression in both normal and diseased livers. Interestingly, obesity and the presence of nonalcoholic steatohepatitis were associated with increased hepatic expression of ghrelin. This interesting result is probably related to the deregulated energetic metabolism in obese subjects and deserves further investigation. We also analyzed serum ghrelin levels in patients with chronic liver diseases. We found that ghrelin serum levels decreased in patients with advanced fibrosis. Our results apparently differ from a recent report showing that ghrelin serum levels are increased in patients with chronic liver diseases.[22] In this latter study, ghrelin serum levels were increased in patients with advanced cirrhosis. This advanced state is associated with profound hepatic failure, caquexia, endotoxinemia, and hemodynamic distur-

Table 1. Effects of Ghrelin Genetic Polymorphisms in the Progress of Fibrosis in Patients with Chronic Hepatitis C

−994 CT Polymorphism					
Sex	**Genotype**	**F0-F2, n (%)**	**F3-F4, n (%)**	**Odds Ratio (95% Confidence Interval)**	***P* Value**
All	CC	134 (84.3)	93 (74.4)	1.00	–
	TT + CT	25 (15.7)	32 (25.6)	1.79 (0.96-3.37)	0.068
Females	CC	55 (90.2)	29 (69)	1.00	–
	TT+ CT	6 (9.8)	13 (31)	9.75 (1.34–71.05)	0.010
Males	CC	79 (80.6)	64 (77.1)	1.00	–
	TT+ CT	19 (19.4)	19 (22.9)	1.01 (0.47–2.19)	0.981

Haplotype						
Sex	**−994 CT**	**−604 GA**	**F0-F2, n (%)**	**F3-F4, n (%)**	**Odds Ratio (95% Confidence Interval)**	***P* Value**
All	C	A	82 (51.64)	63 (50.4)	1.00	–
	C	G	64 (40.5)	44 (35.6)	0.97 (0.66–1.41)	0.850
	T	A	11 (7.17)	17 (14.0)	2.06 (1.08–3.91)	0.028
	T	G	1 (0.7)	0 (0.0)	0.00	1.00
Females	C	A	32 (51.64)	21 (51.19)	1.00	–
	C	G	26 (43.44)	13 (30.95)	0.96 (0.35–2.66)	0.943
	T	A	3 (4.92)	8 (17.86)	8.47 (1.31–54.84)	0.029
	T	G	0 (0.00)	0 (0.00)	0.00	1.00
Males	C	A	51 (51.82)	42 (50.00)	1.00	–
	C	G	38 (38.49)	31 (37.95)	1.12 (0.61–2.05)	0.712
	T	A	8 (8.39)	10 (12.05)	1.40 (0.48–4.05)	0.54
	T	G	1 (1.31)	0 (0.00)	1.08 (0.00–1088)	0.982

bances, which could influence serum levels of cytokines and vasoactive substances. In our series, the vast majority of patients have mild to moderate degree of fibrosis, which could explain the discrepant results. Finally, we studied the role of ghrelin gene variations in the progression of liver fibrosis in a well-characterized series of patients with biopsy-proven chronic hepatitis C. We analyzed *GHRL* polymorphisms and compared their frequencies in patients with mild fibrosis and patients with advanced fibrosis. We found two single-nucleotide polymorphisms in *GHRL* associated with advanced fibrosis in women but not in men. The fact that polymorphisms affect mainly women is a very intriguing question. It is well known that sex is a major factor influencing ghrelin expression and serum levels.[23,24] In fact, previous studies indicate that sex markedly influences the effect of ghrelin polymorphisms in different diseases.[25,26] Therefore, it is not surprising that in our study the influence of ghrelin polymorphisms on liver fibrosis were sex-dependent. Further studies are required to investigate this issue. Moreover, it is well known that fibrosis progression is modulated by estrogens.[27]

Different mechanisms may explain the antifibrotic effects of ghrelin in the injured liver. First, ghrelin seems to protect hepatocytes from cell death, as indicated by decreased necroinflammatory injury and serum levels of aminotransferases in rats subjected to both acute and chronic liver injury. This effect was related to a reduction in the number of infiltrating inflammatory cells as well as decreased apoptosis in hepatocytes in the model of acute liver injury. These results confirm published data indicating that ghrelin prevents parenchymal cell death in different injured tissues.[8,18,28] Interestingly, we found that ghrelin administration to injured rats resulted in increased hepatic expression of hepatoprotective signaling pathways such as phospho-Akt and phospho-extracellular signal-regulated kinase. These results are in keeping with several studies showing that ghrelin induces activation of Akt and extracellular signal-regulated kinase in different cell types.[5,7,29] Second, we found that ghrelin decreases the extent of oxidative stress in the liver, which is a major pathogenic event in the wound healing response to injury. This antioxidant effect of ghrelin has been shown in other organs.[30,31] Whether ghrelin reduces the formation of reactive oxygen species or increases the activity of antioxidant defenses is unknown and deserves further investigation. Third, we provide evidence that ghrelin reduces the accumulation of activated HSCs in the liver and it directly reduces collagen synthesis by cultured HSCs. This effect is associated with decreased transforming growth factor-β1 expression, a major profibrogenic cytokine in the liver. Finally, microarray analysis revealed several potential mechanisms by which ghrelin could exert its antifibrotic effect. Thus, besides reducing expression of genes involved in extracellular matrix synthesis, ghrelin reduced the expression of genes involved in apoptosis

(caspases), inflammation (osteopontin, chemokine receptor 5), and cellular contractility (tropomyosin).

This study has several limitations. First, it is unknown whether locally produced ghrelin or extrahepatic synthesis of ghrelin (e.g., by the stomach) regulate hepatic fibrogenesis. The finding that ghrelin serum levels are decreased in patients with more aggressive fibrosis suggests that extrahepatic sources of ghrelin could be implicated in the progression of fibrosis. Second, further studies using GHS-R antagonists should confirm the involvement of this receptor in the beneficial effects induced by ghrelin. Third, the role of ghrelin in fibrosis resolution and the therapeutic effect of exogenous ghrelin in established cirrhosis should be evaluated. Fourth, because ghrelin requires a posttranslational modification (octanylation) to be active,[32] further analysis of the ghrelin active form should be performed in liver samples and cell types. Fifth, the results in *Ghrl*$^{-/-}$ are less impressive than in rats receiving recombinant ghrelin probably because constitutive knockout mice usually develop strategies to overcome the lack of a given gene. Further studies using ghrelin conditional knockout mice and/or ghrelin receptor knockout mice should clarify this question. Finally, although we provide evidence that ghrelin exerts direct antifibrotic effects in fibrogenic cells, the precise molecular mechanisms by which ghrelin exerts beneficial effects in liver undergoing acute and/or chronic injury should be uncovered in further studies.

The results of our study have potential therapeutic implications. Recombinant ghrelin has been tested in patients with different conditions, including gastroparesis,[11] anorexia,[9] caquexia,[10] and chronic heart failure.[12] In these studies, ghrelin is generally well tolerated and only causes a mild decrease in arterial pressure. Our results suggest that ghrelin could also be useful in patients with liver injury and liver fibrosis. Further studies should evaluate this hypothesis. Moreover, due to the orexigenic properties of ghrelin, ghrelin receptor antagonists have been recently proposed for the treatment of diabetes and obesity.[33] Due to its protective effects, prolonged blockade of ghrelin receptors may cause adverse effects such as accelerated tissue fibrosis, which is commonly seen in the heart and the kidney of patients with metabolic syndrome.

In conclusion, the results of the current study indicate that ghrelin exerts hepatoprotective and antifibrogenic effects in the liver. Further studies should evaluate the safety and efficacy of ghrelin and/or ghrelin agonists in patients with chronic liver diseases.

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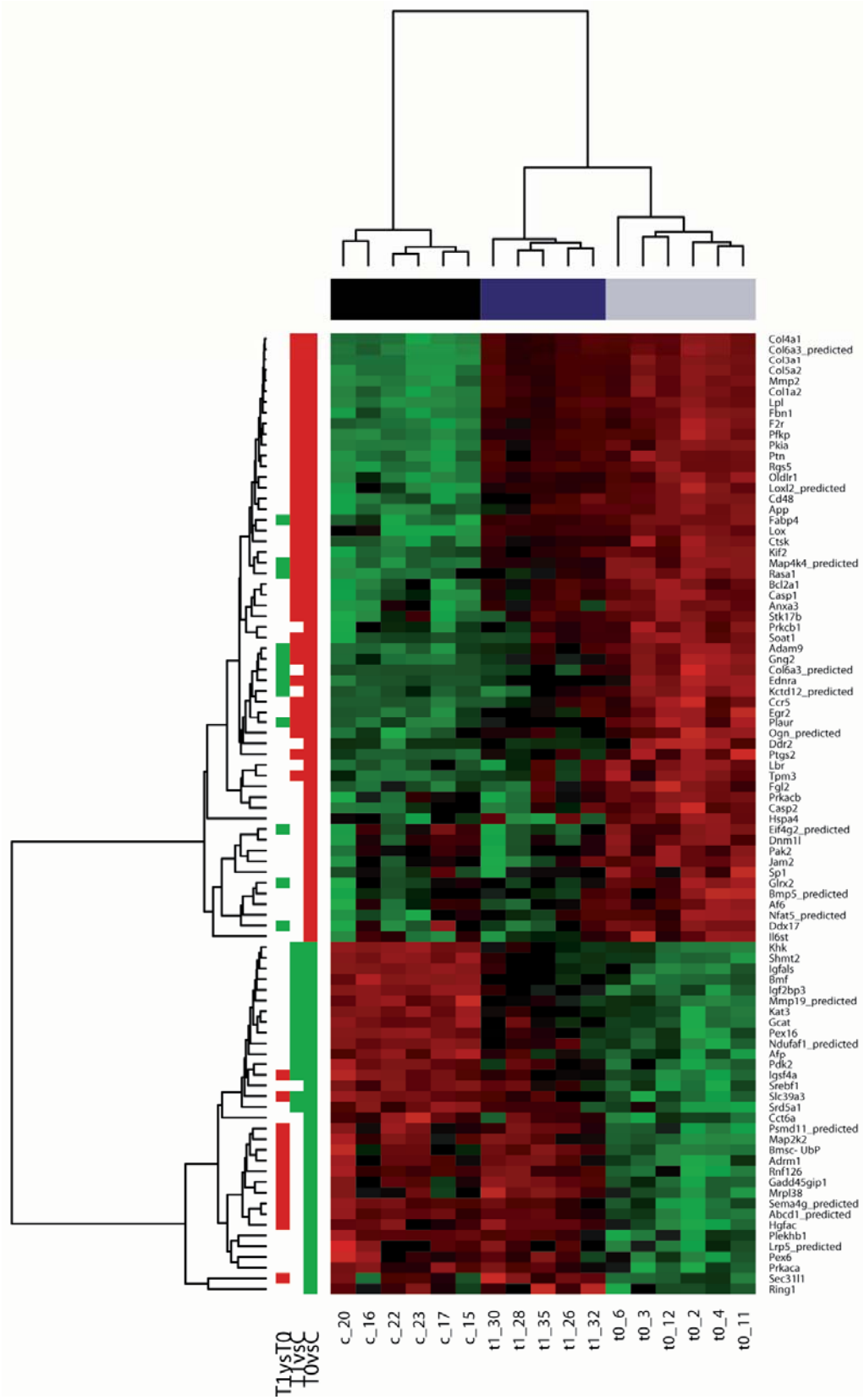
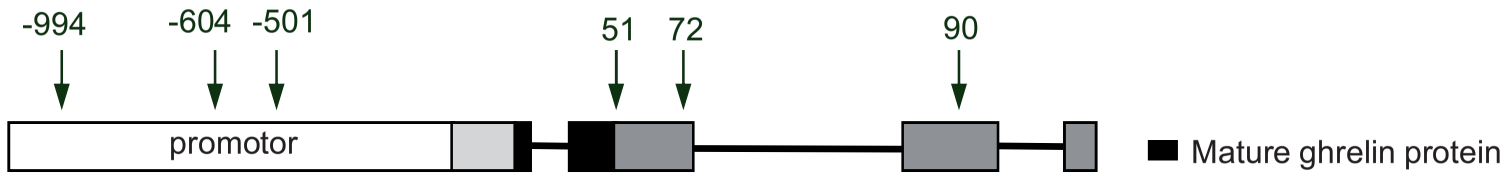


Supplementary Figure 1



Supplementary Figure 2

Supplementary Table 1. Effect of ghrelin on hepatic gene expression in rats with fibrosis as assessed by microarray analysis. Only annotated genes were considered.

Gene symbol	GeneName	BDL-saline vs sham (fold)	BDL-ghrelin vs BDL-saline (fold*)
Extracellular matrix			
Lox	lysyl oxidase	41.97	-2.61
Plaur	plasminogen activator, urokinase receptor	4.17	-2.50
Sparcl1	SPARC-like 1 (mast9, hevin)	5.77	-2.50
Cthrc1	collagen triple helix repeat containing 1	8.17	-2.41
Plod2	procollagen lisine, 2-oxoglutarate 5-dioxygenase 2	25.64	-2.19
Lamc1	laminin, gamma 1	10.48	-1.97
Mmp2	matrix metallopeptidase 2	15.42	-1.95
Fbn1	fibrillin 1	11.87	-1.93
Fgl2	fibrinogen-like 2	2.46	-1.92
Adam9	a disintegrin and metalloproteinase domain 9 (meltrin gamma)	1.58	-1.89
Timp3	tissue inhibitor of metalloproteinase 3 (Sorsby fundus dystrophy, pseudoinflammatory)	1.66	-1.84
Mgp	matrix Gla proteín	18.36	-1.81
Mxra8	matrix-remodelling associated 8	5.31	-1.77
Ermp1	endoplasmic reticulum metallopeptidase 1	1.92	-1.76
Plat	plasminogen activator, tissue	16.99	-1.75
Thbs1	thrombospondin 1	17.34	-1.74
Col5a2	procollagen, type V, alpha 2	11.51	-1.72
Col1a1	procollagen, type 1, alpha 1	24.85	-1.67
Loxl1	lysyl oxidase-like 1	118.96	-1.65
Col4a1	procollagen, type IV, alpha 1	13.60	-1.64
Col12a1	procollagen, type XII, alpha 1	12.64	-1.63
Col3a1	procollagen, type III, alpha 1	5.64	-1.60
Ltbp1	latent transforming growth factor beta binding protein 1	8.72	-1.59

Col5a1	procollagen, type V, alpha 1	5.43	-1.53
Reln	reelin	1.60	-1.50
Inflammation / Immunity			
C7	complement component 7	20.03	-2.85
Colec12	collectin sub-family member 12	12.89	-2.49
Spp1	secreted phosphoprotein 1	46.04	-2.38
Mcam	melanoma cell adhesion molecule	4.45	-2.34
Tnfrsf14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	2.95	-2.34
Cd200	Cd200 antigen	3.15	-2.19
Ahr	aryl hydrocarbon receptor	1.71	-2.16
Cd3g	CD3 antigen, gamma polypeptide	2.15	-1.89
Igsf10	immunoglobulin superfamily, member 10	7.58	-1.83
Ccl2	chemokine (C-C motif) ligand 2	17.57	-1.83
Cd44	CD44 antigen	54.65	-1.78
Tnfrsf11b	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	2.78	-1.70
Catna1	catenin (cadherin-associated protein), alpha 1	1.83	-1.67
Cd38	CD38 antigen	2.19	-1.64
Ccr5	chemokine (C-C motif) receptor 5	2.32	-1.63
Af6	Afadin	1.76	-1.58
Irf8	interferon regulatory factor 8	2.10	-1.57
Tia1	cytotoxic granule-associated RNA binding protein 1	2.06	-1.55
Sla	src-like adaptor	2.79	-1.54
Cd276	CD276 antigen	6.50	-1.50
Igha_mapped	immunoglobulin heavy chain (alpha polypeptide) (mapped)	-4.17	2.73
Energetic metabolism			
Oldlr1	oxidized low density lipoprotein (lectin-like) receptor 1	16.44	-2.87
Fabp4	fatty acid binding protein 4, adipocyte	23.44	-2.55
Vldlr	very low density lipoprotein receptor	5.29	-2.11
B3galt3	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase,	17.61	-2.07

	polypeptide 3		
Mlstd2	male sterility domain containing 2	2.94	-2.07
Lpl	lipoprotein lipase	25.40	-2.01
St3gal2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2	6.55	-1.83
Pfkp	Phosphofructokinase, platelet	5.18	-1.70
Soat1	sterol O-acyltransferase 1	3.72	-1.64
Pdk3	pyruvate dehydrogenase kinase, isoenzyme 3	6.33	-1.57
St3gal4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4	3.40	-1.55
Asah1	N-acylsphingosine amidohydrolase 1	3.33	-1.50
Acly	ATP citrate lyase	-2.88	1.50
Ptms	Parathymosin	-1.99	1.50
Dhcr7	7-dehydrocholesterol reductase	-1.85	1.52
Igfals	insulin-like growth factor binding protein, acid labile subunit	-3.47	1.56
Dcxr	dicarbonyl L-xylulose reductase	-3.61	1.60
Pdk2	pyruvate dehydrogenase kinase, isoenzyme 2	-2.45	1.61
Igf2bp3	insulin-like growth factor 2, binding protein 3	-5.19	1.79
Fasn	fatty acid synthase	-1.87	1.87
Elovl6	ELOVL family member 6, elongation of long chain fatty acids (yeast)	-2.23	1.88
Gcat	glycine C-acetyltransferase (2-amino-3-ketobutyrate-coenzyme A ligase)	-4.28	1.97
Gpd1	glycerol-3-phosphate dehydrogenase 1 (soluble)	-4.26	2.00
Cryl1	Crystallin, lamda 1	-2.44	2.09
Aacs	acetoacetyl-CoA synthetase	-1.59	2.21
Fads2	fatty acid desaturase 2	-3.10	2.24
Metabolism			
Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	2.37	-2.18
Cybb	cytochrome b-245, beta polypeptide	4.30	-2.06
Heph	Hephaestin	6.66	-1.95
Gls	Glutaminase	3.85	-1.83
Cybrd1	cytochrome b reductase 1	1.84	-1.74

Glrx2	glutaredoxin 2 (thioltransferase)	1.91	-1.70
Hprt	hypoxanthine guanine phosphoribosyl transferase	1.84	-1.64
Chdh	choline dehydrogenase	-3.40	1.50
Hfe2	hemochromatosis type 2 (juvenile) homolog (human)	-1.60	1.59
Cyp2t1	cytochrome P450 monooxygenase CYP2T1	-3.69	1.62
Dao1	D-amino acid oxidase 1	-3.99	1.64
Prodh2	proline dehydrogenase (oxidase) 2	-2.67	1.70
Sts	steroid sulfatase	-2.27	1.75
Abat	4-aminobutyrate aminotransferase	-6.01	1.77
Gstm2	glutathione S-transferase, mu 2	-3.46	1.77
Srd5a1	steroid 5 alpha-reductase 1	-4.03	2.29
Signaling			
Rgs4	regulator of G-protein signaling 4	10.05	-4.29
Prkacb	protein kinase, cAMP dependent, catalytic, beta	1.55	-2.73
Egr2	early growth response 2	4.92	-2.51
Zfhx1b	zinc finger homeobox 1b	1.99	-2.26
Arl11	ADP-ribosylation factor-like 11	6.01	-2.25
Pkia	protein kinase inhibitor, alpha	12.72	-2.04
Ptprc	protein tyrosine phosphatise, receptor type, C	4.08	-1.99
Gng2	guanine nucleotide binding proteína, gamma 2	2.76	-1.97
Egr3	early growth response 3	2.07	-1.97
Gadd45b	growth arrest and DNA-damage-inducible 45 beta	1.93	-1.94
Sp1	Sp1 transcription factor	1.70	-1.87
Edg2	endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	3.44	-1.85
Ddit4	DNA-damage-inducible transcript 4	2.84	-1.84
Slk	serine/threonine kinase 2	1.75	-1.80
Bhlhb3	basic helix-loop-helix domain containing, class B3	2.06	-1.74
Adcy3	adenylate cyclase 3	1.96	-1.73
Plcl1	phospholipase C-like 1	3.04	-1.73
Prkcb1	protein kinase C, beta 1	2.71	-1.70

Gucy1a3	guanylate cyclase 1, soluble, alpha 3	1.86	-1.67
Anxa3	annexin A3	2.82	-1.67
Rem1	rad and gem related GTP binding protein 1	1.78	-1.65
Pld1	phospholipase D1	2.31	-1.63
Rgs5	regulator of G-protein signaling 5	8.51	-1.62
Prkaa1	protein kinase, AMP-activated, alpha 1 catalytic subunit	1.88	-1.61
Tcf21	transcription factor 21	4.61	-1.60
Tfec	transcription factor EC	3.08	-1.58
Mtf2	metal response element binding TF 2	1.58	-1.58
Ptprz1	protein tyrosine phosphatise, receptor-type, Z polypeptide 1	9.63	-1.57
Rgs2	regulator of G-protein signaling 2	10.46	-1.57
Stk17b	serine/threonine kinase 17b (apoptosis-inducing)	2.79	-1.56
Arpp19	cAMP-regulated phosphoprotein 19	2.91	-1.56
Hnrpa3	heterogeneous nuclear ribonucleoprotein A3	1.57	-1.56
Ap2b1	adaptor-related protein complex 2, beta 1 subunit	2.00	-1.55
Znf292	zinc finger protein 292	1.60	-1.54
Akap13	A kinase (PRKA) anchor protein 13	3.21	-1.54
Runx3	runt-related transcription factor 3	1.90	-1.54
Atm	ataxia telangiectasia mutated homolog (human)	1.63	-1.54
Pak2	p21 (CDKN1A)-activated kinase 2	1.61	-1.52
Prkch	protein kinase C, eta	1.90	-1.52
Rhoq	ras homolog gene family, member Q	2.53	-1.51
Dab2	disabled homolog 2 (Drosophila)	3.05	-1.51
Anxa2	annexin A2	15.79	-1.50
Rnf39	ring finger protein 39	-2.67	1.99
Nfe2	nuclear factor, erythroid derived 2	-3.17	1.89
Prkaca	protein kinase, cAMP-dependent, catalytic, alpha	-1.95	1.78
Hes6	hairy and enhancer of split 6 (Drosophila)	-3.68	1.71
Rnf126	ring finger protein 126	-1.67	1.70
Srebf1	sterol regulatory element binding factor 1	-2.95	1.67
Rgs3	regulator of G-protein signalling 3	-2.55	1.58

Tcf1	transcription factor 1	-1.72	1.57
Membrane proteins			
Jam2	junction adhesion molecule 2	2.17	-2.29
Abcc5	ATP-binding cassette, sub-family C (CFTR/MRP), member 5	2.65	-2.06
Itga8	integrin alpha 8	14.03	-1.96
Gja1	gap junction membrane channel protein alpha 1	12.37	-1.87
Slc25a4	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	8.49	-1.68
Slc39a6	solute carrier family 39 (metal ion transporter), member 6	2.06	-1.58
Gja7	gap junction membrane channel protein alpha 7	3.18	-1.51
Slc17a5	solute carrier family 17 (anion/sugar transporter), member 5	-1.79	1.51
Atp6v0a1	ATPase, H+ transporting, lysosomal V0 subunit A1	-1.55	1.57
Slc39a3	solute carrier family 39 (zinc transporter), member 3	-2.10	1.57
Abcd3	ATP-binding cassette, sub-family D (ALD), member 3	-2.02	1.58
Slc26a1	solute carrier family 26 (sulfate transporter), member 1	-3.12	1.62
Slc23a1	solute carrier family 23 (nucleobase transporters), member 1	-4.44	1.64
Adrm1	adhesion regulating molecule 1	-1.81	1.90
Vasoactive substances/Coagulation			
Ddr2	discoidin domain receptor family, member 2	3.50	-3.06
Serpine1	serine (or cysteine) peptidase inhibitor, clade E, member 1	2.36	-2.33
Ednra	endothelin receptor type A	2.54	-1.93
Tfpi2	tissue factor pathway inhibitor 2	1.82	-1.86
F2r	coagulation factor II (thrombin) receptor	6.01	-1.82
Ednrb	endothelin receptor type B	9.69	-1.80
Ptafr	platelet-activating factor receptor	2.15	-1.67
Adra1b	adrenergic receptor, alpha 1b	-3.60	1.56
Apoptosis			
Ripk2	receptor (TNFRSF)-interacting serine-threonine kinase 2	2.55	-1.88
Bcl2a1	B-cell leukemia/lymphoma 2 related protein A1	4.99	-1.80
Casp2	caspase 2	2.09	-1.77
Casp1	caspase 1	3.60	-1.68

Bmf	Bcl2 modifying factor	-7.09	1.72
Cytoskeleton			
Eml2	echinoderm microtubule associated protein like 2	3.01	-2.02
Tpm4	tropomyosin 4	7.64	-1.68
Lbr	lamin B receptor	2.05	-1.67
Kif2	kinesin heavy chain family, member 2	3.26	-1.57
Tpm3	tropomyosin 3, gamma	2.09	-1.55
Growth factors			
Fgf13	fibroblast growth factor 13	3.93	-4.03
Pdgfd	platelet-derived growth factor, D polypeptide	7.09	-2.67
Fgfr2	fibroblast growth factor receptor 2	3.26	-2.25
Ptn	Pleiotrophin	6.41	-1.71
Fgfr1	Fibroblast growth factor receptor 1	5.68	-1.68
Hgf	hepatocyte growth factor	2.67	-1.56
Pdgfra	platelet derived growth factor receptor, alpha polypeptide	4.16	-1.51
Others			
Emp1	epithelial membrane protein 1	6.36	-3.05
RT1-Aw2	RT1 class Ib, locus Aw2	2.54	-2.75
Sf3b1	splicing factor 3b, subunit 1	1.56	-2.66
Cdh11	cadherin 11	10.61	-2.53
Nedd4	neural precursor cell expressed, developmentally down-regulated gene 4	2.09	-2.39
Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine:polypeptide-N-acetylglucosaminyl transferase)	1.77	-2.15
Hspa4	heat shock protein 4	2.24	-2.02
Kitl	kit ligand	4.91	-1.99
Crygc	Crystallin, gamma C	2.15	-1.92
Fblim1	filamin binding LIM protein 1	15.74	-1.92
Olfml1	olfactomedin-like 1	3.18	-1.86

Pqlc3	PQ loop repeat containing 3	27.64	-1.84
Tfrc	Transferrin receptor	2.13	-1.80
Fstl1	follistatin-like 1	4.95	-1.78
Ctsk	Cathepsin K	3.98	-1.78
Spnb2	spectrin beta 2	3.64	-1.75
Cugbp2	CUG triplet repeat, RNA binding protein 2	2.68	-1.73
Osbpl5	oxysterol binding protein-like 5	4.03	-1.72
Pcsk1	Proprotein convertase subtilisin/kexin type 1	4.81	-1.70
Clecsf6	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 6	5.00	-1.70
Ssg1	steroid sensitive gene 1	4.68	-1.69
Fhl2	four and a half LIM domains 2	25.51	-1.69
Ddx46	DEAD (Asp-Glu-Ala-Asp) box polypeptide 46	2.49	-1.69
Ppic	peptidylprolyl isomerase C	26.91	-1.68
Ctse	Cathepsin E	5.74	-1.68
RT1-Ba	RT1 class II, locus Ba	3.04	-1.65
Mgl1	Macrophage galactose N-acetyl-galactosamine specific lectin 1	3.26	-1.65
Robo2	roundabout homolog 2 (Drosophila)	12.90	-1.64
Sfpq	splicing factor proline/glutamine rich (polypyrimidine tract binding protein associated)	1.53	-1.63
Ddx17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	1.58	-1.61
Ddx21a	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21a	1.55	-1.61
Lgals1	Lectin, galactose binding, soluble 1	18.79	-1.60
Gpiap1	GPI-anchored membrane protein 1	1.90	-1.59
RT1-N3	RT1 class Ib gene, H2-TL-like, grc region (N3)	1.95	-1.58
S100a6	S100 calcium binding protein A6 (calcyclin)	30.28	-1.55
App	amyloid beta (A4) precursor protein	6.69	-1.55
Mdn1	midasin homolog (yeast)	1.53	-1.54
Cdr2	cerebellar degeneration-related 2	1.97	-1.54
Rab31	RAB31, member RAS oncogene family	5.25	-1.54
RT1-Da	RT1 class II, locus Da	4.03	-1.53

Trip10	thyroid hormone receptor interactor 10	1.65	-1.53
Btg3	B-cell translocation gene 3	7.20	-1.51
Plekhb1	pleckstrin homology domain containing, family B (evectins) member 1	-3.18	2.33
Cct6a	chaperonin subunit 6a (zeta)	-2.53	1.81
Ddhd1	DDHD domain containing 1	-2.65	1.78
Bmsc-UbP	bone marrow stromal cell-derived ubiquitin-like protein	-1.57	1.76
Cml4	camello-like 4	-48.15	1.74
Npy	neuropeptide Y	-1.75	1.72
Xkr8	X Kell blood group precursor related family member 8 homolog	-2.71	1.68
Mig12	MID1 interacting G12-like proteína	-1.98	1.66
Snrpn	small nuclear ribonucleoprotein N	-1.53	1.63
Pex16	peroxisome biogenesis factor 16	-2.82	1.54
Kat3	kynurenine aminotransferase III	-2.90	1.52
Lrp16	LRP16 protein	-3.09	1.51

*At least 50% of variation respect to BDL-saline. FDR < 0.2

Supplementary Table 2. Correlation of *GHRL* hepatic expression with expression of other genes in patients with chronic liver diseases.

Gene Symbol	Gene name	Group	r	P value
SERPINE1	Plasminogen activator inhibitor type 1	A	0.713	<0.0001
TGFB1	Transforming growth factor beta 1	A	0.708	<0.0001
ACE	Angiotensin I converting enzyme	A	0.641	<0.0001
TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	A	0.687	<0.0001
ADIPOR1	Adiponectin receptor 1	B	0.671	<0.0001
IGF1	Insulin-like growth factor 1	B	0.624	<0.0001
IRS1	Insulin receptor substrate 1	B	0.642	<0.0001
PBEF1	Visfatin	B	0.673	<0.0001
ABCG1	ATP-binding cassette, sub-family G member 1	C	0.703	<0.0001
ABCG8	ATP-binding cassette, sub-family G member 8	C	0.671	<0.0001
ABCG5	ATP-binding cassette, sub-family G member 5	C	0.690	<0.0001
SP2	Sp2 transcription factor	D	0.642	<0.0001
JAK1	Janus kinase 1	D	0.694	<0.0001
SREBF1	Sterol regulatory element binding transcription factor 1	D	0.702	<0.0001
SOCS1	Suppressor of cytokine signaling 1	D	0.628	<0.0001
STAT3	Signal transducer and activator of transcription 3	D	0.688	<0.0001
SP1	Sp1 transcription factor	D	0.671	<0.0001
JAK2	Janus kinase 2	D	0.705	<0.0001
PPARG	Peroxisome proliferator-activated receptor gamma	D	0.701	<0.0001
PPARD	Peroxisome proliferator-activated receptor delta	D	0.665	<0.0001
PPARA	Peroxisome proliferator-activated receptor alpha	D	0.631	<0.0001
SREBF2	Sterol regulatory element binding transcription factor 2	D	0.666	<0.0001
ATF4	Activating transcription factor 4	D	0.673	<0.0001
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	E	0.647	<0.0001
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	E	0.646	<0.0001
SMPD1	Sphingomyelin phosphodiesterase 1, acid lysosomal	E	0.699	<0.0001
HSP5A	Heat shock protein 5	E	0.668	<0.0001

A. Fibrosis/inflammation, B. Hormones/adipokines, C. Transporters, D. Intracellular signaling, E. Others

SUPPLEMENTARY MATERIALS AND METHODS.

Cell cultures.

Human hepatic stellate cells (HSC) were isolated from fragments of normal livers obtained from resections of liver metastasis of colon cancer as described in detail previously.(1) Experiments were performed with HSC activated in culture. Cells were cultured in standard conditions in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) containing 15% fetal bovine serum, non essential aminoacids, glutamine, sodium pyruvate and antibiotics. The protocols were approved by the Investigational Review Board of the Hospital Clínic of Barcelona. Huh7 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, glutamine and non essential aminoacids. Cells were serum starved for at least 12 hours before the experiments.

Immuhistochemistry studies.

Paraffine-embedded liver sections were deparaffinized, rehydrated and stained using the DAKO Envision system (DAKO; Carpinteria, CA). To evaluate the degree of liver inflammation, CD43 immunostaining was performed using a monoclonal antibody against CD43 (1:1000, Serotec Inc; Oxford, UK). CD43 positive cells were counted (10 fields per specimen at x200 magnification). p65 immunohistochemistry was performed using a monoclonal antibody against p65 (1:100, Santa Cruz Biotechnology; Santa Cruz, CA). Hepatocytes with p65 positive nuclei per field (30 fields per specimen at x400 magnification) were counted. To evaluate oxidative stress, 4-hydroxynonenal protein adducts (4-HNE) immunostaining was performed (1:200, A.G. Scientific Inc; San Diego, CA). The percentage of positive area (30 fields per specimen at x400 magnification) was calculated as described above for Sirius red. To quantify cell death, Terminal dUTP Nick End Labeling (TUNEL) staining was performed using a commercial kit (Promega Corporation; Madison, WI). Hepatocytes with positive nuclei were counted (10 fields per specimen at x400 magnification). To estimate the amount of fibrogenic myofibroblasts, liver sections

were incubated with a monoclonal antibody against smooth muscle α-actin (α-SMA) (1:1000, DAKO). Positive cells per field were counted (10 fields per specimen at x400 magnification).

Hepatic hydroxyproline content.

Hydroxyproline content was quantified colorimetrically from 80 mg of frozen liver samples. Tissue was homogenized in 300 μl of 6N chloridric acid and hydrolyzed at 100°C overnight. The hydrolysate was filtered, aliquots were evaporated under vacuum, and the sediment was redissolved in 50% isopropanol. Samples were then incubated in a solution containing 0.84% chloramine-T 42 mM sodium acetate, 2.6 mM citric acid, and 39.5% (vol/vol) isopropanol (pH 6.0) for 10 minutes at room temperature. Next, samples were incubated in a solution containing 0.248 g *p*-dimethylaminobenzaldehyde dissolved in 0.27 ml of 60% perchloric acid and 0.73 ml of isopropanol for 90 minutes at 50°C. Hydroxyproline content was quantified photometrically at 558 nm. Results are expressed as μmol hydroxyproline per litre of the last solution.

Gene expression analysis.

RNA was obtained from cultured cells and human liver samples using the TRIzol reagent (Life Technologies Inc; Rockville, MD). RNA was extracted from rat liver samples by the QIAGEN RNeasy kit. In all cases, retrotranscriptions were performed using the High Capacity cDNA Archive Kit (Applied Biosystems; Foster City, CA). Quantitative PCR were performed using TaqMan gene expression assays (Applied Biosystems) for procollagen-α2(I), metallopeptidase 2, endothelin receptor type A and sterol regulatory element binding factor 1 for rat liver samples, procollagen-α2(I) and tissue inhibitor of metaloproteases (TIMP-1) for mice samples and procollagen-α1(I) and transforming growth factor β for samples from hepatic stellate cells (HSC). TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 Machine (Applied Biosystems). For human liver samples, quantitative PCR for 64 genes were performed using pre-designed TaqMan low

density array cards (Applied Biosystems) and carried out in triplicate on an ABI PRISM 7900HT (Applied Biosystems). All data were normalized to *18S* content and were expressed as fold increase over the control group or as correlations with expression of other genes in case of human liver samples. Reactions for qualitative PCR contained 7.5 ng cDNA , 0.75 μM of each primer, 3.2 μmol/L of each deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dTTP), 1X buffer (HotStar), 0.25X Q solution (Qiagen; Hilden, Germany), and 0.04 $U \cdot \mu L^{-1}$ of DNA polymerase HotStarTaq (Qiagen). Water was added to a final volume of 10 μl. An initial denaturation step at 95°C for 15 min was followed by 60 cycles of 95°C for 30 s, annealing temperature for 30 s, and 72°C for 90 s, with a final step at 72°C for 15 min. Annealing temperature was 65ºC for the ghrelin gene (*GHRL)* and 58ºC for the growth hormone secretacogue receptor gene (*GHSR).* The size of the PCR products (2-μL aliquot) was analyzed by electrophoresis on 2% agarose gels. The oligonucleotides used for *GHRL* were: 5'-GAGAGTCCAGCAGAGAAAGGAGTC-3' (forward) and 5'-GACAGCTTGATTCCAACATCAAAG-3' (reverse) and the oligonucleotides used for the ghrelin receptor gene (GHSR) were: 5'-CTCTGGACTGCTCACGGTCAT-3' (forward) and 5'- AACACCACTACAGCCAGCATTTT-3' (reverse).

Microarray studies.

RNA was isolated from rat livers using the QIAGEN RNeasy kit (Qiagen). RNA integrity was checked with the Agilent 2100 Bioanalyser (Agilent Technologies; Santa Clara, CA) and only high quality RNA samples were hybridized to Rat Genome 230 2.0 GeneChips (Affymetrix; Santa Clara, CA). Briefly, 2 μg of total RNA were used to generate double strand complementary DNA (cDNA) using an oligo dT- primer containing the T7 RNA polymerase promoter site and the SuperScript Choice System kit (Invitrogen; Leek, The Netherlands). cDNA was purified by the GeneChip Sample Clean Up Module, followed by *in vitro* synthesis of biotinylated complementary RNA (cRNA) using the BioArray High Yield RNA transcription kit (Affymetrix). The resulting cRNA was purified and fragmented and

15 µg were hybridized to Rat Genome 230 2.0 GeneChips for 16 hours, at 45ºC and 30 *g*. The arrays were then washed and labelled with streptavidin-phycoerythrin (SAPE), and the signal was amplified with an anti-streptavidin biotinylated antibody followed by a second round of staining with SAPE using the Affymetrix fluidics station 450. Finally, the labelled arrays were scanned with a Gene chip scanner 3000. Microarray data from 17 samples (6 for the control group, 6 for BDL-saline group, and 5 for the ghrelin-BDL group) were normalized using the guanidine-cytosine content-adjusted robust multiarray algorithm, which computes expression values from probe-intensity values incorporating probe-sequence information. Next, we employed a conservative probe-filtering step excluding those probes not reaching a $\log_2$ expression value of 5 in at least 1 sample, which resulted in the selection of a total of 15,445 probes out of the original 31,099 set. Differential expression was assessed by using linear models and empirical Bayes moderated t-statistics using LIMMA R-package software.(2) Inter-groups comparisons and determinations of false discovery rates (FDR computation using *Benjamini-Hochberg* procedure) for each comparison were performed and FDR values ≤0.2 were deemed potentially significant and selected for further study.

Assessment of hepatic guanosin 3',5'-cyclic monophosphate (cGMP).

Forty mg of frozen tissue were dropped into 10 volums of 5% trichloroacetic acid (TCA) and homogenized. Precipitated was removed by centrifugation at 1500*g* for 10 min and the supernantant was transferred to a clean test tube. The supernatants were washed with water-saturated diethyl ether three times to remove the TCA. The aqueous suspension and the standars were acetylated and cGMP levels were determined by enzymeimmunoassay (Cayman Chemical Co; Ann Arbor, MI).

Serum biochemical measurements.

Serum aspartate aminotransferase (AST) levels were measured using standard enzymatic procedures.

Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Changes in $[Ca^{2+}]_i$ were measured in Fura-2 (Calbiochem) loaded cells using an inverted epifluorescence microscope as described in detail previously.(3) Cells were pre-incubed for 10 minutes with human recombinant ghrelin (Sigma-Aldrich) or saline and tested with angiotensin-II (Sigma-Aldrich). Cells were considered as responders when $[Ca^{2+}]_i$ increased more than 50% above the resting value.

NF-κB responsive luciferase assay.

Human HSC were infected with a recombinant adenoviral vector expressing a luciferase reporter gene driven by nuclear factor κB (NF-κB) transcriptional activation (Ad5NF-κBLuc) for 12 hours. Medium was replaced and cells stimulated with 12-myristate 13-acetate (PMA, Sigma-Adrich) or vehicle for 8 hours. Human recombinant ghrelin or vehicle was added to the medium 10 minutes before PMA. NF-κB-mediated transcriptional induction was assessed by a luciferase assay system kit (Promega Corporation). Cells were serum starved from 12 hours before the adenoviral infection.

Determination of interleukin 8 secretion.

Human HSC were cultured in 6-well plates at a density of $4x10^5$ cells/well. Medium was removed and cells incubated in serum-free medium for 24 hours in the presence of tumor necrosis factor α (TNF-α, R&D Systems; Minneapolis, MN) or vehicle. Human recombinant ghrelin was added to the medium 10 minutes before TNF-α. Supernatants were collected and stored at –80ºC until analysis. An enzymeimmunoassay for human interleukin 8 (BLK Diagnostics; Barcelona, Spain) was performed.

Western blotting.

Tissue protein extracts were obtained in radio-immunoprecipitation assay (RIPA) lysis buffer containing phosphatase and protease inhibitors. Forty micrograms of protein were loaded onto 10% sodium dodecyl sulfate-acrylamide gels and blotted

onto nitrocellulose membranes. Membranes were then incubated with antibodies against phospho-Akt, Akt, phospho-extracellular signal-regulated kinase (ERK), ERK (Cell Signaling Technology; Berverly, MA), smooth muscle α-actin (DAKO, Carpinteria, CA) or GADPH (Abcam, Cambridge, UK). After extensive washing, membranes were incubated with blocking buffer containing horseradish-peroxidase conjugated secondary antibody. Proteins were detected by chemoluminescence (Amersham Biosciences; Fairfield, CT).

Serum ghrelin levels analysis.

Total ghrelin serum levels were analyzed by radioimmunoassay (Linco Research; St. Charles, MI). Blood samples from all subjects were obtained after an overnight fasting in the early morning. Serum was frozen at –80ºC until analysis.

DNA extraction and genotyping.

DNA was isolated from peripheral blood cells using the Chemagic System (Chemagen; Baesweiler, Germany). Polymerase chain reaction (PCR) amplicons were designed by Primer3 program(4) to completely traverse the promoter, exon 1, exon 3 and exon 4 of *GHRL*. The size of PCR products was analyzed by electrophoresis on 2% agarose gels. Products were treated with Exonuclease I (Amersham Biosciences) and shrimp alkaline phosphatase (Amersham Biosciences) to remove excess primers and deoxynucleotide triphosphates. For the examination of the six single nucleotide polymorphisms (SNPs), extension SNaPshot primers specific to the polymorphic sites (see table below) were used for the SNapshot minisequencing reaction using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems). The resulting products were purified by one unit of Calf Intestine Phosphatase (New England Biolabs, Ipswich, MA). Snapshot products were resuspended in 4,5 μL Hi-Di™ Formamide (Applied Biosystems) and 0.5 μL GeneScan Size Standard. Then, they were electrophoretically analyzed using a DNA Analyzer 3730 (Applied Biosystems). The results of genotyping were analyzed and evaluated by GeneMapper software v. 3.7 (Applied Biosystems).

SNPs statistical analysis.

Allele and genotype frequencies as well as Hardy–Weinberg equilibrium for every SNP were calculated by chi-square test with one degree of freedom. SNPs association with fibrosis were calculated by logistic regression and adjusted by age and gender. Co-dominant, dominant, recessive and over-dominant inheritance models were analyzed for genotype association with fibrosis. The model with lower AIC (Akaike information) was used for every SNP. Haplotypes were stimated by Expectation Maximization algorithm and haplotypes association with fibrosis was calculated by logistic regression models and adjusted by age and gender as covariates. All analysis were performed using SPSS and SNPStats software.(5) We considered statistically significant for association with a *P* value <0.05. Odd ratio was used to evaluate the association of disease state with each SNP or haplotype.

Construction of the human GHRL promoter-Luciferase plasmids.

The fragment containing 599 bp, corresponding to the -1049 to -450 bp upstream region of the human ghrelin gene (*GHRL*), was amplified by PCR and cloned into pcR2.1-Topo vector (Invitrogen). The (−1049 −450) GHRL-luc plasmid was obtained by releasing restriction fragments from the pcR2.1-Topo constructs followed by subcloning into the pGL3-basic vector (Promega Corporation).

Luciferase assay for GHRL promotor activity.

HuH7 cells were seeded in 24-well culture plates and co-transfected using Fugene (Roche applied science, Penzberg, Germany) with 980 ng of GHRL-reporter plasmids and 20 ng of a Renilla luciferase construct (pRL-TK) as an internal control. Transactivation activities were measured 48 h after transfection in a VICTOR3 luminometer (Perkin Elmer, Wellesley, MA) according to the technical manual of the Dual-Luciferase Reporter Assay System (Promega Corporation). The mean firefly luciferase/renilla ratio was considered. Values represent the means ± S.E.M. of four independent transfection experiments run in duplicate.

Single nucleotide polymorphism information for ghrelin gene (*GHRL*)

SNP [A]	Reference [B]	Primers	
rs26312	-994 C>T	Forward	TCCTCGGGAAGGTGTAGAATC
		Reverse	AGGCCCAGAGAGGTTAAACG
		-994 C>T*	tagttatatatattaGCTGTTGCTGCTCTGGCCTCT
rs27647	-604 G>A	Forward	TCCTCGGGAAGGTGTAGAATC
		Reverse	AGGCCCAGAGAGGTTAAACG
		-604 G>A*	atacgttatatTGGGATGGGGTTGCTGGTTTA
rs26802	–501 A>C	Forward	TCCTCGGGAAGGTGTAGAATC
		Reverse	AGGCCCAGAGAGGTTAAACG
		-501 A>C*	agatatatcgtatgatCAGCAGTCACGGACAATAAACCTG
rs34911341	Arg51Gln, 304 G>A (exon 3)	Forward	CCTTCCAGCAGAGAAAGGAG
		Reverse	TGTAGTTGGGACCCTGTTCAC
		R51Q*	atatattctatctCGGAGCCAGCCTGCTAGAGCT
rs696217	Met72Leu, 366 C>A (exon 3)	Forward	CCTTCCAGCAGAGAAAGGAG
		Reverse	TGTAGTTGGGACCCTGTTCAC
		M72T*	cgtcctaGCAGAAGGGGCAGAGGATGAA
rs4684677	Leu90Gln 421 A>T (exon 4)	Forward	CTGACATCTCCTGGGTCCTC
		Reverse	AAACCGAGCAAACCCAGTC
		L90Q*	taatataactccatattacattaTGGAATCAAGCTGTCAGGGGTTC

[A] Single nucleotide polymorphism, [B] Begins in the first nucleotide of first codon of GHRL, accession number of reference sequence was NM_016362 on NCBI.

** SNaPshot primers for GHRL polymorphism detection.*

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4. DISCUSIÓN

En este proyecto se ha investigado el potencial terapéutico para la inflamación y la fibrosis hepática de dos moléculas: la atorvastatina y la ghrelina. Los tratamientos actuales para las enfermedades hepáticas no son siempre efectivos. Es por ello necesario ampliar las posibilidades terapéuticas en hepatología. En el primer estudio se analiza el efecto de la atorvastatina sobre la inflamación y el daño hepático en dos modelos animales (infusión de Ang II y administración de CCl_4) en rata. Además, se analizan algunos efectos de la atorvastatina en HSC. El segundo estudio es un estudio traslacional en el que se investigan los efectos de la ghrelina en modelos animales de daño agudo (CCl_4) y de fibrosis hepática (ligadura del conducto biliar) y el papel de la ghrelina en la progresión de la fibrosis en ratones deficientes en ghrelina inducidos a fibrosis mediante el tratamiento crónico con CCl_4. Por otro lado se investigan algunos efectos de la ghrelina sobre HSC en cultivo. Además, se analizan muestras humanas de pacientes con diferentes enfermedades hepáticas (niveles séricos de ghrelina, expresión hepática de ghrelina y polimorfismos del gen de la ghrelina).

Efecto de la atorvastatina sobre la inflamación hepática.

El primer estudio se centra en analizar los efectos de la atorvastatina sobre las acciones proinflamatorias inducidas por la Ang II en el hígado. La atorvastatina es un fármaco utilizado por su efecto sobre la inhibición de la síntesis de colesterol. Además, de manera independiente a la disminución de la síntesis de colesterol, se ha demostrado que ejerce efectos beneficiosos en diversas enfermedades. Para estudiar si la atorvastatina es hepatoprotectora se investigaron los efectos de la atorvastatina en un modelo de infusión de Ang II bien caracterizado previamente (14), ya que es bien conocido que la Ang II juega un papel importante en la inflamación hepática tanto en roedores como en humanos mediante la activación de los receptores AT1 (62). Además, este modelo ha sido utilizado para el estudio de la inflamación en otros órganos como el riñón o el corazón (241;242). Los

resultados mostrados indican que la atorvastatina atenúa los eventos patogénicos inducidos por la infusión de Ang II en ratas incluyendo el estrés oxidativo, la inflamación y la expresión de genes involucrados en fibrosis. Estos resultados confirman observaciones previas que indicaban un efecto beneficioso de las estatinas sobre las propiedades aterogénicas de la Ang II (236). El efecto hepatoprotector de la atorvastatina se confirmó en otro modelo de daño hepático (administración única de CCl_4), lo cual indica que este efecto de la atorvastatina es independiente del modelo experimental.

Las HSC juegan un papel clave en la fibrogénesis, amplifican la respuesta inflamatoria y son fuente de radicales libres. Es bien conocido que la Ang II ejerce efectos proinflamatorios y profibrogénicos sobre este tipo celular (243). Los resultados presentados en esta tesis muestran que la atorvastatina atenúa la mayoría de estos efectos. Nuevos estudios deberían determinar si la atorvastatina u otras estatinas también bloquean los efectos de la Ang II en otras células no parenquimatosas del hígado como las células de Kupffer o las células endoteliales.

Hay extensas evidencias de que la Ang II es un potente agente pro-oxidante en el hígado. Estimula la producción de ROS en HSC (244) y su infusión en animales induce estrés oxidativo (14). De hecho, se ha demostrado que los ratones deficientes en el receptor AT1 de la Ang II desarrollan menos estrés oxidativo tras un daño hepático crónico, sugiriendo que la Ang II local juega un papel en la generación de ROS en hígados con daño crónico (245). Los resultados presentados aquí demuestran que la atorvastatina reduce los efectos pro-oxidantes de la Ang II en el hígado. Los efectos antioxidantes de las estatinas se habían sugerido previamente en otros órganos (246;247) y el mecanismo responsable descrito es principalmente la inhibición de las proteínas pequeñas de unión a GTP, incluyendo Rac1. Se sabe que la translocación de Rac1 es un requisito para la activación de la NADPH oxidasa, lo cual genera especies reactivas de oxígeno (248). Sin embargo, en este estudio, no se ha demostrado que la atorvastatina module la activación de

Rac1 inducida por Ang II. Nuevos estudios son necesarios para investigar los mecanismos responsables del efecto antioxidante de la atorvastatina en el hígado.

Los resultados presentados en esta tesis demuestran que la atorvastatina reduce la proliferación de las HSC y sus respuestas inflamatorias (secreción de IL-8, expresión de ICAM-1 y activación del factor de transcripción NF-κB). El efecto antiinflamatorio de las estatinas había sido descrito previamente en hepatocitos (249). Además, se demuestra que la atorvastatina bloquea algunos efectos profibrogénicos de la Ang II en las HSC, como son la estimulación de la expresión de genes involucrados en fibrosis (procolágeno 1α(I) y TGF-β) y la secreción de TGF-β. Estos resultados son importantes, ya que las HSC son la principal fuente de colágeno en el hígado dañado y juegan un papel clave en la fibrogénesis hepática. Sin embargo, nuevos estudios con modelos animales de fibrosis deberían definir si la atorvastatina tiene un efecto antifibrogénico.

Este estudio tiene ciertas limitaciones. En primer lugar, quedan sin definir los mecanismos moleculares concretos responsables de los efectos hepatoprotectores de la atorvastatina. En segundo lugar, no se han investigado los efectos de la atorvastatina sobre otras células hepáticas implicadas en inflamación como las células de Kupffer o sobre los propios hepatocitos. En tercer lugar, el posible efecto antifibrogénico de la atorvastatina queda por determinar en nuevos experimentos con modelos animales de fibrosis hepática. Finalmente, ya que la atorvastatina es un tratamiento frecuente entre la población con síndrome metabólico, sería importante evaluar si la atorvastatina también ejerce efectos hepatoprotectores en modelos de esteatohepatitis no alcohólica, una enfermedad relativamente común entre los pacientes con síndrome metabólico.

Papel de la ghrelina en la inflamación y la fibrosis hepática.

En el segundo estudio de esta tesis, se investiga el papel de la ghrelina en la fibrosis y la inflamación hepática. Las hormonas gastrointestinales juegan un papel clave en la regulación de la ingesta de alimentos y en la homeostasis energética a diferentes niveles, desde la regulación del apetito a nivel central hasta la motilidad del tracto gastrointestinal. Además, regulan procesos inflamatorios y fibrogénicos en varios tejidos. La ghrelina es una hormona gastrointestinal que también se expresa en tejidos extraintestinales y ejerce diversos efectos en varios tipos celulares (250). Los resultados de esta tesis, muestran evidencias de que la ghrelina ejerce efectos antiinflamatorios y antifibrogénicos en el hígado en modelos animales de daño hepático. Concretamente, se demuestra que la administración de ghrelina recombinante es capaz de regular la respuesta hepática frente al daño agudo y crónico y que la ghrelina endógena regula la fibrogénesis en ratones. Los efectos hepatoprotectores de la ghrelina confirman estudios previos que indican un papel protector de la ghrelina sobre células parenquimarosas o tejidos dañados como el colon o el corazón (251;252).

El principal resultado de este estudio es que la ghrelina regula la fibrosis hepática. Aunque diversos estudios señalan un efecto protector de la ghrelina sobre la muerte celular (168;253), el presente trabajo expande esta idea demostrando que la ghrelina también previene la formación de tejido fibrótico. Especialmente, se demuestra por primera vez que la ghrelina endógena, regula la fibrogénesis.

En este trabajo se han investigado diversos mecanismos que pueden explicar el efecto antifibrogénico de la ghrelina y se ha llegado a las siguientes conclusiones. Primero, que la ghrelina protege a los hepatocitos de la muerte, como indica el efecto hepatoprotector en el modelo de daño agudo. Esto se asocia a una reducción del infiltrado inflamatorio así como de la apoptosis. Estos resultados confirman datos previos en los que se muestra que la ghrelina protege de la muerte celular de células parenquimatosas en diferentes tejidos dañados (254-256). Como

mecanismos responsables del efecto hepatoprotector se podría indicar que la administración de ghrelina resultó en un incremento en la activación de vías de señalización hepatoprotectoras como Akt y ERK. Estos resultados concuerdan con otros estudios en los que se muestra que la ghrelina induce la activación de estas vías en diversos tipos celulares (257;258). Segundo, que las ratas con daño hepático tratadas con ghrelina tienen un menor grado de estrés oxidativo, que es un evento patogénico clave en la respuesta fibrogénica de un tejido. Este efecto antioxidante también ha sido demostrado en otros órganos (259;260). Nuevos estudios deberían aclarar si este efecto es debido a una inhibición de la formación de ROS o a un aumento de la actividad de los sistemas antioxidantes. Tercero, que la ghrelina reduce la acumulación de HSC en el hígado y la síntesis de colágeno por parte de las HSC. Este efecto está asociado a una menor expression de TGF-β. Finalmente, el análisis de microarrays reveló diversos mecanismos por los cuales la ghrelina podría ejercer su efecto antifibrogénico, por ejemplo se demuestra que, además de reducir la expression de genes involucrados en la síntesis de la matriz extracellular, la ghrelina inhibe la expresión de genes involucrados en apoptosis (caspasas), inflamación (osteopontina, receptor 5 de quimiocinas) y contractilidad celular (tropomiosina).

Con respecto a los análisis de muestras humanas, se analizaron los niveles séricos de ghrelina en pacientes con enfermedades hepáticas. Los resultados muestran que los niveles séricos de ghrelina estan disminuidos en pacientes con fibrosis avanzada. Este resultado difiere de los resultados publicados por otro grupo en el que se mostraba que los pacientes cirróticos tienen concentraciones elevadas de ghrelina en suero (261). Una posible explicación a esta discrepancia es que en este segundo estudio los pacientes estudiados presentaban cirrosis avanzada. Esta situación se asocia con un fallo hepático importante, caquexia, endoteoxemia y alteraciones hemodinámicas, que pueden influir en los niveles séricos de citocinas y sustancias vasoactivas. En los pacientes analizados en el estudio presentado aquí,

la mayoría de pacientes mostraban una fibrosis leve o moderada y por tanto, ambos estudios no son comparables.

Por otro lado, se demuestra que la expresión hepática de ghrelina, es elevada en obesidad y NASH. Probablemente, esto mantiene relación con la desregulación del metabolismo energético presente en personas obesas. La expresión de ghrelina también correlaciona positivamente con la expresión de otros genes relacionados con inflamación o fibrosis.

Finalmente, se estudió el papel de polimorfismos en el gen de la ghrelina en la progresión de la fibrosis en una serie bien caracterizada de pacientes con HVC. Se analizaron 6 polimorfismos y se compararon sus frecuencias entre aquellos pacientes con fibrosis leve o moderada y aquéllos con fibrosis avanzada. El resultado fue que dos polimorfismos del gen de la ghrelina se asociaron con fibrosis avanzada en mujeres. El hecho de que los polimorfismos afecten sobretodo en mujeres es interesante. En otros estudios se ha demostrado que el sexo influencia los efectos de los polimorfismos de la ghrelina en otras enfermedades (262;263). Así, no es sorprendente que en el estudio presentado el efecto de los polimorfismos de la ghrelina fuera dependiente del sexo. Este hecho debería ser investigado en nuevos estudios. Por otro lado, se sabe que los estrógenos modulan la progresión de la fibrosis (264).

Este estudio tiene diversas limitaciones. En primer lugar, no se determina si la ghrelina que regula la fibrosis es la producida localmente en el hígado o la producida fuera del hígado (p. ej. en el estómago). El hecho de que los niveles séricos de ghrelina estén disminuidos en pacientes con fibrosis avanzada indica que fuentes extrahepáticas podrían estar implicadas en la progresión de la fibrosis. En segundo lugar, nuevos estudios utilizando antagonistas del receptor GHS-R1a deberían confirmar la implicación de este receptor en los efectos beneficiosos ejercidos por la ghrelina. En tercer lugar, el papel de la ghrelina en la resolución de la fibrosis y el efecto terapéutico de ésta en la fibrosis establecida debería ser estudiado. Y finalmente, los mecanismos concretos por los que la ghrelina ejerce

efectos beneficiosos en el daño agudo y crónico deberían ser estudiados más minuciosamente.

Resumen final.

En su conjunto, los resultados presentados en esta tesis señalan a la atorvastatina y a la ghrelina como posibles nuevos tratamientos para las enfermedades hepáticas. En el primer estudio, se demuestra que la Ang II tiene propiedades proinflamatorias en el hígado, tanto *in vivo* como *in vitro* y que la administración de atorvastatina reduce estos efectos. Por otro lado, la atorvastatina también previene el daño hepático inducido por la administración de CCl_4. En el segundo estudio, se demuestra que la ghrelina ejerce efectos hepatoprotectores y antifibrogénicos en el hígado. Nuevos estudios deberían evaluar la seguridad y eficacia de estos dos tratamientos en pacientes con enfermedades hepáticas. La atorvastatina es un fármaco muy estudiado y generalmente bien tolerado. En el caso de la ghrelina recombinante, su farmacología no es tan bien conocida pero ha sido utilizada en pacientes con diferentes condiciones clínicas como gastroparesis (265), anorexia (239), caquexia (240) y cardiopatías (217). En todos estos estudios, la ghrelina fue bien tolerada y sólo causó una disminución moderada de la presión arterial. Por otro lado, debido a las propiedades orexigénicas de la ghrelina, recientemente se han propuesto antagonistas de su receptor para el tratamiento de la obesidad y la diabetes (266). Debido a sus efectos protectores y antifibrogénicos, el bloqueo prolongado de los receptores de la ghrelina podría causar efectos adversos como una acelerada fibrosis, que se observa muy comúnmente en corazón y riñón en muchos de los pacientes con síndrome metabólico.

5. CONCLUSIONES

Las principales conclusiones que se han obtenido con los estudios presentados en esta tesis son:

1. La atorvastatina inhibe las acciones patogénicas de la angiotensina II en el hígado, incluyendo el daño hepatocelular y el reclutamiento de células inflamatorias.

2. El tratamiento con atorvastatina inhibe la acumulación de células fibrogénicas y el estrés oxidativo inducida por la angiotensina II.

3. La atorvastatina y el losartan ejercen un efecto hepatoprotector en el modelo de daño agudo en hígado, inducido por tetracloruro de carbono.

4. Las acciones proinflamatorias y profibrogénicas de la angiotensina II sobre las células estrelladas hepáticas en cultivo son bloqueadas por la atorvastatina.

5. La administración de ghrelina disminuye la fibrosis hepática experimental mediante la disminución del acúmulo de células fibrogénicas y la modulación de la expresión génica.

6. El agonista del receptor de la ghrelina $(Des\text{-}Ala)^3$-GHRP-2 reproduce los efectos antifibrogénicos de la ghrelina *in vivo*.

7. Los ratones deficientes en ghrelina son más susceptibles al daño hepático crónico que los ratones salvajes.

8. La administración de ghrelina reduce el daño hepático en un modelo animal de daño agudo en hígado, inducido por tetracloruro de carbono.

9. Los mecanismos por los cuales la ghrelina ejerce un efecto hepatoprotector incluyen una disminución del infiltrado inflamatorio, una disminución del estrés oxidativo y una reducción en la muerte celular.

10. Los niveles séricos de ghrelina son menores en pacientes con fibrosis avanzada que en pacientes con fibrosis leve o moderada.

11. La expresión génica de ghrelina en hígado se correlaciona positivamente con la de otros genes implicados en inflamación y fibrosis.

12. Un haplotipo del gen de la ghrelina se asocia con una mayor progresión de la fibrosis.

En conjunto, estos resultados indican que la atorvastatina podría ser útil en el tratamiento de pacientes inflamación hepática y la ghrelina podría ser útil en pacientes con inflamación y fibrosis hepática.

6. ANEXO

Como resultado de esta tesis existen otras dos publicaciones:

1. **Reduction of advanced liver fibrosis by short-term targeted delivery of an angiotensin receptor blocker to hepatic stellate cells in rats.**

   Estudio colaborativo con la Universidad de Groningen (Holanda) liderado por el laboratorio de fibrosis hepática del IDIBAPS publicado en *Hepatology*.

   El losartan es un antagonista del receptor AT1 de la Ang II que tiene un efecto antifibrogénico en el hígado. En este estudio se evaluó la eficacia antifibrogénica del losartan conjugado a una molécula transportadora (manosa-6-fosfato - albúmina, M6PHSA) que permite su liberación específica a las HSC activadas. En el estudio presentado se demuestra que, tras administrarlo a ratas, el conjugado losartan-M6PHSA se encuentra específicamente localizado en el hígado, y que disminuye la fibrosis hepática en un modelo de fibrosis hepática en ratas de manera más eficaz que el losartan sin conjugar. Esta estrategia permite reducir considerablemente la dosis administrada de losartan y por consiguiente una disminución de sus efectos sistémicos.

2. **Cytokines and renin-angiotensin system signaling in hepatic fibrosis.**

   Capítulo en "The Clinics of North America".

   En este capítulo se revisa el papel del SRA en la fibrosis hepática y las moléculas más importantes implicadas en el proceso de fibrosis hepática (desde citocinas hasta vías de señalización).

Reduction of Advanced Liver Fibrosis by Short-Term Targeted Delivery of an Angiotensin Receptor Blocker to Hepatic Stellate Cells in Rats

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There is no effective therapy for advanced liver fibrosis. Angiotensin type 1 (AT1) receptor blockers attenuate liver fibrogenesis, yet their efficacy in reversing advanced fibrosis is unknown. We investigated whether the specific delivery of an AT1 receptor blocker to activated hepatic stellate cells (HSCs) reduces established liver fibrosis. We used a platinum-based linker to develop a conjugate of the AT1 receptor blocker losartan and the HSC-selective drug carrier mannose-6-phosphate modified human serum albumin (losartan-M6PHSA). An average of seven losartan molecules were successfully coupled to M6PHSA. Rats with advanced liver fibrosis due to prolonged bile duct ligation or carbon tetrachloride administration were treated with daily doses of saline, losartan-M6PHSA, M6PHSA or oral losartan during 3 days. Computer-based morphometric quantification of inflammatory cells (CD43), myofibroblasts (smooth muscle α-actin [α-SMA]) and collagen deposition (Sirius red and hydroxyproline content) were measured. Hepatic expression of procollagen α2(I) and genes involved in fibrogenesis was assessed by quantitative polymerase chain reaction. Losartan-M6PHSA accumulated in the fibrotic livers and colocalized with HSCs, as assessed by immunostaining of anti-HSA and anti–α-SMA. Losartan-M6PHSA, but not oral losartan, reduced collagen deposition, accumulation of myofibroblasts, inflammation and procollagen α2(I) gene expression. Losartan-M6PHSA did not affect metalloproteinase type 2 and 9 activity and did not cause apoptosis of activated HSCs. *Conclusion:* Short-term treatment with HSC-targeted losartan markedly reduces advanced liver fibrosis. This approach may provide a novel means to treat chronic liver diseases. (HEPATOLOGY 2010;51:942-952.)

Hepatic fibrosis is the consequence of most types of chronic liver diseases.[1] There are no effective therapies to treat liver fibrosis in patients in which the causative agent cannot be removed.[2] In experimentally-induced liver fibrosis, several agents reduce progression of the disease.[3] Inhibitors of the renin-angiotensin system (RAS) are probably the most promising drugs. There is extensive evidence indicating

Abbreviations: AT1, angiotensin type 1 receptor; CCl_4, carbon tetrachloride; HSC, hepatic stellate cell; IGF II, insulin-like growth factor II; M6PHSA, mannose-6-phosphate modified human serum albumin; RAS, renin-angiotensin system; ULS, universal linkage system.

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that the RAS regulates liver fibrogenesis.[4] RAS components are overexpressed in livers with fibrosis and angiotensin II induces inflammatory and fibrogenic effects *in vivo* and in activated hepatic stellate cells through AT1 receptors (HSC).[5,6] The blockade of AT1 receptors reduces the accumulation of activated HSCs and attenuates liver fibrosis in rats[7] and AT1 receptor–deficient mice exhibit attenuated response to hepatic inflammation and fibrosis.[8] However, the efficacy of AT1 receptor blockers to reverse established fibrosis is unknown. We propose an innovative approach to deliver drugs to activated HSCs, increasing the concentration in the liver at the sites of active fibrogenesis. Moreover, drug delivery can be useful to avoid systemic undesirable effects such as renal dysfunction.

The drug delivery system applied in this study uses mannose 6-phosphate modified human serum albumin (M6PHSA), a carrier that delivers drugs to activated HSCs.[9] M6PHSA binds to the mannose-6-phosphate/insulin growth factor type II receptor (M6P/IGII-R), a surface exposed receptor that is *de novo* expressed in activated HSCs during liver fibrogenesis.[10] Prior studies demonstrated rapid and efficient accumulation of drug-M6PHSA conjugates in the fibrotic liver.[11,12] To conjugate losartan to M6PHSA, we employed a novel type of platinum linker called ULS (Universal Linkage System), which can bind losartan via a coordinative bond at one of the aromatic nitrogen atoms in the tetrazole group.[13-15] Application of this coordinative linker technology has several important advantages, for instance straightforward coupling of drugs, adequate stability of conjugates, and slow-release of the active pharmacon within target cells.[11]

In the present study, we administered losartan-M6PHSA for a short period of time to rats with advanced fibrosis. We demonstrate that losartan-M6PHSA accumulates exclusively in the fibrotic liver at the sites of activated HSCs. Importantly, treatment with losartan-M6PHSA, but not free losartan given orally, reduced both hepatic inflammation and fibrosis.

Materials and Methods

Synthesis of Losartan-M6PHSA. Losartan and human serum albumin (HSA) were obtained from Synfine (Ontario, Canada) and Sanquin (Amsterdam, The Netherlands), respectively. Losartan was first coupled to Universal Linkage System (ULS; Kreatech BV, The Netherlands). ULS was prepared as described elsewhere.[11] ULS (32 μmol) in dimethylformamide (DMF) was added to a solution of losartan (32 μmol, 10 mg/mL of the potassium salt of losartan in DMF). Mass spectrometry analysis confirmed the presence of the 1:1 losartan-ULS species after completion of the reaction, whereas ^{195}Pt-NMR confirmed the coordination of Pt(II) to a nitrogen donor site. Ion-spray mass spectrometry (ESI^+) mass-to-charge ratio (m/z): 711-717 $[losartan\text{-}ULS\text{-}Cl]^+$, 748-754 $[losartan\text{-}ULS\text{-}DMF]^+$ ^{195}Pt NMR of losartan-ULS (CD_3OD): −2491 and −2658 ppm. M6PHSA was prepared and characterized as described previously.[16] A total of 10 mg M6PHSA (14.3 nmol) was dissolved in 1 mL 20 mM tricine/$NaNO_3$ buffer (pH 8.5) and reacted with losartan-ULS (143 nmol) in 10-fold molar excess overnight at 37°C. The losartan-M6PHSA product was purified by dialysis against PBS at 4°C, filter-sterilized and stored at −20°C. Protein content of the conjugates was assessed by the BCA assay (Pierce, Rockford, IL). ULS content per losartan-M6PHSA was evaluated by inductively coupled plasma–atomic emission spectroscopy (ICP-AES) at 214.424 nm and at 265.945 nm with a VISTA AX CCD Simultaneous ICP-AES (Varian, Palo Alto, CA). Standards (cisplatin) and unknown samples were spiked with yttrium as an internal standard (360.074 nm). Losartan conjugated to M6PHSA was determined after competitive dissociation of drug-ULS bonds by potassium thiocyanate, as described previously.[11,15] High performance liquid chromatography (HPLC) analyses were performed on a thermostated C18 column (Sunfire; Waters Inc., Milford, MA) with an isocratic mobile phase consisting of acetonitrile–water–trifluoroacetic acid (30:70:0.1, vol/vol/vol; pH 2.0). Losartan-M6PHSA and M6PHSA were subjected to anion-exchange and size exclusion chromatography as described.[9]

Animal Experimental Procedures. Liver fibrosis was induced in 250 g male Wistar rats (Harlan, Zeist, The Netherlands) by either bile duct ligation or chronic treatment with CCl_4. For the bile duct ligation,[17] rats were anesthesized with isoflurane (2% isoflurane in 2:1 O_2/N_2O, 1 L/minute; Abbot Laboratories Ltd., Queensborough, UK). The common bile duct was doubly ligated with 4-0 silk and transected between the two ligations. Sham operation was performed similarly with exception of ligating and transecting the bile duct. Animals were sacrificed 15 days after surgery. Arterial blood pressure was measured immediately before tissue harvesting. Animals were anesthesized with pentobarbital (30 mg/kg intraperitoneally) and the right carotid artery was cannulated (PE-90; Transonics Systems Inc., Ithaca, NY). The mean arterial blood pressure was recorded using a pressure analyzer (LPA-200; Digi-Med, Louisville, KY) for 10 minutes. In the model of CCl_4-induced liver fibrosis, rats were fed ad libitum with standard chow and drinking water containing phenobarbital (0.3 g/L). Fibrosis was induced by inhalation of CCl_4 for 8 weeks as

described previously.[18] In both experimental models, rats received a daily injection by the penis vein of saline, losartan-M6PHSA (3.3 mg/kg/day, corresponding to 125 μg losartan/kg), M6PHSA alone (3.3 mg/kg/day), or an oral administration of losartan by gavage (5 mg/kg/day) at 72, 48, and 24 hours before sacrifice. For pharmacokinetic studies, a subset of rats received an additional dose of the treatments 10 minutes before sacrifice. To determine the efectiveness of long-term treatment with losartan-M6PHSA on advanced fibrosis, rats were treated by CCl_4 inhalation for 10 weeks. During the last 3 weeks, rats received saline, losartan-M6PHSA (3.3 mg/kg/day), or M6PHSA alone (3.3 mg/kg/day) by the penis vein twice a week. At least 10 rats were included per group in both models. Animal procedures were approved by the Committee for Care and Use of Laboratory Animals of the Hospital Clínic, Barcelona, and are in accordance with National Institutes of Health guidelines.

Analysis of Losartan-M6PHSA Biodistribution. The presence of losartan-M6PHSA or M6PHSA in tissue cryosections was demonstrated by immunostaining using an anti-HSA antibody (Cappel ICN Biomedicals, Zoetermeer, The Netherlands).[19] The colocalization of losartan-M6PHSA with HSC was assessed by double immunostaining of anti-HSA (Cappel ICN Biomedicals, Zoetermeer, The Netherlands) and anti–α-SMA (Abcam, Cambridge, UK). To avoid cross-reactivity of anti-HSA antibody with rat albumin, normal rat serum was added to the antibody. Sections from rats that did not receive HSA were completely negative after the anti-HSA staining. The amount of losartan in liver tissue homogenates was analyzed by HPLC as described above. Two different procedures were employed to isolate losartan from tissue homogenates. The first method consisted of direct extraction from the livers, whereas the second method comprised an additional incubation of tissues overnight with potassium thiocyanate in order to chemically release conjugate-bound losartan, as described above.

Quantification of Collagen Accumulation and Infiltration by Myofibroblastic Cells and Apoptosis. The degree of hepatic fibrosis was estimated as the percentage of area stained with picrosirius Red (Sirius Red F3B; Gurr-BDH Lab Supplies, Poole, UK).[6] The amount of fibrogenic myofibroblasts was estimated by measuring the percentage of area stained with α-SMA antibody (DAKO, Carpinteria, CA). For morphometric assessment of percentage of area with positive staining, an optic microscope (Nikon Eclipse E600) connected to a high-resolution camera (CC12 Soft-Imaging System, Münster, Germany) was used. Images were analyzed in an automated image-analysis system (AnalySIS, Soft-Imaging System, Münster, Germany). Results are given as percentage of positive area. Cell apoptosis was quantified by using In Situ Death Detection Kit, POD (Roche Applied Science, Barcelona, Spain) based on terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL). TUNEL-positive cells per high-power field (200×) were counted. All measurements were performed blindly.

Statistical Analysis. Results are expressed as the mean ± standard error of the mean. Significance was established using Student *t* test, two-way analysis of variance with Bonferroni's post hoc test and Mann-Whitney assay. Differences were considered significant if $P < 0.05$.

Other methods are shown in Supporting Materials and Methods.

Results

Synthesis of Losartan-M6PHSA and Internalization by HSCs. Losartan was conjugated to manose-6-phosphate coupled to human serum albumin (M6PHSA) (Fig. 1A). After its reaction to the linker at a stoichiometric ratio (Fig. 1B), the losartan-ULS adduct was conjugated to M6PHSA. An average of seven losartan-ULS molecules were coupled to M6PHSA, as assessed by HPLC and confirmed by inductive coupled plasma-atomic emission spectroscopy (ICP-AES) (data not shown). Conjugation of losartan to M6PHSA did not change the charge or size features of M6PHSA, as assessed by anion-exchange chromatography and size exclusion chromatography, respectively (Fig. 1C,D). Because ULS is a derivative of cisplatin, an antitumor agent that may cause cell toxicity, we studied the effects of losartan-M6PHSA on cultured HSCs. Losartan-M6PHSA did not cause cell toxicity, while cisplatin induced cell death, suggesting that occupation of the coordinative sites of platinum with drug and carrier prevents its disruptive reactivity with cellular components (Fig. 1E). To test whether losartan-M6PHSA is biologically active in cultured HSCs, cells were stimulated with angiotensin II in the presence or absence of either free losartan or losartan-M6PHSA. We found that both treatments equally blunted angiotensin II–induced intracellular calcium increase (Fig. 1F). Also, we detected intracellular staining for HSA after incubating HSCs with losartan-M6PHSA for 10 minutes. This staining was strongly blunted by excess of M6P sugars and an antibody against the M6P/IGF II receptor. We found 25.2 ± 2.4, 0.2 ± 0.1, and 5.3 ± 0.6 positive cells in cultures incubated with isotype-matched antibody, excess of M6P, and anti-IGFRII antibody, respectively ($P < 0.001$ of isotype-matched antibody respect to the other two conditions) (Fig. 2A).

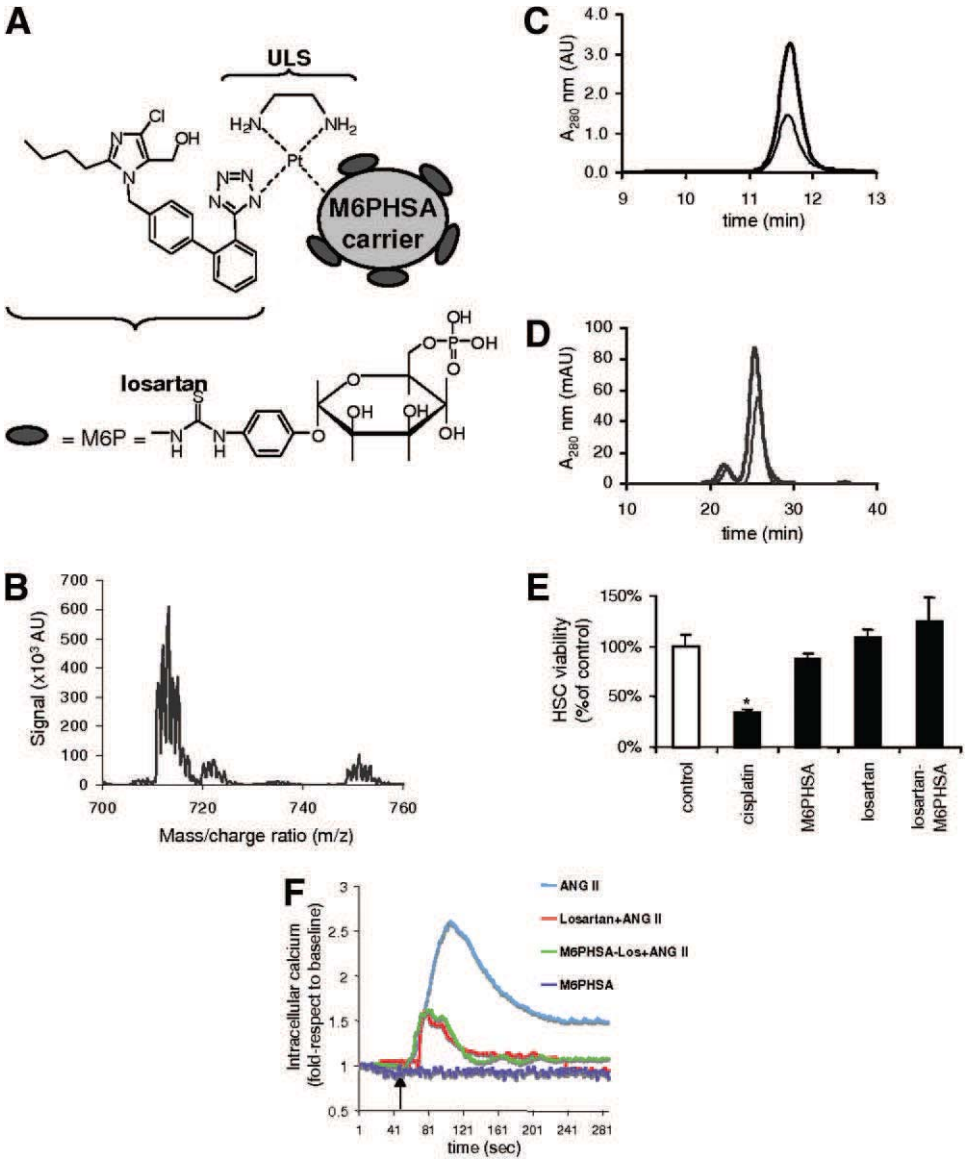

Fig. 1. Synthesis and characterization of losartan-M6PHSA. (A) Coordinative linkage between losartan, ULS, and M6PHSA. M6PHSA consists of an albumin core modified with approximately 28 mannose-6-phosphate (M6P) groups. (B) The formation of losartan-ULS was confirmed by ion-spray mass spectrometry, which demonstrated the typical isotopic pattern of platinum compounds. MonoQ anion exchange chromatography (C) confirmed that the charge of losartan-M6PHSA was not affected by losartan coupling. Bold line: losartan-M6PHSA; thin line: M6PHSA. (D) Size exclusion chromatography showed that losartan-M6PHSA and M6PHSA consisted for >90% of monomeric material and a minor fraction of dimeric albumin. Bold line: losartan-M6PHSA; thin line: M6PHSA. (E) Losartan-M6PHSA induced no cytotoxicity, as assessed by the Alamar Blue viability assay when incubated with rat HSCs for 24 hours. Indicated compounds were equivalent to 100 μM of platinum (cisplatin, losartan-M6PHSA) or equivalent to the amount of drug (100 μM) or carrier (1 mg/mL) in the losartan-M6PHSA preparation. *$P < 0.001$ compared to control HSCs and losartan-M6PHSA using Student t test. (F) Changes in $[Ca++]_i$ in cultured HSCs stimulated with saline or angiotensin II (10^{-8} M) in the presence of different compounds. Results are the mean of at least 15 cells per condition.

These results indicate that losartan-M6PHSA directly interacts with IGF II receptors present in HSCs, and is internalized to inhibit angiotensin II–induced biological actions.

Pharmacokinetics of Losartan-M6PHSA in Bile Duct –Ligated Rats. M6PHSA binds to M6P/IGFII-R, which is expressed in activated HSCs in the fibrotic liver.[16] In the bile duct ligation model, we administered losartan-M6PHSA (3.3 mg/kg, corresponding to 125 μg losartan/kg) daily from day 12-14 and animals were sacrificed at day 15. For pharmacokinetic purposes, a subgroup of the animals received an additional dose of the conjugate at 10 minutes before sacrifice. Control groups were treated with equivalent doses of M6PHSA (3.3 mg/kg), saline, or free losartan given orally at a dose (5 mg/kg) that has been shown to attenuate liver fibrosis when given for a prolonged period of time.[20,21] We first assessed whether losartan-M6PHSA preferentially accumulates in the fibrotic rat liver. The liver and other organs (lungs, heart, spleen, and kidneys) were stained with anti-HSA to detect the presence of the albumin-based conjugate. Losartan-M6PHSA was only detected in the liver (Fig. 2B). Injection of the carrier alone (M6PHSA) followed a similar distribution pattern (not shown). Importantly, losartan-M6PHSA colocalized with activated HSCs, as assessed by double immunostaining with anti-HSA and anti–α-SMA antibodies (Fig. 2C). To further demonstrate the selective homing of losartan-M6PHSA in the liver, tissue levels of losartan were quantified by HPLC. Animals receiving losartan-M6PHSA showed losartan levels which corresponded to 81% of the last injected dose being at least 20% of the cumulative dose (Fig. 2D). In contrast, oral losartan yielded liver tissue levels corresponding to only 4% of the cumulative dose (15% of the

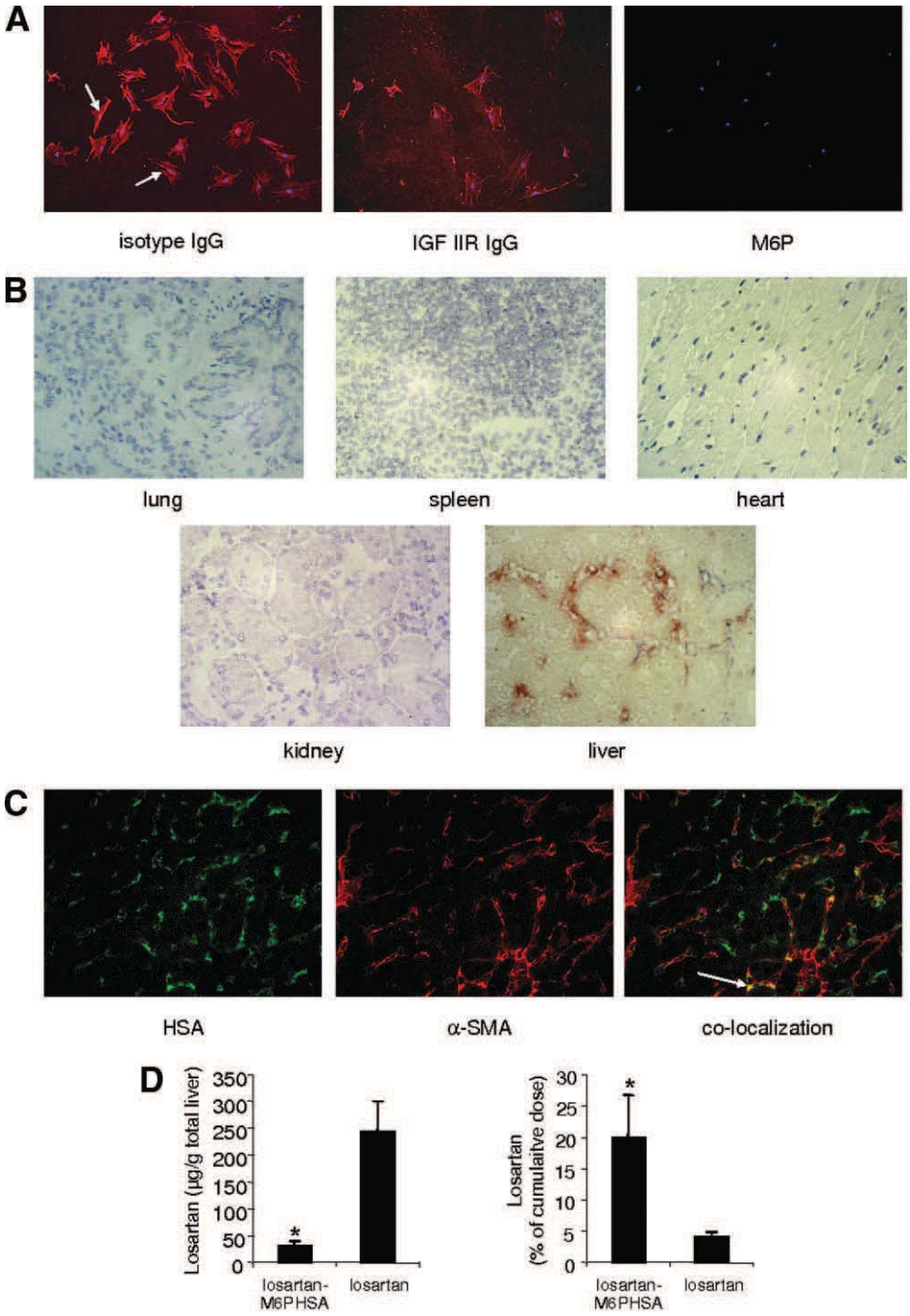

Fig. 2. Analysis of biodistribution of losartan-M6PHSA in HSCs and in rats with advanced fibrosis induced by prolonged bile duct ligation. (A) Representative pictures of cultured hepatic stellate cells (HSCs) visualized with confocal microscopy. Cells were incubated with losartan-M6PHSA (1 mg/mL) for 4 hours in the presence of an isotype-matched antibody (left picture), an anti–IGF-II antibody (middle picture), and with an excess of M6P, a ligand for the M6P/IGF-II receptor (right picture). Losartan-M6PHSA was clearly seen inside HSCs treated with isotype antibody (arrows), whereas both an anti–IGF-II antibody and M6P-HSA markedly prevented losartan-M6PHSA uptake. (B) Losartan-M6PHSA was not detected in the lung, spleen, heart, or kidney, but was detected in the liver within the nonparenchymal cells of rats treated with losartan-M6PHSA (magnification 40×). Staining was absent in rats treated with saline (not shown). (C) Losartan-M6PHSA colocalized with stellate cells in rat liver (arrow), as assessed with double immunostaining with anti-HSA and anti–α-SMA. Epifluorescence microscopy verified the colocalization of anti–α-SMA (red fluorescence color) and anti-HSA (green fluorescence color) in the regions colored in yellow (arrow) (magnification 400×). Five rats were studied per group. (D) Quantification of losartan in liver homogenates by HPLC. Absolute levels of losartan in the liver were highest for orally administered losartan (left panel), but represented a five-fold lower relative accumulation (right panel) in view of the different doses administered. *$P < 0.05$ (losartan).

last dose administered). These results illustrate the preferential hepatic accumulation of losartan-M6PHSA. However, because free losartan was administered at a 40-fold higher dose as compared to targeted losartan, the control treatment yielded nine-fold higher absolute concentrations.

Treatment with Losartan-M6PHSA, but not Oral Losartan, Reduces Advanced Liver Fibrosis. Rats were submitted to prolonged ligation of the common bile duct, which induces profound changes in the hepatic architecture including bridging fibrosis.[17] As expected, bile duct ligation for 15 days resulted in a marked increase in serum bilirubin and aminotransferase levels, which were unaffected by any of the treatments. Bile duct–ligated rats treated with saline or M6PHSA alone showed severe septal fibrosis (Fig. 3A). Hepatic collagen, as assessed by morphometric analysis of Sirius red staining and hydroxyproline content, was markedly increased in these rats as compared to sham-operated rats (Fig. 3A,B). In contrast, bile duct–ligated rats treated with losartan-

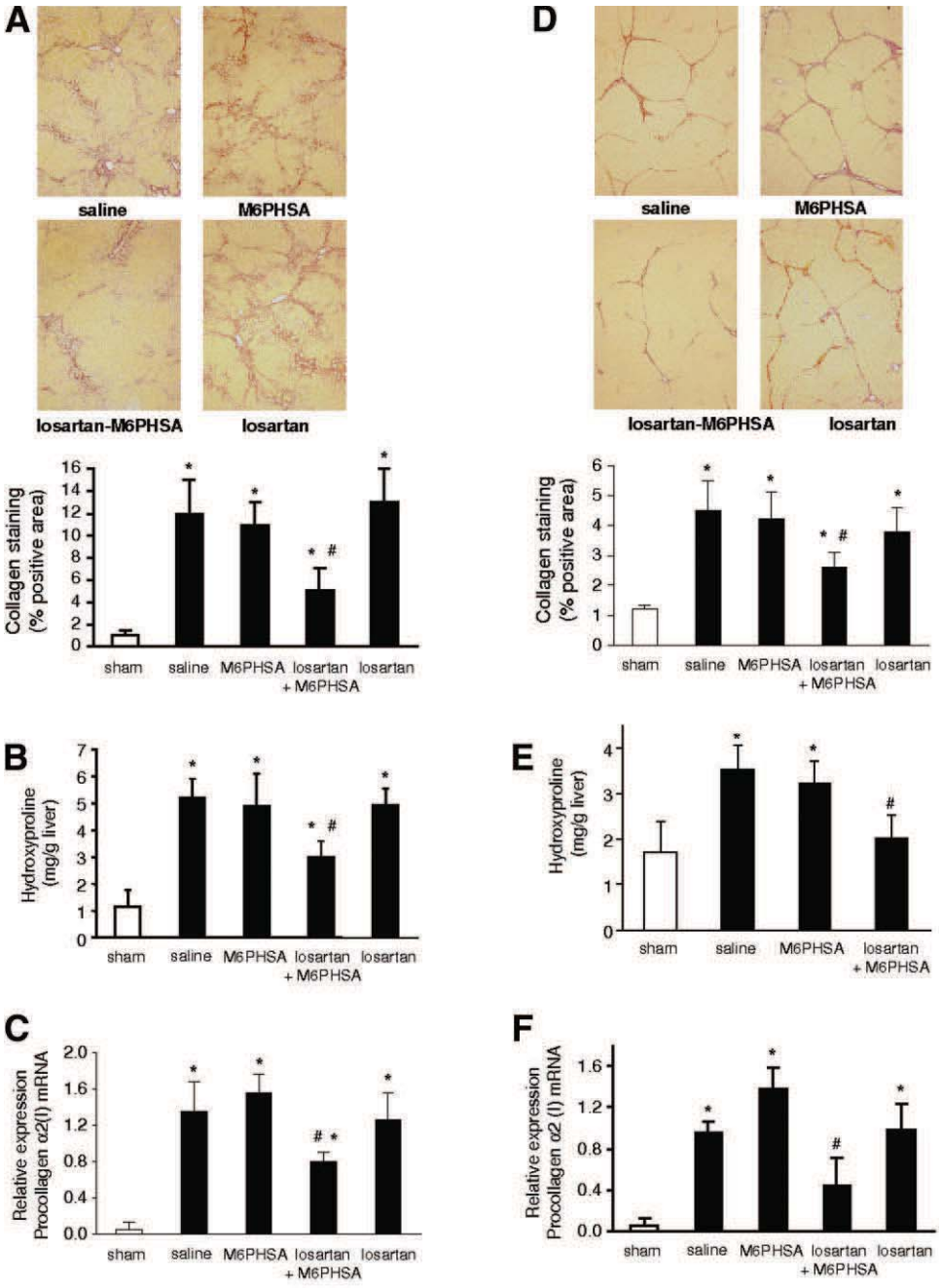

Fig. 3. Effect of different treatments on the degree of liver fibrosis in two different experimental models of liver fibrosis. (A) Bile duct ligation model. Severe bridging was observed in rats receiving saline, M6PHSA, or oral losartan. In contrast, rats treated with losartan-M6PHSA showed fewer areas with collagen accumulation (magnification 40×). Morphometric quantification of the area with Sirius red staining in rat livers showed significant inhibitory effects by losartan-M6PHSA, but not by other treatments. Pictures represent a reconstruction of 16 different areas of the liver biopsy; 40× magnification. (B) Analysis of collagen deposition in bile duct–ligated rats by measuring hydroxyproline content. Hepatic hydroxyproline markedly increased in rats with bile duct ligation compared to controls. Losartan-M6PHSA, but not oral losartan, reduced hydroxyproline content. (C) Quantification of the messenger RNA expression of procollagen α2(I). Expression was reduced by losartan-M6PHSA treatment. Losartan-M6PHSA but not oral losartan also reduced liver fibrosis in rats treated with carbon tetrachloride (CCl_4) as assessed by (D) Sirius red staining, (E) hydroxyproline content, and (F) procollagen α2(I) gene expression. Pictures represent a reconstruction of 16 different areas of the liver biopsy; 40× magnification. $^{*}P < 0.05$ versus sham; $^{\#}P < 0.05$ versus other fibrotic groups. Results are the mean of at least five different samples per condition.

M6PHSA showed less collagen deposition with less frequent formation of bridging fibrosis. Importantly, short-term oral treatment with losartan alone did not reduce histological fibrosis or the amount of collagen content. To confirm these results, hepatic procollagen α2(I) gene expression was quantified. Procollagen α2(I) was up-regulated 10-fold in bile duct–ligated rats treated with saline compared with sham-operated animals. Losartan-M6PHSA, but not oral losartan or M6PHSA alone, reduced procollagen α2(I) by 60% (Fig. 3C). These results indicate that short-term treatment with losartan-M6PHSA, but not oral losartan, attenuates advanced liver fibrosis. To provide additional evidence of the antifibrotic effects of HSC-targeted losartan, liver fibrosis was also induced by CCl_4 for 8 weeks.[18] Rats treated with CCl_4 for 8 weeks showed a marked distortion of the hepatic architecture with bridging fibrosis. At the end of the treatment period, rats received three consecutive daily doses of saline, oral losartan, losartan-M6PHSA, or M6PHSA alone. Similar to bile duct–ligated rats, we administered a final dose 10 minutes before sacrifice, to enable the detection of losartan-M6PHSA in the tissues. Losartan-M6PHSA accumulated in the fibrotic liver to a similar extent (13% ± 6% of the cumulative dose, n = 10, data

not shown) as observed in bile duct–ligated rats. Hepatic collagen content, as assessed by morphometric analysis of Sirius red staining, hydroxyproline content, and procollagen α2(I) gene expression, was reduced in rats treated with losartan-M6PHSA (Fig. 3D,E,F). Finally, none of the treatments in both experimental models induced changes in renal function, as indicated by normal serum creatinine levels, nor histological changes in the heart or the kidney (data not shown). Both losartan-M6PHSA and oral losartan induced a slight decrease in arterial pressure (data not shown). All together, these results demonstrate that short-term treatment with losartan targeted to HSCs is highly effective in attenuating liver fibrosis in rats. To investigate whether long-term treatment with losartan-M6PHSA was also effective, a new experimental procedure was carried out. Advanced liver fibrosis was established by CCl_4 inhalation for 10 weeks. During the last 3 weeks, rats were given saline, losartan-M6PHSA, or M6PHSA alone twice a week. We found that losartan-M6PHSA was able to reduce collagen synthesis, as assessed by reduced expression of procollagen α1(I) and procollagen α2(I). However, the amount of activated HSCs (as assessed by α-SMA expression) and the degree of collagen accumulation (as assessed by Sirius red staining) were not significantly reduced (Supporting Fig. 1). Further studies identifying the ideal route and drug dosage from long-term studies are clearly required.

Mechanisms of the Antifibrotic Effect of Losartan-M6PHSA. To explore the mechanisms involved in the potent antifibrotic effect of losartan-M6PHSA, we first assessed the accumulation of fibrogenic myofibroblasts by morphometric quantification of α-SMA–positive cells. Bile duct ligation resulted in the accumulation of abundant α-SMA–positive cells around proliferating bile ducts as well as in the hepatic sinusoids (Fig. 4A,B). Treatment with losartan-M6PHSA, but not oral losartan or M6PHSA alone, was associated with a significant reduction in the accumulation of myofibroblasts, as determined by morphometric analysis of the positively stained area (Fig. 4C). This effect was not associated with increased HSC apoptosis (data not shown). In the CCl_4 model of liver fibrosis, α-SMA hepatic immunostaining was also reduced by losartan-M6PHSA treatment.(Fig. 4D,E) Next, we assessed hepatic expression of metalloproteinases (MMP) 3 and 9 and tissue inhibitor of metalloproteinase-1 (TIMP-1). Bile duct ligation resulted in a marked increase in these four genes, which was not reduced by losartan-M6PHSA or oral losartan (Fig. 5A,B,D). However, TIMP-1 protein expression was reduced, as assessed by immunohistochemistry (Supporting Fig. 2). We also assessed the activity of metalloproteinases MMP2 and MMP9 by gelatin zymography. We found that losartan-M6PHSA did not modify MMP2 and MMP9 activity in bile duct-ligated rats (Fig. 5C). Also, we explored the hepatic expression of transforming

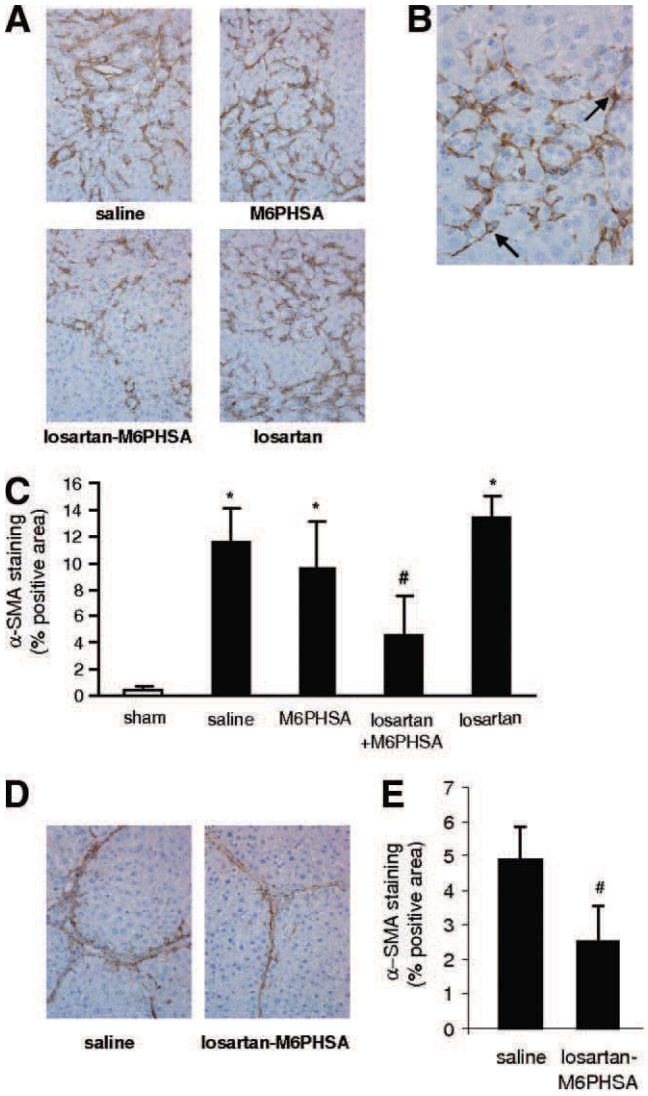

Fig. 4. Effect of different treatments on α-SMA–positive cells in fibrotic livers. (A) Effect of different treatments on the accumulation of myofibroblasts and activated HSCs. Liver sections of bile duct–ligated animals stained with anti–smooth muscle α-actin expression (α-SMA) antibody. Bile duct–ligated animals showed a marked accumulation of α-SMA–positive cells. Rats treated with losartan-M6PHSA showed fewer α-SMA–positive cells. Pictures represent a reconstruction of 16 different areas of the liver biopsy, 40× magnification. (B) High power magnification (400×) photomicrograph of a liver from a bile duct–ligated rat treated with saline. α-SMA staining was detected in cells located in the sinusoids corresponding to activated HSCs as well as in myofibroblasts around proliferating bile ducts. (C) Morphometric quantification of the area with α-SMA staining in rat liver specimens ($^*P < 0.05$ versus sham; $^\#P < 0.05$ versus saline, M6PHSA and oral losartan). (D) In the CCl_4 model, treatment with losartan-M6PHSA reduced α-SMA staining in the liver as compared to diseased animals treated with saline. (E) Quantification of the area with α-SMA staining in liver specimens ($^\#P < 0.05$).

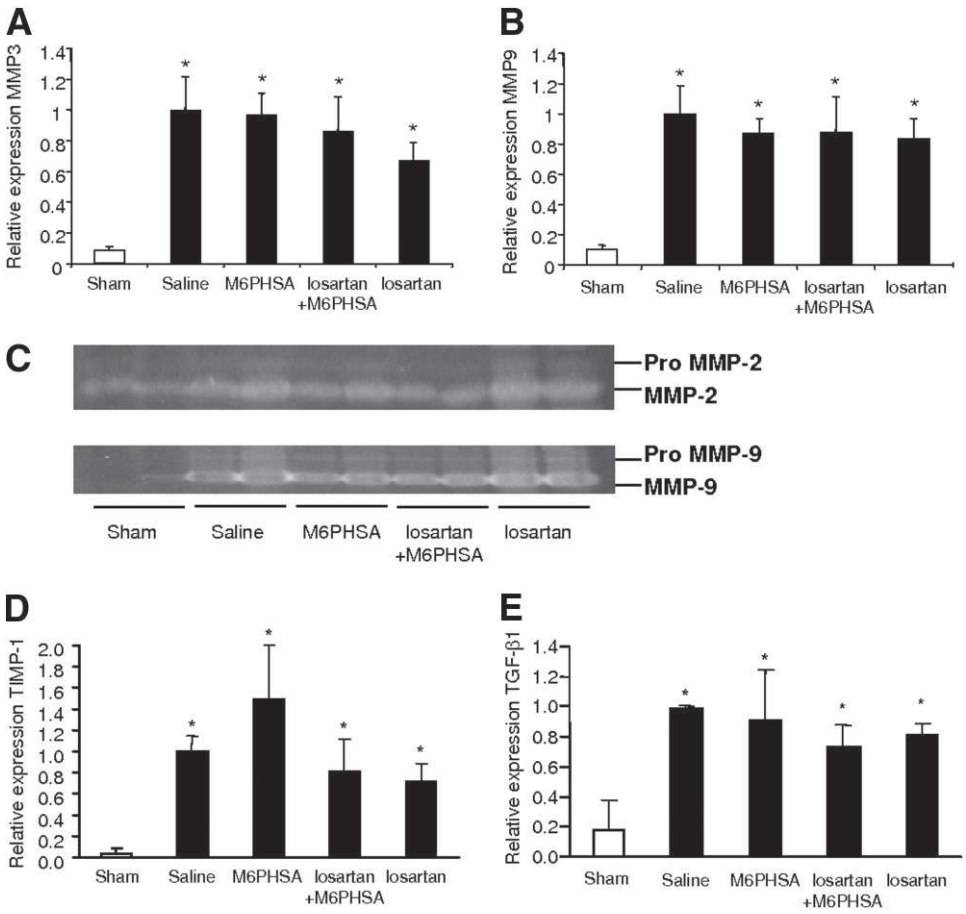

Fig. 5. Gene expression and metalloproteinase activity in the liver of rats submitted to different treatments. (A,B) Matrix metalloproteinases 3 and 9 (MMP3 and MMP9) expression in sham-operated rats and bile duct–ligated rats receiving various treatments. (C) Zymogram analysis showing activity for MMP2 and MMP9 in bile duct–ligated rats submitted to different treatments. (D) TIMP1 expression in sham-operated rats and bile duct–ligated rats. (E) TGF-β1 in sham-operated rats and rats with bile duct ligation receiving various treatments. Results are the mean of four independent experiments. *$P < 0.05$ versus sham-operated rats.

growth factor β1 (TGF-β1), a cytokine that mediates the fibrogenic actions of angiotensin II.[22] Bile duct ligated rats showed increased TGF-β1 gene expression, which was not reduced in rats treated with losartan-M6PHSA (Fig. 5E). Further studies should analyze protein expression of TGF-β1 to confirm these results. Furthermore, we explored whether losartan-M6PHSA reduces hepatic inflammation. First, we analyzed in HSCs the expression of proinflammatory genes (ICAM-1 and interleukin-8 [IL-8]). Both genes were up-regulated by angiotensin II treatment. Treatment by losartan and losartan-M6HSA reduced this effect (Fig. 6A,B). Next, *in vivo* liver inflammation was assessed by quantifying the infiltration of inflammatory cells (CD43-positive) in the hepatic parenchyma by immunohistochemistry. Compared to sham-operated rats, bile duct–ligated rats showed a marked increase in the infiltration of CD43-positive inflammatory cells (Fig. 7A). This effect was blunted by treatment with losartan-M6HSA and, to a lesser extent, by oral losartan. In contrast, monocyte chemotactic protein 1 expression was not modified by any of the treatments (Fig. 7C). The number of CD43-positive cells was also decreased in CCl_4-treated rats (Fig. 7B).

Discussion

This study demonstrates that advanced liver fibrosis can be attenuated by short-term administration of an antifibrotic drug selectively targeted to activated HSCs. We provide evidence that the delivery of the AT1 receptor blocker losartan to activated HSCs reduces hepatic inflammation and collagen deposition. This novel approach appears to be more effective than conventional treatment with oral losartan.

The new drug conjugate losartan-M6PHSA was successfully synthesized by applying a novel linker system that binds losartan via a transition-metal coordination bond. Traditionally, linking drugs to carrier moieties represents a complex issue involving tedious drug-derivatization reaction steps.[23] A key property of our platinum linker, ULS, is that it can be applied for conjugation of many valuable drug molecules containing aromatic nitrogens, forming a bond of intermediate binding strength. The ligand-exchange behavior of platinum compounds is quite slow, giving them a high kinetic stability.[24] The slow rate of drug release from the linker[11,15] will cause sustained drug release within target cells and will effectuate

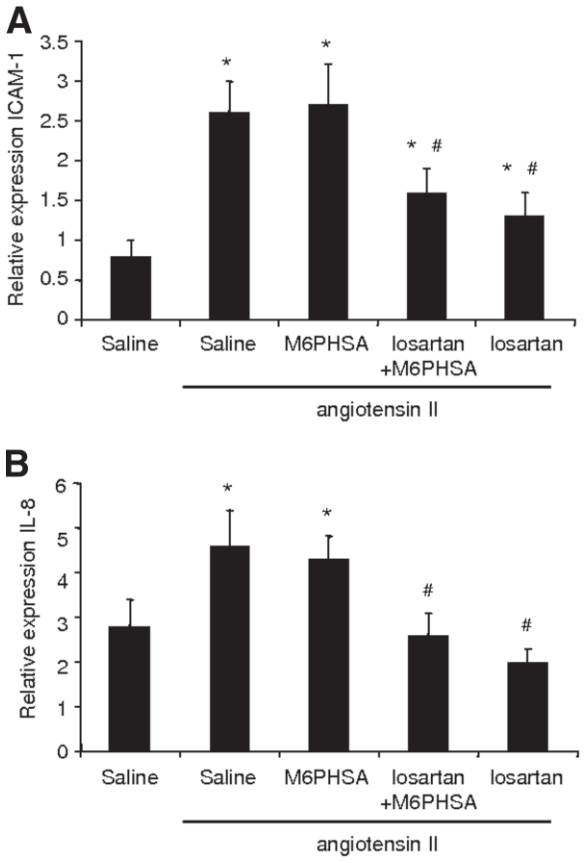

Fig. 6. Effects of different treatments on expression of proinflammatory genes in cultured primary hepatic stellate cells (HSCs). Gene expression of (A) intercellular adhesion molecule-1 (ICAM-1) and (B) interleukin-8 were measured by quantitative polymerase chain reaction in cultured HSCs. Angiotensin II (10^{-8} M) stimulated the expression of both genes. This effect was attenuated by both free losartan and losartan-M6PHSA. Results are the mean of four independent experiments.*$P <$ 0.05 versus saline; $^{\#}P < 0.05$ versus angiotensin alone.

only very low concentrations of reactive platinum in target cells, which are orders of magnitude lower than applied in cisplatin cancer therapy. One therefore would predict rapid detoxification of ULS by binding to cytosolic platinophilic ligands. The HSC viability studies with losartan-M6PHSA are in agreement with the safety data of other drug-M6PHSA conjugates prepared with the ULS linker.[11,25]

An important finding of the current study is that oral losartan given for short periods of time, did not reduce established fibrosis. This is not surprising, since in the vast majority of studies in which losartan reduces the extent of liver fibrosis, losartan is given concomitantly with the agent causing liver injury, and for prolonged periods of time (i.e., several weeks).[26,27] This finding suggests that antifibrotic drugs may be not as active as expected when administered to rats with established fibrosis, which is in line with the poor clinical usefulness of many preclinical drug-candidates. Here, we demonstrate that the selective delivery of antifibrotic drugs to the main fibrogenic cell type in the liver (i.e., activated HSCs) markedly increased the antifibrotic effect.

Different mechanisms may explain the strong antifibrotic effect achieved with our drug-targeting construct. First, targeting losartan to activated HSCs via the modified albumin, M6PHSA, increases the fraction of the dose that accumulates within the fibrogenic cells. Since HSCs only represent a small fraction of the total liver, the drug levels found in liver homogenates may underestimate the actual accumulation of losartan-M6PHSA within HSCs. However, orally administered losartan resulted in higher hepatic concentrations due to the much higher dose, which however produced weaker antifibrogenic effects. Thus, the strong effects of losartan-M6PHSA cannot be attributed to an increase in drug concentrations within the liver, but to the selectivity of losartan to activated HSCs. Secondly, the activity of losartan-M6PHSA may be enhanced by the specific interaction that M6PHSA provides. The M6P/IGFII receptor participates in the activation of latent TGF-β1, which may be affected by M6PHSA.[10] However, the finding that treatment with M6PHSA alone did not affect fibrosis or inflammation in bile duct-ligated rats does not support this hypothesis. Thirdly, we show that targeted losartan rapidly reduces the accumulation of activated HSCs in the fibrotic liver. This is consistent with previous reports showing that angiotensin II is a powerful mitogen for HSCs.[28] And finally, targeted losartan strongly attenuated infiltration of inflammatory cells, a major pathogenic event in liver fibrogenesis.[29] This latter effect is consistent with previous reports showing that Ang II exerts pro-inflammatory actions both in cultured cells and *in vivo*.[6,30] Although the cell type mediating the anti-inflamatory effect is unknown, activated HSCs are potential candidates. In fact, losartan-M6PHSA attenuated the inflammatory effects induced by angiotensin II on cultured HSCs. The beneficial effect of losartan-M6PHSA is not related with increased expression or activity of the collagenolytic enzymes MMP2, MMP3, and MMP9.

Our results may have implications for the treatment of chronic liver diseases. First, we provide evidence that short-term treatment with a highly active oral compound—losartan—is capable to attenuate the inflammatory response but it is not strong enough to reduce liver fibrosis. Therefore, the current assumption that RAS blockers are highly effective in attenuating experimental liver fibrosis should be tempered. Secondly, our results support the current research to develop innovative systems to deliver drugs to activated HSCs. This approach would be particularly useful in conditions with rapidly aggressive hepatic fibrosis (e.g., acute alcoholic hepatitis)

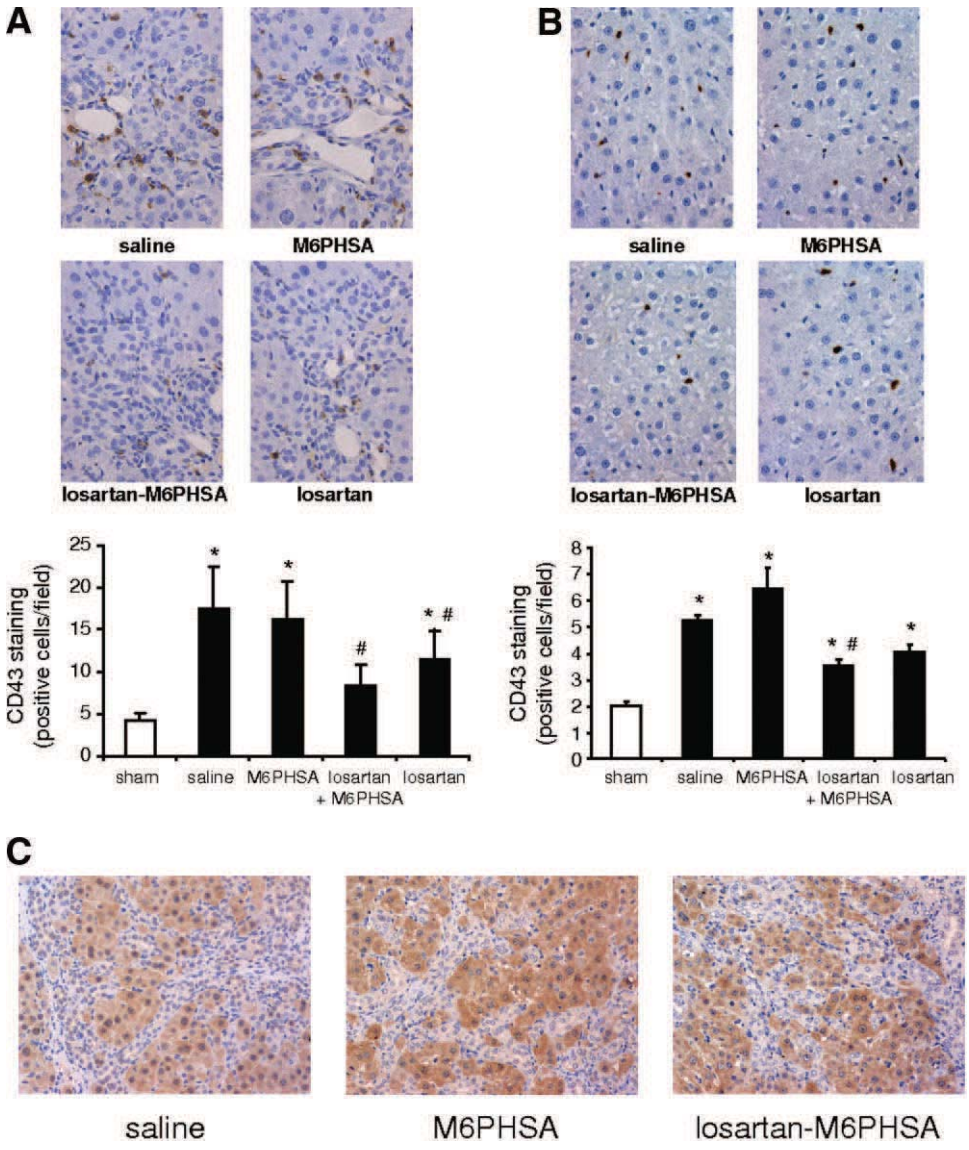

Fig. 7. Treatment with losartan-M6PHSA and, to a lesser extent, oral losartan reduced the number of infiltrating leukocytes in the liver parenchyma. Liver sections of (A) bile duct–ligated rats or (B) CCl_4-treated rats receiving the specified treatments were processed for immunohistochemistry and stained with anti-CD43 (magnification 40×). Rats receiving saline or M6PHSA showed intense infiltration of CD43-positive leukocytes. Treatment with losartan-M6PHSA reduced the inflammatory infiltrate. Quantification of the number of positive cells in 20 randomly chosen high-power fields (*$P < 0.05$ versus sham; #$P < 0.05$ versus saline and M6PHSA). (C) Representative pictures of immunostaining for monocyte chemotactic protein 1 in bile-duct ligated rats. No differences were detected between groups.

in which the use of AT1 receptors blockers may induce undesirable side effects such as renal failure. Thirdly, our results suggest the possibility to use drugs known to block other pathogenic functions of activated HSCs, such as cell contractility and angiogenic effects. These pathogenic actions of activated HSCs could participate in the pathogenesis of portal hypertension and the progression of hepatocellular carcinoma, respectively.[28,31]

Although the current study demonstrates that a short treatment of an antifibrotic drug to HSCs is able to reduce liver fibrosis, further studies should be performed to assess whether this strategy is also feasible for long periods of time. This aim includes initial pharmacodynamic studies to investigate the optimal route and dosage to ensure a stable and continuous release of the compounds to the fibrotic liver. We attempted to address this issue by giving losartan-M6PHSA for 3 weeks in rats with advanced fibrosis. This regime was able to reduce collagen synthesis but not the degree of fibrosis. This partial result can be explained by the lack of previous studies identifying the best regime for chronic administration of targeted drugs to HSCs. It is plausible that more frequent injections or the use of alternative routes (e.g., subcutaneous osmotic pumps) would have yielded positive results. We are currently performing complex pharmacological studies to address this issue.

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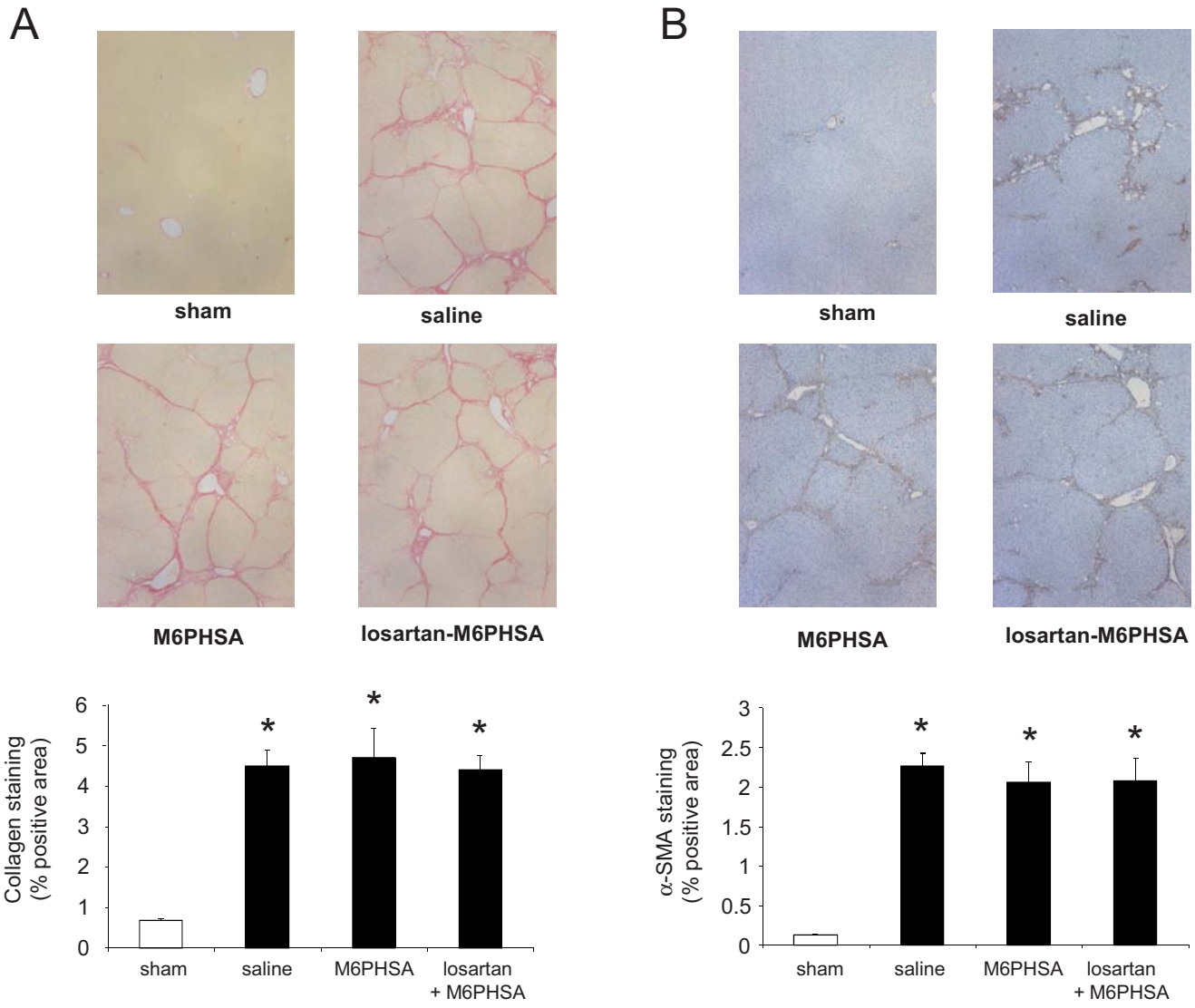
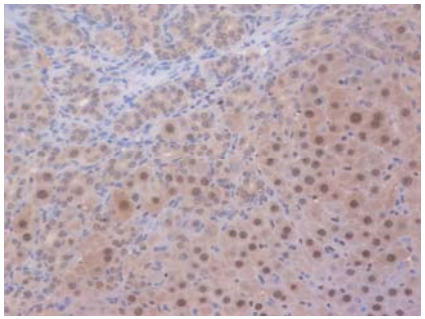


Supplementary Figure 1



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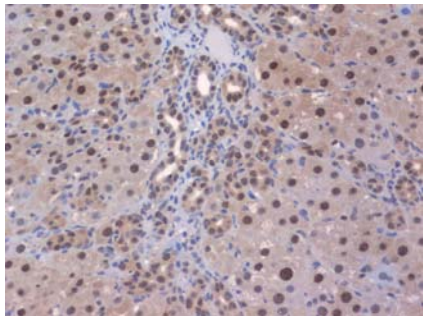
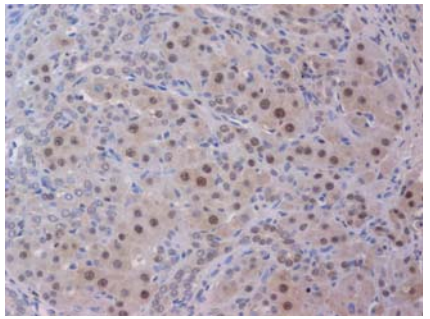


M6PHSA



losartan-M6PHSA

Supplementary Figure 2

SUPPLEMENTARY MATERIALS AND METHODS.

Cell toxicity studies.

Hepatic Stellate Cells (HSC) were freshly isolated from rat livers and purified with 12% nycodenz gradient centrifugation (1). Cells were studied after 10 days in culture (activated myofibroblastic HSC). Cells were cultured in 96 well-plates (10,000 cells/well) and then incubated for 24h at 37°C in culture medium containing either cisplatin (100 μM), M6PHSA (1 mg/ml), losartan (100 μM) or losartan-M6PHSA (1 mg/ml based in protein concentration, 100 μM in platinum concentration). Cell viability was determined using the Alamar Blue viability assay (Serotec Ltd, Kidlington, UK).

Internalization of losartan-M6PHSA by HSC.

HSCs were isolated from Wister rats and cultured until day 10, when all cells show a phenotypic activation into myofibroblastic cells. Cells were transferred to a labtek (8 chambers) and they were allowed to attach. At day 12, cells were incubated with 100 μg/ml losartan-M6PHSA (concentration was based on protein content) for 2 hrs at 37°C. Cells were pretreated 5 minutes prior to the losartan-M6PHSA conjugate with anti-IGFRII antibody (Santa-Cruz Biotechnology, Santa Cruz, CA), isotype-matched IgG or an excess of M6PHSA. After the 2 hr incubation, cells were fixed with methanol/acetone (1:1 v/v) for 15 minutes and stained with polyclonal rabbit-anti-human HSA (Cappel ICN Biomedicals, Zoetermeer, The Netherlands) and subsequently with TRITC-labeled swine-anti-rabbit (Dako, Carpinteria, CA). Labteks were examined with a fluorescence microscope. Cells with positive HSA staining were counted in 10 different high power fields. We found 25.2±2.4, 5.3±0.6 and 0.2±0.1 positive cells in cells incubated with isotype-matched antibody, anti-IGFRII antibody and excess of M6P, respectively ($p<0.001$ of isotype-matched antibody respect to the other 2 conditions).

Measurement of intracellular Ca^{++} concentration ($[Ca^{++}]_i$).

Intracellular calcium concentration was measured as described (2). Briefly, cells were cultured in glass bottom microwell dishes (MatTek, Ashland, MA) for 24 hours and then loaded with Fluo-4 (10 μM, Molecular Probes, Eugene, OR) for 20 min at 37°C. Cells were then rinsed twice with DMEM and stimulated with angiotensin II (10^{-8}M). Changes in $[Ca^{++}]_i$ was measured using a Zeiss LSM-510 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany). Results are expressed as changes in cell fluorescence over basal levels.

Immunohistochemistry studies

Paraffine-embedded liver sections were deparaffinized, rehydrated and stained using the DAKO Envision system (DAKO, Carpinteria, CA). To evaluate the degree of liver inflammation, CD43 immunostaining was performed using a monoclonal antibody against CD43 (1:1000, Serotec Inc; Oxford, UK). CD43 positive cells were counted (10 fields per specimen at x200 magnification). TIMP-1 expression was estimated by immunostaning using a polyclonal antibody against TIMP1 (1:20, Santa Cruz Biotechnology, Santa Cruz, CA). Monocyte chemotactic protein (MCP-1) expression was analyzed by immunostaining with using a polyclonal antibody against MCP-1 (1:50, Abcam, Cambridge, MA). All measurements were performed blindly.

Serum biochemical measurements.

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine and bilirubin levels were measured using standard enzymatic procedures.

Hepatic hydroxyproline content.

Hydroxyproline content was quantified colorimetrically from 0.2 g liver samples as described in detail previously (3). The results were expressed as μg hydroxyproline/g liver.

Analysis of hepatic gene expression.

RNA was isolated from frozen liver samples using Trizol (Life Technologies Inc., Rockville, MD). Quantitative PCR was performed with pre-designed Assays-on-Demand TaqMan probes and primer pairs for rat procollagen α2(I), procollagenα1(I), transforming growth factor β1 (TGF-β1), matrix metalloproteinase 3 and 9 (MMP 3 and 9), intercellular adhesion molecule type 1 (ICAM-1), interleukin-8, tissue inhibitor metalloproteinase type 1 (TIMP-1), tumor necrosis factor alpha (TNF-α) and ribosome subunit 18s (Applied Biosystems, Foster City, CA). Information on these Assay-on-Demand is available at: http://www.appliedbiosystems.com. TaqMan reactions were carried out in duplicate on an ABI PRISM 7900 machine (Applied Biosystems).

Zymogram

Gelatin zymography was performed in 7.5% SDS-PAGE containing 0.2% of gelatin. After electrophoresis, SDS was removed by 2.5% Triton X-100 to renature the gelatinases. Gels were then incubated in developing buffer (50 mM Tris-HCl buffer, 5 mM $CaCl_2$ and 2.5% of Triton X-100, pH 8) at room temperature for 30 minutes. Buffer was replaced by fresh developing buffer and incubated overnight at 37°C. Gels were then stained with 0.25% Coomassie Blue R-250 for 2 hours. Gels were destained gradually with destaining solution (50% Methanol, 10% acetic acid and 40% water). Bands were visulaized with a trasilluminator.

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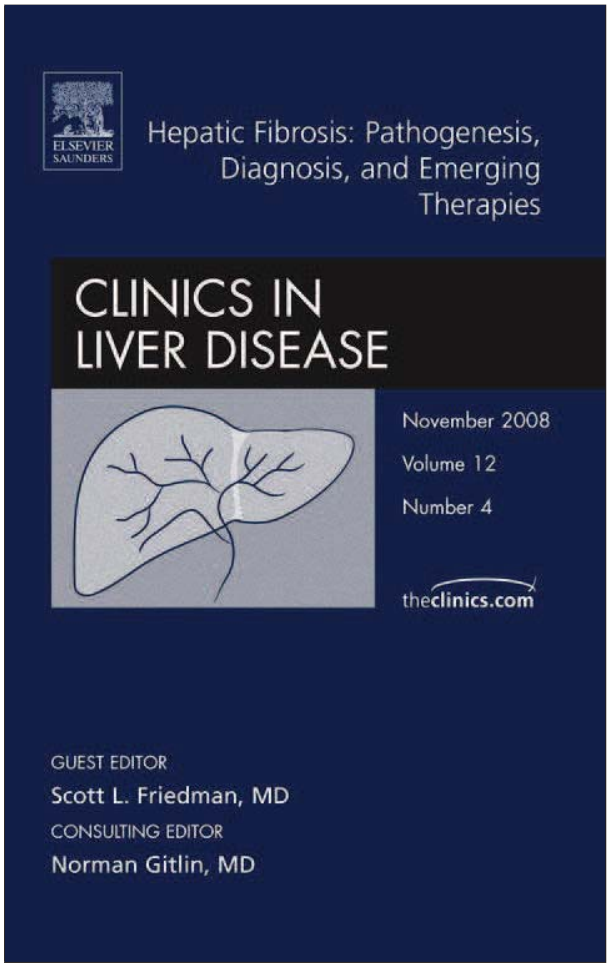

Cytokines and Renin-Angiotensin System Signaling in Hepatic Fibrosis

Montserrat Moreno, PhD, Ramon Bataller, MD*

KEYWORDS

• Liver fibrosis • Cytokines • Angiotensin II
• Hepatic stellate cells • Renin-angiotensin system

Hepatic fibrosis is the wound healing response of the liver to repeated injury.[1] Fibrosis is the result of a complex interplay among resident hepatic cells, infiltrating inflammatory cells, and several locally acting peptides called *cytokines*. Cytokines are a family of proteins that function as mediators of cell communication.[2] They include chemokines, interleukins, interferons, growth factors, angiogenic factors, vasoactive substances, soluble receptors, and soluble proteases. Unregulated cytokine synthesis and release coordinate the hepatic response to injury and participate in the initiation, progression, and maintenance of fibrosis. Understanding the complexity of the cytokine-driven mechanisms of fibrosis is important for identifying potential molecular targets for future pharmacologic interventions in prevention and treatment. Key mediators include transforming growth factor β1 (TGF-β1), platelet derived growth factor (PDGF), adipokines, and several inflammatory cytokines and chemokines.

The cellular source of cytokines in liver diseases probably depends on the type of disease. In chronic viral diseases, infected hepatocytes and infiltrating lymphocytes release reactive oxygen species (ROS), inflammatory chemokines, and fibrogenic mediators.[3] In alcoholic liver disease, damaged hepatocytes, Kupffer cells, and infiltrating neutrophils secrete large amounts of ROS and cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-8, favoring hepatocellular death and myofibroblast accumulation.[4] Recent data indicate that adipokines also play an important role in liver fibrogenesis.[5,6] They are locally produced by liver resident cells (eg, activated hepatic

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stellate cells [HSCs]) and amplify inflammatory and fibrogenic signals in fibrogenic myofibroblats.

The past few years have seen an explosion of knowledge about signal transduction pathways in liver fibrogenesis, involving virtually all events in tissue repair, such as myofibroblast accumulation, hepatocyte regeneration, and scar tissue formation.[6] Most of these pathways have been identified in cultured HSCs, the main target cell for fibrogenic cytokines. Most importantly, drugs interfering with intracellular pathways involved in increased collagen production are considered potential therapies for liver fibrosis.

Accumulating evidence indicates that the renin–angiotensin system (RAS) is a major mediator in liver fibrogenesis.[7] Key components of the RAS are locally expressed in chronically injured livers and activated HSCs de novo generate angiotensin II, the main effector peptide of this system.[8] Angiotensin II induces an array of fibrogenic actions in activated HSCs, including cell proliferation, migration, secretion of proinflammatory cytokines, and collagen synthesis.[9,10] These actions are largely mediated by ROS generated by a nonphagocytic form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase.[1] Pharmacologic or genetic ablation of the RAS attenuates experimental liver fibrosis.[11–14] Currently, RAS inhibitors are being tested as antifibrotic drugs in patients who have chronic liver diseases.

This article provides a succinct and current overview of cytokines implicated in liver fibrogenesis and the signaling pathways involved, and describes the role of the RAS and angiotensin II.

CYTOKINES INVOLVED IN LIVER FIBROGENESIS

Hepatic fibrosis is regulated by host of factors, including interactions with the extracellular matrix, surface of inflammatory cells, hormones, and an extremely complex and redundant network of profibrogenic cytokines.[1,15] The nature of mechanisms through which cytokines regulate fibrosis is dual: indirect, through attraction of inflammatory cells, and direct, through binding to specific receptors on myofibroblasts and stimulating proliferation, collagen production, and secretion of autocrine factors.

Main cytokines involved in liver fibrogenesis are depicted in **Table 1**. They include classical proinflammatory cytokines and chemokines and growth factors. Moreover, vasoactive substances traditionally considered hormones regulating arterial pressure homeostasis are currently viewed as true cytokines that participate in the wound healing response to injury.[16]

Adipokines are another family of cytokines that are locally produced in the liver and regulate liver fibrosis.[5] All of these mediators do not work alone but rather in a complex network of intracellular signaling and interaction with cells and extracellular matrix components.

Inflammatory Cytokines

During liver inflammation and fibrosis, secretion of cytokines is dysregulated, promoting an inflammatory state. Potential sources of inflammatory cytokines in the hepatic wound healing response are Kupffer cells, hepatocytes, HSCs, natural killer cells, and lymphocytes, including $CD4^+$ T helper (Th).[1] Th cells can differentiate into Th1 and Th2 subsets, a classification that is based on the pattern of cytokines produced. In general, Th1 cells produce cytokines that promote cell-mediated immunity (interferon [IFN]-γ, TNF-α, and IL-2) and protect against fibrosis, whereas Th2 cells promote humoral immunity (IL-4, IL-5, IL-6, and IL-13) and induce fibrosis, as evidenced by a study using

two mice strains with different polarity of Th cells and different susceptibility to liver fibrosis.[17]

Classic cytokines may be divided into chemokines (monocyte chemotactin protein 1 [MCP-1], RANTES, IL-8), interferons (IFN-α, IFN-γ) and interleukins (IL-1, IL-6, IL-10). Chemokines are divided into four groups depending on the spacing of their first cysteine residues: CC (eg, MCP-1, RANTES), CXC (eg, IL-8, GRO-α), C (lymphotactins), and CX_3C (fractalkine).[18] TNF-α participates in the activation process of HSCs and is a critical factor for the proinflammatory role of HSCs.[19,20] Another powerful cytokine, IL-1, also exerts profibrogenic actions by stimulating metalloproteinase secretion.[21] Administration of IL-1 receptor antagonist reduces matrix deposition in a rat model of liver fibrosis.[22] In contrast, IL-10 exerts net antifibrogenic effects in the liver in vivo[23] and in vitro.[24] IFN-α has been shown to exert a direct antifibrotic effect in vitro over HSCs[25] and in vivo in different animal models of hepatic fibrosis, and is used as antiviral therapy in patients who have chronic hepatitis C.[26–28] IFN-γ inhibits HSC proliferation and procollagen mRNA expression in vitro and reduces liver fibrosis in rodents.[29,30]

Among CC chemokines, MCP-1 is a profibrogenic chemokine overexpressed in the injured liver.[31] It induces chemotaxis of HSCs[32] and participates in experimentally induced fibrosis in rats.[33] RANTES is produced by HSCs, is up-regulated in livers of patients who have HCV, and induces HSC proliferation in vitro.[34,35] CXC chemokines are also involved in liver fibrosis, as evidenced by several studies. For instance, serum IL-8 is increased in alcoholic patients[36] and those who have nonalcoholic steatohepatitis (NASH), and primary biliary cirrhosis,[37] and GRO-α expression is up-regulated in livers of patients who have alcoholic hepatitis.[17,38]

Growth Factors

Growth factors play a key role in liver fibrogenesis by promoting activation and accumulation of HSCs and stimulating collagen synthesis. PDGF and TGF-β are the most important mediators because of their effects on HSC proliferation and extracellular matrix protein production, respectively. PDGF is a dimeric protein composed of two polypeptide chains (mainly A and B) that can combine to form PDGF-A and PDGF-B.[39,40] They signal through the tyrosine kinase receptors PDGF receptor α and β (PDGFRα and PDGFRβ, respectively). PDGFβ is the most potent mitogen factor for HSCs by acting through PDGFRβ.[41] All isoforms of PDGF and PDGFR are up-regulated in injured livers and correlate with the degree of inflammation and fibrosis.[42–44] Moreover, inhibition of PDGF-B attenuates experimental liver fibrogenesis.[45,46]

TGF-β1 is a key mediator of liver fibrogenesis. In the injured liver, TGF-β1 is up-regulated[47] and favors the transition of resident HSCs into myofibroblast-like cells, stimulating synthesis of extracellular matrix proteins and inhibiting their degradation. Strategies aimed at disrupting TGF-β1 synthesis or signaling pathways markedly decreased fibrosis in experimental models.[48,49] A newly discovered regulator of TGF-β activity is bone morphogenic protein and activin membrane-bound inhibitor (BAMBI), which is a TGF-β pseudoreceptor that inhibits TGF-β signaling by preventing the formation of receptor complexes.[50] Down-regulation of BAMBI seems to be a mechanism of fibrogenesis induced by lipopolysaccharide through toll-like receptor (TLR)-4.[51]

Established angiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) play a central role in not only angiogenesis but also chronic wound-healing conditions. VEGF and its receptors (VEGFR-1 and VEGFR-2) are up-regulated in chronic liver injury and promote fibrogenic effects in HSCs by stimulating cell proliferation, collagen production, and migration.[52]

Table 1
Main cytokines regulating liver fibrogenesis

	Expressed by Hepatic Stellate Cells	Effect in Hepatic Stellate Cells	Effect on Animal Models in Rodents
Inflammatory cytokines			
MCP-1	Yes	Profibrogenic	Blockade of MCP-1 suppresses hepatic fibrosis
RANTES	Yes	Profibrogenic	?
IL-1	?	Profibrogenic	Antagonism of IL-1 reduces liver fibrosis
IL-4	?	Controversial	?
IL-8	Yes	?	?
IL-10	Yes	Antifibrogenic	IL-10 treatment reduces hepatic fibrosis
IL-13	?	Controversial	?
TNF-α	Yes	Controversial	Treatment with an anti–TNF-α agent reduces liver fibrosis
IFN-γ	?	Antifibrogenic	IFN-γ treatment attenuates liver fibrosis
IFN-α	?	?	IFN-α attenuates liver fibrosis
Growth factors			
TGF-β1	Yes	Profibrogenic	Inhibition of TGF-β1 signaling decreases liver fibrosis
PDGF-BB	Yes	Profibrogenic	Inhibition of PDGF-B prevents liver fibrosis
CTGF	Yes	Profibrogenic	Down-regulation of CTGF by siRNA reduces liver fibrosis
VEGF	Yes	Profibrogenic	Antibodies against VEGF receptors inhibit liver fibrosis
FGF-1 and -2	?	Profibrogenic	Double knockout exhibit reduced liver fibrosis
HGF	Yes	Antifibrogenic	HGF gene therapy attenuates progression of liver fibrosis
IGF-1	Yes	Antifibrogenic	IGF-1 treatment attenuates liver fibrosis

Vasoactive substances			
Angiotensin II	Yes	Profibrogenic	RAS inhibition attenuates liver fibrosis
Endothelin-1	Yes	Profibrogenic?	ETA receptor antagonism reduces hepatic fibrosis
Vasopressin	?	Profibrogenic	?
Thrombin	Yes	Profibrogenic	Thrombin antagonism reduces liver fibrosis
Norepinephrine	Yes	Profibrogenic	α-adrenoreceptor blocker treatment attenuates liver fibrosis
Nitric oxide	Yes	Antifibrogenic	Mice lacking iNOS exhibit exacerbated liver fibrosis
Relaxin	?	Antifibrogenic	Relaxin administration reduces hepatic fibrosis
Prostaglandin E2	Yes	Antifibrogenic	Hepatoprotection in the liver
Adrenomedullin	Yes	Antifibrogenic	?
Atrial natriuretic peptide	Yes	Antifibrogenic	?
Adipokines			
Leptin	Yes	Profibrogenic	Leptin deficient rats do not develop liver fibrosis
Resistin	?	Profibrogenic?	?
Adiponectin	Yes	Antifibrogenic	Adiponectin-deficient mice show enhanced liver fibrosis
Others			
Tetrahydrocannabinol	Yes	Profibrogenic	CB1 receptor antagonism reduces liver fibrosis

Abbreviations: CTGF, connective tissue growth factor; ETA, endothelin A; HGF, hepatocyte growth factor, IGF-1, insulin-like growth factor 1; iNOS, inducible nitric oxide synthase; MCP-1, monocyte chemotactin protein 1; siRNA, small interfering RNA strands; VEGF, vascular endothelial growth factor.

Moreover, VEGFR-1 and VEGFR-2 signaling is required for liver fibrosis development.[53] FGF-1 and -2 exert profibrogenic effects in vivo as double knockout mice exhibit reduced liver fibrosis.[54] Other growth factors, including hepatocyte growth factor (HGF) and insulin-like growth factor 1 (IGF-1), attenuate liver fibrosis in rodents.[55–57]

Vasoactive Substances

Several vasoactive substances are locally produced in the injured liver and have an autocrine or paracrine effect on HSCs. Vasodilator substances (eg, nitric oxide, prostaglandin E_2, atrial natriuretic peptide, adrenomedullin, and relaxin) exert antifibrotic effects, whereas vasoconstrictors (eg, endothelin-1, norepinephrine, angiotensin II, and thrombin) have opposite effects.[1]

Livers with advanced fibrosis have a predominance of vasoconstrictors compared with vasodilators, favoring collagen deposition. Among vasodilatory substances, nitric oxide has received special attention. It is produced by all nonparenchymal cells and inhibits liver fibrosis in vitro and in vivo. Advanced fibrosis is associated with endothelial dysfunction and decreased nitric oxide production, which may contribute to disease progression.[58]

Prostaglandin E2 is a vasodilatory molecule synthesized by virtually all liver cells that inhibits HSC proliferation and TGF-β1–mediated collagen synthesis and attenuates fibrosis in vivo.[59,60] Drugs delivering either NO or prostaglandin E2 have been proposed to treat patients who have liver fibrosis. Among vasoconstrictors, angiotensin II is the most widely studied and is discussed extensively later.

Thrombin is a multifunctional serine protease that binds to specific cell surface receptors called *protease-activated receptors* (PAR). Thrombin is produced by activated HSCs to regulate cell migration, growth, and fibrogenic actions. Both thrombin and PAR-1, its main receptor, are overexpressed in fibrotic livers. Moreover, antagonism of thrombin attenuates liver fibrosis in animal models.[61–63]

Endothelin is another important vasoconstrictor implicated in liver fibrosis. Three isoforms of endothelin[1–3] act through two receptors (ET_A and ET_B). Endothelin and its receptors are up-regulated in the fibrotic liver and their expression correlates with the severity of the disease.[64] In the early phase of activation, HSCs have most ET_A receptors, which stimulate an increase in intracellular-free calcium in HSCs coupled with cell contraction and proliferation. This process is linked to stimulation of fibrogenesis. In later stages, ET_B receptors become more abundant and their stimulation promotes an antiproliferative effect. The use of ET_A/ET_B receptor blockers have yielded conflicting results,[16,65] possibly because of the different relative activities toward each of the two receptors.

Norepinephrine is a catecholamine with a dual role as a neurotransmitter and a hormone. Evidence indicates that norepinephrine stimulates liver fibrogenesis. Activated HSCs are capable of secreting mature norepinephrine, which induces proinflammatory and fibrogenic effects. Moreover, α_1 adrenoreceptors are up-regulated in livers with advanced fibrosis and its blockade attenuates the development of liver fibrosis in rats with chronic liver injury.[66,67]

Adipokines

Adipokines are biologically active peptides mainly secreted by adipose tissue. Main adipokines include leptin, resistin, visfatin, and adiponectin. Circulating adipokines secreted by excessive fat accumulation may regulate hepatic fibrosis in diseases such as NASH.[5,68] Moreover, several adipokines are locally synthesized in the liver and may regulate fibrogenesis in an autocrine/paracrine manner. Leptin is secreted by activated HSCs and stimulates cell proliferation, secretion of chemokines, and collagen

synthesis. Moreover, leptin is required for fibrosis development.[69,70] In contrast, adiponectin markedly inhibits liver fibrogenesis in vitro and in vivo.[71] Resistin is up-regulated in alcoholic liver disease and exerts proinflammatory effects on HSCs, suggesting a role in liver fibrogenesis.[68]

Endogenous Cannabinoids

In addition to their central effects, cannabinoids display a wide variety of peripheral functions, including regulation of wound healing response to injury. The endogenous cannabinoid system has been implicated in liver fibrosis. Both CB1 and CB2 receptors and endocannabinoids are up-regulated in chronic liver diseases.[72] Pharmacologic or genetic inactivation of CB1 reduces fibrosis in different models of chronic liver injury.[73] In contrast, activation of CB2 receptors attenuates liver injury, inflammation, and oxidative stress, and CB2 knockout mice exposed to carbon tetrachloride (CCl_4) show enhanced liver fibrosis.[74] Globally, cannabinoids may worsen liver injury because daily cannabis use exacerbates liver fibrosis in patients who have chronic hepatitis C.[75]

CYTOKINE SIGNALING PATHWAYS INVOLVED IN LIVER FIBROGENESIS

All molecules implicated in liver fibrosis activate different intracellular pathways (**Fig. 1**). A complex cross-talk exists between them that determines the global effect on liver fibrosis. Data on intracellular pathways regulating liver fibrogenesis are mainly derived from studies using cultured HSCs, whereas understanding of their role in vivo is progressing through experimental fibrogenesis studies using knockout mice.

PI3K/Akt Pathway

The focal adhesion kinase (FAK) phosphoinositol-3-phosphate kinase (PI3K)/Akt–signaling pathway mediates various profibrogenic actions in HSCs, including proliferation, chemotaxis, and transcription of profibrogenic genes.[76,77] This pathway may be activated by growth factors that trigger tyrosine kinase activity (PDGF, VEGF) or activation of cytokine receptors (MCP-1), but also by other signals, including integrins, stimulators of G-protein-coupled receptors (angiotensin II, thrombin), and adipokines (leptin).[10,78,79] As an example, when PDGF binds to its receptor (a tyrosine kinase receptor), the receptor dimerizes and autophosphorylates.[80] Then, PI3K associates with the activated receptor and becomes activated by phosphorylation. PI3K activation results in the phosphorylation of inositol lipids.

Phosphatase and tensin homolog (PTEN) functions as an antagonist of PI3K, thereby impairing the generation of phosphoinositol-3,4,5-triphosphate (PIP_3) from phosphoinositol-4,5-biphosphate (PIP_2).[81] The phosphoinositols bind to Akt and induce its translocation to the plasma membrane where it becomes phosphorylated by the phosphoinositide-dependent kinase, and thus activated. Activated Akt induces mammalian target of rapamycin (mTOR) activity, and signals through mTOR increase the phosphorylation of p70S6 kinase, which phosphorylates a ribosomal subunit and 4E-BP1 leading to up-regulation of protein synthesis and stimulation of cell growth signals. Rapamycin, which inhibits mTOR activity, attenuates liver fibrosis, possibly through decreasing growth of HSCs.[82] Moreover, inhibition of PI3-K by wortmannin blocks mitogenesis and chemotaxis in response to PDGF, supporting the involvement of this pathway in HSC accumulation in vivo.[77]

Mitogen-Activated Protein Kinase Pathway

Members of the mitogen-activated protein kinases (MAPK) family, including extracellular-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK), and p38 MAPK, are

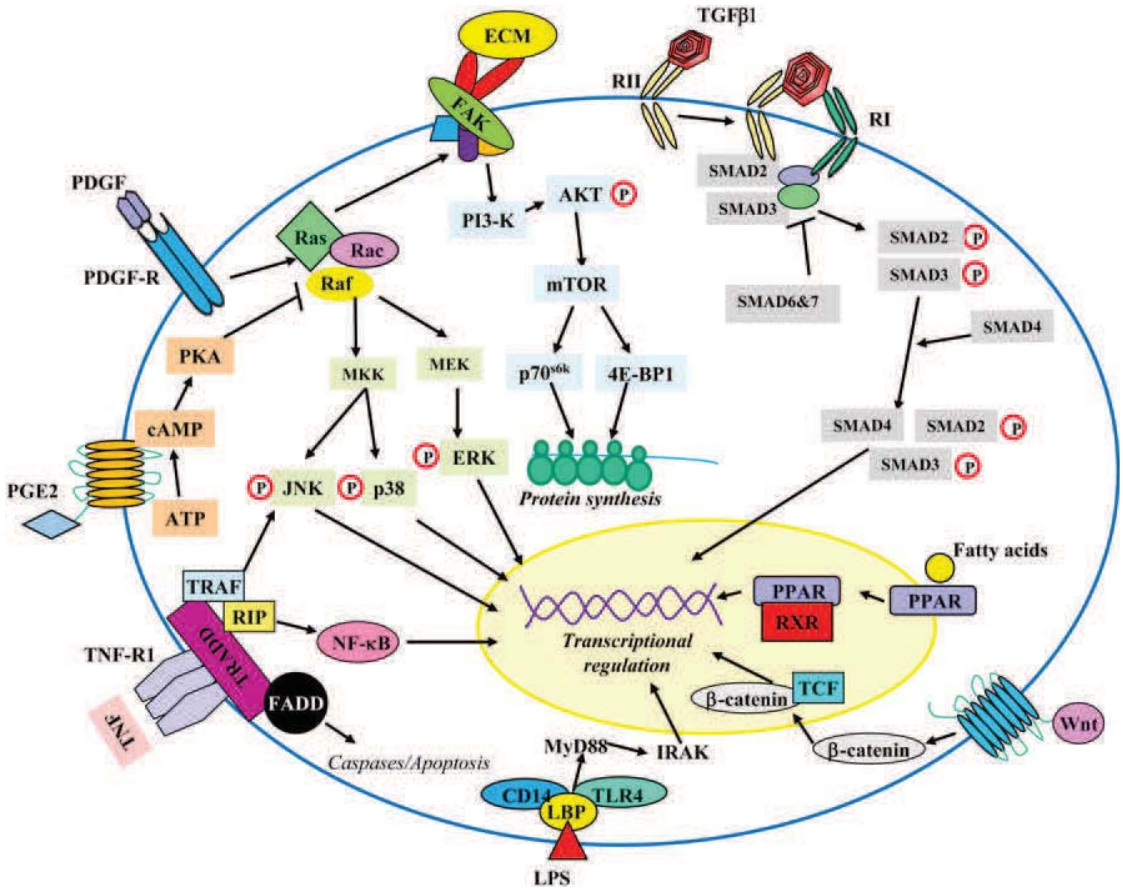

Fig. 1. Main cytokine signaling pathways in the hepatic stellate cell. After binding of cytokines such as TGF-β1, PDGF, or TNF-α to their cell surface receptors, activation of several intracellular signaling pathways occurs. The PDGF stimulation can induce activation of mitogen-activated protein kinase (MAPK) signaling and the phosphatidylinositol 3-kinase/Akt/p70S6 kinase (PI3K/Akt/p70S6K) signaling pathway. The matrix-associated focal adhesion kinase also stimulates the PI3K/Akt/p70S6K signaling pathway. TGF-β1 stimulates transcription of profibrogenic genes through activating the Smad signaling pathway. Fatty acids and other agonists activate peroxisome proliferator–activated receptors to regulate gene expression. The Wnt/β-catenin pathway is also involved in transcriptional regulation. Bacterial products such as lipopolysaccharide bind to TLR4 and stimulate IL-1 associated kinase to induce fibrogenic signals. TNF-α binds to the protein TNFR-associated death domain to activate c-Jun N-terminal kinase and nuclear factor-κB. Finally, agonists such as prostaglandin E2 induce cAMP production and protein kinase A activation to inhibit MAPK signaling.

activated by several growth factors and vasoactive peptides and subsequently translocated to the nucleus where they phosphorylate several transcription factors, resulting in cellular responses.[83] In HSCs, ERKs regulate cell proliferation, secretion of chemokines, cell migration, and collagen synthesis. This pathway is basically activated by peptides that induce proliferation (PDGF, thrombin, angiotensin II, VEGF, and leptin) and by chemokines.[10,80,84] On activation, tyrosine kinase and G-protein–coupled receptors recruit the signaling molecule Ras, causing the sequential activation of Raf, MEK, and ERK1 and -2.

Activated ERK induces activation of transcription factors implicated in cell proliferation, such as activating protein type-1 (AP-1). ERK activation is induced in vivo in rats with chronic liver injury and after chronic exposure to angiotensin II.[85,86] In HSCs, JNK or stress-activated protein kinase (SAPK) is activated in response to cellular stress, bacterial products, FasL, oxidative stress, vasoactive substances (angiotensin II), adipokines (leptin), chemokines (RANTES), and growth factors (TGF-β1 and PDGF).[78]

JNK activity is regulated upstream by MAPK kinase 4 (MKK4) and MAPK kinase 7 (MKK7); it is a profibrogenic pathway in HSCs through modulating cell growth and secretion of inflammatory cytokines.[10,87–89]

MAPK kinase 6 (MKK6) and MKK3 act directly upstream p38 MAPK and lead to the phosphorylation of p38, which subsequently regulates gene expression through activating transcription factors. The p38 pathway seems to have an antiproliferative role in HSCs, because blocking p38 activity increases cellular proliferation[89] Moreover, p38 activity has profibrogenic effects, because collagen expression induced by TGF-β or other molecules is partially mediated by p38 MAPK signaling in HSCs.[90,91]

Smad Pathway

Smad pathway plays a major role in liver fibrosis through signaling TGF-β1 in activated HSC16397841. TGF-β1–dependent Smad signaling also mediates other fibrogenic factors, such as hypoxia.[92] TGF-β1 binds to its type II receptor that becomes activated and dimerized with type I receptor. Smad2 and -3 bind the resulting complex to become phosphorylated, and are then released to the cytosol and associate with Smad4. The heterotrimer translocates into the nucleus and activates profibrogenic transcription factors (eg, Sp1), which bind to the promoter region of target genes.[93] Smad6 and -7 are endogenous inhibitors of Smad signaling through preventing the binding of Smad2 and -3 to the TGF-β receptor.

Inhibitory Smad proteins mediate the effect of IFN-γ on TGF-β signaling in the liver.[94] Smad signaling participates in TGF-β1–dependent mesenchymal-to-epithelial transition in hepatocytes, a novel mechanism implicated in liver fibrogenesis.[95] In vivo, Smad signaling mediates liver fibrogenesis induced by chronic cholestasis,[96] and inhibition of Smad signaling suppresses collagen gene expression and hepatic fibrosis in mice.[97]

Nuclear Factor-κB Signaling

Nuclear factor-κB (NF-κB) is a major downstream effector of proinflammatory cytokines such as TNF-α.[98] Other peptides, such as angiotensin II and leptin, also activate NF-κB signaling.[91] NF-κB is a transcription factor composed of homo- or heterodimers of the Rel protein family (p65, p50, p52, c-Rel, and RelB). NF-κB activity in the cytoplasm is regulated by its inhibitor, IκBα. After IκBα degradation, the active form of NF-κB translocates into the nucleus where it regulates transcriptional activity of target genes. After HSC activation, NF-κB becomes persistently activated and many NF-κB–responsive genes (eg, IL-6, intercellular adhesion molecule-1, ICAM-1) are constitutively expressed.[99,100] NF-κB plays a pivotal role in the inflammatory effects of TNF-α and other mediators on HSCs. Its activity is not required for proliferation or activation, but it protects activated HSCs against TNF-α–induced apoptosis.[100]

Nuclear Receptors

Nuclear receptors can directly bind to DNA and regulate the expression of adjacent genes, and therefore are classified as transcription factors. Several nuclear receptors have been described in HSCs. The pregnane X receptor (PXR) is a nuclear receptor that seems to exert an antifibrotic role. Pregnane X receptor activators inhibit the proliferation, transdifferentiation, and expression of TGF-β1 in HSCs.[101,102] In addition, treatment with a PXR activator markedly reduces the degree of liver fibrosis in animal models.[103]

Peroxisome proliferator–activated receptors (PPARs) regulate HSC's biologic actions and are potential targets for antifibrotic therapy.[104] Three isoforms are encoded by three different genes: PPARα, PPARβ, and PPARγ. Fatty acids and eicosanoids

bind to PPAR, which dimerizes with the retinoid receptor and migrates into the nucleus, where they bind to peroxisome proliferator response elements in the promoter region of target genes and recruit cofactors that modulate transcriptional activity. PPARs regulate mainly metabolic functions in the liver but also inflammation and fibrogenesis. After HSC activation, expression of PPARγ diminishes as PPARβ expression increases. PPARγ activation inhibits the proinflammatory and profibrogenic actions in HSCs and attenuate liver fibrosis in vivo,[105,106] whereas PPARβ seems to exert opposite effects.[107] The mechanisms involve attenuation of TGF-β signaling in HSC.[108] Most importantly, PPARγ ligands (eg, thiazolidinediones) are currently being tested to treat liver fibrosis in the context of NASH.

Wnt/β-Catenin Pathway

The Wnt/β-catenin pathway is crucial in normal development, including embryogenesis. This pathway also signals cytokines and promotes inflammation.[109] It was recently implicated in hepatic fibrogenesis 17,464,972. Wnt is an extracellular-secreted glycoprotein that binds to the cell surface receptor Frizzled (Fz) and induces specific downstream events.[110] In a normal state, the monomeric form of β-catenin in the cytoplasm is targeted for degradation by ubiquitinitation, keeping free levels of β-catenin low and preventing it from translocating to the nucleus to induce target gene transcription.

When any Wnt proteins bind to their seven-transmembrane receptor, a complex cascade of reactions occurs until β-catenin becomes hypophosphorylated and released and translocated into the nucleus, where it binds to T-cell factor/lymphoid-enhancing factor. Once formed, this complex transcriptionally regulates several target genes. In the liver, evidence indicates that Wnt signaling has a profibrogenic role. In cultured activated HSCs, mRNA for Wnt genes and coreceptors increase and protect cells from apoptosis.[111] Moreover, Wnt activity is enhanced in liver cirrhosis. These observations suggest that Wnt signaling promotes hepatic fibrosis through enhancing HSC activation and survival.[111,112]

CD14/TLR-4 Pathway

Toll-like receptors (TLRs) are pattern recognition receptors that recognize pathogen-associated molecular patterns and signal through adaptor molecules, including myeloid differentiation factor 88 (MyD88) and TRIF-related adaptor molecule (TRAM), to activate transcription factors, NF-κB, and interferon regulatory factors (IRFs), leading to innate immunity. Although Kupffer cells are considered the primary cells to respond to pathogen-associated molecular patterns in the liver, recent studies show TLR signaling in hepatic nonimmune cell populations, including hepatocytes and hepatic stellate cells.[113]

Recent studies suggest a role for intracellular pathways driven by TLRs in liver inflammation.[114] In particular, TLR4 is implicated in liver fibrogenesis and lipopolysaccharide signaling. Mice lacking TLR4 have a reduced liver fibrosis compared with wild-type mice. The mechanism showing a role for TLR4 in liver fibrogenesis was recently uncovered. TLR4 activation in HSCs reduces BAMBI expression, which is a TGF-β pseudoreceptor, and therefore TLR4 activation enhances TGF-β signaling in HSCs.[51] The intracellular domain of TLR is similar to that of IL-1 receptor, and thus they share intracellular pathways. Stimulated Toll/IL-1 receptors activate MyD88, and then the receptor recruits IL-1–associated kinase that becomes activated.[115] This process leads to phosphorylation of the TNF receptor–associated factor (TRAF), which then activates proinflammatory transcription factors (AP-1 and NF-κB).

Death Pathways

Several pathways implicated in cell death mediate cytokine signaling in activated HSCs (see article by Gieling and Mann elsewhere in this issue). TNF receptors (TNFR) belong to a superfamily that includes several transmembrane molecules that bind cytokines and other molecules.[116] Receptors with a dead domain include TNFR1, Fas, and p75 receptor for nerve growth factor. TNFR2 and CD40 lack the death domain.

TNFR1 plays a major role in mediating biologic actions of TNF-α.[117] In HSCs, TNF-α activates pathways that regulate gene transcription and inflammation and other pathways leading to cell death.[118] Binding of TNF-α induces homotrimerization of TNFR1, which binds to the death domain containing protein TNFR-associated death domain (TRADD). It associates with receptor-interacting protein and TNFR-associated factor-2 (TRAF2) to activate NF-κB and JNK, respectively.

TNF-α is a critical factor for the proinflammatory role of HSCs. Quiescent HSCs express TNFR1, and TNF binds to the cell surface. However, the receptor in quiescent cells seems to be only partially functional, because activity of NF-κB in response to TNF-α is only seen in activated HSCs. TNF-α activates JNK both in quiescent and activated HSC. TNF-α also activates ERK1/2 and p38 MAPK, which regulates collagen synthesis in HSC.[119] Other receptor, CD40, interacts with its ligand to amplify the inflammatory behavior of HSC through TRAF2- and IKK2-dependent pathways.[87] Cell death is mediated by the interaction of TRADD with Fas-associated protein with dead domain (FADD), which stimulates caspases leading to apoptotic cell death. Fas (CD95) is also expressed in quiescent HSCs and drives proliferation and resistance to apoptosis.[120] Another ligand, TNF-related apoptosis-inducing ligand (TRAIL) binds to TRAIL receptor 2 in activated HSCs to induce apoptosis.[121]

JAK/STAT Pathway

Janus kinases (JAKs) can bind to both tyrosine receptors and G-protein–coupled receptors. They phosphorylate tyrosine residues on the receptor and create sites for interaction with proteins that contain phosphotyrosine-binding SH2 domain. Signal transducer and activator of transcription (STAT) proteins possessing SH2 domain are recruited to the receptors and are phosphorylated at tyrosine residues by JAKs. Activated STAT dimers accumulate in the cell nucleus and activate transcription of their target genes. In HSCs, this pathway is stimulated by various cytokines and mediators, including INF-γ and leptin.[122] Activation of STAT1 plays an important role in liver injury, inflammation, and inhibition of liver regeneration.[123] Mice lacking STAT1 exhibit accelerated liver fibrosis from inhibition of HSC proliferation, suppression of PDGF expression, and inhibition of TGF-β/Smad3 signaling.[124]

AMP-Activated Protein Kinase Pathway

AMP-activated protein kinase (AMPK) is a fuel-sensing enzyme that can cellular metabolism in response to different stimuli. Once activated, AMPK activates catabolic pathways, leading to ATP generation, and inactivates ATP-consuming processes not essential for short-term survival. AMP-activated protein inhibits cell proliferation, migration, chemokine secretion, and collagen production in HSCs.[125] This pathway mediates the antifibrogenic effect of adiponectin in HSCs.[126]

THE RENIN–ANGIOTENSIN SYSTEM AND LIVER FIBROSIS

General Concepts

The RAS has been traditionally considered an endocrine system that regulates arterial pressure homeostasis.[127] According to this concept, the precursor angiotensinogen is

synthesized by the hepatocytes and released into the bloodstream, where it is transformed to angiotensin I by renin. Angiotensin I is then cleaved to angiotensin II by angiotensin-converting enzyme (ACE), an ectoenzyme abundant in endothelial cells. Angiotensin II binds to angiotensin type 1 (AT1) receptors to induce vasoconstriction and, in glomerulosa cells, to release aldosterone, which causes sodium and water reabsorption in the kidneys (**Fig. 2**).

Besides this endocrine action, the RAS components are expressed in damaged tissues that de novo generate mature angiotensin II.[128] Key enzymes for local synthesis of angiotensin II include ACE type 1 (ACE-1) and chymase. Angiotensin II accumulates at the sites of parenchymal injury and binds to AT1 receptors in myofibroblasts to promote recruitment of inflammatory cells, secretion of extracellular matrix proteins, and inhibition of collagen degradation.[129] Moreover, angiotensin II regulates the local microcirculation through inducing contraction of vascular cell types.[130] Angiotensin II also binds to angiotensin type 2 (AT2) receptors that are typically found in many organs during embryogenesis and are re-expressed in chronically inflamed tissues.[131] These receptors oppose the actions of AT1 receptors by inducing vasodilatation and tissue growth inhibition.[132] ACE type 2 is overexpressed in tissues with fibrosis and converts angiotensin II into angiotensin,[1–7] a smaller peptide with vasodilatory actions that counteracts the actions of angiotensin II.[133]

The RAS is currently viewed as part of a system of interconnected cytokines that become activated after tissue injury to promote tissue repair.[134] This new understanding of the RAS has important clinical implications. It explains why blockade of the RAS with ACE inhibitors, the newer AT1 receptor antagonists, or both together significantly

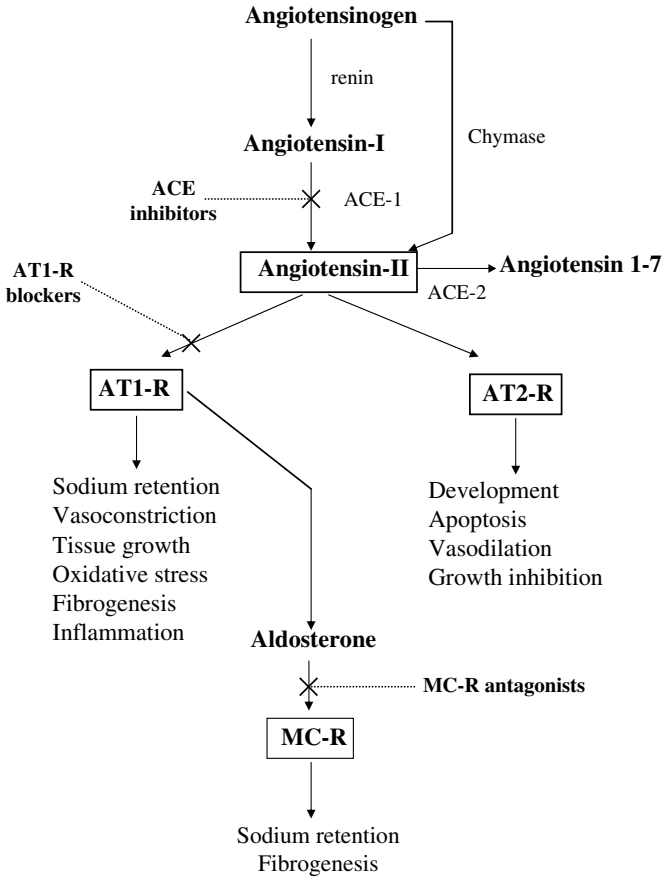

Fig. 2. The renin–angiotensin pathway and related pathogenic actions. ACE, angiotensin-concerting enzyme; AT1-R, angiotensin type 1 receptor; AT2-R, angiotensin type 2 receptor; MC-R, mineralocorticoid receptor.

slows the progression of many fibrotic diseases. This antifibrotic effect has been shown in coronary heart disease, heart failure, diabetic nephropathy, and stroke.[135] The beneficial effect of RAS inhibitors in reducing morbidity and mortality in these patients is not necessarily associated with a reduction in arterial pressure, indicating that angiotensin II induces tissue injury through mechanisms other than arterial hypertension.

Role of the Renin-Angiotensin System in Liver Fibrosis: Intracellular Mechanisms

The role of RAS in liver fibrosis was first suggested when HSCs were found to bear functional AT1 receptors.[9,136] After phenotypic activation, HSCs highly express AT1 receptors, the activation of which mediates cell contraction, migration, and proliferation. Moreover, angiotensin II stimulates collagen synthesis and TGF-β1 expression. Angiotensin II also induces proinflammatory effects in HSCs, stimulating the expression of cell adhesion molecules and the secretion of inflammatory chemokines.[9] All these effects are blocked by AT1 receptor antagonists and are blunted in mouse HSCs lacking AT1a receptors.[137,138]

An important finding is that an intrahepatic RAS is expressed in the fibrotic liver.[139] Although angiotensinogen is the only component of the RAS expressed in the normal rat liver, ACE and AT1 are markedly expressed in fibrotic rat livers. In humans, ACE and chymase are up-regulated in livers with significant fibrosis, whereas AT1 receptor expression is shifted to fibrotic areas.[38,140,141] The cellular source of the RAS in the injured liver is uncertain. In other tissues (eg, heart), myofibroblasts accumulated at the areas of tissue remodeling express all components of the RAS and generate angiotensin II, which participates in the tissue repair process. In the human liver, quiescent HSCs neither express the RAS components nor secrete angiotensin II.[8] In contrast, after cell activation in culture and in vivo, myofibroblastic HSCs express key components of the RAS and generate mature angiotensin II.

The molecular mechanisms mediating the inflammatory and fibrogenic effects of angiotensin II in HSC have been partially uncovered (**Fig. 3**).[10,142,143] Angiotensin II increases intracellular calcium concentration and induces ROS formation, stimulating key intracellular pathways, such as PI3k/AKT, Rho kinase, and MAPKs. It increases AP-1 DNA binding, whereas NF-κB activity is stimulated in rat but not human HSCs. Angiotensin II also stimulates the Smad signaling pathway through up-regulation of TGF-β1.[142] As a consequence, angiotensin II stimulates the expression of numerous genes involved in hepatic tissue remodeling, such as extracellular matrix components, inflammatory cytokines, and collagenolysis inhibitors.

The stimulation of intracellular signaling pathways and the biologic actions stimulated by angiotensin II are redox-sensitive. In HSCs, a nonphagocytic form of NADPH oxidase mediates angiotensin II–induced ROS formation. NADPH oxidases present in vascular cell types are constitutively active, producing relatively low levels of ROS under basal conditions and generating higher levels of oxidants in response to cytokines such as angiotensin II, stimulating redox-sensitive intracellular pathways.[144] Disruption of active NADPH oxidase protects mice from developing severe fibrosis after bile duct ligation, indicating that NADPH oxidase plays an important role in liver fibrosis.[10]

The most convincing evidence supporting a role for the RAS in experimental liver fibrosis is the finding that blockade of the generation of angiotensin II or its binding to AT1 receptors markedly attenuates experimental liver fibrosis. Remarkably, at least 27 studies using different experimental models of liver fibrosis have yielded similar results (**Table 2**).[11–14,141,143,145–161]

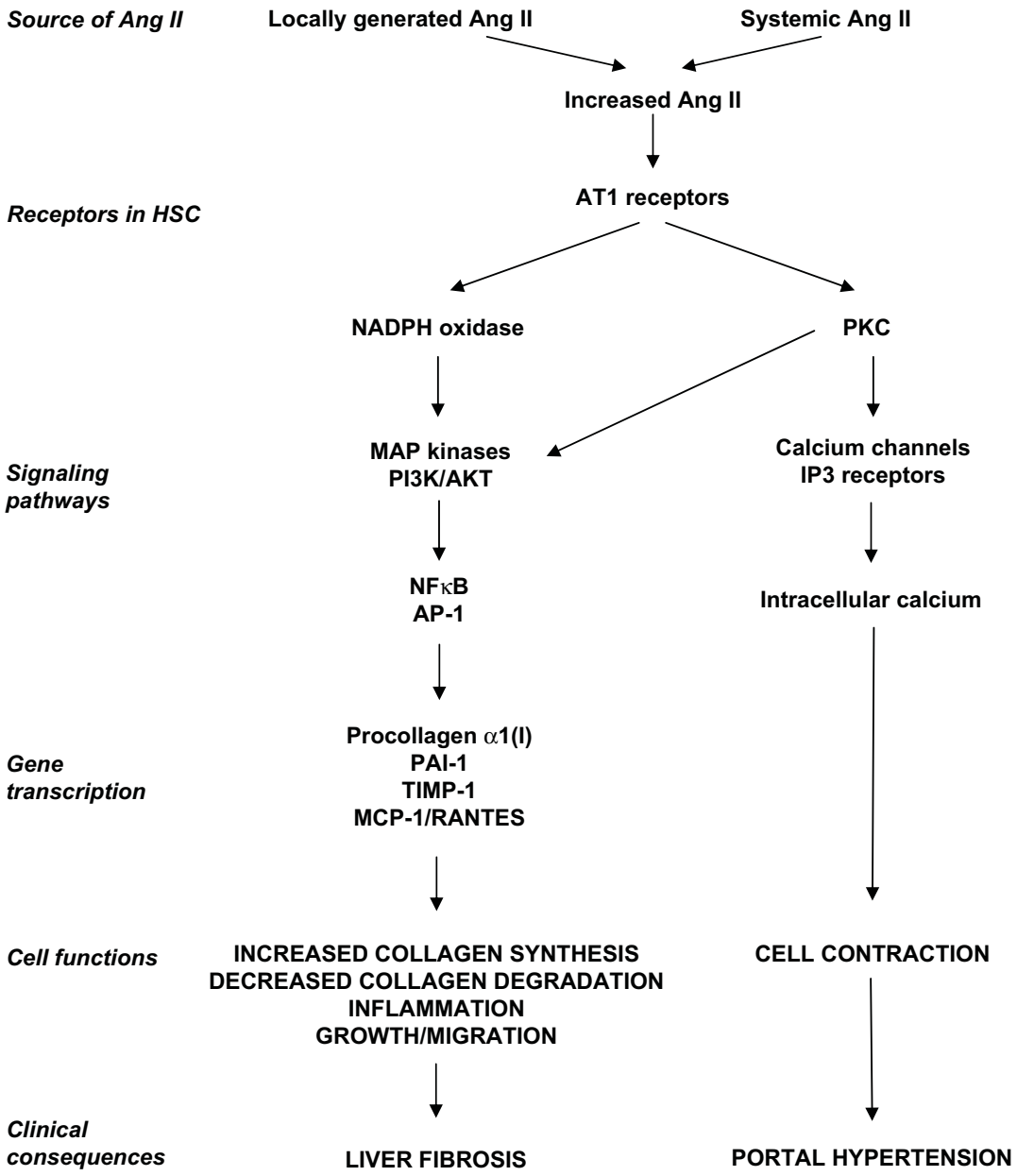

Fig. 3. Mechanisms of the pathogenic effect of the renin–angiotensin system in the liver. Increased angiotensin II binds to AT1 receptors located in activated HSCs. AT1 receptors activate a nonphagocytic NADPH oxidase to generate ROS that stimulate redox-sensitive intracellular pathways. Increased gene transcription leads to mitogenic, fibrogenic, and inflammatory properties, promoting fibrogenesis. Angiotensin II increases intracellular calcium and induces cell contraction, increasing intrahepatic vascular resistance and participating in the pathogenesis of portal hypertension. AP-1, activating protein type-1; MAPk, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein type 1; NF-κB, nuclear factor κB; PI3k, phosphoinositol 3 kinase; PAI-1, plasminogen activator inhibitor type 1; PKC, protein kinase C; TIMP-1, tissue inhibitor of metalloproteinases type 1.

RAS inhibition is associated with a decrease in TGF-β1 expression in the injured liver, a key effector mediating the fibrogenic effect of angiotensin II in other tissues.[162] Moreover, it causes a decrease in connective tissue growth factor and AT1 receptor expression.[149] RAS inhibition prevents the accumulation of myofibroblasts positive for smooth muscle α–actin.[13,14,149,154,156,159,160] A role for AT1 receptors in liver fibrosis is also supported by the finding that mice lacking AT1a receptors are protected to develop liver fibrosis after prolonged bile duct ligation.[137,138] In contrast, mice lacking AT2 receptors show increased susceptibility to liver fibrosis.[150]

The Renin-Angiotensin System and Hepatic Fibrosis: Clinical Implications

Although the experimental evidence supporting a role for the RAS in liver fibrogenesis is overwhelming, clinical evidence is limited. Components required to synthesize angiotensin II, such as ACE-1 and chymase, are expressed in the livers of patients who have alcoholic liver disease and chronic hepatitis C.[38,163] A polymorphism in the angiotensinogen gene that confers increased angiotensin II synthesis influences fibrosis progression in patients who have chronic hepatitis B, hepatitis C, and NASH.[157,164,165]

Few reports have investigated the potential antifibrotic effect of RAS inhibition in patients who have chronic liver diseases. Preliminary uncontrolled studies suggest that losartan, an AT1 receptor blocker, may attenuate fibrosis progression in patients who have chronic hepatitis C or NASH.[147,166–168] Moreover, a retrospective study in patients who underwent transplantation and experienced hepatitis C recurrence showed that those receiving RAS inhibitors as antihypertensive therapy showed less fibrosis progression than patients receiving other types of drugs.[169] This study is potentially relevant, because fibrosis progression is aggressive in patients who underwent transplantation who experienced hepatitis C recurrence and is the main cause of graft loss.[170]

The rationale supporting the use of RAS inhibitors is that they markedly attenuate experimentally induced liver fibrosis and are safe and effective in preventing renal or cardiac fibrosis progression in patients who have type II diabetes and arterial hypertension.[7,171] Moreover, RAS inhibitors are reasonably inexpensive and can be safely administered for prolonged periods. However, clinical evidence supporting their use in patients who have liver diseases is only based on pilot studies that included a low number of patients and retrospective data. Randomized clinical trials are needed before this approach can be recommended.

The target population for clinical trials is patients who have chronic liver diseases for which the causative agent cannot be removed (eg, chronic hepatitis C not responding to antiviral therapy, chronic cholestatic conditions). Alcohol-induced liver fibrosis and NASH, conditions associated with marked oxidative stress, are also potential targets for RAS blockade. The expected benefits in these patients include decreased fibrosis progression and decreased inflammation. As a result, RAS inhibitors may slow progression to advanced fibrosis and therefore prevent development of portal hypertension and related complications. The duration of antifibrotic therapy to show changes in liver fibrosis should be considered, depending on the rate of fibrosis progression of the underlying disease. Obviously, patients who have undergone liver transplantation represent the ideal population to evaluate the efficacy of antifibrotic drugs, including RAS inhibitors.

Finally, experts have suggested that the RAS may participate in the development and progression of hepatocellular carcinoma through promoting fibrosis and angiogenesis, respectively.[160] Although experimental studies indicate that RAS inhibition prevents liver carcinogenesis,[160] no clinical data support this hypothesis. Whether this approach is useful in patients who have advanced cirrhosis is unknown and should be evaluated in clinical trials.

Side effects are a potential limitation for the use of ACE inhibitors in patients who have chronic liver diseases. The antifibrotic profile of ACE inhibitors and AT1 antagonists is similar, but AT1 antagonists are usually better tolerated. Hepatotoxicity can occur, although its incidence in patients who have chronic hepatitis is unknown.[172] RAS inhibitors can cause arterial hypotension or renal impairment in patients who have advanced cirrhosis and subsequent activation of the systemic RAS. In this clinical setting, the efficacy of RAS inhibitors is probably very limited.

Table 2
Studies assessing the role of the renin-angiotensin system in liver fibrosis in rats: proposed mechanisms

Reference	RAS Inhibitor	Experimental Model	Proposed Mechanism
Ramos et al.[153]	Captopril	Pig serum	Decreased mast cell accumulation
Ohishi et al.[151]	Lisinopril	CCl_4	Decreased stimulation of HSCs Decreased TGF-β expression
Jonsson et al.[13]	Captopril	BDL	Decreased TGF-β expression Regulation MMPs/TIMPs
Yoshiji et al.[159]	Candesartan Perindopril	Pig-serum	Reduced αSMA–positive cells Decreased TGF-β expression
Paizis et al.[152]	Irbesartan	BDL	Decreased TGF-β expression AT1 down-regulation
Wei et al.[156]	Losartan	CCl_4	Reduced αSMA–positive cells Decreased TGF-β expression
Ramalho et al.[14]	Losartan	BDL	Reduced αSMA–positive cells
Croquet et al.[145]	Losartan	CCl_4	Not assessed
Yoshiji et al.[173]	PerindoprilCandesartan	Pig serum	Decreased TIMP-1 expression
Li et al.[174]	Perindopril	CCl_4	Decreased MMP2,9 expression Decreased TGF-β, NF-κB
Ueki et al.[175]	Candesartan	BDL	Decreased CTGF expression Decreased TGF-β expression
Yoshiji et al.[176]	Perindopril	CCl_4	Decreased αSMA expression
Li et al.[174]	Perindopril Candesartan	CCl_4	Decreased MCP-1 expression Decreased TGF-β expression
Xu et al.[158]	Perindopril Valsartan	CCl_4	Decreased Smad3 expression
Kurikawa et al.[149]	Olmesartan	BDL	Reduced αSMA–positive cells Decreased TGF-β expression Decreased CTGF expression Effect in HSCs

Tuncer et al.[154]	Candesartan Captopril	CCl_4	Reduced αSMA-positive cells
Ibanez et al.[148]	Losartan	CDD	Reduced TGF-β expression
El Demerdash et al.[146]	Lisinopril Losartan	CCl_4	Reduced oxidative stress
Kitamura et al.[143]	TCV-116	CCD	Decreased activation Rho kinase
Turkay et al.[155]	Enalapril	BDL	Decreased TGF-β/MMP2 expression
Hirose et al.[11]	Olmesartan	CDD	Decreased TGF-β expression Rseduced αSMA–positive cells
Jin et al.[12]	Telmisartan	CDD	Decreased TIMP-1/MMP13 expression
Nabeshima et al.[150]	Losartan	CCl_4	Decreased TGF-β and TNF-α expression

Abbreviations: αSMA, α-smooth muscle actin; BDL, bile duct ligation; CCl_4, carbon tetrachloride; CDD, choline deficient diet; MMP, matrix metallopeptidase; TIMP-1, tissue inhibitor of metalloproteinases type 1.

CONCLUSIONS

Hepatic wound healing response to injury involves several cytokines that regulate myofibroblast proliferation, angiogenesis, and collagen synthesis. Dysregulated cytokine synthesis contributes to the initiation and progression of fibrosis. Multiple signaling pathways are activated by cytokines liver fibrogenesis. Most of these pathways have been identified in cultured HSCs, the main target cell for fibrogenic cytokines. Understanding the complexity of the cytokine-driven mechanisms of fibrosis and related signaling pathways is important for identifying potential molecular targets for future pharmacologic interventions in prevention and treatment. A growing body of evidence indicates that the RAS plays a key role in liver fibrogenesis. Angiotensin II exerts prooxidant, fibrogenic, and proinflammatory actions in the liver. Although the molecular mechanisms underlying the fibrogenic effect of angiotensin II in the liver are unknown, NADPH oxidase–derived ROS seem to play an important role. Although preliminary clinical data suggest that RAS inhibition can be useful as an antifibrotic therapy in patients who have chronic liver diseases, randomized clinical trials are needed before this approach can be routinely recommended.

SUMMARY

Hepatic fibrosis is the result of a complex interplay between resident hepatic cells, infiltrating inflammatory cells, and several locally acting peptides called cytokines. Dysregulated cytokine release underlies the hepatic response to injury and participates in the initiation, progression, and maintenance of fibrosis. Key mediators include TGF-β1, vasoactive substances, adipokines, and several inflammatory cytokines and chemokines.

Multiple signal transduction pathways are involved in cytokine signaling. Most pathways have been identified in cultured hepatic stellate cells, the main target cell for fibrogenic cytokines. Drugs interfering intracellular pathways involved in increased collagen production are potential therapies for liver fibrosis. Accumulating evidence indicates that angiotensin II, the main effector of the RAS, is a true cytokine that plays a major role in liver fibrosis. An intrahepatic RAS is expressed in chronically damaged livers, and angiotensin II induces an array of fibrogenic actions in hepatic stellate cells, including increased collagen synthesis and secretion of inflammatory mediators. These effects are mediated by NADPH oxidase–generated ROS and are prevented by angiotensin type 1 receptor blockers.

Inhibition of the RAS markedly attenuates experimentally induced liver fibrosis in rodents. Preliminary studies in patients who have chronic hepatitis C and NASH suggest that RAS blocking agents may have beneficial effects on fibrosis progression. This article outlines the main cytokines involved in liver fibrosis, including angiotensin II, and describes the signaling pathways involved.

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