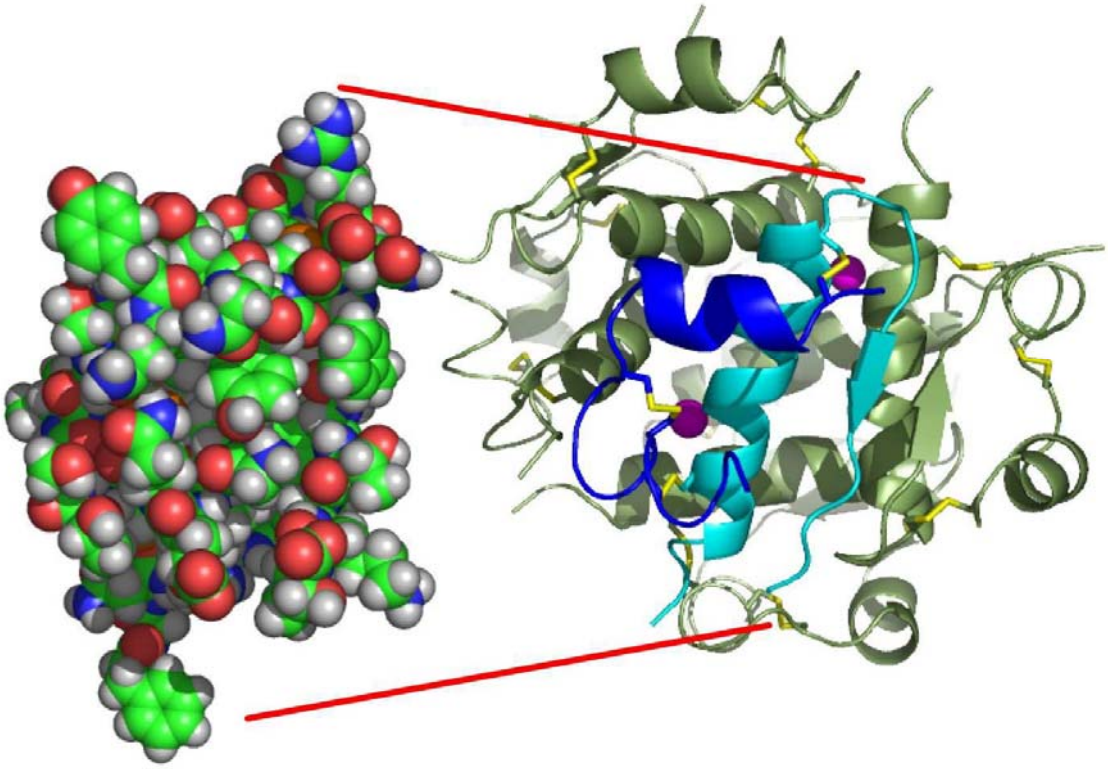
Resistència a la insulina i disfunció endotelial sinusoïdal a la malaltia hepàtica per dipòsit de greix

Marcos Pasarín Castellanos

RESISTÈNCIA A LA INSULINA I DISFUNCIÓ ENDOTELIAL SINUSOÏDAL A LA MALALTIA HEPÀTICA PER DIPÒSIT DE GREIX



Tesi doctoral presentada per **Marcos Pasarín Castellanos**

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UNIVERSITAT DE BARCELONA

Facultat de Medicina

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Tesi presentada per

MARCOS PASARÍN CASTELLANOS

Per optar al títol de Doctor per la Universitat de Barcelona

Programa de Doctorat de Medicina

Directors: Dr. Juan González-Abraldes Iglesias / Prof. Jaume Bosch i Genover

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A mis padres

Índex

ÍNDEX

INFORME DELS DIRECTORS DE TESI

Barcelona, 25 d'Octubre del 2011.

Juan González-Abraldes Iglesias, Especialista del Servei d'Hepatologia de l'Hospital Clínic de Barcelona, i Jaume Bosch Genover, catedràtic de la Facultat de Medicina de la Universitat de Barcelona i Consultor Sènior del Servei d'Hepatologia de l'Hospital Clínic de Barcelona,

CERTIFIQUEN:

Que la tesi doctoral *RESISTÈNCIA A LA INSULINA I DISFUNCIÓ ENDOTELIAL SINUSOÏDAL A LA MALALTIA HEPÀTICA PÈR DIPÒSIT DE GREIX,* presentada per Marcos Pasarín Castellanos per a optar al títol de Doctor per la Universitat de Barcelona s'ha realitzat sota la nostra direcció i compleix tots els requisits necessaris per ser defensada davant el Tribunal d'avaluació corresponent.

Juan González-Abraldes Iglesias **Jaume Bosch i Genover**

1. Introducció

1.1. La Síndrome Metabòlica

La síndrome metabòlica és un conjunt de factors de risc que prediuen la malaltia cardiovascular i la diabetis millor que qualsevol dels seus components individualment (1). La resistència a la insulina (IR) sembla tenir un paper rellevant tant en l'inici com en el desenvolupament de la malaltia.

La primera vegada que va aparèixer el concepte d'una síndrome (Síndrome X, terme que es va utilitzar en un primer moment) unida a la resistència a la insulina va ser gràcies a la menció de Reaven el 1988 (2), el qual va proposar que una acció deficient de la insulina era el component central d'una sèrie d'alteracions metabòliques (nivells elevats de triglicèrids, colesterol HDL baix, hiperglucèmia en dejú i pressió sanguínia elevada). També va fer notar que aquestes alteracions es presentaven en absència de factors de risc clàssics com concentracions elevades de colesterol LDL.

La prevalença d'aquesta síndrome s'ha estimat entre un 35.2 i un 40.1% de la població dels EEUU majors de 20 anys, convertint-se en un problema de primera magnitud als països occidentals i també als emergents, com la Xina.

1.1.1. Malaltia Hepàtica per Dipòsit de Greix

La malaltia hepàtica per dipòsit de greix (MHDG) és considerada la manifestació hepàtica de la síndrome metabòlica (3) i es refereix a l'acumulació de greix, principalment triglicèrids, als hepatòcits, de forma que aquests superen el 5% del pes total del fetge.

Només recentment, Ludwig i col·laboradors (4) han identificat una síndrome caracteritzada per l'associació de fetge gras, hepatitis lobular i uns nivells plasmàtics permanentment elevats d'alanina aminotransferasa en pacients on l'ingesta d'alcohol era negligible. El terme malaltia hepàtica per dipòsit de greix (MHDG o NAFLD) s'ha adoptat per cobrir tot l'espectre de desordres metabòlics del fetge gras (5;6), que va des de la simple acumulació de greix al

fetge, passant per la inflamació (esteatohepatitis no-alcohòlica o NASH) i fins la cirrosi.

La prevalença de la MHDG en la població general sembla situar-se al voltant del 30% (7), percentatge que pot ascendir fins al 90% si parlem de persones obeses (8) o d'entre el 50 i el 75% en persones diabètiques (9).

A l'igual que passa en la síndrome metabòlica, la resistència a la insulina és el factor que més es reprodueix en el desenvolupament de la MHDG (10).

1.2. Resistència a la Insulina

La resistència a la insulina (IR) és la condició en la qual les quantitats normals d'insulina són insuficients per a produir una resposta d'insulina normal al greix, cèl·lules del múscul i fetge (11).

El fetge és el principal òrgan on la insulina fa el seu efecte, a més del múscul esquelètic i el teixit adipós (12). En dejú, la insulina limita la producció de glucosa hepàtica, la qual cosa manté la concentració plasmàtica de glucosa a nivells normals. En estadis postpandrials, la insulina activa la transcripció de l'enzim glucocinasa, que catalitza la reacció de glucosa a glucosa-6P, la qual no inhibeix la glucocinasa. Tanmateix, la insulina actua inhibint l'acció de la glucosa-6 fosfatasa, la qual defosforila la glucosa-6P per convertir-la en glucosa. La insulina també estimula l'acumulació de glucosa en forma de glucogen. Quan la concentració plasmàtica de glucosa cau, la producció d'insulina resta inhibida. Una altra acció de la insulina és la de limitar la producció de lipoproteïnes de molt baixa densitat (VLDL) (13). Per contra, quan el fetge té les reserves de glucogen plenes, tota la glucosa que entra en excés és derivada cap a la síntesi d'àcids grassos, els quals seran exportats des del fetge en forma de VLDL.

Quan el fetge es torna gras a causa de la MHDG, l'acció de la insulina, inhibint la producció hepàtica de glucosa, queda malmesa. Aquesta resistència a la insulina condueix a un lleuger increment en els nivells de glucosa plasmàtica i a

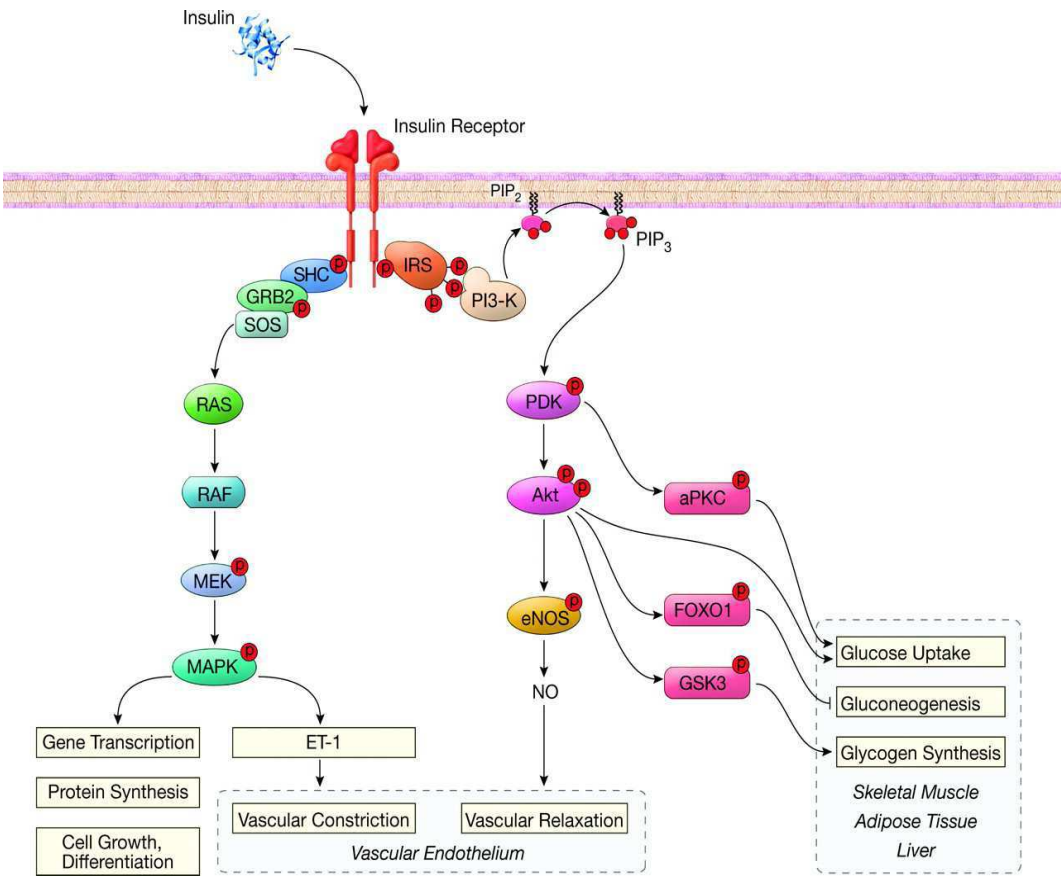
una estimulació de la secreció d'insulina. Probablement, la hiperinsulinèmia és una conseqüència, i no una causa, de la MHDG.

En pacients amb MHDG (i per tant, amb resistència a la insulina hepàtica), el fetge produeix VLDL riques en triglicèrids en dejú (14) i en condicions d'hiperinsulinèmia (15). Això comporta una hipertrigliceridèmia i una concentració plasmàtica baixa de colesterol HDL (16).

1.2.1 Resistència Vascular a la Insulina

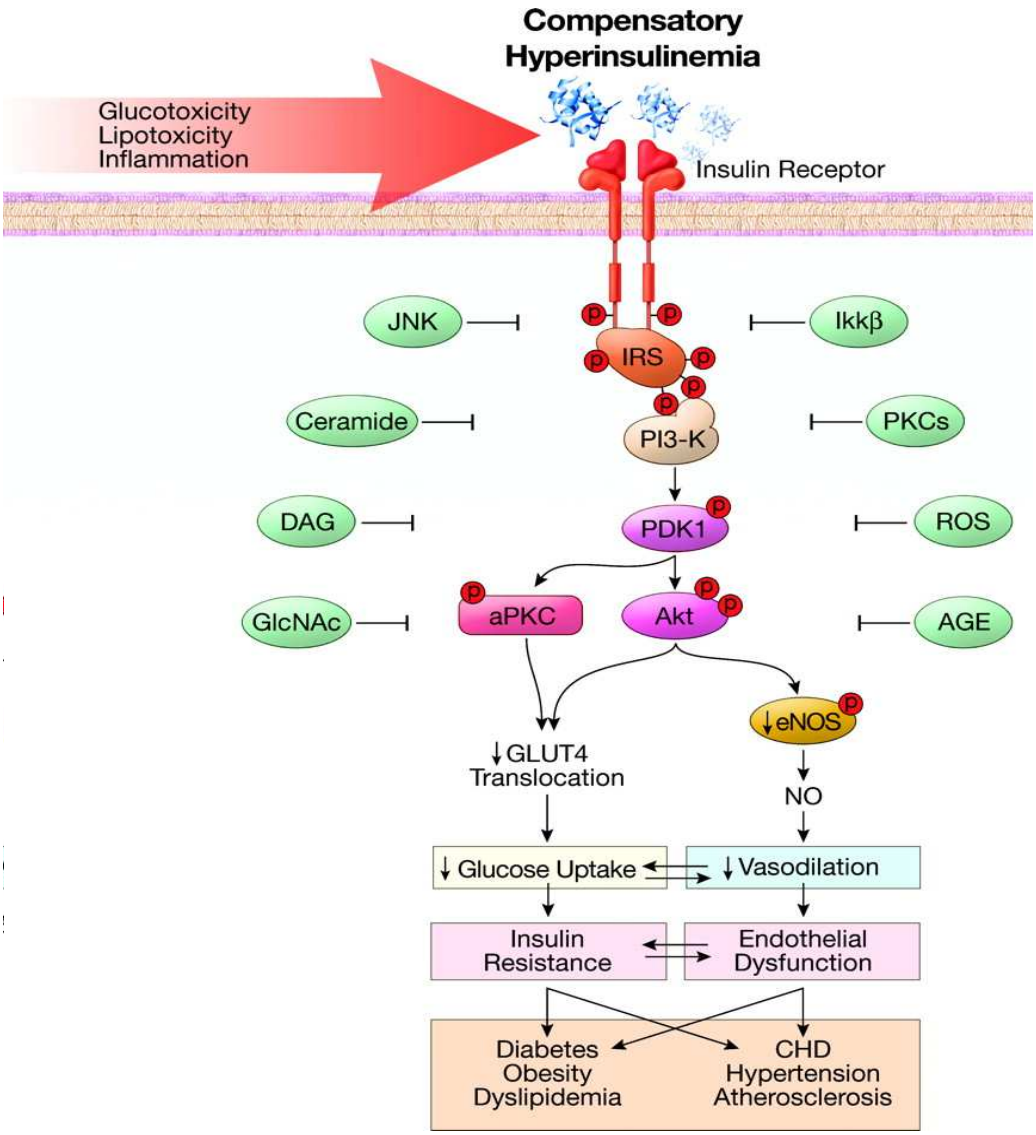
Molts estudis han demostrat un paper de la resistència a la insulina sistèmica en el desenvolupament de complicacions cardiovasculars. De fet, la majoria de les grans complicacions en pacients amb síndrome metabòlica tenen un origen a nivell vascular (17;18).

A nivell de l'endoteli perifèric, la insulina s'uneix al seu receptor (19), el qual fa que es fosforili el substrat del receptor de la insulina (20), activant PI3K (21), la qual cosa comportarà la fosforilació i activació de PDK-1 (22) que, a la vegada, fosforilarà i activarà AKT, la qual fosforilarà directament la sintasa endotelial de l'òxid nítric (eNOS) en la serina en posició 1176 en rates (Ser^{1176}, Ser^{1177} en humans) (23;24). El resultat d'aquest conjunt de reaccions produeix, en últim terme, un increment d'activitat d'eNOS i un augment de la producció d'òxid nítric (NO), el que condueix a la vasodilatació. A més, la insulina també produeix vasoconstricció a causa de l'activació d'endotelina, mitjançant la via RAS/RAF/MEK i MAPK (25;26),



Visió general de les vies de senyalització de la insulina (Muniyappa R. Endocrine Reviews 2007).

En condicions de resistència a la insulina, l'alteració de la via de PI3K és un element clau que la caracteritza, mentre que altres branques de la senyalització de la insulina no es veuen afectades, incloent la via de Ras/MAPK (27;28). Això té importants implicacions fisiopatològiques ja que la resistència a la insulina metabòlica està acompanyada, normalment, d'hiperinsulinèmia compensatòria per tal de mantenir l'euglicèmia. A més, tant a la vasculatura com si no, la hiperinsulinèmia sobrecarregarà la via dependent de MAPK, la qual no estarà afectada per la resistència a la insulina, la qual cosa conduirà a un desequilibri entre les funcions de la insulina que es realitzen mitjançant les vies de PI3K (recordem, afectada) i la de MAPK (no afectada) (29).



Els mecanismes de glucotoxicitat, lipotoxicitat i inflamació provoquen relacions recíproques entre resistència a la insulina i disfunció endotelial (Muniyappa R. Endocrine Reviews 2007).

Aquest desequilibri portarà a una menor activació de l'eNOS i, per tant, a una menor producció d'NO i una conseqüent disfunció endotelial.

S'han implicat molts mecanismes en el desenvolupament de disfunció endotelial dins la resistència a la insulina, com la lipotoxicitat (mitjançant una trasmissió deficient del senyal de la insulina (30), estrés oxidatiu (31;32), alteracions locals del sistema renina-angiotensina (33) i una sensibilitat

adrenèrgica augmentada de les cèl.lules vasculars musculars llises (34)), glucotoxicitat (a través d'estrés oxidatiu, flux incrementat, activació de diacilglicerol, entre d'altres (35;36)) i inflamació (37;38).

1.3. Endoteli i Disfunció Endotelial

Al ser un dels principals reguladors de l'hemostasi vascular, l'endoteli manté un equilibri entre les substàncies vasodilatadores i vasoconstrictores produïdes per (o que actuen a) l'endoteli, entre la inhibició i l'estimulació de la proliferació i migració de la cèl.lula muscular llisa, i entre la trombogènesi i la fibrinolisi (39).

Quan aquest desequilibri es trenca parlem de disfunció endotelial.

L'endoteli sa regula el to vascular i té accions anticoagulants, antiplaquetàries, antiproliferatives i fibrinolítiques. El manteniment del to vascular és regulat per la secreció de numeroses substàncies vasodilatadores i vasoconstrictores.
El vasodilatador més important secretat per l'endoteli és l'NO, originalment descrit com a *factor relaxant derivat de l'endoteli* (EDRF en anglès). Uns altres vasodilatadors són la bradicinina i la prostaciclina.

L'endoteli també produeix substàncies vasoconstrictores, com l'endotelina (el vasoconstrictor endogen més important) i l'Angiotensina II. Aquests dos vasoconstrictors promouen la proliferació de les cèl·lules musculars llises i, per tant, la formació de placa (40). Els macròfags activats i les cèl·lules musculars llises vasculars, components cel.lulars característics de la placa ateroscleròtica, produeixen grans quantitats d'endotelina (41).

a) Òxid Nítric (NO)

L'òxid nitric és una substància clau d'entre les produïdes per l'endoteli. La característica principal de la disfunció endotelial és la pèrdua de capacitat vasodilatadora de l'endoteli, la qual és mitjançada per NO. S'ha proposat que un defecte en la producció de NO o en la seva activitat pot ser un dels grans mecanismes responsables de la disfunció endotelial i un contribuent per l'aterosclerosi.

L'NO és sintetitzat com a producte de la formació de L-citrulina per part de les 3 isoformes de la proteïna sintasa del NO (NOS): la NOS neuronal (nNOS), la induïble (iNOS) i la endotelial (eNOS) (42). Les nNOS i l'eNOS produeixen NO de forma constitutiva i en petites quantitats, mentre que la iNOS ho fa de manera induïble en resposta a citocines, lipopolisacàrids i molts altres agents en quantitats fins a 1000 vegades superiors a les que pot produir l'eNOS (43). Encara que el terme constitutiu implica que la seva expressió no està regulada, l'ARN missatger (mRNA) i els nivells proteics, així com les activitats enzimàtiques poden ser modificades (sobretot de l'eNOS).

a.1) eNOS

La producció hepàtica fisiològica de NO és derivada de l'eNOS en resposta a estímuls com l'estrés de fregament i la presència de vasoconstrictors (44;45).
L'activació clàssica de l'eNOS (per exemple, per acetilcolina (ACh)), implica un increment intracel.lular de calci (Ca^{2+}) i la unió de Ca^{2+}/calmodulina a l'enzim. Recentment s'ha descrit una via de regulació d'eNOS independent de Ca^{2+}. Aquesta via és estimulada per *shear stress* o insulina (46;47). Tant el *shear stress* com la insulina incrementen la producció endotelial d'NO via l'activació de PI 3-kinase i protein kinase B (PKB/Akt), que fosforila eNOS en Ser1176 (48). A més, la insulina regula a l'alça la transcripció d'eNOS en les cèl.lules endotelials (49).

El mecanisme d'acció de l'NO és paracrí, possiblement (al fetge) sobre les cèl.lules estrellades hepàtiques (CHE), promovent la síntesi de GMP cíclic (GMPc) (50). La diana més important del GMPc és una proteïna cinasa anomenada GMPc-dependent (PKG) que fosforila nombroses proteïnes involucrades en la regulació de la homeostasi del Ca^{2+}, entre les quals trobem el receptor Inositol 1,4,5 trifosfat. Aquesta fosforilació comporta una disminució de la concentració de Ca^{2+} intracel.lular que produirà relaxació de les CHE i vasodilatació (51).

El paper de l'NO en el fetge sa és de compensació vascular enfront un estímul vasoconstrictor (52). Diversos estudis, a través d'estratègies ben diferents, han aconseguit incrementar la producció hepàtica d'NO en la cirrosi. D'una banda,

la infecció gènica del fetge amb l'adenovirus que conté la nNOS o l'eNOS (53;54); de l'altra, administrant donadors d'NO específicament hepàtics (55) i molt recentment, augmentant la síntesi d'NO mitjançant estatines (56).

a.2) iNOS

Aquest enzim, identificat inicialment pel seu paper vital en el sistema immunològic, pot produir NO contínuament si el substrat i els cofactors no són limitants, al contrari del que passa amb la molt regulada producció de NO mediada per l'eNOS. Està sobreexpressat en teixits metabòlics sota diferents condicions d'estrés metabòlic (57). La iNOS, encara que és important pel sistema immunitari, quan la seva expressió es troba induïda, pot ser perjudicial per altres tipus cel.lulars, com ara les cèl.lules β del pàncrees (58) i les cèl.lules vasculars (59). Estudis recents han demostrat que l'NO derivat de la INOS pot tenir un paper en la fisiopatologia de la disfunció metabòlica induïda per obesitat (60); la seva inhibició millora la hiperglucèmia, hiperinsulinèmia i la sensibilitat a la insulina en el fetge (61) i és un modulador crític de l'activació de PPAR-γ (podent restar eficàcia a l'efecte de fàrmacs sensibilitzadors de la insulina) (62), a part està implicada en un nou mecanisme de resistència a la insulina (63). A més, l'NO derivat de la iNOS causa dany vascular. Diversos estudis en diferents models animals han demostrat com la iNOS, quan està induïda, provoca disfunció endotelial (64-66), majoritàriament a causa de la producció d'estrés oxidatiu (67). Aquest estrés oxidatiu, provocat per una sobreproducció d'NO per part de la iNOS, podria estar suprimint l'expressió de l'eNOS (68). Tanmateix, la inhibició d'iNOS en models animals on es troba sobreexpressada restaura la correcta funció endotelial (69-72). En condicions normals, l'única NOS expressada a l'endoteli dels vasos és l'eNOS. Durant la inflamació, els vasos sanguinis expressen iNOS així com eNOS (73). La sobreexpressió d'iNOS, doncs, contribueix a la disfunció vascular.

3.1 L'endoteli Sinusoïdal Hepàtic

L'endoteli sinusoïdal hepàtic mostra característiques diferencials respecte a l'endoteli perifèric: manca de membrana basal, està fenestrat i no expressa basalment el factor de von Willebrand (vWF) ni molècules d'adhesió com el CD31. En condicions normals manté un ambient antiinflamatori, antitrombòtic,

antifibròtic i vasodilatador en el sinusoide hepàtic, principalment a través de la producció de NO. Per una altra part, l'endoteli sinusoïdal juga un paper fonamental en la resposta al dany hepàtic (74). A la seva vegada, factors sintetitzats a l'hepatòcit i a les cèl·lules estrellades son fonamentals per mantenir la integritat i característiques diferencials de l'endoteli sinusoïdal. El principal d'aquests factors és el VEGF, i el seu efecte està mediat a través de l'activació dels seus dos receptors endotelials: VEGF-R1 (que media l'estímul de la producció d'HGF (factor de creixement hepatocitari) i IL-6, (factors protectors de l'hepatòcit i que promouen la regeneració hepàtica en resposta al dany hepàtic) (75) i VEGF-R2 (que media l'estímul de la producció de NO) (76). Per tot això, alteracions en la funció hepatocitària o de les cèl.lules estrellades hepàtiques podrien resultar en alteracions de la funció sinusoïdal, la qual cosa podria accentuar a la seva vegada, el dany hepatocitari. És possible, per tant, que la pèrdua d'una adequada funció de l'endoteli sinusoïdal actuï com a amplificador del dany hepatocitari una vegada aquest s'inicia.

La rellevància patogènica a la malaltia hepàtica crònica de la disfunció endotelial sinusoïdal ha estat recentment demostrada en la cirrosi hepàtica (77). La circulació intrahepàtica en la cirrosi és caracteritzada per una resposta exagerada de la vasculatura hepàtica a vasoconstrictors i una resposta disminuïda a vasodilatadors dependents d'endoteli (78), la qual cosa contribueix al desenvolupament d'hipertensió portal. S'ha postulat, a més, que mitjançant la facilitació de fenòmens trombòtics (79) i de la pèrdua de l'efecte antifibrogènic del NO (80), la disfunció endotelial contribueix a la progressió de la cirrosi (81;82).

2. Justificació i Objectius

JUSTIFICACIÓ I OBJECTIUS DE LA PRESENT TESI

Justificació i objectius generals

La MHDG té una prevalença del 25.8% en la població general espanyola (33.4% entre els homes i un 20.3% entre les dones) (83) i del 30% a la població estadounidenca (84). Els pacients amb MHDG tenen una esperança de vida menor respecte la població control (85), sobretot en el rang de 45 a 54 anys (86), encara que aquest increment en la mortalitat sigui petit (87;88). Entre aquests pacients, la incidència d'una mort a causa de la malaltia hepàtica passa a ser la tercera causa (després del càncer i de la malaltia coronària isquèmica), respecte a la tretzena de la població general (89).

La MHDG pot evolucionar cap a esteatohepatitis (o NASH) en un 5% dels casos (90), dels quals fins un 15% pot acabar desenvolupant cirrosi (91;92). Dels pacients amb MHDG, un 3% acabarà desenvolupant també cirrosi (93).

A més, cada cop hi ha més evidències que la MHDG és la causa més freqüent de cirrosi criptogènica (94;95), essent, aquesta, la tercera o quarta causa d'indicació per a trasplantament hepàtic (96).

Per tant, encara el risc de mortalitat entre els pacients amb MHDG i el de mort a causa de malaltia hepàtica sigui modest, l'alta prevalença de la malaltia fa que sigui una càrrega molt important per al sistema sanitari.

Fins ara, no hi ha una teràpia farmacològica per a la MHDG; la teràpia establerta consisteix en mantenir una dieta saludable i fer exercici. Només alguns estudis experimentals, en un petit nombre de pacients, han assajat amb diferents fàrmacs (97), sense que encara se n'hagi recomanat cap.

La disfunció endotelial apareix abans que apareguin altres complicacions vasculars a la perifèria en condicions de síndrome metabòlica. En el fetge, la disfunció endotelial sinusoïdal hepàtica contribueix a la hipertensió portal, però la situació de l'endoteli sinusoïdal en la MHDG no estat estudiada.

Els treballs de recerca de la present tesi estan orientats, globalment, a estudiar la situació de l'endoteli sinusoïdal hepàtic en condicions d'esteatosi simple i els mecanismes moleculars implicats en una possible disfunció endotelial hepàtica.

Estudi 1: Insulin resistance and liver microcirculation in a rat model of early NAFLD, role of Inducible Nitric Oxide Synthase.

Com s'ha comentat anteriorment, la resistència a la insulina és una de les principals característiques fisiopatològiques implicades en la MHDG i es creu que contribueix tant en l'inici com en la progressió de la mateixa. Malgrat tot, no es coneix en profunditat com la resistència a la insulina pot contribuir al dany hepàtic. Un millor coneixement d'aquests mecanismes possibilitaria l'ús racional de tractaments específics que previnguessin la hipertensió portal i la cirrosi en pacients amb MHDG.

Només molt recentment s'han demostrat anormalitats vasculars en models de fetge gras (98;99) en forma d'una reduïda perfusió sinusoïdal i una disfunció sinusoïdal causa per l'acumulació parenquimal de lípids i l'acumulació de col.lagen a l'espai de Disse. Mai, però, s'han explorat les relacions patogèniques entre la resistència a la insulina i la disfunció endotelial sinusoïdal hepàtica.

La insulina té efectes beneficiosos sobre l'endoteli (100), promou la producció de NO per la via de PI3K/Akt/eNOS, la qual cosa provocarà vasodilatació i protecció vascular (101;102). En condicions de resistència a la insulina a nivell vascular es perdran els efectos beneficiosos de l'NO, i aquesta condició serà suficient per a causar disfunció endotelial. Com ja s'ha explicat anteriorment, una adequada funció de l'endoteli sinusoïdal hepàtic és necessari per un bon funcionament de tot l'òrgan. Una disfunció de l'endoteli podria contribuir a la hipertensió portal (103-107).

Un dels mecanismes proposats en el desenvolupament de resistència a la insulina després de l'administració d'una dieta rica en greix és la regulació a l'alça d'iNOS en el fetge (108). Aquesta sobreexpressió podria causar dany endotelial en els vasos de rosegadors (109;110), però, la contribució de la iNOS en el dany vascular hepàtic en la MHDG no es coneix.

Per tant, les hipòtesis d'aquest estudi van ser, en primer lloc, avaluar si les respostes vascular a la insulina es troben alterades en un model animal de MHDG; avaluar si l'administració d'un fàrmac sensibilitzador de la insulina millora aquestes anormalitat; i, per últim, investigar el paper d'iNOS en aquestes alteracions.

Estudi 2: Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD

La MHDG és la manifestació hepàtica de la síndrome metabòlica.

No es coneixen els mecanismes pels quals la MHDG pot progressar des d'esteatosi simple fins a esteatohepatitis o cirrosi.

Per un costat, se sap que la majoria de complicacions de la síndrome metabòlica semblen tenir un origen vascular (111). Un dels principals motius d'aquestes complicacions vasculars és la disfunció endotelial amb una menor producció d'NO (112), que s'ha observat abans que es puguin demostrar complicacions patològiques a nivell vascular (113). Això suggereix que la disfunció endotelial és un esdeveniment primerenc en el curs de les complicacions vasculars. La correcció de la funció endotelial s'associa a una disminució dels esdeveniments vasculars i, per tant, la disfunció endotelial es considera una diana terapèutica útil en aquesta síndrome (114;115). A més, els pacients amb MHDG presenten disfunció endotelial sistèmica i un augment del risc cardiovascular (116).

Malgrat això, mai s'ha investigat la presència de disfunció endotelial sinusoïdal en el fetge. També es desconeix si aquesta disfunció endotelial apareix abans que altres signes de la malaltia, tal i com passa en la circulació sistèmica.

Per tant, els objectius d'aquest estudi foren caracteritzar els canvis a nivell histològic i vascular d'un model animal d'obesitat, que presenta la majoria de les característiques de la síndrome metabòlica.

3. Còpies dels Articles Originals

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Insulin resistance and liver microcirculation in a rat model of early NAFLD

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Background & Aims: Insulin contributes to vascular homeostasis in peripheral circulation, but the effects of insulin in liver microvasculature have never been explored. The aim of this study was to assess the vascular effects of insulin in the healthy and fatty liver.

Methods: Wistar rats were fed a control or a high fat diet (HFD) for 3 days, while treated with a placebo, the insulin-sensitizer metformin, or the iNOS inhibitor 1400W. Vascular responses to insulin were evaluated in the isolated liver perfusion model. Insulin sensitivity at the sinusoidal endothelium was tested by endothelium-dependent vasodilation in response to acetylcholine in the presence or absence of insulin and by the level of liver P-eNOS after an insulin injection.

Results: Rats from the HFD groups developed liver steatosis. Livers from the control group showed a dose-dependent hepatic vasodilation in response to insulin, which was blunted in livers from HFD groups. Metformin restored liver vascular insulin-sensitivity. Pre-treatment with insulin enhanced endothelium-dependent vasodilation of the hepatic vasculature and induced hepatic eNOS phosphorylation in control rats but not in HFD rats. Treatment with metformin or 1400W restored the capacity of insulin to enhance endothelium dependent vasodilation and insulin induced eNOS phosphorylation in HFD rats.

Conclusions: The administration of a HFD induces insulin resistance in the liver sinusoidal endothelium, which is mediated, at least in part, through iNOS upregulation and can be prevented by the administration of metformin. Insulin resistance at the hepatic vasculature can be detected earlier than inflammation or any other sign of advanced NALFD.

Keywords: Insulin resistance; NAFLD; Sinusoidal endothelium; iNOS.
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Abbreviations: ACh, acetylcholine; AMPK, 5′ adenosine monophosphate-activated protein kinase; CD, Control diet; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; HFD, High fat diet; IR, Insulin resistance; NAFLD, Non-alcoholic fatty liver disease; NO, Nitric oxide; PI3K, Phosphoinositide 3-kinase.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent liver diseases world-wide [1,2], and is an increasingly frequent cause of cirrhosis. Insulin resistance is one of the major pathophysiological features implicated in NAFLD [3,4] and it is thought to contribute both to the initiation of the disease and to the progression to advanced forms of NAFLD. However, the mechanisms linking insulin resistance (IR) to liver injury are still largely unknown. A better understanding on how insulin resistance contributes to progressive liver injury and fibrogenesis is pivotal to devise rational treatments that could prevent portal hypertension and cirrhosis in these patients.

Some recent data have shown the presence of microvascular abnormalities in models of fatty liver [5–7] characterized by the presence of reduced sinusoidal perfusion [6] and dysfunctional sinusoids due to lipid accumulation in parenchymal cells and to collagen deposition in the space of Disse [5]. However, it has never been explored whether these vascular abnormalities have any pathogenic link to IR, or are a non-specific consequence of inflammation, fibrosis, and liver injury.

Insulin has beneficial vascular effects, specifically on the endothelium [8]. It promotes nitric oxide (NO) production through the activation of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) signaling pathway, which results in vasodilation and vascular protection [9,10]. When insulin resistance develops, not only the metabolic effects of insulin are impaired, but also its beneficial vascular effects, which might lead to the development of endothelial dysfunction, contributing to further vascular damage [8].

The effects of insulin on hepatic vasculature in healthy conditions and in conditions associated with insulin resistance have never been addressed, but could be of relevance to the pathogenesis and progression of NAFLD. An adequate function of the sinusoidal endothelial cells is capital to maintain an anti-inflammatory, anti-fibrogenic, and anti-thrombotic environment in the liver [11–14], and contributes to liver regeneration [15,16]. Therefore, the development of endothelial dysfunction in the liver circulation might promote a pro-thrombotic, pro-fibrogenic, and pro-inflammatory environment, and impair regeneration after liver injury, thus contributing to the progression of liver disease [17]. In addition, liver endothelial dysfunction has been shown to increase hepatic resistance in advanced chronic liver disease, contributing to the development of portal hypertension [18–22].

Research Article

One of the mechanisms proposed to account for the development of insulin resistance after the administration of a high fat diet is iNOS upregulation in the liver [23]. This would occur through s-nitrosilation/nitrotyrosination of proteins from the insulin signaling cascade [23,24]. In addition, recent evidence suggest that iNOS overexpression could contribute to endothelial dysfunction in vessels from diabetic rodents [25,26], but the potential contribution of iNOS induction to vascular abnormalities in liver diseases is largely unknown.

The aims of this study were: (1) to evaluate whether the vascular responses to insulin are impaired in a model of early NAFLD associated with insulin resistance, (2) to evaluate whether the administration of an insulin sensitizer might reverse or improve the liver vascular insulin resistance observed in this model, and (3) to investigate the contribution of iNOS upregulation to these abnormalities.

Materials and methods

Animals, diets, and treatments

Male Wistar rats, weighing 275–300 g, were caged individually in a 12:12-h light–dark cycle, temperature- and humidity-controlled environment. Animals were divided into six groups. The first group (CD; n = 30) was fed with standard chow diet for 3 days and treated with a placebo during the same period. The second group (CD-Met; n = 28) was fed with standard chow diet and treated with the insulin sensitizer Metformin (Sigma–Aldrich, Madrid, Spain), 300 mg/kg/day per gavage with the final dose 1 h prior to the studies. The third group (CD-1400W; n = 28) was fed with standard chow diet and treated with the iNOS inhibitor 1400W (Cayman Chemical, Ann Arbor, Michigan, USA), 5 mg/kg/day ip with the final dose 1 h prior to the studies. The fourth group (HFD; n = 30) was fed with a high fat diet (26% carbohydrates, 59% fat, and 15% protein; #112245, Dyets Inc., Bethlehem, PA) as previously described [27], and treated with a placebo. The fifth group (HFD-MET; n = 28) was fed with the high fat diet and treated with Metformin. The sixth group (HFD-1400W; n = 28) was fed with the high fat diet and treated with 1400W. The high fat diet used in this study has safflower oil as the major constituent. Given for three days, it has been shown to induce an increase in circulating free fatty acids, liver steatosis, a three-fold increase in hepatic triglycerides content, and impaired insulin signaling in the liver, but not in peripheral tissues [27]. Two additional groups of rats (CD and HFD) were treated with Pioglitazone (10 mg/kg/day) (see Supplementary data).

The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were approved by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, NIH Publication 86-23, revised 1996).

Liver vascular studies

Liver vascular responses were assessed in the isolated, *in situ* liver perfusion system, as described previously [28,29]. Briefly, 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 (Edwards Lifesciences, Irvine, CA) 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 afterward blindly analyzed under code. The perfused rat liver preparation was allowed to stabilize for 20 min before the studied substances were added. A normal gross appearance of the liver and a stable perfusion pressure and perfusate pH (7.4 ± 0.1) were required during this period. If any viability criterion was not satisfied, the experiment was discarded. To evaluate the vascular effects of insulin on the liver circulation, livers from each of the four groups (n = 6 per group) were preconstricted with methoxamine (10^{-4} M), an α-adrenergic agonist. After maximum vasoconstriction, we added increasing doses (3×10^{-7}, 3×10^{-6} and 3×10^{-5} M) of insulin (Actrapid, NovoNordisk, Novo Allé, Denmark) every 1.75 min. To evaluate the impact of insulin pretreatment on endothelium dependent vasodilation of the liver vasculature, we perfused livers from the six study groups (n = 6 for each condition). After stabilization, we added to the reservoir a single dose of insulin 3×10^{-5} M or vehicle 10 s before pre-constricting the liver with methoxamine. After that, cumulative doses of acetylcholine (ACh; 10^{-7}, 10^{-6}, and 10^{-5} M) were added to the system.

Western blotting

To evaluate the effects of insulin on eNOS phosphorylation, rats from the six study groups (n = 6 per group) were anesthetized with ketamine (80 mg/kg) and midazolam (5 mg/kg). Rats were injected with insulin (5 UI) or a similar volume (500 μl) of saline through the ileocolic vein [30]. Five minutes later the rats were euthanized and liver samples were obtained and immediately frozen in liquid nitrogen and kept at −70 °C until processing. The samples were processed as previously described [29]. Aliquots from each sample containing equal amounts of protein (40–100 μg) were run on an 8% SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. Equal loading was insured by Ponceau staining. The blots were subsequently blocked for 1 h with Tris-buffered saline and probed overnight at 4 °C with a mouse antibody recognizing eNOS, iNOS (BD Transduction Laboratories, Lexington, KY) or a rabbit antibody recognizing phosphorylated eNOS at Ser^{1176} (BD Transduction Laboratories, Lexington, KY). This was followed by incubation with rabbit anti-mouse (1:10,000) or goat anti-rabbit (1:10,000) HRP-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) for 1 h at room temperature. Blots were revealed by chemiluminescence and digital images were taken by a luminescent image analyzer LAS-3000 (Fujifilm Life Science, Tokyo, Japan). Protein expression was determined by densitometric analysis using the Science Lab 2001, Multi Gauge V2.1 (Fuji Photo Film Gmbh, Düsseldorf, Germany). Quantitative densitometry values of eNOS and iNOS were normalized to β-actin or GAPDH and displayed in histograms. The degree of eNOS phosphorylation at Ser^{1176} was calculated as the ratio between the densitometry readings of P-eNOS and eNOS blots.

Histopathology

Liver samples were fixed in 10% formalin, embedded in paraffin, sectioned (thickness of 2 μm), and slides were stained with hematoxylin and eosin (H&E) to analyze the hepatic parenchyma [31]. To detect neutral lipids, livers were frozen in liquid nitrogen, fixed in a freezing medium (Jung, Leica Microsystems, Nussloch, Germany) and stained with Oil Red O for 2 h at room temperature.

The samples were photographed and analyzed using a microscope (Zeiss, Jena, Germany) equipped with a digital camera.

Immunohistochemistry

Immunostaining of paraffin-embedded liver sections was performed with anti-iNOS diluted 1:100 or, as a negative control, with phosphate-buffered saline in CD and HFD groups. Bound antibody was visualized using diaminobenzidine as chromogen, and slides were then counterstained with hematoxylin. Images were taken using AxioVision software.

Liver transaminase levels on the perfusate

Buffers from the liver perfusion studies were taken at the end of each experiment to analyze AST and ALT levels (as markers of liver damage) with standard methods at our institution's CORE lab.

Analysis of hepatic triglyceride, free fatty acids, and cholesterol content

One gram samples of frozen livers were homogenized in 3 ml (1:3 w:v) of HEPES buffer composed of 50 mM Tris, 150 mM NaCl, and 5 mM EDTA (Sigma–Aldrich, Madrid, Spain) and analyzed with standard methods at the Hospital Clinic's CORE lab.

Statistics

Statistical analysis was performed using the SPSS 16.0 statistical package (SPSS Inc., Chicago, IL). Comparisons of the baseline characteristics between control and high fat diet groups were performed with the unpaired Student's *t*-test after

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confirming the assumptions of normality. We analyzed the dose response curves with repeated measures ANOVA introducing type of diet and pre-treatment with insulin/vehicle as the between-subjects factors. All data are reported as means ± SD. Differences were considered significant at a p value <0.05.

Results

A 3 day high fat feeding period induces liver steatosis and liver endothelial insulin resistance

Table 1 shows the baseline characteristics of the rats. Rats from the placebo group fed a HFD for three days developed liver steatosis without signs of inflammation (Fig. 1A and Supplementary Fig. S1). This was associated with an increase in the liver content of triglycerides, cholesterol, and free fatty acids as compared with CD. There were no changes in liver transaminase levels in the perfusion buffer.

Rats from the control group and HFD group pre-treated with placebo had a similar baseline portal perfusion pressure and hepatic vascular resistance (Table 1). Both groups of rats exhibited a dose-dependent vasodilation to insulin (Fig 2A). However, the vasodilating response to insulin was blunted in HFD rats ($p = 0.03$), indicating the presence of insulin resistance at the hepatic vasculature.

To evaluate if insulin resistance occurred specifically at the liver endothelium, we performed a series of functional and molecular studies. We first evaluated the endothelium-dependent vasodilation of the liver in the presence or absence of insulin. Insulin significantly enhanced the endothelium dependent vasodilation to increasing doses of ACh in livers from control rats but not in livers from the HFD group (Fig. 3A). To evaluate whether HFD impairs insulin to eNOS signaling at the liver endothelium, we assessed the degree of eNOS phosphorylation (at Ser^{1176}) after a portal injection of insulin (5UI) or saline. Hepatic eNOS is majorly expressed at the liver endothelium, and, therefore, it can be safely assumed that changes in eNOS phosphorylation in the total liver are representative of changes at the endothelium. Insulin increased P-eNOS/eNOS ratio in CD rats (×3.5-fold; $p = 0.004$) but not in HFD rats (×0.96-fold; $p = 0.20$) (Fig. 4A). Altogether, these results show that a HFD induces insulin resistance at the liver endothelial cells.

The administration of an insulin-sensitizer prevents the development of liver endothelial insulin-resistance

Rats treated with metformin during the 3 day of the HFD administration still developed liver steatosis, but the increase in liver triglycerides, cholesterol, or free fatty acids was non-significant as compared to CD. Thus, metformin attenuated hepatic lipid accumulation (Table 1), (Fig. 1B).

In rats treated with metformin the vasodilation of the liver vasculature in response to increasing doses of insulin was similar between CD and HFD rats, ($p = 0.99$; Fig. 2B), suggesting that metformin prevented the development of hepatic vascular insulin-resistance in HFD rats. In addition, in rats pre-treated with metformin, insulin significantly enhanced the endothelium dependent vasodilation both in control (Fig 3B) and HFD rats (Fig. 3B). Furthermore, metformin treatment restored the capacity of insulin to increase P-eNOS/eNOS ratio in HFD rats (×2.84-fold; $p = 0.014$) (Fig. 4B). Altogether, these results demonstrate that metformin acts as an insulin-sensitizer at the liver endothelium in rats fed a HFD. Comparable results were obtained with another insulin sensitizer (pioglitazone) (Supplementary data, and Supplementary Figs. S1–S3).

iNOS is upregulated by HFD administration and contributes to the development of liver endothelial insulin resistance

Previous studies have shown that the administration of a high fat diet upregulates iNOS in the liver [22] and that this contributes to the development of insulin resistance by interfering with insulin signaling. To assess whether this occurred also in our model, we first evaluated whether a 3 day HFD upregulates iNOS. HFD increased liver iNOS expression, prominently localized in hepatocytes (Fig. 5A and B). We further evaluated the contribution of iNOS upregulation to liver endothelial insulin resistance, by treating CD and HFD rats with the iNOS inhibitor 1400W. This molecule has a ×5000-fold selectivity for iNOS as compared to eNOS [32]. Treatment with 1400W during the administration of a HFD attenuated the development of hepatic steatosis, as showed by a mild, non-significant increase in liver triglycerides, cholesterol, and free fatty acids (Table 1, Fig 1C).

In liver perfusion studies, the vasodilation of the liver vasculature in response to increasing doses of insulin was similar

Table 1. Baseline characteristics of rats fed a control or a high fat diet for 3 days, treated with vehicle, metformin or 1400W. Data is presented as mean ± SD. (FFA: free fatty acids).

	CD	HFD	p	CD-Met	HFD-Met	p	CD-1400W	HFD-1400W	p
Rat Weight (g)	317 ± 4	298 ± 13	0.19	340 ± 10	316 ± 9	0.098	345 ± 4.7	345 ± 3.9	0.982
Liver Weight (g)	10.7 ± 0.2	12.1 ± 0.6	0.041	10.8 ± 0.4	9.9 ± 0.4	0.199	11.8 ± 0.4	12.9 ± 0.3	0.055
% Liver/Total Weight	3.4 ± 0.06	4.1 ± 0.1	0.0001	3.2 ± 0.1	3.1 ± 0.07	0.924	3.4 ± 0.09	3.7 ± 0.08	0.02
Cholesterol (mg/g Liver)	2.9 ± 0.16	3.59 ± 0.31	0.092	3.34 ± 0.27	2.8 ± 0.5	0.314	2 ± 0.4	2.2 ± 0.3	0.664
Triglycerides (mg/g Liver)	9.7 ± 0.6	23.1 ± 1.5	0.004	13.5 ± 1.4	15.38 ± 1.6	0.465	6.5 ± 1.7	9.7 ± 2.2	0.109
FFA (μmol/g Liver)	8.1 ± 1.1	12.3 ± 0.7	0.01	9.5 ± 1.1	8.6 ± 0.6	0.584	10 ± 3	9.2 ± 1	0.653
Baseline Portal Perfusion Pressure (mmHg)	4.8 ± 0.08	4.8 ± 0.05	0.901	5.5 ± 0.12	5.5 ± 0.11	0.569	5.2 ± 0.06	5.2 ± 0.1	0.973
Baseline Intrahepatic Resistance (mmHg*g of Liver*min*ml^{-1})	1.4 ± 0.01	1.5 ± 0.12	0.601	1.7 ± 0.17	1.7 ± 0.09	0.968	1.7 ± 0.11	1.8 ± 0.41	0.686
AST (U/L) levels in perfusing buffer	4.2 ± 0.66	4.8 ± 0.73	0.559	2 ± 0.7	2.8 ± 0.86	0.437	3.6 ± 0.5	2.5 ± 1.2	0.315
ALT (U/L) levels in perfusing buffer	0.4 ± 0.24	0.2 ± 0.2	0.61	0.4 ± 0.4	0 ± 0	0.311	0.4 ± 0.24	0.25 ± 0.25	0.718

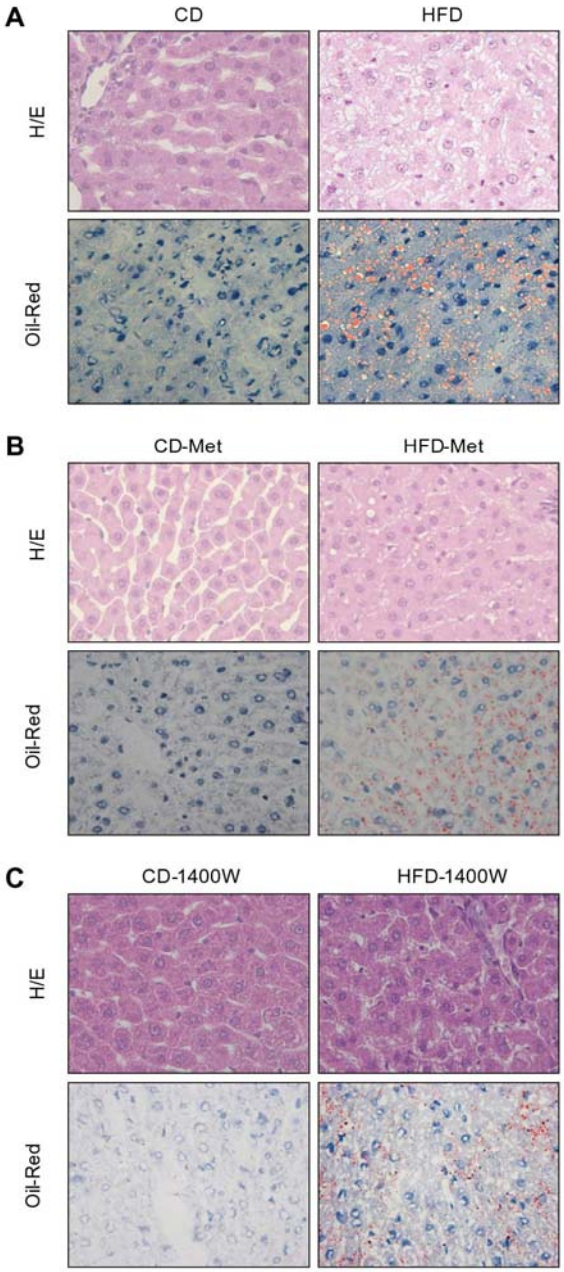

Fig. 1. Hematoxylin/eosin (H/E) and oil-red images from livers of rats fed a control diet (CD) or a high fat diet (HFD) treated with placebo (A), metformin (B), or the iNOS inhibitor 1400W (C) (original magnification, 400×).

between CD and HFD rats treated with 1400W, ($p = 0.244$; Fig. 2C), indicating that 1400W prevents HFD induced liver vascular insulin-resistance. In addition, insulin significantly enhanced endothelium dependent vasodilation both in control and in HFD rats treated with 1400W (Fig. 3C). Furthermore, and contrary to what was observed in rats treated with placebo, in rats fed a HFD during treatment with 1400W, insulin enhanced eNOS phosphorylation (×2.19-fold; $p = 0.028$) (Fig. 4C), again showing that iNOS upregulation interferes with insulin signaling at the liver endothelium.

Discussion

This study reports three important and novel findings that add to our understanding of the regulation of hepatic vasculature in conditions of insulin resistance: (a) liver steatosis induced by a high fat diet is associated with impaired insulin-induced liver vasodilation, (b) the steatotic liver exhibits insulin resistance specifically at the liver endothelium that is related to iNOS induction, and (c) treatment with an insulin sensitizer improves liver endothelial insulin resistance and attenuates liver fat accumulation. These findings were documented after only 3 days of HFD administration, which is enough to induce liver steatosis and to impair insulin signaling, but not to cause inflammation, fibrosis or other features of advanced NAFLD [27]. This indicates that these are early events in the evolution of NAFLD that could be targeted early in the course of the disease by directed treatments.

About 20% of the patients with NAFLD end up developing cirrhosis and complications of portal hypertension [33,34], but the mechanisms of disease progression in NAFLD are still poorly understood. The present study addresses the possible contribution of insulin resistance to vascular dysfunction within the liver, which could have an impact on a number of events involved in disease progression. Indeed, endothelial dysfunction with decreased NO production is thought to contribute to the progression of cirrhosis, since it is associated with increased vascular resistance [18,19] and hepatic stellate cell activation [14].

In the endothelium of peripheral vessels, insulin stimulates endothelial NO release through a Ca^{2+} independent pathway, via the activation of PI3-kinase and Akt, which phosphorylates eNOS on Ser^{1176} [36]. Disruption of insulin signaling, specifically at the endothelium, impairs both insulin induced vasodilation and ACh (endothelial) dependent vasodilation [8]. Furthermore, it has been shown that insulin resistance is associated with endothelial dysfunction [8–37], which is a key early event in the development of atherosclerosis [38]. In the present study, we evaluate whether these abnormalities, well described in peripheral vessels, also occur in the liver vasculature in an experimental model of NAFLD. For this purpose we evaluated, in a model of diet induced liver steatosis, whether the insulin signaling is impaired at the liver endothelium, both from a functional and molecular point of view. We choose to evaluate this in a model that does not exhibit inflammation or features of advanced liver disease (fibrosis), since it is well documented that liver endothelial dysfunction is present in advanced liver disease [18,19]. The complexity of the potential mechanisms involved in endothelial dysfunction at such advanced stages would preclude a direct evaluation of the role of insulin resistance. We demonstrate in the current study that the liver endothelium from HFD fed rats exhibits functional insulin resistance, demonstrated by an impaired ability of insulin to potentiate endothelium dependent vasodilation. Furthermore, we show impaired insulin signaling at the liver endothelium, as demonstrated by impaired eNOS phosphorylation at Ser^{1176} in response to an *in vivo* portal injection of insulin. We did not specifically evaluate Akt phosphorylation in response to insulin, an intermediate step in the insulin/PI3K/Akt/eNOS signaling cascade, since Akt

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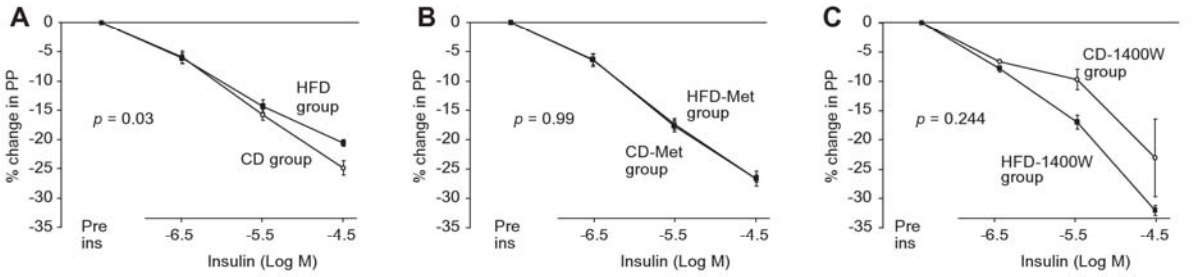

Fig. 2. Vasodilatory response to insulin in livers pre-constricted with methoxamine from rats fed a control diet (CD; black squares) or a high fat diet (HFD; white circles). (A) Livers from rats fed a HFD showed a blunted vasodilation to insulin as compared to rats fed a CD. Both (B) metformin and (C) 1400W treatment equaled liver vasodilatory response to insulin in HFD and CD groups. (PP, portal perfusion pressure).

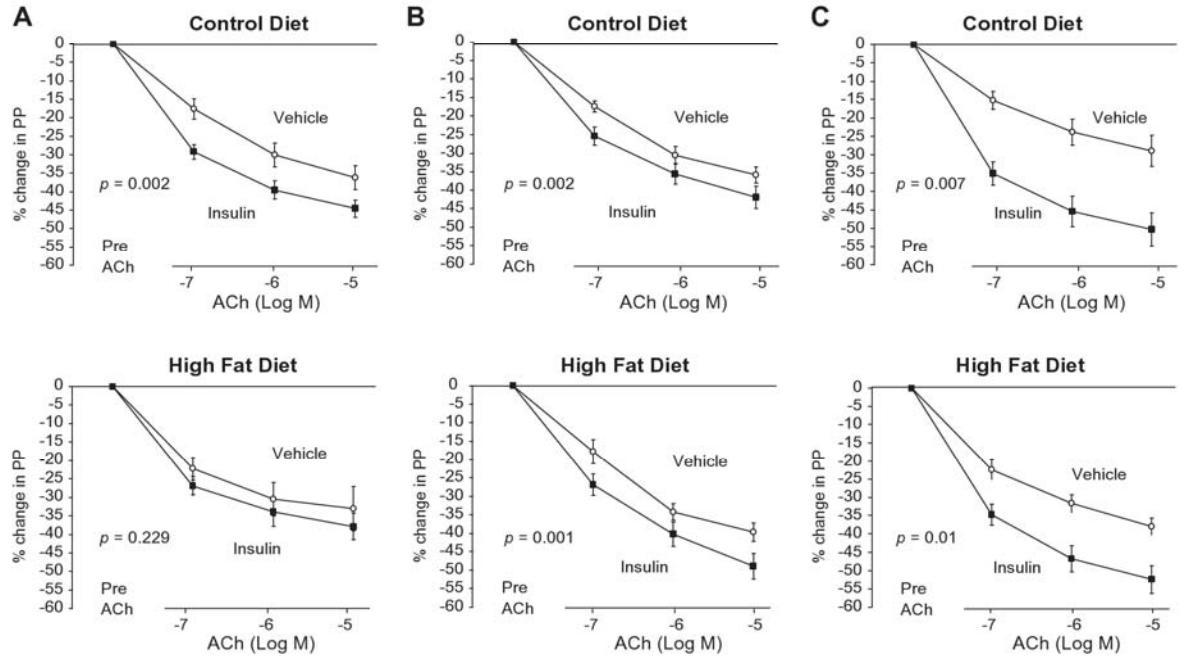

Fig. 3. Response to ACh in livers from control diet rats (CD) and high fat diet rats (HFD), in the presence (black squares) or absence (white circles) of insulin. (A) In rats treated with placebo, insulin enhanced endothelium dependent vasodilation in livers, in rats fed a control diet, but not in rats fed a HFD. (B) In rats treated with metformin, insulin significantly enhanced the endothelium dependent vasodilation both in control and in HFD rats. (C) In rats treated with the iNOS inhibitor 1400W, insulin significantly enhanced the endothelium dependent vasodilation both in control and in HFD rats. (ACh: acetylcholine; PP: portal perfusion pressure; Met: metformin).

is ubiquitously expressed in all liver cell types, while eNOS is selectively expressed in endothelial cells. In addition, we did not directly evaluate the effects of insulin on other cells with potential vasoactive relevance, such as hepatic stellate cells or vascular smooth muscle cells, so we cannot exclude the impairment in insulin signaling also extend to these cell types [39]. Finally, it is important to note that our model does not develop muscle or adipose insulin resistance [27]. Thus, our results are in keeping with previous data showing that a HFD induces insulin resistance in the vasculature before it develops in any other tissue [40].

In the present study, we further demonstrate that when the HFD is concomitantly administered with an insulin sensitizer, the development of hepatic vascular resistance to the effects of insulin is prevented. Metformin acts through a number of mechanisms (not completely understood) to improve glucose metabolism [41–43]. It has been also shown to improve vascular insulin sensitivity [44]. Our data clearly show that it improves insulin signaling at the liver endothelium, since it restores insulin induced eNOS phosphorylation. Whether this was a direct effect on insulin signaling or an indirect consequence of an improvement in the lipid deposit in the liver remains unexplained. In addition, metformin can induce eNOS phosphorylation through an insulin-independent mechanism [45,46]. In our experimental setting, this occurred in rats fed a control diet, which showed increased eNOS phosphorylation in baseline conditions, but not in those fed a HFD. Though this was not the primary aim of the

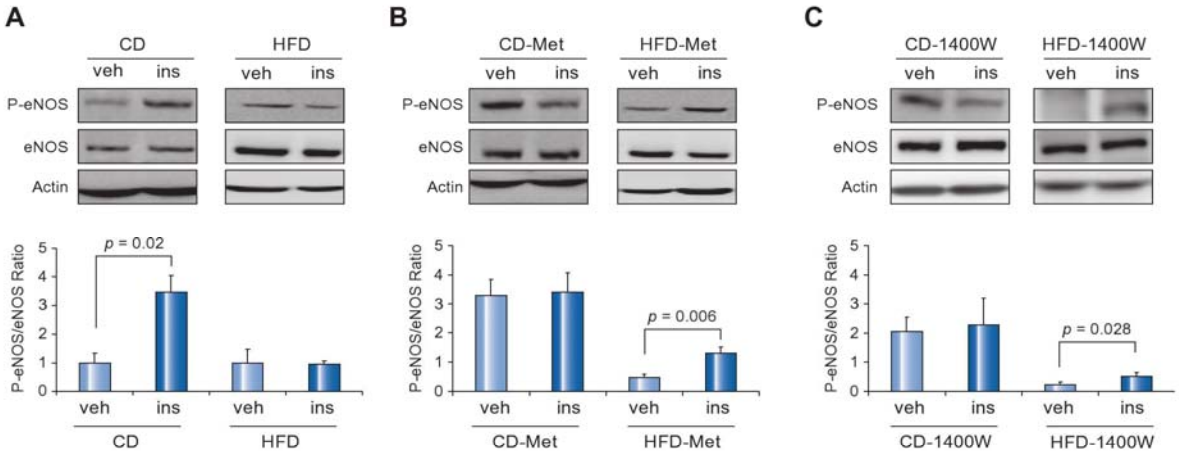

Fig. 4. Representative blots and densitometry readings of liver P-eNOS (at Ser^{1176}) to eNOS ratio (Western blotting), five minutes after a portal injection of vehicle (light blue bars) or insulin (dark blue bars). (A) Insulin increased eNOS phosphorylation in CD rats but not in HFD rats treated with placebo. (B) Treatment with metformin restored insulin induced eNOS phosphorylation in HFD rats. In the CD group, metformin increased baseline eNOS phosphorylation, that was not further enhanced by insulin. (C) Treatment with 1400W also restored insulin induced eNOS phosphorylation in HFD rats. All results were normalized to the control diet-vehicle group, which was run in every Western Blotting gel.

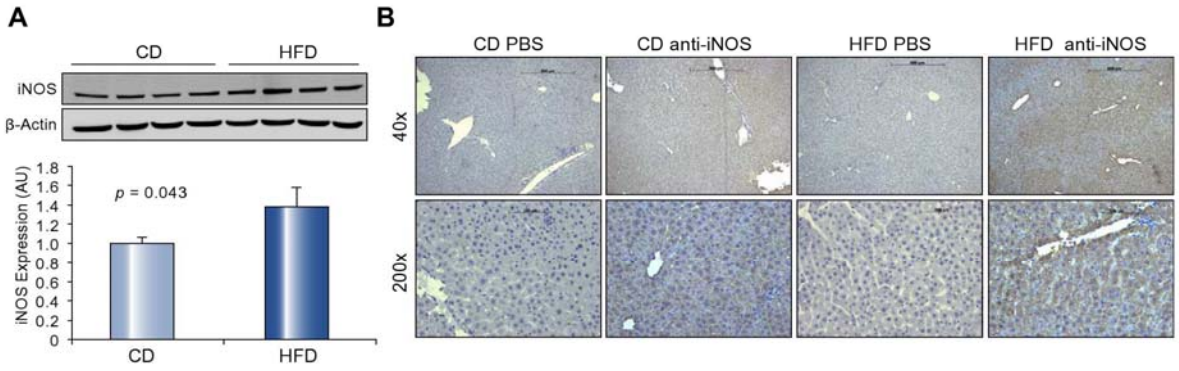

Fig. 5. A high fat diet (HFD) increases inducible Nitric Oxyde Synthase (iNOS) expression. (A) Representative blots and densitometry readings of liver iNOS to GAPDH in rats fed a control (CD) or a high fat diet (HFD) AU: Arbitrary Units. (B) Immunohistochemistry (iNOS immunostaining) in livers from HFD and CD rats, (original magnification: 40×; 200×). PBS: negative control.

study, these data suggest that such a short course of metformin is unable to improve hepatic eNOS phosphorylation in rats fed a HFD, but it is enough to restore, at least in part, the insulin sensitivity at the liver sinusoidal endothelial cells. This should not be taken as evidence of a clinical benefit of metformin in NAFLD, but does support the notion that metformin targets liver vascular insulin resistance in NAFLD, which might be of potential benefit in halting or slowing NAFLD progression. In addition, our preliminary data show that pioglitazone, an insulin sensitizer that acts through a different mechanism of action, also prevents the development of endothelial insulin resistance and, thus, might also share this beneficial effect on the liver endothelium.

Another finding of our study is a potential role of iNOS upregulation in the development of liver endothelial insulin-resistance. We confirm in our model other's findings showing increased iNOS expression in the liver after the administration of a high fat diet [47,48]. This was shown to impair insulin signaling by s-nitrosilation/nitrotyrosination of several proteins of the insulin signaling cascade [24]. Our data suggest that this also occurs at the liver endothelium, since iNOS inhibition restored insulin induced eNOS phosphorylation.

Our study has limitations inherent to the methodology we use. First, the isolated liver perfusion system is ideal to explore the responses of the liver vasculature to individual compounds, or to explore the interaction between the effects of two or more vasoactive mediators. However, it must be taken into account that the isolated perfused liver is deprived of the influence of the substances that reach the liver through the portal vein and the hepatic artery *in vivo*, and that might modulate the effects of the vasoactive mediators tested in the isolated system. In our case, non-fasting rats fed a HFD have a marked increase in the portal vein levels of free-fatty acids, which have been shown to be potent inducers of endothelial insulin resistance [49,50]. In the isolated liver perfusion, the liver is deprived from the contact

with plasma FFA for at least 25 min before the experiment is conducted and this might attenuate the effect of FFA on insulin resistance. Another limitation of this study is that we do not provide data on the effects of insulin in isolated liver endothelial cells. It must be noted, however, that the biological behavior of the liver endothelial cells is extremely dependent on the local milieu within the liver, and many of the specificities of these cells are lost early upon culture [51–54]. We believe that our functional experiments (endothelium dependent vasodilation with or without insulin) and the *in vivo* evaluation of insulin-induced eNOS phosphorylation at the liver tissue level (where eNOS is majorly expressed at the endothelium) are strong arguments to support the presence of liver endothelial insulin resistance.

In conclusion, the administration of a high fat diet induces hepatic steatosis and insulin resistance in the liver sinusoidal endothelium, which is mediated, at least in part, through iNOS upregulation and can be prevented by the administration of the insulin sensitizer metformin. Our findings further show that insulin resistance at the hepatic vasculature can be detected earlier than inflammation or any other sign of advanced NALFD, and suggest that it could contribute to disease progression. Further studies are needed to delineate the pathogenic relevance of these abnormalities in the progression of NALFD.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.01.053.

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Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD

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All authors declare that they have nothing to disclose.

Keywords: insulin resistance; NAFLD; sinusoidal endothelial dysfunction; eNOS

Abstract

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome. Most morbidity associated with the metabolic syndrome is related to vascular complications, in which endothelial dysfunction is a major pathogenic factor. However, whether NAFLD is associated with endothelial dysfunction within the hepatic vasculature is unknown. The aims of this study were to explore, in a model of diet-induced overweight that expresses most features of the metabolic syndrome, whether early NAFLD is associated with liver endothelial dysfunction. Wistar Kyoto rats were fed a cafeteria diet (CafD; 65% of fat, mostly saturated) or a control diet (CD) for 1 month. CafD rats developed features of the metabolic syndrome (overweight, arterial hypertension, hypertryglyceridemia, hyperglucemia and insulin resistance) and liver steatosis without inflammation or fibrosis. CafD rats had a significantly higher *in vivo* hepatic vascular resistance than CD. In liver perfusion livers from CafD rats had an increased portal perfusion pressure and decreased endothelium-dependent vasodilation. This was associated with a decreased Akt-dependent eNOS phosphorylation and NOS activity. In summary, we demonstrate in a rat model of the metabolic syndrome that shows features of NAFLD, that liver endothelial dysfunction occurs before the development of fibrosis or inflammation.

1. Introduction

The metabolic syndrome is defined as a combination of abnormalities including central obesity, hypertriglyceridemia, low levels of HDL cholesterol, hypertension and hyperglycemia [1]. Insulin resistance (IR) is thought to be the pathophysiological hallmark of the syndrome [2,3]. Non-alcoholic fatty liver disease (NAFLD) is the hepatic expression of the metabolic syndrome and has an increasing prevalence in the western population [4]. The spectrum of NAFLD lesions is wide, and goes from simple steatosis, non-alcoholic steatotohepatitis (inflammation, features of hepatocyte injury with or without fibrosis), to overt cirrhosis [5]. The mechanisms that account for disease progression in NAFLD are still poorly understood.

Most complications leading to morbidity in patients with the metabolic syndrome are of vascular origin [6]. One of the factors contributing to vascular disease in this setting is the presence of endothelial dysfunction, with decreased nitric oxide (NO) production [7], which has been consistently observed before cardiovascular events occur, and even before any pathological abnormalities in the vascular tree can be demonstrated [8]. This suggests that endothelial dysfunction is an early pathogenic event in the course of the vascular complications that occur in these patients. In keeping with this concept, correction of endothelial dysfunction is associated with an improvement in the rates of vascular events and, therefore, it is considered a useful therapeutic target in this syndrome [9,10]. Interestingly, patients with NAFLD exhibit systemic endothelial dysfunction and a increased cardiovascular risk [11].

The liver sinusoidal endothelium is a very specialized and phenotypically differentiated endothelium, being its major specificities the presence of fenestrae and the absence of basal membrane [12]. Among other functions, an adequately functioning sinusoidal

endothelium maintains an anti-inflammatory, anti-thrombotic and anti-fibrotic milieu within the liver parenchyma [13–15].

Some recent data have shown the presence of microvascular abnormalities in models of fatty liver, characterized by the presence of reduced sinusoidal perfusion [16] and structurally abnormal sinusoids due to lipid accumulation in parenchymal cells and to collagen deposition in the space of Disse [17]. However, the presence of liver endothelial dysfunction has not been specifically investigated. In addition, whether endothelial dysfunction might occur earlier than other features of advanced NAFLD (as it occurs in the peripheral circulation where endothelial dysfunction precedes the development of arteriosclerosis) is largely unknown.

The aims of this study were to characterize the changes in liver histology and liver microcirculatory function in a model of diet-induced obesity that expresses most features of the metabolic syndrome.

2. Materials and Methods

2.1 Animals, Diets, and Induction of Obesity

10 weeks old male Wistar Kyoto rats, weighing 225-250 grams, were caged individually in a 12:12-hour light-dark cycle, temperature -and humidity-controlled environment. The rats were divided into two dietary conditions. The first group (CD; n=15) was fed with standard chow diet for 1 month (which supplied 8% of calories as fat, type AO4; Panlab, Barcelona, Spain). The second group (CafD; n=15) was fed with cafeteria diet for 1 month. Cafeteria diet is a highly palatable diet consisting of a daily offering of cookies, liver pate, bacon, standard chow, and whole milk supplemented with 333 g/litter of sucrose and 10 g/litter of a mineral and vitamin complex (Meritene; Nestle HealthCare Nutrition, Esplugues de Llobregat, Spain) as previously described [18,19]. This diet contains 65% of the energy derived from fat that is predominantly saturated. All of the food items were weighed daily and presented in excess. Body weight was recorded daily. All rats were put on standard chow diet 24 hours before the experiments, to prevent any direct influence from the diet itself in the experimental results.

The animals were kept in environmentally controlled animal facilities at the Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS). All experiments were approved (ID: 3142, approved on 03/June/2005) by the Laboratory Animal Care and Use Committee of the University of Barcelona and were conducted in accordance with *Guide for the Care and Use of Laboratory Animals* (National Insitutes of Health, NIH Publication 86-23, revised 1996).

2.2 Biochemical Measurements

Blood samples were taken in fasting conditions from the tail vein. Plasma was separated within 15 minutes and frozen at -80°C for subsequent analysis. Liver transaminases,

glucose, bilirubin, triglycerides, free fatty acids (FFA) and insulin were analyzed with standard methods at the Hospital Clinic's CORE lab.

To determine the liver triglycerides and FFA content 1g of frozen liver tissue was homogenized in 3mL of HEPES (1:3 w/v) buffer composed of 50mM Tris, 150mM NaCl and 5mM EDTA (Sigma-Aldrich, Madrid, Spain) and analysed with standard methods at the Hospital Clinic's CORE lab.

2.3. Glucose tolerance test (GTT)

Rats were fasted for 4 hours before the administration of a glucose bolus (2 g/kg, i.p.; Braun Medical, Rubí, Spain). Glycemia was determined at 0, 15, 30, 60, 105 minutes after glucose administration with the AccuTrend glucose sensor (Roche Diagnostics, Sant Cugat del Valles, Spain).

2.4. Intraperitoneal Insulin Sensitivity Test

Non-fasted rats were given an i.p. injection of insulin (1 U/kg; Actrapid, NovoNordisk, Novo Allé, Denmark), and blood glucose levels were measured at 0, 15, 30, 60, 120, and 180 minutes after injection.

2.5. In vivo Hemodynamic Studies

Rats were anesthetized with ketamine (100 mg/kg, i.p., Imalgene, Barcelona, Spain) and midazolam (5 mg/kg, i.p., Laboratorio Reig Jofre, Barcelona, Spain) and maintained at constant temperature of 37 ± 0.5 ºC (continuously monitored during the experiment). A tracheostomy and cannulation with a PE-240 catheter (Portex, Kent, UK) was performed in order to maintain adequate respiration during the anaesthesia. Thereafter, PE-50 catheters were introduced into the femoral artery and the ileocolic vein, in order

to measure arterial pressure (MAP; mmHg) and portal pressure (PP, mmHg) respectively. A perivascular ultrasonic flow probe (2PR, 2-mm diameter. Transonic Systems Inc., Ithaca, NY, USA) placed around the portal vein measured the portal vein blood flow (PBF). Hepatic vascular resistance (HVR) was calculated as: $PP*PBF^{-1}$. Results of flow and resistance were indexed to liver weight. All measurements were continuously registered on a multichannel computer based recorder (Powerlab 4SP, ADInstruments, Mountain View, LA).

2.6. Isolated-Perfused Liver System

After haemodynamic measurements in vivo, livers were quickly isolated and perfused with Krebs' buffer in a recirculation fashion with a total volume of 100 mL at a constant flow rate of 35 mL/min [20]. 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 20 min before the studied substances were added. To assess the integrity of endothelial function, livers were preconstricted with metoxamine (Mtx) (10^{-4} M), an α-adrenergic agonist. After maximum vasoconstriction, increasing doses of the endothelium dependent vasodilator acetylcholine (ACh) (10^{-7}, 10^{-6} and 10^{-5} M) were added. Another group of livers (CD

n=6; CafD n=6) were perfused in the presence of the NO donor sodium nitroprusside (SNP; 10^{-3} M; Sigma-Aldrich, Madrid, Spain).

2.7. Western blot analysis

After haemodynamic studies, liver samples were immediately frozen in liquid nitrogen and kept at −70 °C until processing. The samples were processed as previously described [21]. Briefly, aliquots from each sample containing equal amounts of protein (60–80 μg) were run on a SDS–polyacrylamide gel, and transferred to a nitrocellulose membrane. Equal loading was ensured by Ponceau staining. The blots were subsequently blocked for 1 h and probed overnight (at 4 °C) with a mouse antibody recognizing eNOS (BD Transduction Laboratories, Lexington, KY), phosphorylated eNOS at Ser^{1176} (BD Transduction Laboratories, Lexington, KY), Akt (Cell Signaling Technology, Beverly, MA), P-Akt at Ser^{473}, SE-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or CD31 (Santa Cruz Biotechnology, California,CA). This was followed by incubation with rabbit anti-mouse (1:10,000) or goat anti-rabbit (1:10,000) HRP-conjugated secondary antibodies (Stressgen, Glandford Ave, Victoria, BC, Canada) for 1 h at room temperature. Blots were revealed by chemiluminescence and digital images were taken by a luminescent image analyzer LAS-3000 (Fujifilm Life Science, Tokyo, Japan). Protein expression was determined by densitometric analysis using the Science Lab 2001, Image Gauge (Fuji Photo Film Gmbh, Düsseldorf). Quantitative densitometry values of proteins were normalized to β-Actin or GAPDH and displayed in histograms. The degree of eNOS phosphorylation at Ser^{1176} and Akt phosphorylation at Ser^{473} was calculated as the ratio between the densitometry readings of P-eNOS/eNOS and P-Akt/Akt bands.

To evaluate the effects of insulin on eNOS phosphorylation, rats (n= 12) from the two study groups were anesthetized with ketamine (80 mg/Kg) and midazolam (5 mg/Kg). Rats were injected with insulin (5 UI) or a similar volume (500 μL) of saline through the ileocolic vein [22]. Five minutes later the rats were euthanized and liver samples were obtained and immediately frozen in liquid nitrogen and kept at −70 °C until processing.

2.8. Measurement of nitric oxide synthase activity

Nitric oxide synthase (NOS) activity was measured in homogenized livers from CafD and CD rats, by determining the conversion of 14C-labeled L-arginine to 14C-labeled L-citrulline, according to a previously reported method [23–25]. Enzymatic activity was expressed as nmol*min^{-1}*mg^{-1}protein.

2.9. Histopathology

Liver samples were fixed in 10% formalin, embedded in paraffin and sectioned (thickness of 2 μm). Slides were stained with hematoxylin and eosin (H&E) and Mason's Trichrome. Additional liver samples were frozen in liquid nitrogen, fixed in a freezing medium (Jung, Leica Microsystems, Nussloch, Germany) and stained with *Oil Red O* for 2 h at room temperature to detect neutral lipids. The samples were photographed, and analyzed using a microscope (Zeiss, Jena, Germany) equipped with a digital camera with the assistance of AxioVision softwares. The area of steatosis was quantified in 6 random photographs of each sample using AxioVision software.

2.10. Immunohistochemistry

Immunostaining of paraffin-embedded liver sections was performed with anti-CD43 (a pan-leucocyte marker) [26], anti-α-smotth muscle actin (α-SMA) or anti-CD34. Phosphate-buffered saline was used as a negative control. Bound antibody was visualized using diaminobenzidine as chromogen, and slides were then counterstained with hematoxylin. The number of CD43 positive cells was quantified using AxioVision software.

2.11. Statistics

Statistical analysis was performed using the IBM SPSS 19.0 statistical package (IBM, Armonk, NY). Comparisons of the baseline characteristics between groups were performed with the unpaired Student's *t*-test after confirming the assumptions of normality. Dose response curves were analysed with repeated measurements ANOVA introducing the type of diet as the between-subjects factor. All data are reported as means ± SD. Differences were considered significant at a p value <0.05.

3. Results

3.1 One-month cafeteria diet induces features of the metabolic syndrome and NAFLD.

Administration of high-fat palatable diet (cafeteria diet; CafD) for 30 days led to overweight, increasing the body weight gain by 33% as compared to rats fed the CD ($p=0.034$). This was associated with other features of the metabolic syndrome: fasting hyperglycemia, a markedly abnormal glucose tolerance test, fasting hyperinsulinemia, impaired response to insulin (insulin resistance) and arterial hypertension (Suppl fig 1 and Table 1). Plasma free fatty acids and triglycerides were markedly increased. (Table 1).

CafD increased liver weight, even after adjusting for body weight ($p=0.015$) (Table 1). This was associated with an increase in liver triglyceride and FFA content. Bilirubin levels were slightly increased, AST was not changed and ALT was decreased. CafD rats showed marked liver steatosis (fig 1), mainly located at the pericentral areas, without inflammation (assessed with IHC for CD43) (Fig 2A) or fibrosis (fig 1). There was no increase in alpha smooth muscle actin expression (α-SMA), suggesting that at this early stage of NAFLD HSCs were not activated (Fig 2B).

3.2 Splanchnic and systemic hemodynamics after cafeteria diet.

CafD induced arterial hypertension and a slight, but not significant, increase in *in vivo* portal pressure (Table 1). This was associated with a decrease in portal blood flow, indicating that CafD significantly increased *in vivo* intrahepatic vascular resistance (Table 1).

3.3. CafD induces endothelial dysfunction at the liver microvasculature.

To further characterize the abnormalities of the intrahepatic microcirculation induced by CafD we conducted experiments in the isolated perfused liver. *Ex- vivo* liver perfusion evaluates the liver microcirculation devoid of extra-hepatic influences, such as vasoactive mediators or changes in portal blood inflow, since the liver is perfused with a *clean* buffer at a constant flow. The *ex-vivo* portal perfusion pressure (PPP) was significantly increased in CafD rats as compared to CD rats (Fig. 3A). This difference disappeared when livers were perfused in the presence of the NO donor SNP (Fig 3B), suggesting that the increase in PPP was due to an increased hepatic vascular tone.

One of the canonic features of endothelial dysfunction is a decreased response to the endothelium dependent vasodilator acetylcholine (ACh). Therefore, after pre-constriction with methoxamine, we tested the response of the liver vasculature to increasing doses of ACh. The vasodilating response to ACh was significantly blunted in livers from CafD rats as compared to CD rats, thus showing that one-month cafeteria diet induces sinusoidal endothelial dysfunction (Fig 4A).

A well characterized mechanism that leads to systemic endothelial dysfunction in the metabolic syndrome is a decreased Akt-dependent eNOS phosphorylation (at Ser^{1176}) at the vascular endothelium, with ensuing decreased eNOS activity. To evaluate whether this was also the case at the intrahepatic circulation in our NAFLD model, we assessed Akt and eNOS phosphorylation in liver samples from CafD and CD rats. Hepatic eNOS is majorly expressed at the liver endothelium, and therefore it can be safely assumed that changes in eNOS phosphorylation in total liver are representative of changes at the endothelium (Suppl fig 2). Both P-Akt (at Ser^{473}, the active form of Akt) and P-eNOS were significantly decreased in CafD (Fig 4C and 4D). Furthermore, liver NOS activity

was decreased in CafD as compared to CD fed rats (Fig. 4B). Altogether, these results reinforce the concept that the liver endothelium exhibits endothelial dysfunction in our experimental model of early NAFLD.

To evaluate whether CafD impairs insulin to eNOS signalling at the liver endothelium we assessed the degree of eNOS phosphorylation (at Ser^{1176}) after a portal injection of insulin (5UI) or saline. Insulin increased P-eNOS/eNOS ratio in CD rats but not in CafD rats (Fig. 5), indicating that CafD induces liver endothelial insulin resistance.

The liver sinusoidal endothelium has differential characteristics from the vascular endothelium, among others the presence of fenestrae. One of the changes associated with advanced liver disease is a phenotypical shift in LSECs, including a loss in fenestrations. In vitro data suggest that the degree of LSEC fenestrations is closely reflected by the expression of SE-1 [27]. CafD did not decrease SE-1 expression, indirectly suggesting intact fenestrae in our model (Fig 6A). An additional feature that reflects the loss of the typical LSEC phenotype is the *de novo* expression of CD31 and CD34 at the sinusoids [28–33]. Both are expressed by vascular endothelial cells, but not LSECs [34]. There were no changes in liver CD31 expression between the two groups (fig 6B). In addition, IHC did not show *de novo* expression of CD34 at the sinusoids after one-month CafD (Fig 6C). Altogether, therefore, our data suggest that liver endothelial dysfunction occurs in our model of NAFLD earlier than inflammation, fibrosis or morphological changes in LSEC.

4. Discussion

Endothelial dysfunction is a major factor implicated in the development of arteriosclerosis and vascular complications in patients with the metabolic syndrome [35,36]. However, the endothelial phenotype and the regulation of endothelial function might have significant dissimilarities among different organs and tissues. Even within a single vascular territory there might be major differences in endothelial function between macro and microcirculations. Therefore, the pathophysiological events present in the peripheral vasculature cannot be directly extrapolated to the liver vasculature. The integrity of the liver sinusoidal endothelium is of utmost relevance for the maintenance of liver physiology, and disruption of sinusoidal endothelial function might have a prominent role in liver pathophysiology. Liver sinusoidal endothelial dysfunction, with decreased intrahepatic NO production, has been considered for years a relevant pathogenic factor in the progression of liver cirrhosis [37]. It has been demonstrated that decreased NO production contributes to increased intrahepatic vascular resistance and, therefore, to portal hypertension, but it is also though to contribute to other relevant mechanisms implicated in disease progression. An adequately functional sinusoidal endothelial cell tonically inhibits, through NO production, the activation of hepatic stellate cells (HSC) [14,38], thus being a potent natural antifibrotic. In addition, endothelial derived NO protects from microthrombotic events within the sinusoids [15], a well described mechanism of cirrhosis progression [39,40]. Further, a healthy sinusoidal endothelium is essential for liver regeneration [41]. Though intrahepatic endothelial dysfunction is most severe in advanced phases of cirrhosis (with ascites), less advanced disease (established cirrhosis without ascites) is still associated with (milder) endothelial dysfunction [42]. However, it is unknown whether sinusoidal endothelial dysfunction might precede the development of fibrosis at

the liver circulation., as it occurs in peripheral vascular disease in which endothelial dysfunction occurs earlier than structural changes of arteriosclerosis and is believed to represent the initial pathogenic event [43,44]. This could have a major therapeutic relevance, since liver sinusoidal endothelial dysfunction constitutes a druggable target with compounds already available in the market, such as statins [21,45].

In the present study we show, for the first time, functional features of intrahepatic endothelial dysfunction in a model of early NAFLD. We first show, in the complex *in vivo* setting, an increased hepatic vascular resistance (calculated from directly measured portal blood flow and portal pressure). To more precisely assess the abnormalities in the liver microcirculation we performed further studies in the isolated and perfused liver, demonstrating an impaired vadodilatory response of the liver vascular bed to acetyl-choline, the hallmark feature of endothelial dysfunction. In addition, we provide evidence of impaired endothelial function at the molecular level, showing decreased Akt-dependent eNOS phosphorylation. We and others [46] have demonstrated that liver eNOS expression is negligible outside endothelial cells, and so it can be safely assumed that changes in eNOS phosphorylation in liver homogenates represent changes at the liver endothelium. Specific phenotypical markers of LSECs, such as absence of CD34 and CD31 expression, were maintained in livers from CafD group. In addition, there were no changes in SE-1 expression, which has been shown (in *in-vitro* experiments) to closely reflect the degree of fenestrations. Thus, we provide here functional and molecular evidence of a dysfunctioning liver sinusoidal endothelium in the presence of phenotypically normal LSECs.

A major mechanisms that has been implicated in the development of endothelial dysfunction in the metabolic syndrome at peripheral vessels is insulin resistance itself,

since insulin, via Akt [47], stimulates endothelial NO release through a Ca^{2+} independent pathway. Disruption of insulin signalling specifically at the endothelium impairs endothelial dependent vasodilation [48,49]. We have recently shown that insulin signalling at the liver endothelium is disrupted as early as after 3 days of a high fat administration [50], and we confirm here this finding in our CafD model, suggesting that this mechanism could probably be a major contributor to the development of liver endothelial dysfunction in NAFLD.

Our model was appropriate to test our hypothesis, since it reproduces most features of the metabolic syndrome, such as overweight, arterial hypertension, hypertrigliceridemia and insulin resistance, and that causes liver steatosis not associated with inflammation or fibrosis, thus mimicking early human NAFLD. This model, based in the administration of a high fat diet with a lipid content that resembles that of ”fast food”, has been thoroughly used in metabolic studies, but data concerning the liver abnormalities associated with “cafeteria diet” are scant. The high content of saturated fat in this model, in contrast to high fat diets with high polyunsaturated fat content, that are associated with markedly increased levels of oxidative stress and which rapidly induce inflammation [51], probably reflects better the type of unhealthy diets that lead to NAFLD and the metabolic syndrome in clinical practice. In addition, this model could compare favourably as a model of NAFLD with genetic models of obesity such as *fa/fa* rats or *ob/ob* mice, in which critical pathways involved in liver injury are inactive, or with other widely used models such as methionine-choline deficient diet, which does not exhibit insulin resistance [52].

In summary, in this study we demonstrate, in a rat model of the metabolic syndrome that shows features of NAFLD, that liver endothelial dysfunction occurs before the development of fibrosis or inflammation. Therefore, liver endothelial dysfunction might be an early event implicated in disease progression in NAFLD, and might constitute a useful target for devising therapies for this disease.

Acknowledgements

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Figure legends

Figure 1. Hematoxylin/eosin (H/E), oil-red, Mason's Thricrome images and steatosis area from livers from rats fed a control diet (CD) or a high fat diet (CafD). (original magnification, x100 or x200 as displayed in the figures).

Figure 2. Liver inflammation and hepatic stellate cells phenotype. A) Immunohistochemistry showing CD43 (a pan-leukocyte marker) immunostaining in livers from high fat feeding (CafD) and control rats (CD). The administration for 1 month of a high fat diet did not induce liver inflammation (original magnification: x200). **B)** Cafeteria diet did not induce activation of HSC as assessed by immunohistochemistry for α-SMA.

Figure 3. Ex-vivo assessment of liver circulation. Portal Perfusion Pressure (PPP) in CafD (n=12) and CD rats (n=12) in the absence **(A)** or the presence **(B)** of the NO donor sodium nitroprusside (SNP). Livers from rats fed cafeteria diet showed an increased PPP. No differences were observed in the presence of SNP (CD: control diet; CafD: cafeteria diet).

Figure 4. CafD rats show liver endothelial dysfunction A) Response to ACh in livers from control diet rats (CD; black squares; n=6) and high fat diet rats (CafD; white circles; n=6). **B)** NOS activity in liver homogenates from control rats (CD; n=4) and cafeteria fed-rats (CafD; n=4). **C)** Representative blots and densitometry readings of liver P-Akt (at Ser^{473}) to Akt ratio and **D)** P-eNOS (at Ser^{1176}) to eNOS ratio (western blotting). AU: Arbitrary Units.

Figure 5. Representative blots and densitometry readings of liver P-eNOS (at Ser^{1176}) to eNOS ratio (western blotting), five minutes after a portal injection of vehicle (white bars and -) or insulin (black bars and +). Insulin increased eNOS phosphorylation in CD rats **(A)** but not in CafD rats **(B)**.

Figure 6. Endothelial cells phenotype. Cafeteria diet did not induce changes in **A)** SE-1 expression nor **B)** CD31, which are closely correlated with fenestrations and capillarization at the sinusoidal endothelial cells. **C)** Cafeteria diet did not induce *de novo* expression of CD34, a marker of loss of LSEC phenotype.

Supplementary data

Suppl figure 1. Rats fed a high fat diet (black squares) developed overweight and an impaired response to the glucose tolerance and insulin sensitivity tests. A) Body weight in CD and CafD during the study period. B) Glucose tolerance test. Blood glucose levels after an intraperitoneal (i.p.) injection of glucose (2 g/Kg) (n= 4 rats per group). C) Insulin sensitivity test. Glucose levels after an i.p. of insulin (5UI) (n= 4 rats per group).

Suppl figure 2. Protein expression of eNOS and Akt in the different liver cell types. GAPDH was used as a loading control: eNOS is detected selectively in LSECs. **(LSEC: liver sinusoidal endothelial cells. HSC: Hepatic stellate cells).**

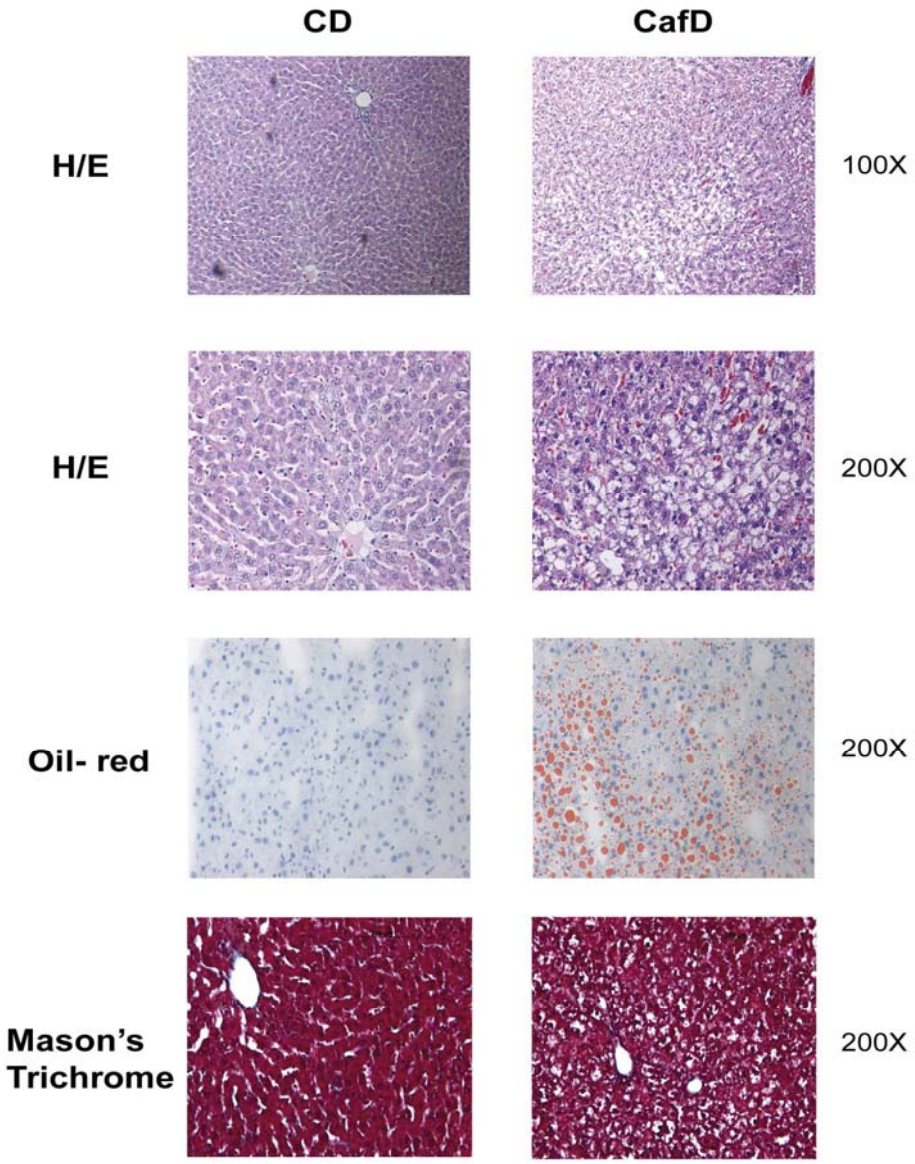
Methods: LSEC, Kuppfer cells and hepatocytes were isolated from CD rat livers (n=3) as described previously *(Gracia-Sancho J. et al. Hepatology 47: 1248-1256)*. Briefly, after perfusion of the livers with collagenase, hepatocytes and non-parenchymal cells were separated by centrifugation. Hepatocytes were washed twice with PBS and immediately lysed. Kuppfer cells and LSEC were isolated by isopycnic sedimentation (through a two-step density gradient Percoll) and pure monolayers were established by selective attachment on plastic or on collagen I,respectively. Cells were lysed after 12h of culture. Hepatic stellate cells were isolated from CD livers (n=3) as described previously *(Rodriguez-Vilarrupla A, et al. Liver Int 28: 566-573)*. Briefly, livers were perfused with Gey's balanced salt solution (GBSS), and digested at 37 °C with 0.01% collagenase, 0.01% DNAse and 0.004% pronase in GBSS. Cells were centrifuged at 50*g*, the supernatant was centrifuged at 800*g* and the pellet was then washed two times with Roswell Park Memorial Institute medium. Cells were grown in Iscove's modified Dulbecco's medium and lysed 3–5 days after isolation. Hepatic primary cells were homogenized in triton-lysis buffer. Aliquots from each sample containing equal

amounts of protein (20 μg) were run on an 8% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane.

Table 1. Baseline characteristics and hemodynamics of rats fed for 1 month a control (CD) or a cafeteria (CafD) diet. Data is presented as mean ± SD. (FFA: free fatty acids).

	Number of rats evaluated (CD/CafD)	CD	CafD	p
Body weight (g): Baseline	7 / 7	240 ± 5	239 ± 4	0.850
Body weight (g): 4 weeks	7 / 7	307 ± 5	328 ± 6	**0.001**
Liver weight (g)	7 / 7	9.1 ± 0.3	11.1 ± 0.4	**<0.001**
% liver/total weight	7 / 7	2.9 ± 0.1	3.3 ± 0.1	**0.005**
Blood glucose (mg/dL)	4 / 4	117 ± 8	151 ± 10	**0.003**
Plasma insulin (µU/mL)	4 / 4	35.9 ± 4.5	56.9 ± 8.4	**0.004**
Bilirubin (mg/dl)	4 / 4	0.10 ± 0.04	0.28 ± 0.10	**0.033**
AST (U/L)	4 / 4	85 ± 14	88 ± 8	0.656
ALT (U/L)	4 / 4	52 ± 9	32 ± 6	**0.014**
Plasma FFA (µM)	4 / 4	1613 ± 274	3214 ± 334	**0.001**
Plasma Triglycerides (mg/dL)	4 / 4	75 ± 13	200 ± 42	**0.026**
Liver FFA (umol*g liver^{-1})	4 / 4	9.2 ± 1.1	13.0 ± 1.1	0.070
Liver Triglycerides (mg*g of liver^{-1})	4 / 4	6.0 ± 0.2	8.8 ± 0.9	**0.035**
Mean Arterial Pressure (mmHg)	7 / 7	126.1 ± 5	153.1 ± 8	**0.005**
***In Vivo* Portal Pressure (mmHg)**	7 / 7	7.5 ± 2.1	9.7 ± 0.6	0.062
Portal Blood Flow (mL*min^{-1}*g of liver^{-1})	7 / 7	1.0 ± 0.2	0.7 ± 0.2	**0.050**
Hepatic Vascular Resistance (mmHg* g of liver*min* ml^{-1})	7 / 7	6.9 ± 0.9	15.4 ± 2.6	**0.027**

Figure 1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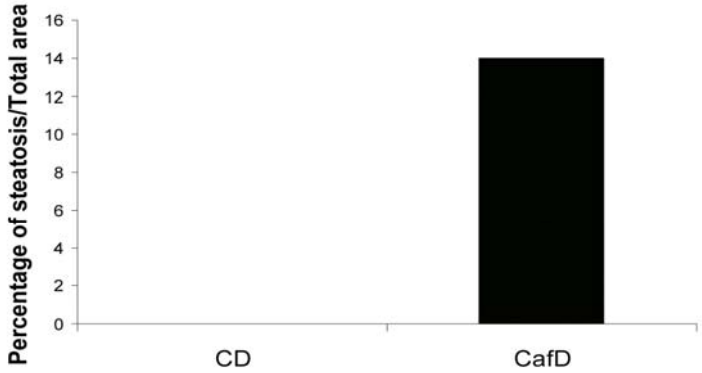
Percentage of steatosis/Total area
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CD
CafD

Figure 2

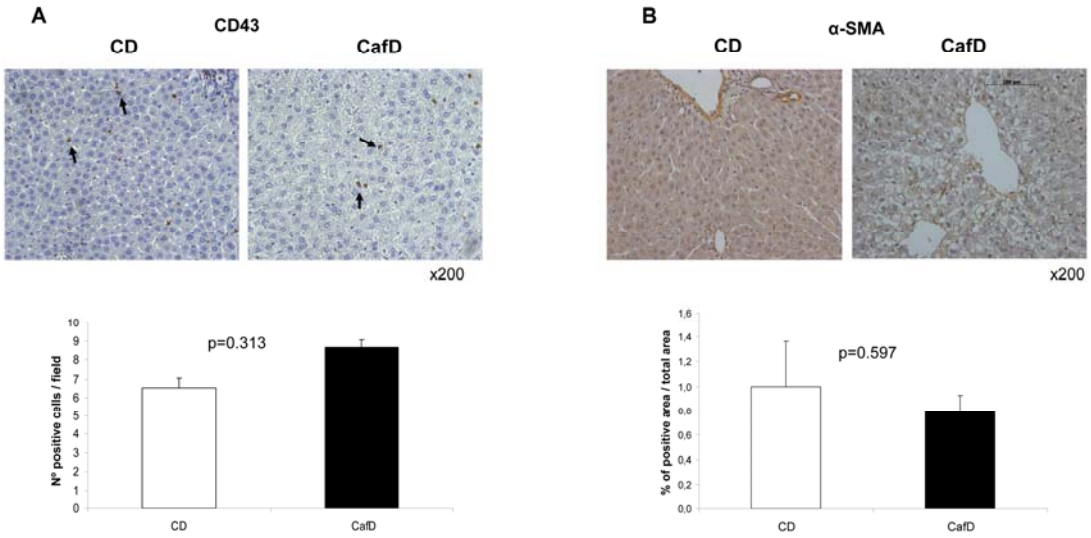


Figure 3

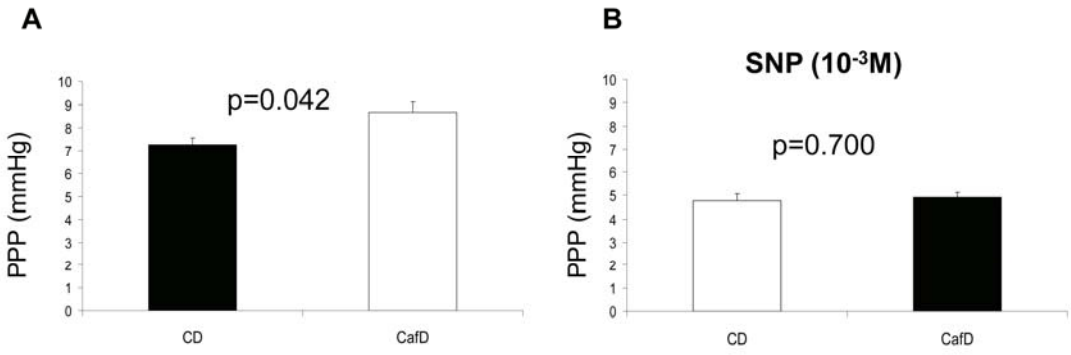


Figure 4

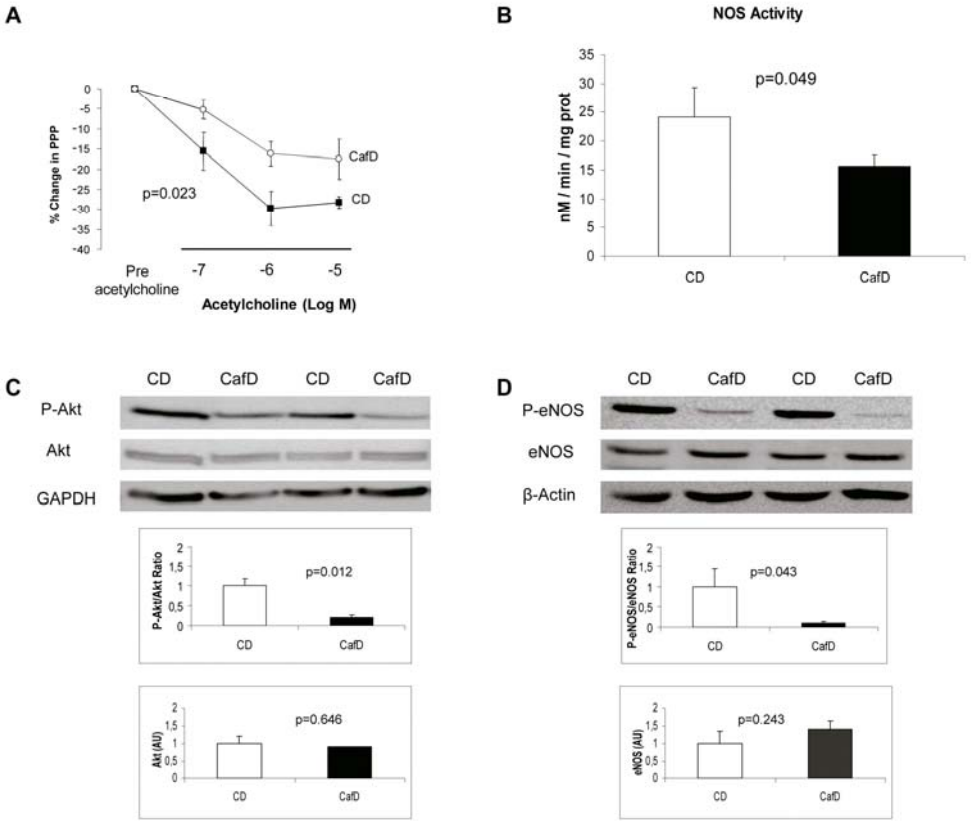


Figure 5

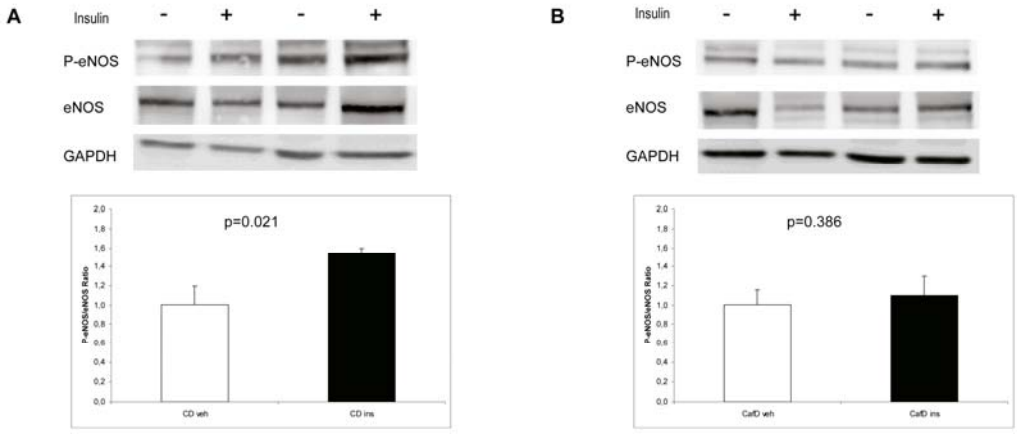
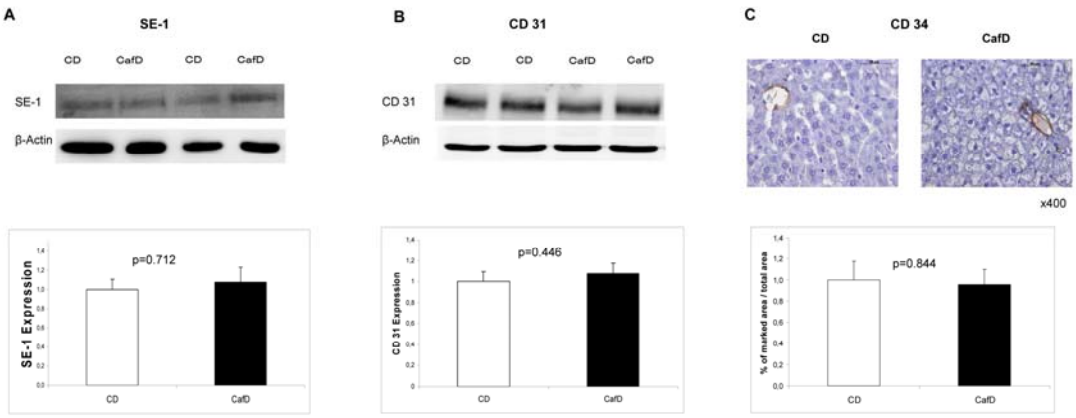


Figure 6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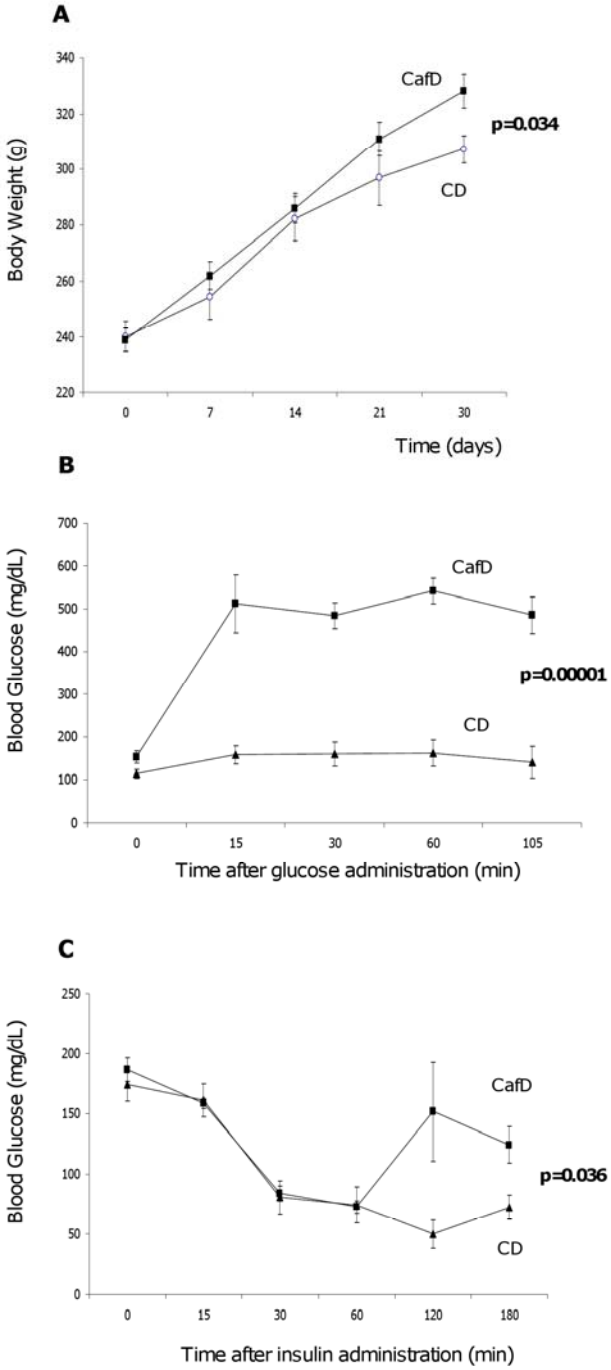
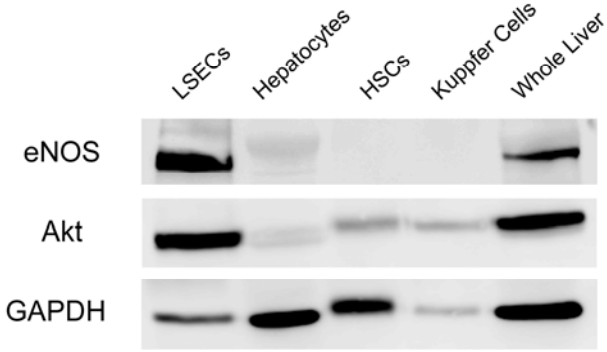
Supplemental Figure 1



Supplemental Figure 2



4. Resum dels Resultats

4.1- **Estudi 1: Insulin resistance and liver microcirculation in a rat model of early NAFLD, role of Inducible Nitric Oxide Synthase.**

- L'administració, durant 3 dies d'una dieta rica en greix en rates provoca esteatosi sense que s'apreciï cap signe d'inflamació. Això es troba associat a un increment intrahepàtic de triglicèrids, colesterol i àcids grassos lliures.
- Les rates esteatòsiques no presenten basalment un augment de la resistència vascular intrahepàtica.
- La insulina provoca vasodilatació dosi dependent en el fetge control, atenuada, però, en el fetge gras, la qual cosa suggereix la presència de resistència vascular a la insulina.
- Les rates esteatòsiques presenten resistència endotelial a la insulina, mesurada com una menor capacitat de la insulina per a potenciar la vasodilatació endoteli-dependent.
- A nivell molecular, això es tradueix a una menor capacitat de la insulina per augmentar la fosforilació d'eNOS (forma activa) respecte el vehicle en rates alimentades amb una dieta rica en greix respecte les rates alimentades amb una dieta control.
- El tractament amb un fàrmac sensibilitzador a la insulina (metformina o pioglitazona) atenua l'acumulació lipídica intrahepàtica en rates alimentades amb una dieta rica en greix.
- Tant la metformina com la pioglitazona prevenen el desenvolupament de resistència vascular a la insulina en rates esteatòsiques. A més, i al contrari que en les rates amb fetge gras no tractades, la insulina és capaç de potenciar la vasodilatació endoteli-dependent i augmentar la fosforilació d'eNOS després d'una injecció portal d'insulina.
- La inhibició de la iNOS en rates tractades durant 3 dies amb una dieta rica en greix atenua l'acumulació intrahepàtica de lípids, prevé el desenvolupament de resistència vascular a la insulina, restaura l'acció de la insulina potenciant la vasodilatació endoteli-dependet i augmenta

- la fosforilació d'eNOS després d'una injecció portal d'insulina respecte el vehicle.

4.2- **Estudi 2: Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD**

- En rates, l'administració durant 1 mes d'una dieta rica en greix (dieta de cafeteria) indueix un conjunt d'alteracions que pertanyen a la síndrome metabòlica: obesitat, hiperglicèmia, intolerància a la glucosa, resistència a la insulina i hipertensió arterial. A més a més, els nivells plasmàtics de triglicèrids i àcids grassos lliures estan augmentats.
- Les rates alimentades amb dieta de cafeteria presenten una relació pes del fetge/pes total augmentada respecte les rates alimentades durant un mes amb una dieta control.
- Aquesta dieta provoca un augment de nivells plasmàtics de bilirrubina, mentre que els de transaminases no estan elevats. Els fetges presenten esteatosi sense fibrosi, confirmat per la manca d'activació de les cèl.lules estrellades hepàtiques.
- La dieta de cafeteria indueix un lleuger increment de la pressió portal, associat a un menor flux portal, la qual cosa suggereix un augment de la resistència vascular intrahepàtica.
- A més, aquesta dieta provoca, en les rates, un increment significatiu en la pressió portal de perfusió a causa dels canvis funcionals, ja que aquest increment desapareix quan s'administra un vasodilatador.
- Les rates esteatòsiques presenten disfunció endotelial sinusoïdal, sense que es presentin canvis morfològics en les cèl.lules endotelials sinusoïdals.
- Aquesta disfunció endotelial sinusoïdal s'associa a una menor activació de la via AKT/eNOS, on les rates alimentades amb la dieta de cafeteria presenten una menor fosforilació tant d'Akt com d'eNOS, així com una menor activitat de l'eNOS.
- Tanmateix, en les rates esteatòsiques, la insulina no és capaç d'augmentar la fosforilació d'eNOS, la qual cosa sí succeeix en les rates alimentades amb dieta control.

5. Discussió dels Resultats

Els resultats del primer estudi aporten noves i importants dades fins ara no conegudes sobre els efectes de la insulina a nivell vascular, tant en fetges sans com en aquells esteatòsics. Per primera vegada demostrem que els fetges de rates alimentades durant només 3 dies amb una dieta rica en greix, presenten una menor resposta vasodilatadora a la insulina respecte a les rates alimentades durant tres dies amb una dieta normal. A més, els fetges esteatòtics presenten resistència a la insulina a nivell específicament de l'endoteli sinusoïdal hepàtic, relacionada amb una inducció de la iNOS. El tractament amb un fàrmac sensibilitzador de la insulina millora la resistència a la insulina a nivell endotelial i redueix l'acumulació de greix. Aquests canvis es van produir, com ja hem dit, després de només 3 dies d'administració d'una dieta rica en greix, els quals són suficients per a induir esteatosi i per a alterar la via de senyalització de la insulina, però abans que aparegui inflamació, fibrosi o altres signes que indiquin que ens trobem davant un estat avançat de la MHDG (117). Això vol dir que aquests successos primerencs podrien ser dianes per a possibles tractaments.

Una part dels pacients amb MHDG acaben desenvolupant cirrosi (118), però els mecanismes involucrats en aquest procés no estan molt definits. Aquest estudi incideix sobre la possible contribució de la resistència a la insulina en la disfunció vascular en el fetge. A més, es creu que la disfunció endotelial, caracteritzada per una menor producció endotelial de NO, contribueix a la progressió en la cirrosi, ja que està associada a un increment en la resistència vascular (119;120) i a una activació de les cèl.lules estrellades hepàtiques (121).

En l'endoteli dels vasos perifèrics, la insulina estimula la producció de NO d'origen endotelial mitjançant un mecanisme Ca^{2+}-independent, a través de l'activació de PI3K i Akt, la qual fosforila eNOS. La inactivació de la via de senyalització de la insulina, específicament en l'endoteli, afecta tant la vasodilatació induïda per insulina com la vasodilatació endoteli dependent (122). També s'ha vist que la resistència a la insulina està associada a disfunció endotelial (123;124), la qual sembla ser un element clau en el

desenvolupàment de l'aterosclerosi (125). En aquest estudi, vàrem estudiar si aquestes anormalitats, ben descrites en els vasos perifèrics, també ocorren a la vasculatura hepàtica en un model experimental de MHDG. Vam decidir avaluar aquestes alteracions en un model que no presenta ni fibrosi ni inflamació, degut a que està ben documentada que la disfunció endotelial hepàtica està present a fases avançades de la malaltia hepàtica (126;127). En aquest primer estudi demostrem que l'endoteli hepàtic de fetges amb MHDG presenten resistència funcional a la insulina, demostrada per una menor capacitat de la insulina per potenciar la vasodilatació dependent d'endoteli. A nivell molecular, demostrem que la via de senyalització de la insulina està alterada, a causa d'una menor fosforilació de l'eNOS (en Ser^{1176}) en resposta a una injecció portal *in vivo* d'insulina. No hem avaluat la fosforilació d'Akt en resposta a insulina, un pas intermig de la cascada de senyalització de la insulina, perquè mentre que eNOS s'expressa específicament a l'endoteli, Akt ho està ubiquament. A més, no vam avaluar els efectes de la insulina sobre altres cèl.lules amb potencial efecte vascular, com les cèl·lules estrellades hepàtiques o les cèl.lules muscular llises, per la qual cosa no podem excloure que l'afectació de la via de senyalització de la insulina no ocorri també en aquests tipus cel·lulars (128). Finalment, és important remarcar que el nostre model no desenvolupa resistència a la insulina ni a nivell muscular ni adipós (129). Aquesta dada va en concordància amb un altre estudi que indica que la resistència a la insulina induïda per una dieta rica en greix apareix abans a la vasculatura que no a qualsevol altre teixit (130).

Una altra troballa important d'aquest estudi és que quan la dieta rica en greix és administrada conjuntament amb un fàrmac sensibilitzador de la insulina, es prevé l'aparició de resistència vascular hepàtica a la insulina. La metformina actua a través d'una sèrie de mecanismes (molts d'ells encara desconeguts) per a millorar el metabolisme de la glucosa (131-133) així com també la funció vascular (134;135). Diversos grups han demostrat que els efectes de la metformina es donen tan aviat com en 24 hores en cultius cel·lulars (136;137) i 3 dies en un altre model murí (138). Les nostres dades mostren com la metformina restaura la fosforilació d'eNOS induïda per insulina la qual cosa demostra clarament com la metformina millora la senyalització de la insulina a l'endoteli hepàtic. Queda per demostrar, però, si aquests efectes són causats

per un efecte directe sobre la via de senyalització de la insulina o a la reducció del contingut lipídic intrahepàtic. La metformina també pot promoure la fosforilació d'eNOS mitjançant un mecanisme independent de la insulina (139;140). En la nostra aproximació experimental això va ocórrer en les rates alimentades amb dieta control, les quals van mostrar un augment en la fosforilació d'eNOS en condicions basals, però no en aquelles alimentades amb una dieta rica en greix. Aquestes dades suggereixen que un tractament tan curt de metformina és incapaç de millorar la fosforilació a nivell hepàtic d'eNOS en rates alimentades amb una dieta rica en greix, però que és capaç de restaurar, com a mínim en part, la sensibilitat a la insulina en cèl.lules sinusoïdals endotelials hepàtiques. Aquests resultats no impliquen de forma directe un benefici clínic de la metformina en la MHDG, però dona suport a la idea que la metformina actua sobre la resistència a la insulina vascular hepàtica en la MHDG. A més, les nostres dades preliminars indiquen que la pioglitazona, un fàrmac sensibilitzador de la insulina, que actua per mecanismes diferents a la metformina, també prevé el desenvolupament de resistència endotelial a la insulina, i, per tant, pot compartir aquest efecte beneficiós sobre l'endoteli hepàtic.

Una altra troballa clau del nostre estudi és el paper potencial de la regulació a l'alça de la iNOS en el desenvolupament de la resistència endotelial hepàtica a la insulina. En el nostre model confirmem els resultats d'altres estudis que demostren una sobreexpressió d'iNOS en el fetge després de l'administració d'una dieta rica en greix (141;142). Aquesta regulació a l'alça d'iNOS pot alterar la via de senyalització de la insulina a causa de la nitrosilació i nitrotironització de moltes proteïnes de la cascada de senyalització de la insulina tant a múscul esquelètic (143) com al fetge (144). Les nostres dades suggereixen que això també succeeix a l'endoteli hepàtic, ja que la inhibició d'iNOS va restaurar la fosforilació d'eNOS induïda per la insulina.

En el nostre model, les rates alimentades amb una dieta rica en greix van tenir un increment significatiu dels nivell d'àcids grassos lliures a la vena porta, els quals, s'ha vist que són potents inductors de resistència a la insulina (145;146).

En resum, l'administració d'una dieta rica en greix indueix esteatosi hepàtica i resistència a la insulina en l'endoteli sinusoïdal hepàtic, la qual està mitjançada, com a mínim en part, per la regulació a l'alça de la iNOS, i pot ser previnguda

per l'administració del fàrmac sensibilitzador de la insulina, la metformina. Les nostres troballes demostren també que la resistència a la insulina a la vasculatura hepàtica pot ser detectada abans que la inflamació o qualsevol altre signe que indiqui una fase avançada de la MHDG, i suggereixen que podria contribuir a la progressió de la malaltia.

El segon treball presentat en aquesta tesi doctoral es centrà en estudiar els canvis histològics i la funció vascular hepàtica en un model d'inducció d'obesitat causada per l'administració d'una dieta rica en greixos saturats amb la majoria de característiques de la síndrome metabòlica, com són la hipertensió, hipertensió arterial, hipertrigliceridèmia i resistència a la insulina, que causa esteatosi no associada amb inflamació o fibrosi.

La disfunció endotelial és un dels principals factors implicats en el desenvolupament de l'arterosclerosi i complicacions vasculars en pacients amb síndrome metabòlica (147;148). Però, el fenotip endotelial i la regulació de la funció endotelial poden tenir diferències importants entre els diferents òrgans i teixits. Inclòs en un mateix territori vascular poden existir diferències en la funció endotelial entre la micro i la macrocirculació. Per tant, els esdeveniments fisiopatològics que poden succeir en la vasculatura perifèrica no poden ser extrapolats directament a la vasculatura hepàtica. La integritat de l'endoteli sinusoïdal hepàtic és de vital importància per a la fisiologia hepàtica. L'alteració en el correcte funcionament de l'endoteli sinusoïdal podria tenir un paper en la fisiopatologia del fetge. La disfunció endotelial sinusoïdal hepàtica, amb una menor producció de NO d'origen endotelial intrahepàtic, ha estat considerada durant anys un factor patogènic molt rellevant en la progressió de la cirrosi hepàtica (149). S'ha demostrat que una menor producció d'NO contribueix a una resistència vascular intrahepàtica augmentada i, per tant, a l'hipertensió portal; però també es creu que podria contribuir a altres mecanismes rellevants implicats en el desenvolupament de la malaltia. Una funcionalitat adequada de la cèl·lula endotelial sinusoïdal inhibeix tònicament, mitjançant el NO, l'activació de les cèl.lules estrellades hepàtiques (150;151); per tant, el NO actua com a potent antifibròtic. A més, el NO d'origen endotelial protegeix de microtrombos als sinusoïds (152), i aquest és un mecanisme descrit implicat en la progressió de la cirrosi (153;154). Un endoteli sa és essencial per a la regeneració

hepàtica (155). Encara que la disfunció endotelial intrehepàtica és més severa a les fases avançades de la cirrosi (amb ascitis), també es troba present en fases més primerenques (156;157). No es coneix, però, si la disfunció endotelial hepàtica podria precedir l'aparició de fibrosi, com sí que s'observa a la malaltia vascular perifèrica en la qual la disfunció endotelial apareix abans que els canvis estructurals arterioscleròtics i es creu que representa el primer esdeveniment patogènic (158;159). Aquest fet podria ser d'una gran importància terapèutica, a causa que la disfunció endotelial sinusoïdal hepàtica pot ser tractada farmacològicament amb drogues ja existents al mercat, com ara les estatines (160;161).

En aquest estudi, mostrem, per primer cop, la presència de disfunció endotelial intrahepàtica en un model de MHDG en fases primerenques. Aquest model presenta una resistència vascular hepàtica incrementada (calculada a través de la mesura directa de flux i pressions portals). Per a aprofundir en les anormalitats de la microcirculació intrahepàtica, vam realitzar experiments de perfusió de fetge aïllat, demostrant una resposta vasodilatadora disminuïda de la vasculatura hepàtica a l'acetilcolina, la principal prova de disfunció endotelial. A més, mostrem evidències d'una funció endotelial alterada a nivell molecular, mostrant una fosforilació reduïda d'eNOS dependent d'Akt. Encara que aquests experiments s'han realitzat en homogenats de fetge total, demostrem con l'expressió d'eNOS és negligible fora de la cèlul.la endotelial; dades que van en concordància amb les trobades per altres grups (162). Per tant, podem assumir que els canvis en la fosforilació d'eNOS en els homogenats hepàtics representen canvis que es produeixen en l'endoteli hepàtic. No es trobaren canvis en marcadors específics de cèl.lules endotelials sinusoïdals hepàtiques, com la presència de fenestres (en forma d'absències de canvis al marcador SE-1) o l'absència d'expressió dels marcadors CD34 i CD31 en els fetges de rates alimentades amb una dieta rica en greix.

Un dels principals mecanismes implicats en el desenvolupament de disfunció endotelial en la síndrome metabòlica és la resistència a la insulina, a causa que la insulina, via Akt (163), estimula la producció de NO endotelial de manera Ca^{2+}-independent. La intervenció a l'endoteli de la via de senyalització de la insulina redueix la vasodilatació dependent d'endoteli (164;165). Tal com hem demostrat en l'article anterior, la via de senyalització de la insulina en l'endoteli

es troba afectada ja als 3 dies d'administrar una dieta rica en greix (166) i ho confirmem en el present treball, demostrant que aquest mecanisme podria contribuir de manera molt important en el desenvolupament de la disfunció endotelial en la MHDG.

En resum, en aquest estudi demostrem que en un model de síndrome metabòlica induïda per l'administració durant un mes d'una dieta rica en greix a rates, la presència de disfunció endotelial hepàtica abans de l'aparició d'inflamació o fibrosi. Per tant, la disfunció endotelial hepàtica podria ser un factor primerenc implicat en la progressió de la MHDG, convertint-se en una diana potencial per a tractar aquesta malaltia.

6. Conclusions

4.1- **Estudi 1: Insulin resistance and liver microcirculation in a rat model of early NAFLD, role of Inducible Nitric Oxide Synthase.**

- L'administració durant 3 dies d'una dieta rica en greix produeix esteatosi hepàtica sense que s'observi cap signe d'inflamació.
- Les rates esteatòtiques presenten resistència vascular a la insulina.
- Més concretament, les rates alimentades amb una dieta rica en greix durant 3 dies presenten resistència endotelial a la insulina.
- El pretractament amb un fàrmac sensibilitzador de la insulina en fetges de rates alimentades amb una dieta rica en greix prevé el desenvolupament de resistència vascular a la insulina.
- Aquest pretractament prevé també l'aparició de resistència endotelial a la insulina en rates esteatòtiques.
- Aquestes alteracions estan mitjançades, com a mínim en part, per una regulació a l'alça de la iNOS.
- Els resultats obtinguts en aquest estudi suggereixen que la resistència vascular a la insulina és una alteració que apareix en les primeres fases de la MHDG i que podria contribuir a la progressió de la malaltia.

4.2- **Estudi 2: Sinusoidal endothelial dysfunction precedes inflammation and fibrosis in a model of NAFLD**

- L'administració durant un mes d'una dieta rica en greixos saturats en rates provoca alteracions de la síndrome metabòlica així com resistència a la insulina.
- Després d'un mes de dieta rica en greixos, les rates presenten un augment de la pressió portal de perfusió, resistència endotelial a la insulina i disfunció endotelial sinusoïdal, caracteritzada per una menor activació de la via Akt/eNOS i una conseqüent menor producció d'NO.
- Aquests canvis es produeixen en absència d'inflamació, d'activació de les cèl.lules estrellades hepàtiques i de canvis morfològics de les cèl.lules endotelials sinusoïdals.

- Sempre tenint en compte les limitacions a l'hora de traslladar les dades obtingudes en models animals als humans, els resultats obtinguts en aquest estudi reforçarien la idea de considerar la disfunció endotelial hepàtica com un esdeveniment primerenc que podria constituir una diana terapéutica per tal de tractar aquesta malaltia.

7. Referències Bibliogràfiques

Referències Bibliogràfiques

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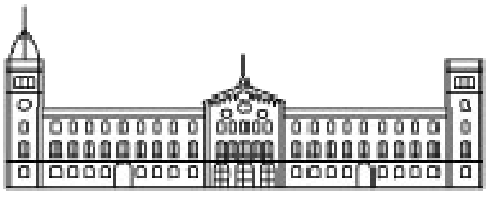

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