Tesi Doctoral

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MECANISMES INVOLUCRATS EN LA REGULACIÓ DEL TO VASCULAR HEPÀTIC EN LA CIRROSI: PAPER DELS PROSTANOIDS VASOCONSTRICTORS I DE L'ESTRÈS OXIDATIU

Tesi presentada per

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Per optar al grau de

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Als meus pares

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INFORME DELS DIRECTORS DE TESI

Barcelona, a 30 de Juliol de 2007.

Joan Carles García-Pagán, consultor del Servei d'Hepatologia de l'Hospital Clínic de Barcelona, i Jaume Bosch i Genover, catedràtic de la Facultat de Medicina de la Universitat de Barcelona i Consultor Senior del Servei d'Hepatologia de l'Hospital Clínic de Barcelona,

CERTIFIQUEN:

Que la tesi doctoral *MECANISMES MOLECULARS INVOLUCRATS EN LA REGULACIÓ DEL TO VASCULAR HEPÀTIC EN LA CIRROSI: PAPER DELS PROSTANOIDS VASOCONSTRICTORS I DE L'ESTRÈS OXIDATIU*, presentada per Jorge Gracia-Sancho per optar al grau de Doctor en Bioquímica ha estat realitzada sota la nostra direcció i compleix tots els requisits necessaris per ser defensada davant el Tribunal d'avaluació corresponent.

Joan Carles García-Pagán **Jaume Bosch i Genover**

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1. Introducció

1- INTRODUCCIÓ

La síndrome clínica anomenada hipertensió portal es caracteritza per un increment patològic de la pressió hidrostàtica del sistema venós portal per sobre del seu valor normal d'1-5 mmHg. Aquest increment origina la formació d'una extensa xarxa de venes col·laterals portosistèmiques que deriven una part del flux sanguini portal cap a la circulació sistèmica, evitant que circuli pel fetge. Bona part de les complicacions derivades de la cirrosi hepàtica, com l'hemorràgia digestiva pel trencament de varius esofàgiques, l'encefalopatia hepàtica, l'ascites o la insuficiència renal, estan directament relacionades amb la presència d'hipertensió portal. A causa de la gravetat d'aquestes complicacions, la hipertensió portal representa la primera causa de mort i de trasplantament hepàtic en malalts cirròtics (1).

1.1- Bases fisiopatològiques de la hipertensió portal

Com en qualsevol sistema hidrodinàmic, la pressió [portal] ve determinada per la interrelació entre el flux [sanguini portal] i la resistència que s'oposa a aquest flux. Mitjançant la Llei d'Ohm, el gradient de pressió entre dos punts en un vas sanguini queda definit per la següent equació:

$$\Delta P = Q \times R$$

On **ΔP** seria el gradient de pressió portal o pressió de perfusió hepàtica (la diferència entre la pressió de la vena porta i la pressió de la vena cava inferior), **Q**, el flux sanguini de tot el sistema venós portal (que en la patologia inclou el flux de la vena porta i el corresponent a les venes col·laterals portosistèmiques) i **R**, la resistència vascular exercida pel sistema venós portal, per les venes col·laterals i pel mateix fetge (2).

1.1.1- Resistència vascular intrahepàtica (RVI)

L'augment de la RVI al flux portal constitueix el fenomen fisiopatològic primari de la hipertensió portal. En la cirrosi aquest augment té lloc al sinusoide hepàtic (hipertensió portal sinusoïdal).

Durant llarg temps s'havia considerat que l'elevada RVI únicament era conseqüència de la distorsió de l'arquitectura vascular del fetge cirròtic causada per la malaltia i que, per tant, aquesta no era modificable mitjançant fàrmacs.

L'any 1985 Bathal i Grossmann (3) canviaren aquest dogma demostrant que una part de l'augmentada RVI podia ser modificada farmacològicament. A partir d'aquell moment, diversos estudis van confirmar i ampliar aquesta troballa (4; 5). Avui en dia és acceptat que una part significativa de la RVI en la cirrosi és modificable per drogues i és denominada component "dinàmic" de la RVI i podria arribar a representar fins el 40% de la RVI total.

1.1.2- Component dinàmic de la RVI

Amb l'objectiu de caracteritzar el component dinàmic de la RVI, diferents estudis han cercat quins són els elements contràctils intrahepàtics, i si en la cirrosi, existeixen anormalitats en la regulació dels mecanismes de contracció cel·lular. Fins a l'actualitat s'han descrit diversos tipus de cèl·lules hepàtiques amb capacitat contràctil com els miofibroblasts portals i septals i les vènules portals (6) però majoritàriament s'ha postulat que les principals cèl·lules involucrades en la regulació del to vascular hepàtic són les cèl·lules hepàtiques estelades (CHE) (7).

a) CHE, executores de l'increment en la RVI

Les CHE, també conegudes com a cèl·lules d'Ito o lipòcits, representen aproximadament el 5% del total de cèl·lules hepàtiques i són el principal magatzem de vitamina A de l'organisme. Es troben localitzades a l'espai de Disse (espai virtual entre les cèl·lules endotelials i les cèl·lules hepàtiques) i presenten extensions citoplasmàtiques que rodegen els sinusoides, característica que els permet regular el flux sinusoïdal per contracció. En situacions de dany hepatocel·lular, les CHE sofreixen un ràpid canvi, tant morfològic com funcional, que comporta la seva "activació", donant lloc a cèl·lules amb un fenotip tipus miofibroblast amb propietats típiques d'aquest tipus cel·lular, és a dir, amb potencial pro-fibrogènic i altes capacitats contràctil, immuno-moduladora i migratòria. Quan el dany hepàtic es converteix en crònic, les CHE es perpetuen en l'estat activat (7-9).

b) Substàncies vasoactives, reguladores de l'increment en la RVI

Un ampli ventall de molècules amb capacitat vasomotora s'han descrit com a moduladores de la RVI en la cirrosi (10-13). Aquestes substàncies poden

procedir de la circulació sistèmica (com l'angiotensina II o la vasopressina), poden originar-se a l'endoteli i actuar de forma paracrina (com l'òxid nítric [ON], la prostaciclina [PGI_2] o l'endotelina) o tenir un origen neuronal (norepinefrina). Tinguin l'origen que tinguin, un fet ben conegut i característic de la cirrosi és el marcat desequilibri en la concentració d'aquests mediadors vasoactius en la circulació hepàtica: es troben elevats nivells de vasoconstrictors (com la endotelina o el **tromboxà**) i reduïdes quantitats de vasodilatadors (principalment l'**ON**) (14-17). A més, s'ha demostrat que els fetges cirròtics hiperresponen als estímuls vasoconstrictors i hiporesponen als vasodilatadors (10-13; 18; 19).

Tromboxà A2 (TXA_2)

Aquesta molècula de 20 àtoms de carboni, derivada de l'àcid araquidònic per acció dels enzims ciclooxigenasa (COX) i tromboxà sintasa (TXS), té com a principals característiques ser un potent vasoconstrictor i induir l'agregació plaquetària (el seu nom prové d'aquesta última propietat [induir trombosi]). És sintetitzat principalment per plaquetes, tot i que diversos autors han demostrat que alguns tipus de cèl·lules endotelials també en poden produir (20-23).

Els primers estudis que involucraren el TXA_2 en la hipertensió portal foren publicats pels grups de Clemens i Zhang als EUA i de García-Pagán i Bosch a Barcelona. Ambdós grups, treballant amb diferents models experimentals de cirrosi (lligadura del conducte biliar els primers i inhalació de CCl_4 els segons) evidenciaren que els fetges de rates amb hipertensió portal sintetitzen majors quantitats de TXA_2, tant basalment com a l'estimular la circulació intrahepàtica amb un agonista alfa-1-adrenèrgic (metoxamina), i que aquest augment en la síntesi de TXA_2 s'acompanya d'un increment significatiu en la pressió portal (13; 24).

Estudis posteriors ampliaren aquest coneixement demostrant que la modulació del to vascular intrahepàtic cirròtic per l'acció de prostanoids vasoconstrictors (principalment TXA_2) és dependent de la isoforma 1 de la COX (COX-1) i que les cèl·lules endotelials sinusoïdals (CES) podrien ser les responsables de la seva síntesi (25).

Òxid nítric (ON)

Aquest gas, fonamental en varietat de processos biològics, ha estat definit com el vasodilatador més important d'origen endotelial. En el camp de la hipertensió portal, des del pioner estudi de Bathal i Grossman fins avui en dia, s'han publicat més de 350 estudis avaluant el rol de l'ON sobre la regulació del to vascular hepàtic i la forma de modular-lo.

L'ON és sintetitzat com a subproducte de la formació de L-citrulina per part de tres isoformes de la proteïna sintasa d'ON (NOS), la NOS neuronal (nNOS), la forma induïble (iNOS) i l'endotelial (eNOS) (26). La producció hepàtica fisiològica d'ON és derivada de la eNOS en resposta a estímuls com l'estrès de fregament i la presencia de vasoconstrictors (27; 28). El mecanisme d'acció de l'ON és paracrí, possiblement sobre les CHE, promovent la síntesi de GMP cíclic (GMPc) (29).

El seu paper en el fetge normal és de compensació vascular enfront un estímul vasoconstrictor i per tant, la seva biodisponibilitat és correcta i plenament efectiva (30). En el fetge cirròtic, en canvi, la síntesi d'ON es troba fortament alterada per diferents desregulacions post-traduccionals que condueixen a un estat de baixa biodisponibilitat intrahepàtica (16; 31-33).

El dèficit d'ON en els fetges cirròtics va proporcionar la base racional per al tractament de la hipertensió portal amb donants d'ON, primerament no selectius i més recentment, fetge selectius, per tal d'evitar la hipotensió arterial provocada pels primers. Diversos estudis, a través d'estratègies ben diferents, han aconseguit incrementar la producció hepàtica d'ON i reduir així significativament la RVI i la pressió portal de rates amb cirrosi sense modificar la pressió arterial. D'una banda, la infecció gènica del fetge amb l'adenovirus que conté la nNOS o l'eNOS (34; 35); de l'altra, administrant donants d'ON específicament hepàtics (36) i molt recentment augmentant la síntesi d'ON mitjançant el tractament amb estatines (37).

En resum, el component "dinàmic", modificable i reversible de la RVI en la cirrosi ve determinat per un desequilibri en les substàncies vasoactives intrahepàtiques (excés de vasoconstrictors enfront dèficit de vasodilatadors), que, a l'actuar sobre les CHE provocarien la seva contracció i conseqüentment, l'increment del to vascular hepàtic.

1.1.3- El segon factor de l'equació, el flux portal

El segon factor que contribueix a l'existència d'hipertensió portal és l'augment del flux del sistema portal. Aquest fet és secundari a una marcada vasodilatació esplàcnica causada per un alliberament exagerat de mediadors vasodilatadors endògens i circulants i a una hipo-resposta als agents vasoconstrictors presents (38).

Diverses substàncies com el glucagó (39; 40), la prostaciclina (PGI_2) (41; 42) o el monòxid de carboni (43) s'han descrit com a causes de la marcada vasodilatació esplàncnica en la cirrosi, però el mediador més important sembla ésser l'ON.

Els primers estudis dirigits a la recerca de factors vasodilatadors esplàncnics es van centrar en el **glucagó**. Elevats nivells d'aquesta proteïna foren descrits en pacients cirròtics i en models experimentals de cirrosi. A més, la inhibició de la síntesi de glucagó redueix parcialment el flux esplàncnic i l'administració de glucagó a rates sanes comporta un augment significatiu en la vasodilatació esplàncnica (39; 40; 44-46). Aquest conjunt de troballes proporcionaren la base racional per a l'ús d'inhibidors de la síntesi de glucagó (somatostatina i anàlegs) en el tractament de les complicacions de la hipertensió portal.

Més recentment, el possible paper de l'**ON** regulant el to vascular mesentèric i els seus mecanismes d'acció foren i continuen sent investigats. Hom pot trobar una extensa bibliografia en referència a aquesta recerca. S'ha descrit que pacients amb cirrosi i animals amb hipertensió portal experimental mostren elevats nivells plasmàtics d'ON, els quals són més elevats en la zona portal, suggerint una producció mesentèrica. A més, la producció esplàncnica d'ON en rates cirròtiques és superior a les controls, i la inhibició de la seva síntesi redueix el flux de l'artèria mesentèrica superior, el grau de col·lateralització i preveu parcialment el desenvolupament de la circulació hiperdinàmica típica de la hipertensió portal (47-51).

Diferents estudis han hipotetitzat sobre l'origen i els mecanismes moleculars responsables dels elevats nivells sistèmics i esplàncnics d'ON en la cirrosi. Ja fa gairebé dues dècades, Vallance i Moncada proposaren que l'endotoxèmia sovint present en la cirrosi activava l'expressió vascular de la isoforma induïble de la sintasa d'ON (iNOS), i augmentava d'aquesta forma la producció d'ON

(52). Posteriorment, aquesta hipòtesi fou desestimada a l'observar que en la cirrosi, els nivells d'expressió i l'activitat enzimàtica d'aquesta isoforma no es troben augmentats i que, la seva inhibició selectiva no prevén el desenvolupament de la circulació hiperdinàmica. A més, fou demostrat que l'isoforma endotelial de la sintasa d'ON (eNOS) és la veritable responsable mitjançant un augment en la seva activitat enzimàtica (53; 54).
Els mecanismes que explicarien la sobre-activació de l'eNOS en el territori esplàncnic són diversos i possiblement, encara no els coneixem tots. L'augmentat estrès per fregament present al territori esplàncnic cirròtic, juntament amb elevats nivells de diferents factors humorals, proteïnes estabilitzadores i citosines pro-inflamatòries podrien ser alguns d'aquests mecanismes (50; 55-58).

L'ON té un paper antagònic en la cirrosi (38). En la circulació intrahepàtica existeix un dèficit en la seva producció que provoca una alteració marcada del to vascular. En canvi, en la circulació esplàncnica i sistèmica la producció d'ON es troba augmentada, la qual cosa condueix a una forta vasodilatació arterial i a una hipo-resposta a vasoconstrictors. Així, ambdues situacions contribueixen a mantenir i a empitjorar la síndrome d'hipertensió portal. Aquesta dualitat d'efectes negatius ha comportat la ineficàcia dels tractaments amb donants d'ON no selectius pel fetge, ja que redueixen la RVI i la pressió portal, però alhora augmenten la vasodilatació perifèrica, la qual s'acompanya d'una disminució de la pressió arterial sistèmica (59).

1.2- Circulació hiperdinàmica

Els pacients en fases avançades de cirrosi presenten la síndrome anomenada circulació hiperdinàmica de la hipertensió portal. Aquesta es caracteritza per una vasodilatació generalitzada acompanyada d'un descens en la pressió arterial i resistència vascular perifèrica juntament amb un augment en la despesa cardíaca i el volum plasmàtic (60).
Aquests canvis hemodinàmics a nivell sistèmic són derivats de l'exagerada vasodilatació esplàncnica típica de la cirrosi. Es creu que part dels vasodilatadors esplàncnics passarien a la circulació general (ja sigui per no haver estat degradats hepàticament o per haver evitat el fetge via venes

col·laterals portosistèmiques) comportant una vasodilatació arterial generalitzada, seguida d'una hipovolèmia relativa de la circulació arterial. En aquest punt l'organisme activaria una sèrie de sistemes reguladors, com el sistema renina-angiotensina, el sistema nerviós o la secreció de vasopressina, que activarien la retenció d'aigua i sodi per part del ronyó, augmentant així el volum plasmàtic i mantenint la pressió arterial perifèrica (60-63).

Com a conseqüència d'aquesta adaptació, l'alteració de la funció renal més precoç i comuna dels pacients amb hipertensió portal és l'elevada retenció de sodi. A més, diverses complicacions en altres òrgans s'han relacionat amb la síndrome de la circulació hiperdinàmica, com la síndrome hepatopulmonar, la progressió d'encefalopatia hepàtica, la síndrome d'isquèmia i formació d'estrès oxidatiu en diferents teixits, etc. (64; 65). Diversos autors han relacionat la severitat de la circulació hiperdinàmica amb la prognosi i supervivència de pacients amb hipertensió portal (66).

Per últim, en relació amb el tractament de la circulació hiperdinàmica la restricció de sodi en rates amb hipertensió portal experimental evita l'expansió del volum plasmàtic i normalitza l'índex cardíac i el flux esplàncnic. A més, en pacients cirròtics, l'administració de diürètics disminueix la pressió portal (67; 68).

2. Justificació i objectius

2- JUSTIFICACIÓ I OBJECTIUS DE LA PRESENT TESI

2.1- Justificació i objectius generals

La cirrosi hepàtica és la tercera causa de mort en homes d'entre 35 i 64 anys a Catalunya i a Espanya (cinquena causa en dones de la mateixa franja d'edat) i setena en persones d'entre 25 i 64 anys als Estats Units (69; 70). La hipertensió portal i les seves complicacions (trencament de varius gastroesofàgiques, ascites, síndrome hepatorrenal, encefalopatia hepàtica...) són la principal causa de mort i trasplantament en pacients cirròtics. La hipertensió portal pot aparèixer per un augment de la RVI, per un augment del flux portal o per un augment d'ambdós factors. Tanmateix, l'augment de la RVI és el factor fisiopatològic primari i per tant, aquelles teràpies que disminueixin la RVI produiran un descens de la pressió portal i reduiran el risc de patir complicacions.

Fins ara, a causa de la inexistència d'alternatives fiables, segures i efectives, la teràpia majoritàriament emprada en els pacients amb cirrosi és l'ús de beta-bloquejants, els quals redueixen la pressió portal al disminuir els fluxos col·lateral i portal. Tot i això, aquelles estrategies capaces de reduïr el flux portal tenen com a efecte secundari una menor irrigació hepàtica i per tant, un possible efecte deleteri sobre la funció hepàtica (71; 72).

Per contra, s'ha demostrat que també és possible manipular farmacològicament el component dinàmic de la RVI (sense necessitat de modificar el flux portal) (73; 74). Aquest increment de la resistència dinàmica és derivat de tres mecanismes fisiopatològics diferents: a) un augment en la concentració dels vasoconstrictors hepàtics, b) un augment en la resposta a aquests vasoconstrictors, i c) una marcada disminució en la biodisponibilitat de vasodilatadors hepàtics. Fins a dia d'avui, encara no coneixem tots els mediadors vasomotors que contribueixen a incrementar la resistència dinàmica ni quins mecanismes regulen la seva acció.

Els treballs de recerca de la present tesi estan globalment orientats a ampliar el coneixement dels mecanismes moleculars responsables de l'augment del component dinàmic de la RVI.

2.2- Estudi 1: Enhanced vasoconstrictor prostanoid production by sinusoidal endothelial cells increases perfusion portal pressure in cirrhotic rat livers.

Com s'ha mencionat anteriorment, dos fets responsables de la incrementada RVI en la cirrosi són l'elevada producció intrahepàtica de vasoconstrictors i l'exagerada resposta vascular a aquests. Repassant la recent bibliografia al voltant d'aquest tema, l'estudi del possible paper dels prostanoids derivats de l'àcid araquidònic (AA) sobre la RVI en la cirrosi ocupa una remarcable posició (12; 13; 21; 24; 25; 75-78).

Graupera i col·laboradors varen demostrar que, tant la hiperresposta a vasoconstrictors com la disfunció endotelial presents en la cirrosi són conseqüència, en part, d'una exagerada producció de prostanoids vasoconstrictors derivats de l'acció de COX-1. A més, en el seu últim estudi publicat el 2005 varen mostrar que l'únic tipus cel·lular hepàtic que sobre-expressa l'isoforma 1 de la COX en la cirrosi són les cèl·lules endotelials sinusoïdals (CES), hipotetitzant així que serien aquestes les responsables de la exagerada síntesi.

Diversos estudis realitzats en cèl·lules *in vitro* han mostrat que l'activació de l'enzim fosfolipasa A2 mitjançant un agonista del receptor lligat a proteïna G, promou l'alliberament de molècules d'àcid araquidònic (AA) procedents dels fosfolípids de membrana (79; 80). Una vegada lliure, l'AA pot ser transformat per l'acció de COX a prostaglandina H2 (PGH_2; intermediari comú de la resta de prostanoids i amb propietats vasoconstrictores) i posteriorment aquesta PGH_2 pot convertir-se en l'ampli ventall de prostanoids mitjançant l'activitat de diferents sintases (Fig 1).

Segons els antecedents presentats, la **hipòtesi** del present estudi fou que en el fetge cirròtic un augment en la disponibilitat d'AA en un ambient de sobre-expressió de COX-1, com el present en les CES cirròtiques, comportaria una exagerada producció de prostanoids vasoconstrictors que promourien un marcat augment de la RVI.

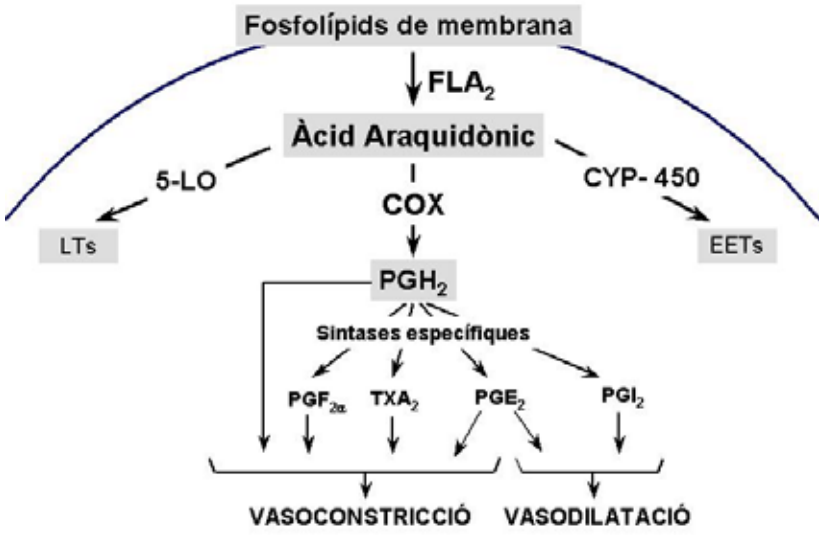

Figura 1: Via metabòlica de l'àcid araquidònic (AA). L'AA, sintetitzat per l'enzim fosfolipasa A2 (FLA2), pot ser transformat en tres famílies diferents de compostos mitjançant l'acció dels enzims: 5-Lipooxigenasa (5-LO) per donar lloc a leucotriens (LTs), ciclooxigenasa (COX) per formar el precursor comú prostaglandina (PG) H2, que pot ser ulteriorment transformat en un altre tipus de prostaglandina o en tromboxà A2; i per últim pel citocrom P-450 (CYP-450) sintetitzant àcids epoxieicosatrienoics (EETs).

Per tant, els **objectius** del present estudi foren: avaluar l'efecte d'una elevada disponibilitat d'AA sobre la modulació del to vascular intrahepàtic en la cirrosi i sobre la síntesi de prostanoids per part de les CES. A més, l'enzim responsable de la formació de l'AA fou estudiat amb l'objectiu de conèixer si aquest es troba sobre-activat en la cirrosi i, per tant, seria un nou mecanisme a tenir en compte.

2.3- Estudi 2: Evidence against a role for NADPH oxidase modulating hepatic vascular tone in cirrhosis.

La disfunció endotelial que presenten els fetges cirròtics es caracteritza per una deficient resposta endoteli dependent a estímuls vasodilatadors. Es creu que aquesta deficiència és causada per una disminució en la biodisponibilitat d'ON (38; 81; 82) així com una exagerada síntesi de vasoconstrictors derivats de COX-1 (13; 25; 75).

La reducció en la biodisponibilitat intrahepàtica d'ON ha estat atribuïda principalment a una reducció en l'activitat de l'enzim responsable de la seva síntesi (eNOS) (16; 83). Tot i això, en altres territoris com el cardiovascular la

reducció en la biodisponibilitat d'ON s'ha relacionat amb un augment del segrest d'aquest per part del radical superòxid (O_2^-) derivat de l'activitat de l'enzim NADPH oxidasa (84-86). A més, s'ha atribuït un important rol a la NADPH oxidasa en patologies hepàtiques que cursen amb presència d'estrès oxidatiu (87-90).

És important afegir que estudis del nostre grup han demostrat que el fetge cirròtic presenta elevats nivells de O_2^- (vegeu estudi 3 de la present tesi) i que l'ús d'antioxidants millora la vasorelaxació hepàtica en pacients amb cirrosi (74).

Amb els antecedent descrits, la **hipòtesi** del present estudi fou que, en el fetge cirròtic, un augment en la formació de O_2^- per part de l'enzim NADPH oxidasa comportaria un increment en el segrest d'ON i per tant, una disminució en la seva biodisponibilitat, empitjorant així la deficient vasodilatació intrahepàtica. Per tant, la inhibició selectiva de l'enzim NADPH oxidasa milloraria la hipertensió portal i la disfunció endotelial en la cirrosi.

En conseqüència, els **objectius** del present estudi foren caracteritzar bioquímica i biològicament l'enzim NADPH oxidasa en el fetge cirròtic i avaluar el seu possible rol modulant el to vascular hepàtic.

2.4- Estudi 3: Increased oxidative stress in cirrhotic livers. A potential mechanism contributing to reduce nitric oxide bioavailability.

Anteriorment s'ha descrit que la baixa biodisponibilitat d'ON en el fetge cirròtic, responsable parcialment de la disfunció endotelial, és causada per una menor activitat enzimàtica d'eNOS secundària a una sèrie de desregulacions post-traduccionals .

En malalties cardiovasculars s'ha demostrat que un increment en els nivells de l'espècie radical d'oxigen, superòxid (O_2^-), al reaccionar amb l'ON, redueix marcadament la biodisponibilitat d'aquest vasodilatador i s'acompanya d'un augment significatiu en el to vascular (84; 91-94).

A més, en diferents desordres hepàtics s'han descrit elevats nivells de O_2^-, conseqüència d'una exagerada síntesi i/o d'una deficient eliminació (95-99).

La **hipòtesi** del present estudi va ser que, en els fetges cirròtics, un increment en els nivells de O_2^-, al reaccionar amb l'ON, contribuiria a reduir la biodisponibilitat d'ON i per tant, l'eliminació del O_2^- del fetge cirròtic seria una nova estratègia terapèutica encaminada a incrementar la biodisponibilitat intrahepàtica d'ON.

Per tant, els **objectius** d'aquest estudi foren conèixer els nivells d'ON en el fetge cirròtic, el grau d'estrès oxidatiu i les seves possibles causes a més d'avaluar l'efectivitat d'una teràpia antioxidant sobre la biodisponibilitat d'ON.

3. Còpies dels articles originals

3- CÒPIES DELS ARTICLES ORIGINALS

3.1- Enhanced vasoconstrictor prostanoid production by sinusoidal endothelial cells increases perfusion portal pressure in cirrhotic rat livers.
Gracia-Sancho J, Laviña B, Rodríguez-Vilarrupla A, García-Calderó H, Bosch J, García-Pagán JC.
Journal of Hepatology 2007;47 (2): 220-227. IF: 6.07

3.2- Evidence against a role for NADPH oxidase modulating hepatic vascular tone in cirrhosis.
Gracia-Sancho J, Laviña B, Rodríguez-Vilarrupla A, Brandes RP, Fernández M, Bosch J, García-Pagán JC.
Gastroenterology 2007; en premsa. IF: 12.46

3.3- Increased oxidative stress in cirrhotic livers. A potential mechanism contributing to reduce nitric oxide bioavailability.
Gracia-Sancho J*, Rodríguez-Vilarrupla A*, Laviña B, García-Calderó H, Fernández M, Bosch J, García-Pagán JC.
Pendent d'acceptació

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Enhanced vasoconstrictor prostanoid production by sinusoidal endothelial cells increases portal perfusion pressure in cirrhotic rat livers☆

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***Background/Aims*: Cyclooxygenase-1 (COX-1) is overexpressed in sinusoidal endothelial cells (SEC) of cirrhotic rat livers, and through an enhanced production of vasoconstrictor prostanoids contributes to increase intrahepatic resistance. Our study was aimed at investigating the role of enhanced AA bioavailability modulating the hepatic vascular tone of cirrhotic livers and identifying which prostanoid is involved.**

***Methods*: SEC isolated from control and cirrhotic rat livers were incubated with AA, methoxamine or vehicle. TXA_2 was quantified. In addition, portal perfusion pressure (PP) response curves to AA were performed in rat livers pre-incubated with vehicle, SC-560 (COX-1 inhibitor), Furegrelate (inhibitor of TXA_2 synthesis) and SQ-29548 (PGH_2/TXA_2 receptor blocker). cPLA2 activity was determined in control and cirrhotic livers.**

***Results*: AA and methoxamine incubation promoted a significant increase in TXA_2 release by Cirrhotic-SEC, but not in Control-SEC. AA produced a dose-dependent increase in the PP, associated with increased TXA_2 release. These responses were significantly greater in cirrhotic livers. COX-1 inhibition and PGH_2/TXA_2 receptor blockade, but not TXA_2 synthase inhibition, markedly attenuated the PP response to AA of cirrhotic livers. Additionally, cirrhotic livers exhibited significantly increased cPLA2 activity.**

***Conclusions*: An enhanced production of vasoconstrictor prostanoids, probably PGH_2, by SEC contributes to increase vascular tone of cirrhotic livers.**

Keywords: **Intrahepatic resistance; Phospholipase A2; Sinusoidal endothelial cells; Arachidonic acid; PGH_2**

1. Introduction

In cirrhotic livers, increased vascular resistance to portal blood flow is the initial factor leading to the development of portal hypertension [1]. This increase in resistance is, in part, due to reversible functional alterations including a deficient response of the hepatic vascular bed to vasodilators or an exaggerated response to vasoconstrictors [2–4]. Increased production of arachidonic acid (AA) derived vasoconstrictors via the cyclooxygenase-1 (COX-1) pathway has been shown to increase hepatic vascular resistance in cirrhosis [5–8].

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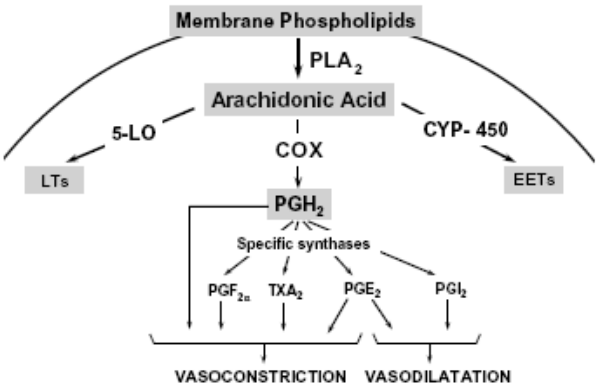

Fig. 1. Arachidonic acid (AA) metabolism pathway. AA is synthesized from membrane phospholipids by phospholipase A2 (PLA2). Once free AA could be transformed through three pathways forming different product families. 1 AA could be metabolised by 5-lipooxygenase (5-LO) to form leukotrienes (LTs), essential eicosanoid lipid mediators involved in inflammation and in vascular tone regulation. 2 By cyclooxygenase (COX) activity AA could be transformed to the common prostaglandin (PG) precursor and vasoconstrictor PGH2. PGH2 is rapidly metabolised by specific synthases to form the broad spectrum of prostaglandins family, including PGF2α, PGE2, PGI2 and thromboxane A2 (TXA2), all of them with potent vasoactive properties. 3 Finally AA could also be transformed to epoxyeicosatrienoic acids (EETs), metabolites with some vascular and tubular functions, by cytochrome P-450 (CYP-450).

Moreover, it has been suggested that these vasoconstrictor prostanoids would be mainly synthesized by cirrhotic sinusoidal endothelial cells (SEC) that overexpress COX-1 [7].

In several cell systems activation of cytosolic phospholipase A2 (PLA_2) by a G-protein coupled receptor-dependent mechanism promotes the release of AA from membrane phospholipids [9,10]. AA would then be metabolised to PGH_2 by COX. PGH_2 is the common precursor for prostaglandin (PGs) and thromboxane (TXs) by action of different specific PG and TX synthases [11] (Fig. 1). We hypothesize that, in the cirrhotic liver, an increase in AA bioavailability in an environment of increased COX-1 expression, such as in cirrhotic SEC, will contribute to enhanced production of vasoconstrictor prostanoids that would lead to increased hepatic vascular resistance.

Thus, the aim of the present study was to investigate the role of an enhanced AA bioavailability modulating the hepatic vascular tone of cirrhotic livers, regulating vasoconstrictor-prostanoid synthesis by SEC and identifying which is the main vasoconstrictor prostanoid involved.

2. Materials and methods

2.1. Induction of cirrhosis by CCl_4

Male Wistar rats weighing 175–200 g underwent inhalation exposure to CCl_4 and received phenobarbital in the drinking water as previously described [5]. Once the cirrhotic rats developed ascites, usually at week 16, administration of CCl_4 and phenobarbital was stopped and experiments were performed 1 week later. Control animals received only phenobarbital. The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in the Hospital Clínic and IDIBAPS.

2.2. Liver sinusoidal endothelial cells isolation

Liver sinusoidal endothelial cells (SEC) were isolated from control ($n = 7$) and cirrhotic ($n = 7$) rat livers as described elsewhere [7,12] with minor modifications. Briefly, livers were perfused through the portal vein for 10 min at a flow rate of 20 mL/min at 37 °C with Hanks' Balanced Salt Solution (HBSS) without calcium and magnesium containing 12.5 mM Hepes (pH 7.4), 0.5 mM EGTA and 2% BSA. Then, 30 min at a flow rate of 5 mL/min at 37 °C with 0.015% collagenase A (for cirrhotic livers the concentration was increased by 25%), HBSS containing 12.5 mM Hepes (pH 7.4) and 4 mM $CaCl_2$. The resultant digested liver was excised and in vitro digestion was performed at 37 °C with 0.01% collagenase A, HBSS containing 12.5 mM Hepes (pH 7.4) and 4 mM $CaCl_2$ for 10 min. The cells were passed through nylon filters, collected in cold Krebs' buffer and centrifuged at 50*g* for 3 min. The supernatant was centrifuged at 800*g* for 10 min at 4 °C and the obtained pellet was resuspended in Dulbecco's PBS (DPBS) and centrifuged at 800*g* for 25 min through a two-step Percoll gradient (25–50%) at 4 °C. The interface of the gradient was enriched in Kupffer cells and SEC. This cell fraction was diluted in DPBS and centrifuged at 800*g* for 10 min. The cell pellet was resuspended in RPMI medium, seeded in plastic dishes and incubated for 30 min at 37 °C in humid atmosphere with 5% CO_2 in order to enhance SEC purity by selective adherence of Kupffer cells. Non-adherent cells were seeded in collagen-coated 24 multiwell dishes at a concentration of 10^6 cells/mL per well and incubated for 1 h (37 °C, 5% CO_2). After this time the medium was discarded and adhered cells were washed twice with DPBS and cultured for 12 h (37 °C, 5% CO_2) in RPMI-1640 as previously described [13]. Specific immunocytochemical marking using rat endothelial cell antigen RECA-1 showed that almost 93% of these cells were SEC with a viability of 95% (evaluated by trypan blue exclusion).

Studies were performed 12 h after SEC isolation to preserve its typical phenotype [14].

2.3. Isolated perfused liver system

A flow-controlled perfusion system was employed in this study, as described previously [15]. Livers were perfused with Krebs' buffer in a recirculation fashion with a total volume of 100 mL at a constant flow rate of 35 mL/min. An ultrasonic transit-time flow probe (model T201; Transonic Systems, Ithaca, NY) and a pressure transducer were placed on line, immediately ahead of the portal inlet cannula, to continuously monitor portal flow and perfusion pressure. Another pressure transducer was placed immediately after the thoracic vena cava outlet for measurement of outflow pressure. The flow probe and the two pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart version 5.0.1 for Windows software (ADInstruments, Mountain View, LA). The average portal flow, inflow and outflow pressures were continuously sampled, recorded and afterwards analyzed.

The perfused rat liver preparation was allowed to stabilize for 30 min before the studied substances were added. The gross appearance of the liver, stable perfusion pressure and a stable buffer pH (7.4 ± 0.1) were measured during this period. If any viability criteria were not satisfied, the experiment was discarded.

2.4. SEC prostanoids production

Twelve hours monolayer cultures of SEC isolated from control and cirrhotic rat livers were pre-incubated for 15 min with vehicle or with the selective COX-1 inhibitor SC-560 (5 μM) then AA (40 μM) or its vehicle (ethanol 0.1%) was added. After 20 min 250 μL aliquots of

media were collected and stored at −80 °C until analysis of TXB_2 (the end metabolite of TXA_2), $PGF_{2\alpha}$ and 6-keto $PGF_{1\alpha}$ (the end metabolite of PGI_2) by enzyme immunoassays.

In additional experiments, SEC isolated from cirrhotic rat livers pre-incubated with vehicle or with the COX inhibitor Indomethacin (10 μM) were stimulated with the alpha 1-adrenergic agonist methoxamine (10^{-4} M) or its vehicle. After 20 min the media were collected and TXB_2 was analyzed as described above.

2.5. Portal perfusion pressure dose–response curve to arachidonic acid

A portal perfusion pressure (PP) response curve to AA was performed by adding increasing doses of AA (2×10^{-5}, 1×10^{-4}, 2×10^{-4}, 2×10^{-3} M) to the perfusion system every 3 min in different groups of perfused control and cirrhotic rat livers pre-incubated with vehicle (ethanol, $n=6$), the selective COX-1 inhibitor SC-560 (5 μM, $n=6$), the selective TXA_2 synthase inhibitor Furegrelate (50 mg/kg body wt i.p. 14 h before and 20 μM in the perfusion buffer, $n=6$) or with SQ 29,548, a blocker of the common receptor for PGH_2 and TXA_2 (1 μM, $n=6$). These doses have been previously shown to selectively inhibit COX-1 prostanoid production, TXA_2 synthesis and block the PGH_2/TXA_2 receptor, respectively [5,16,17].

2.6. Measurement of TXB_2

In all the liver-perfusion experiments, samples of the perfusate were obtained before and after the dose–response curve. The samples were stored at −80 °C and TXB_2 was quantified in duplicate as described above.

2.6.1. cPLA2 expression and activity

Total cPLA2 and phospho-cPLA2 protein expression in rat livers from eight cirrhotic and eight control rat livers was assessed by Western blot as follows. Livers were collected, snap-frozen in liquid N_2 and stored at −80 °C until analyzed. Livers were minced thoroughly with mortar and pestle under liquid nitrogen. For each sample, a similar amount of obtained powder was collected in 200 μl Triton-lysis buffer containing Tris/HCl (pH 7.4, 20 mM), NaCl (150 mM), NaF (20 mM), $Na_4P_2O_7$ (10 mM), okadaic acid (10 nM), Na_3VO_4 (2 mM), antipain (2 μg/ml), aprotinin (2 μg/ml), chymostatin (2 μg/ml), leupeptin (2 μg/ml), pepstatin (2 μg/ml), trypsin inhibitor (2 μg/ml), phenylmethylsulfonyl fluoride (40 μg/ml), and Triton X-100 (1% v/v), left on ice for 10 min, and then centrifuged at 10,000*g*/10 min. Protein concentration was assessed by the Bradford method. Aliquots from each sample, containing equal amounts of protein (100 μg), were run on an 8% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. The efficiency of the transfer was visualized by Ponceau staining. The blots were subsequently blocked at room temperature for 1 hour with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20, 5% (wt/vol) bovine serum albumin (BSA), and subsequently incubated with primary anti-cPLA2 (Santa Cruz Biotechnology) or anti-phospho-cPLA2 (Cell Signaling Technologies) antibodies overnight at 4 °C. Then, membranes were incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were revealed by chemiluminescence. Protein expression was determined by densitometric analysis using the Science Lab, Image Gauge. Images were obtained using Science Lab 2001 Image Gauge (Fuji Photo Film GmbH, Düsseldorf). After stripping, blots were assayed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative densitometric values of each protein were normalized to GAPDH and displayed in histograms. To estimate cPLA2 activity, the ratio of normalized p-cPLA2 to total cPLA2 was calculated [18].

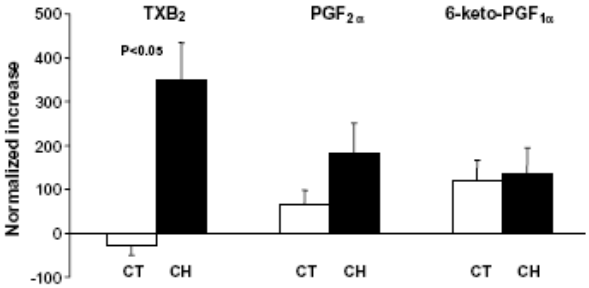

Fig. 2. Vasoconstrictors thromboxane A2 (as its stable form TXB_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and vasodilator prostacyclin (6-keto-$PGF_{1\alpha}$) production by sinusoidal endothelial cells (SEC) isolated from control (CT) and cirrhotic (CH) livers after incubation with arachidonic acid (AA, 40 μM). AA promoted a significant increase in TXB_2 release by cirrhotic-SEC, that was not observed in control-SEC. $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ production was similar. Values represent means ± SEM of prostanoid production increase normalized to vehicle.

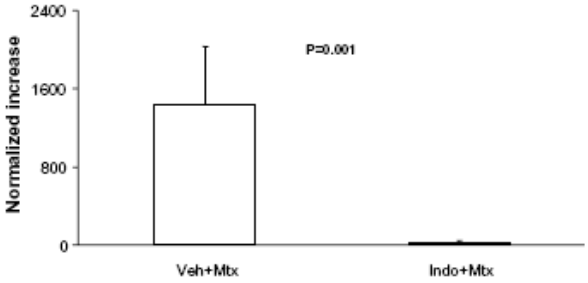

Fig. 3. TXB_2 production by sinusoidal endothelial cells (SEC) isolated from cirrhotic livers pre-incubated with the non-specific COX inhibitor indomethacin (Indo, 10 μM) or its vehicle (Veh) and stimulated with the α1-adrenergic agonist methoxamine (Mtx, 10^{-4} M). Mtx induced a significant increase in the production of TXB_2 which was significantly abolished when COX was inhibited. Values represent means ± SEM of prostanoid production increase normalised to vehicle.

2.7. Drugs and reagents

AA, prostanoid enzyme immunoassay kits, SC-560, Furegrelate and SQ 29,548 were from Cayman Chemical (Ann Arbor, MI). Mouse anti-RECA monoclonal antibody was from Serotec (Oxford, UK). Collagenase A was from Roche Diagnostics (Mannheim, Germany).

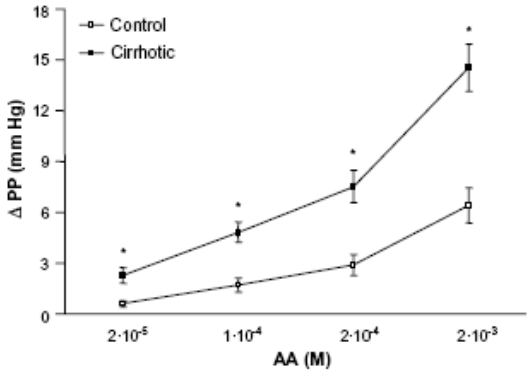

Fig. 4. Portal perfusion pressure (PP) dose–response curve to cumulative doses of arachidonic acid (AA) in control (CT) and cirrhotic (CH) rat livers. CH livers exhibited a hyperresponse to AA ($^*p < 0.05$ vs. CT).

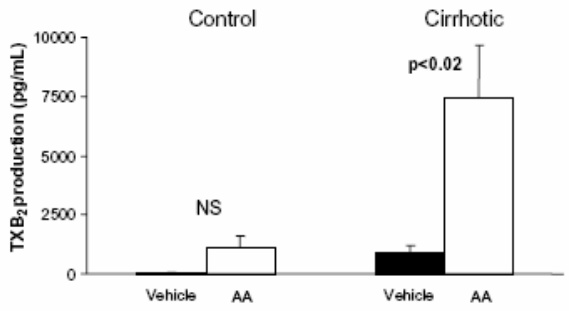

Fig. 5. TXB_2 production in perfused control (CT) and cirrhotic (CH) livers. Arachidonic acid markedly increased TXB_2 production in CH livers after arachidonic acid (AA).

Percoll was from Amersham Biosciences (Uppsala, Sweden). RPMI medium and culture complements were from Biological Industries (Israel). Nylon filters (100 μm) were from Becton–Dickinson Labware (Franklin Lakes, NJ). Methoxamine, indomethacin, DPBS and all other chemical reagents were obtained from Sigma (St. Louis, MO).

2.8. Statistical analysis

Statistical analysis was performed using the SPSS 10.0 for windows statistical program (SPSS Inc. Chicago, IL). The unpaired Student's *t*-test and ANOVA were used as adequate. All data are reported as means ± SD. Differences were considered significant at a p value <0.05.

3. Results

3.1. Prostanoid production by sinusoidal endothelial cells (SEC)

AA incubation promoted a significant increase in TXB_2 release by cirrhotic-SEC that was not observed in control-SEC. However, the production of $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ was similar in SEC isolated from control or cirrhotic livers (Fig. 2).

Preincubation of cirrhotic SEC with the selective COX-1 inhibitor SC-560 completely abolished the formation of TXB_2 stimulated by AA (99 ± 0.7% of abolition vs. SEC-AA).

3.2. Thromboxane production by cirrhotic SEC after methoxamine stimulation

Methoxamine preincubation induced a large and significant increase in the production of TXB_2 by cirrhotic-SEC. Preincubation of cirrhotic-SEC with indomethacin completely blunted TXB_2 release (Fig. 3).

3.3. Cirrhotic livers exhibit an enhanced vasoconstrictive response to AA

AA produced a significant, dose-dependent increase in PP in control and cirrhotic livers. However, the response at each tested dose of AA was significantly greater in cirrhotic than in control livers (Fig. 4). TXB_2 levels, measured before and after AA administration, increased in the perfusate of control and cirrhotic livers stimulated with AA, however the increase was statistically significant (and much greater) only in the cirrhotic group (Fig. 5).

3.4. Effects of COX-1 inhibition with SC-560, TXA_2 synthase inhibition with Furegrelate or PGH2/TXA_2 receptor blockade with SQ 29,548 on the portal perfusion pressure response to AA

Preincubation with SC-560 attenuated the PP response to arachidonic acid both in control and in cirrhotic livers. However, while in control livers this was significant only with the higher dose of AA, in the cirrhotic livers this was much more pronounced and significant at all tested doses (Fig. 6). Actually, this attenuation in cirrhotic livers was so marked that the hyperresponse to AA completely disappeared (Fig. 6).

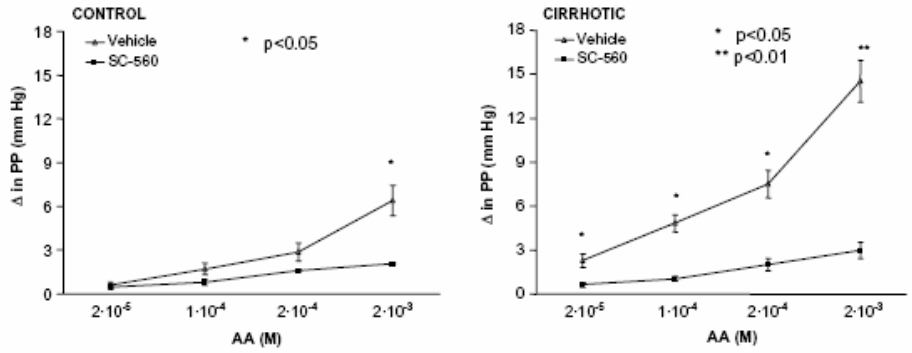

Fig. 6. Effect of SC-560 (5 μM) preincubation or its vehicle on the Portal perfusion pressure (PP) dose–response curve to arachidonic acid (AA) in control (CT) and cirrhotic (CH) rat livers.

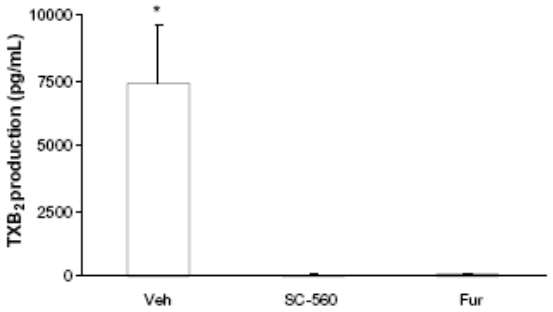

Fig. 7. TXB_2 production in AA stimulated perfused cirrhotic livers pre-incubated with the COX-1 selective inhibitor (SC-560, 5 μM), the thromboxane synthase inhibitor Furegrelate (Fur, 20 μM) or vehicle (Veh). TXB_2 production was completely blunted when COX-1 or the thromboxane synthase was inhibited. (*$p < 0.05$ vs. livers receiving SC-560 or Furegrelate).

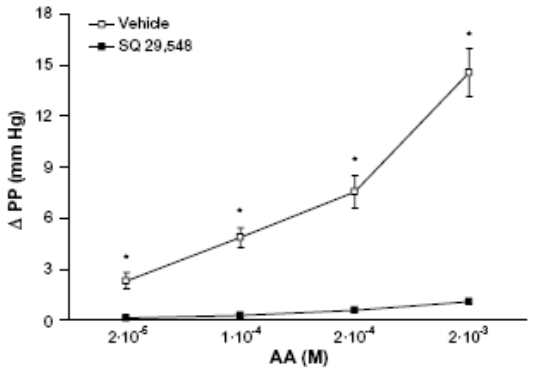

Fig. 9. Effect of SQ 29,548 (1 μM) preincubation or its vehicle on the portal perfusion pressure (PP) dose–response curve to arachidonic acid (AA) in cirrhotic rat livers. SQ 29,548 corrected the hyperresponse to AA (*$p < 0.05$ vs. vehicle).

SC-560 preincubation was associated with an almost total prevention in the increase of TXB_2 in the perfusate (Fig. 7).

Furegrelate preincubation completely blunted TXB_2 production (Fig. 7). However, this did not significantly modify the response to AA in cirrhotic livers and only a slight attenuation was observed in control livers (Fig. 8).

By contrast, blockade of the PGH_2/TXA_2 receptor with SQ 29,548 almost completely prevented the increase in PP observed after AA administration in cirrhotic livers (Fig. 9). As expected, SQ 29,548 did not prevent the increase in TXB_2 production (data not shown).

3.4.1. cPLA2 expression and activity

Total cPLA2 protein expression was similar in cirrhotic and in control rat livers. However, the phosphorylated and therefore active form of cPLA2 was significantly higher in cirrhotic than in control rat livers (Fig. 10).

4. Discussion

The description of a dynamic, and therefore reversible, component of the increased intrahepatic resistance of cirrhotic livers opened the rationale to use drugs that may reduce portal hypertension while improving liver perfusion through the reduction of the hepatic vascular resistance [1]. Subsequent research explored the possibility to reduce intrahepatic resistance by acting on its pathophysiological mechanisms: decreased availability of NO and enhanced production of hepatic vasoconstrictors [3,19–27]. Regarding the latter mechanism, our group has described a role for 5-lipooxygenase (5-LO) derived cysteinyl-leukotrienes [15] and for COX-1 derived vasoconstrictor prostanoids [5–7] modulating the increased vascular tone of the cirrhotic livers. Thus, in cirrhotic livers COX-1 inhibition normalized the endothelium-dependent vasodilatory response to acetylcholine as well as the response to the α-1 adrenergic agonist methoxamine which is abnormally enhanced in the cirrhotic liver [5,6]. COX-1 inhibition was further

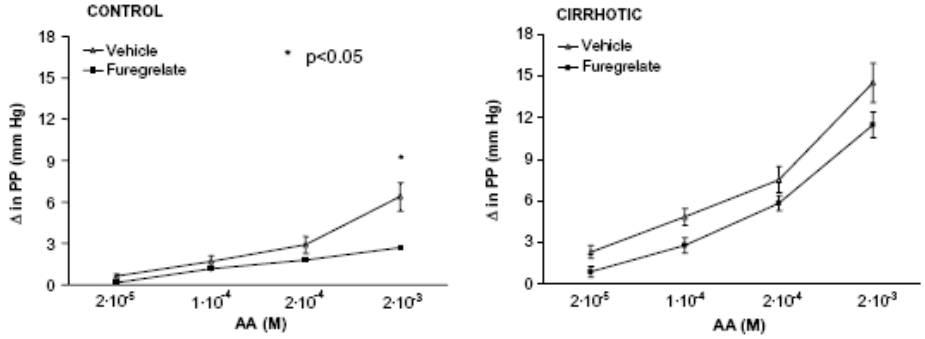

Fig. 8. Effect of Furegrelate (20 μM) preincubation or its vehicle on the portal perfusion pressure (PP) dose–response curve to arachidonic acid (AA) in control (CT) and cirrhotic (CH) rat livers. Thromboxane synthase inhibition attenuated the response to the higher dose of AA in CT, but did not significantly modify it in CH livers.

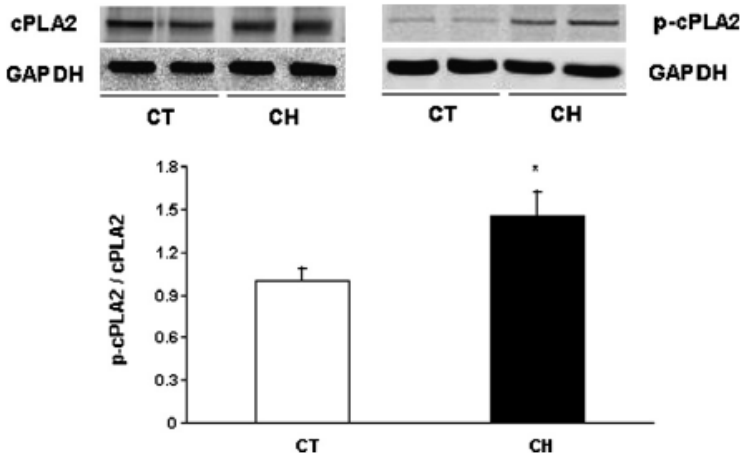

Fig. 10. Top, representative western blot of cPLA2 and phospho-cPLA2 in livers from control (CT) and cirrhotic (CH) rats. Bottom, densitometry analysis of Western blot for cPLA2 and p-cPLA2 from CT ($n = 8$) and CH ($n = 8$) rat livers (normalized to GAPDH; values represent means ± SEM; $^{*}p < 0.05$ vs. CT).

found to prevent the marked increase in TXA_2 observed in cirrhotic livers [5,6]. In addition, sinusoidal endothelial cells (SEC) from cirrhotic livers were shown to overexpress COX-1 [7]. Our previous findings strongly suggested that COX-1 derived prostanoids produced by SEC were playing a prominent role in the exaggerated vasoconstrictive response to adrenergic stimulation as well as in the impaired endothelial-dependent vasorelaxation.

The present study confirms and extends these findings by demonstrating that SEC from cirrhotic rat livers are primed to synthesize increased amounts of vasoconstrictor prostanoids such as TXA_2, when they are incubated with the prostanoid precursor AA. This increased production of vasoconstrictors was completely abolished after pre-incubation of cirrhotic SEC with SC-560, a selective COX-1 inhibitor. The capacity of cirrhotic SEC to produce TXA_2 was also triggered by methoxamine incubation and again this response was blunted after COX inhibition. These findings strongly support that SEC are indeed a major contributor to the increased production of vasoconstrictors observed in cirrhotic livers, although it is possible that Kupffer cells may also be implicated in the enhanced production of vasoconstrictive prostanoids [17,28]. The observed increase in the phosphorylation and therefore active form of PLA2 further emphasizes the role of arachidonic acid derived metabolites within the cirrhotic liver. Thus, increased PLA2 activity may be an additional mechanism contributing to the exaggerated vasoconstrictor prostanoid formation in cirrhotic livers by increasing AA bioavailability.

Our findings further suggest that TXA_2 may be a relevant factor. However, the observation that preincubation with the TXA_2 synthase inhibitor Furegrelate, albeit abolishing the formation of TXA_2, did not correct the hyperresponse to AA of cirrhotic livers suggests that a constrictor prostanoid other than TXA2, presumably the TXA_2 precursor and also potent vasoconstrictor prostaglandin endoperoxide H_2 (PGH_2) [29], could be involved in the increased vascular tone of cirrhotic livers. In fact, PGH_2 shares with TXA_2 its cell-membrane receptor [30] and this may explain why the blockade of the PGH_2/TXA_2 common cell-membrane receptor by means of SQ-29,548 completely blunted the hyperresponse to AA of cirrhotic livers. It might also be possible that although TXA_2 could be the major activator of the PGH_2/TXA_2 receptor in normal conditions, when TXA_2 production is blocked by TXA_2 synthase inhibitors its precursor PGH_2 may accumulate with ensuing TXA_2 receptor activation. In either case, blocking the common receptor would be an effective way to block the vasoconstrictor prostanoid pathway within the liver. Our findings may be relevant when considering blockade of enhanced hepatic vasoconstrictor prostanoids as a potential target for therapy since blockade of the common PGH_2/TXA_2 receptor should not be associated with deterioration of renal function [31–33]. This is not the case with strategies based on COX-1 blockade, that is effective in blocking the production and effects of vasoconstrictive prostanoids in the cirrhotic liver, but with a potential for causing renal failure [34].

In conclusion, this study provides further evidences supporting that SEC are playing a major role in the increased hepatic vascular tone of cirrhosis. This effect of SEC would not be only due to the well-known reduction in NO bioavailability, but also by a marked increase in vasoconstrictor prostanoid production. It is therefore possible that, strategies directed not only to increase intrahepatic NO bioavailability, but also to

block the increased vasoconstrictor prostanoid pathway might be an attractive therapeutic approach for decreasing intrahepatic vascular resistance and reducing portal hypertension in cirrhosis.

Acknowledgments

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Evidence Against A Role For Nadph Oxidase Modulating Hepatic Vascular Tone In Cirrhosis

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EVIDENCE AGAINST A ROLE FOR NADPH OXIDASE MODULATING HEPATIC VASCULAR TONE IN CIRRHOSIS

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Short title: NADPH oxidase in cirrhosis.

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Abbreviations: endothelial nitric oxide synthase (eNOS), nitric oxide (NO), cycloxygenase (COX), nicotinamide adenine dinucleotide phosphate (NADPH), superior mesenteric artery blood flow (SMABF), superior mesenteric artery resistance (SMAR), acetylcholine (Ach), non-phagocytic NADPH oxidase (NOX).

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Abstract

Background/Aims: Increased hepatic vascular resistance in cirrhosis is in part due to reduced nitric oxide (NO) bioavailability. This is related to insufficient NO synthesis from eNOS and to enhanced NO scavenging by superoxide radicals (O_2^-). NADPH-oxidase, an important source of O_2^- that increases vascular tone in different cardiovascular disorders. Thus, our aims were to study the molecular and biochemical state of NADPH-oxidase in cirrhotic livers, and to investigate its possible role modulating hepatic vascular tone in cirrhosis. *Methods*: NADPH-oxidase expression and enzymatic activity were determined in control (n=8) and CCl_4-cirrhotic (n=8) rat livers. Additional control (n=6) and CCl_4-cirrhotic (n=10) rats were treated with apocynin (a selective NADPH-oxidase inhibitor) or its vehicle. Mean arterial pressure, portal pressure (PP) and superior mesenteric arterial blood flow (SMABF) were measured "in vivo". Moreover hepatic endothelial function was evaluated in isolated and perfused rat livers by dose-response curves to acetylcholine. In addition, in 6 control and 6 cirrhotic human livers NADPH-oxidase activity and expression were evaluated.

Results: Rat cirrhotic livers had no increased NADPH-oxidase protein expression or activity in relation to control livers. NADPH-oxidase inhibition did not modify splanchnic or systemic hemodynamics in control or cirrhotic rats and did not improve the impaired endothelial-dependent vasodilatory response to acetylcholine of cirrhotic livers. Human cirrhotic livers did not also exhibit increased NADPH-oxidase expression or activity.

Conclusions: Our study shows that NADPH-oxidase activity is decreased in the cirrhotic livers, and therefore can not explain increased hepatic O_2^-, endothelial dysfunction and increased vascular tone in cirrhotic livers.

Introduction

In cirrhotic livers, increased resistance to portal blood flow is the primary factor in the pathophysiology of portal hypertension (1). This increased resistance is in part due to changes in the hepatic vascular architecture but also to an increase in hepatic vascular tone. Endothelial dysfunction, characterized by an impaired endothelium-dependent response to vasodilators, is considered one of the main mechanism involved in the increased hepatic vascular tone of cirrhotic livers (2) and has been related to both a reduction in NO bioavailability (3) and to increased synthesis of COX-1-derived vasoconstrictor prostanoids (4; 5).

The reduced NO bioavailability of cirrhotic livers has been attributed to a decrease in endothelial-NO-synthase activity (2; 6). However, in several vascular disorders such as hypercholesterolemia, hypertension, diabetes, atherosclerosis, and heart failure reduced NO bioavailability has also been related to increased scavenging by NADPH oxidase dependent superoxide production (7-9). Recent data from our group has demonstrated that cirrhotic rat livers present high levels of superoxide radical (10) and that antioxidants improve flow-mediated vasorelaxation of the hepatic vasculature in patients with cirrhosis (11). Moreover, an important role for NADPH oxidase has been hypothesized in different liver pathologies, including early alcohol-induced hepatitis, hepatic fibrosis, non-alcoholic fatty liver disease, and ischemia-reperfusion injury (12-15).

The present study was aimed at characterizing the molecular and biochemical state of NADPH oxidase in cirrhotic livers and to investigate its possible role modulating hepatic vascular tone.

Materials and methods

Induction of cirrhosis by CCl_4

Male Wistar rats weighing 175-200g underwent inhalation exposure to CCl_4 and received phenobarbital in the drinking water as previously described (16). Once the cirrhotic rats developed ascites, administration of CCl_4 and phenobarbital was stopped and experimental protocols were started 1 week later. Control animals received only phenobarbital. The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in the Hospital Clínic and IDIBAPS.

Experimental protocols

Animals were treated during 7 days with the selective NADPH oxidase inhibitor Apocynin (1.5mM in the drinking water, Sigma, Tres Cantos, Madrid, Spain; control rats: n= 6; cirrhotic rats: n= 10) or its vehicle (control rats: n= 6; cirrhotic rats: n= 10).

Previous studies have shown that this dose of apocynin and the route of administration is effective at inhibiting NADPH oxidase activity in rats (17-19).

In vivo hemodynamic studies

Under anaesthesia with i.p. ketamine hydrochloride (Ketalar ®, 100mg/kg b.w.; Parke-Davis S.L. El Prat de Llobregat, Barcelona, Spain) and xylazine hydrochloride (Sigma, 5 mg/kg body weight), a tracheotomy was performed and a polyethylene PE-240 tubing was inserted into the trachea to ensure a patent

airway. PE-50 catheters were introduced into the femoral artery, for arterial pressure recording (mmHg), and into the portal vein through an ileocolic vein, to measure portal pressure (mmHg). Then, the superior mesenteric artery was carefully dissected free from connective tissue, and a non-constrictive perivascular transit-time ultrasonic flow probe (1PR, 1-mm diameter; Transonic Systems, Ithaca, New York, USA) was placed around this vessel close to its aortic origin. The flow probe was connected to a flow meter, to measure the superior mesenteric artery blood flow ($mL \cdot min^{-1} \cdot 100g^{-1}$) (20). Resistance in the superior mesenteric artery ($mmHg \cdot mL^{-1} \cdot min^{-1} \cdot 100\ g^{-1}$) was calculated as: (mean arterial pressure – portal pressure)/superior mesenteric artery blood flow. Blood pressures and flows were registered on a multichannel computer-based recorder (PowerLab; ADInstruments, Colorado Springs, Colorado, USA). The external zero reference point was placed at the midportion of the animal. Hemodynamic data was collected after a 30-min stabilization period.

Evaluation of endothelial function in perfused rat livers. Effect of NADPH oxidase inhibition

In a sub-group of animals livers were isolated and perfused by a flow-controlled perfusion system as described previously (21). Briefly, livers were perfused with Krebs' buffer in a recirculation fashion with a total volume of 100mL at a constant flow rate of 35 mL/min. An ultrasonic flow probe (model T201; Transonic Systems, Ithaca, NY) and a pressure transducer were placed on line, immediately ahead of the portal inlet cannula, to continuously monitor portal flow and perfusion pressure. Another pressure transducer was placed immediately after the thoracic vena cava outlet for measurement of outflow

pressure. The flow probe and the two pressure transducers were connected to a PowerLab (4SP) linked to a computer using the Chart version 5.0.1 for Windows software (ADInstruments, Mountain View, LA). The average portal flow, inflow and outflow pressures were continuously sampled, recorded and afterwards analyzed. The perfused rat liver preparation was allowed to stabilize for 30 min before the studied substances were added. The gross appearance of the liver, stable perfusion pressure and a stable buffer pH (7.4 ± 0.1) were measured during this period. If any viability criteria were not satisfied, the experiment was discarded.

The intrahepatic microcirculation was preconstricted by adding to the reservoir, during a 3 min period, the α_1-adrenergic agonist methoxamine to achieve the final concentration of 10^{-4} M. Five minutes later, concentration-effect curves to cumulative doses of acetylcholine (Ach; 10^{-8}, 10^{-7}, 10^{-6} mol/L) were evaluated in control (n= 8) and cirrhotic (n= 12) livers treated with vehicle (n= 10) or apocynin (n= 10). The concentration of acetylcholine was increased by one log unit every 1.5 min. Response to cumulative doses of acetylcholine was calculated as percent change in perfusion pressure as previously described (2; 21).

Quantification of NADPH oxidase-dependent superoxide production

NADPH oxidase-dependent superoxide anion production, was assessed in control (n=9) and cirrhotic (n=9) rat livers by lucigenin-enhanced chemiluminescence (22; 23). Briefly, tissues were excised, immediately snap-frozen in liquid nitrogen, and stored at –80°C for analysis. Tissues were minced thoroughly with mortar under liquid nitrogen. A 10% homogenate was prepared

by homogenizing the obtained powder in 1 mL Krebs-HEPES buffer, containing 0.01 M EDTA and 0.01 M EGTA (pH 7.4) (Sigma, Tres Cantos, Madrid, Spain), by use of a glass-to-glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 minutes, to remove unbroken cells and debris. Protein quantification was performed by the Lowry method, and the final concentration adjusted to 10 µg/mL. For the chemiluminescence assay, 100 µL aliquots were added to 400 µL of a Krebs-HEPES assay solution containing lucigenin (5 µM; Sigma) as the electron acceptor. After equilibration and background counts, NADPH (0.1 mM; Sigma) was added as the substrate, and the luminescence counts (relative light units) were monitored continuously over a 3-min period in a luminometer (Lumat LB 9507, Berthold Technologies, GmbH & Co. KG, Bad Wildbad, Germany), at 37°C. Then, superoxide dismutase (400 U/mL; Sigma) was added and counts were measured again during 3 minutes.

In additional experiments, apocynin (100µM) was added in the assay mixture before NADPH addition, luminescence counts were measured as described above.

RNA isolation and reverse transcription

Total RNA was isolated from frozen control (n=8) or cirrhotic (n=8) livers using the Trizol method (Invitrogen, El Prat de Llobregat, Barcelona, Spain). RNA was treated with DNAse (Ambion, Austin TX) to eliminate contaminating DNA. For cDNA synthesis, 1 µg of total RNA was retrotranscribed using MLV reverse transcriptase and random hexamers, as described by the manufacturer (Invitrogen).

Real time Quantitative PCR (RT-PCR) of NADPH oxidase subunits

cDNA templates were amplified by RT-PCR using the fluorescent TaqMan technology (Applied Biosystems, Foster City, CA) on an ABI Prism 7900 sequence Detection System (Applied Biosystems). The probes and primers for the quantification of rat NOX2, rat p22phox and rat p67phox were designed using Primer Express software (Applied Biosystems), while the quantification of rat NOX1, rat NOX4, rat p47phox and the endogenous control 18S RNA was performed using pre-designed Gene-Expression-Assays obtained from Applied Biosystems according to the manufacturer's protocol.

Each PCR reaction was carried out with 2 µL of the hepatic cDNA sample, 1X TaqMan Universal PCR Master Mix (Applied Biosystems), and primers and probe in a final volume of 20 µL, as recommended by the manufacturer. After an initial denaturation step at 95º C for 10 minutes, 40 cycles were performed as follows: 95º C for 15 s and 60º C for 1 min.

All experiments were performed in duplicate and several negative controls were included. NADPH oxidase sub-units expression was related to a standard curve derived from serial dilutions (10^{-1}-10^{-4}) of a random sample cDNA. Standard curves were constructed by plotting the log of standard dilutions *vs* the threshold cycle (C_T) values, C_T being the fractional cycle number at which the fluorescence passes a fixed threshold. The mRNA concentration of each NADPH oxidase subunit in hepatic samples was calculated referring the sample C_T to the standard curve, and normalized with the corresponding value of endogenous control C_T as recommended in the TaqMan user's manual. Values were expressed as relative units.

Western Blot analysis of NADPH oxidase subunits

Protein expression for NOX2, NOX4 and p22phox in rat livers from six cirrhotic and six control rat livers was assessed by Western blot (the 3 NADPH oxidase subunits for which there are commercially available antibodies for tissue blotting) as follows. Livers were collected, snap frozen in liquid N_2 and stored at –80ºC until analysed. Livers were minced thoroughly with mortar and pestle under liquid nitrogen. For each sample, a similar amount of obtained powder were collected in 200 µL triton-lysis buffer containing Tris/HCl (pH 7.4, 20mM), NaCl (150 mM), NaF (20 mM), $Na_4P_2O_7$ (10 mM), okadaic acid (10 nM), Na_3VO_4 (2 mM), antipain (2 µg/mL), aprotinin (2 µg/mL), chymostatin (2 µg/mL), leupeptin (2 µg/mL), pepstatin (2 µg/mL), trypsin inhibitor (2 µg/mL), phenylmethylsulfonylfluoride (40 µg/Ll), and Triton-X100 (1%v/v), left on ice for 10 min, and then centrifuged at 10000*g*/10 min. Protein concentration was assessed by the Bradford method. Aliquots from each sample, containing equal amounts of protein (100µg), were run on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. The efficiency of the transfer was visualized by Ponceau staining. The blots were subsequently blocked at room temperature for 1 hour with phosphate-buffered saline (PBS) containing 0.1% (v/v) Tween 20, 5% (wt/vol) bovine serum albumine (BSA), and subsequently incubated with primary antibodies overnight at 4ºC. Then, membranes were incubated with the appropriate HRP-conjugated secondary antibodies for 1 hour at room temperature. Blots were revealed by chemiluminiscence. Protein expression was determined by densitometric analysis using the Science Lab, Image Gauge. Images were obtained using Science Lab 2001 Image Gauge (Fuji Photo Film GMBH, Düsseldorf). After stripping, blots were assayed for

glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Quantitative densitometric values of each NADPH oxidase subunit were normalized to GAPDH and displayed in histograms.

NADPH oxidase activity and protein expression in control and cirrhotic human livers

NADPH oxidase activity and NOX2 and NOX4 protein expression were determined, as described above, in liver tissue specimens from six patients with cirrhosis (3 men and 3 women, aged from 44 to 63 years) who received orthotopic liver transplantation for end-stage liver disease between the months of May 2004 - October 2004 (3 alcoholic and 3 postviral cirrhosis). As controls, biopsy specimens were obtained from non-tumoral, normal liver tissue from hepatectomy specimens from 6 patients (3 men and 3 woman, aged from 29 to 78 years) who underwent partial liver resection surgery for different reasons (1 patient with focal nodular hyperplasia, metastatic adenocarcinoma of intestinal origin in 3 cases and resection of hepatocarcinoma over normal liver in 2 patients). The study was approved by the Ethical Committee of the Hospital Clinic i Provincial de Barcelona.

Drugs and reagents

Methoxamine and other chemical reagents were purchased from Sigma (Tres Cantos, Madrid, Spain).

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 for windows statistical package (SPSS Inc. Chicago, IL). The unpaired Student's *t*-test and ANOVA were used as adequate. All data are reported as means ± SD. Differences were considered significant at a *P* value < 0.05.

Results

NADPH oxidase mRNA expression

Control and cirrhotic rat livers expressed NOX2, NOX4, p22phox, p47phox and p67phox mRNA, but not NOX1 mRNA.

The levels of NOX2, p22phox, p47phox and p67phox mRNA were significantly increased but NOX4 mRNA levels were significantly reduced in cirrhotic in comparison to controls livers (Fig 1).

By contrast, expression of the mRNA NOX1 subunit was almost undetectable, both in control and in cirrhotic livers. Indeed, the expression was about two hundred times lower than those observed in mRNA from colon tissue used as positive control (data not shown).

NADPH oxidase protein expression

Protein expression of both NOX2 and NOX4 was significantly decreased in cirrhotic in comparison to control rat livers (Fig 2). We were unable to detect expression of p22phox protein.

NADPH oxidase activity

NADPH oxidase dependent superoxide generation, measured by lucigenin-enhanced chemiluminiscence, was not increased, but significantly reduced, in cirrhotic in comparison to control rat livers (Fig 3).

Hemodynamic effects of NADPH oxidase inhibition

Cirrhotic rats had significantly higher portal pressure and superior mesenteric artery blood flow and significantly lower superior mesenteric artery resistance than control rats, without significant differences in mean arterial pressure (Table 1). Treatment with apocynin did not significantly modify any splanchnic or systemic hemodynamic parameters either in control or in cirrhotic rats (Table 1).

Effects of NADPH oxidase inhibition on intrahepatic endothelial function

Cirrhotic livers had a significantly greater baseline portal perfusion pressure (PP; 9.4 ± 3.1 vs. 6.6 ± 2.0 mmHg; p=0.03) and intrahepatic vascular resistance (2.8 ± 0.8 vs. 2.0 ± 0.6 mmHg. g. min/ml; p=0.01) than control livers. As expected, control livers showed a dose-dependent vasorelaxation to cumulative doses of Ach. However, cirrhotic livers exhibited endothelial dysfunction as shown by the impaired vasodilatory response to Ach: 10^{-8} (-6.8 ± 3 vs. –15.4 ± 8% in controls; p<0.05), 10^{-7} (-15.3 ± 8 vs. –29.6 ± 9%; p=0.05), 10^{-6}mol/L (-19.7 ± 3 vs. –49.1 ± 12%; p=0.004) (Fig. 4).

Treatment with apocynin did not significantly modify baseline portal perfusion pressure or the dose response to Ach either in control or in cirrhotic perfused rat livers (Fig. 4).

Human NADPH oxidase activity and protein expression

NOX4 protein expression was significantly reduced in cirrhotic in comparison to control human livers, while no significant differences were observed in NOX2 protein expression and NADPH oxidase enzymatic activity (Fig 5).

Discussion

Increased hepatic vascular resistance in cirrhosis has been related to reduced NO bioavailability within the cirrhotic liver (2; 6; 24), mainly attributed to reduced eNOS activity (2; 6; 24; 25). It has been recently proposed that, in analogy with other vascular disorders (7-9), the reduced NO bioavailability in the cirrhotic liver would be further aggravated by NO scavenging due to its binding to superoxide, which is increased in several liver disorders (10; 12-15). It could be therefore possible to increase NO bioavailability and ameliorate hepatic vascular tone by reducing hepatic superoxide. Supporting this concept, it has been shown that the acute administration of high doses of the potent antioxidant vitamin C is able to improve hepatic endothelial dysfunction in patients with cirrhosis (11).

NADPH oxidase is an important source of superoxide that has well characterized as a key enzyme determining endothelial dysfunction in cardiovascular diseases (7-9). In these conditions, activation of the enzymatic NADPH oxidase complex generates large amounts of superoxide radical that reacts with NO reducing its bioavailability (26). Accordingly, the aims of the present study were to study the molecular and biochemical state of NADPH oxidase in cirrhotic livers, and to investigate its possible role modulating hepatic vascular tone in cirrhosis.

NADPH oxidase activity was evaluated by the lucigenin-enhanced chemiluminiscence assay. Contrary to our hypothesis, NADPH oxidase activity was significantly lower in cirrhotic than in controls rat livers. This result is quite robust, as it was confirmed in 3 independent experiments (with a total of nine livers per each group). In accordance with this finding, we observed that effective inhibition of NADPH oxidase with apocynin did not cause any significant effect on hepatic or systemic hemodynamics in rats with established cirrhosis. Thus, it appears that in advanced liver disease the NADPH oxidase complex plays no role promoting or aggravating the abnormally elevated hepatic vascular tone and impaired vasorelaxation characteristic of cirrhosis. However, we can not discard that in early stages of the disease, where inflammation processes are more relevant, NADPH oxidase might be increased. In fact, it has been shown that hepatic stellate cells activation by various humoral stimuli, as it happens at initial stage of fibrogenesis, is NADPH oxidase mediated and that mice lacking NADPH oxidase are protected from liver fibrosis development (13; 27). Additionally, in non-alcoholic fatty liver disease, hepatic NADPH oxidase activity and NADPH oxidase expression have been shown to be increased and implicated in the fibrotic process (12; 27-29).

Similarly, our group described that development of portal hypertension in the partial portal vein ligation model is associated with increased NADPH oxidase activity in the mesenteric territory and that early NADPH oxidase inhibition prevents splanchnic angiogenesis in this model (30). However, the results of the present study clearly show that NADPH oxidase can not be considered a player in the intrahepatic hemodynamic abnormalities observed in well established cirrhosis.

To understand why cirrhotic livers exhibited low NADPH oxidase activity, we studied the hepatic gene and protein expression of this enzymatic complex. Protein expression of the NADPH oxidase subunits NOX2 and NOX4 was significantly down regulated in cirrhotic rat livers in comparison to controls, and we could not detect expression of p22phox. Reduced protein expression of NOX4 in cirrhotic livers is remarkable because its expression has been well described in vascular endothelium (26; 31-34). In fact, NOX4 down regulation has been shown to markedly reduce superoxide production in endothelial cells both in vivo and in vitro suggesting that NOX4 is a major catalytic component of endothelial NADPH oxidase (35).

The reduced protein expression of all the subunits evaluated is in accordance and explains our finding of reduced NADPH oxidase activity in cirrhotic tissue, as well as the lack of effect of NADPH oxidase inhibition. For some of the studies subunits (NOX4 and NOX1) reduced/absent protein expression was likely due to reduced/absent mRNA expression. However, and unexpectedly, p47phox, p67phox, p22phox and NOX2 mRNA expression was increased in cirrhotic livers in comparison to controls, despite low NOX2 and no p22phox protein expression, suggesting that in cirrhotic livers mRNA transcription of these subunits could be deregulated.

Measurement of NADPH oxidase activity or expression on the whole liver might not be representative of what happen in the hepatic vascular system. Unfortunately, and contrary to what happens with arteries or veins of different vascular territories, because of the architectural nature of the liver it is not feasible to isolate the intrahepatic vascular bed (where most of the hepatic resistance to portal blood flow is generated) to be able to selectively

characterize molecular and biochemically the NADPH oxidase system. However, the finding of NOX4 down expression (the main NADPH oxidase subunit expressed in endothelial cells and not in kupffer cells or macrophages) that is the best molecular approach to characterize the NADPH oxidase in the intrahepatic vascular system argues against a role for NADPH oxidase in this setting.

Nevertheless, the most convincing evidence that NADPH oxidase would not be playing a role modulating the intrahepatic vascular tone in cirrhosis are the physiological studies showing lack of effect NADPH oxidase inhibition with apocynin.

It is always risky to extrapolate data between experimental models and even more from experimental models to humans. Thus, to check if there is a rationale to think that our observations in CCl_4 cirrhotic rats are also feasible in human cirrhosis, NADPH oxidase enzymatic activity and protein expression were evaluated in liver samples from cirrhotic and control patients. NADPH oxidase enzymatic activity and NOX2 protein expression were not increased in human cirrhotic livers and even NOX4 protein expression was significantly reduced. Thus, supporting the lack of role for NADPH oxidase in established cirrhosis.

In conclusion, the results of the present study clearly shows that NADPH oxidase activity is decreased in the cirrhotic livers, and therefore can not explain increased hepatic superoxide radical, endothelial dysfunction and increased vascular tone in cirrhotic livers. Furthermore, this study opens the rationale to challenge and clarify the role of this enzyme in other liver diseases.

Tables

	CT-Veh (n=6)	CT-Apo (n=10)	CH-Veh (n=6)	CH-Apo (n=10)
Bd wt g (mean±SD)	455 ± 57	416 ± 48	422 ± 75	393 ± 51
Liver wt g (mean±SD)	12.7 ± 1.9	11.8 ± 2.1	11.1 ± 1.3	12.1 ± 1.8
MAP mmHg (mean±SD)	112 ± 18	110 ± 11	105 ± 18	107 ± 10
PP mmHg (mean±SD)	8.9 ± 1.2	8.0 ± 1.9	13.7 ± 2.5*	14.0 ± 3.2
SMABF/100g (mean±SD)	2.2 ± 0.4	2.8 ± 0.7	3.8 ± 1.2*	3.9 ± 1.3
SMAR (mean±SD)	47.6 ± 12.6	39.4 ± 14.8	26.9 ± 16.9*	22.2 ± 12.7

Table 1. Effects of NADPH oxidase inhibition by Apocynin treatment (1.5mM, during 7 days) on mean arterial pressure (MAP), portal pressure (PP), superior mesenteric artery blood flow (SMABF) and resistance (SMAR) in control (CT) and CCl_4-cirrhotic (CH) rats. Results are shown as mean ± SD. (*$p<0.05$ vs. CT-Veh).

Figure Legends

Figure 1: NADPH oxidase sub-units mRNA expression levels in cirrhotic (n=8) rat livers. Values for NADPH oxidase amplification from liver cDNA have been normalized to an endogenous reference gene (RNA 18S). Values (mean ± SEM) are normalized to CT livers expression. (*p<0.01 vs. CT).

Figure 2: Top: Representative western blot analysis of NOX2 and NOX4 in livers from control (CT) and cirrhotic (CH) rats. Detection of NOX2 revealed three bands with an apparent molecular mass around 90 and 70 kDa in liver homogenates. Bottom: Densitometry analysis of western blots for NOX2 and NOX4 from CT (n=6) and CH (n=6) rat livers (normalized to GAPDH; values represent mean ± SEM).

Figure 3. NADPH oxidase activity of control (n=9) and cirrhotic (n=9) livers. Relative light unit values of cirrhotic livers are normalized to control livers activity.

Figure 4. Effect of NADPH oxidase inhibition with apocynin in the portal perfusion pressure (PP) dose-response curve to acetylcholine, in control (A) and cirrhotic (B) rat livers.

Figure 5. A) *Top:* Representative western blot analysis of NOX2 and NOX4 in control (CT) and cirrhotic (CH) human livers. *Bottom:* Densitometry analysis of western blots for NOX2 and NOX4 from CT (n=6) and CH (n=6) humans livers (normalized to GAPDH; values represent mean ± SEM). **B)** NADPH oxidase activity of CT (n=6) and CH (n=6) human livers. Relative light unit values of CH livers are normalized to CT livers activity.

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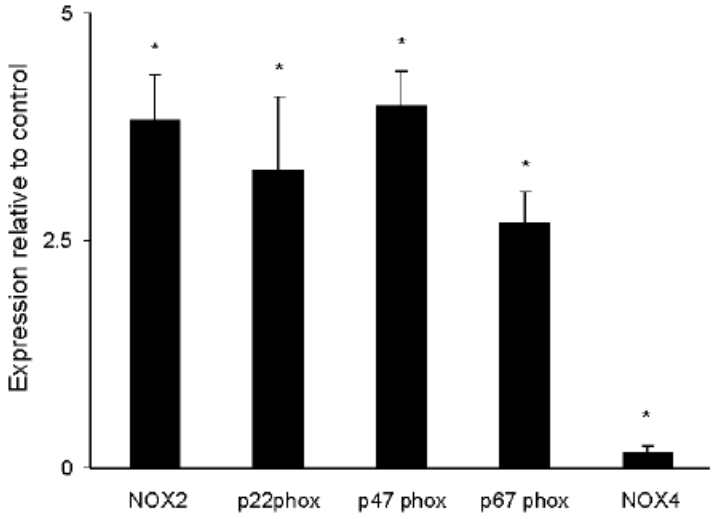
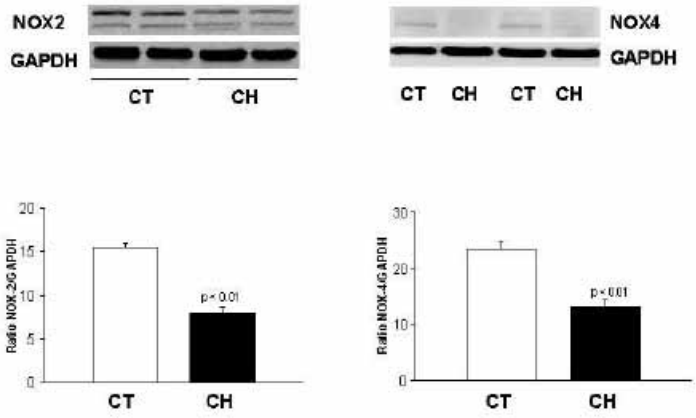


Figure 1



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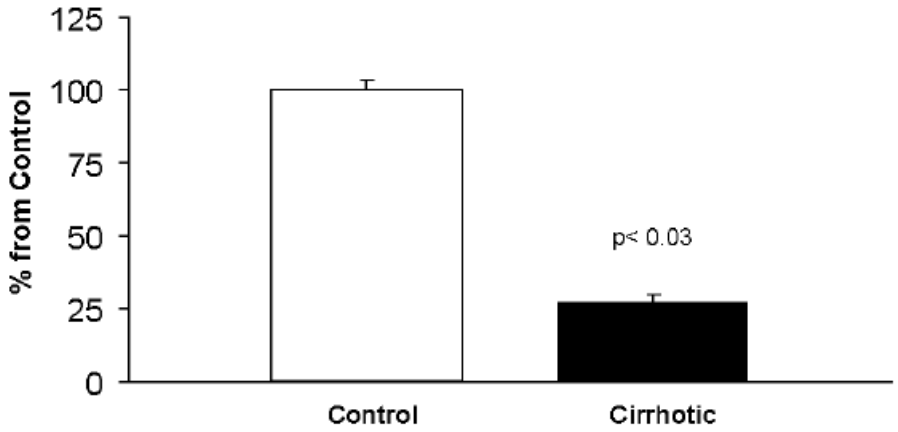

Figure 3

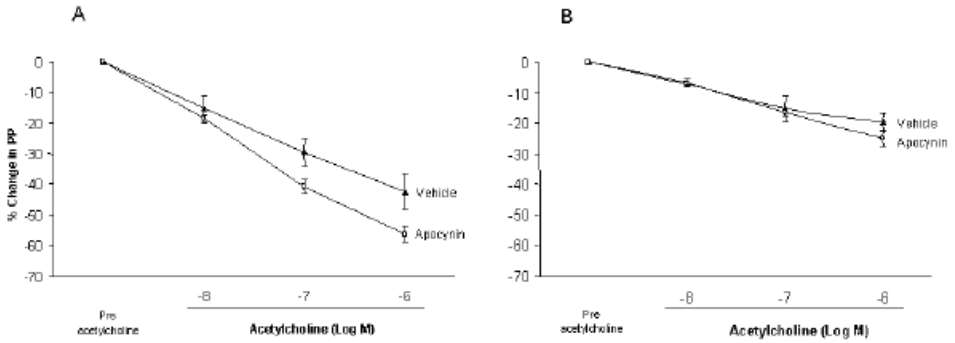

Figure 4

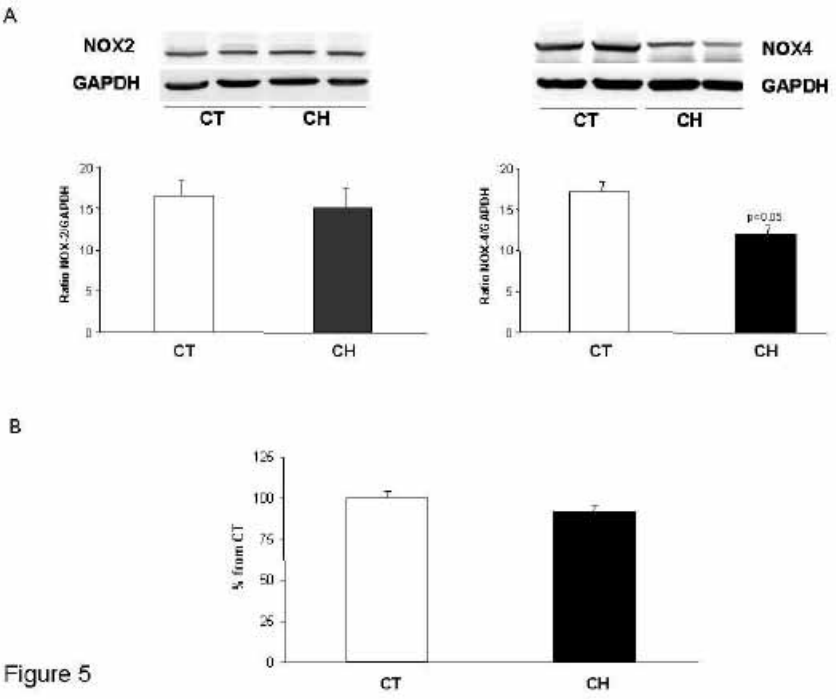

Figure 5

INCREASED OXIDATIVE STRESS IN CIRRHOTIC LIVERS. A POTENTIAL MECHANISM CONTRIBUTING TO REDUCE NITRIC OXIDE BIOAVAILABILITY

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Key words: reactive oxygen species, superoxide, endothelial dysfunction, portal hypertension, nitrotyrosine

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Abbreviations

NO, nitric oxide; eNOS, endothelial nitric oxide synthase; SEC, sinusoidal endothelial cells; O_2^-, superoxide; COX, cyclooxygenase; SOD, superoxide dismutase; XO, xanthine oxidase; DAF-FM-DA, 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate; DHE, dihydroethidium; NBT, nitro blue tetrazolium; DDC, diethyldithiocarbamate

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Abstract

In cirrhotic livers, decreased nitric oxide (NO) bioavailability is a major factor increasing intrahepatic vascular tone. Moreover, in several vascular disorders, an increase in superoxide radical (O_2^-) has been shown to contribute to reduce NO bioavailability through its reaction with NO to form peroxynitrite. This study examined the hypothesis that, in cirrhotic livers, increased O_2^- by reacting with NO, reduces NO bioavailability. In control and in cirrhotic rat livers, NO bioavailability was evaluated by measuring cGMP in liver tissue and by DAF fluorescence in isolated sinusoidal endothelial cells (SEC); O_2^- content was determined by DHE-staining in fresh liver sections and SEC. Hepatic superoxide dismutase (SOD) enzymatic activity and protein expression was also determined. In addition, protein-nitrotyrosination, a marker of NO-O_2^- reaction, was evaluated in liver homogenates. Cirrhotic livers exhibited increased O_2^- levels. This was due, at least in part, to increased production and to reduced scavenging by SOD. Increased O_2^- was associated with a significant reduction in NO bioavailability and increased nitrotirosinated proteins. In SEC, enhanced O_2^- levels was followed by decreased NO bioavailability. In conclusion, oxidative stress may contribute to reduce NO bioavailability in cirrhotic livers.

Introduction

Portal hypertension is a leading cause of morbidity and mortality in patients with cirrhosis of the liver (1;2). Endothelial dysfunction, characterized by an impairment in the endothelium-dependent response to vasodilators, is considered to be one of the mechanisms leading to the increased vascular tone of cirrhotic livers that contributes to the development of portal hypertension. Endothelial dysfunction is partly due to increased release of cyclooxygenase-1 (COX-1)-derived vasoconstrictive prostanoids (3;4) and to reduced nitric oxide (NO) bioavailability within the liver (5-7), which has been attributed to decreased endothelial nitric oxide synthase (eNOS) activity (8) secondary to several disturbances in the posttranslational regulation of the enzyme (9-12).

In several vascular disorders it has been demonstrated than an increase in the reactive oxygen specie superoxide (O_2^-), by rapidly reacting with NO (13), promotes a marked reduction in NO bioavailability followed by an increase in vascular tone (14-17).

An imbalance between O_2^- production and degradation systems could lead to an increase in oxidative stress. In fact, an increase in O_2^- levels due to increased production by xanthine oxidase (XO), NADPH oxidase or COX, among other enzymatic systems, and a reduced O_2^- scavenging by superoxide dismutase has been suggested to play a pathophysiological role in different liver disorders such as in alcoholic and non-alcoholic liver disease (18-22).

We hypothesize that in cirrhotic livers increased O_2^-, by reacting with NO, would contribute to reduce NO bioavailability. As a consequence, removing O_2^- from the cirrhotic livers could be a new therapeutic strategy to improve intrahepatic

NO bioavailability. The present study was designed to be a proof of concept of this hypothesis.

Material and Methods

Induction of cirrhosis by carbon tetrachloride (CCl_4)

Male Wistar rats weighing 175 to 200 g underwent inhalation exposure to CCl_4. Phenobarbital (0.3 g/L) was added to the drinking water as previously described (23). A high yield of micronodular cirrhosis was obtained after approximately 12 to 15 weeks of CCl_4 inhalation. When the cirrhotic rats developed ascites, administration of phenobarbital was stopped and the subsequent experiments were performed 1 week later. Control animals received only phenobarbital. The animals were kept in environmentally controlled animal facilities at the IDIBAPS. All experiments were performed according to the criteria of the Committee for the Care and Use of Laboratory Animals in the Hospital Clínic and IDIBAPS.

Liver sinusoidal endothelial cell isolation and culture

Sinusoidal endothelial cells (SEC) were isolated from control and cirrhotic rat livers as described previously (24;25). Briefly, after collagenase perfusion of the livers, and isopycnic sedimentation of the resulting dispersed cells through a two-step density gradient of Percoll, pure monolayer cultures of SEC were established by selective attachment on a substrate of fibronectin. Afterwards, cells were cultured for 12 h (37ºC, 5% CO_2) in RPMI-1640 as previously described (25). Almost 93% of these cells were SEC, as assessed by specific immunocytochemical marking using rat endothelial cell antigen RECA-1 (26)

and had a viability of 95% (by trypan blue exclusion). All studies were performed on cells from the first passage, 12 h after SEC isolation, to preserve its typical phenotype (27).

Evaluation of NO bioavailability in liver tissue and SEC of cirrhotic and control rats

Measurement of NO levels in SEC

In situ NO levels in SEC were assessed using 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate (DAF-FM-DA) (Molecular Probes, Inc, Eugene, OR) as described (28;29). DAF-FM-DA is a cell-permeable NO-sensitive dye that is virtually nonfluorescent until it reacts with NO to form benzotrizole. Isolated SEC were washed in RPMI 1640 without phenol red and loaded with DAF-FM-DA (final concentration 10 μM, 20 min, 37ºC). Then SEC were rinsed three times with PBS, kept in the dark, and maintained at 37ºC with a warm stage on a laser scanning confocal microscope (model TCS-SL DMIRE2, Leica, Wetzlar, Germany) and images were obtained using a 488 nm (excitation) and 505-530 nm (emission) filter set for DAF-FM-DA, with a 40x1.3 oil objective. Quantitative analysis was obtained by averaging the peak relative fluorescent intensity (optical density arbitrary units) of each cell using Image J 1.33u software (NIH, USA) (30). In some experiments the NOS inhibitor L-NAME (1.5 mM) or the NO donor DETA-NONOate (500 μM) were added 20 min before loading DAF-FM DA.

cGMP

Measurements of cGMP, a marker of NO bioavailability, were performed in liver homogenates (31). Briefly, equal amounts of liver tissue (200 mg) were drop

into 10 volumes of 5% trichloroacetic acid and homogenized at 4ºC. The precipitate was removed by centrifugation at 2000 *g* for 15 min at 4ºC. The supernatant was transferred to a clean test tube, washed with water-saturated diethylether for three times and lyophilised. The dried extract was dissolved in assay buffer, and cGMP levels were determined by enzyme immunoassay (Cayman Chemical Co., Ann Arbor, MI). Results were expressed as pmol/ml.

Evaluation of superoxide

Measurement of superoxide content in liver tissue of cirrhotic and control rats

In situ O_2^- levels were evaluated with the oxidative fluorescent dye dihydroethidium (DHE; Molecular Probes, Inc, Eugene, OR) (32). DHE specifically reacts with intracellular O_2^- and is converted to the red fluorescent compound ethidium bromide (EtBr), which then binds irreversibly to double-stranded DNA and appears as punctuate nuclear staining (33). EtBr is excited at 488 nm with an emission spectrum of 610 nm. Liver cryosections (10 μm) were incubated with DHE (10 μmol/L) in PBS. In order to demonstrate the specificity of the assay for O_2^-, parallel incubations with SOD (200 U; Applichem, Darnstadt, Germany) were performed. Fluorescence images were obtained with laser scanning confocal microscope (TCS-SL DMIRE2, Leica, Wetzlar, Germany), and quantitative analysis was performed using Image J 1.33 u software (NIH, USA).

Superoxide generation

To determine which proteins are responsible for O_2^- generation, cirrhotic rats (n=2 per group) were pre-treated with the specific xanthine oxidase (XO)

inhibitor, allopurinol (50 mg/kg i.p., 18 h and 1 h before experiment), with the cyclooxygenase (COX) inhibitor, indomethacin (20 mg/kg p.o., 5 h before experiment), the NO synthase (NOS) inhibitor, L-NAME (15 mg/kg i.v., 15 min before experiment) or with vehicle (saline) and the hepatic O_2^- content was evaluated in fresh liver sections by DHE staining as described above. The efficacy of these doses inhibiting their respective enzymatic systems has been previously demonstrated (34-36).

Superoxide dismutase (SOD) activity assay

Total SOD activity was measured in liver homogenates by an indirect assay previously described (37). The "NBT method" is based on the competition reaction between SOD and the indicator molecule, nitro blue tetrazolium (NBT). In brief, NBT is reduced to formazan by O_2^-. The production of formazan is photometrically quantified at 560 nm. Dilutions of SOD were used to generate a standard curve. One unit of SOD activity is defined as the amount of protein required to give half-maximal inhibition of NBT reduction. SOD activity was expressed as U/mg total tissue.

Western blot analysis of SOD isoforms

Liver frozen samples from control and cirrhotic rats were crashed to powder and subsequently homogenized in triton-lysis buffer as previously described (38). Aliquots from each sample containing equal amounts of protein (20-100 μg) were run on a 10-12% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. After the transfer, the blots were subsequently blocked for 2 hours with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk, and probed with rabbit antibodies against CuZnSOD (Stressgen, Victoria, BC, Canada), MnSOD (Upstate Biotechnology,

Lake Placid, NY), and extracellular SOD (ECSOD; Stressgen) overnight at 4ºC followed by incubation with goat anti-rabbit HRP-conjugated secondary antibodies (1:10000, 1 h, room temperature; Stressgen). Blots were revealed by chemiluminiscence.

Protein expression was determined by densitometric analysis using the Science Lab, Image Gauge (Fuji Photo Film GMBH, Düsseldorf). After stripping, blots were assayed for GAPDH (Santa Cruz Biotec., Santa Cruz, CA) expression as standarization of sample loading. Quantitative densitometric values of all proteins were normalized to GAPDH.

NO-O_2^- interaction

Nitrotyrosine protein detection

Protein nitrotyrosination, a marker of peroxynitrite production, was determined in blots blocked for 2 hours at room temperature with Tris-buffered saline containing 0.05% (vol/vol) Tween 20 and 3% (wt/vol) BSA, and probed with a mouse anti-nitrotyrosine (1 μg/ml) antibody (Cayman Chemical Co., Ann Arbor, MI) overnight at 4ºC followed by incubation with rabbit anti-mouse HRP-conjugated secondary antibody (1:10000, 1 h, room temperature; Stressgen). Blots were revealed by chemiluminescence as described above.

NO modulation by superoxide

SEC isolated from control rat livers were incubated for 6 h with vehicle or with the cytoplasmatic SOD inhibitor diethyldithiocarbamate (DDC, 25 μM) (39) alone or in association with the O_2^- scavenging enzyme SOD (300 U). Then, O_2^- and NO levels were evaluated in different subsets of SEC by DHE (10 μmol/L) and DAF-FM-DA staining, respectively.

Drugs and reagents

Mouse anti-RECA monoclonal antibody was from Serotec (Oxford, UK). Collagenase was from Roche Diagnostics (Mannheim, Germany). Percoll was from Amersham Biosciences (Uppsala, Sweden). Reagents for cell culture were provided by Biological Industries Ltd. (Kibbutz Beit Haemek, Israel). L-NAME and other chemical reagents were purchased from Sigma (Tres Cantos, Madrid, Spain).

Statistical analysis

Statistical analysis was performed using SPSS 10.0 for Windows statistical package (SPSS Inc., Chicago, IL). All results are expressed as mean ± SEM. Comparisons between groups were performed with the Student's *t*-test or Mann-Whitney *t*-test for unpaired data when adequate. Differences were considered significant at a P value < 0.05.

Results

NO bioavailability is reduced in SEC from cirrhotic livers.

SEC isolated from cirrhotic rat livers had decreased NO bioavailability in comparison to SEC isolated from control livers as shown by the significant and marked reduction in DAF fluorescence (46.3±8% vs 100±17% in SEC from control rat livers; $p<0.01$) (Fig 1).

No DAF fluorescence was observed in SEC pre-treated with the NOS inhibitor L-NAME (1.5 mM, data not shown) and a strong cytoplasmic fluorescence was observed in SEC isolated from both control and cirrhotic rats pre-treated with the NO donor DETANONOate (500 μM, data not shown).

Reduced NO bioavailability within the cirrhotic liver was further confirmed by measuring hepatic cGMP levels, a surrogate marker of NO bioavailability. Indeed, hepatic cGMP levels were significantly lower in cirrhotic than in control rat livers (2.5±0.2 vs 5.0±1.1 pmol/ml; $p<0.05$) (Fig 2).

Superoxide is increased in cirrhotic livers by an increased production by COX and XO and a reduced degradation by SOD

Along with reduced NO bioavailability, cirrhotic livers exhibited an increase in superoxide content. Thus, confocal microscopy showed a marked increase in DHE fluorescence in tissue sections from cirrhotic in comparison to control rat livers (Fig 3). SOD preincubation, the enzyme that metabolises superoxide to H_2O_2, markedly attenuates DHE fluorescence, demonstrating the specificity of the assay for O_2^- (data not shown).

Specific inhibition of XO or COX resulted in a significant reduction in cirrhotic hepatic O_2^- levels comparing to those animals receiving vehicle. However, no differences were observed when animals were treated with the NOS inhibitor L-

NAME (Fig 4).

Total SOD activity was significantly reduced in cirrhotic (n=10) compared to control rat livers (n=8) (2.1±0.2 vs. 4.2±0.4 U/mg tissue; $p<0.01$) (Fig 5). This was associated with a significant reduction in cytoplasmatic (CuZn) SOD and mitochondrial (Mn) SOD protein expression (Fig 6), without significant changes in EcSOD expression (data not shown).

NO bioavailability can be modulated by superoxide

Decreased NO bioavailability and increased superoxide content in cirrhotic livers coexist with an increase in hepatic nitrotyrosinated proteins (16.2±8.1 vs 1±0.6 AU in control livers; $p=0.025$; Fig 7). Nitrotyrosinated proteins are considered a fingerprint of peroxynitrite formation, the result of superoxide reacting with, and therefore scavenging, NO.

SEC isolated from control rat livers treated with the SOD inhibitor DDC displayed a marked and significant increase in O_2^- levels, as shown by DHE staining at confocal microscopy, in comparison to vehicle-treated SEC (Fig 8A). Increase in O_2^- was associated with a significant reduction in NO bioavailability as exposed by DAF-FM-DA staining (Fig 8B). Co-administration of SOD attenuated both the increase in O_2^- levels observed with DDC (Fig 8A) and the reduction in NO bioavailability (Fig 8B).

Discussion

Reduced NO bioavailability within the liver plays a major role increasing hepatic vascular tone in cirrhosis. Up to now it has been attributed to decreased NO production due to reduced eNOS activity in the liver (40). Our present results

confirmed diminished NO bioavailability within cirrhotic livers as shown by the observed reduced hepatic cGMP content. In addition, by using DAF staining and confocal microscopy, we were able to directly demonstrate reduced NO bioavailability in SEC from cirrhotic livers. This is the first time that NO content has been directly quantified in SEC only 12 h after its isolation when they still preserve its typical phenotype.

Reduced NO content was associated with increased O_2^- levels. Based in indirect data, such as the increase in plasma and tissue levels of lipid peroxidation markers and the observation of reduced hepatic and plasma antioxidant content, it has been previously suggested that there is an increase in oxidative stress in several liver disorders (41;42). However, this is the first study that, by using DHE staining and confocal microscopy, specifically demonstrates a marked increase in O_2^- levels in livers of rats induced to cirrhosis by CCl_4 administration.

In agreement with a previous report (43), we found a reduced SOD activity, the enzyme dismutating O_2^- to H_2O_2, as a possible mechanism underlying the observed increase in O_2^- in cirrhotic livers. Furthermore, our study clarifies that reduced SOD activity is, at least in part, due to decreased protein expression of the cytoplasmic and mitochondrial SOD, but not of the extracellular SOD isoform.

In addition to reduced metabolisation, increase in O_2^- content may also be due to enhanced generation. In a previous study we demonstrated that eNOS uncoupling, secondary to tetrahydrobiopterin deficiency, contributed to the reduced NO bioavailability of the cirrhotic liver (44). Uncoupled eNOS may also produce O_2^-; however, in our study, no reduction in O_2^- was observed after NOS

inhibition, suggesting that eNOS is not a significant contributor of the increased O_2^- content of cirrhotic livers.

By contrast, our results showing that COX or XO inhibition markedly reduced intrahepatic O_2^- levels points out, for the first time, that these enzymatic systems are potential sources of O_2^- in cirrhosis, which provides the rationale for further investigations of potential conceptual and therapeutic relevance. The possible role of another potential source of superoxide in cirrhosis, the NADPH oxidase system, has been recently discarded (45).

The pathophysiological role of increased O_2^- reducing NO bioavailability has been extensively demonstrated in several vascular disorders (46-48). Our finding of an increase in nitrotyrosinated proteins, a well recognized marker of the reaction of O_2^- with NO, strongly supports that this mechanism of reduction in NO bioavailability also occurs in the cirrhotic liver. The relationship between NO bioavailability and O_2^- content in the liver is further supported by our experiments in SEC demonstrating that NO bioavailability is modulated by O_2^-. Indeed, increasing O_2^- content in SEC by incubating with the SOD inhibitor DDC was associated with a prominent reduction in NO bioavailability. Further, abolition of the increase in O_2^- using SOD supplementation was followed by a partial restoration in NO bioavailability.

Altogether, these findings strongly support the concept that NO scavenging by O_2^- may be an important determinant of decreased NO bioavailability, endothelial dysfunction and increasing hepatic vascular tone in cirrhosis.

The role of increased O_2^- impairing NO biology within cirrhotic livers probably goes beyond its direct reaction with NO. Indeed O_2^- could oxidize, and therefore inactivate, the NO synthase cofactor tetrahydrobiopterin (49) or adjust

interactions of eNOS with other inactivating or activating proteins (50). These considerations further emphasize that antioxidant therapy, by removing O_2^- from the cirrhotic livers, could be a new therapeutic strategy to improve intrahepatic NO bioavailability and to ameliorate hepatic vascular tone in cirrhosis, and encourage further studies elucidating the mechanism of the O_2^--NO interaction and testing antioxidants as adjunctive therapy in the medical treatment of portal hypertension.

Figure Legends

Figure 1: Fluorescent detection of intracellular nitric oxide (NO) in sinusoidal endothelial cells (SEC) isolated from control (CT) and cirrhotic (CH) rat livers. *Top:* Representative image of DAF fluorescence from CT SEC (A) and CH SEC (B) visualized and collected with a confocal laser-scanning microscopy. Original magnification, 40x; bar, 10 μm. *Bottom:* Fluorescence intensity of DAF in arbitrary units (AU) indicating NO bioavailability. The data shown are from 779 individual CT SEC and 555 CH SEC obtained from two different experiments. DAF-FM-DA fluorescence intensity was significantly lower in SEC isolated from CH rats (*$p<0.01$ vs CT).

Figure 2: cGMP levels in liver homogenates from control (CT; n=5) and cirrhotic (CH; n=5) rats. cGMP levels were significantly reduced in CH rat livers (*$p=0.05$ vs CT).

Figure 3: *Top:* Representative confocal microscopy images of *in situ* detection of superoxide in fresh liver sections from control (CT; n=3) and cirrhotic (CH; n=3) rats with the oxidative dye dihydroethidium (DHE; bar, 10 μm). *Bottom:* Fluorescence intensity of DHE in arbitrary units (AU) indicates superoxide production. Analysis showed increased superoxide levels in CH livers (*$p<0.05$ vs CT).

Figure 4: *In situ* superoxide (O_2^-) detection in fresh liver sections from cirrhotic rats treated with vehicle, with the nitric oxide synthase (NOS) inhibitor L-NAME, with the xanthine oxidase (XO) inhibitor allopurinol or with the cyclooxygenase (COX) inhibitor indomethacin (n=2 per each condition). A significant reduction in cirrhotic intrahepatic O_2^- content was observed when XO or COX were

selectively inhibited (Values represent arbitrary units normalized to cirrhotic livers treated with vehicle; *p<0.01 vs. Cirrhotic-Vehicle).

Figure 5: Total SOD activity in liver homogenates from control (CT; n=8) and cirrhotic (CH; n=10) rats. Total SOD activity was significantly reduced in CH rat livers (*p<0.01 vs CT).

Figure 6: Representative western blots and analysis of CuZnSOD and MnSOD protein expression in liver homogenates of control (CT; n=4) and cirrhotic (CH; n=4) rats. Numbers below the lanes (expressed in arbitrary units) indicate the results of the relative densitometry, normalized to GAPDH for CT and CH, showing a decreased expression of both isoforms in CH livers (p<0.01 vs CT).

Figure 7: *Top:* Representative western blot of nitrotyrosine (3-NT) abundance in liver homogenates from control (CT; n=4) and cirrhotic (CH; n=4) rats. *Bottom:* Densitometry quantification in arbitrary units (AU), normalized to GAPDH showing more than a 20-fold increase in the main nitrated protein band in CH livers(*p<0.01 vs CT).

Figure 8: *In situ* superoxide (O_2^-) detection by DHE staining (10 μM) and NO quantification by DAF staining (10 μM) in SEC isolated from control rat livers treated with the specific SOD inhibitor DDC or in association with SOD (DDC + SOD). A: DDC-treated SEC had markedly increased intracellular O_2^- levels. This was significantly inhibited by co-incubating with SOD (*p<0.01 vs Veh; #p<0.01 vs DDC). Data shown are from 498 individual Veh-SEC, 372 DDC-SEC and from 357 DDC+SOD-SEC obtained from two different experiments. B: NO levels were significantly reduced when intracellular oxidative stress was induced by DDC. This low NO content was significantly ameliorated when cells were co-incubated with SOD. (*p<0.01 vs Veh; #p<0.01 vs DDC). Data shown are from

509 individual Veh-SEC, 282 DDC-SEC and from 376 DDC+SOD-SEC obtained from two different experiments

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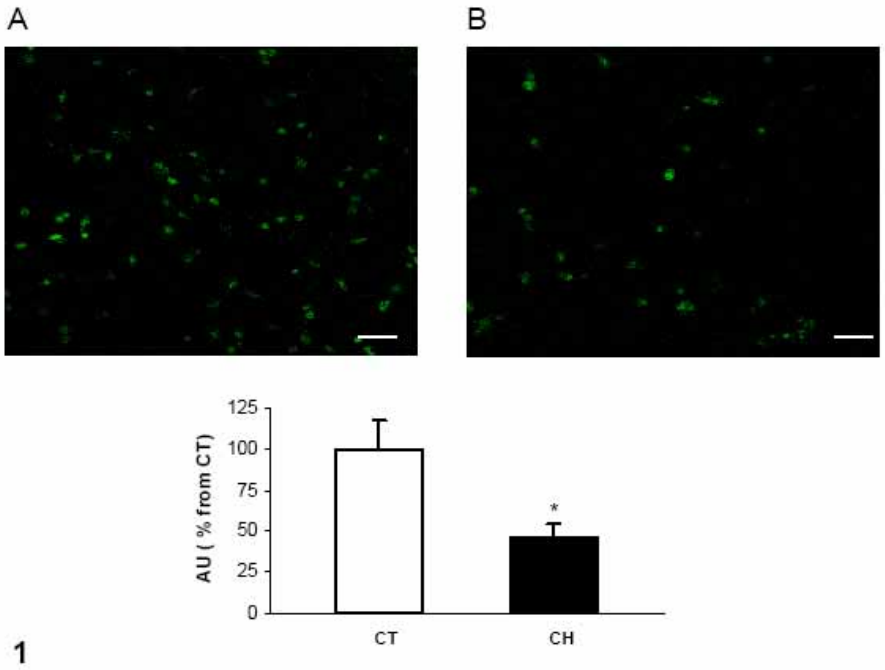

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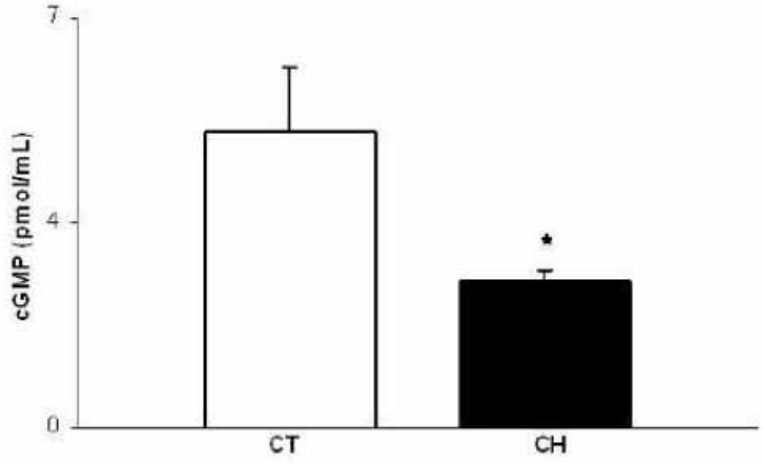

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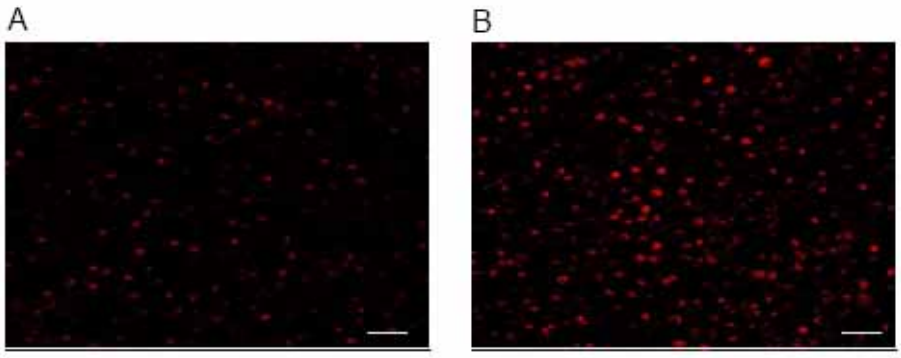
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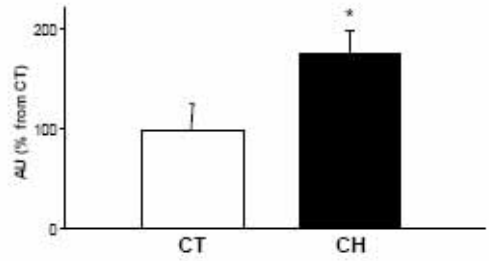
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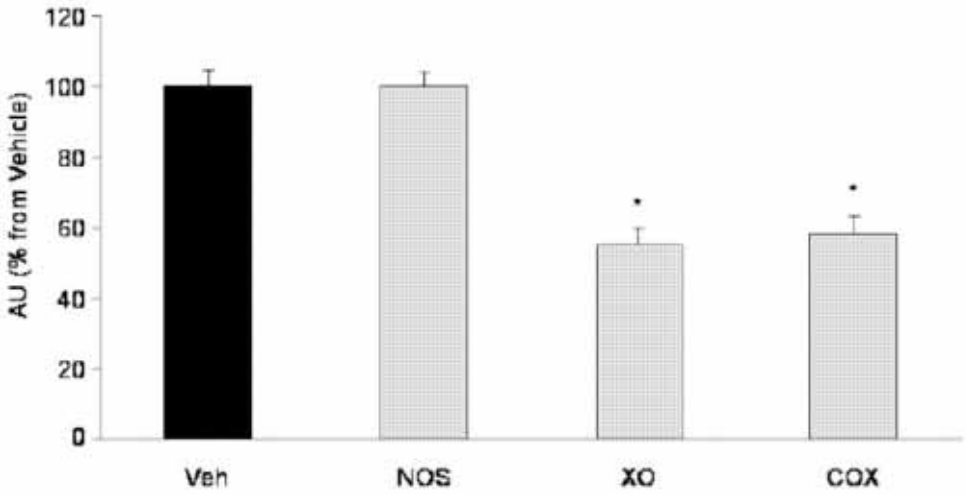
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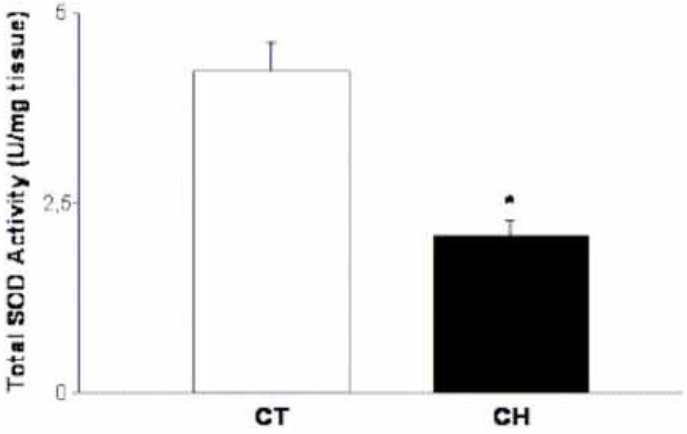
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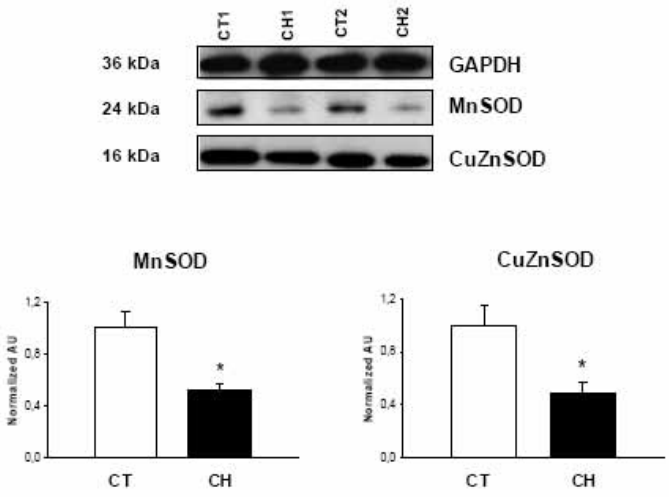
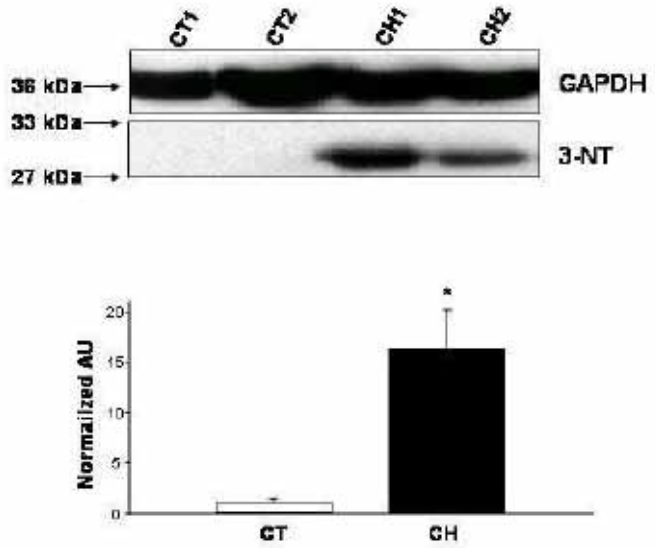
CH

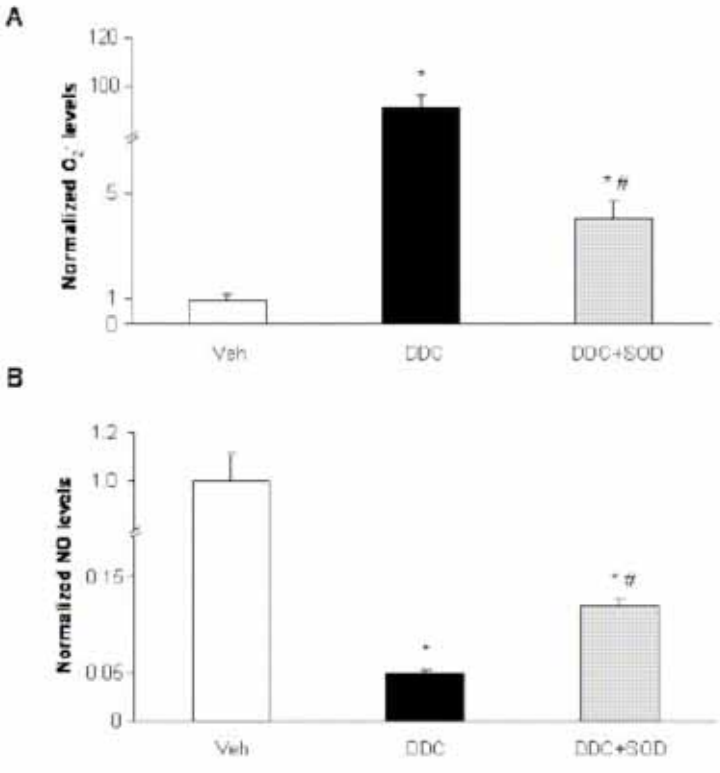
5


CT1
CH1
CT2
CH2
36 kDa
GAPDH
24 kDa
MnSOD
16 kDa
CuZnSOD
MnSOD
CuZnSOD
Normalized AU
1,2
0,8
0,4
0,0
*
CT
CH

6


CT1
CT2
CH1
CH2
36 kDa
33 kDa
27 kDa
GAPDH
3-NT
Normalized AU
20
15
10
5
0
*
CT
CH

7


A
Normalized $O_2^{\cdot-}$ levels
120
100
5
1
0
*
*#
Veh
DDC
DDC+SOD
B
Normalized NO levels
1.2
1.0
0.15
0.05
0
*
*#
Veh
DDC
DDC+SOD

8

4. Resum de resultats

4- RESUM DELS RESULTATS

4.1- Estudi 1: Enhanced vasoconstrictor prostanoid production by sinusoidal endothelial cells increases perfusion portal pressure in cirrhotic rat livers.

- Les CES de fetges cirròtics són capaces de sintetitzar el prostanoid vasoconstrictor TXA_2, tant a partir de l'administració exògena del precursor de prostanoids, l'AA, com al promoure l'alliberació endògena d'aquest, mitjançant l'estimulació amb l'agonista alfa-1-adrenèrgic metoxamina. Aquest fet no s'observa en les CES de fetges controls.
- L'administració exògena d'AA promou l'augment de la pressió portal de perfusió (PPP) tant en fetges controls com en cirròtics. Tot i això, l'augment de la PPP en els fetges cirròtics és significativament superior als controls. Aquest fet s'acompanya d'un increment en la síntesi de TXA_2, essent també significativament superior en els fetges de rates cirròtiques.
- La inhibició selectiva de l'enzim COX-1 atenua totalment, tant la resposta de la PPP com la producció de TXA_2 enfront l'administració exògena d'AA dels fetges controls i especialment dels cirròtics.
- La inhibició selectiva de l'enzim responsable de la formació del TXA_2, la tromboxà sintasa, aboleix totalment la síntesi d'aquest prostanoid. Malgrat això, no s'observen canvis en l'augment de la PPP quan s'administra exògenament AA.
- El bloqueig del receptor cel·lular comú pel TXA_2 i per la PGH_2 inhibeix totalment l'augment de la PPP quan s'administra AA, tant en fetges controls com particularment en cirròtics, sense modificar la síntesi de TXA_2.
- L'activitat enzimàtica de la fosfolipasa A2, enzim responsable d'incrementar la biodisponibilitat intracel·lular d'AA, es troba significativament augmentada en els fetges procedents de rates cirròtiques respecte els controls.

4.2- Estudi 2: Evidence against a role for NADPH oxidase modulating hepatic vascular tone in cirrhosis.

- Els fetges procedents de rates cirròtiques sobre-expressen significativament l'mRNA de les sub-unitats NOX2, p22phox, p47phox i p67phox i infra-expressen NOX4 en comparació amb fetges controls.
- L'expressió proteica de NOX2 i NOX4 es troba significativament disminuïda en els fetges procedents de rates cirròtiques respecte els controls.
- Els fetges cirròtics presenten una activitat enzimàtica de NADPH oxidasa significativament inferior respecte els controls.
- La inhibició selectiva i crònica de l'enzim NADPH oxidasa no modifica cap paràmetre de l'hemodinàmica sistèmica o hepàtica en les rates controls ni en les cirròtiques.
- La inhibició selectiva i crònica de l'enzim NADPH oxidasa no modifica la PPP basal ni la vasodilatació endoteli dependent a acetilcolina de les rates controls ni de les cirròtiques.
- En humans, l'expressió proteica de NOX4 és significativament inferior en fetges procedents de pacients cirròtics respecte individus sans. No s'observen diferencies en l'expressió de NOX2 ni en l'activitat enzimàtica de NADPH oxidasa entre ambdós grups.

4.3- Estudi 3: Increased oxidative stress in cirrhotic livers. A potential mechanism contributing to reduce nitric oxide bioavailability.

- Les CES procedents de rates cirròtiques presenten menors nivells d'ON, mesurats en directe i en viu, respecte les procedents de rates controls. Aquesta dada es confirma amb el fet que els fetges de rates cirròtiques presenten significativament menor quantitat de GMPc comparant-los amb controls.
- La quantitat d'estrès oxidatiu, mesurat com a quantitat de radicals O_2^-, present en els fetges de rates cirròtiques és marcada i significativament superior al present en controls. Dues de les possibles fonts són

identificades, les proteïnes COX i XO, en canvi el paper de l'eNOS en la formació de espècies lliures d'oxigen no és significativa.

- L'activitat de degradació de radicals O_2^- per part de la SOD en els fetges cirròtics es troba significativament disminuïda respecte als controls, probablement per la inferior expressió proteica de les isoformes citoplasmàtica i mitocondrial de la SOD.
- El marcador per excel·lència de la reacció entre el radical O_2^- i l'ON, la nitrotirosinació proteica, es troba significativament augmentat en els fetges cirròtics comparant-los amb els controls.
- La inhibició de la SOD en CES aïllades de rates controls indueix un augment significatiu en els nivells de radicals O_2^- intracel·lulars que s'acompanya d'una marcada reducció en la biodisponibilitat d'ON. Aquests dos fets són corregits si les cèl·lules són suplementades exògenament amb l'eliminador de O_2^-, SOD, demostrant l'íntima i alhora oposada relació entre els nivells de O_2^- i d'ON.

5. Discussió

5- DISCUSSIÓ DELS RESULTATS

La hipertensió portal és la més greu complicació de la cirrosi, en ser responsable del sagnat per varius esofàgiques, l'ascites, la disfunció renal i l'encefalopatia. El factor primari del desenvolupament d'hipertensió portal és aquell que els últims avenços en el coneixement de la fisiopatologia de la malaltia indiquen com a futura teràpia: l'augmentada resistència vascular intrahepàtica (RVI). L'augment en la RVI en la cirrosi té dues causes: primerament, una distorsió en l'arquitectura vascular del fetge deguda al transcurs de la pròpia malaltia, i que, fins a dia d'avui és pràcticament irreversible, i segon, un component dinàmic secundari a la contracció de diferents estructures contràctils de localització sinusoïdal i extrasinusoïdal, principalment cèl·lules hepàtiques estelades (CHE), resultat del desequilibri entre forces vasoconstrictores i vasodilatadores intrahepàtiques.

El fet que aquest component dinàmic de la RVI sigui reversible, i per tant, potencialment manipulable, ha constituït la base racional per al tractament farmacològic de la hipertensió portal (1).

Conseqüentment, en els presents treballs d'investigació ens vàrem proposar ampliar el coneixement entorn la regulació del to vascular intrahepàtic per part dels metabòlits vasomotors derivats de l'AA, així com indagar nous mecanismes que podrien reduir la biodisponibilitat intrahepàtica del vasodilatador ON.

Els resultats del primer estudi amplien els coneixements ja existents al voltant de l'efecte dels prostanoids sobre la RVI, alhora que aporten noves dades fins ara desconegudes. Per primera vegada hem demostrat que les cèl·lules endotelials sinusoïdals (CES) procedents de fetges cirròtics tenen la capacitat de produir el vasoconstrictor TXA_2, quan se les incuba amb el precursor de prostanoids AA. Aquesta producció és totalment abolida quan l'enzim COX-1 és inhibit. De forma semblant, la capacitat de síntesi de TXA_2 per part de les CES de fetges cirròtics quan els seus receptors cel·lulars acoblats a proteïna G són estimulats amb un agonista alfa-1-adrenèrgic, metoxamina, queda demostrada i de nou s'aboleix quan l'enzim COX és inhibit.

Aquests primers resultats reforcen el paper que tenen les CES en l'elevada producció de prostanoids vasoconstrictors observada en els fetges cirròtics, cal

remarcar que altres tipus cel·lulars, com les cèl·lules de kupffer, també podrien contribuir a la síntesi global de prostanoids (21; 100).

Una altra troballa important del primer estudi ha estat la demostració que els fetges cirròtics exhibeixen una major activitat enzimàtica de la fosfolipasa A_2, la proteïna responsable de proporcionar AA intracel·lular. Una major biodisponibilitat d'AA en el fetge cirròtic podria donar lloc a una elevada síntesi de prostanoids vasoconstrictors derivats i per tant, constitueix una nova diana terapèutica a considerar.

Els resultats obtinguts en els experiments de perfusió hepàtica ens mostren que el TXA_2 tindria un destacat paper en la modulació de la RVI. Tot i això, la manca d'efecte de la inhibició de la sintasa del TXA_2 en l'augment del to vascular hepàtic en resposta a l'administració d'AA ens indica que algun altre prostanoid constrictor, hipotèticament el precursor i també potent vasoconstricor PGH_2, podria estar involucrat en l'augment de la RVI dels fetges cirròtics. De fet, PGH_2 comparteix amb el TXA_2 el seu receptor cel·lular (101). Això explicaria per què el bloqueig del receptor PGH_2/TXA_2 resulta en una total inhibició de la hiper-resposta a l'AA observada en els fetges cirròtics. També seria concebible que, en condicions normals, fóra el TXA_2 el responsable d'activar el seu receptor, però en situacions d'inhibició selectiva de la producció de TXA_2 l'acumulació del precursor PGH_2 donaria lloc igualment a l'activació del receptor PGH_2/TXA_2. Sigui com sigui, el bloqueig efectiu del receptor comú PGH_2/TXA_2 seria una forma ideal d'inhibir l'exagerada resposta a prostanoids vasoconstrictors del fetge cirròtic.

Aquest conjunt de troballes poden ser d'important interès terapèutic considerant que la inhibició selectiva de l'altre enzim involucrat en la formació de PGH_2 i TXA_2, la COX-1, pot comportar fallida renal (102). Aquest problema no existiria al bloquejar selectivament el receptor per PGH_2/TXA_2.

El segon i tercer treballs de la present tesi es centraren en l'estudi del possible paper que pot exercir l'estrès oxidatiu sobre la biodisponibilitat d'ON i per tant, en la regulació de la RVI en el fetge cirròtic.

El complex enzimàtic NADPH oxidasa és una important font d'estrès oxidatiu, en forma de radicals O_2^-, que ha estat identificada com a enzim clau en el desenvolupament i manteniment de la disfunció endotelial present en diferents patologies cardiovasculars (84-86). S'ha proposat que una exagerada

producció de O_2^- reaccionaria amb l'ON present a l'endoteli vascular i comportaria una marcada disminució en la biodisponibilitat d'aquest (103).

La caracterització bioquímica i biològica de l'enzim NADPH oxidasa en el fetge cirròtic ens mostra que la seva activitat enzimàtica, determinada mitjançant quimioluminiscència, és significativament menor que en els fetges controls. Aquest resultat lliga totalment amb el fet que la inhibició selectiva de la NADPH oxidasa no produeix canvis hemodinàmics hepàtics o sistèmics en rates amb cirrosi avançada. Tot i això, no podem descartar que aquest enzim jugui un paper clau en estadis previs de la malaltia, on els processos inflamatoris són més importants. Així ho han posat de manifest alguns estudis que han demostrat que la NADPH oxidasa està implicada en la formació de fibrosi (88; 104), a més d'en altres hepatopaties que cursen amb alts nivells d'inflamació (87; 104-106).

De fet, un recent estudi publicat per la Dra. Fernández i col·laboradors va evidenciar que, durant el desenvolupament d'hipertensió portal en el model experimental de lligadura parcial de vena porta, l'activitat de l'enzim NADPH oxidasa augmenta i, que la seva inhibició prevén l'angiogènesi esplàncnica (107).

Per tal d'entendre les raons de la baixa activitat enzimàtica hepàtica de la NADPH oxidasa en la cirrosi, van ser determinades l'expressió gènica i proteica de diferents sub-unitats d'aquest complex. Les expressions proteiques de NOX2 i NOX4 es troben significativament disminuïdes en els fetges cirròtics comparant-los amb controls. Caldria remarcar la troballa de baixa expressió de NOX4 ja que aquesta sub-unitat ha estat àmpliament descrita en l'endoteli vascular (103; 108-111). A més, s'ha demostrat que la regulació a la baixa de NOX4 redueix de forma significativa la producció de O_2^- en cèl·lules endotelials tant *in vitro* com *in vivo*, reforçant la hipòtesi que NOX4 és el principal component de la NADPH oxidasa endotelial (112).

La reduïda expressió proteica de NADPH oxidasa obtinguda en el fetge cirròtic concorda amb les dades prèvies d'aquest estudi: la baixa activitat enzimàtica i la manca d'efecte a l'inhibir l'enzim. Al quantificar l'expressió gènica de les diferents sub-unitats de la NADPH oxidasa vàrem obtenir interessants resultats, així com la quantitat de mRNA de la sub-unitat NOX4 és significativament menor en els fetges cirròtics (mateix resultat que expressió proteica), les sub-

unitats NOX2, p22phox, p47phox i p67phox es troben significativament sobre-expressades en el fetges de rates cirròtiques respecte els controls, indicant per tant, que aquest enzim pateix algun tipus de regulació post-transcripcional.

Som conscients que la mesura de l'activitat enzimàtica i l'expressió proteica i gènica de la NADPH oxidasa en teixits hepàtics totals (entenent total com a una mostra representativa de tot el fetge, sense aïllar vasos, tipus cel·lulars o lòbuls hepàtics en particular) pot no ser representatiu del que succeiria al territori vascular hepàtic (allà on es genera la major part de RVI). Malauradament, a causa de la particular arquitectura del fetge, no és possible obtenir el llit vascular intrahepàtic aïllat de la resta de l'òrgan per tal de caracteritzar bioquímica i biològicament el sistema NADPH oxidasa. Tot i aquesta limitació, la troballa de baixa expressió gènica i proteica de la sub-unitat NOX4 (la més important sub-unitat expressada en cèl·lules endotelials i no en cèl·lules de kupffer ni en macròfags) és la millor aproximació molecular al coneixement de l'estat de la NADPH oxidasa al territori vascular intrahepàtic i suggereix també, la manca de rol de NADPH oxidasa en estadis avançats de cirrosi.

No obstant, els resultats més convincents en contra d'un possible paper de la NADPH oxidasa en la regulació del to vascular hepàtic són els obtinguts en els estudis fisiològics, mostrant que la inhibició crònica i selectiva del complex enzimàtic no modifica cap paràmetre hemodinàmic hepàtic o sistèmic.

L'extrapolació de resultats entre diferents models experimentals i encara més entre diferents espècies és complexa. Per això, i amb l'objectiu de conèixer si els resultats obtinguts en el present estudi serien aplicables a la cirrosi humana, vàrem caracteritzar bioquímica i biològicament la NADPH oxidasa en mostres hepàtiques procedents de pacients cirròtics i individus sans. Els resultats d'aquest estudi ens mostren que els fetges cirròtics humans no presenten superiors activitat enzimàtica o expressió proteica de NOX2 al comparar-los amb els controls, a més l'expressió proteica de NOX4 es troba significativament disminuïda, la qual cosa reforçaria la idea que la NADPH oxidasa no tindria un important rol en la cirrosi.

Així doncs, la importància atribuïda al complex NADPH oxidasa com a catalitzador del segrest d'ON al formar elevades quantitats de O_2^- sembla no confirmar-se en la cirrosi avançada. Malgrat aquesta troballa, els resultats del tercer estudi ens mostren que els fetges cirròtics presenten una baixa

biodisponibilitat d'ON, determinada al quantificar el missatger secundari GMPc. A més, es demostra mitjançant microscòpia confocal, que les CES en cultiu primari aïllades de fetges cirròtics també presenten baixa biodisponibilitat d'ON. Aquesta reduïda quantitat d'ON intrahepàtic es troba associada a un augment significatiu en els nivells d'O_2^-. Estudis previs han suggerit que existeix un elevat estrès oxidatiu en el fetge cirròtic (96; 113), no obstant aquest és el primer cop que emprant la tinció específica de dihidroetidi es demostra que els fetges cirròtics presenten elevats nivells de O_2^- comparant-los amb fetges sans. D'acord amb els resultats d'un estudi previ (81), hem demostrat que els fetges cirròtics presenten l'activitat enzimàtica de SOD disminuïda respecte als controls. Aquest fet podria representar una de les causes de l'elevat O_2^- intrahepàtic cirròtic. No obstant, per primer cop hem esbrinat que la baixa activitat enzimàtica seria conseqüència d'una expressió proteica reduïda de les isoformes citoplasmàtica i mitocondrial de la SOD.

A més d'una reducció en la seva eliminació, l'increment en el O_2^- intrahepàtic també podria ser causat per un excés en la seva formació. El recent estudi publicat per Matei i col·laboradors va demostrar que el "desacoblament" de l'enzim eNOS, secundari a la deficiència en BH_4, està involucrat en la reducció de la generació d'ON (114). L'eNOS desacoblada és capaç de produir O_2^-, però els resultats del nostre estudi mostren que la inhibició selectiva d'aquest enzim no redueix els nivells intrahepàtics de O_2^-, indicant que l'eNOS no seria un dels responsables de l'elevat estrès oxidatiu existent en el fetge cirròtic.

En canvi, els resultats de la marcada reducció en el contingut de O_2^- quan els enzims COX o XO són inhibits, suggereixen que aquests són fonts efectives de O_2^- i requereixen per tant, un estudi futur més profund.

En l'àmbit cardiovascular, s'ha demostrat el paper fisiopatològic d'un augment de O_2^- que redueix la biodisponibilitat d'ON (85; 115; 116). La troballa d'una marcada nitrotirosinació proteica, marcador específic de la reacció de O_2^- amb l'ON, ens demostra que aquest mecanisme de reducció dels nivells d'ON també succeeix al fetge cirròtic. Addicionalment, la relació entre la presència de O_2^- i la biodisponibilitat d'ON es demostra, més encara, amb els experiments realitzats en CES, on s'observa *in vivo* que la biodisponibilitat d'ON és modulada per la presència de O_2^-. De fet, l'increment de radicals O_2^-, obtingut a l'inhibir selectivament la SOD, s'acompanya d'una reducció marcada en la

biodisponibilitat d'ON. A més, la reducció dels nivells d'estrès a l'afegir exògenament SOD promou una recuperació parcial en els nivells d'ON intracel·lulars.

Els resultats d'aquest estudi evidencien que el segrest de l'ON per part dels radicals O_2^- podria ser un factor contribuent a la reducció de la biodisponibilitat d'ON i en conseqüència a l'increment del to vascular intrahepàtic en la cirrosi.

6. Conclusions

6- CONCLUSIONS

- Les cèl·lules endotelials sinusoïdals del fetge cirròtic tenen la capacitat de sintetitzar el prostanoid vasoconstrictor tromboxà A2, tant a partir del precursor àcid araquidònic, com a l'estimular els seus receptors de membrana units a proteïna G amb un agonista alfa-adrenèrgic.
- En el fetge cirròtic, l'administració exògena d'àcid araquidònic promou un increment significatiu de la pressió portal de perfusió que s'acompanya d'una exagerada síntesi de prostanoids vasoconstrictors.
- El bloqueig selectiu del receptor pel tromboxà A2/prostaglandina H2 inhibeix totalment la resposta a l'administració d'àcid araquidònic. Això no s'observa quan la sintasa del tromboxà és inhibida.
- Els fetges cirròtics presenten una sobre-activació de l'enzim generador d'àcid araquidònic, la fosfolipasa A2.

- El complex enzimàtic generador de radicals superòxid, NADPH oxidasa, es troba infra-activat en el fetge cirròtic. A més, la seva inhibició selectiva i crònica no modifica cap paràmetre hemodinàmic sistèmic o hepàtic.

- El fetge cirròtic presenta elevats nivells d'estrès oxidatiu. Aquests, al reaccionar amb l'òxid nítric present i formar radicals peroxinitrit, contribuirien a reduïr la biodisponibilitat hepàtica d'òxid nítric.
- Les causes dels elevats nivells d'estrès oxidatiu del fetge cirròtic serien: la reduïda eliminació per part de l'enzim superòxid dismutasa i l'elevada síntesi per part de la ciclooxigenasa i la xantina oxidasa.

7. Epíleg

7- EPÍLEG

La present tesi doctoral, englobada dins la recerca de quins són els mecanismes moleculars que regulen el to vascular hepàtic en la cirrosi, no tan sols aporta nou coneixement, sinó que alhora, il·lumina nous camins a explorar al voltant de la recerca bàsica i aplicada sobre la hipertensió portal.

Les troballes descrites demostren que, les cèl·lules endotelials sinusoïdals desenvolupen un important paper en l'increment del to vascular en la cirrosi. No tan sols a causa d'una reduïda producció del vasodilatador òxid nítric, sinó també per un marcat increment en la síntesi de prostanoids vasoconstrictors. Noves estratègies encaminades al bloqueig de les vies de formació de prostanoids i, especialment al bloqueig del receptor cel·lular PGH_2/TXA_2, serien d'alt interès terapèutic.

El complex enzimàtic generador de radicals superòxid, la NADPH oxidasa, no seria responsable de l'elevat estrès oxidatiu hepàtic, de la disfunció endotelial ni de l'incrementat to vascular intrahepàtic del fetge cirròtic.

L'ús d'antioxidants, a l'eliminar l'excés de radicals lliures d'oxigen, seria una nova estratègia terapèutica per incrementar la biodisponibilitat d'òxid nítric intrahepàtic i reduir així, l'elevat to vascular hepàtic en la cirrosi. El desenvolupament d'estudis que resolguin els mecanismes de reacció entre el radical superòxid i l'òxid nítric en el fetge cirròtic, així com l'assaig d'antioxidants com a teràpia en la hipertensió portal són necessaris.

8. Altres publicacions

8- ALTRES PUBLICACIONS

8.1- Heme oxygenase attenuates oxidative stress and inflammation, and increases VEGF expression in portal hypertensive rats.

Angermayr B, Mejías M, **Gracia-Sancho J**, García-Pagán JC, Bosch J, Fernández M.

Journal of Hepatology, 2006 June; 44(6): 1033-1039 IF:6.07

8.2- NAD(P)H oxidase modulates angiogenesis and the development of portosystemic collaterals and splanchnic hyperaemia in portal hypertensive rats.

Angermayr B, Fernández M, Mejías M, **Gracia-Sancho J**, García-Pagán JC, Bosch J.

Gut 2007;56:560-564. IF:9.00

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