UNIVERSITAT DE BARCELONA

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5 años, 60 meses, 21900 días, 524.600 horas, 31.536.000 minutos, 1.892.160.000 segundos…es el tiempo que he dedicado en acabar este proyecto de vida que es la tesis.

Han sido años, meses, días, horas, minutos y segundos a veces complicados a veces insoportables a donde solo quería dejar, pero al final todo ha ido a su lugar, todo ha encajado para que este proyecto pudiera convertirse en realidad.

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5 años, 60 meses, 21900 días, 524.600 horas, 31.536.000 minutos, 1.892.160.000 segundos …soy más viejo, más maduro, más luchador y totalmente cierto que…siempre acabamos llegando a donde nos esperan.

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ABREVIATURAS

11β-HSD1	11β-hidroxiesteroide deshidrogenasa de tipo I
11β-HSD2	11β-hidroxiesteroide deshidrogenasa de tipo II
ABCA1	*ATP-binding cassette transporter* A1
ABCG1	*ATP-binding cassette transporter* G1
ACAT	Acil-CoA:colesterol-aciltransferasa
ACTH	Hormona adenocorticotropina
AP-1	Proteína activadora 1
apoE	Apolipoproteína E
BVT.2733	Inhibidor de la enzima 11β-HSD1
CBG	Globulina ligadora de corticosteroides
CD163	Cluster de diferenciacion 163
CETP	Proteína de transferencia de ésteres de colesterol
CKO	Grupo de ratones *knockout* con dieta control
CLS	*Crown-like structures*
COX-2	Ciclooxigenasa 2
CRH	Hormona liberadora de corticotropina
CWT	Grupo de ratones *wild type* con dieta control
DM2	Diabetes mellitus tipo 2
DUSP-1	*Dual specificity phophatase 1*
ECV	Enfermedad cardiovascular
EMR1	*Epidermal growth factor module-containing mucin-like receptor 1*
FAT/CD36	Translocasa de ácidos grasos
G6PT	Transportador de glucosa-6-fosfato
GCr	Receptor de glucocorticoides
GCs	Glucocorticoides
GILZ	*Glucocorticoid-induced leucine zipper*
GLUT4	Transportador de glucosa 4
GRE	*Glucocorticoid-response elements*
H6PDH	Hexosa-6-fosfato deshidrogenasa
HDL	Lipoproteínas de alta densidad
HLKO	Grupo de ratones *knockout* con dieta hiperlipídica
HLWT	Grupo de ratones *wild type* con dieta hiperlipídica
HPA	Eje hipotálamo-hipófisis-adrenal
HSL	Lipasa sensible a hormonas
IkB	Inibidor de NF-κB
IL-1	Interleucina 1
IL-10	Interleucina 10
IL-13	Interleucina 13
IL-1β	Interleucina 1β
IL-2	Interleucina 2
IL-6	Interleucina 6
IL-8	Interleucina 8
IMC	Índice de masa corporal
JNKs	*c-Jun NH2-terminal kinases*
LDL	Lipoproteínas de baja densidad

LPL	Lipoproteína lipasa
LPS	Lipopolisacáridos
LXRα	Receptor hepático X alfa
M1	Macrófagos activados clásicamente
M2	Macrófagos activados alternativamente
MAPK	*Mitogen-activated protein kinase*
MC4R	Receptor de melanocortina 4
MCP-1	Proteína quimioatrayente de monocitos 1
MKP-1	*Mitogen-activated protein kinase phosphatase 1*
MMR	Receptor de manosa de macrófagos
MPO	Mieloperoxidasa
MRr	Receptor de mineralocorticoides
NCEH	*Neutral cholesterol ester hydrolase*
NEFA	Ácidos grasos no esterificados
NF-kB	Factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas
NLR	NOD-like receptors
NPY	Neuropéptido Y
OMS	Organización mundial de la salud
OxLDL	Lipoproteínas de baja densidad oxidadas
P2Y2R	Receptor purinérgico 2Y2
PAI-1	Inhibidor del activador del plasminógeno-1
PC1	Proconvertasa 1
PEPCK	Fosfoenolpiruvato carboxiquinasa
PMA	Ésteres de forbol
POMC	Proopiomelanocortina
PPAR-α	*Peroxisome proliferator-activated receptor α*
PPAR-β	*Peroxisome proliferator-activated receptor* β
PPAR-γ	*Peroxisome proliferator-activated receptor* γ
rIL10	Receptor de IL-10
SEGRA	Agonistas selectivos de los receptores de glucocorticoides
SERPIN	Inhibidores de serina proteasas
SM	Síndrome metabólico
SNPs	Polimorfismos de un único nucleótido
SRA1	*Scavenger receptor* A1
SRB1	*Scavenger receptor* B1
TA	Tejido adiposo
TAB	Tejido adiposo blanco
TAM	Tejido adiposo marrón
TAS	Tejido adiposo subcutáneo
TAV	Tejido adiposo visceral
TCR	Receptor de células T
TGFβ	Factor de crecimiento transformante β
THP-1	Línea celular monocítica humana
TNF-α	Factor de necrosis tumoral α
TLR2	*Toll-like receptor 2*

VEGF	Factor de crecimiento endotelial vascular
VLDL	Lipoproteínas de muy baja densidad

ÍNDICE

INTRODUCCIÓN

1. Glucocorticoides

1.1 Síntesis adrenal y extraadrenal

Los glucocorticoides (GCs) son una clase de hormonas esteroideas que se sintetizan en la zona fasciculada de la corteza de la glándula suprarrenal a partir del colesterol. Su secreción está regulada por el eje hipotálamo-hipófisis-adrenal (HPA) sobre el que los GCs ejercen una retroalimentación negativa (figura 1)[1]. La activación del eje comienza con la secreción de la hormona liberadora de corticotropina (CRH) en el núcleo paraventricular del hipotálamo. La CRH viaja a través del sistema porta-hipofisario y estimula a la hipófisis anterior para que sintetice y libere la hormona adenocorticotropina (ACTH) que a su vez viaja por el torrente sanguíneo y estimula, a nivel de la glándula adrenal, la síntesis de glucocorticoides[2,3].

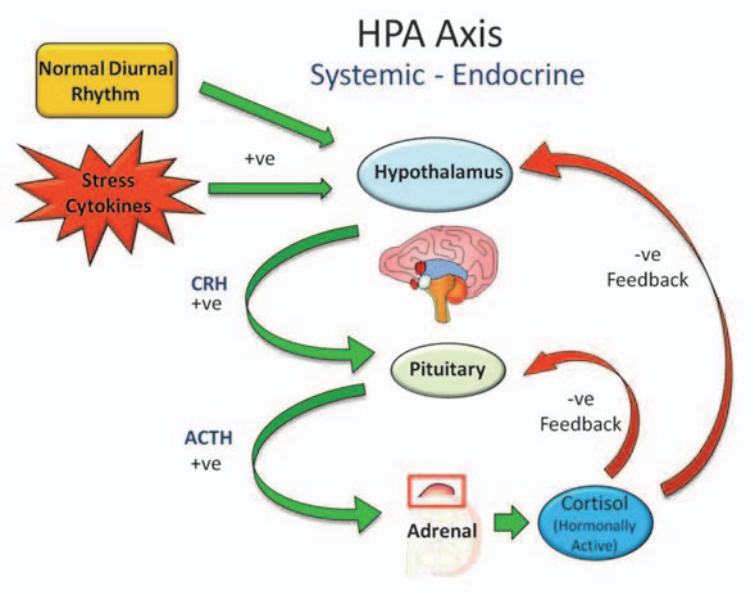


Figura 1. Regulación sistémica de los niveles circulantes de cortisol por el eje HPA[1] .

El principal glucocorticoide activo en los seres humanos es el cortisol (corticosterona en roedores) y su secreción por la glándula suprarrenal sigue un ritmo circadiano muy marcado. Situaciones de estrés tanto físico como psicológico activan el eje HPA y los niveles de GCs circulantes incrementan y provocan una pérdida del ritmo circadiano.

Los GCs circulan en sangre unidos a la globulina ligadora de corticosteroides (CBG), de la que se hablará extensamente más adelante, que regula su biodisponibilidad dado que sólo una pequeña fracción de la concentración total de la hormona se encuentra libre en

sangre[4] y es la que se considera puede acceder al interior tisular y ejercer el efecto correspondiente.

La denominación GCs se refiere tanto a las formas activas (cortisol en humanos y corticosterona en roedores) como a las formas inactivas (cortisona en humanos y 11-dehidrocorticosterona en roedores). Al margen de la síntesis adrenal, existe una vía de síntesis/degradación tisular constituida por las enzimas 11β-hidroxiesteroide deshidrogenasa de tipo I (11β-HSD1) y de tipo II (11β-HSD2). *In vivo*, la enzima 11β-HSD1 convierte predominantemente glucocorticoides inertes en las formas activas correspondientes (figura 2). La enzima 11β-HSD1 tiene una amplia distribución en el organismo, estando presente mayoritariamente en el hígado, el tejido adiposo, gónadas, sistema nervioso central, riñón, piel, músculo, hueso, ojo, glándula adrenal, pituitaria y tejidos linfoides[4,5].

La expresión de la enzima 11β-HSD2 (figura 2), que cataliza la reacción opuesta[1], está restringida a tejidos diana de mineralocorticoides como el riñón, piel, pulmón y la corteza suprarrenal[6]. Tanto la presencia de CBG como de ambos enzimas constituyen un punto esencial de regulación de la biodisponibilidad de los GCs y por tanto, de regulación de la acción glucocorticoide a nivel local[4].

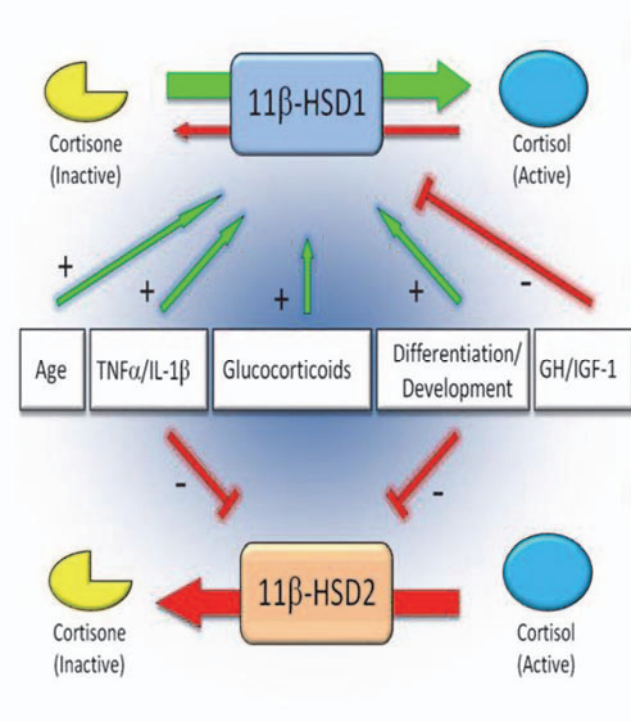


Figura 2. Actividad de la enzima 11β-HSD1 que activa cortisona a cortisol, y la enzima 11β-HSD2, que cataliza la reacción inversa [1].

La función de la 11β-HSD1 es la de amplificar la señal de los glucocorticoides circulantes y permitirla/intensificarla en el interior celular. Recientemente se ha demostrado que la ausencia de 11β-HSD1 en ratones transgénicos es capaz de impedir los efectos del exceso de GCs circulantes, como esteatosis, deposición del tejido adiposo blanco, hipertensión y resistencia a la insulina[7]. Estos efectos también revierten en ratones que presentan la enzima 11β-HSD1 inactivada únicamente en tejido adiposo mientras que se reproducen cuando se bloquea la presencia de la enzima únicamente en hígado[7] destacando el papel de la 11β-HSD1 en tejido adiposo como principal reguladora de los efectos metabólicos de los glucocorticoides[8]. Apoya también la función mediadora de la 11β-HSD1 el hecho de que los GCs incrementen la actividad y expresión de la enzima[9].

1.2 Receptores de los glucocorticoides y mecanismo de acción

Los GCs ejercen sus acciones en los tejidos diana utilizando mecanismos genómicos y no genómicos. Mientras que los primeros precisan horas o días para manifestarse, los mecanismos no-genómicos pueden darse a los pocos minutos de la presencia de GCs[10].

Los mecanismos no-genómicos que utilizan los GCs para actuar están aún sujetos a clarificación pero se han identificado varios de ellos: interacciones inespecíficas con componentes de la membrana celular, efectos no-genómicos mediados por la unión a receptores citosólicos específicos e interacciones con receptores específicos presentes en la membrana plasmática[11]. Por ejemplo, a dosis farmacológicas, los GCs se intercalan entre la bicapa lipídica de las membranas plasmática y mitocondrial, alterando su permeabilidad y modificando la funcionalidad de algunas de las proteínas ancladas[10]. Ello constituye algunas de las acciones no-genómicas que se incluyen dentro de las interacciones inespecíficas a nivel membrana. Una consecuencia sería la disminución del gradiente de protones a nivel mitocondrial y el consiguiente descenso de la síntesis de ATP en células del sistema inmune, cuya respuesta inflamatoria es altamente dependiente de ATP[12]. Otro ejemplo de acción no genómica se da a través de la unión al GCr pero sin regulación de la transcripción génica. Las proteínas que conforman el complejo multiproteico citosólico con el GCr inactivo, se separan del receptor al unirse al ligando y pueden inhibir diversas vías de señalización, entre ellas la formación de ácido araquidónico, que constituye un importante mediador de algunas reacciones inflamatorias y la división celular[11].

Los efectos mediados por la vía genómica son dependientes de la unión de los GCs al receptor citosólico específico[11]. Los GCs actúan a través de la unión a dos receptores citosólicos: el receptor de mineralocorticoides (MRr) y el receptor de glucocorticoides (GCr). El MRr constituye el mecanismo a través del que actúan también los

mineralocorticoides y se da la paradoja de que presentan una afinidad 10 veces mayor por los GCs que el propio receptor específico GCr. Es por esta razón, y el hecho de que los GCs circulantes estén más concentrados que los mineralocorticoides, que se considera a la enzima 11β-HSD2 preservadora de la actividad específica mineralocorticoide a través de la inactivación de los GCs en los tejidos diana de mineralocorticoides[9]. El GCr está codificado por el gen NR3C1, situado en el locus cromosómico 5q31-q32 y constituido por 9 exones. Inicialmente se identificaron dos isoformas del receptor pero actualmente las isoformas identificadas suman ya más de 8, de las que 5 se obtienen por *splicing* y traducción diferencial del gen (Figura 3)[13].

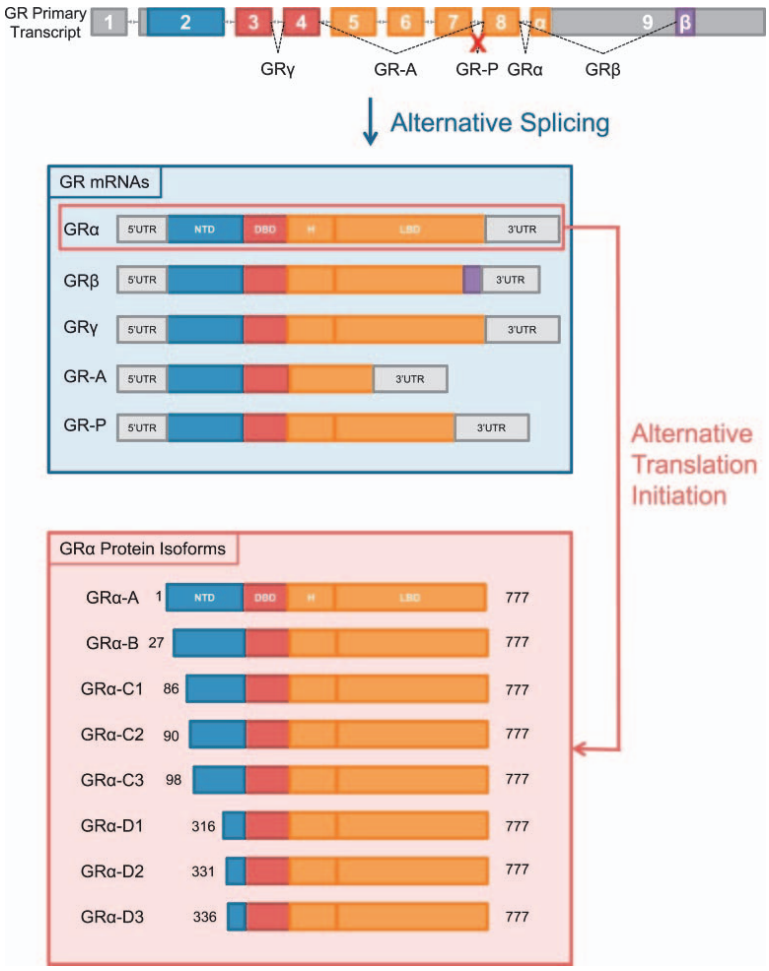


Figura 3. Isoformas identificadas del GCr con diferente funcionalidad obtenidas a través de splicing diferencial (5 de ellas) y posterior traducción diferencial[13].

La isoforma αGCr es prácticamente ubicua y la más abundante, capaz de unirse a los GCs y considerada la forma clásica del receptor. La isoforma βGCR, por el contrario, constituye apenas el 1% de la totalidad del receptor para GCs expresado, y al principio se consideró que actuaba como inhibidor dominante negativo del αGCr y se le asociaba a

modelos patológicos de resistencia a glucocorticoides pero recientemente se ha demostrado que ejerce regulación transcripcional, activando e inhibiendo genes diana propios no regulables por αGCr[14]. Del resto de isoformas aún no se conoce con claridad su función, aunque se han identificado en líneas celulares y patologías con marcada resistencia a la acción de los glucocorticoides y se han identificado genes específicamente regulables por cada isoforma[15].

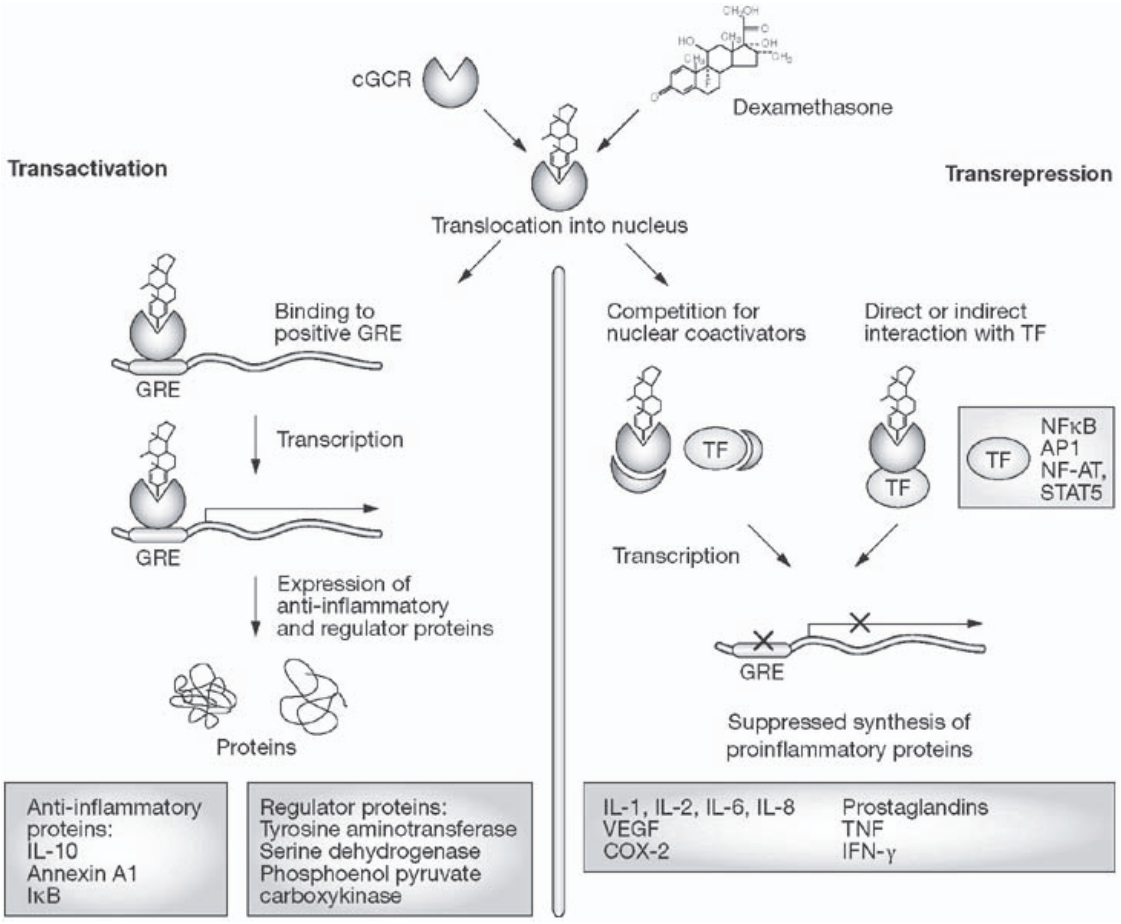


Figura 4. Los efectos genómicos de los glucocorticoides se dan a través de la transactivación y la transrepresión[11].

El GCr pertenece a la súper familia de los receptores nucleares[16]. En ausencia de ligando, se encuentra en el citosol donde interactúa con diversos tipos de *heat-shock proteins* y diversas quinasas de la vía de señalización de las *mitogen-activated protein kinase* (MAPK) entre otras. Al unirse al ligando, el GCr se disocia del resto de proteínas y transloca al núcleo celular donde puede actúar a través de dos mecanismos bien caracterizados: la transactivación y la transrepresión (figura 4). La transactivación se da cuando homodímeros del GCr se unen a secuencias específicas del DNA llamadas *glucocorticoid-response elements* (GRE) que se hallan en la zona promotora de genes diana y estimulan o inhiben su transcripción. Algunos de los efectos antiinflamatorios de

los glucocorticoides se llevan a cabo a través de este mecanismo de acción, así como la mayoría de acciones metabólicas, el *feed-back* negativo ejercido sobre el eje HPA y los efectos secundarios provocados por su administración farmacológica[11].

La transrepresión ocurre cuando el GCr nuclear interacciona con otros factores de transcripción como la proteína activadora 1 (AP-1) o el factor nuclear potenciador de las cadenas ligeras kappa de las células B activadas (NF-kB) impidiendo su unión al DNA y evitando por tanto que ejerzan la regulación génica correspondiente. De esta forma, evitan la expresión de proteínas con actividad proinflamatoria lo cual constituye uno de los mecanismos que utilizan para reprimir la inflamación[17].

1.3 Funciones de los glucocorticoides

Los glucocorticoides son esenciales para la adaptación a un ambiente cambiante a través de lo que denominamos "estrés" y a la vez, son fundamentales para restablecer la homeostasis una vez adaptados, es decir, para finalizar con la situación de "estrés"[18]. Asimismo participan en las fluctuaciones de nuestro ritmo circadiano para poder llevar a cabo todas nuestras funciones[19]. Todo ello exige actuar tanto a nivel de sistema nervioso central como en tejidos periféricos. Los efectos más importantes de los GCs incluyen el control de funciones cognitivas y memoria en el hipocampo, creación de hábitos y recompensa en el sistema límbico, la regulación de los ejes tiroidales, reproductivo y del crecimiento, el desarrollo y maduración del pulmón así como la regulación del funcionamiento del sistema gastrointestinal, cardiorespiratorio, el metabolismo energético y el sistema inmune[19]. En esta introducción nos centraremos en las funciones de los glucocorticoides el sistema inmune y el metabolismo energético.

1.3.1 Glucocorticoides e inflamación

Los glucocorticoides se utilizan farmacológicamente como antiinflamatorios e inmunosupresores en muchas enfermedades. Sin embargo, su uso farmacológico está limitado, debido a los efectos metabólicos adversos asociados con dosis altas y tratamientos crónicos. Algunos de estos efectos son, por ejemplo, la promoción de la resistencia a la insulina y por tanto de la diabetes de tipo 2, la hiperfagia, redistribución de la grasa corporal de depósitos subcutáneos a viscerales y aumento de peso, aumento del riesgo de aterosclerosis, la hipertensión y enfermedades asociadas al síndrome metabólico que paradójicamente se asocia a un estado inflamatorio leve y crónico[20–24].

Tanto los GCs endógenos como los exógenos realizan diversas acciones anti-inflamatorias sobre el sistema inmune (figura 5). Estos efectos están mediados a través de gran número de mecanismos diferentes y actúan sobre células del sistema inmune

innato y adaptativo. Los GCs actúan específicamente sobre ellas para combatir la hiperactivación del sistema inmune, tanto a nivel transcripcional como celular. Por ejemplo, bloquean la expresión de la ciclooxigenasa *2* (Cox-2), impiden la hiperactivación de linfocitos T en respuesta a una excesiva estimulación del *T cell receptor* (TCR), inducen apoptosis de macrófagos para garantizar la supervivencia en respuesta a la sepsis inducida por LPS o para suprimir la inflamación asociada a alergias[17]. Sobre la transcripción, pueden activar la expresión de proteínas con actividad antiinflamatoria (IL-10), reprimir de la actividad de factores de transcripción, como la AP-1 y NF-kB, impidiendo por lo tanto, la expresión de proteínas con acción proinflamatoria como TNF-α, IL-1, IL-2 o IL-6[23–26].

La respuesta proinflamatoria está íntimamente ligada a la antiinflamatoria. Un ejemplo de ello, es que los *toll-like receptors* (TLR2 y TLR4), implicados en la recepción de estímulos inflamatorios (LPS y ácidos grasos saturados serían algunos de ellos) y en la iniciación de la respuesta inflamatoria, son los responsables de estimular la secreción de glucocorticoides en la glándula adrenal[27]. Las vías de señalización a través de las cuales los glucocorticoides ejercen su acción antiinflamatoria son muy diversas. A destacar, la inducción de la expresión de *dual specificity phophatase 1* (DUSP-1), una fosfatasa específica de la vía de las *mitogen-activated protein kinases* (MAPK) o de la expresión de *glucocorticoid-inducible leucine zipper* (GILZ) que promueven la pérdida de actividad de NF-kB y AP-1.

Todas estas acciones poseen en común el freno a la inflamación, el efecto más utilizado y estudiado de los GCs. En los últimos años, sin embargo, diversos estudios han evidenciado acciones proinflamatorias llevadas a cabo por los glucocorticoides Se considera que los glucocorticoides estimulan el sistema inmune antes de la llegada del estímulo inflamatorio para que puedan hacerle frente con garantías[10,17]. Algunos de los efectos descritos proinflamatorios son por ejemplo, la estimulación de la expresión de TLRs, especialmente TLR2[27] que conlleva la amplificación de la respuesta inflamatoria. También inducen la expression de NLRP3 en macrófagos, perteneciente a la familia de *NOD-like receptors* (NLRs) que sensibilizan las células a estímulos inflamatorios. En concreto, NLRP3 sensibiliza a los macrófagos a activarse ante bajas concentraciones de ATP aumentando la secreción de citoquinas como TNF-α e IL-6[28]. Asimismo, estimulan la aparición en membrana del receptor purinérgico P2Y2R, aumentando la secreción de IL-6 también a consecuencia de la estimulación por ATP[29].

Todas estas funciones, que pueden parecer antagónicas, se circunscriben en lo que se ha dado en llamar las *5Rs*, que resumen los efectos globales que pueden ejercer los GCs

sobre el sistema inmune: *reinforce, ready, repress, resolve* y *restore.* No únicamente colaboran en reprimir y resolver la inflamación y restaurar la homeostasis, sino que también refuerzan y permiten al sistema inmune estar preparado para afrontar cualquier ataque[17].

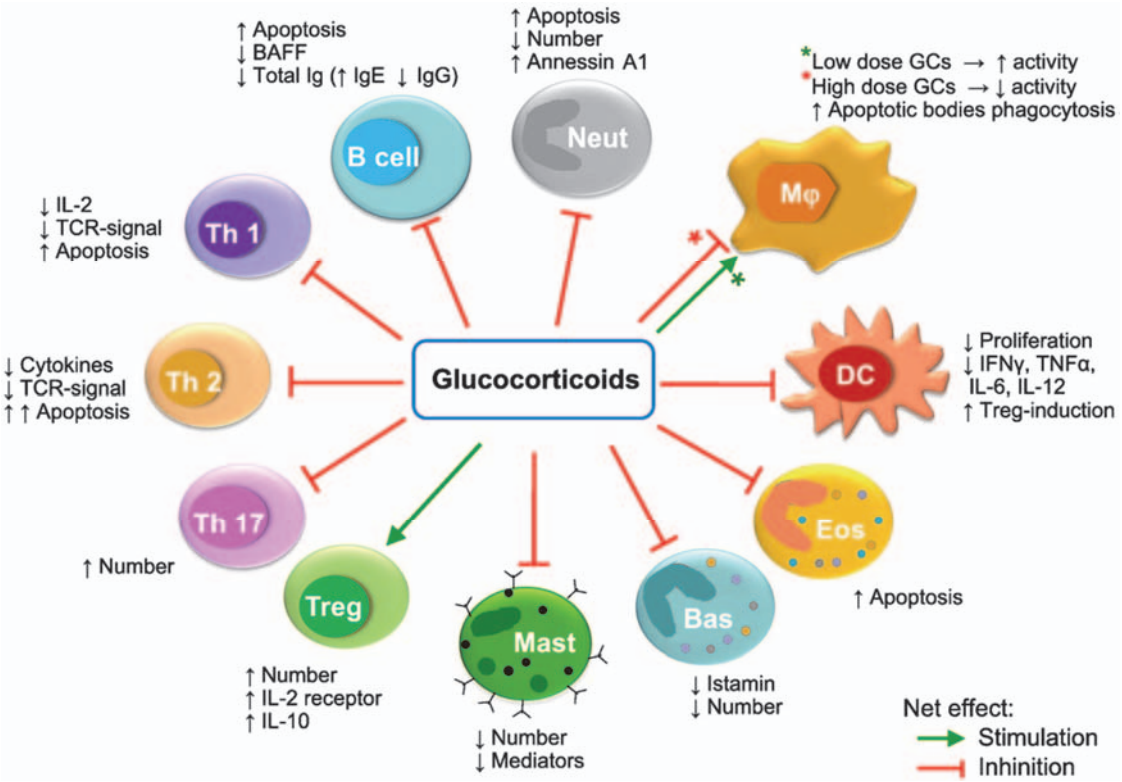


Figura 5. Efectos de los glucocorticoides sobre las células del sistema inmune. GS: glucocorticoides, Th: células T helper, Treg: células T reguladoras, Neut: neutrófilos, DC: células dendríticas, Mφ: macrófagos, Eos: eosinófilos, Bas: basófilos, Mast: mastocitos, TCR: receptor de células T, Ig: inmunoglobulina, IFN: interferón, BAFF: factor estimulante de linfocitos B[10].

Recientemente la enzima 11β-HSD1 se ha implicado en la regulación de las respuestas inmunitarias locales a través de su actividad regeneradora de glucocorticoide activo intracelular. La 11β-HSD1 se expresa en gran número de tipos celulares relacionados con la inmunidad innata y adaptativa. Estos incluyen macrófagos, células dendríticas, mastocitos y células T, así como en timo, médula ósea, piel y membrana sinovial[9]. Su expresión se ha identificado en las células del estroma y en los leucocitos en zonas inflamadas. La actividad de la enzima aumenta fuertemente por la regulación ejercida por citoquinas pro-inflamatorias tales como TNF-α e IL-1β[1,30,31]. Además, la combinación de citoquinas proinflamatorias y GCs aumenta dramáticamente la expresión de la enzima. Las células mononucleares de sangre periférica circulantes no presentan expresión de 11β-HSD1, que sin embargo aumenta significativamente durante la diferenciación a macrófagos. En la tipología de macrófagos proinflamatorios M1, la expresión elevada de

11β-HSD1 aumenta su sensibilidad a los GCs endógenos[1]. Ello a su vez regula negativamente su comportamiento pro-inflamatorio y reduce su supervivencia[32–34].

1.3.2 Glucocorticoides y metabolismo energético

Las acciones de los glucocorticoides sobre el metabolismo energético difieren según se trate de efectos a corto plazo o a largo plazo (figura 6). A corto plazo, inhiben la captación de glucosa periférica y la secreción de insulina, estimulan la gluconeogénesis en el hígado y facilitan la lipólisis en el tejido adiposo a través de la inducción de la lipasa sensible a hormonas (HSL) y la proteólisis muscular. A medio y largo plazo, provocan resistencia a la insulina e hiperinsulinemia, con la cual actúan sinérgicamente estimulando la acumulación de triacilgliceroles en el tejido adiposo a través de la inducción de la lipoproteína lipasa (LPL) y pueden causar un aumento de peso corporal e hiperfagia[4]. Los glucocorticoides contraregulan la acción de las hormonas de adelgazamiento, como la leptina[9] y el oleato de estrona[35].

La leptina reduce la ingesta a nivel central a través de la inhibición de la expresión de neuropéptido Y (NPY) en contraste con la acción de los GCs que estimulan el apetito (figura 7) a través de la potenciación de la grelina y el incremento de los niveles de NPY en el núcleo arcuato hipotalámico donde se integran todas las señales reguladoras de la ingesta para elaborar una única respuesta[36]. Sin embargo, el tratamiento con glucocorticoides produce hiperleptinemia, por lo que se ha planteado la hipótesis de que pueden provocar un estado de resistencia a la leptina[4]. Además, los glucocorticoides se han relacionado con la motivación en la selección de alimentos palatables, por su participación en la secreción de dopamina en el núcleo accumbens[37–39].

Las acciones biológicas de los GCs en tejido adiposo tienen lugar a través de los receptores MCr y GCr, ya que ambos se expresan en los adipocitos. Los GCs promueven la captación de ácidos grasos, a través de la estimulación tanto de la expresión como de la actividad de la LPL y también activan la lipólisis, a través de la inducción de la expresión de HSL[24]. Se ha identificado mayor número de GCr en tejido adiposo visceral que en subcutáneo, por lo que se considera al visceral el tejido adiposo donde los GCs ejercen mayor influencia (figura 7). Además promueven la diferenciación de preadipocitos a adipocitos[3].

El potente efecto de los glucocorticoides en la acumulación de tejido adiposo se evidencia observando las consecuencias de la adrenalectomía, que consiste en la extirpación quirúrgica de ambas glándulas suprarrenales. La adrenalectomía impide el desarrollo de la obesidad en ratas, efecto que se anula con la restauración de los niveles de

corticosterona[40]. La adrenalectomía también provoca un aumento de la sensibilidad del organismo al efecto adelgazante de la leptina, que se invierte mediante la administración subcutánea o intracerebroventricular de dexametasona, un ligando específico y con alta afinidad por el GCr. Sin embargo, en ratas normales que no han sido sometidas a adrenalectomía, el tratamiento subcutáneo con cortisol causa la inducción de la expresión de la leptina en el tejido adiposo y la consecuente reducción del peso corporal. La infusión intracerebroventricular causa el efecto contrario, incrementando la ingesta y el peso corporal[40]. En pacientes con síndrome de Cushing, una enfermedad causada por una sobreproducción de cortisol de diversa etiología, muestran las consecuencias del exceso de GCs: presentan obesidad central, con un aumento del tejido adiposo visceral y una disminución del tejido adiposo subcutáneo, así como hiperfagia, resistencia a la insulina e hipertensión, es decir, un cuadro de síndrome metabólico[41–44].

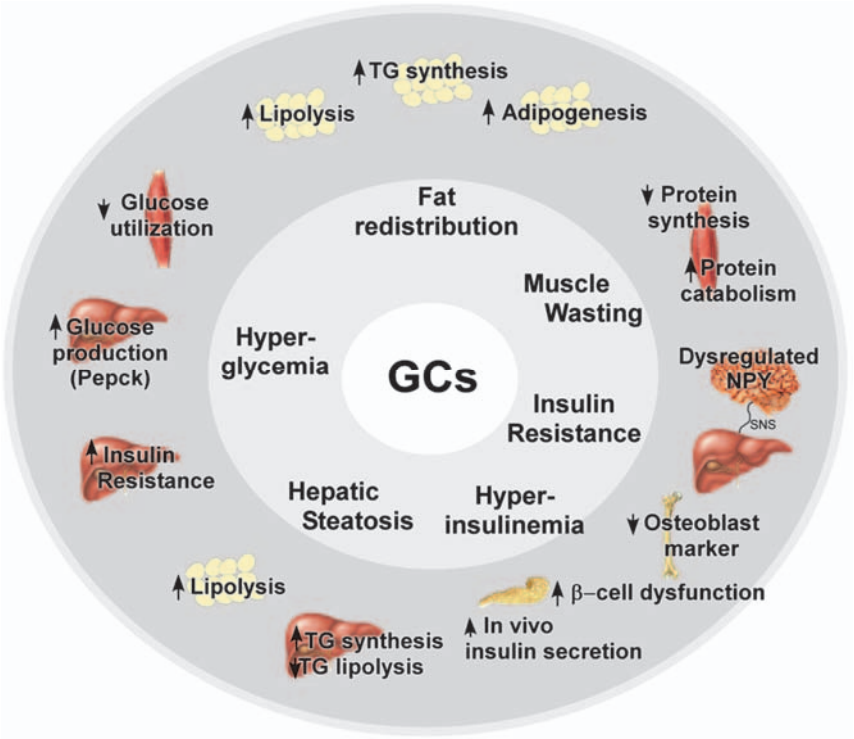


Figura 6. Esquema de los efectos de los glucocorticoides sobre el metabolismo energético en tejidos sensibles y las vías inducidas. TG, triglicéridos; SNS, sistema nervioso simpático[45].

Por todo ello, la desregulación de los glucocorticoides y el eje HPA se ha considerado como una de las posibles causas de la obesidad visceral y las patologías asociadas. En la obesidad, el recambio de cortisol o la actividad del eje HPA aumenta en las personas obesas. El exceso o la falta de secreción de cortisol en respuesta al estrés se ha

asociado con diferentes grados de obesidad y así como la pérdida de la capacidad de retroalimentación negativa de los glucocorticoides sobre el eje[46]. A pesar de que en pacientes obesos se han descrito tanto niveles normales como incrementados como disminuidos de GCs circulantes, la actividad 11β-HSD1 aumenta tanto en el tejido adiposo subcutáneo como en el visceral, lo que implica una mayor exposición a los GCs que, combinado con la mayor densidad de receptores específicos en el tejido adiposo visceral, puede causar o contribuir a las mayorías de alteraciones metabólicas presentes en la obesidad central[47–51].

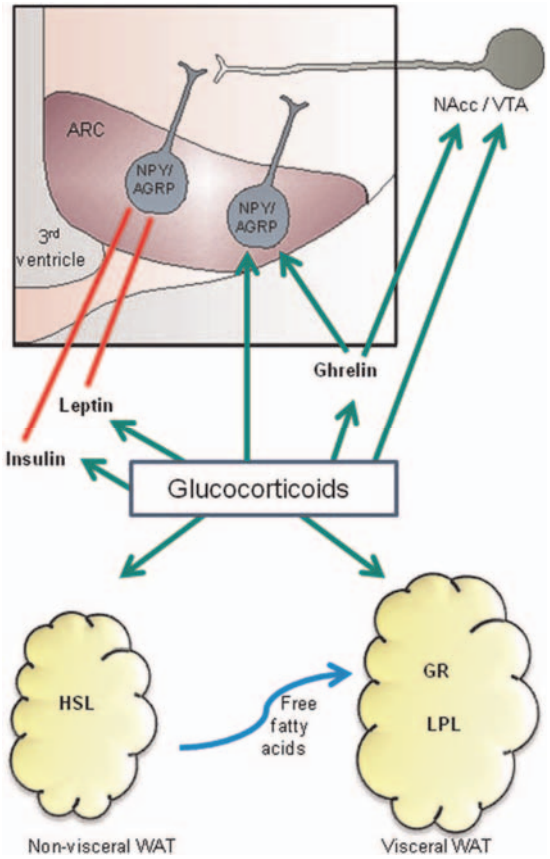


Figura 7. La acción de los GCs tiene lugar tanto a nivel central, donde regulan la ingesta, como a nivel periférico. En general, estimulan la lipólisis pero, en presencia de insulina, actúan sinérgicamente estimulando la actividad de la LPL en los depósitos viscerales[3].

Ratones transgénicos que sobreexpresan la 11β-HSD1 en tejido adiposo, desarrollan síndrome metabólico sin que los niveles de corticosterona circulante resulten modificados. Por el contrario, los ratones *knockout* para 11β-HSD1 son resistentes a la obesidad inducida mediante dieta hiperlipídica, mejorando la sensibilidad a la insulina y la lipidemia lo que refuerza el papel de la 11β-HSD1 y de los GCs en el desarrollo de obesidad[52,53]. Actualmente, la inhibición de la actividad 11β-HSD1 se considera una posible diana terapeútica contra la obesidad[9].

1.4 Globulina ligadora de corticosteroides (CBG)

La CBG o transcortina, se identificó hace casi 60 años[54–57]. Es una glicoproteína monomérica formada por 383 aminoácidos[58] con un peso molecular de 50-60 kDa[59] y miembro de la superfamilia de los inhibidores de serina proteasas (SERPIN) aunque la CBG no posee esta actividad[57,58]. La CBG es codificada por un solo gen que abarca 19 kilobases, se encuentra en el cromosoma 14 (q31-q32.1) y consiste en cinco exones, de los cuales cuatro son codificantes[60].

En situación basal, el 80-90% del cortisol viaja unido a la CBG con gran afinidad, un 10-15% se une a la albúmina y sólo el 5-10% restante se encuentra en el torrente sanguíneo en forma libre[61]. Según la "hipótesis de la hormona libre" formulada por Mendel en el año 89[62] y aceptada ampliamente, es la fracción de hormona libre la que es biológicamente activa. El cortisol unido a la CBG es pues biológicamente inactivo y constituye una reserva de hormona fácilmente movilizable a través de la regulación de los niveles de CBG. La CBG se encarga pues, de transportar los GCs por el torrente sanguíneo, actuando como un tampón en situaciones en que aumenta desmesuradamente la concentración de GCs o como un reservorio[63]. Existe una ligera variación circadiana en los niveles de CBG plasmáticos que varían en sentido opuesto al de los GCs, acentuando así el perfil diario de hormona libre[64].

La CBG se sintetiza principalmente en el hígado desde donde es secretada al torrente sanguíneo[58,65]. La síntesis hepática está regulada por diversas moléculas: los estrógenos son potentes inductores, lo que podría justificar la mayor cantidad circulante de CBG en las hembras que en los machos de muchas especies, mientras que la IL-6, los GCs y la insulina juegan un papel inhibitorio[66–68]. La expresión de CBG ha sido detectada también en la placenta, riñón, endometrio, pulmón, tejido adiposo[58,68] y mediante inmunohistoquímica, se ha identificado en cerebro, en zonas tales como hipotálamo e hipófisis[69]. La función de la CBG en tejidos extrahepáticos aún no ha sido esclarecida aunque se ha hipotetizado que puede actuar modulando el *pool* intratisular de glucocorticoides[4].

Estudios *in vitro* han demostrado que la CBG es sustrato de la elastasa secretada por neutrófilos[70] una vez activados por un estímulo inflamatorio. La elastasa hidroliza un pequeño fragmento de la CBG lo cual provoca cambios conformacionales permanentes que causan la pérdida de afinidad de la CBG por el cortisol y su consiguiente liberación en el preciso lugar donde la inflamación está progresando[64]. Por ello, un rol emergente

de la CBG es la liberación dirigida de cortisol hacia los tejidos inflamados, facilitando su acción inmunomoduladora[71].

1.4.1 Deficiencia de CBG

Dos modelos de ratones *knockout* (KO) para la CBG se han obtenido hasta el momento[72,73]. En el primero, obtenido por el equipo de Thomas Willnow en 2006, se observaron niveles reducidos de corticosterona total circulante pero por el contrario, altos niveles de corticosterona libre en comparación con los ratones WT y un eje HPA hiperactivo, con una alta concentración circulante de ACTH. Pese a ello, no se evidenció ninguna alteración en el peso corporal y la ingesta en respuesta al ayuno o bien a una dieta con un 30% de la energía en forma de lípidos, ni un incremento de la expresión de genes diana de glucocorticoides en hígado, como la fosfoenolpiruvato carboxiquinasa (PEPCK). En este modelo, también se evaluó la respuesta a LPS. Tras una inyección de LPS, los ratones KO mostraron un aumento de la susceptibilidad de sufrir un shock séptico agudo en comparación con los WT y mayor mortalidad a las 48 horas de la inyección, como si la actividad antiinflamatoria se hallara bloqueada. Otra característica detectada fue la presencia de fatiga, que es característica de pacientes con la enfermedad de Addison cuya glándula adrenal es incapaz de sintetizar glucocorticoides. Ello llevó a los autores a hipotetizar que la falta de CBG provoca la incapacidad de responder adecuadamente a la elevada corticosterona libre circulante que presentan y por tanto, que la CBG es necesaria, de alguna forma, para que los glucocorticoides consigan actuar[72].

En el modelo KO para CBG obtenido por el equipo de Marie Pierre Moisan en 2010[73], también observaron la reducción de la corticosteronemia pero sin alteración de los niveles de hormona libre. Esta discrepancia ha sido explicada por los autores a que en el trabajo de Willnow solo se evalúan niveles de corticosterona matinales y no se realiza un seguimiento de la variación del ritmo circadiano y que los niveles hallados son demasiado altos y corresponden a niveles de respuesta a estrés, quizás debido al momento del sacrificio, por lo que las condiciones en la que se midió la hormona libre podrían no ser basales. Otro hallazgo de este equipo fue la falta de reactividad del eje HPA en respuesta al estrés en los ratones KO, por lo que hipotetizan que la CBG es necesaria para garantizar el funcionamiento del eje.

Además de las funciones fisiológicas conocidas, hay posibles roles de la CBG que están todavía sujetos a investigación, como el control de la absorción de sodio intestinal a través de un mecanismo dependiente de aldosterona[74]. Al disminuir la concentración de sodio en la dieta, los ratones deficientes en CBG no son capaces de reducir la excreción

fecal de sodio para optimizar su captación, una acción que en el organismo realiza la aldosterona. Es importante hacer notar que la aldosterona no es trasportada en sangre por la CBG. Esta observación revela otra función potencial de la CBG que, de confirmarse en humanos, puede contribuir a la hipotensión y la fatiga observadas en sujetos portadores de mutaciones en la CBG[75].

Un papel interesante y poco comprendido de la CBG se relaciona con el dolor crónico y la fatiga, que son algunas de las características clínicas de los sujetos que portan algunas mutaciones en la CBG. En un estudio se ha identificado CBG en estructuras neuronales implicadas en la regulación del dolor, tales como el área gris periacueductal, el bulbo raquídeo y el cuerno dorsal de la médula espinal[76].

1.4.2 Evidencias de la asociación entre CBG y obesidad e inflamación

La relación entre la concentración y la actividad de CBG y la incidencia de la obesidad ha sido objeto de investigación. Varios estudios indican que la alteración de la concentración de CBG y su capacidad de unión al cortisol están relacionados con la obesidad y la resistencia a la insulina. Estudios en especies porcinas evidencian que la presencia de polimorfismos y de mutaciones que disminuyen la afinidad de la CBG a los GCs está asociada a un mayor riesgo de padecer obesidad[77,78].
En ratas genéticamente obesas de la cepa Zucker, cuyos niveles circulantes de corticosterona son normales, se ha descrito la disminución significativa de la capacidad de unión de la CBG plasmática, junto a un aumento de su afinidad por la corticosterona[4].

En humanos, se ha asociado una disminución de la presencia de CBG al desarrollo de la obesidad[79]. La falta completa de CBG en humanos no resulta ser fatal, aunque se asocia con hipotensión y fatiga[63]. Se ha hallado una familia italiana con una mutación en el gen de la CBG que impide su expresión en individuos homocigotos y que como consecuencia presentan niveles reducidos de cortisol y además tienen una mayor tendencia a la obesidad[80]. De hecho, sus preadipocitos muestran una mayor capacidad de proliferación y diferenciación al compararlos con pre-adipocitos normales[81]. Por otro lado, varios estudios poblacionaesl han establecido la correlación negativa entre la concentración de CBG circulante y el índice de masa corporal, la relación cintura-cadera, la presión sanguínea diastólica y sistólica así como con varios marcadores de síndrome metabólico entre ellos la sensibilidad a la insulina[82].

Recientemente, un nuevo estudio ha puesto de nuevo de manifiesto la relación entre la CBG circulante y la obesidad central, donde se confirman bajos niveles de CBG en persona con obesidad central con la novedad de aportar un nuevo dato: con anticuerpos

específicos, han sido capaces de distinguir la CBG nativa con alta afinidad por el cortisol de la CBG hidrolizada por alguna proteasa y que por tanto, presenta un tamaño inferior (apenas 5 kDa) y menor afinidad por el cortisol. En personas con obesidad abdominal han detectado menor presencia de la CBG con baja afinidad respecto la de alta afinidad. La resistencia a la escisión de la CBG puede obstaculizar la entrega de cortisol a los tejidos inflamados y por tanto perpetuar la inflamación en la obesidad central y por consiguiente, el síndrome metabólico[83].

2. Obesidad y síndrome metabólico

"….On the morning of the 9th March 1877, while engaged in private practice in Preston, I received a hurried message to go at once and see a man who had been found dead in bed. When I reached the house I found a huge man lying dead. He had evidently been dead a few hours. As he had gone to bed on the previous evening apparently well, I acquainted his medical man with the circumstances of the case, and at his request made a post-mortem examination for him on the following day. The subject of these remarks, W. S., aged 50 years-had previously been an over looker in one of the cotton factories of the town, but owing to his increasing bulk he had been obliged to give up his situation, and for a few years before his death he had followed no occupation whatever. He was not a tall man (I should think about 5 feet 4 inches), but was extremely fat. He was very lazy; everything in the shape of movement was a trouble to him-so much so that for months before his death he seldom rose from his chair, unless it was at times, and that very occasionally, to stand at the open door of his house. His daughter and his sister-in-law informed me that a year before his death he weighed 30 St. 6 lbs.…."

Post – Mortem in a case of Extreme Obesity. Thomas Oliver, 1880 [84]

Era el 14 de abril del 1880 cuando Thomas Oliver publico el primer trabajo relacionado con la obesidad que se conoce[84]. Después de 136 años de estudios, se considera que la obesidad es una entidad clínica muy compleja y heterogénea, caracterizada por un exceso de grasa corporal junto una inflamación leve y crónica[85], con un importante componente genético, cuya expresión está influida por varios factores ambientales: dieta, actividad física, clase socio-económica, estrés, desarrollo perinatal y factores culturales entre otros[86]. Desde 1980, se ha casi duplicado su prevalencia mundial, hasta convertirse en una epidemia que amenaza la salud pública[87–89]. Los datos de la literatura científica lo demuestran: la palabra clave "obesity" en *PubMed* produjo 19.857 resultados en el año 2014, 20.683 resultados en 2015 y 14.079 resultados hasta agosto de 2016. Son

números asombrosos que nos dan una idea del interés que esta enfermedad suscita a nivel científico.

2.1 Epidemiología de la obesidad

Durante la década de los años ochenta, se introdujo el concepto de índice de masa corporal (IMC) para evaluar el peso corporal de los individuos, calculado como peso /estatura^2 (kg/m^2) y se delimitaron los rangos para definir el sobrepeso y la obesidad en adultos[90]. Posteriormente se introdujeron y delimitaron los percentiles para evaluar el peso en niños y adolescentes (Tabla 1)[91,92].

Categoría	Niños y adolescentes*	Adultos**
Peso insuficiente	<p5	<18,5
Peso normal	p5-85	18,5-24,9
Sobrepeso	p85-p95	25-29,9
Obesidad de grado 1	>p95	30-34,9
Obesidad de grado 2		35-39,9
Obesidad mórbida		40-49,9
Obesidad supermórbida		>50

Tabla 1. Evaluación del peso corporal según el índice de masa corporal. En niños y adolescentes, *percentil (p) de IMC para su edad (según *Centers for Disease Control and Prevention*, 2016)[92]. En adultos, **IMC (según *National Institute of Health* y Organización Mundial de la Salud, 1998)[91].

En 1997, la Organización Mundial de la Salud (OMS) propuso diagnosticar obesidad a partir de un IMC ≥30 en un individuo adulto[89,93]. En 2015, más de 1900 millones de adultos presentaban un peso superior al deseable de los cuales 600 millones (13% de la población adulta mundial, 11% de los hombres y 15% de las mujeres) eran obesos. En 2014, más de 42 millones de niños menores de cinco años presentaron sobrepeso u obesidad.

Actualmente la obesidad es el factor de riesgo más importante asociado a las enfermedades cardiovasculares que constituyen la principal causa de mortalidad a nivel mundial. Si antes se consideraba un problema de los países ricos o desarrollados, donde se aunaban un estilo de vida sedentario y la amplia disponibilidad de alimentos de alta

densidad energética, actualmente el problema se ha extendido también a los países con ingresos bajos y medianos. Según estimaciones de la OMS, África será el continente con mayor incremento de la mortalidad por enfermedades cardiovasculares durante la próxima década[94,95].

2.2 Etiología de la Obesidad

A medida que el número de personas con obesidad alcanza proporciones epidémicas, existe una fuerte necesidad de entender completamente la etiología de la obesidad. Los estudios realizados hasta ahora han llevado a la conclusión de que las formas más comunes de la obesidad tienen un origen multifactorial que probablemente es el resultado de una compleja interacción entre la biología del metabolismo energético y factores ambientales, a través de una posible predisposición genética, condicionamiento epigenético, aspectos hormonales, metabólicos y de comportamiento[96].

Se sabe que las mutaciones de un solo gen como en el caso de la leptina[97–103], el receptor de leptina[104–106], receptor de melanocortina-4[107–110], proopiomelanocortina[111–114] y la enzima proconvertasa-1[115–118] son responsables de formas raras monogénicas de obesidad[100].

Sin embargo, las formas más comunes de obesidad son poligénicas[119] y no responden a mutaciones con pérdida de función sino que la base genética consiste en la presencia de polimorfismos de un único nucleótido (SNPs) [120] que amplifican o limitan la acción final de la proteína que codifica el correspondiente gen. Un estudio de 2013 identifica 1.515 genes codificantes de proteínas y 221 miRNAs, secuencias de RNA cortas complementarias a los mRNA de los genes que regulan impidiendo su traducción, potencialmente implicados en fenotipos obesos comunes en cuatro especies de mamíferos, número que sigue creciendo[121].

Otros estudios también han evaluado la influencia de la epigenética, que consiste en cambios producidos sobre la cadena de nucleótidos que modulan la expresión génica como metilaciones, acetilaciones, fosforilaciones, etc o a través de la expresión diferencial de microRNAs, Estas marcas epigenéticas serían consecuencia de determinados factores ambientales: dietas desequilibradas con carencias o exceso de micronutrientes y/o macronutrientes, contaminantes ambientales como el bisfenol A, alteraciones de la microbiota intestinal, etc[122].

A pesar de que las marcas epigenéticas tienen lugar a lo largo de toda la vida, existen las llamadas “ventanas críticas” que son épocas vitales de especial susceptibilidad ante los

factores ambientales con capacidad de afectar epigenéticamente el genoma: una de ellas es durante el desarrollo embrionario y el primer año de vida. Acontecimientos como una dieta materna inadecuada, tanto por exceso como por defecto, o incluso el estrés materno durante el embarazo pueden causar cambios persistentes en la expresión de ciertos genes e influir en el riesgo de enfermedad en la edad adulta, un concepto conocido como "programación fetal"[96]. Además, incluso la dieta paterna durante la espermatogénesis puede tener un impacto en las alteraciones epigenéticos de la obesidad. Actualmente existen evidencias de que las marcas epigenéticas se pueden transmitir incluso de generación en generación, pero todavía no se conoce qué papel juega esta herencia en la obesidad humana[96].

Los cambios en la composición de la dieta a consecuencia de la introducción de los alimentos procesados y la mayor disponibilidad y accesibilidad a alimentos altamente palatables pero pobres en nutrientes, así como los cambios en la agricultura y el tipo de alimentos producidos, han contribuido al gran aumento de la obesidad en los últimos 30-40 años. A ello se ha añadido el sedentarismo consecuencia de la tecnología disponible en la vida cotidiana, la urbanización urbana, la utilización de vehículos privados y el ocio asociado a actividades audiovisuales no activas, que ha provocado un descenso de las necesidades energéticas del organismo[123].

El número cada vez mayor de las experiencias estresantes de los individuos en la sociedad moderna influencia la conducta alimentaria a través del aumento del apetito asociado a los alimentos llamados "comfort foods" que se caracterizan por un alto contenido en grasas y azúcares y gran densidad energética. Por ello y por la acciones metabólicas a largo plazo de los GCs seretados en respuesta al estrés, se ha relacionado también al estrés creciente que sufre la población con el aumento de casos de obesidad[39,124,125].

El estrés de la madre durante del embarazo o experiencias traumáticas durante los primeros años de vida pueden dejar una huella epigenética que altere el funcionamiento de diversos sistemas fisiológicos. Puede influir en el umbral de sensibilidad del eje hipotálamo-pituitario-adrenal (HPA), a través de cambios en el nivel de expresión de los GCr en hipocampo e hipotálamo y de la CRH[39], así como la señalización de las hormonas relacionadas con la saciedad como leptina, insulina y grelina, alterando el comportamiento a largo plazo.

Dentro el concepto de obesidad hay que tener en cuenta las diferencias biológicas de género entre mujeres y hombres. Las mujeres muestran cambios endocrinos más

drásticos que dejan huella en el cuerpo relacionados con la reproducción durante toda la vida. Sin embargo, las diferencias de género no son exclusivamente biológicas, sino que también dependen de la interacción entre la biología y el ambiente. Surgen también de los procesos socio-culturales, como los diferentes comportamientos de mujeres y hombres, la exposición a las influencias específicas del entorno, diferentes formas de nutrición, estilos de vida o el afrontamiento al estrés[126].

2.3 Síndrome metabólico

En 1988, Reaven propuso el término "Síndrome X" para describir un grupo de anomalías metabólicas asociadas a la obesidad que incluían la intolerancia a la glucosa, la dislipemia, la hipertensión, y la resistencia a la insulina[127]. Todos estos trastornos metabólicos, son factores de riesgo independientes de sufrir complicaciones cardiovasculares y, de hecho, su coexistencia se correlaciona con una alta morbilidad y mortalidad por estas enfermedades[128].

Desde entonces, el término "Síndrome metabólico" se ha adoptado para ilustrar esta agrupación de factores de riesgo cardiometabólico, abriendo nuevas perspectivas para el estudio de sus interrelaciones[128].

El síndrome metabólico (SM) se caracteriza por una combinación de factores de riesgo subyacentes que cuando aparecen juntos, culminan en resultados adversos, incluyendo la diabetes mellitus tipo 2 (DM2) y la enfermedad cardiovascular (ECV)[129] que provocan un aumento de aproximadamente 1,6 veces en la mortalidad[130]. Tanto en hombres como en mujeres, es la cantidad de tejido adiposo visceral la que correlaciona de manera directa con este perfil de riesgo metabólico, que precede al desarrollo de DM2 y enfermedad cardiovascular. Así, se ha demostrado que la acumulación regional de grasa en los depósitos viscerales es factor predictivo de riesgo cardiovascular y resulta más fiable para evaluarlo que medir la cantidad total de grasa corporal[86,131].

Aunque el síndrome metabólico está totalmente reconocido, no existe un único criterio para su diagnóstico entre las diversas organizaciones sanitarias de prestigio internacional, como la OMS, el Grupo Europeo para el Estudio de la Resistencia a la Insulina (EGIR), el *National Cholesterol Education Program-Third adult treatment Panel* (NCEP: ATPIII, USA), la Asociación Americana de Endocrinología Clínica (AACE) y la Federación Internacional de Diabetes (FID). Como se puede observar en la Tabla 2, cada una de estas organizaciones ha desarrollado sus propios criterios para la definición del síndrome metabólico, con el fin de identificar con precisión a las personas con un riesgo más alto de desarrollar DM2 y ECV[132].

OMS	EGIR	NCEP:ATPIII	AACE	FID
High insulin level + *Two of the following:* 1. Abdominal obesity WC > 37", BMI > 30 kg m^{-2}	High fasting insulin concentrations – insulin resistance + *Two of the* 1. *following:* WC ≥ 94 cm (male) ≥80 cm (female)	*Any three of the following:* 1. WC > 40" (male) >35" (female)	Impaired glucose tolerance + *Two of the* 1. *following:* Triglycerides ≥150 mg dL^{-1} Cholesterol – HDL <40 mg dL^{-1} (male) <50 mg dL^{-1} (female)	Central obesity = WC (ethnicity and gender specific)+ *Two of the following:* 1. Triglycerides ≥150 mg dL^{-1} Cholesterol – HDL <40 mg dL^{-1} (male) <50 mg dL^{-1} (female)
2. Triglycerides >150 mg dL^{-1} Cholesterol – HDL <35 mg dL^{-1} (male) <39 mg dL^{-1} (female)	2. Triglycerides >2 mmol L^{-1} Cholesterol – HDL <1 m g d L^{-1}	2. Triglycerides ≥150 mg dL^{-1} Cholesterol – HDL <40 mg dL^{-1} (male) <50 mg dL^{-1} (female)	2. BP ≥ $\frac{130}{85}$ mm Hg	2. BP ≥ $\frac{130}{85}$ mm Hg
3. BP ≥ $\frac{140}{90}$ mm Hg	3. BP ≥ $\frac{140}{90}$ mm Hg or hypertensive medication	3. BP $\frac{130}{85}$ mm Hg		3. Fasting plasma glucose ≥5.6 mmol L^{-1} or T2DM
4. Microalbuminuria >30 mg g^{-1}	4. Fasting glucose ≥6.1 mmol L^{-1}	4. Fasting plasma glucose ≥110 mg dL^{-1}		

Tabla 2. Criterios establecidos por las diferentes asociaciones para la definición y el diagnóstico del síndrome metabólico. WC= perímetro de la cintura; BP= presión sanguínea.

La presencia de obesidad (IMC>30) no es un requisito imprescindible para sufrir síndrome metabólico. Se ha observado la existencia de individuos con IMC normal pero metabólicamente obesos, es decir, con las alteraciones típicas de los pacientes con obesidad y síndrome metabólico: resistencia a la insulina, adiposidad central, bajos niveles de colesterol asociados a lipoproteínas de alta densidad (HDL) y elevadas concentraciones de triglicéridos, así como hipertensión arterial[133]. Al mismo tiempo, existen los que se han denominado obesos metabólicamente sanos que presentan un IMC>30, pero ninguna de las alteraciones metabólicas típicas del síndrome metabólico y sin ninguna patología asociada[134].

2.4 Aterosclerosis

La aterosclerosis es el activador principal de las enfermedades cardiovasculares y consiste en un proceso inflamatorio crónico de origen multifactorial con etiología genética y ambiental que se desarrolla lentamente durante décadas en respuesta a los efectos biológicos de los factores de riesgo cardiovasculares[135–137], entre los que destacan la hipercolesterolemia, la hipertensión, el estrés y el tabaquismo. La aterosclerosis afecta a las arterias de diferentes lechos vasculares y se caracteriza por el engrosamiento de la capa íntima y media con pérdida de la elasticidad. Su lesión básica es la placa de ateroma compuesta fundamentalmente de lípidos, tejido fibroso y células inflamatorias, y su desarrollo sufre diferentes estadios[135,138].

Una de las principales causas del daño es la acumulación de lipoproteínas oxidadas de baja densidad (OxLDL). Como consecuencia de la lesión, el endotelio disminuye la síntesis de óxido nítrico e induce la secreción de numerosos factores proinflamatorios[139]. Asimismo, se reclutan monocitos al endotelio, donde polarizan a un perfil de macrófagos proinflamatorio. La absorción incontrolada por parte de los macrófagos de OxLDL conduce a la formación subsiguiente de células espumosas (*foam cells*), los componentes principales de las placas ateroscleróticas[140].

La oclusión arterial completa con isquemia y necrosis celular del órgano perfundido, es la consecuencia patológica extrema de la aterosclerosis. La oclusión no se produce por la obstrucción provocada por la placa debido a su crecimiento sobre la pared, sino por la ruptura de una placa que entra en el torrente sanguíneo y provoca la formación de un trombo y la consiguiente oclusión de la luz arteriolar[141].

Los macrófagos desempeñan un papel fundamental en el desarrollo y la progresión de la aterosclerosis y el conocimiento de los factores que regulan su metabolismo son cruciales para controlar el proceso. Un paso clave en la formación de macrófagos en células espumosas es la internalización de OxLDL a través de los *scavenger receptors A1* (SRA1) y la FAT/CD36. El grado de acumulación de lípidos en los macrófagos es además dependiente de la esterificación intracelular del colesterol, regulado por la Acil-CoA:colesterol-aciltransferasa (ACAT) y la *neutral cholesterol ester hydrolase* (NCEH) y del eflujo de colesterol que depende de la actividad de los *ATP-binding cassette transporter A1 y G1* (ABCA1, ABCG1) y el *scavenger receptor BI* (SR-BI) que interactúa con las HDL para permitir la salida del colesterol intracelular[142].

En consecuencia, la transformación de macrófagos en células espumosas es el resultado del desequilibrio entre la entrada de colesterol, esterificación intracelular y el eflujo, y se produce cuando los macrófagos sometidos a una gran entrada de colesterol no logran restablecer la homeostasis interna a través de la estimulación del transporte inverso de colesterol al exterior celular y empiezan a acumular ésteres de colesterol[142].

2.4.1 Las fases de la aterosclerosis

El inicio de la enfermedad, normalmente, se localiza en las bifurcaciones y curvaturas de las arterias, donde el endotelio está expuesto a la presión ejercida por el flujo sanguíneo y que depende de la viscosidad de la sangre y su velocidad, lo que se ha dado en llamar tensión de cizallamiento. Una tensión disminuida provoca la reducción de la producción endotelial de óxido nítrico, el aumento de la expresión de moléculas de adhesión, de citoquinas inflamatorias como el TNF-α[143,144] y citoquinas que aumentan la permeabilidad

y la migración de monocitos siendo ésta la primera etapa de la formación de la lesión aterosclerótica (figura 8A)[145]. Todo ello permite la entrada de lipoproteínas y de otros constituyentes del plasma por la pared arterial, así como la acumulación en la matriz subendotelial de colesterol procedente de las LDL. Cuando existe un exceso de LDL circulantes, aumenta el transporte y la retención de las mismas en los lugares donde la permeabilidad es mayor. Las LDL difunden a través del endotelio y quedan retenidas en la íntima debido a la interacción entre la molécula de la apolipoproteína B de las LDL y los proteoglicanos de la matriz subendotelial lo que favorece su degradación proteolítica, oxidación y consiguiente captación por los macrófagos tisulares polarizados[136,146].

En un estadio posterior (figura 8B) y una vez constituida la estría grasa, se forma la placa fibrosa, caracterizada por la migración de las células musculares lisas desde la capa media hacia la matriz subendotelial donde proliferan y captan lipoproteínas modificadas contribuyendo así, junto con las células espumosas, al engrosamiento del núcleo lipídico, que se asocia a una disminución del grosor de la capa media. Estas células musculares lisas secretan proteínas de la matriz extracelular como colágeno y proteoglicanos, lo que aumenta la retención y agregación de lipoproteínas modificadas y conduce al desarrollo de la placa fibrosa. Esta fase del desarrollo de la lesión está influenciada por las interacciones entre monocitos/macrófagos y células T, que dan como resultado una amplia gama de respuestas humorales y celulares y la adquisición de muchas características de un estado inflamatorio crónico[136,145].

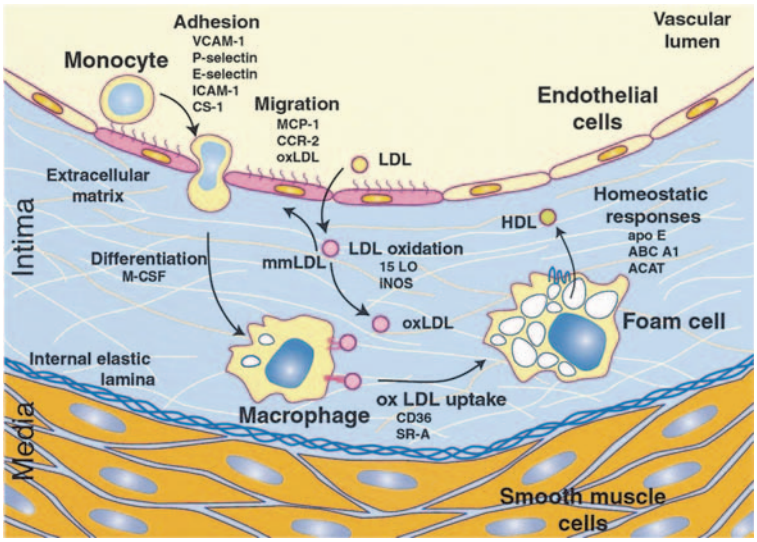
2.4.2 Macrófagos en la aterosclerosis

Una de las funciones más críticas de los macrófagos, en el contexto de la aterosclerosis, es la captación y deposición de lípidos que tiene un fuerte impacto en la progresión subsiguiente de la enfermedad. Durante la última década, ha sido ampliamente documentada y estudiada la heterogeneidad de las poblaciones de macrófagos[147] en la lesión aterosclerótica.

Normalmente los macrófagos residentes se encuentran en estado de reposo y se activan cuando son estimulados por varios factores tales como TNF-α, IL-1β, IL-6, interferón γ, etc. Esta tipología de activación de los macrófagos se conoce como activación clásica y es promovida por la secreción de moléculas producidas por las células T-*helper* en respuesta a una lesión o infección[148]. Los macrófagos activados de esta forma se denominan de tipo 1 (M1) proinflamatorios y proaterogénicos con una elevada actividad microbicida. Los macrófagos se pueden activar en forma alternativa (M2) en respuesta a las interleucinas IL-4 o IL-13 o glucocorticoides, muestran un programa de expresión

génica antiinflamatorio y contribuyen activamente a la resolución de la inflamación. Siendo como son células con un alto nivel de plasticidad, los macrófagos pueden cambiar del estado M1 a M2 y viceversa en función del microambiente endocrino que las rodea[147,149,150]. La realidad hasta ahora se ha mostrado más compleja que el paradigma de los macrófagos M1 o M2 identificándose un fenotipo heterogéneo de los macrófagos presentes que no encaja completamente en uno u otro de los grupos[140].

A



B

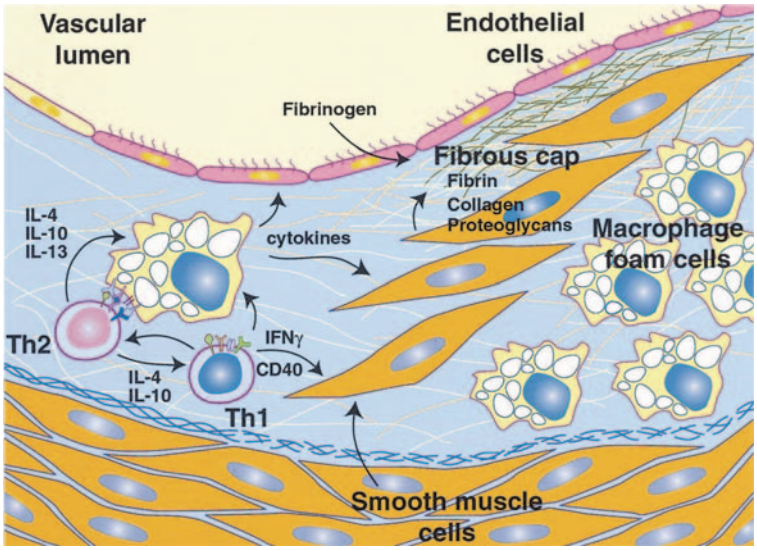


Figura 8. Fase inicial (A) y avanzada (B) en la formación de la lesión aterosclerótica y la formación de la placa fibrosa[145].

Se ha sugerido que la aterosclerosis puede ser causada no sólo por una reacción pro-inflamatoria sino también por su ausencia[151]. Por ejemplo, el bloqueo parcial de NF-kB en macrófagos, una vía claramente proinflamatoria, incrementa el tamaño de la lesión aterosclerótica, contrariamente a lo esperable[152]. Aunque no se ha aclarado el mecanismo, se ha atribuido al menor aumento de la expresión de IL-10 hallada en estos macrófagos, que tiene un claro efecto protector y que se da en los macrófagos durante la progresión de la inflamación simultáneamente al incremento de marcadores inflamatorios.

Placas de pacientes con aterosclerosis sintomática reciente de la carótida, presentan un predominio de macrófagos M1 y contenido de lípidos superior en comparación con las placas femorales de pacientes asintomáticos[153]. Además, se ha relacionado la polarización de los macrófagos con la vulnerabilidad de las placas ateroscleróticas. Las placas de pacientes sintomáticos contienen una mayor concentración de macrófagos M1. Por el contrario, el aumento de expresión de marcadores asociados con los macrófagos M2, se ha observado en placas de pacientes asintomáticos[147].

3. El tejido adiposo

En el organismo los lípidos se almacenan en 2 tipos de tejido adiposo (TA): el tejido adiposo blanco (TAB) y el tejido adiposo marrón (TAM)[154]. El TAB es el principal tejido de almacén de energía del organismo, además tiene la función de aislamiento y protección mecánica de algunos órganos vitales[155] y su distribución es más amplia (figura 9)[156]. El TAM en los seres humanos es abundante en los recién nacidos, pero luego se reduce progresivamente[157] aunque no llega a desaparecer contrariamente a lo que se creía.

Los adipocitos maduros del TAB muestran el perfil de expresión requerido para la síntesis de triacilgliceroles, captación de glucosa y ácidos grasos y lipogénesis, así como de lipólisis[155]. Este fenotipo permite que cuando el aporte de energía al organismo es excesivo y/o el gasto energético disminuye, el exceso de energía se deposite eficientemente en el TAB en forma de triacilgliceroles. Por otro lado, en el caso de escasez de ingesta energética y/o incremento del gasto energético el TAB moviliza los depósitos de lípidos liberando ácidos grasos y glicerol que a través de la sangre son transportados a los tejidos, donde serán oxidados para obtener energía[158] .

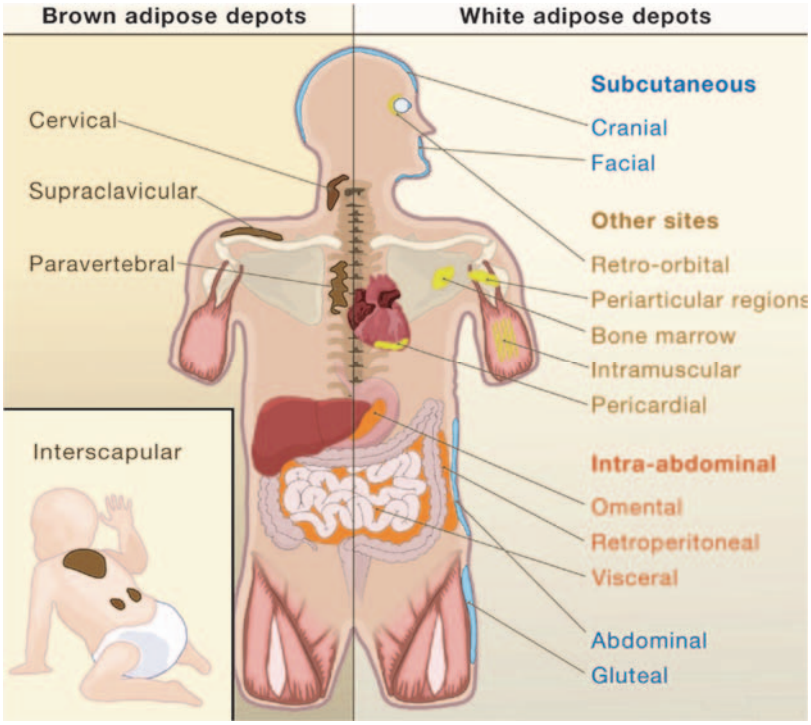


Figura 9. Distribución corporal del TAB y del TAM en humanos[156].

3.1 El tejido adiposo es un órgano endocrino

Al margen de su función metabólica, el tejido adiposo se ha revelado como un órgano endocrino capaz de secretar moléculas con múltiples funciones en el resto del organismo[159]. Por otra parte, también un hallazgo de la última década sido la comprensión de que el TAB presenta una gama compleja y dinámica de componentes celulares más allá de los adipocitos, que juegan papeles críticos en el mantenimiento de la homeostasis de nutrientes[159]. Los adipocitos supondrían alrededor del 50% del total de células y el resto constituye la fracción estromal vascular que comprende fibroblastos, células endoteliales, preadipocitos y macrófagos infiltrados[160] entre otros.

Hoy se sabe que el TAB es un tejido altamente dinámico que sintetiza y secreta numerosos factores de naturaleza lipídica y peptídica, que intervienen en la regulación de un amplio rango de procesos fisiológicos y metabólicos. A pesar de que a finales de la década de los ochenta ya se describe que el TAB secreta hormonas esteroideas[161], no es hasta 1994 con el descubrimiento de la leptina[162], que se reconoce al TAB como órgano endocrino. Posteriormente se han identificado numerosos factores liberados por el TAB con funciones autocrinas, paracrinas y endocrinas[154].

De las numerosas sustancias secretadas por el TAB, los ácidos grasos son cuantitativamente las moléculas más importantes y son liberados en periodos de balance

energético negativo, como en el ayuno[158]. Otras moléculas de naturaleza lipídica son también secretadas por el TAB, incluyendo prostaglandinas sintetizadas por el propio tejido, colesterol y retinol que se almacenan para ser liberados posteriormente[163] y hormonas esteroideas (estrógenos y glucocorticoides), que en el TAB pueden experimentar transformaciones de formas inactivas a activas o viceversa, con un importante papel autocrino y paracrino, como es el caso de la enzima 11β-HSD1 y 11β-HSD2 ya detallado anteriormente[154].

Aparte de sustancias de naturaleza lipídica, el tejido adiposo secreta un número considerable de proteínas que se designan bajo la denominación común de adipoquinas (figura 10). De forma estricta, el término «adipoquina» debería utilizarse para designar las proteínas que son sintetizadas y secretadas por el adipocito[164,165]. Sin embargo, de forma genérica se utiliza para referirse a las proteínas sintetizadas y secretadas por el TAB en su conjunto, aunque su síntesis principal sea a cargo de otros tipos celulares presentes en el tejido, como los macrófagos infiltrados. Las adipoquinas son muy diversas en cuanto a la estructura química y a la función fisiológica [166,167]. Muchas de ellas están relacionadas con el sistema inmunitario, incluyendo citoquinas clásicas como TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-4, IL-13 y MCP-1, pudiéndose establecer un nexo entre la inflamación y la obesidad, situación en que se incrementa la secreción de adipoquinas proinflamatorias[168]. Las adipoquinas también incluyen proteínas que intervienen en la regulación de la ingesta y del balance energético (leptina), en la regulación de la presión sanguínea (angiotensinógeno), en la hemostasia vascular (PAI-1), en el metabolismo lipídico (RBP-4, CETP), en la homeostasis glucídica (adiponectina, resistina, visfatina), en la angiogénesis (VEGF), así como factores de crecimiento (TGFβ) y proteínas de fase aguda y respuesta al estrés (haptoglobulina, α1-acid glycoprotein) con un amplio e importante papel regulador a distintos niveles fisiológicos[154].

3.2 Tejido adiposo visceral y subcutáneo

Es necesario mencionar la diferencia entre el tejido adiposo visceral y el tejido adiposo subcutáneo glúteo-femoral: el primero se encuentra en la zona intraabdominal y el segundo es más superficial y se distribuye principalmente en la parte inferior del cuerpo (figura 11). Es importante tener en cuenta que el SM está especialmente asociado al tejido adiposo visceral, y no al glúteo-femoral. Este es un factor que puede explicar el mayor riesgo de padecerlo en los hombres que en las mujeres: la grasa visceral es de hecho el mayor depósito en la distribución de grasa masculina, en comparación con el tejido subcutáneo glúteo-femoral, que está más presente en mujeres[169] (figura 11).

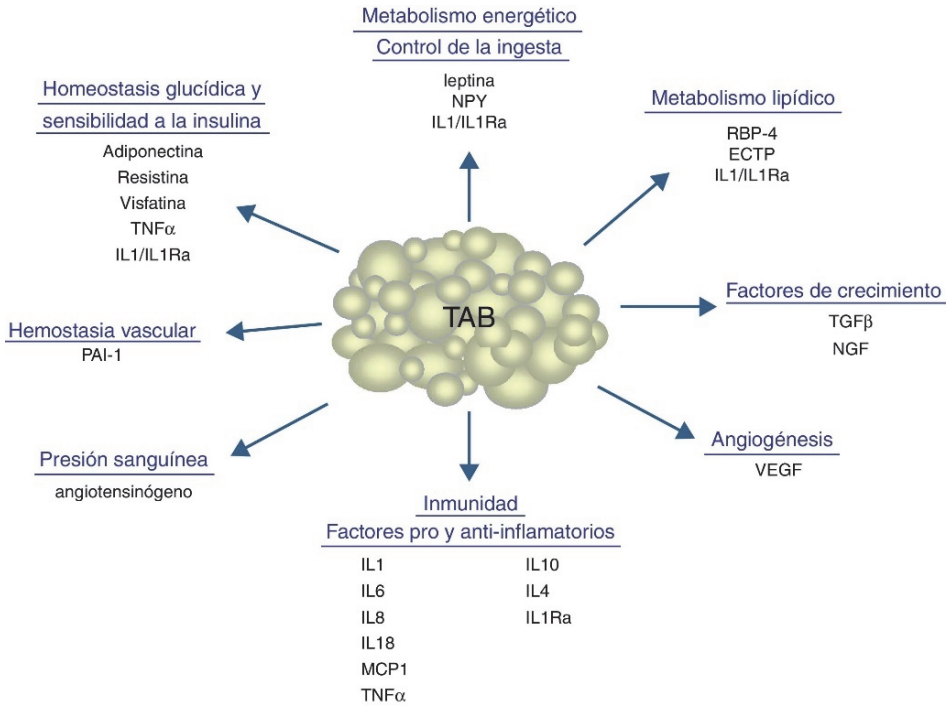


Figura 10. Esquema de los procesos fisiológicos y metabólicos regulados por el TAB mediante la secreción de adipoquinas[154].

La obesidad abdominal se asocia con el desarrollo de enfermedades cardiovasculares, resistencia a la insulina, diabetes tipo 2 y mortalidad. Por el contrario, la obesidad glúteo-femoral muestra una asociación inversa con enfermedades cardiovasculares y diabetes tipo 2[170,171]. Los depósitos abdominales se caracterizan por una rápida acumulación de la grasa de la dieta a la vez que son fácilmente estimulables a través de la activación de los receptores adrenérgicos con el consiguiente aumento de la lipólisis. Los depósitos glúteo-femorales evidencian un intercambio lento de los lípidos y parecen tener mayor capacidad para reclutar nuevos adipocitos, mostrando menos signos de inflamación. La vía metabólica de la acumulación inmediata de los depósitos en la parte superior del cuerpo consiste en la recuperación de ácidos grasos a través de las lipoproteínas de muy baja densidad (VLDL)[172,173] y los ácidos grasos no esterificados (NEFA)[174–179].

3.3 El tejido adiposo blanco en la obesidad: un órgano inflamado

La obesidad afecta a la función de muchos tejidos del cuerpo, no solo el adiposo, incluyendo el páncreas[180–182], el hígado[183,184], músculo-esquelético[185], el corazón[186,187], las articulaciones y el sistema nervioso central[188]. Como se mencionó anteriormente, a nivel clínico la acumulación de tejido graso contribuye al desarrollo de la diabetes tipo 2, hipertensión, hipercolesterolemia, aterosclerosis, aumenta el riesgo de cáncer, artritis y enfermedades neurodegenerativas como la enfermedad de Alzheimer. Aunque hay varios

mecanismos moleculares que asocian a la obesidad con sus complicaciones, actualmente se considera la inflamación crónica que se da en la obesidad como la mayor causante de tal asociación[26,148,189–191].

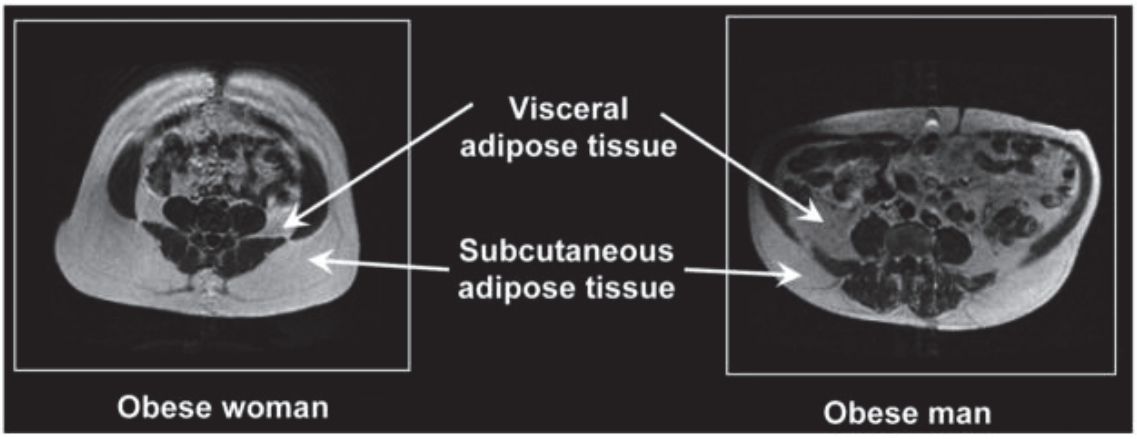


Figura 11. Sección transversal de resonancia magnética abdominal, imágenes de niveles de gris de una mujer obesa y un hombre obeso[169].

Los macrófagos del tejido adiposo constituyen entre el 5 y 10% del total de las células presentes, pero la ganancia de peso inducida por la dieta puede generar un aumento significativo de la infiltración de macrófagos[192]. Fueron detectados por primera vez en el año 2000 con la utilización de técnicas inmunohistoquímicas empleando anticuerpos anti-CD68 o anti-α-quimiotripsina. La inflamación se identificó inicialmente por la elevada expresión de citoquinas inflamatorias tales como TNF-α[193], IL-6[194], MCP-1[195,196] y PAI-1[197] en tejido adiposo de pacientes obesos. Basándose en estas observaciones, los estudios iniciales se centraron en cómo estos mediadores inflamatorios se generaban por los adipocitos. Sin embargo, en 2003, dos artículos demostraron que la mayoría de los factores inflamatorios, no eran producidos por los adipocitos, sino por las células del estroma, entre las que destacaban los macrófagos, una importante fuente de factores de inflamación[192,198]. Esto condujo a una serie de estudios que demostraron que los macrófagos se acumulan en el tejido adiposo en sujetos obesos, son prominentes en el tejido adiposo visceral, y disminuyen con la pérdida de peso[199–202].

Estos resultados permitieron hipotetizar que la interacción entre los adipocitos y los macrófagos en tejidos adiposo durante la obesidad forman parte de los mecanismos fisiopatológicos que permiten el establecimiento de la resistencia a la insulina y posteriormente la DM2. Adicionalmente, plantearon que probablemente el deterioro al endotelio, producto del daño oxidativo resultante de un marcado ambiente lipolítico,

jugaba un rol importante en el reclutamiento de los macrófagos similar al observado en la ateroesclerosis[203].

Los estudios de Cinti en 2005 y Strissel en 2007 evidencian que la hipoxia tiene lugar cuando el TA se expande en un periodo breve de tiempo, que contribuye a la necrosis de los adipocitos que, a su vez, atrae a los macrófagos a fin de eliminar las células muertas y retirar su contenido lipídico potencialmente citotóxico. Se ha demostrado que la hipoxia podría participar activamente en el desarrollo de inflamación asociada a la obesidad, con un notable papel en la alteración de la secreción de adipoquinas, en el aumento en la expresión de genes proinflamatorios y en la muerte de los adipocitos[204–206].

La naturaleza proinflamatoria del tejido adiposo incrementa en proporción a la acumulación de grasa y muestra una correlación positiva con el aumento de IMC y en particular del tejido adiposo visceral. En el TA de personas con normopeso (figura 12A), la IL-4 producida por los eosinófilos estimula la producción por parte de los macrófagos M2 residentes, de citoquinas antiinflamatorias tales como IL-10. Este fenotipo establece un entorno inmunológico antiinflamatorio y promueve directamente la sensibilidad a la insulina de los adipocitos. A su vez, los adipocitos producen adiponectina, que colabora con IL-4 en promover la polarización alternativa de macrófagos M2. En el TA de personas con obesidad (figura 12B), los macrófagos inflamatorios M1, activados por el exceso de nutrientes, producen citoquinas proinflamatorias que exacerban la resistencia a la insulina, aumentan el estrés celular, y reclutan monocitos adicionales. Los adipocitos, a su vez, también secretan citoquinas inflamatorias y liberan al entrar en necrosis ácidos grasos saturados que refuerzan el entorno inflamatorio[207].

Los *peroxisome proliferator-activated receptors* (PPAR-α,-β y –γ) están involucrados en la regulación y la homeostasis del metabolismo lipídico y glucídico pero también de la inflamación. Los PPAR-γ se pueden encontrar en diversos tejidos, pero especialmente en adipocitos, en las células intestinales y en los macrófagos. Su activación provoca el aumento de la transcripción de muchos genes adipogénicos (tales como la LPL, FAT/CD36, GLUT4, acil-CoA sintetasa) que tienen en general un efecto hipoglucémico e hipolipemiante. Los agonistas sintéticos de PPAR-γ, las glitazonas, se utilizan en el tratamiento de la DM2 y de hecho, reducen la respuesta inflamatoria a través de la reducción directa de la transcripción de genes proinflamatorios, e indirectamente a través de su efecto sobre el metabolismo lípidico y glucídico. En ambos casos, como resultado, hay una reducción de la infiltración de macrófagos en el tejido adiposo[208].

Estudios en ratones tratados con dieta hiperlipidica y con macrófagos deficientes en PPAR-γ -/-, mostraron que estos macrófagos eran incapaces de diferenciarse a M2, y los

ratones portadores presentaban obesidad exacerbada y una resistencia a la insulina más acentuada. En el hígado, mostraron esteatosis agravada y mayor presencia de macrófagos (células de Kupffer) inflamatorios M1. En el tejido adiposo se observó una menor sensibilidad a la insulina y mayor infiltración de macrófagos de tipo M1. Esto demuestra que la presencia de macrófagos M1 y la incapacidad de activación de la vía alternativa juegan un papel importante en el mantenimiento de la inflamación y en el establecimiento de la resistencia a la insulina y la obesidad[208].

Además de las citoquinas inflamatorias y las moléculas de señalización intercelular, también hay que tener en cuenta las vías intracelulares que regulan la respuesta inflamatoria en el desarrollo de la obesidad[148]. En estas vías, NF-kB es un factor de transcripción de múltiples proteínas que tiene como objetivo la inducción de proteínas inflamatorias, tales como el TNF-α y MCP-1[148,209–211]. Muchos estímulos inflamatorios, incluyendo la activación de receptores TLR, especies reactivas del oxígeno, luz ultravioleta y citoquinas proinflamatorias, llevan a la fosforilación y la consiguiente activación de NF-kB. Una vez liberado del inhibidor de NF-kB (IkB) que al fosforilarse inicia su degradación, el complejo proteico NF-kB migra del citosol al núcleo celular donde activa la transcripción de genes diana[148,212,213].

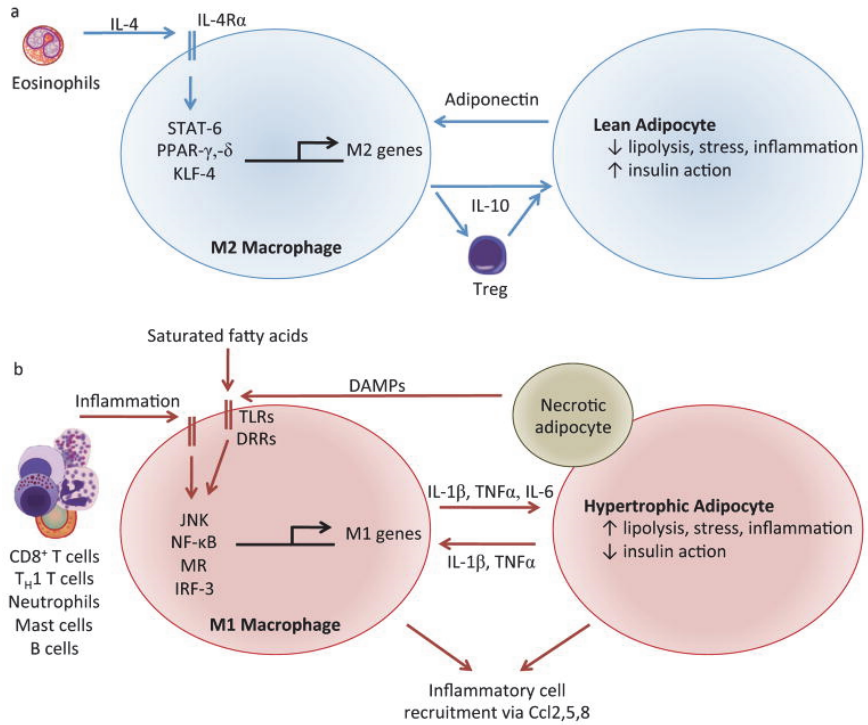


Figura 12. En el tejido adiposo de personas con normopeso y personas con obesidad se encuentran mayoritariamente macrófagos M2 y M1 respectivamente que se diferencian a su fenotipo en función del ambiente endocrino y expresan proteínas con actividad mayoritariamente anitinflamatoria o proinflamatoria[207].

La obesidad provoca el aumento de la presencia de NF-kB en el núcleo celular de células hepáticas y en músculo esquelético además de tejido adiposo[148,214,215]. De manera análoga también se eleva en la obesidad la actividad de las *c-Jun NH2-terminal kinases* (JNKs), una familia de tres serina/treonina quinasas, estructuralmente relacionadas (JNK1-3)[216], en músculo y tejido adiposo pero es su incremento en hígado, macrófagos y cerebro el que se ha relacionado directamente con el establecimiento de la inflamación, resistencia a la insulina y esteatosis hepática[217]. La supresión de JNK1 en ratones atenúa el desarrollo de la obesidad inducida por la dieta y reduce la inflamación en tejido adiposo, la esteatosis hepática i la resistencia a la insulina, aunque produce el aumento del daño oxidativo en la piel[218].

En la respuesta inflamatoria que se produce en tejido adiposo a consecuencia de la obesidad inducida por la dieta, los neutrófilos son las primeras células del sistema inmune reclutadas lo cual se produce tan solo entre los 3 y 7 días de iniciarse el seguimiento de la dieta. Cuando la respuesta inflamatoria progresa a consecuencia del mantenimiento de la dieta, se produce un cambio gradual en el tipo de células presente el tejido inflamado, aumentando la proporción de macrófagos solucionadores del daño y de la inflamación. Si persiste el estímulo inflamatorio se llega a un estado inflamatorio crónico que rompe el equilibrio entre las células proinflamatorias y antiinflamatorias[219].

Incluso tejidos periféricos alejados del tejido adiposo sufren las consecuencias de la inflamación en tejido adiposo a través de la secreción de adipoquinas al torrente sanguíneo. Diversos estudios con animales obesos mostraron que una respuesta inflamatoria exagerada en tejido adiposo puede causar disfunción en diversos órganos, incluyendo el cerebro, el corazón y el intestino[220].

OBJETIVOS

La actividad antiinflamatoria de dosis farmacológicas de glucocorticoides los ha convertido en una de las familias de fármacos más utilizados en las últimas décadas a pesar de que su prescripción se haya sujeta a restricciones debido a los efectos indeseados sobre el metabolismo, entre otros. La acción sobre la inflamación de los glucocorticoides endógenos no está bien caracterizada, siendo antiinflamatoria en algunas ocasiones, pero también proinflamatoria en otras. Parte de la dificultad reside justamente en la multiplicidad de funciones que realizan. Sobre el metabolismo, promueven la deposición central de lípidos, que claramente desemboca en inflamación, así como también causan resistencia a la insulina, una de las consecuencias de la inflamación. Sin embargo, también presentan acción antiinflamatoria. ¿Cómo disociar ambos efectos?

A nivel fisiológico, los glucocorticoides siguen un ritmo circadiano con niveles variables según el momento del día. Por otra parte, su acción depende de la modulación de su biodisponibilidad a través de la capacidad de la proteína trasportadora, CBG que regula los niveles de hormona libre en sangre y la actividad de las enzimas 11β-HSD1 y 11β-HSD2 que activan la cortisona a cortisol e inactivan cortisol a cortisona respectivamente en el medio intracelular. Recientemente, además, se ha atribuido a la 11-βHSD1 el papel de auténtica traductora de la acción glucocorticoide en los tejidos, mediante la síntesis intracelular estimulada por los glucocorticoides circulantes.

También ha adquirido en los últimos tiempos un creciente interés el rol de la CBG regulando o mediando las acciones de los glucocorticoides y su implicación en patologías donde los glucocorticoides intervienen tanto como promotores, sería el caso de la obesidad, o como atenuadores, que sería el caso de la artritis reumatoide. Ello es debido a la pertenencia de la CBG a la familia de inhibidores de las serina proteasas lo que la convierte en sustrato de enzimas como la elastasa de neutrófilos, que proliferan en las zonas de inflamación y la hidrolizan provocando la liberación del corticoide que transporta, justamente en el lugar donde su acción antiinflamatoria es necesaria.

Por todo ello, nos hemos planteado como objetivo de esta tesis caracterizar la acción de los glucocorticoides en diversos modelos de patologías asociadas a inflamación y como ésta se ve afectada por la modulación de su biodisponibilidad. En el grupo de investigación en el que se ha desarrollado esta tesis doctoral, la investigación sobre los desencadenantes y consecuencias de la obesidad son motivo de estudio desde hace tiempo. Teniendo en cuenta la implicación de los glucocorticoides en el desarrollo de la obesidad y el síndrome metabólico, decidimos estudiar en primer lugar, la patología con

una de las mayores prevalencias mundiales y desencadenante de las enfermedades cardiovasculares, la aterosclerosis, en la que el papel de los glucocorticoides sobre su progresión y concretamente sobre los macrófagos, principales protagonistas, no estaba todavía clarificado.

En segundo lugar, nos planteamos analizar la evolución de la inflamación asociada a la obesidad inducida por la dieta en el modelo de ratones deficientes en CBG, con la alteración de la biodisponibilidad de glucocorticoides que supone y en ausencia de CBG, que se ha implicado en la mediación de la actividad antiinflamatoria de los glucocorticoides. El modelo que utilizamos es el desarrollado por Thomas Willnow, que nos cedió 4 ratones heterozigotos con los que montamos una colonia en Barcelona.

En este mismo modelo de ratón deficiente en CBG, un estudio previo había mostrado como estos ratones presentaban mayor inflamación y menor supervivencia ante una exposición a LPS que ratones WT. Ello llevó a los autores a hipotetizar que su respuesta antiinflamatoria estaba deteriorada a causa de la ausencia de CBG. Por ello, nos planteamos utilizar otro modelo diferente donde también se produce una inflamación aguda a corto plazo como es el caso de la pancreatitis. El daño pancreático provoca la fuga de enzimas pancreáticas al resto de tejidos y una inflamación generalizada. La mortalidad asociada a esta patología se produce a causa de un fallo pulmonar provocado por una inflamación exacerbada en este tejido.

Por todo ello, los objetivos específicos planteados han sido:

1) Determinar los efectos de los glucocorticoides y la 11β-HSD1 en el control de la homeostasis del colesterol en la línea celular de macrófagos humanos THP-1 sometidos a un estímulo proinflamatorio desencadenante de la formación de las placas de ateroma causantes de la aterosclerosis.

2) Analizar los cambios en la biodisponibilidad de corticosterona en la obesidad y evaluar la progresión y establecimiento de la inflamación asociada a la obesidad inducida por la dieta en ausencia de CBG, utilizando un modelo de ratón KO para CBG.

3) Estudiar la evolución de una patología asociada a inflamación aguda, la pancreatitis, en el modelo murino de déficit de CBG e identificar si la alteración de la biodisponibilidad de los glucocorticoides y la ausencia de CBG afecta a la progresión de la enfermedad

.

RESULTADOS

1. *Data related with inflammation and cholesterol deposition triggered by macrophages exposition to modified LDL*

Este artículo complementa los datos presentados en el apartado 2 de este capítulo y publicados bajo el título: *Decreased OxLDL uptake and cholesterol efflux in THP1 cells elicited by cortisol and by cortisone through 11β-hydroxysteroid dehydrogenase type 1.*

Ambos estudios se desarrollaron en colaboración entre nuestro grupo de investigación y el grupo de la Dra. Marina González del Departamento de Bioquímica Celular del Instituto de Investigación Bioquímica (INIBIOLP) de La Plata (Argentina), donde realicé una estancia de dos meses.

En este trabajo se detallan los experimentos previos que fueron necesarios para la realización de los estudios sobre la función de los glucocorticoides y de la 11β- HSD1 en la línea celular de macrófagos humanos THP-1. Consistió en la puesta a punto del protocolo de oxidación de LDL humanas con diversa intensidad (baja, media o alta oxidación), su verificación y el ensayo de los efectos que causaban sobre la viabilidad celular de los macrófagos, comparándolos con los efectos de LDL nativa.

Una vez determinado que el grado medio de peroxidación era efectivo para conseguir la formación de células espumosas sin alterar la viabilidad celular, se realizaron experimentos de *time-course* para determinar cuándo se iniciaba la respuesta inflamatoria y comenzaba la alteración de la expresión de los transportadores membranales de lipoproteínas y el enzima responsable de la esterificación de colesterol intracelular, ACAT, implicados en la acumulación de colesterol intracelular.

Todo ello nos llevó a constatar que 24 horas era el tiempo suficiente de incubación para evaluar las alteraciones provocadas por las OxLDL en THP-1, puesto que ya se podían detectar células espumosas en este período así como la modificación de la expresión de los genes involucrados.

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Data Article

Data related to inflammation and cholesterol deposition triggered by macrophages exposition to modified LDL

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ABSTRACT

This article supports experimental evidence on the time-dependent effect on gene expression related to inflammation and cholesterol deposition in lipid-loaded cells. The cells employed were human monocytes THP1 line transformed into macrophages by treatment with phorbol esters. Macrophages were treated at different times with oxidized low density lipoprotein (Ox-LDL) and then gene expression was measured. We also include data about the different types of oxidized lipoprotein obtained (low, media or high oxidation) for differential exposure with Cu ions. These data include characterization to lipid and protein peroxidative damage and also quantification of cell viability by exposure to native and modified LDL. The present article complements data published in "Decreased OxLDL uptake and cholesterol efflux in THP1 cells elicited by cortisol and by cortisone through 11β-hydroxysteroid dehydrogenase type 1" Ledda et al. (in press) [1].

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Specifications Table

Subject area	Biology
More specific subject area	Biochemistry and Molecular Biology
Type of data	Figures
How data was acquired	Images were obtained at transmission electron microscope JEM 1200 EX II (JEOL Ltd., Tokyo, Japan) and then photographed by an Erlangshen camera ES1000W (Model, 785 Gatan Inc., Pleasanton, California, USA) Analytical measures were registered in a two-beam spectrophotometer -Cintra-20, Sydney, Australia. RT-PCR reactions were run on ABI PRISM 7900 HT detection system (Applied Biosystems)
Data format	Analyzed
Experimental factors	THP1 cells line from ATCC® Number: TIB-202™. LDL as obtained from healthy human donors from Instituto de Hemoterapia de la Provincia de Buenos Aires, Argentina
Experimental features	Cellular RNA was extracted with Trizol according to the technique supplied by the manufacturer and cDNA synthesis was performed employing iScript cDNA Synthesis Kit. Real-Time PCR was carried out using SYBR Green PCR Master Mix on an ABI PRISM 7900 HT detection system using an annealing temperature of 60 °C.
Data source location	La Plata, Argentina and Barcelona, Spain
Data accessibility	Data are with this article

Value of the data

- These data show the characterization of different types of oxidized LDL that refers to protein and lipid lipoprotein peroxidation. This information could be considered in studies employing Ox-LDL.
- Data presented in this article also show that the degree of LDL oxidation is directly related to damage and viability cell. This result could be potentially important for further investigations related to the initiation of plaques in atherosclerotic lesions.
- Our data show that the expression of genes related to inflammation and lipid accumulation in human macrophages is time-dependent. These data could be helpful for other researchers in the development of experiments related to atherosclerotic diseases.

1. Data

The data presented in this article provide information about the characterization of different types of oxidized LDL as well as how modified LDL alters the gene expression related to inflammation process. Dataset shows the methodology used to obtain different types of oxidized low density lipoproteins (Ox-LDL) in order to produce low (L), medium (M), or high (H) peroxidation degree of LDL. We characterized Ox-LDLs (L, M, and H) using quantitative techniques for lipid determination and protein peroxidation. To perform experiments, we selected medium peroxidation degree LDL (M) based on cell viability. We show data about the time-dependency of gene expression involved in the inflammatory process and cholesterol loading.

2. Experimental design, materials and methods

2.1. Ox-LDL characterization

We have obtained Ox-LDL employing the Cu^2 oxidation method (see Ref. [21] in [1]). Native human (N-LDL, 3 mg/ml) in a volume of 15 ml was treated with 0.5 ml copper sulfate (3.6 mg/ml) at a final concentration of 5 μM at 37 °C under gentle agitation for 4, 8, or 12 h in order to produce L, M, or H peroxidation degree of LDL. To stop the peroxidation process, each preparation was treated with a solution of butylhydroxytoluene (BHT) in PBS at a final concentration of 0.1 mM and immediately subjected to dialysis against PBS (50 mM, pH 7.40, changed every 8 h) for 24 h to eliminate BHT and Cu ions. Peroxidative damage infringed to lipid and protein components of the native LDL induced by copper treatment was evaluated by thiobarbituric acid reactive substances (TBARS) [3], conjugated dienes formation [2] and protein carbonyls (PCs) [4].

TBARS was fluorimetrically determined to estimate the extent of lipid peroxidation in homogenates [3]. An aliquot of cellular homogenates (50–100 μL) was reacted with 200 μL of SDS (8.10%, W/V) and 1.5 mL of acetic acid 10% (V/V) (pH 3, 5). Subsequently, 1.5 mL of thiobarbituric acid (TBA 0.8%) and 600 μL of water were incorporated and the mixture was heated in sealed tubes at 95 °C for 60 min. Under these conditions TBARS (mainly malondialdehyde (MDA) generated by lipid peroxidation) reacted with TBA to yield TBA-MDA adducts which were quantified at 515 nm excitation and 553 nm emission. The concentration of the chromophore was calculated from a calibration curve prepared with fresh tetrametoxipropane (TMP) solutions (TMP was purchased from Sigma Chem. Co., Buenos Aires, Argentina).

PCs were determined by the method of Reznick and Packer [4]. Aliquots of cellular homogenates were incubated with dinitro-phenylhydrazine in HCL 2 N at 37 °C in the dark for 30 min. The corresponding hydrazone-derivatives present in the proteins were revealed after the addition of excess NaOH and measured at 505 nm. The concentration of PCs was calculated from a calibration curve prepared with a stock solution of sodium pyruvate (Sigma Chem. Co., Argentina).

TBARS formation was expressed as *m* moles of malondialdehyde (MDA)/mg protein (first bar) and conjugated dienes as optical density units (ODU)/mg protein (second bar) are shown in.

Fig. 1A Carbonylation of aminoacyl residues in LDL protein after pro-oxidant treatment is shown in Fig. 1B and expressed as *n* moles of pyruvate (Pyr)/mg protein. Degree of peroxidation was denoted by capital letters (N, native LDL; L, light-; M, medium-, and H, high-peroxidized LDL). Each bar represents the mean of three independent experiments assayed in triplicate (mean ± SD).

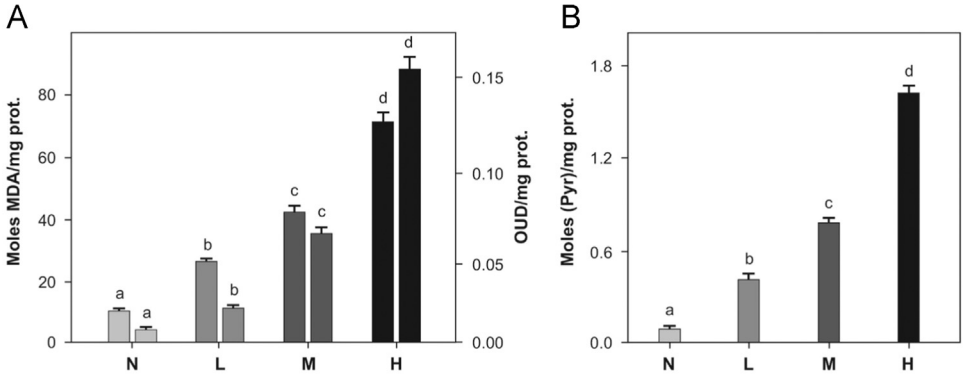

Fig. 1. Peroxidative damage infringed to lipid and protein components of the native LDL induced by copper treatment. TBARS formation (A) and carbonylation of aminoacyl residues in LDL protein (B). Each bar is the mean of three independent experiments assayed in triplicate (mean ± SD). Statistical differences among data of the same type are indicated with different letters on the top of the bars. Data with distinct letters are statistically different between them at $p < 0.01$ signification level (ANOVA + Turkey test).

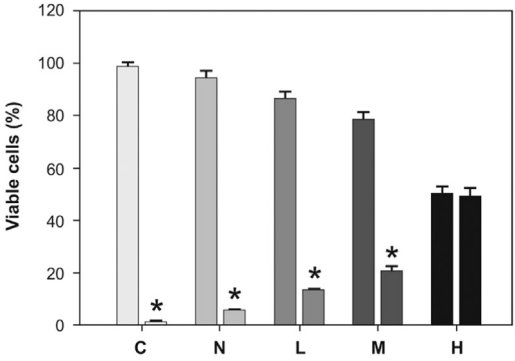

Fig. 2. Cell viability by trypan blue dye (TBD) exclusion test in macrophages treated without (C) or with native (N) or different degree of peroxidized LDL (L, M, or H). First bar corresponds to viable cells and second bar to non-viable cells in each condition. $^{*}p < 0.01$ viable respect non-viable cells.

2.2. Cytotoxic effect of different types of Ox-LDLs (N, L, M and H)

We have evaluated cell viability by trypan blue dye (TBD) exclusion test in macrophages treated with native or peroxidized LDL for 24 h (Fig. 2**)**. The Ox-LDL preparation was diluted 1/10 in culture medium (RPMI). Ox-LDL was added in the culture medium at a final protein concentration of 100 μg/mL at 2 ml final volume for well. According to the evaluation of peroxidation degree of LDL and cells survival test, we have chosen medium (M) peroxidation degree of the LDL to perform experiments described in the present DiB and in the original research article. Briefly, cells were washed with PBS and treated with 100 μL of 0.1% solution of trypan blue dye (in PBS, pH 7.40). After one-min incubation at room temperature (gentle orbital agitation) they were examined under optical microscopy to determine the percentage of viable cells according to the method described by Jauregui et al. [5]. At least four fields of one hundred cells per field were counted and the results were expressed as the percentage of non-viable cells.

2.3. Gene expression involved in inflammatory processes

We selected medium (M) peroxidation degree of the LDL to evaluate the expression of genes involved in the inflammatory process. Monocytes humans cells (THP1) were grown in RPMI medium containing 10% of serum fetal bovine (SFB) at 37 °C in a 5% CO_2 atmosphere. Later, they were stimulated with phorbol esters (PMA-200 nM) for 24 h and transformed into macrophage type. Then, macrophages THP1 were treated with Ox-LDL for 4, 8, 12, and 24 h at a final concentration of 100 μg protein/ml. The expression level of messenger RNA (mRNA) of different genes was quantified by real-time PCR (RT PCR) (see original research article). As a housekeeping gene, the expression of ribosomal protein L4 (RPL4) was measured. To evaluate the inflammation related to the atherogenic process we determined the expression of the genes: epidermal growth factor like module-containing mucin-like hormone receptor-like1 (EMR1), macrophage mannose receptor (MMR) (Fig. 3A) and tumor necrosis factor α (TNFα) (Fig. 3B panel left). To quantify the TNFα secretion in the medium when the cells were treated with Ox-LDL we used a specific enzyme immunoassay from Becton Dickinson Co. (Durham, USA) according to the manufacturer's instructions. Captured antibody was Anti-Human TNF-α monoclonal antibody, the detection antibody was Biotinylated anti-Human TNF monoclonal antibody and the enzyme reagent was conjugated with streptavidin. Biotin–streptavidin interaction increased significantly affinity and specificity of the assay (Fig. 3B panel right).

For the study of genes involved in cholesterol influx from macrophages and foam cells formation we analyzed fatty acid translocase (FAT/CD36) and acyl-CoA: cholesterol acyl transferase (ACAT) (Fig. 3C).

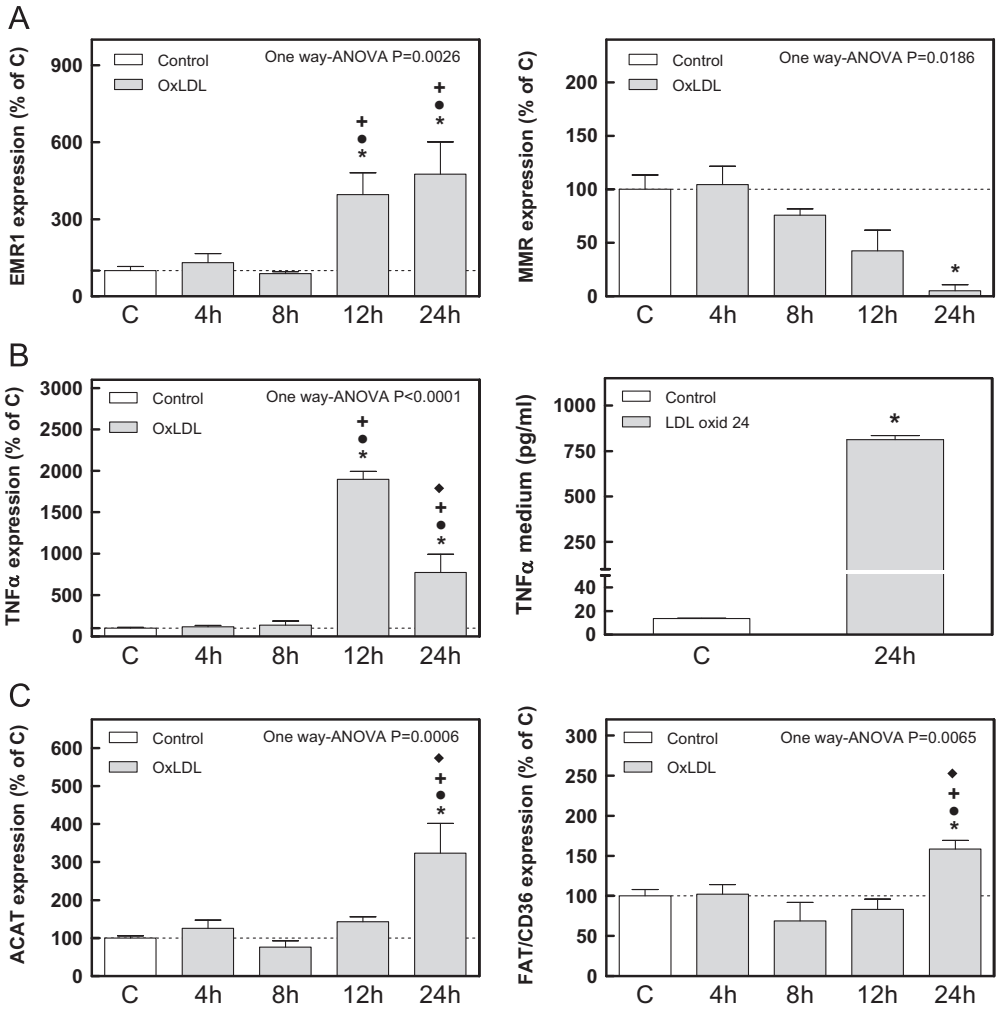

Fig. 3. Gene expression related to inflammatory processes in THP1 cells. A. Expression of EMR1 and macrophage mannose receptor (MMR). B Expression of tumor necrosis factor α (TNFα) (left panel) and TNFα secretion (right panel). C. Expression of fatty acid translocase (FAT/CD36) and acyl-CoA:cholesterol acyltransferase (ACAT). Statistical comparisons were performed by one way-ANOVA and Bonferroni post Test analysis: $^{*}p < 0.05$ vs. C; $\bullet p < 0.05$ vs. 4h; $+p < 0.05$ vs. 8h; $\blacklozenge p < 0.05$ vs. 12h.

2.4. Electron microscopy

The samples treated with or without Ox-LDL were fixed with 2% glutaraldehyde in buffer phosphate (PH 7.2–7.4) for 2 h at 4 °C. Then, cells were centrifuged at 1500 rpm for 10 min. The secondary fixation was performed with osmium tetroxide (1%) for 1 min at 4 °C and then samples were dehydrated with increasing alcohol series and subsequently included in epoxy resin. Ultrathin sections (90 nm) were contrasted with uranyl acetate and lead citrate and examined in a transmission electron microscope JEM 1200 EX II (JEOL Ltd., Tokyo, Japan) and then photographed by an Erlangshen camera ES1000W (Model, 785 Gatan Inc., Pleasanton, California, USA), Central Electron Microscopy service of the Faculty of Veterinary Science, UNLP (Fig. 4).

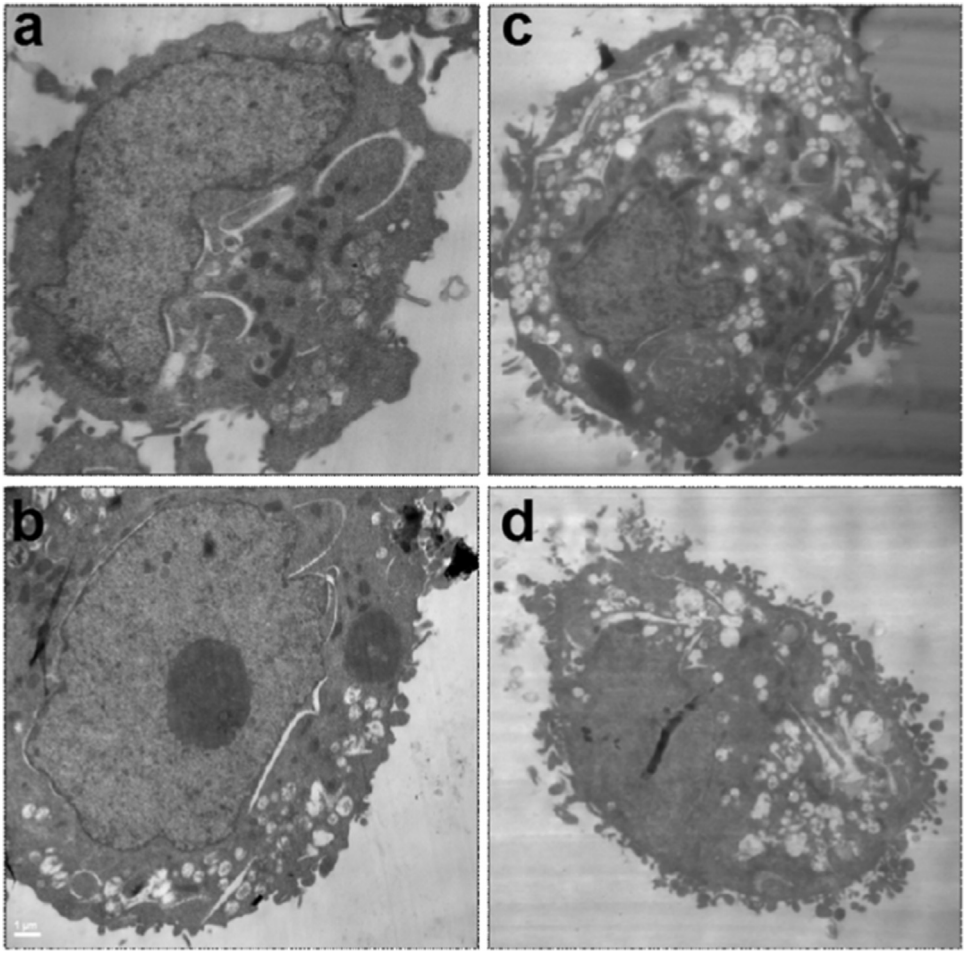

Fig. 4. THP cells electron microscopy. Cells were fixed with glutaraldehyde for electron microscopy, a and b were control without Ox-LDL, c and d were treated with Ox-LDL.

Acknowledgements

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Transparency document. Supplementary Material

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2. *Decreased OxLDL uptake and cholesterol efflux in THP1 cells elicited by cortisol and by cortisone through 11β-hydroxysteroid dehydrogenase type 1*

El objetivo de este estudio fue comprender el papel que juegan los glucocorticoides en la modulación de la respuesta de los macrófagos a un potente estímulo inflamatorio, las OxLDL durante la cual se convierten en células espumosas y pueden acabar provocando aterosclerosis.
Se incubaron células THP1 diferenciadas a macrófagos con ésteres de forbol con OxLDL durante 24 horas en las condiciones experimentales determinadas en el trabajo anterior. La incubación se realizó en ausencia o en presencia de cortisol, cortisona o bien cortisona más un inhibidor de la actividad de la 11β-HSD1, BVT.2733, para determinar el papel de la enzima en los efectos de los glucocorticoides durante este proceso.

Los datos obtenidos demostraron que el cortisol y la cortisona disminuyeron significativamente la inflamación promovida por la incubación con OxLDL, y también la expresión de los genes implicados en la entrada y la salida del colesterol celular lo cual resultó en una menor acumulación de lípidos intracelulares. La presencia del inhibidor de 11β-HSD1 suprimió todos los efectos provocados por la cortisona. El cortisol y la cortisona disminuyeron la expresión de la enzima 11β-HSD1 previamente estimulada de forma potente por la incubación con OxLDL.

Nuestros resultados indicaron un efecto directo de los glucocorticoides frenando la fase inicial de la aterosclerosis, provocando la reducción de los marcadores proinflamatorios, reduciendo la absorción de OxLDL y re-esterificación de colesterol así como su eflujo. Estos efectos podían estar mediados, al menos en parte, por la actividad 11β-HSD1 que incrementó fuertemente a consecuencia de la exposición a OxLDL.

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Decreased OxLDL uptake and cholesterol efflux in THP1 cells elicited by cortisol and by cortisone through 11β-hydroxysteroid dehydrogenase type 1

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ABSTRACT

Background and aims: Data about glucocorticoids role in the development of atherosclerosis are controversial showing different effects in human than in experimental animal models. Atherosclerosis is the result of a chronic inflammatory response to an injured endothelium where an uncontrolled uptake of OxLDL by macrophages triggers the development of foam cells, the main component of fatty streaks in atherosclerotic plaque. There are few data about the direct effect of glucocorticoids in macrophages of atherosclerotic plaque. The aim of the study was to elucidate the role of glucocorticoids in the development of foam cells in atherosclerosis initiation.
Methods: For this purpose we used THP1 cells differentiated to macrophages with phorbol esters and incubated with OxLDL alone or with cortisol or cortisone. THP1 cells were also incubated with cortisone plus an inhibitor of 11β-hydroxysteroid dehydrogenase 1 (11βHSD1) activity to determine the role of this enzyme on glucocorticoid action in this process.
Results: Ours results showed that cortisol and cortisone decreased significantly the inflammation promoted by OxLDL, and also diminished the expression of genes involved in influx and efflux of cholesterol resulting in a reduced lipid accumulation. Likewise cortisol and cortisone decreased 11βHSD1 expression in THP1 cells. The presence of the inhibitor of 11βHSD1 abolished all the effects elicited by cortisone.
Conclusion: Our results indicate a direct effect of glucocorticoids on macrophages braking atherosclerosis initiation, reducing pro-inflammatory markers and OxLDL uptake and cholesterol re-esterification, but also inhibiting cholesterol output. These effects appear to be mediated, at least in part, by 11βHSD1 activity.

1. Introduction

Atherosclerosis is an essential trigger for the development of cardiovascular disease (CVD), which is the main cause of mortality in both developed and developing countries alike [1]. Atherosclerosis occurs as a result of a chronic inflammatory response to an injured vessel wall [2]. One of the main causes of this vessel wall damage is the accumulation of oxidized lipids in low density lipoproteins (OxLDL). As a consequence of lesion, monocytes are recruited to the endothelium, where they polarize to a pro-inflammatory macrophage profile. Then, uncontrolled macrophage uptake of OxLDL leads to the subsequent formation of foam cells, the main components of fatty streaks in atherosclerotic plaque [3].

Abbreviations: EMR1, epidermal growth factor like module-containing mucin-like hormone receptor-like1; CD163, Cluster of Differentiation 163; MMR, macrophage mannose receptor; IL-12b, interleukin-12b; IL-6, interleukin-6; TNFα, tumor necrosis factor α; rIL-10, interleukin-10 receptor; PLA2, phospholipase A2; FAT/CD36, fatty acid translocase; SRA1, Scavenger receptor class A type1; ACAT, acyl-CoA:cholesterol acyltransferase; NCEH1, neutral cholesterol ester hydrolase1; LXRα, liver X receptor α; ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; APO E, apoprotein E; 11βHSD1, 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2; G6PT1, glucose-6-phosphate translocase 1; H6PDH, hexose-6-phosphate dehydrogenase; RPL4, ribosomal protein L4.

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Thus, macrophages play a pivotal role in the development and progression of atherosclerosis, and the knowledge of the factors that regulate their metabolism is crucial to control the process. A key step in the formation of foam cell macrophages is the internalization of OxLDL through specific scavenger receptors (mainly SRA1 and FAT/CD36), but the degree of lipid accumulation is also dependent of cholesterol esterification (regulated by ACAT and NCEH) and cholesterol efflux that involves the activity of ATP-binding cassette transporters (ABCA1, ABCG1), scavenger receptor class B (SR-BI) and high density lipoproteins (HDL). Consequently, foam cell formation is the result of a disrupted balance between cholesterol influx, esterification and efflux in macrophages, which occurs when macrophages fail to restore their cellular cholesterol homeostasis via regulation of reverse cholesterol transport [4].

Stress and visceral obesity are main risk factors for CVD [5,6]. Stressful situations are known to activate the hypothalamic-pituitary-adrenal axis (HPA), inducing increased levels of circulating glucocorticoids; however, the role of glucocorticoids in the development of atherosclerosis remains controversial [7,8]. Whereas circulating glucocorticoids correlate positively with CVD in humans [9,10], animal studies (e.g. on rabbits and dogs) suggest an atheroprotective role of both natural and synthetic glucocorticoids [8,11]. An excess of glucocorticoids, as in Cushing's syndrome or with the pharmacological therapy common in autoimmune diseases, is associated with an increase in atherosclerotic and cardiovascular events [12,13]. Normalization of cortisol levels in patients with Cushing's syndrome largely reverses pathophysiological changes in vascular function and structure [14]. However, most atherosclerosis occurs independently of exogenous glucocorticoid administration, and plasma cortisol levels are not normally elevated in atherosclerosis.

Glucocorticoid availability in tissues could differ from circulating levels due to 11β-hydroxysteroid dehydrogenase type 1 and 2 (11βHSD1 and 11βHSD2) activity. In vivo, the 11βHSD1 enzyme predominantly converts inert glucocorticoids (cortisone in humans, 11-dehydrocorticosterone in rodents) into the corresponding active forms (cortisol in humans, corticosterone in rodents). 11βHSD1 is widely expressed, mainly in liver but also at more modest levels in classical glucocorticoid target cells and tissues, e.g. adipose tissue and immune cells [15]. In 11βHSD1 knockout mice, the absence of 11βHSD1 is atheroprotective [16]. Expression of the 11βHSD2 enzyme, which catalyzes the opposite reaction, is restricted to mineralocorticoid target cells or tissues, mainly in kidney but also in skin, lung and adrenal cortex [17]. 11βHSD2 deficient mice show increased atherosclerotic plaque development, probably due to increased activation of mineralocorticoid receptors by glucocorticoids [18].

Few studies have addressed the direct role of glucocorticoids and 11βHSD1 in the control of cholesterol homeostasis on macrophages throughout the atherogenic process. To elucidate it, we studied the changes caused by cortisol and cortisone in THP1 macrophages incubated in the presence of OxLDL (a potent atherogenic stimulus) on internalization of OxLDL, lipid accumulation and cholesterol efflux. In addition, THP1 macrophages were also incubated with an inhibitor of 11βHSD1 activity to unravel its mediating function of glucocorticoid actions in the macrophages involved in the initiation of atherogenic process.

2. Materials and methods

2.1. Isolation of LDL, preparation of oxidized LDL (OxLDL) and acetylated LDL (AcLDL)

The LDL fraction was isolated from plasma obtained from clinically healthy human volunteers after a 12 h fast. The isolation method employed is described elsewhere [19], with some modifications. The density of the isolated plasma was adjusted to 1.21 g/mL with NaBr in the presence of EDTA 1%. Throughout purification, the resulting plasma was kept on ice and protected from light. After ultracentrifugation at 55,000 rpm for 36 h, the lipoproteins were separated by gel permeation on a 1 mL Sephacryl S-300 column. The LDL subfraction was dialyzed against TRIS/HCl buffer (10 mM pH 7.40, containing 1 mM EDTA) overnight, and adjusted to a final protein concentration of 3 mg/mL. The pooled preparations were aliquoted after nitrogen bubbling into cryovials at −70 °C until use (no more than one month) [20].

To obtain OxLDL, 15 mL of human isolated LDL (3 mg protein/mL) were treated in vitro with 0.5 mL copper sulfate at a final concentration of 5 μM at 37 °C under gentle agitation for 8 h in order to produce a medium peroxidation degree of the LDL lipids (about 45 nmol malondialdehyde/mg protein). To stop the peroxidation process, each preparation was treated with a solution of butylhydroxytoluene (BHT; 2,6-di-tbutyl-p-cresol) in PBS at a final concentration of 0.1 mM [21] and immediately subjected to dialysis against PBS (50 mM, pH 7.40, changed every 8 h) for 24 h to eliminate BHT and Cu ions. Cu elimination was tested by atomic absorption spectrometry as described elsewhere [22]. In brief, the samples were diluted with ultrapure water (18 mΩ cm, Carlo Erba) and ultrafiltered using a 0.22 μm Millipore membrane (Milli-Q Purification System, Millipore). Ultrafiltered dissolutions were directly aspirated into the flame 1100 B Spectrophotometer equipped with a cathode lamp (Perkin-Elmer) at a spectral width of 1 nm. Calibrations were performed with a standard solution of $Cu(NO_3)_2$ in HNO_3 0.5 N (Tritrisol from Merck Co.) and 18 Ω cm water ultrafiltered through a Millipore membrane. All measurements were performed in peak height mode (324.7 nm line). The intra- [(SD/ξ).100] and inter- [ΔSD/Δξ).100] assay coefficients of variations were 15.5 and 6.0%, respectively. We routinely obtained a similar equation for the calibration curve (IR = 0.00055 + 0.04788 [Cu, mg/L]) and statistical analyses routinely demonstrated a correlation coefficient of between 0.956 and 0.991.

The LDL were acetylated by the Fraenkel-Conrat method [23]. Firstly, 5 mL of human isolated LDL (3 mg protein/mL) were added to a 5 mL of a saturated solution of sodium acetate with continuous stirring in an ice water bath. Then acetic anhydride was added in multiple small aliquots (2 μL) over 1 h with continuous stirring. After the addition of a total mass of acetic anhydride equal to 1.5 times the mass of protein used, the mixture was stirred for an additional 30 min without further additions. Then the reaction solution was dialyzed for 24 h at 4 °C against a buffer containing 150 mM NaCl and 0.3 mM EDTA, pH 7.4. Preparations of OxLDL and AcLDL were then sterilized by ultrafiltration under vacuum using a 0.22 μm membranes (milli-Q purification System, Millipore), fractioned into small aliquots, stored under nitrogen atmosphere at 4 °C (for no more than 2 weeks), and used for the experimental protocols.

2.2. Cell culture and treatment

About 1×10^6 human monocytes (THP1) were seeded on 6-well plates and incubated for 1 day with 2 mL of RPMI medium supplemented with penicillin/streptomycin (100 units/mL) and 10% FBS at 37 °C in a 5% CO_2 atmosphere. Phorbol esters at 200 nM (PMA) were added to the medium for 24 h to transform monocytes into macrophage type cells.

THP1 macrophages were seeded and grown for 24 h in disposable culture dishes (Falcon) in a humidified atmosphere with PMA. Then, the medium was collected and remaining cells were used for the different experiments. The experiments conducted in presence of OxLDL or AcLDL were carried out at a final concentration in the medium of 100 μg protein/mL in a total volume for well of 2 mL.

Cells were treated for 24 h with OxLDL and increasing concentrations of cortisol, cortisone or cortisone plus 100 μM of BTV.2733 (cat. nº 1756, Axon Medchem) to inhibit 11βHSD1 activity [24]. Cortisol and corticosterone concentrations ranging from physiological to pharmacological were used (from 0.1 to 1000 nM). In addition, a set of cells were treated for 24 h with AcLDL or AcLDL plus 1000 nM of cortisol. The glucocorticoids were dissolved in dimethylsulfoxide (DMSO) and immediately diluted in the culture medium (RPMI). The final concentration of DMSO was 0.1% in the culture medium, and control culture dishes were also supplemented with an equivalent aliquot of DMSO. After the corresponding treatment, the medium was collected and frozen at −80 °C. The cell monolayers were washed three times with cold sterile PBS (5 mL), and Trizol (Invitrogen) reagent was added for RNA extraction.

2.3. Trypan blue dye (TBD) assay test

Cell viability was assessed for each experimental condition. Attached cells were washed with PBS and incubated with 100 μL of a 0.1% solution of trypan blue dye (in PBS, pH 7.4). The cells were examined under optical microscopy to determine viability according to the method previously described [25]. At least four fields of one hundred cells per field were counted and the results were expressed as the percentage of non-viable cells.

2.4. Quantitative real-time PCR

Total RNA (1 μg) was used to generate cDNA using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR (RT-PCR) amplification was carried out using 10 μL of amplification mixture containing SYBR Green PCR Master Mix (Life Technologies), 10 ng of cDNA and 300 nM of primers. The primers used are shown in Table 2 (supplemental material). Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using an annealing temperature of 60 °C. To determine amplification sensitivity and efficiency, PCR linear ranges were previously established for each gene pair of primers and cells cDNA. To evaluate inflammation related to the atherogenic process, we determined the expression of epidermal growth factor like module-containing mucin-like hormone receptor-like 1 (EMR1), Cluster of Differentiation 163 (CD163), interleukin-12b (IL-12b), macrophage mannose receptor (MMR), interleukine-6 (IL-6), tumor necrosis factor α (TNFα), interleukin-10 receptor (rIL-10) and phospholipase A2 (PLA2). For the study of genes involved in cholesterol influx and efflux from macrophages we analyzed fatty acid translocase (FAT/CD36), scavenger receptor class A type 1 (SRA1), liver X receptor α (LXRα), ATP-binding cassette transporters A1 and G1 (ABCA1, ABCG1) and apoprotein E (APO E). To evaluate the cholesterol esterification and hydrolysis of cholesterol esters we determined the expression of acyl-CoA:cholesterol acyltransferase ACAT) and neutral cholesterol ester hydrolase 1 (NCEH1) respectively. To investigate the availability of glucocorticoid in cells, we determined the expression of 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11βHSD1, 11βHSD2), glucose-6-phosphate translocase 1 (G6PT1) and hexose-6-phosphate dehydrogenase (H6PDH). As a housekeeping gene, the expression of ribosomal protein L4 (RPL4) was measured. Ct values were obtained using an automatically calculated threshold and the relative expression of target genes to RPL4 was calculated using the ΔC(t) formula. Data were expressed as the % with respect to OxLDL-THP1 cells without hormone.

2.5. Oil-red staining

Cells were fixed to glass plates by covering them with 10% formaldehyde in PBS overnight at room temperature. Oil red O working solution was prepared from a stock solution (0.5 g Oil Red O in 100 mL isopropanol, Sigma HT50-1-640) by adding 6 mL of stock to 4 mL of bidistilled water. After mixing, it was filtered through Whatman 1 filter paper. Subequently removing fixing buffer carefully, cells were covered with fresh Oil Red O working solution at least one hour at room temperature. Then cells were rinsed several times, carefully with bidistilled water and were allowed to air dry [26]. The macrophages were visualized under optical microscope (Olympus BX-51), and image stacks were captured (Olympus DP-70). Lipid droplets areas were analyzed using the Fiji ImageJ [27].

2.6. TNFα quantification

Determination of TNFα in the cells medium was performed by Human TNF ELISA set (BD Biosciences) following supplier instructions.

2.7. Statistical analysis

The data were analyzed using the GraphPad software program version 5.0 and were expressed as the mean ± SEM. Statistical comparisons for different concentrations of cortisol, cortisone or cortisone plus BT2733 were made by one-way ANOVA and Bonferroni post Test. The Student's *t*-test was used to compare Control with OxLDL and discrete concentration points of cortisol and cortisone or cortisone and cortisone plus BTV.2733. P values <0.05 were considered.

3. Results

The viability of cultured cells treated with OxLDL in all conditions was expressed as percentage of dead cells (Fig. 1) and did not exceed 7–8% of total cells, confirming that our results were not affected by the addition of DMSO, hormones or the inhibitor BTV.2733.

Gene expression of macrophage and inflammation mediator markers as a response to incubation of THP1 cells with OxLDL and cortisol, cortisone and cortisone plus BTV.2733 is given in Table 1. The presence of OxLDL increased the expression of macrophage marker EMR1 and of pro-inflammatory interleukins IL-6, IL-12b

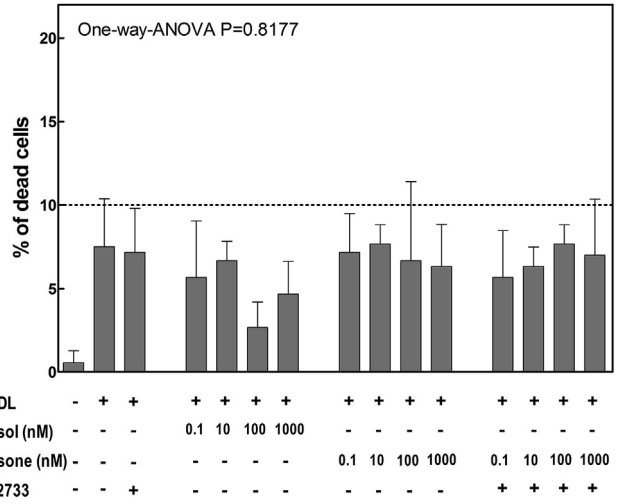

Fig. 1. Viability of cultured THP1 macrophages for all conditions tested expressed as dead cells versus total cells (%). The One way ANOVA significance is shown in the graphic. Bonferroni post Test analysis was not significant in any case.

Table 1
Expression of inflammatory markers in THP1 macrophages treated with OxLDL and glucocorticoids during 24 h.

	Control	With OxLDL	With OxLDL										One way ANOVA		
			Cortisol (%OxLDL)			Cortisone (%OxLDL)			Cortisone plus BVT.2733 (%OxLDL)						
			0.1 nM	10 nM	100 nM	1000 nM	0.1 nM	10 nM	100 nM	1000 nM	0.1 nM	10 nM	100 nM	1000 nM	Cortisol	Cortisone	Cortisone plus BVT.2733
EMR1	2.3 ± 0.7*	100 ± 15	100 ± 8	45.5 ± 6.7^{+}	44.5 ± 5.9^{+}	18.4 ± 0.7	94.3 ± 7.6	98.9 ± 11.7	88.9 ± 11.9●	30.3 ± 7.5●	95.2 ± 16.2	99.8 ± 15.2	137 ± 15	124 ± 15	<0.0001	0.0004	0.3885
TNFα	5.9 ± 1.6*	100 ± 12	112 ± 9	47.2 ± 5.8^{+}	25.8 ± 5.8^{+}	5.9 ± 0.9	104 ± 13	108 ± 10	72.2 ± 16.8	7.6 ± 1.3●	104 ± 11	54.3 ± 16.5	71.1 ± 1.3	63.9 ± 9.3	<0.0001	<0.0001	0.2751
IL-12b	34.6 ± 3.0*	100 ± 10	97.8 ± 10.2	110 ± 17	62.4 ± 12.4	30.1 ± 6.7^{+}	129 ± 13	132 ± 14	133 ± 4	76.7 ± 4.7	139 ± 8	111 ± 6	134 ± 55	77.0 ± 7.3	0.0011	0.6331	0.5973
IL-6	0.9 ± 0.0*	100 ± 2	67.1 ± 8.3	20.2 ± 2.8^{+}	23.8 ± 5.7^{+}	7.0 ± 0.7^{+}	65.6 ± 5.9	89.7 ± 8.3	60.2 ± 10.7	19.3 ± 3.6●	47.2 ± 10.4	86.1 ± 12.8	91.1 ± 13.5	61.1 ± 15.9	<0.0001	<0.0001	0.0525
MMR	4080 ± 88*	100 ± 20	74.0 ± 27.9	96.8 ± 18.6	490 ± 65^{+}	1605 ± 138^{+}	206 ± 97	191 ± 65	155 ± 59	88.6 ± 46.6	203 ± 95	65.7 ± 21.4	105 ± 44	108 ± 22	<0.0001	0.7556	0.3760
CD163	39.8 ± 4.2*	100 ± 12	78.1 ± 1.1	66.7 ± 7.2^{+}	310 ± 2^{+}	269 ± 17	78.3 ± 9.4●	125 ± 6.8●	159 ± 6●	323 ± 84●	52.0 ± 2.5	80.4 ± 3.5	109 ± 13.1	157 ± 4	<0.0001	<0.0001	<0.0001
rIL-10	10.0 ± 1.8*	100 ± 6	75.4 ± 1.6^{+}	52.4 ± 6.1^{+}	76.9 ± 10.3	32.8 ± 1.3	96.7 ± 5.6●	90.6 ± 6.8	81.5 ± 11.0	22.5 ± 5.9●	142 ± 14	95.7 ± 26.4	112 ± 26	127 ± 18	<0.0001	<0.0001	0.5277

The data are the mean ± sem. Statistical significance performed by One Way Anova is shown in the last columns of the table. Student's *t*-test is denoted by symbols: *P < 0.05 control vs OxLDL; +P < 0.05 cortisol vs cortisone; ● P < 0.05 cortisone vs cortisone plus BVT.2733.

and TNFα. In addition, OxLDL strongly decreased the expression of anti-inflammatory marker MMR but increased the expression of rIL-10 and CD163 also related to M2 macrophage profile. Cortisol markedly decreased the expression of EMR1 and pro-inflammatory cytokines such as IL-6, IL12b and TNFα when added together with OxLDL. Consistently with this, cortisol promoted an increase in the expression of MMR and CD163, markers for the alternative pathway (M2) of macrophage differentiation. We observed a significant dose-response effect for cortisol in all cases. Cortisol also diminished the expression rIL-10 but to a lesser extent than for the pro-inflammatory markers mentioned above. At the highest concentration tested, cortisone completely mimicked the effects of cortisol on the expression of EMR1, TNFα, CD163 and rIL-10, but only partially for IL-6. In the case of MMR, and IL12b cortisone had no effect on its expression. The presence of BTV.2733 blocked the effect of cortisone in all cases. In addition, levels of TNFα were measured in culture medium of cells treated with OxLDL or AcLDL and a similar increase was found for both modified LDL, of about 100 fold compared to the control without modified LDL. Cortisol decreased TNFα production in the medium in a 42% and 66% respectively.

To check if PLA2 was involved in the anti-inflammatory effect of cortisol we determined its expression. The OxLDL increased PLA2 expression 7 fold that found in unstimulated THP1 macrophages (14.2 ± 1.3% in control and 100.0 ± 7.6% in OxLDL treated). Cortisol strongly decreased PLA2 expression only at 1000 nM to a 12.5 ± 0.8% and cortisone showed no effect.

Fig. 2 shows the expression of genes coding for proteins involved in macrophage modified LDL uptake (FAT/CD36 and SRA1), esterification of cholesterol (ACAT) and hydrolysis of cholesterol esters (NCEH1). The expression of FAT/CD36, SRA1 and ACAT, was strongly increased by OxLDL. The expression of NCEH1 was also significantly increased by OxLDL to a lesser extent. Cortisol reduced the expression of FAT/CD36, SRA1 and ACAT in a dose-dependent manner, but had no effect on NCEH1. Cortisone at the highest dose exerted the same effect that cortisol, blunted by the addition of the 11βHSD1 inhibitor. The expression of genes that encode for the key transporters involved on efflux of cholesterol (ABCA, ABCG1), as well as LXRα, that up regulates their expression, and ApoE is shown in Fig. 3. ABCA1, ABCG1, LXRα and ApoE expression were increased by OxLDL effect. Cortisol reduced the expression of all genes studied in a dose-dependent manner. In all cases, the effect of cortisol at the highest dose tested was to reduce the expression of these genes to below 50% compared to cells incubated with OxLDL. The weakest effect exerted by cortisol was on ApoE, and the strongest effect was on LXR, with a decrease in expression to 57% and 81%, respectively. Cortisone only mimicked the effect of cortisol at the highest dose, and the presence of BVT2733 abolished this effect. In summary, it is observed a decreased expression of genes involved in OxLDL uptake and cholesterol esterification, but also of those involved in cholesterol efflux without effect in the expression of the main enzyme involved in hydrolysis of cholesterol esters.

Intracellular lipids droplets, measured by oil-red staining (Fig. 4A and B), were increased 3.3 fold by effect of OxLDL, whereas when cortisol and cortisone were also present the increase was lower, about 1.7 and 2.4 fold respectively. Thus, the presence of glucocorticoids caused a significant decrease of cell cholesterol accumulation induced by OxLDL. The presence of BTV.2733 blocked the effect of cortisone. The effect of AcLDL on the charge of lipids was also evaluated (Fig. 4C). In our hands, AcLDL promoted an increase of 3.1 fold compared to that found in control macrophages without AcLDL. Cortisol reduce the lipids charge but to a lesser extents that found under OxLDL incubation.

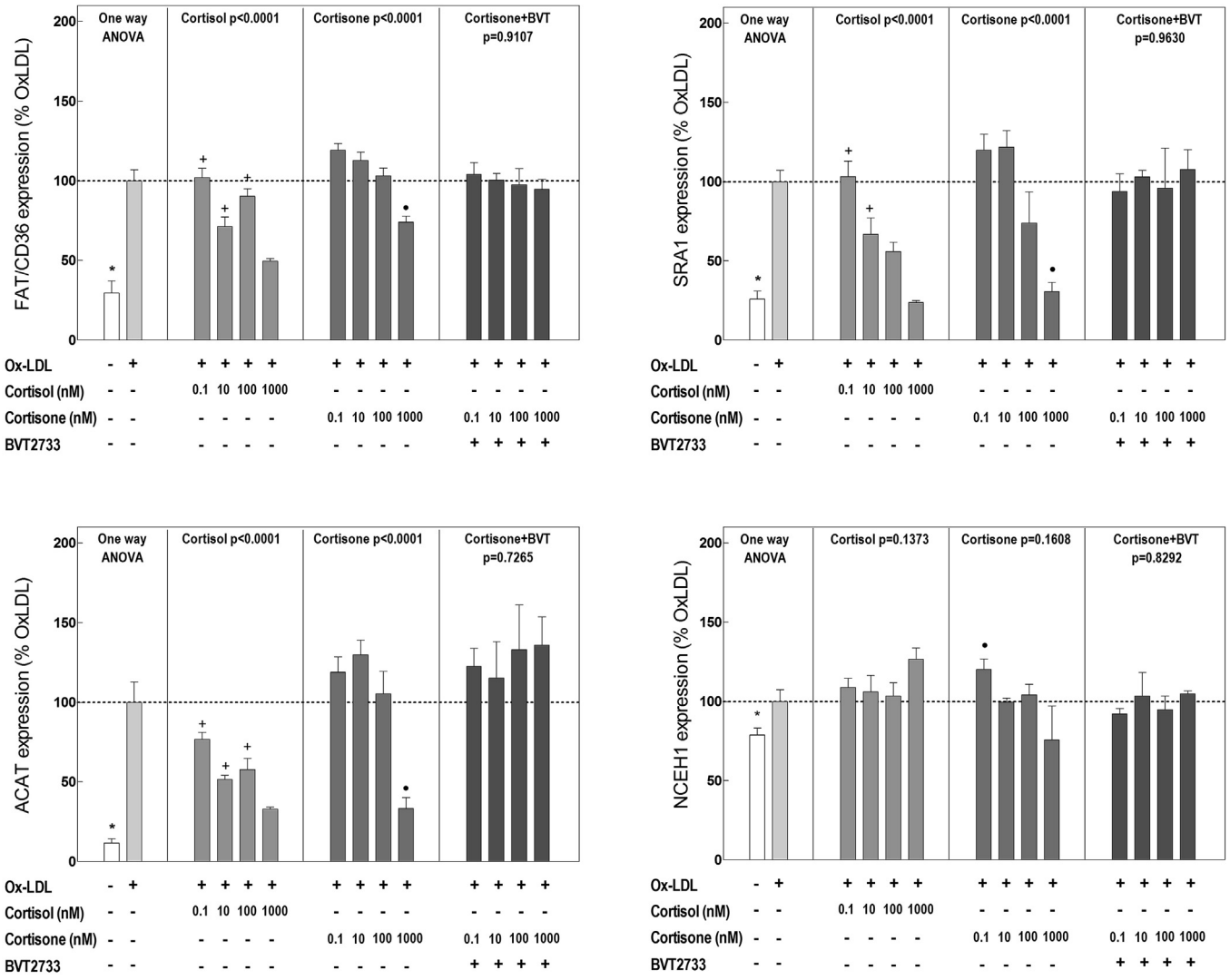

Fig. 2. Expression of genes involved on influx of OxLDL, esterification of cholesterol and hydrolysis of cholesterol esters in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733. Data are expressed as percentage of the expression found in cells incubated only with OxLDL. Fatty acid translocase (FAT/CD36), Scavenger receptor class A type 1 (SRA1), acyl-CoA:cholesterol acyltransferase (ACAT) and neutral cholesterol ester hydrolase 1 (NCEH1). One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

The expression of 11βHSD1 and 11βHSD2 is shown in Fig. 5. Interestingly, the expression of both was increased by OxLDL. Cortisol blocked the increased expression of 11βHSD1 elicited by OxLDL in a dose-dependent manner. The expression of 11βHSD2 was unaffected by cortisol at the lowest dose, but was inhibited at a concentration of 10 nM; in contrast, 100 and 1000 nM of cortisol promoted an increase in this expression proportional to the hormone concentration. In addition, the expression of G6PT1 and H6PDH, which are important to supply reduced substrate (NADH + H) for the 11βHSD1 reaction, was decreased by cortisol. The effect of cortisone on 11βHSD1 expression was similar to that of cortisol but weaker, except at 0.1 nM, when it was the same. Cortisone had no effect on the expression of 11βHSD2 with respect to cells incubated with OxLDL alone. Cortisone increased the expression of G6PT1 at 0.1 nM with respect to the same concentration of cortisol or with OxLDL alone; however, increasing doses of cortisone induced inhibition of G6PT1 expression. Cortisone had the same effect as cortisol on the expression of H6PDH.

4. Discussion

The aim of this study was to elucidate the direct effect of glucocorticoids on the macrophages in the atherogenic process. For this purpose we used THP1 macrophages incubated with OxLDL to promote the accumulation of lipids. Our results highlight that cortisol, and to a lesser extent cortisone, arrested the progression of inflammation in a dose-dependent manner, assessed by the reduction in EMR1, TNFα, IL-12b and IL-6 whereas the anti-inflammatory macrophage profile M2 markers as MMR and CD163 [28–30] were increased by cortisol, but only at high doses. It is well established that glucocorticoids exert an anti-inflammatory effect [31] and activate the alternative pathway of macrophage differentiation. Glucocorticoids and IL-10 activate the alternative differentiation to specific M2 macrophages which express several scavenger receptors such as SRA1 and CD163. The increased expression of SRA1 and CD163 contributes to M2 macrophage functions such as apoptotic cell clearance [32]. These subset of specialized M2 macrophages are the cells involved in foam cell

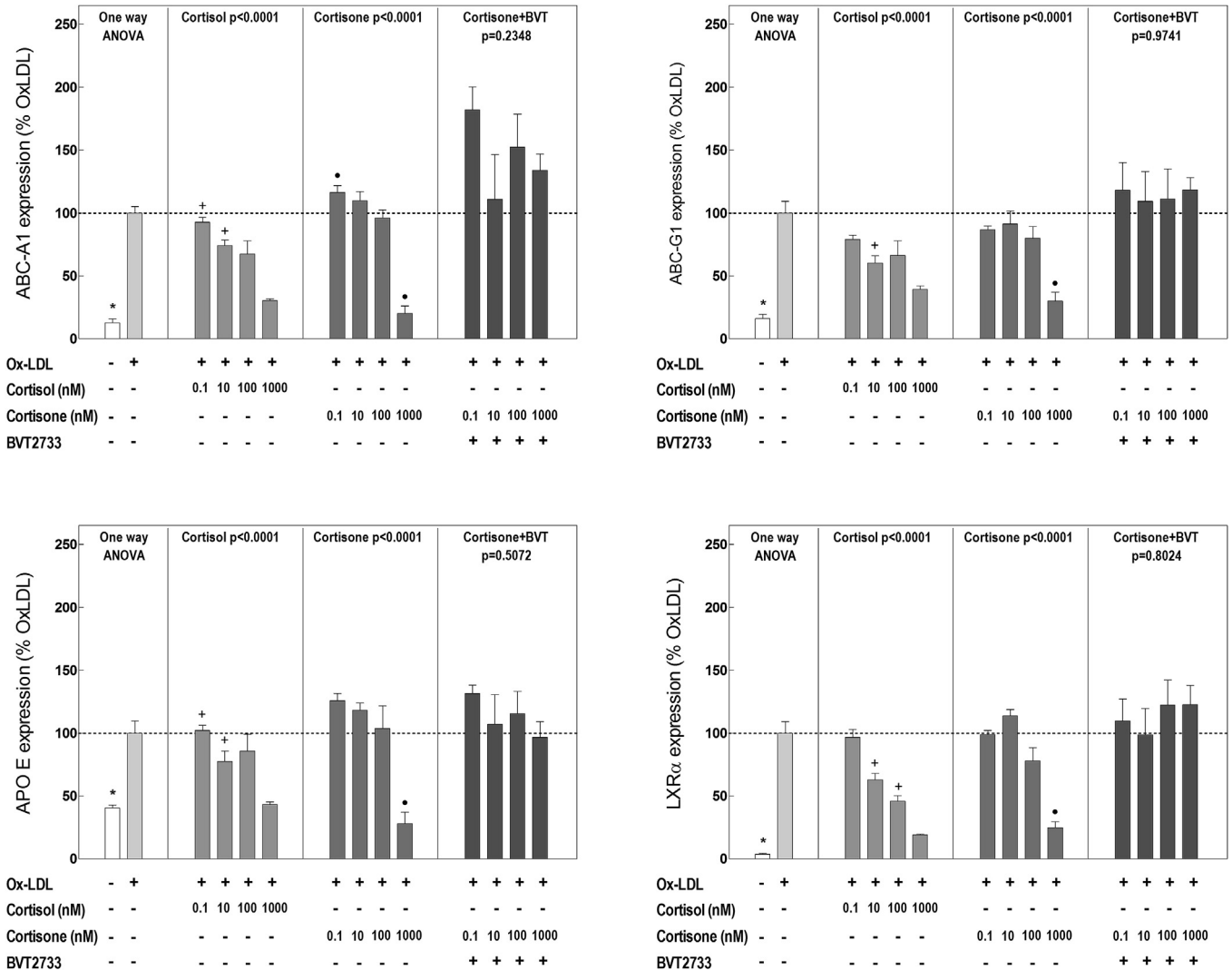

Fig. 3. Expression of genes involved on efflux of cholesterol in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733. Data are expressed as percentage of the expression found in cells incubated only with OxLDL. ATP-binding cassette transporters A1 and G1 (ABC-A1, ABC-G1), apoprotein E (APO E) and liver X receptor a (LXRa). One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

formation as a pathological consequence of a normal anti-inflammatory response [32].

In the present study, OxLDL promoted the uptake of cholesterol and its esterification in THP1 macrophages through increased gene expression of CD36 and SRA1, the main pathways of cholesterol uptake in macrophages [33], and of ACAT, which re-esterifies cholesterol inside macrophages [34]. Cortisol inhibited the expression of CD36, SRA1 and ACAT, thus limiting the influx of cholesterol. ATP-binding cassette transporters, specifically ABCA1 and ABCG1, are responsible for most cholesterol efflux from macrophages [35] and to a lesser extent scavenger receptor-BI [36]. In addition, increased apoE secretion could be linked to an augmented cholesterol efflux through ABCA1/ABCG1 in macrophages [37]. Our data show that OxLDL increased gene expression of ABCA1, ABCG1, apoE and LXRα, the transcription factor that regulate its expression [38]. Nevertheless, the presence of the active glucocorticoid cortisol in the culture medium inhibited the expression of genes involved in cholesterol efflux, probably through a reduction in LXRα expression. Cortisone had a more attenuated effect compared with cortisol, which was abolished by the presence of the inhibitor of 11βHSD1 (BVT.2733) due the impossibility of activating cortisone to cortisol. A recent study [39] showed that cortisol decreased cholesterol efflux in THP1 macrophages, in agreement with our results, but the authors also reported a higher influx. Our results indicate that glucocorticoids have the capacity to inhibit the expression of genes related to the influx and the efflux of cholesterol in macrophages, resulting in a reduction of their net lipid content.

The mechanism of action of cortisol is probably multi-factorial, because glucocorticoids can act through multiple systems. First, the reduced efflux could be partially consequence of a minor lipid content inside the cell due to reduced influx, in addition of a direct glucocorticoid action on gene expression supported by the finding of negative glucocorticoid response elements in the promoter region of some of the genes involved, such as ABCA1 that in liver is repressed by glucocorticoid through a direct action [40]. Second, it is known that glucocorticoids mediate their anti-inflammatory action, clearly observed in our data, through transrepression of the NF-kβ transcription factor, which also has a role in foam cell development [41]. It has been described that macrophages from

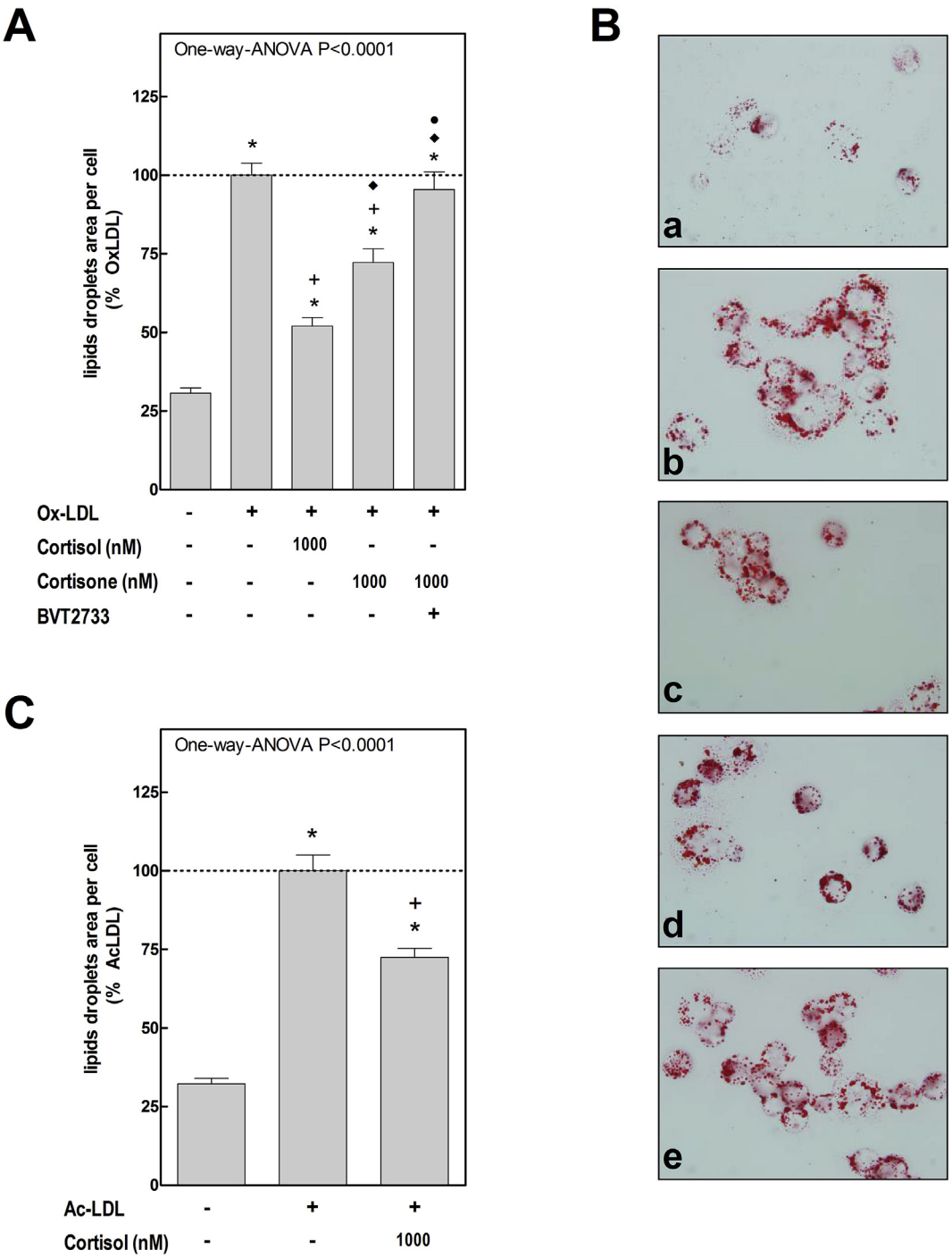

Fig. 4. Intracellular lipids droplets, measured by oil-red staining of THP1 macrophages. A) Lipid droplets deposition in THP1 macrophages incubated without OxLDL, with OxLDL and with OxLDL plus cortisol or cortisone or cortisone and BTV.2733. B) Representative pictures of THP1 macrophages stained with oil-red at 100X. a) THP1 cultured without OxLDL, b) THP1 cultured with OxLDL, c) THP1 cultured with OxLDL plus cortisol, d) THP1 cultured with OxLDL plus cortisone and e) THP1 cultured with OxLDL plus cortisone and BVT.2733. C) Lipid droplets deposition in THP1 macrophages incubated without AcLDL, with AcLDL and with AcLDL plus cortisol. In all cases cortisol and cortisone were used at 1000 nM. One way Anova significancy and Bonferroni post Test analysis are included in the graph: *$p < 0.05$ vs without OxLDL or AcLDL; + $p < 0.05$ vs with OxLDL or AcLDL; ◆ $p < 0.05$ vs OxLDL plus cortisol and ● $p < 0.05$ vs with OxLDL plus cortisona.

p50-KO mice (without transcriptional activity of NF-kβ) show, in agreement with our data, reduced SRA1 expression [42]. In addition, ACAT can be upregulated through activation of NF-kβ [42]. In consequence, NF-kβ repression would result in a reduced ACAT expression. However, data on NF-kβ role on foam cell development is conflicting. Mice overexpressing p65 (with increased transcriptional activity of NF-kβ) show reduced expression of FAT/CD36 and a limited foam cell development [43]. A third option would be that cortisol effects occur through PPRAγ. PPAR inhibition would result in the decrease of FAT/CD36, LXRα, ABCA1 and ABCG1 [44]. Finally, ACAT is upregulated also through MAPK which in turn is inhibited by glucocorticoids [41,44].

The concentration of active glucocorticoids, i.e. cortisol or corticosterone, in tissue depends on 11βHSD1 and 11βHSD2

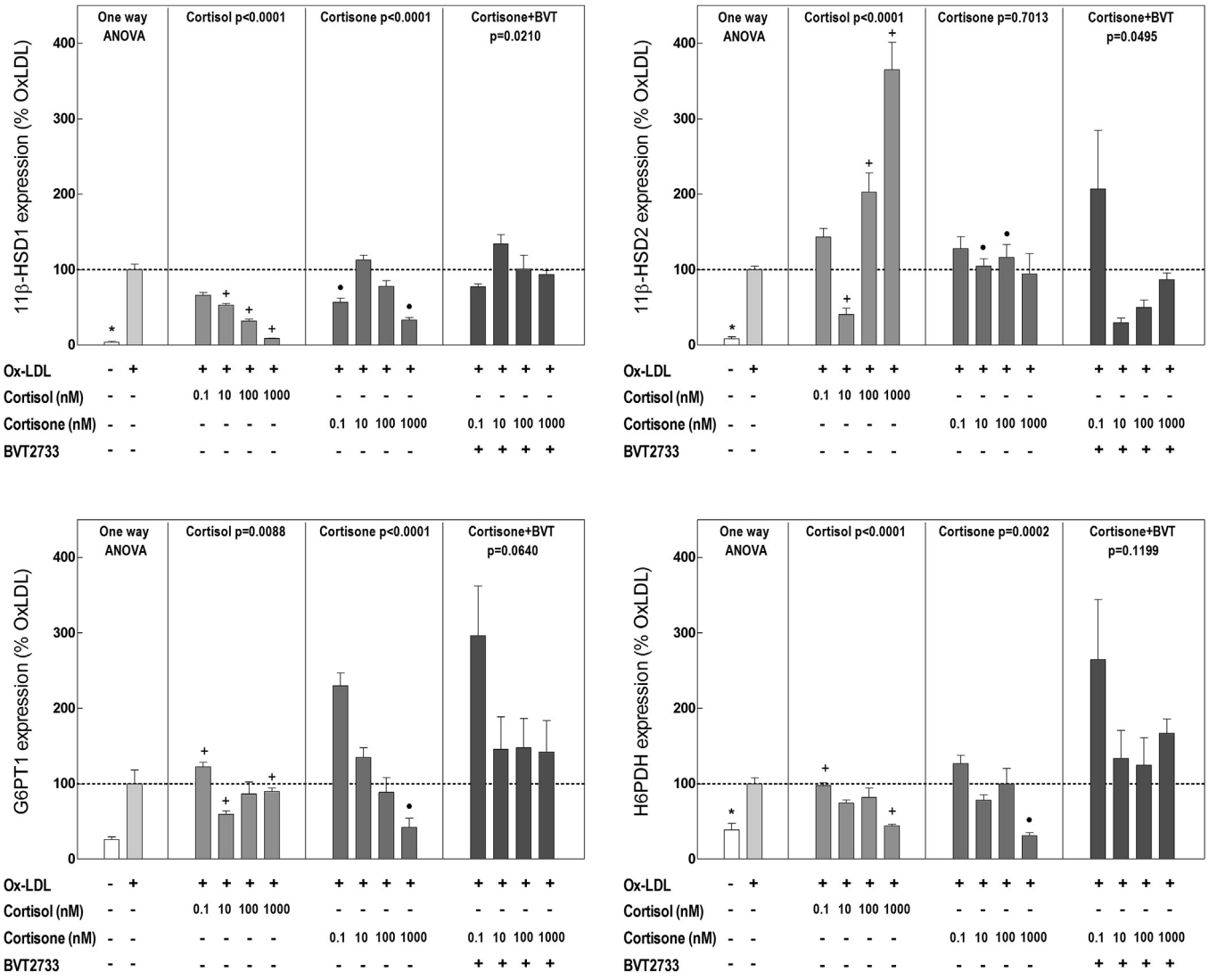

Fig. 5. Expression of genes involved in glucocorticoid availability in THP1 macrophages incubated with OxLDL or with OxLDL plus cortisol or cortisone or cortisone with BVT.2733: 11β-hydroxysteroid dehydrogenase type 1 and type 2 (11βHSD1, 11βHSD2), glucose-6-phosphate translocase 1 (G6T1), hexose-6-phosphate dehydrogenase (H6PDH). Data are expressed as percentage of expression in cells incubated with OxLDL. One way Anova significancy for each group of hormone treatment is shown in the graph. Student's *t*-test for discrete points was used. Symbols denote significativity between: * without OxLDL vs with OxLDL; + Cortisol vs Cortisone; ● Cortisone vs Cortisone with BVT.2733. P values <0.05 were considered significant.

activity, and it has been reported that 11βHSD1 deficiency/inhibition in the organism is atheroprotective whereas 11βHSD2 deficiency/inhibition accelerates atherosclerosis [45]. However, 11βHSD1 and 11βHSD2 expression in macrophages, and their contribution to the development of foam cells and progression of atherosclerosis, remain unclear. The expression of 11βHSD1 in macrophages is dependent on their activation state [46]. Thus, M1 polarization of native macrophages by lipopolysaccharides induces 11βHSD1 expression; in contrast, polarization to M2 by IL4/IL13 has little effect on 11βHSD1 expression. However, if monocytes are differentiated to macrophages in presence of IL4/IL13, the activity of 11βHSD1 is even higher than in M1 macrophages [47]. Here, we found an increase in both isozyme expression elicited by OxLDL but to a greater extent in 11βHSD1. This increase in 11βHSD1 is concordant with the observed elevated expression of TNFα, which is a potent inducer of 11βHSD1 expression [48,49]. On the other hand, the expression of 11βHSD2 in immune cells has only been described in some human diseases, for example synovial macrophage expression [50] and transient expression in peripheral blood mononuclear cells [51] of patients with arthritis rheumatoid. The synovial macrophages in rheumatoid arthritis could reflect an adaptation to enable an altered proliferation and differentiation in a chronically inflamed environment [52] as in the formation of atherosclerotic plaque.

Our results show that cortisol has an inhibitory effect on 11βHSD1 expression and a dual effect on 11βHSD2. Low concentrations did not affect or inhibited 11βHSD2 expression, but the highest cortisol concentration strongly induced its expression. This profile is coherent with a protective response to excessive cortisol levels; thus, when the concentration of cortisol increases, cells tend to reduce its levels through repressing 11βHSD1 and increasing 11βHSD2. Interestingly, cortisone had a different effect. It did not affect 11βHSD2 expression, and the inhibition of 11βHSD1 expression was more attenuated, since cortisone must be activated to cortisol by 11βHSD1 activity. It could be expected cortisone inhibiting 11βHSD2 expression due to an excess of product, but we did not find any effect.

In conclusion, our results indicate a direct effect of

glucocorticoids on macrophages in atherosclerosis progression limiting the accumulation of lipids. Thus, in presence of OxLDL, cortisol reduce the expression of genes involved in OxLDL uptake and cholesterol re-esterification, but also inhibited those involved in cholesterol output perhaps as a consequence of a minor influx (Fig. 6). In addition, cortisone can mimic all the effects of cortisol and the inhibition of 11βHSD1 activity blocked the cortisone action in macrophages. All the facts would indicate a key role of 11βHSD1 modulating the availability and local action of glucocorticoids in atherogenic progression. Our observations point to a direct anti-atherogenic role of glucocorticoids on THP1 macrophages. The molecular mechanisms mediating those effects on cholesterol homeostasis deserve further research.

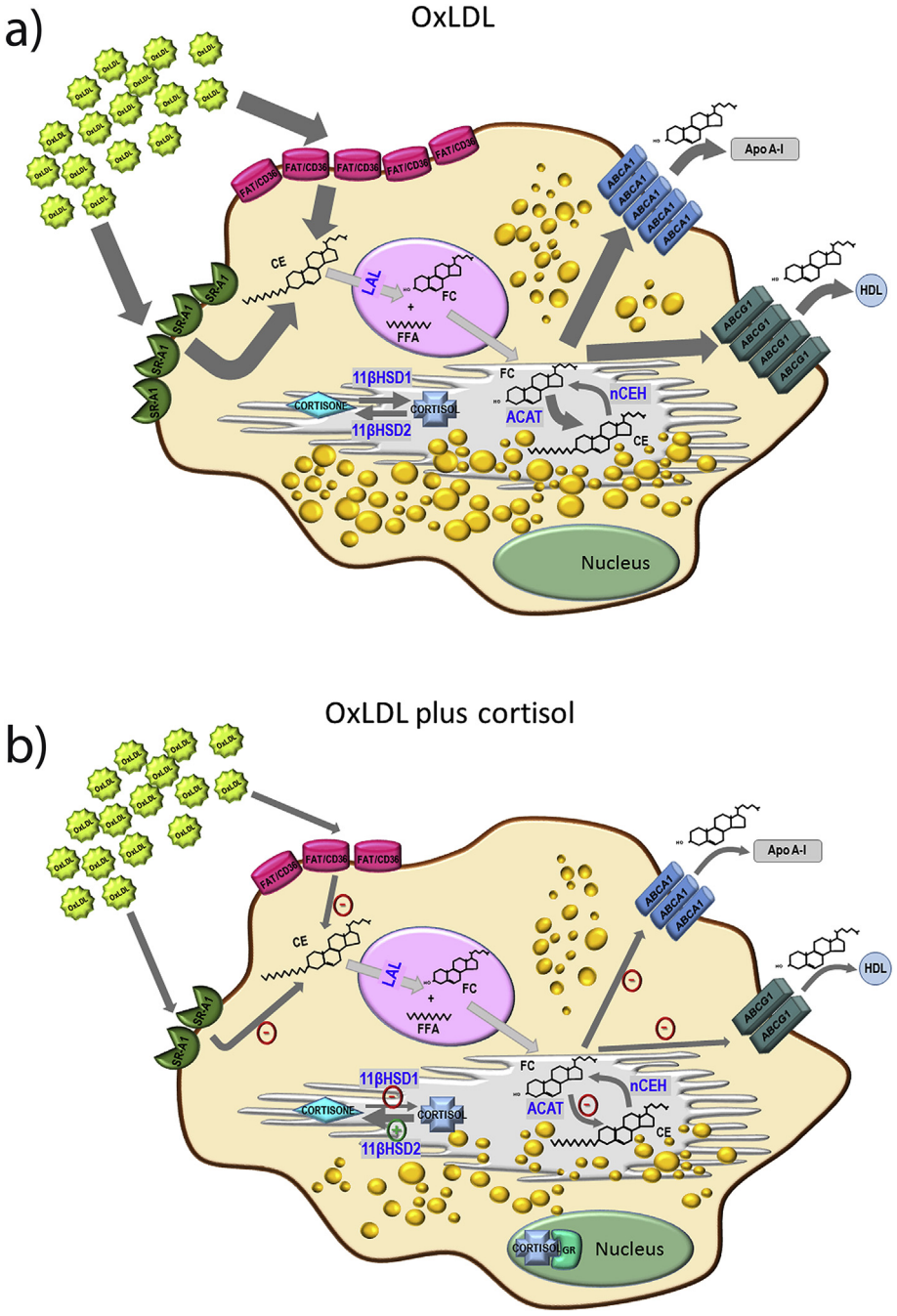

Fig. 6. Schematic representation of the effects caused by OxLDL(a) and OxLDL plus cortisol (b) in THP1 macrophages. The arrows thickness indicates the intensity of the pathway. In b), the pathways are marked in red (−) when cortisol inhibits them and in green (+) when cortisol activates them. Cortisol decreased the influx of OxLDL, reducing SRA1 and FAT/CD36 expression, decreased cholesterol esterification by ACAT and finally diminished the efflux of cholesterol trough ABCA1 and ABCG1 resulting in a minor lipid accumulation inside cells. FAT/CD36 = fatty acid translocase; SRA1 = Scavenger receptor class A type1; ACAT = acyl-CoA:cholesterol acyltransferase; nCEH = neutral cholesterol ester hydrolase 1; ABCA1 = ATP-binding cassette transporter A1; ABCG1 = ATP-binding cassette transporter G; 11βHSD1 = 11β-hydroxysteroid dehydrogenase type 1; 11βHSD2 = 11β-hydroxysteroid dehydrogenase type 2; FC= Free cholesterol; CE= Cholesterol esters; FFA= Free fatty acids; LAL = Lysosomal acid lipase; Apo A-I: Apolipoprotein A1; HDL= High density lipoprotein.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.04.020.

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3. *Altered lipid partitioning and glucocorticoid availability in CBG-deficient male mice with diet-induced obesity*

En este estudio, se investigaron las alteraciones provocadas por la deficiencia de CBG sobre la evolución de la obesidad inducida por la dieta. Además, se caracterizó también cómo la obesidad afectaba la biodisponibilidad de glucocorticoides en plasma y en el tejido adiposo en presencia y ausencia de CBG.

Para ello se utilizaron ratones machos C57BL/6 WT y KO para la CBG de la colonia propia establecida en Barcelona a partir de ratones heterozigotos enviados desde Berlin por el Dr. Thomas Willnow. Él y su equipo previamente habían observado que el seguimiento durante 6 semanas de una dieta con un 30% de contenido energético aportado por los lípidos no modificaba el peso ni la ingesta en ratones KO respecto WT. Así que nos propusimos evaluar qué ocurría si ofrecíamos una dieta con un 60% de contenido graso (HL) desde el punto de vista calórico durante 12 semanas.

El peso corporal y la ingesta de alimento se incrementaron ligeramente pero significativamente en los ratones KO alimentados con una dieta estándar en comparación con los WT, pero sin embargo no hubo diferencias cuando se alimentaron con la dieta HL. A pesar de la falta de efecto sobre el peso corporal, el depósito subcutáneo se desarrolló menos en ratones KO obesos que en WT obesos, lo que se asoció a una menor área de los adipocitos y la reducida expresión del receptor PPAR-γ. Por el contrario, los depósitos viscerales aumentaron ligeramente su peso y el análisis del tejido adiposo epididimal reveló el aumento del área de los adipocitos de los ratones KO respecto los WT y un incremento de la expresión de PPAR-γ principalmente en ratones KO con dieta estándar.

Por inmunohistoquímica, RT-PCR, y *Western blot*, se encontró expresión de CBG en tejido adiposo blanco de ratones WT. La obesidad causó el aumento de su expresión, tanto en hígado como en tejido adiposo, pero solo aumentó la CBG en plasma, a la vez que lo hacían los niveles de corticosterona totales, con lo cual la hormona libre permaneció inalterada.

La ausencia de CBG causó una caída de la expresión hepática de la enzima 11β-HSD2 y un aumento en el tejido adiposo epididimal, en particular en los ratones HL.

En conclusión, los datos presentados indican que, en un contexto de exceso de lípidos, la deficiencia de CBG impulsa la acumulación de lípidos a depósitos viscerales en detrimento de la acumulación en el tejido adiposo subcutáneo y modula la expresión de 11β-HSD2 diferencialmente en función del tejido.

Original Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Altered Lipid Partitioning and Glucocorticoid Availability in CBG-Deficient Male Mice with Diet-Induced Obesity

José Gulfo[1,2,3], Angelo Ledda[1,2,3], Elisabet Serra[1], Cristina Cabot[1], Montserrat Esteve[1,2,3], and Mar Grasa[1,2,3]**

Objective: To evaluate how deficiency in corticosteroid-binding globulin (CBG), the specific carrier of glucocorticoids, affects glucocorticoid availability and adipose tissue in obesity.
Methods: C57BL/6 (WT) and CBG-deficient (KO) male mice were fed during 12 weeks with standard or hyperlipidic diet (HL). Glucocorticoid availability and metabolic parameters were assessed.
Results: Body weight and food intake were increased in KO compared with WT mice fed a standard diet and were similar when fed a HL diet. Expression of CBG was found in white adipose tissue by immunochemistry, real-time PCR, and Western blot. In obesity, the subcutaneous depot developed less in KO mice compared with WT, which was associated with a minor adipocyte area and peroxisome proliferator-activated receptor-γ expression. Conversely, the epididymal depot displayed higher weight and adipocyte area in KO than in WT mice. CBG deficiency caused a fall of hepatic 11β-hydroxysteroid dehydrogenase type 2 expression and an increase in epidymal adipose tissue, particularly in HL mice.
Conclusions: Deficiency in CBG drives lipid partitioning from subcutaneous to visceral adipose depot under a context of lipid excess and differentially modulates 11β-hydroxysteroid dehydrogenase type 2 expression.

Obesity (2016) **24**, 1677-1686. doi:10.1002/oby.21543

Introduction

Corticosteroid-binding globulin (CBG) is the specific carrier of glucocorticoids in the blood. Although glucocorticoids are bound unspecifically to albumin, 80% to 90% of glucocorticoids bind to CBG with high affinity. The free hormone hypothesis attributes to CBG the ability to control the free fraction of glucocorticoids, able to enter into the cells and act (1,2). This control is possible through the regulation of the binding capacity and hormone affinity of CBG (3). However, some evidence points to a more active role for CBG, modulating directly the hormone action (4). Among them and the first to be established, the cleavage of CBG by serine proteases is a way to release cortisol exactly where these proteases are released, as elastase from neutrophils, in inflamed tissues (5). For that, CBG must be inside the tissue where inflammation develops. Evidence of membrane receptors has also been reported (6), and CBG expression has been detected in glucocorticoid target and producer tissues, such as the central nervous system, lung, and white adipose tissue (WAT) (7-9).

Glucocorticoids promote obesity in rodents and humans. Cushing syndrome, which is characterized by the uncontrolled production of glucocorticoids, elicits central obesity and insulin resistance, among other pathologies (10).

Data from humans carrying CBG mutations decreasing its affinity for cortisol indicate the lack of strong effects but subtle abnormalities, the most frequent of which are fatigue, increased sensitivity to pain, and hypotension (11). In some cases of null mutations, overweight or obesity have been reported (12).

Mice deficient in CBG failed to show alterations in body weight when submitted to a hyperlipidic diet (HL) providing 30% of energy from lipids (13). However, KO mice exhibited higher mortality in response to septic shock that pointed out an impaired anti-inflammatory action of glucocorticoids. Other authors reported in CBG-deficient mice an impaired response of the hypothalamic-pituitary-adrenal (HPA) axis to emotional stress (14).

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Disclosure: The authors declare no conflict of interest.
Author contributions: ME and MG conceived and design the experiments. JG, AL, ES, and CC carried out the experiments. JG and AL calculated data. ME, MG, JG, and AL analyzed data. All authors were involved in writing the paper and had final approval of the submitted and published versions.
*Montserrat Esteve and Mar Grasa contributed equally to this work.
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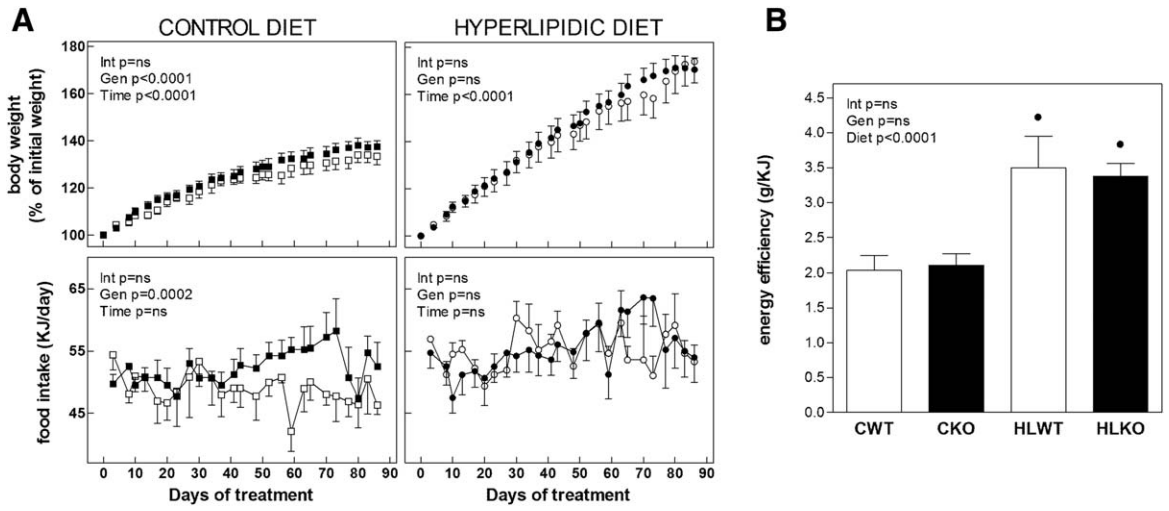

Figure 1 Changes in the (**A**) body weight and food intake and (**B**) energy efficiency of WT mice (open symbols) and KO mice (solid symbols) fed a control diet or a hyperlipidic diet. The numerical results of two-way ANOVA are specified only in cases in which $P < 0.050$. Genotype (Gen) and time (Time) were the two variables assessed. The interaction (Int) is also shown; ns = not significant. Significant Bonferroni post-test: •significant difference between control (**C**) and hyperlipidic (HL) -fed mice of the appointed genotype.

In this study, we aimed to address the consequences of CBG deficiency in the WAT features of mice submitted to diet-induced obesity. We subjected CBG-KO mice to a very high-fat diet (60%) and analyzed the changes in the metabolism and glucocorticoid signaling in WAT and also in liver, because of its importance in the overall glucocorticoid availability as the main source of plasma CBG and the high 11β-hydroxysteroid dehydrogenase type 1 (11HSD1) activity.

Methods

Animals and experimental protocol

Six-week-old wild-type (WT) and CBG-deficient (KO) male mice (C57BL/6) were used. The colony of WT and KO mice was established crossing heterozygous animals (generously provided by Dr. T.E. Willnow).

The WT and KO mice were distributed into two groups of 6 to 10 animals, 2 to 4 mice per cage. During 12 weeks, the mice were fed a control diet (C) or a HL that provided 18% and 60% of the energy from fat, respectively (Teklad Global 2018 and TD.06414, Harlan-Interfauna Ibérica). Mice were sacrificed under isoflurane anesthesia between 9.00 and 11.00 a.m. The procedures were conducted in accordance with the guidelines for the use of experimental animals established by the European Union, Spain, and Catalonia and were approved by the Animal Handling Ethics Committee of the University of Barcelona.

Determination of total and free corticosterone

Pools of serum from mice belonging to the same group were centrifuged in Centrifree micropartition devices (YM membranes, MW cutoff of 30,000; Merck Millipore). Ultrafiltrates and intact serum were assayed for free and total corticosterone using an enzyme immunoassay (DetectX® Kit K014-H1, Arbor Assays).

CBG binding capacity

CBG binding capacity was evaluated through ^{3}H-corticosterone binding as previously reported (15).

Western blot of CBG

Proteins from WAT were isolated using the TRI-Reagent (Life Technologies) and quantified with a QuantiPro BCA Assay Kit (Sigma). Tissue protein (25 μg) and serum (1 μL) were separated by SDS/PAGE and electrotransferred onto a PVDF membrane (Millipore). Membranes were incubated overnight with anti-CBG antibody (LS-C39044, LifeSpan) and as loading control, anti-β-actin antibody, (A-5316, Sigma), or anti-albumin (sc-46293, Santa Cruz Biotechnology).

Immunodetection was performed with Pierce ECL (Thermo Fisher Scientific) and quantified using Total Lab v2003.3 (Non-Linear Dynamics).

Serum metabolites and hormones

Glucose, triacylglycerols, cholesterol, urea, (Biosystems), NEFAs (Wako), insulin, and leptin (Ultra-Sensitive ELISA, Crystal Chem Inc.) were measured in serum.

Liver glycogen and lipid content

Liver samples were used for the determination of glycogen as glycosyl residues (16). Lipids from liver were extracted with trichloromethane-methanol (2:1), dried and weighed (17).

Real-time PCR expression analysis

Total RNA from liver, subcutaneous, and epididymal WAT was extracted using TRI-Reagent, reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo-dT primers (Attendbio). Real-time PCR was conducted with SYBRGreen Master Mix (Life Technologies) on an ABI PRISM 7900 HT system (Applied

TABLE 1 Tissue weights

Group	Liver	Thymus	Adrenal (mg, %)	Epididymal WAT	Inguinal sc WAT	Retroperitoneal WAT	Mesenteric WAT
CWT							
g	1.41 ± 0.06	0.085 ± 0.015	3.83 ± 0.21	0.232 ± 0.049	0.146 ± 0.028	0.076 ± 0.020	0.293 ± 0.036
%	4.65 ± 0.14	0.274 ± 0.041	0.127 ± 0.009	0.748 ± 0.134	0.468 ± 0.074	0.244 ± 0.057	0.949 ± 0.087
CKO							
g	1.37 ± 0.06	0.065 ± 0.007	5.4 ± 0.8	0.276 ± 0.055	0.157 ± 0.019	0.099 ± 0.015	0.308 ± 0.032
%	4.49 ± 0.21	0.215 ± 0.025	0.172 ± 0.028	0.864 ± 0.146	0.499 ± 0.049	0.315 ± 0.042	0.975 ± 0.083
HLWT							
g	1.72 ± 0.12	0.123 ± 0.018	4.31 ± 0.32	0.766 ± 0.092•	0.467 ± 0.080•	0.251 ± 0.027•	0.670 ± 0.097•
%	4.32 ± 0.20	0.310 ± 0.045	0.109 ± 0.005	1.89 ± 0.17•	1.13 ± 0.14•	0.620 ± 0.045•	1.64 ± 0.16•
HLKO							
g	1.56 ± 0.10	0.088 ± 0.013	5.23 ± 0.49	0.811 ± 0.069•	0.297 ± 0.055*•	0.285 ± 0.017•	0.503 ± 0.063•
%	4.19 ± 0.14	0.232 ± 0.029	0.141 ± 0.012	2.176 ± 0.154•	0.764 ± 0.115*•	0.779 ± 0.063•	1.33 ± 0.12•
ANOVA							
Int							
g	ns	ns	ns	ns	0.0895	ns	ns
%	ns	ns	ns	ns	0.0621	ns	ns
Gen							
g	ns	0.0487	0.0419	ns	ns	ns	ns
%	ns	0.0550	0.0482	ns	ns	0.0473	ns
Diet							
g	0.0108	0.0301	ns	<0.0001	0.0001	<0.0001	<0.0001
%	0.0979	ns	ns	0.0014	0.0001	<0.0001	0.0001

Tissue weights of WT mice or KO mice fed a control diet (C) or a high-fat diet (HL), expressed as grams and percentages respective to body weight (in the case of adrenal gland, in mg and per thousand respective to body weight). The numerical results of two-way ANOVA are specified only in cases in which $P < 0.100$. Genotype (Gen) and diet (Diet) were the two variables assessed. The interaction (Int) is also shown when $P < 0.100$. All valyes are mean ± SEM.
Significant Bonferroni post-test: *significant difference between WT and KO under the appointed diet and •significant difference between C and HL-fed mice of the appointed genotype.

Biosystems). The primers used for glucose-6-phosphate transporter (G6PT), hexose-6-phosphate dehydrogenase (H6PDH), CBG, 11HSD1 or 11HSD2, fatty acid translocase (FAT/CD36), 5α-reductase (5αR), lipoprotein lipase (LPL), leptin (LEP), phosphoenolpyruvate carboxykinase (PEPCK), peroxisome proliferator-activated receptor (PPAR)-γ and ribosomal protein L32 as housekeeping are listed in the Supporting Information (Table S1). The ΔΔCt method was used for relative quantifications. Data were calculated as percentage respective to CWT group, which was normalized to 100.

Histological and immunohistochemical analysis of WAT

Liver, epididymal, and subcutaneous WAT were dissected and fixed in 40 g/L paraformaldehyde. Then, samples were embedded in paraffin and sections of 5 μm were stained with eosin-hematoxylin and visualized under optical microscope (Olympus BX-51). Image stacks were captured (Olympus DP-70) and adipocyte areas were analyzed using the Fiji ImageJ (18). A minimum of 100 adipocytes per mouse and four mice per group were measured. A manual revision was performed of the automatically detected adipocytes by the software in order to exclude broken or cut cells.

For immunohistochemical detection of CBG, sections were incubated with anti-CBG and the detection was performed with an avidin-biotin peroxidase complex (Vecstatin ABC kit, Vector Labs) using diaminobenzidine. Slides were scanned and visualized using the Pannoramic Viewer 1.15.2 software (3DHistech Ltd).

Statistics

The data were analyzed using GraphPad 5.0 and are expressed as mean ± SEM. Normality of the data was assessed by the Shapiro-Wilk normality test. Statistical comparisons were obtained by two-way ANOVA to evaluate the impact of time or diet and genotype (named Time, Diet, and Gen, respectively in the graphs). Interaction significativity is also included (Int). A significant interaction would denote a different response in mice of each genotype depending on the diet. The Bonferroni post-test was used as a multiple comparisons method to evaluate significant differences between pairs of data. Student's *t*-test was used to compare CWT and HLWT mice when KO data was missing. Significance was considered with *P* values below 0.05.

Results

Body and tissue weights and food intake

The KO mice fed with the control diet (CKO) showed body weights slightly but consistently higher than WT (CWT) mice (Gen $P < 0.0001$). Accordingly, KO mice exhibited an increased energy intake (Figure 1A)

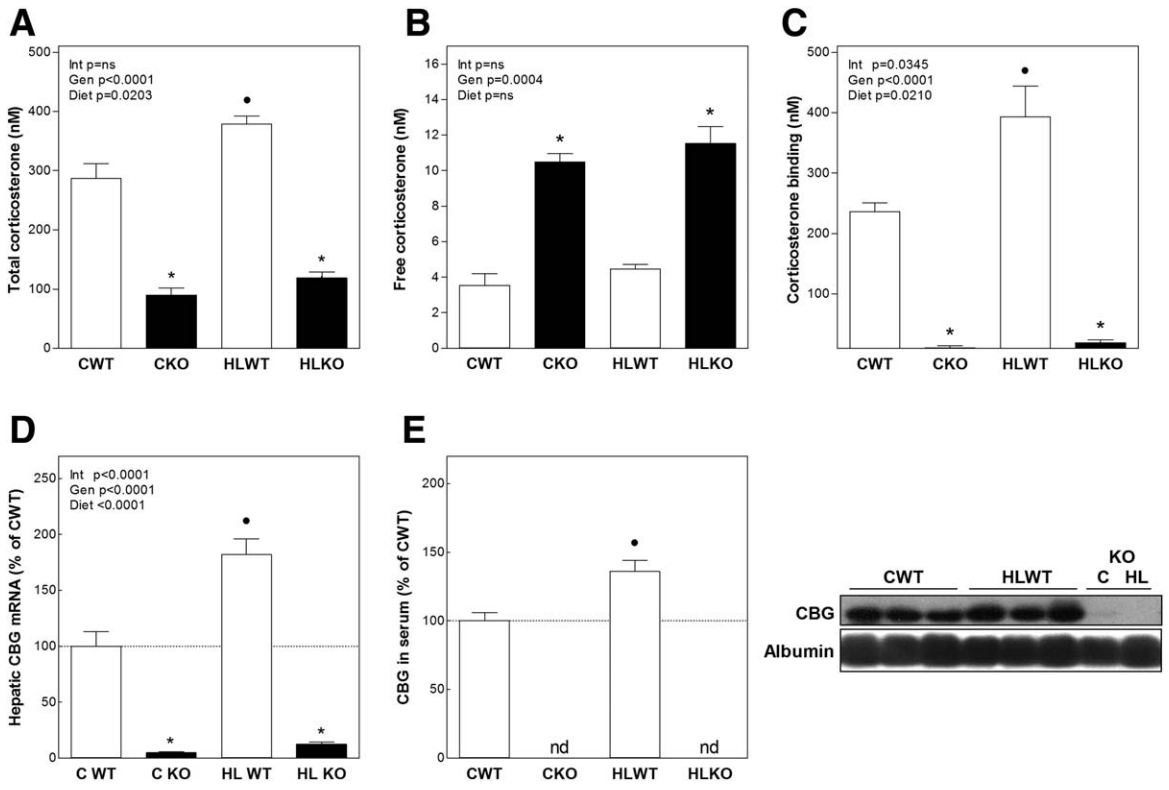

Figure 2 Levels of (**A**) total and (**B**) free serum corticosterone, (**C**) serum corticosterone binding, (**D**) corticosteroid-binding globulin (CBG) mRNA expression in the liver, which is the main source of blood CBG, and (**E**) serum CBG protein of WT and KO mice fed a control diet (CWT and CKO) or a hyperlipidic diet (HLWT and HLKO). The numerical results of two-way ANOVA are specified only in cases in which $P < 0.050$. Genotype (Gen) and diet (Diet) were the two variables assessed. The interaction (Int) is also shown; ns = not significant. Significant Bonferroni post-test: *Significant difference between WT and KO under the appointed diet and •significant difference between C and HL-fed mice of the appointed genotype.

and similar energy efficiency (Figure 1B). The HL diet induced the same increase in body weight and food intake in mice of both genotypes. WT and KO mice fed with HL diet (HLWT and HLKO) exhibited the same energy efficiency, which was 1.75-fold higher than in mice fed with control diet (Figure 1A, B).

The tissue weights of the different groups are shown in Table 1. Changes in thymus and adrenal weights were observed in the KO mice compared with WT. Thymus weight showed a significant reduction in KO (Gen $P = 0.0487$), whereas adrenals were larger (Gen $P = 0.0419$). HL diet caused an increase of the thymus in

TABLE 2 Serum parameters

	Glucose (mM)	Insulin (ng/mL)	Leptin (ng/mL)	Triacylglicerols (mM)	NEFA (mM)	Cholesterol (mM)	Urea (mM)
CWT	9.3 ± 0.7	0.525 ± 0.076	3.35 ± 0.31	1.34 ± 0.32	0.128 ± 0.022	3.37 ± 0.17	8.4 ± 0.3
CKO	9.3 ± 0.3	0.589 ± 0.093	5.82 ± 1.02	0.99 ± 0.08	0.145 ± 0.019	3.51 ± 0.26	8.1 ± 0.6
HLWT	12.0 ± 0.5•	0.956 ± 0.241	44.2 ± 8.0•	1.09 ± 0.12	0.134 ± 0.014	4.15 ± 0.21	9.3 ± 0.5
HLKO	11.8 ± 0.6•	0.996 ± 0.317	37.5 ± 5.4•	0.91 ± 0.07	0.162 ± 0.014	4.40 ± 0.40	7.7 ± 0.4
ANOVA							
Int	ns	ns	ns	ns	ns	ns	ns
Gen	ns	ns	ns	ns	ns	ns	ns
Diet	<0.0001	0.0503	<0.0001	ns	ns	0.0172	ns

Serum parameters evaluated in WT mice and KO mice fed a standard diet (CWT and CKO) or a high-fat diet (HLWT and HLKO). The numerical results of two-way ANOVA are specified only in cases in which $P < 0.100$. Genotype (Gen) and diet (Diet) were the two variables assessed. The interaction (Int) is also shown; *ns* = not significant. All values are mean ± SEM.
Significant Bonferroni post-test: •significant difference between C and HL-fed mice of the appointed genotype.

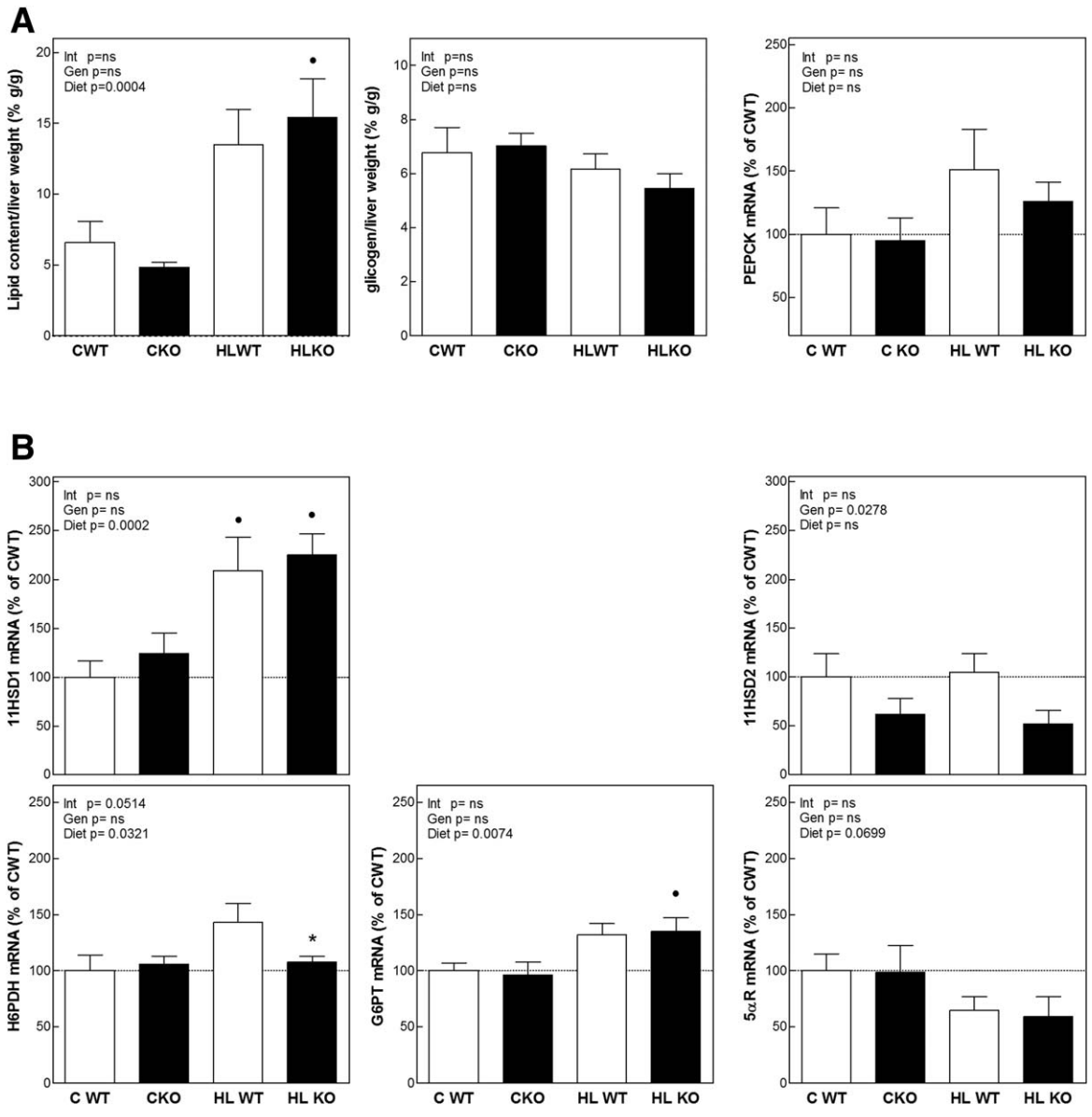

Figure 3 Lipid and glycogen liver content and hepatic gene mRNA expression in WT mice and KO mice fed a standard diet (CWT and CKO) or a hyperlipidic diet (HLWT and HLKO). (**A**) Lipid and glycogen in liver and hepatic PEPCK mRNA expression and (**B**) expression of genes involved in glucocorticoid pre-receptor metabolism in the liver (11HSD1, 11HSD2, H6PDH, and G6PT). The two-way ANOVA statistical *P* values are depicted in each graph only in cases in which $P < 0.050$. Genotype (Gen) and diet (Diet) were the two variables assessed. The interaction (Int) is also shown; ns = not significant. Significant Bonferroni post-test: *Significant difference between WT and KO under the appointed diet and •significant difference between C and HL-fed mice of the appointed genotype.

both genotypes (Diet $P = 0.0301$) but did not affect adrenal weight. The liver remained unchanged in KO mice respective to WT but increased in both groups of mice with HL diet (Diet $P = 0.0108$).

All WAT depots shared similar weights in WT and KO mice under control diet. However, when fed HL diet, KO mice exhibited a significantly lower subcutaneous WAT accumulation than WT mice (Bonferroni test between HLWT and HLKO $P < 0.05$), whereas among visceral depots, the retroperitoneal WAT was significantly higher in KO mice when expressed as % of total body weight (Gen $P = 0.0473$) and the epididymal showed a trend to increase.

Circulating corticosterone and CBG levels

Total serum corticosterone was markedly reduced in KO mice (Gen $P < 0.0001$, Figure 2A) whereas free corticosterone was increased (Gen $P = 0.0004$, Figure 2B) compared with WT irrespective of the diet consumed. HL diet consumption increased total corticosterone more intensively in WT mice (Diet $P = 0.0203$, Bonferroni between CWT and HLWT $P < 0.05$) but without altering free corticosterone. This could be due to the increased corticosterone binding (Diet $P = 0.0210$, Figure 2C), in agreement with the raise of serum CBG (Student's *t*-test $P < 0.05$, Figure 2E) and the hepatic CBG expression (Diet $P < 0.0001$, Figure 2D). In the KO mice, as expected, there

was no significant specific corticosterone binding in the serum, and CBG band was not observed by Western blot.

Serum metabolites and hormones

Values of serum metabolites and hormones are included in Table 2. CBG deficiency did not significantly alter any plasma parameter. The HL diet elicited similar increases in serum glucose, insulin, leptin, and cholesterol levels of WT and KO mice.

Liver metabolic parameters

Lipid and glycogen liver content did not change as a consequence of CBG deficiency, irrespective of the diet provided. As expected, HL diet spurred the hepatic lipid accumulation (Diet $P = 0.0004$, Figure 3A) more sharply in KO (Bonferroni between CKO and HLKO $P < 0.05$).

Expression of the glucocorticoid target gene PEPCK, the main regulator of gluconeogenic activity in the liver, did not change as a consequence of diet or CBG deficiency.

Expression of genes involved in glucocorticoid availability

We assessed the mRNA expression of the main enzymes involved in glucocorticoid reactivation (11HSD1, H6PDH, and G6PT) and inactivation (11HSD2 and 5αR) in liver (Figure 3B). The only change caused by CBG deficiency was a fall of 11HSD2 mRNA in both mice fed control and HL diets (Gen $P = 0.0278$). HL diet boosted 11HSD1 (Diet $P = 0.0002$) and G6PT expression (Diet $P = 0.0074$) in both WT and KO mice. Expression of H6PDH was only increased in WT but not in KO mice (Diet $P = 0.0321$, Int = 0.0514). In contrast, the HL diet tended to reduce 5αR expression (Diet $P = 0.0699$), whereas 11HSD2 remained unaltered.

With the exception of 5αR, the same gene expressions were quantified in epididymal and subcutaneous WAT (Figure 4). CBG deficiency elicited two depot-specific changes: a sustained increase in 11HSD2 expression in KO compared with WT (Gen $P = 0.0035$ and Bonferroni test between HLWT and HLKO $P < 0.05$) in epididymal WAT, and in subcutaneous, 11HSD1 expression was markedly reduced in KO mice fed control diet respective to WT (Gen = 0.067 and Int = 0.0497, Bonferroni test between CWT and CKO $P < 0.05$). HL diet consumption triggered the reduction of 11HSD1 mRNA in epididymal tissue of WT and KO mice (Diet $P = 0.0242$) and in the WT subcutaneous depot (Diet $P = 0.0196$ and Int = 0.0497). Changes due to HL diet in 11HSD2 expression were depot-dependent: in the epididymal depot, 11HSD2 expression increased in WT and KO mice (Diet $P = 0.013$), but in subcutaneous, 11HSD2 expression decreased in a similar extent in both WT and KO mice (Diet $P = 0.0367$). H6PDH expression increased in the subcutaneous WAT of all HL-fed mice (Diet $P = 0.0031$) but remained unaltered in the epididymal WAT. G6PT expression did not change in any depot.

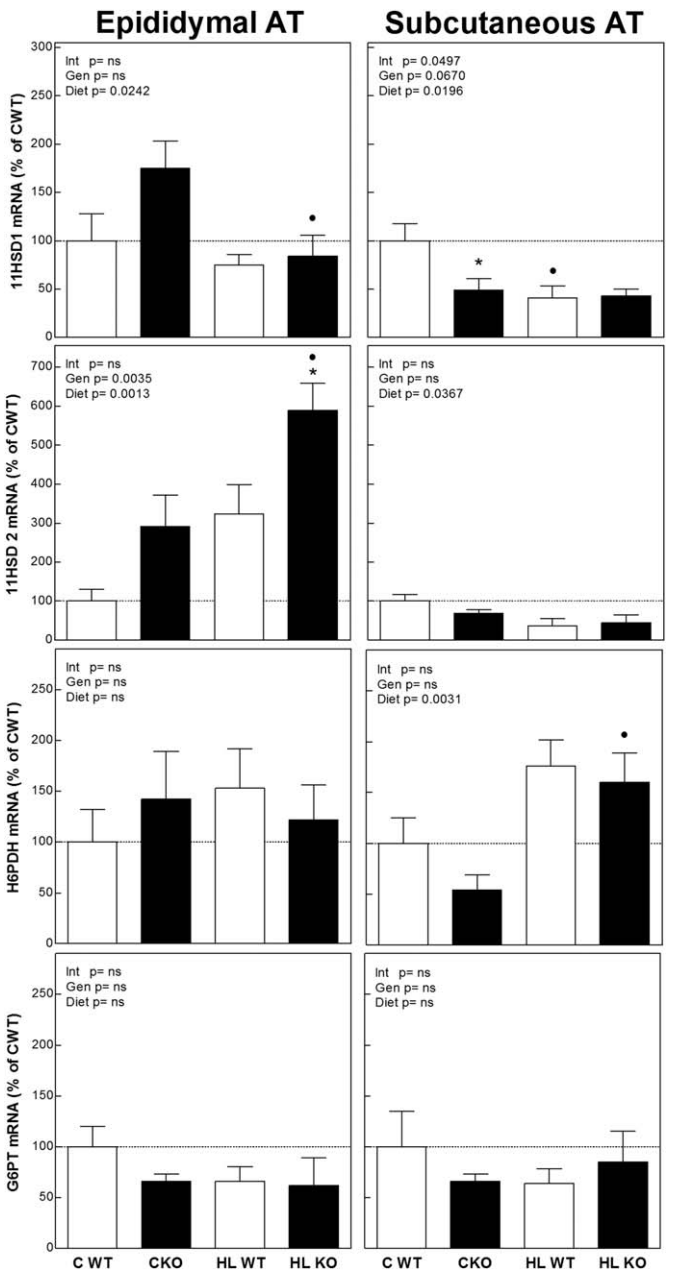

Figure 4 Expression of genes involved in glucocorticoid pre-receptor metabolism in epididymal and subcutaneous adipose tissue (AT) in WT mice and KO mice fed a standard diet (CWT and CKO) or a hyperlipidic diet (HLWT and HLKO). Expression of 11HSD1, 11HSD2, H6PDH, and G6PT in epididymal adipose tissue (left column) and subcutaneous adipose tissue (right column). The two-way ANOVA statistical *P* values are depicted in each graph only in cases in which $P < 0.050$. Genotype (Gen) and diet (Diet) were the two variables assessed. The interaction (Int) is also shown; ns = not significant. Significant Bonferroni post-test: *Significant difference between WT and KO under the appointed diet and •significant differences between C and HL-fed mice of the appointed genotype.

CBG in WAT

CBG was detected by immunohistochemistry in sections of liver and WAT from WT mice fed with control and HL diets (Figure 5A). Liver and WAT from KO mice and from WT mice where the primary antibody was omitted were used as negative controls (in Figure 5A labeled as C−). CBG mRNA was found in both epididymal and subcutaneous WAT (Figure 5B). HL diet heightened CBG mRNA in both depots, significantly in the epididymal. Despite this, CBG protein showed a nonsignificant decrease either in epididymal and subcutaneous WAT.

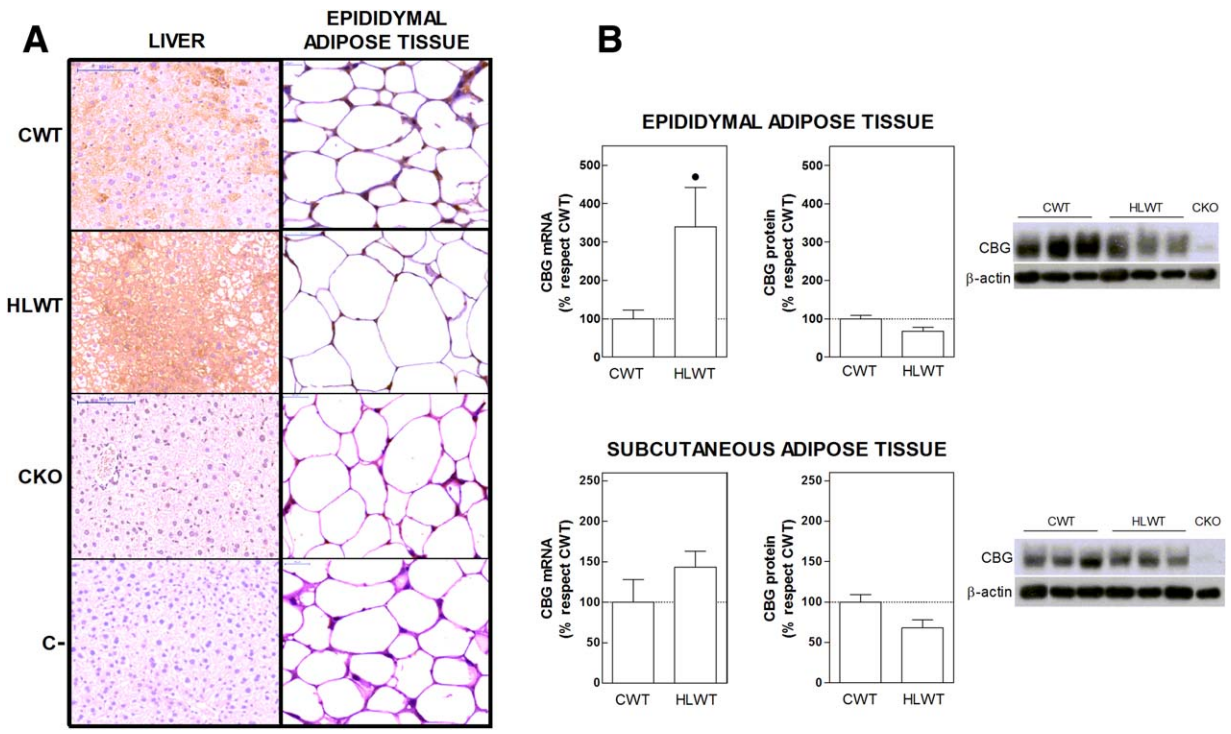

Figure 5 Corticosteroid-binding globulin (CBG) in white adipose tissue. (**A**) The immunohistochemistry results of CBG are shown in liver, which was used as a positive control, and epididymal adipose tissue. The subcutaneous adipose tissue was also analyzed, and similar results were obtained. Negative controls are adipose tissue of KO mice fed with the control diet (CKO) and adipose tissue of WT mice fed with the control diet (CWT) without primary antibody (C−). (**B**) CBG mRNA (left) and CBG protein (right) in epididymal and subcutaneous adipose tissue. Significant Student's *t*-test: •significant difference ($P < 0.050$) between CWT and WT mice fed hyperlipidic diet (HLWT).

Adipocyte area and expression of metabolic genes in WAT

Mean area and the frequency of distribution by area of epididymal and subcutaneous adipocytes are shown in Figure 6A. Adipocytes from epididymal WAT were larger than those from the subcutaneous, irrespective of the diet or mice genotype. Adipocytes from both depots of KO mice fed with control diet tended to be smaller than those of WT mice. However, with HL diet, epididymal adipocytes from KO mice exhibited a 2.5-fold increase in area, whereas adipocytes from WT presented only a 1.5-fold increase (Gen $P <$ 0.00001 and diet $P < 0.0001$). In contrast, adipocytes from the subcutaneous depot of KO mice showed a minor increase—1.9-fold—than the 2.3-fold increase in those from WT (Gen $P < 0.00001$ and diet $P < 0.0001$).

The mRNA expression of LPL, FAT/CD36, leptin, and PPAR-γ in both depots was also assessed (Figure 6B). CBG deficiency failed to change LPL expression, whereas the HL diet increased LPL expression only in epididymal adipose tissue. In both depots of WT and KO mice, the HL diet increased the FAT/CD36 and LEP mRNA levels. PPAR-γ expression increased in epididymal WAT of WT mice fed with HL diet, whereas in KO there was a sustained higher expression level, regardless of the diet (Diet = 0.0645, Int = 0.02 and Bonferroni between CWT and CKO $P < 0.05$). Conversely, in the subcutaneous depot of KO mice, a sustained decrease in PPAR-γ expression was observed (Gen = 0.0443).

Discussion

Mutations that affect CBG binding affinity, capacity, or protease sensitivity are emerging targets for clinical assessment in diseases associated with abnormalities in cortisol levels or activity (19). Human carriers of these mutations usually present hipocortisolemia or low cortisol levels within the normal range (11). In most cases of human CBG null mutations, fatigue and hypotension have been reported suggesting adrenal insufficiency. In some cases, overweight or obesity (12) as well as redistribution of adipose tissue to the visceral depot are present (20), typical features of glucocorticoid excess, even subtle, like those observed in patients with adrenal incidentalomas (21).

Our model of CBG-deficient mice presented high free corticosterone (2.5-fold more than WT) but low total corticosterone levels (three to fourfold lower) as previously reported (13). According to the free hormone hypothesis, this would be a model of glucocorticoid excess. Thymus involution, observed in KO mice, is the expected response to sustained high glucocorticoid levels (16). Conversely, the adrenal increased in KO mice, a feature associated with adrenal hyperactivity in response to an active HPA axis. An increased adrenal gland has been associated with high circulating corticosterone levels (22,23). The reason of the fall in total corticosterone in CBG-deficient mice could be the accelerated blood corticosterone clearance previously described (13). Adrenal hypertrophy in KO mice could be an attempt to compensate the depleted corticosterone levels

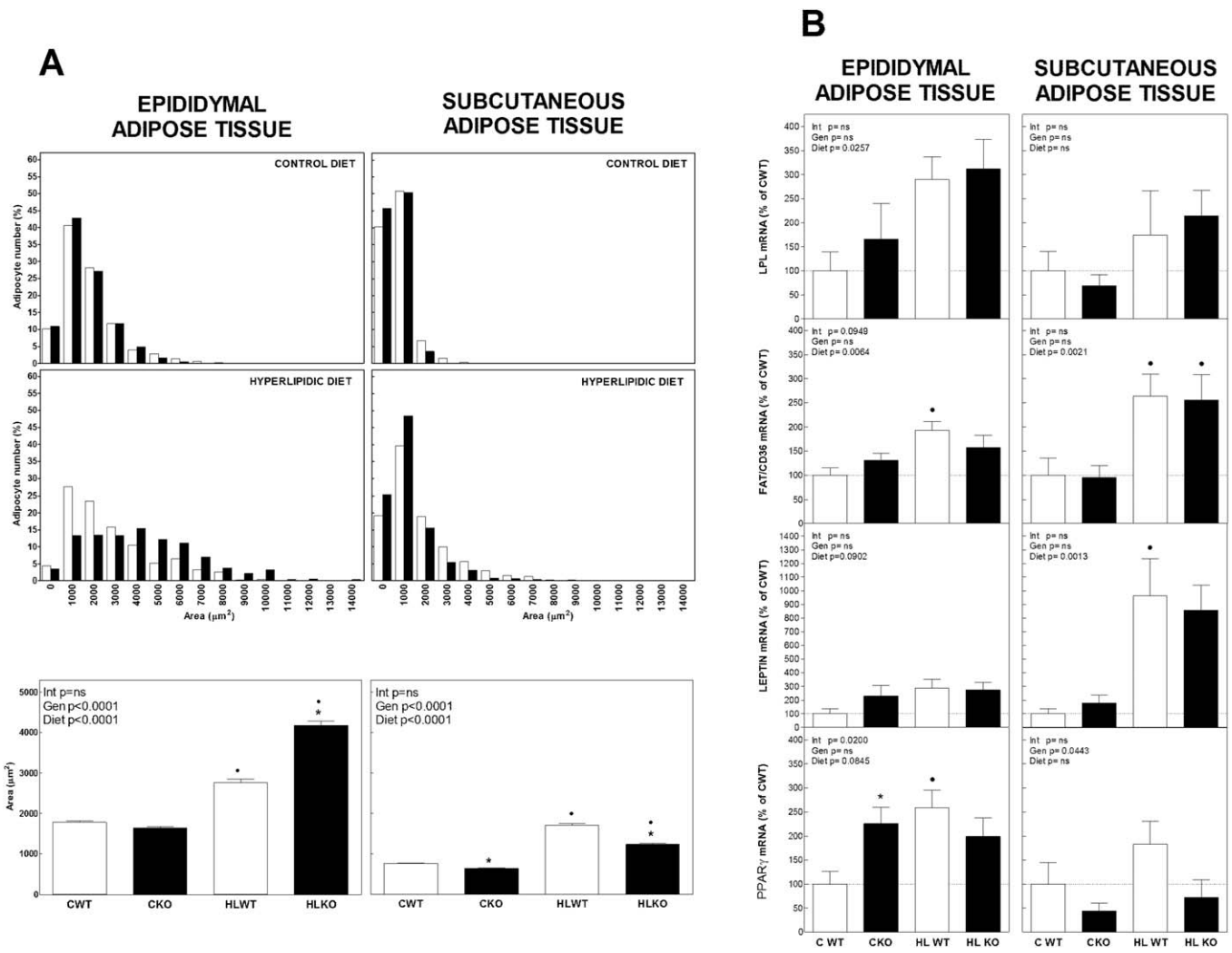

Figure 6 Adipocyte area and expression of metabolic genes in epididymal and subcutaneous adipose tissue in WT mice and KO mice fed a standard diet (CWT and CKO) or a hyperlipidic diet (HLWT and HLKO). (**A**) The adipocyte area measurements from epididymal and subcutaneous adipose tissue were evaluated. The frequency of area distribution (upper graph for control diet and intermediate graph for HL diet) and total mean area (bottom graphs) are depicted. (**B**) The epididymal and subcutaneous mRNA expression levels of some metabolic genes are also shown. The two-way ANOVA statistical *P* values are depicted in each graph (ns = not significant). The significant differences determined by Bonferroni method are: *significant difference between WT and KO of the appointed diet and •significant difference between C and HL-fed mice of the appointed genotype. The open bars correspond to the WT data, and the black bars correspond to the data from the KO mice.

triggered by its high turnover. In any case, further research is needed to assess adrenal functioning in CBG-deficient mice.

It is known that the glucocorticoid action on rodent body weight follows a bell-shaped curve (24,25), in which sustained moderate levels of total corticosterone render an increase in body weight, whereas pharmacological chronic levels causes body weight reduction (26). Previously published data (13) show that CBG-deficient mice presented a body weight comparable to WT mice, even after an overnight fasting or after having been fed with HL diet (30% of the energy derived from fat) during a period of 6 weeks. In our study, body weight and food intake were recorded over a period of 12 weeks in mice under a standard diet or a more extreme HL diet containing 60% of the energy derived from fat. CBG-deficient mice subjected to the control diet showed a slightly larger body weight than WT mice, beside a higher food intake. This is a corticosterone effect observed when normal insulin levels are present (27), i.e., under the control diet. It was reasonable to expect a further increase in the body weight of CBG-deficient mice fed with HL diet taking into account that corticosterone, in the presence of high insulin levels, boosts the drive to eat (27). However, no differences in body weight or food intake were observed. In WT mice, diet-induced obesity elicited serum corticosterone and binding increases at the same time that CBG increased in serum and liver. Although free corticosterone did not change, it is possible that the increase in total corticosterone levels observed in WT matched the corticosterone effect on HLKO mice. Glucocorticoids are known inhibitors of CBG expression (28). Therefore, the corticosterone increase observed in WT mice fed with HL diet could be secondary to the CBG upregulation, a consequence of diet-induced obesity.

Immunohistochemistry revealed locally established CBG in WAT, as previously reported in rats (9). We detected limited CBG mRNA expression (around 30 cycles vs. 19 in liver) but significant CBG protein. Hence CBG entry from blood into adipose tissue cannot be ruled out. In addition, the fact that CBG protein in adipose tissue did not change in HLWT mice despite the expression increase in the epydidimal depot and plasma, points to a post-translational regulation or a CBG secretion to the blood. The function of CBG in WAT remains uncertain, but taking into account its function in plasma, CBG may also regulate the free glucocorticoid fraction in the adipose tissue.

CBG deficiency promoted the storage of lipids in visceral instead of subcutaneous adipose tissue. This is in agreement with the known glucocorticoid regulation of lipid partitioning, i.e., in a model of glucocorticoid and insulin excess (29) such as the HL diet. Our results point out that reduced subcutaneous weight and adipocyte area in KO mice can be mediated at least in part by the blunted PPAR-γ expression found in this depot.

The glucocorticoid action is highly modulated by its local availability, consequence of circulating glucocorticoid levels, and the intracellular activation/inactivation balance. It is well established that the decay of 11HSD1 expression in WAT of mice with diet-induced obesity (30), is a way to protect the tissue against further metabolic derangements. Despite the fact that 11HSD2 is mainly found in mineralocorticoid target tissues, a minor but significant expression has been found in liver (31) and WAT (32-34). In male rats WAT, 11HSD2 expression correlated with body weight and fat content (33). However, the scarce literature available is inconclusive: after diet-induced obesity, authors have reported normal 11HSD2 expression in the subcutaneous depot but high in the retroperitoneal fat pad (33) and others, a reduced 11HSD2 expression in subcutaneous adipose tissue in mice submitted to HL diet (34). In our hands, WT mice with obesity showed an increased 11HSD2 expression in epididymal WAT and a decreased expression in subcutaneous WAT whereas hepatic 11HSD2 did not change. CBG deficiency reduced 11HSD2 expression in liver irrespective of the diet and in the epididymal depot, spurred a further increase in 11HSD2 expression in addition to the increase promoted by the HL diet previously described (33). The consequence of 11HSD2 reduction would be the increase in corticosterone availability due to its reduced inactivation. But recently, the analysis of a KO mice deficient in 11HSD2 has revealed reduced adipose tissue probably induced by the drop in glucocorticoid signaling (35). The authors propose that the absence of 11HSD2 would limit substrate availability for 11HSD1 and therefore local regeneration to active glucocorticoid. In the liver, the HL diet promoted corticosterone availability through increased 11HSD1, as previously reported (30), and decreased the 5α-reductase, which is involved in its inactivation without changes in KO mice. In this context, and taking into account the lack of up-regulation of PEPCK expression and the normal accumulation of lipids and glycogen in CBG-deficient mice, suggesting normal glucocorticoid signaling, the decrease of 11HSD2 mRNA would reduce 11-dehydrocorticosterone availability. In the KO mice, the epididymal 11HSD2 mRNA increase in WAT would react by inactivating glucocorticoids and counteract the enlargement of adipocyte area. Petersen et al. also found normal hepatic PEPCK expression in CBG-deficient mice suggesting the need of CBG to mediate glucocorticoid action in liver (13). Regulation of 11HSD2 would be a mechanism of glucocorticoid availability control inside tissues other than mineralocorticoid targets as recently observed in lung of pancreatitis-induced mice deficient in CBG (15).

In conclusion, CBG deficiency causes an environment high in free glucocorticoids although low in circulating total corticosterone which, under a context of lipid excess, drives lipid partitioning from subcutaneous to visceral adipose tissue without further alterations in food intake or in body weight. In addition, the fact that CBG localizes in WAT beyond blood suggests that WAT is a sensitive tissue to CBG alterations where it would act as a controller of glucocorticoid availability inside the tissue. Our data highlight the role of CBG in the fine control of optimal corticosterone availability and point to the need to evaluate CBG in tissues to completely understand glucocorticoid action. Thus, under some environmental disturbances where the HPA axis is activated, such as unbalanced diet, septic shock (13), or stress (14), alterations in binding and capacity properties of CBG would magnify glucocorticoid action inside target tissues and as a consequence have an impact on adipose tissue architecture. O

Acknowledgments

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4. *Exacerbated proinflammatory and antiinflammatory mechanisms in epididymal adipose tissue of CBG-deficient male mice with diet-induced obesity*

El objetivo de este trabajo fue determinar las consecuencias de la deficiencia de CBG en la progresión y la gravedad de la inflamación crónica de baja intensidad asociada a la obesidad inducida por la dieta (la misma dieta que en el trabajo anterior) en tejido adiposo blanco epididimal.

Ratones machos de 6 semanas de edad WT y deficientes en CBG (KO) se alimentaron durante 1 o 12 semanas con dieta control estándar (grupos CWT y CKO) o una dieta hiperlipídica (grupos HLWT y HLKO). Se evaluó la concentración circulante de IL-6 e IL-10, la capacidad de unión de la CBG y se realizó un test intraperitoneal de tolerancia a la glucosa. En el tejido adiposo blanco epididimal se evaluó la infiltración de neutrófilos a 1 semana, *crown-like structures* (CLS) a las 12 semanas y la expresión de marcadores inflamatorios y antiinflamatorios a 1 y 12 semanas de iniciar el consumo de la dieta. A las 12 semanas, se cuantificaron e identificó la tipología de los macrófagos en epididimal y se evaluó la expresión génica de algunos genes involucrados en la respuesta inflamatoria. En adipocitos se determinó la actividad lipolítica y la activación de NFkB.

La deficiencia de CBG promovió una mezcla de acciones proinflamatorias y antiinflamatorias en función del tiempo de exposición a la dieta y del tipo de dieta. El tejido adiposo de los ratones deficientes en CBG, con dieta estándar, presentó más signos de inflamación que el de ratones WT, como mayor expresión de TNF-α e IL-6, mayor infiltración de neutrófilos y mayor proporción de macrófagos M1 activados aunque no cambió el número total de macrófagos ni de CLS. Al mismo tiempo, también se observó una elevada expresión del mediador de la acción antiinflamatoria de los glucocorticoides DUSP-1. Todo ello indicaría que en ausencia de CBG y de estímulo inflamatorio, esta potenciada la acción de los glucocorticoides preparando y potenciando el sistema inmune.

En la obesidad inducida por la dieta, los ratones KO presentaron una respuesta temprana, a la semana de inicio de la dieta, con mayor respuesta inflamatoria que se mitiga con el tiempo progresando hacia un estado más antiinflamatorio que el observado en ratones WT y caracterizado por mayor expresión de DUSP-1 y el aumento de macrófagos de tipo M2 así como la contención de la activación de NF-kB en adipocitos. En este caso, los glucocorticoides en presencia del estímulo inflamatorio, estarían promoviendo la resolución de la inflamación. Efectos metabólicos característicos de los glucocorticoides, tales como la inducción de resistencia a la insulina o la estimulación de la lipólisis, también se observaron en ratones deficientes en CBG.

En conclusión, la deficiencia de CBG promovió un conjunto de acciones proinflamatorias y antiinflamatorias en función del tiempo de exposición a la dieta y la propia dieta, acorde con un modelo de exceso moderado de glucocorticoides circulantes.

Manuscrito en preparación

Exacerbated pro-inflammatory and anti-inflammatory activities in epididymal adipose tissue of CBG-deficient male mice with diet-induced obesity

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Introduction

Glucocorticoids are a group of steroid hormones that include corticosterone in rodents and cortisol in humans. They are secreted by the adrenal gland and transported in blood by binding to corticosteroid-binding globulin (CBG). Glucocorticoids control the mobilization and deposition equilibrium of body nutrient stores (triacylglycerol (TAG) in adipose tissue, glycogen in liver and muscle, and, in exceptional circumstances, protein in muscle), playing important roles in meeting the energy demands during responses to physical and psychological stress, as well as replenishing energy stores for future challenges (1). Chronic elevation in glucocorticoid levels is the primary cause of Cushing's syndrome, which presents central obesity and some features of metabolic syndrome.

Glucocorticoids are also efficient anti-inflammatory molecules that suppress the immune system at inflammation sites. CBG, a member of the serine protease inhibitor family (2), regulates glucocorticoid availability. At inflammation sites, elastase, a serine protease secreted by neutrophils, cleaves a small peptide from CBG, thereby reducing its ligand affinity and promoting the release of glucocorticoid (3). Although glucocorticoids are anti-inflammatory during inflammation, their role in the normal physiology of the immune system is unclear. Under basal conditions, glucocorticoids may play a protective role by ensuring the immune system is ready to respond to pathogens, which appears to be the case with the innate immune system where glucocorticoids are not strictly immunosuppressive (4).

In peripheral blood mononuclear cells from healthy human donors, glucocorticoids induce the expression of chemokines, cytokines, and complement family members, while repressing the expression of genes associated with adaptive immunity (5). In activated inflammatory T-helper subsets, glucocorticoids downregulate the expression of specific genes that upregulate in resting cells (5). In addition, some effects can appear/disappear in a dose-dependent manner. For example, corticosterone has been reported to exert immunostimulatory effects on macrophage function at low concentrations, but not at high concentrations (6).

CBG-deficient mice display a small chronic excess of free corticosterone and markedly low concentrations of total hormone (7). Lack of CBG does not affect the progression of inflammation 12 hours after the induction of acute pancreatitis (8), but LPS-injected CBG-deficient mice have

been reported to show lower survival rates and more exacerbated inflammatory responses (9), suggesting a key role of CBG in the anti-inflammatory response. Previously, we observed that CBG deficiency increased adipocyte volume in visceral epididymal adipose tissue and decreased subcutaneous stores without affecting body weight or food intake (7).

The aim of this study was to elucidate the effects of CBG deficiency on the progression and severity of chronic low-grade inflammation in epididymal white adipose tissue (EWAT) associated with diet-induced obesity.

Materials and Methods

Animals and experimental protocols

Six-weeks-old wild-type (WT) and CBG-knockout (KO) male mice were used. The CBG-KO colony was established through crossing heterozygous animals (kindly provided by Dr. Willnow). The WT mice used were also obtained from the colony. The procedure for disrupting the CBG gene to generate KO mice has been previously described (9).

WT and KO mice were divided into two groups containing 6 to 10 animals. Each cage housed 2 to 4 mice. Mice were fed either a control (C) diet (CWT and CKO groups), which provided 18% of the energy from fat (Teklad Global 2018), or a hyperlipidic (HL) diet (HLWT and HLKO groups), which gave 60% of the energy from fat (TD.06414) for 1 or 12 weeks. All the diets were supplied by Harlan-Interfauna Ibérica (Sant Feliu de Codines, Spain). The animals were provided food and water *ad libitum* until they were sacrificed, and maintained under a 12-hour light cycle (from 08.00 to 20.00) in a temperature-controlled environment (20–22°C). Their body weight and food intake were periodically measured. At the end of the dietary treatment, mice were fasted overnight and the animals were sacrificed under isoflurane anesthesia between 07:00 and 09:00. Blood from the cava vein was obtained during a 2-minute procedure and maintained on ice until centrifugation at 12,000 *g* for 20 minutes to obtain the serum fraction. EWAT was then excised, weighed and immediately submerged in 4% paraformaldehyde or collected for collagenase digestion and the rest frozen in liquid nitrogen.

Animal maintenance and the experimental procedures were conducted in accordance with the guidelines for the use of experimental animals established by the European Union, Spain, and

Catalonia, and were approved by the Animal Handling Ethics Committee of the University of Barcelona.

CBG binding assay

The binding capacity of CBG was evaluated using ^{3}H-corticosterone, as previously reported (8). Briefly, 10 µl of serum samples were stripped by adding 0.5 ml of a charcoal-dextran suspension (0.05% dextran and 0.5% charcoal in phosphate-buffered saline (PBS) with 0.1% gelatin (PBSG) at pH 7.4) and incubated at room temperature for 30 minutes with occasional shaking. Samples were then centrifuged and the clear supernatants further diluted 1:5 in PBSG buffer before use in corticosterone binding assays. The final concentration of labeled corticosterone in the tubes was 15 nM. The tubes were left to incubate at 37ºC for 30 minutes and then put on ice for 2 hours. The charcoal-dextran suspension was then added to each tube and the mixture centrifuged for 10 minutes. Clear supernatants were added to 4 ml of a water-miscible scintillation cocktail (EcoLite, MP Biomedicals, USA) and counted in a standard scintillation counter. In all the samples, individual non-specific binding (measured in a 500-fold excess of non-radioactive corticosterone) was measured and subtracted from the total amount of bound radioactive corticosterone.

Serum cytokine measurements

Serum samples from mice belonging to each group were pooled to obtain sufficient volume for the analysis of cytokine levels using a commercial enzyme immunoassay for IL-6 (KMC0061), monocyte chemoattractant protein 1 (MCP1) (KMC1011), and IL-10 (KMC0101, Invitrogen).

Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IGTT) was performed 11 weeks after starting the dietary treatments, as described previously (10). Briefly, after 4 hours of fasting, a bolus of glucose (2g/kg body weight) was injected into the intraperitoneal cavity and a few µl of blood were obtained through a small cut in the tail at 0 (just before the injection), 15, 30, 45, 60, and 90 minutes after the injection for blood glucose measurements (Glucocard G+Meter, Menarini Diagnostics).

Immunohistochemical analysis of EWAT

Small amounts of EWAT were dissected and fixed by immersion in 40 g/L paraformaldehyde in 0.1M phosphate buffer, pH 7.4, at 4°C overnight. The samples were maintained at 4°C in phosphate buffer and later dehydrated and embedded in paraffin blocks.

Serial 5-µm sections were dewaxed in xylene and stained with eosin-hematoxylin. The immunohistochemical detection of elastase or F4/80 was performed with the avidin-biotin-peroxidase method. Endogenous peroxidases were blocked with a 3% solution of hydrogen peroxide and then diluted 1:19 in horse serum (HS) to reduce non-specific staining. Sections were incubated overnight at 4°C in a high-humidity environment with goat anti-mouse elastase antibody (sc-9521, Santa Cruz Biotechnology) or rabbit anti-mouse F4/80 antibody (MAB10058, Abnova) diluted 1:100 or 1:200 in HS with background-reducing components, respectively (Dako, Barcelona, Spain). Biotinylated horseradish peroxidase (HRP)-conjugated anti-goat IgG was used as the secondary antibody (Vector Labs, Burlingame, CA, USA). Detection was performed with an avidin-biotin-peroxidase complex (Vectastain® ABC kit, Vector Labs) using diaminobenzidine (DAB) as the substrate (DAB Peroxidase Substrate Kit, Vector Labs). The slides were visualized under an optical microscope (Olympus BX-51, Olympus Iberia, Barcelona, Spain), and image stacks were captured (Olympus DP-70). A minimum of 100 adipocytes per mouse distributed in 10 fields and 3 mice per group were evaluated.

Real-time PCR expression analysis

Total RNA was extracted from EWAT using the TRI Reagent Solution (Life Technologies, Waltham, MA, USA) and quantified with an ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were reverse-transcribed using the MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primers.

RNA from macrophages was extracted with RNeasy Micro Kit (cat. 74004, Qiagen) and the samples reverse-transcribed with the QuantiTect Reverse Transcription Kit (cat. 205311, Qiagen).

Real-time PCR (RT-PCR) amplification was conducted using 10 µl of the amplification mixtures containing SYBR Green PCR Master Mix (Life Technologies) and 300 nM primers. The primers

used for tumor necrosis factor α (TNFα), dual-specific phosphatase 1 (DUSP1), elastase, IL-10 receptor (recIL10), macrophage transcription factor PU1m (PU1m), angiopoietin-like 4 (ANGPTL4), arginase, IL-6, and F4/80 are listed in Supplementary Table 1. The reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems), using an annealing temperature of 60°C. To determine the sensitivity and efficiency of the amplification, PCR linear ranges were established for each gene with tissue cDNA. We measured the expression of the housekeeping gene ribosomal protein L32 (RPL32) as control. Ct values were obtained using an automatically calculated threshold, and the ΔΔCt method was used for relative quantifications. Final data were calculated as percentages with respect to the WT group following the control diet for 12 weeks (CWT group), which was normalized to 100%.

Isolation of adipocytes and cells from stromal vascular fractions

EWAT from 2-3 mice was collected to achieve 1 g of tissue, which was then minced and incubated for 45 minutes at 37°C with gentle agitation in Krebs-Ringer buffer (KRB, 3 mL/g adipose tissue), containing 10 mM glucose, 4% bovine serum albumin (BSA) and 1 mg/ml collagenase type IV (Worthington Chemicals, Lakewood, NJ, USA). The digested tissue was filtered through a chiffon mesh into a graduated cylinder. The lipid-laden adipocytes floated, and the infranatant fraction was removed and centrifuged at 200 *g* twice to pellet cells from the stromal vascular fraction (SVF). The adipocyte fraction was resuspended in DMEM containing 3.5% of defatted BSA and without phenol red, and allowed to stand to enable the isolation and removal of the infranatant. This wash step was repeated once again. The number of adipocytes was counted using a Neubauer chamber. SVF cells were quantified using a Gallios multi-color flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) set up with the 3-laser/10-color standard configuration.

Flow cytometry and sorting

Aliquots of SVF cells in 100 µL of PBS were incubated with 0.2 µg of FITC-labeled anti-F4/80, anti-CD206 (mannose receptor), anti-CD210 (IL-10 receptor) or isotype-matched control antibodies (Biolegend) for 15 minutes. Cells were then washed with 2% HS-PBS, incubated with fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences), and resuspended in 200 µl of 2%

HS-PBS. Flow cytometric analysis was carried out using a FACSAria I SORP sorter (Beckton Dickinson). The gate for the total number of macrophages was defined by F4/80. The gate for M2-polarized macrophages was defined by F4/80, CD206 and/or CD210, whereas that for M1-polarized macrophages was obtained by subtracting the results for M2-polarized macrophages from those for the total number of macrophages. The data obtained were analyzed with Summit software version 4.3 (Dako Colorado, Inc.).

Adipocyte lipolysis assays

Adipocyte concentration was adjusted to 100,000 adipocytes per ml of DMEM containing 3.5% of defatted BSA and without phenol red. Aliquots of 1 ml were distributed into polypropylene tubes and incubated at 37ºC for 90 minutes under constant shaking in the presence of buffer or 10 µM isoproterenol. The infranatant was collected and kept at -80ºC for glycerol quantification (F6428, Sigma). Adipocytes were resuspended in lysis buffer containing DMEM (without phenol red) and phosphatase and protease inhibitor cocktails (Sigma), frozen in liquid N_2 and thawed on ice. Adipocytes were homogenized by passing through an insulin-needle syringe, aliquoted, and kept at -80ºC until use.

Western blot analysis of NF-kB

Protein concentrations of lysed adipocytes were quantified with the QuantiPro BCA Assay Kit (Sigma), and samples containing 25 µg of total protein were separated by SDS-PAGE in a 10% gel and electrotransferred onto a PVDF membrane (Millipore, Billerica, MA, USA). After blocking non-specific binding sites with 5% non-fat milk in 0.05% TBS-Tween for 90 minutes at room temperature, the membranes were incubated overnight with polyclonal goat anti-mouse nuclear factor kappa-light-chain-enhancer (NFκB) antibody (diluted 1:1,000; Santa Cruz Biotechnology) or anti-mouse p65 NFκB antibody. An anti-lamin antibody (diluted 1:50,000; sc-46293, Santa Cruz Biotechnology) was used as a loading control. Immunoreactive proteins were detected with an anti-goat or anti-rabbit HRP-conjugated secondary antibody (diluted 1:20,000; Santa Cruz Biotechnology). Immunodetection was performed with Pierce ECL Western Blotting Substrate

(Thermo Fisher Scientific), and protein levels were quantified using the TotalLab Quant software version 2003.3 (Non-Linear Dynamics, Newcastle, UK).

Statistical analysis

Data were analyzed using the GraphPad software (version 5.0) and are expressed as means ± SEM. Statistical comparisons were made using two-way ANOVA to evaluate the effect of genotype (Gen) and diet (Diet), with Bonferroni post-hoc tests used to compare WT and KO pairs for each type of diet, and C- and HL-diet pairs for each type of genotype. The interaction significance was also determined (referred to as 'Int' in the figures and tables). P values less than 0.05 were considered statistically significant.

Results

Body weight and food intake

Body weight and food intake were recorded once a week. The effects of overnight fasting before sacrifice on body weight and food intake were also analyzed. A week after starting the HL diet, there was increased food energy intake in HL mice (two-way ANOVA, Diet, p=0.0391), but no changes in body weight (Table 1) in both WT and KO mice. Overnight fasting triggered the largest body weight loss in mice on the C diet (Diet, p=0.0385), which was more dramatic when expressed as a percentage of the body weight (Diet, p=0.0040). After 12 weeks of dietary treatment (Table 2), the mean energy intake continued to be higher in HL-fed than C-fed mice (Diet, p=0.0312), KO mice presenting lower values than WT animals (Gen, p=0.0198) that were not significant after Bonferroni analysis and also not observed in a former study (7). Body weight at 12 weeks increased to a similar extent in both WT and KO mice fed the HL diet (Diet, p=0.0007), as previously reported (7). The impact of fasting on body weight loss was significantly lower in HL-fed mice compared to C-fed mice when expressed as a percentage at 1 week (Diet, p=0.0040) and 12 weeks (Diet, p=0.0346). The effects of fasting on body weight were not affected by genotype. EWAT weight significantly increased as a consequence of the HL diet both at 1 week (Diet, p=0.0094) and 12 weeks (Diet, p=0.0003).

Blood cytokine levels and IGTT

Serum IL-6 concentration increased significantly in mice on the HL diet irrespective of genotype (Figure 1; Diet, $p<0.0001$). Serum IL-10 levels showed a different response to the HL diet depending on the genotype. HLWT mice showed a non-significant reduction in serum IL-10 concentrations, whereas HLKO mice displayed significantly elevated IL-10 levels (Gen, $p=0.0780$; Diet, p=ns; Int, $p=0.0048$). MCP1 concentrations remained unaltered in all the experimental groups.

IGTT was performed 11 weeks after starting the dietary treatments. Glucose levels in HLKO mice were significantly higher at 45 and 90 minutes after starting the IGTT (Figure 2) compared to the rest. The area under the curve was significantly higher due to the HL diet (Diet, $p=0.0008$) mainly in CBG-deficient mice (Int, $p=0.0385$) which showed significant higher values than HLWT by Bonferroni post-test analysis.

CBG binding in serum

CBG binding was measured in the serum of WT mice 1 and 12 weeks after starting the diets (Figure 1B). CBG binding increased with time (Time, $p=0.0067$) mainly in HL-fed mice (significant in Bonferroni post-hoc test).

Neutrophil infiltration and crown-like structures in EWAT

We assessed the presence of neutrophils in EWAT which are the first immune cells to respond to inflammation, by quantifying elastase expression in mice following the diets for 1 or 12 weeks (Figure 3A). We also recorded the number of immunoreactive neutrophils stained with the elastase antibody in EWAT slides from mice submitted to each diet for 1 week (Figure 3B). Elastase mRNA levels showed a non-significant increase above basal levels in KO and HL-fed mice, especially in HLKO mice 1 week after following the diet, but not after 12 weeks (Figure 3A and 3B). Neutrophil counting in the EWAT slides, expressed as elastase-positive cells per 100 adipocytes, revealed the same pattern, which resulted statistically significant (Figure 3C).

The HL diet increased the number of crown-like structures (CLS) in the adipose tissue of both WT and KO mice compared to the C diet (Figure 4A) quantified as CLS per 100 adipocytes (Figure 4B).

The number of CLS in the adipose tissue was slightly higher in KO mice than in WT in both HL- and C-fed mice, without reaching statistical significance (Figure 4B).

Expression of inflammatory markers in EWAT

Expression of some of the inflammatory markers in EWAT was also evaluated from the beginning (1 week of diet) to the end (12 weeks of diet) of the study period (Figure 5). One week of the HL diet had a larger effect on expression levels than 12 weeks of the diet. Using two-way ANOVA, we identified significant changes in the mRNA levels of TNFα ($p=0.035$), PU1m ($p=0.0143$), IL-6 ($p<0.0001$), arginase ($p=0.0053$) and F4/80 ($p=0.0404$) after 1 week on the diets. ANGPTL4 expression was also significantly affected ($p=0.0425$), increasing in WT mice and decreasing in KO animals. After 12 weeks, the only significant changes due to HL diet were the elevations in TNFα ($p=0.035$) and ANGPTL4 ($p=0.0026$) mRNA levels.

CBG-deficient mice showed significantly increased EWAT TNFα ($p=0.0014$), DUSP1 ($p=0.021$), arginase ($p=0.0304$) and IL-6 ($p=0.0011$) mRNA levels after 1 week on the C and HL diets, with expression being greater in the HL- than C-fed mice, except for DUSP1 mRNA, which was more increased in C- than HL-fed mice. At 12 weeks, only DUSP1 mRNA levels remained significantly higher in CBG-deficient mice than in WT (Gen, $p=0.0125$).

Characterization of macrophages in EWAT

Figure 6A shows the total macrophage content in EWAT for each group of mice. The only significant difference was increased macrophage infiltration in HL-fed WT and KO mice (two-way ANOVA, Diet, $p=0.0227$).

The type of macrophages present in EWAT after 12 weeks was assessed by FACS (Figure 6B). Macrophages were identified as F4/80-positive cells. The ratio of M1- to M2-polarized macrophages in the adipose tissue varied according to diet and genotype (two-way ANOVA, Int, $p=0.0026$) (Figure 6B). CWT mice showed a majority of M2-polarized macrophages (around double the number of M1-polarized macrophages), whereas HLWT mice displayed an increase in the number of M1-polarized macrophages. The opposite pattern was observed in CBG-deficient mice,

with CKO mice showing similar numbers of M1- and M2-polarized macrophages and HLKO mice presenting more M2- than M1-polarized macrophages (Figure 6B).

Due to inconsistent data in the literature about the role of 11HSD1 in macrophages, we investigated 11HSD1 mRNA expression in M1- and M2-polarized macrophages from adipose tissue. The data obtained gave a Ct in the range of 31-36. Some samples from each group generated values out of the range of detection and were therefore unreliable. Thus, we were unable to determine whether 11HSD1 expression in resident macrophages was affected by inflammation in the adipose tissue of HL-fed mice, as has been observed *in vitro* in THP-1 macrophages (11). Furthermore, we could not detect 11HSD2 mRNA expression. TNFα mRNA levels were assessed as a marker of pro-inflammatory activity in M1-polarized macrophages (Figure 6C). TNFα expression increased in M1-polarized macrophages in CKO and HLKO mice compared to WT mice (two-way ANOVA, Gen p=0.0308), with the most significant difference observed between macrophages from CWT and CKO mice (significant difference according to the Bonferroni post-hoc test). ANGPTL4 is a multifunctional protein that is downregulated in activated macrophages through Toll-like receptors (TLR). In M2-polarized macrophages, ANGPTL4 expression was lower than in M1-polarized macrophages and was not affected by either the HL diet or CBG deficiency (Figure 6C). In M1-polarized macrophages, ANGPTL4 expression decreased sharply in response to the HL diet (two-way ANOVA, Diet p=0.0004) mainly in WT mice (Int p=0.0071). On the C diet, macrophages from KO mice showed a significant reduction in ANGPTL4 expression compared to WT mice.

DUSP1 is a key mediator of the anti-inflammatory effects of glucocorticoid in macrophages (12). DUSP1 mRNA levels were highly elevated in pro-inflammatory M1- and anti-inflammatory M2-polarized macrophages (Figure 6C) from KO mice compared to those from WT mice (two-way ANOVA, Gen, p=0.0010 for M1- and p=0.0029 for M2-polarized macrophages), particularly in animals on the C diet (significantly different according to the Bonferroni post-hoc test), which decreased in HL-fed animals, also in KO mice (Figure 6C). In macrophages from WT mice, the HL diet increased DUSP1 expression in M1- (two-way ANOVA, Diet, p=ns; Int, p=0.0052) and decreased DUSP1 mRNA levels in M2-polarized macrophages (two-way ANOVA, Diet, p=0.0180).

Inflammation in adipocytes

To assess the specific inflammatory status of EWAT adipocytes, we evaluated the amount of total and phosphorylated NFκB (Figure 7A) and the lipolysis activity of adipocytes (Figure 7B) in mice 12 weeks after starting the diets. The HL diet enhanced total (p=0.036) and phosphorylated (p=0.0601) levels of NFκB in adipocytes, with CBG deficiency further increasing the amount of total NFκB irrespective of diet (p=0.0022). CBG deficiency promoted NFκB phosphorylation in CKO mice, but did not trigger NFκB phosphorylation in HLKO mice respect to HLWT. The ratio of phosphorylated NFkB to total NFkB showed an almost significant increase due to diet (Diet, p=0.0539), but not to genotype. Basal lipolysis was not affected by either diet or genotype, but isoproterenol-induced lipolysis was much higher in KO than WT mice on the HL diet (Diet, p=0.0025; Int, p=0.0080; Bonferroni post-hoc test between CKO and HLKO mice, p<0.001).

Discussion

The CBG-knockout mice used in this work has been previously characterized (7, 9). Compared to WT, CBG-deficient mice showed reduced levels of total corticosterone, but small increases in free hormone levels that were sufficient to elicit a small but significant decrease in thymus weight (7), which is an immunosuppressive effect of sustained glucocorticoid levels (13). However, LPS-injected CBG-deficient mice exhibit greater mortality and pro-inflammatory cytokine secretion, explained as an absence of the anti-inflammatory properties of glucocorticoids after acute inflammation (9). Moreover, elastase-mediated cleavage of CBG leads to the release of glucocorticoids at the site of inflammation, where its anti-inflammatory effects are required to resolve inflammation and restore homeostasis. All these data suggest that CBG regulates the anti-inflammatory activities of glucocorticoids (9, 14).

The aim of this study was to elucidate the effects of CBG deficiency on the progression and severity of chronic low-grade inflammation associated with diet-induced obesity. The serum CBG increase previously found after 12 weeks of HL diet (7) have not been observed at 1 week of starting the HL diet and perhaps is a secondary consequence of the altered endocrine environment. After 12 weeks, the larger IGTT area under the curve (Figure 2) and the increased isoproterenol-induced lipolysis in the adipocytes (Figure 7) of HLKO compared to HLWT mice

would be the consequences of glucocorticoid excess (15, 16), only manifested during the altered metabolism triggered by the hyperlipidic diet, as previously reported (7).

To assess the inflammation status, we first quantified neutrophil infiltration in EWAT from mice fed an HL diet for one week. Neutrophil infiltration is considered the first step of inflammation in obesity, which is then followed by macrophage infiltration and CLS formation (17). Despite similar numbers of macrophages (Figure 6A) and CLS (Figure 4) at 12 weeks, CBG-deficient mice showed increased numbers of neutrophils (Figure 3) compared to WT mice, suggesting an early exacerbated response to the unbalanced diet (in HLKO mice), but also a reinforced innate immune system that is able to act if necessary (in CKO mice).

A similar ratio of M1- to M2-polarized macrophages (Figure 6B) and the increased NFkB phosphorylation in CKO and HLWT mice after twelve weeks (Figure 7A) indicate the occurrence of inflammation in the EWAT of both groups, which did not lead to elevated pro-inflammatory cytokine expression in CKO mice after 12 weeks of diet (Figure 5). By contrast, HLKO mice showed a decreased M1/M2 ratio, but similar levels of phosphorylated NFkB to those in CKO mice despite higher amounts of total NFkB. Like CKO mice, HLKO animals did not present increased expression of pro-inflammatory cytokines. It is well established that prolonged glucocorticoid exposure induces macrophages to become M2-polarized cells or anti-inflammatory, secreting IL-10, removing dead cells through phagocytosis, and promoting tissue healing (18). It is generally considered that obesity-associated adipose M1-polarized macrophages are pro-inflammatory and have detrimental effects, whereas normoweight-associated adipose M2-polarized macrophages are anti-inflammatory and linked to the development of healthy tissue (19). A recent study investigating macrophages from animals after 8 and 12 weeks on an HL diet demonstrated a mixed profile of macrophages that were initially pro-inflammatory, but became anti-inflammatory with time. In fact, CLS in long-term diet experiments have been observed to be formed by macrophages resembling M2- more than M1-polarized cells (20). Furthermore, recent findings indicate that inflammation in adipose tissue is an essential requirement for adipose tissue expansion in an HL diet, avoiding lipotoxicity and systemic inflammation (21).

Glucocorticoids are anti-inflammatory during sustained inflammation. However, they are not always immunosuppressive. Within the range of endogenous corticosterone levels released during the

hypothalamic-pituitary-adrenal axis circadian rhythm, glucocorticoids prime the innate immunity for inflammatory responses (18). Three different levels of glucocorticoid activity on macrophages have been suggested: basal levels that maintain an inactive system in the absence of stimuli; medium levels that promote immune responses to injury; and high levels that are immunosuppressive in order to help restore homeostasis (22).

TLRs are the best characterized mediators that initiate and perpetuate the inflammatory response. Stimulation of TLRs activates intracellular signaling pathways, including the NF-κB and mitogen-activated protein kinase (MAPK) pathways, which lead to the expression of a large number of genes, including cytokines. The anti-inflammatory effects of glucocorticoids are achieved through suppressing NFκB transcription, as well as through inhibiting or stimulating the expression of pro-inflammatory or anti-inflammatory factors, respectively (23). Glucocorticoids directly promote the expression of DUSP1, a pivotal mediator of the anti-inflammatory effects of glucocorticoids that inhibit the MAPK pathway through its phosphatase activity (24, 25). DUSP1 overexpression blocks the production of the cytokines TNFα and IL-6 (24).

In CBG-deficient mice, irrespective of diet, DUSP1 expression remained elevated throughout the entire duration of the study, most likely due to the presence of high glucocorticoid levels. After 1 week on the diets, the increase in DUSP1 expression was similar to the increases seen with TNF and IL-6 expression in both CKO and HLKO mice (Figure 5). However, after 12 weeks, the only significant difference that remained was the increased DUSP1 expression. The main sources of DUSP1 in EWAT were the SVF cells (data not shown). The M1- and M2-polarized macrophages obtained from adipose tissue also expressed DUSP1 in a similar pattern (Figure 6C), with CBG-deficient mice showing higher expression, especially those fed the C diet. This trend was also observed with TNFα expression in M1-polarized macrophages. These findings are not consistent with DUSP1 being characteristic of anti-inflammatory M2-polarized macrophages and TNFα being characteristic of pro-inflammatory M1-polarized cells (26). Therefore, our findings demonstrate the versatility shown by macrophages in their ability to alter their phenotype depending on the microenvironment, even performing pro-inflammatory and anti-inflammatory roles simultaneously (27).

ANGPTL4 is a direct glucocorticoid target that inhibits lipoprotein lipase (LPL) activity and drives lipolysis (28). Although its expression is activated by inflammatory stimuli in most tissues, including white adipose tissue, ANGPTL4 expression is reduced in activated macrophages exposed to LPS (29). After 1 week, we observed a different pattern of EWAT ANGPTL4 expression between CBG-deficient and WT mice. The expected increase in ANGPTL4 expression was observed in HLWT mice, but CBG-deficient mice showed increased expression that was sustained regardless of diet, which again could be triggered by the higher levels of free corticosterone. After 12 weeks, this pattern disappeared and the increased ANGPTL4 expression was seen only in HL-fed mice. Since ANGPTL4 diminution is a sign of macrophages activation by inflammation, its pattern of expression in EWAT macrophages suggests that in M1-polarized macrophages ANGPTL4 response is more sensitive than in M2 and that macrophages from CKO mice are more activated than those from CWT mice. The HL diet was a strong activating stimulus in M1-polarized macrophages in both WT and KO mice.

NFκB transrepression through tethering or repressive interaction is another mechanism underlying glucocorticoid anti-inflammatory activity that enables glucocorticoids to repress the expression of the pro-inflammatory TNFα and IL-6, among others (18). In this study, CBG-deficient mice presented greater NFkB expression than WT. This difference disappeared between WT and KO mice fed the HL diet when taking into account the active phosphorylated NFkB, but remained for WT and KO mice fed the C diet. NFkB activation, however, only translated into higher TNFa expression in HL-fed mice, thus leading to both pro-inflammatory and anti-inflammatory responses in CBG-deficient mice fed the control diet.

Recently, CBG has been detected in mouse adipose tissue, where it has limited mRNA expression compared to the liver (7). CBG protein levels increase in the EWAT of diet-induced obese mice, suggesting a role of CBG in either facilitating glucocorticoid delivery or acting as an *in-situ* controller of glucocorticoid availability in adipose tissue (7). Reduced CBG cleavage has been recently associated with human abdominal obesity and metabolic syndrome, where it is thought to contribute to the chronic inflammatory conditions seen in metabolic syndrome (30).

In this study, we observed that CBG deficiency elicited both pro-inflammatory and anti-inflammatory responses, depending on the time of exposure to the inflammatory stimuli and diet.

According to the model of the 5 R's (ready, reinforce, repress, resolve, and restore) proposed to explain the complex effects of glucocorticoids on inflammation (18), we can conclude that CBG-deficiency, in absence of inflammatory stimuli, prime and ready the innate immunity into the EWAT in young mice. During early responses to inflammatory stimuli, such as a hyperlipidic diet, free glucocorticoids, in the absence of CBG, elicit a more inflammatory response that becomes attenuated with time through the presence of more resolving immune cells and the persistence of key anti-inflammatory proteins such as DUSP1.

Acknowledgments

We thank to Dra. Laura Herrero her valuable help with the FACS protocol and Dr. Jaume Comas from the Centres Científics i Tecnològics of the University of Barcelona for his technical guide with FACS experiments.

Table 1. Mean body weights and food intake in mice submitted to control or HL diet for 1 week.

	CWT	CKO	HLWT	HLKO
BW day 0 (g)	22.4±0.5	21.9±0.7	23.7±0.7	22.4±1.2
BW 1 week (g)	23.9±0.6	23.0±0.8	25.4±0.5	23.9±1.2
BW 1 week (%)	106±3	105±1	107±1	107±2
Loss BW after fasting (g)	4.01±0.1	3.3±0.2	1.8±0.2	2.5±0.9
Loss BW after fasting(%)	16.7±2.4	18.5±2.0	7.1±0.7	11.3±4.2
Food intake (kJ/day/mice)	50±6	56±7	61±3	69±1
EWAT weight (g)	0.157±0.016	0.130±0.021	0.229±0.027	0.259±0.072

Significativity of two-way ANOVA was obtained for loss of body weight (BW) after wasting (in g and %) due to diet (p=0.0385 and p=0.0040), for food intake due to diet (p=0.0391) and for EWAT weight due to diet (p=0.0094).

Table 2. Mean body weights and food intake in mice submitted to control or HL diet for 12 weeks.

	CWT	CKO	HLWT	HLKO
BW day 0 (g)	25±1	21±1	21±1	20±1
BW 12 week (g)	40±1	36±1	43±3	40±2
BW 12 week (%)	166±8	169±5	203±16	196±7
Loss BW after fasting (g)	3.0±0.3	2.2±0.2	2.0±0.6	1.6±0.8
Loss BW after fasting(%)	7.4±0.6	6.3±0.5	4.6±1.5	3.8±2.3
Food intake (kJ/day/mice)	56±1	52±2	62±1	55±1
EWAT weight (g)	0.98±0.11	0.98±0.09	1.34±0.07	1.50±0.12

Significativity of two-way ANOVA was obtained for body weight (BW) at 12 weeks (in %) due to diet (p=0.0007), for loss of BW after wasting (in %) due to diet (p=0.0346), for food intake due to genotype (p=0.0198) and diet (p=0.0312) and for EWAT weight due to diet (p=0.0003).

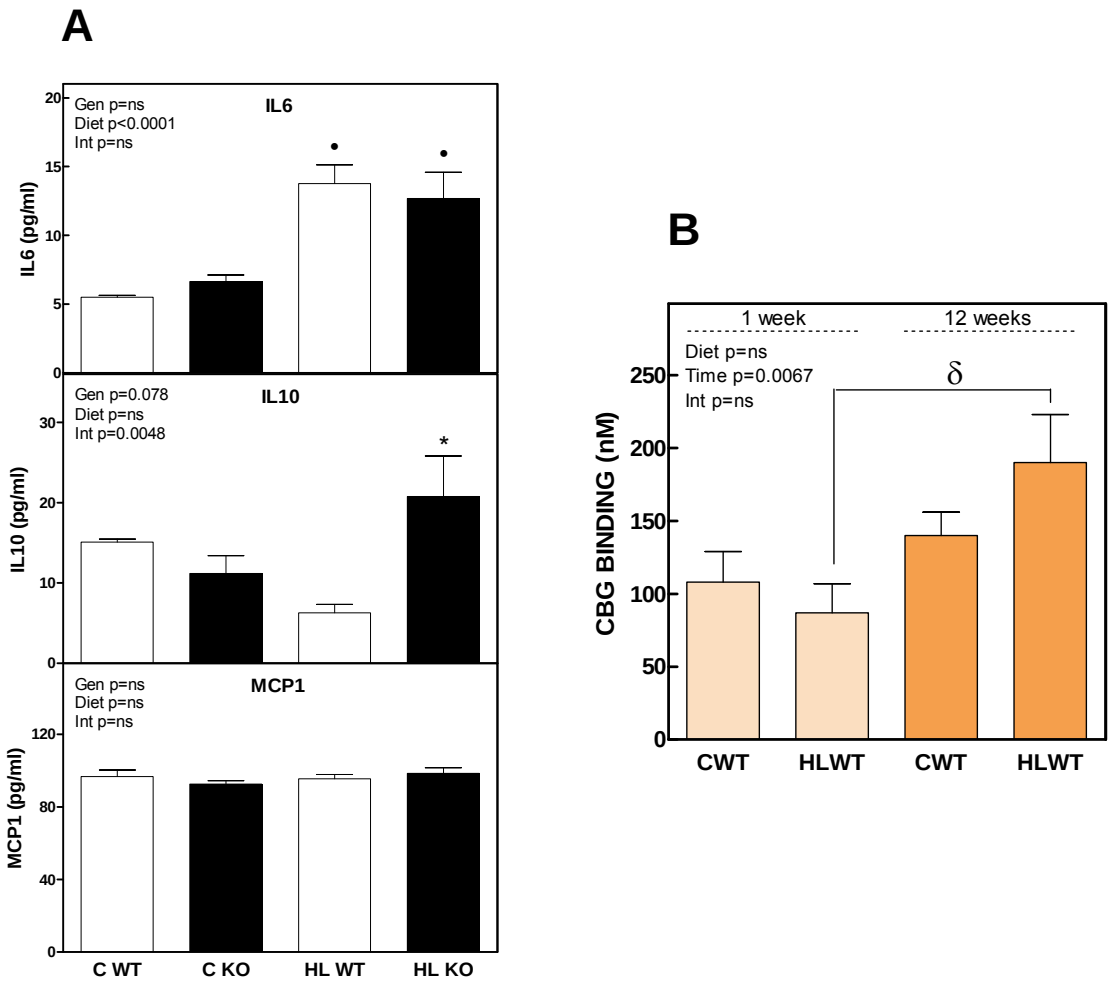

Figure 1. Serum measurement of cytokines in mice submitted for 12 weeks to control or HL diet (A) and CBG binding (B) in WT mice submitted for 1 and 12 weeks to a control or an HL diet. Significativity of two-way ANOVA is depicted in each graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*) or between C and HL of the corresponding genotype (●) in the case of cytokines graph. In B, (δ) denotes statistical significativity between mice submitted during 1 week and 12 weeks to hyperlipidic diet.

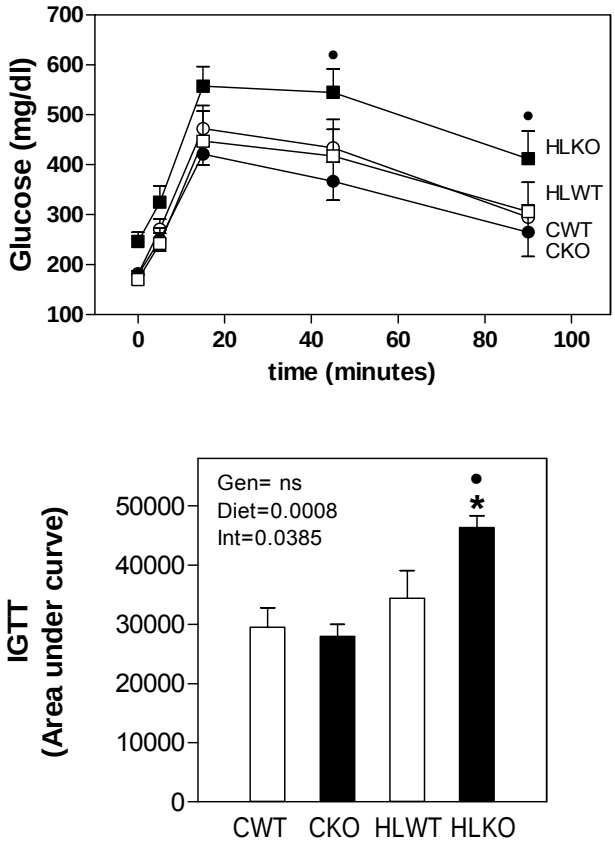

Figure 2. Intraperitoneal glucose tolerance test (IGTT) performed in mice at 12 weeks of started the diet. In the upper graph, blood glucose values obtained during the 90 minutes of IGTT. In the graph below, the area under the curve calculated for each mice group. Significativity of two-way ANOVA is depicted in IGTT graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*) or between C and HL of the corresponding genotype (●).

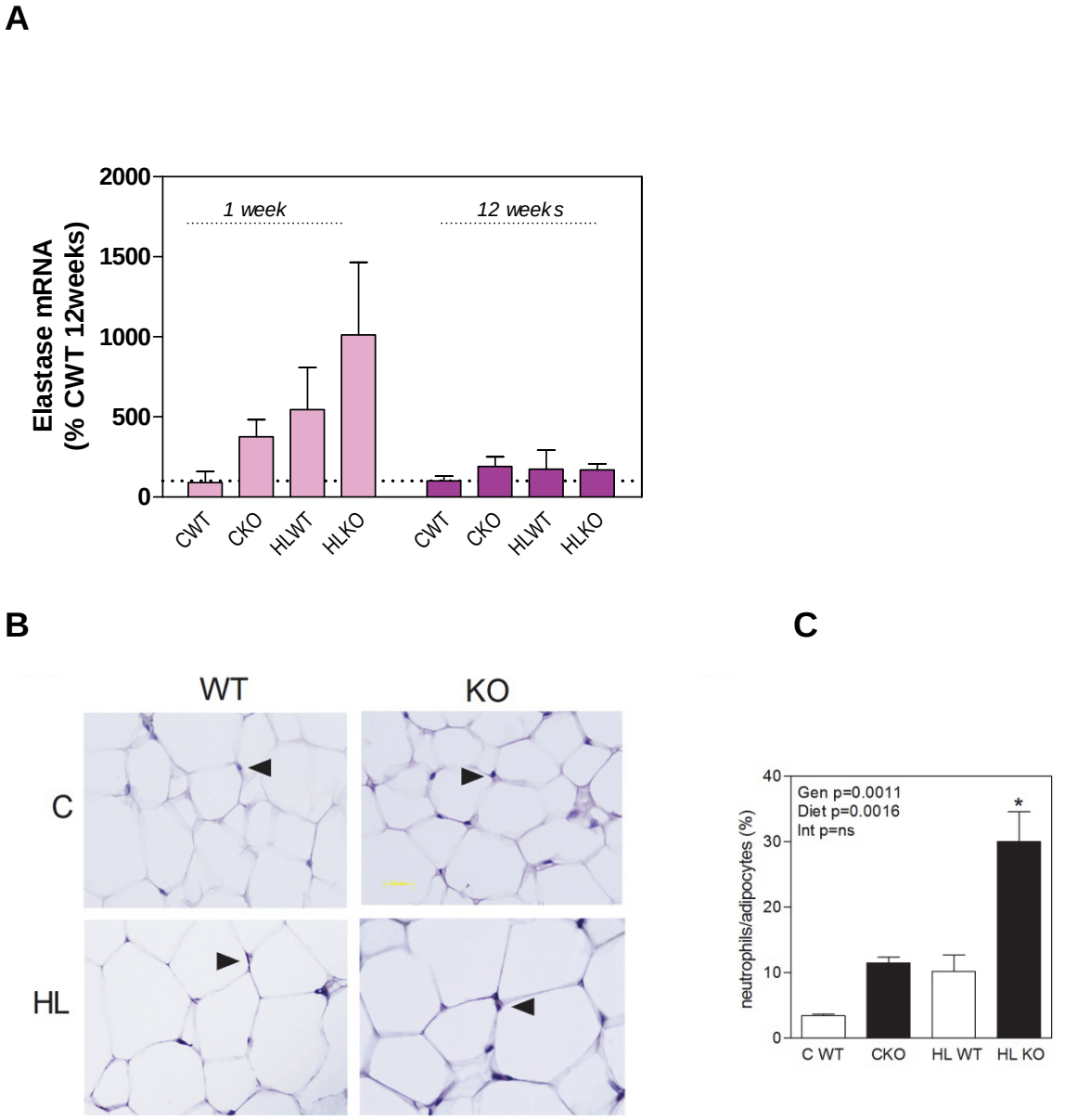

Figure 3. Neutrophil infiltration in EWAT after 1 and 12 weeks of started the consumption of the HL diet. A) Elastase mRNA in epididymal adipose tissue at 1 week or 12 weeks after starting the diet; B) immunohistochemistry of elastase in epididymal adipose tissue slides quantified as C) elastase positive cells found per 100 adipocytes. Significativity of two-way ANOVA is depicted in each graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*).

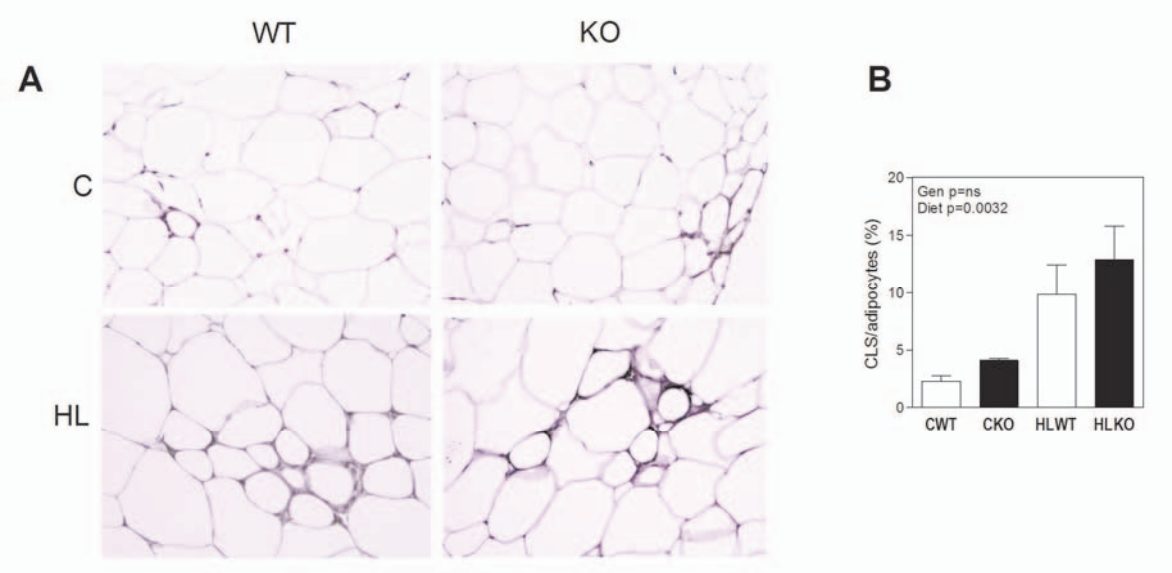

Figure 4. A) Immunohistochemistry of F4/80 in epididymal adipose tissue slides quantified as B) crown-like structures (CLS) surrounding adipocytes per each 100 adipocytes counted. Significativity of two-way ANOVA is depicted in the graph.

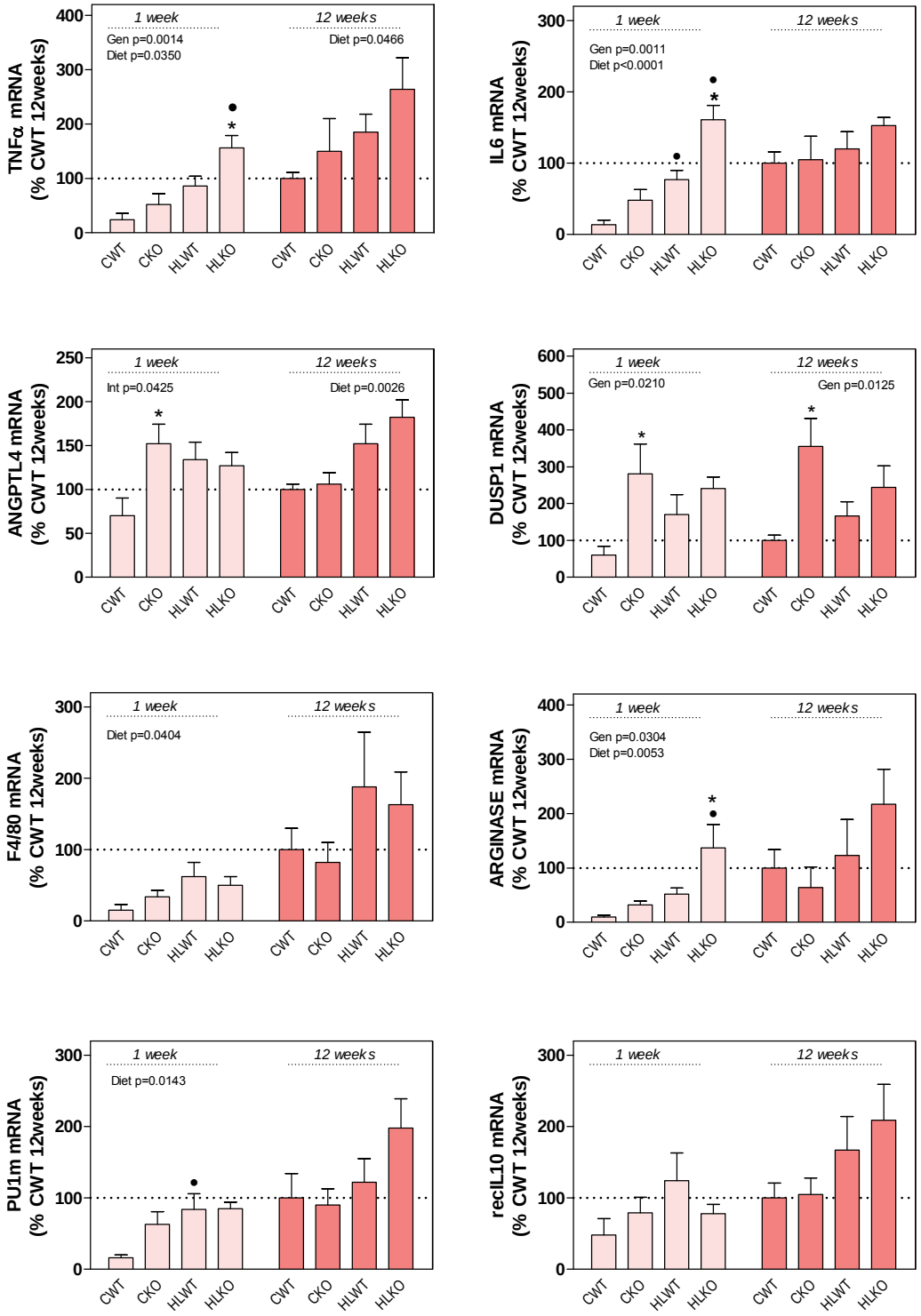

Figure 5. Expression of some inflammatory markers in whole EWAT from mice submitted to a standard (CWT and CKO) or HL diet (HLWT and HLKO) during 1 week or 12 weeks. The gene studied were, as proinflammatory markers, TNFα, PU1.m, IL6 and F4/80 and as anti-inflammatory DUSP1, recIL10, Arginase and ANGPTL4. Significativity of two-way ANOVA is depicted in each graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*) or between C and HL of the corresponding genotype (●) in each graph.

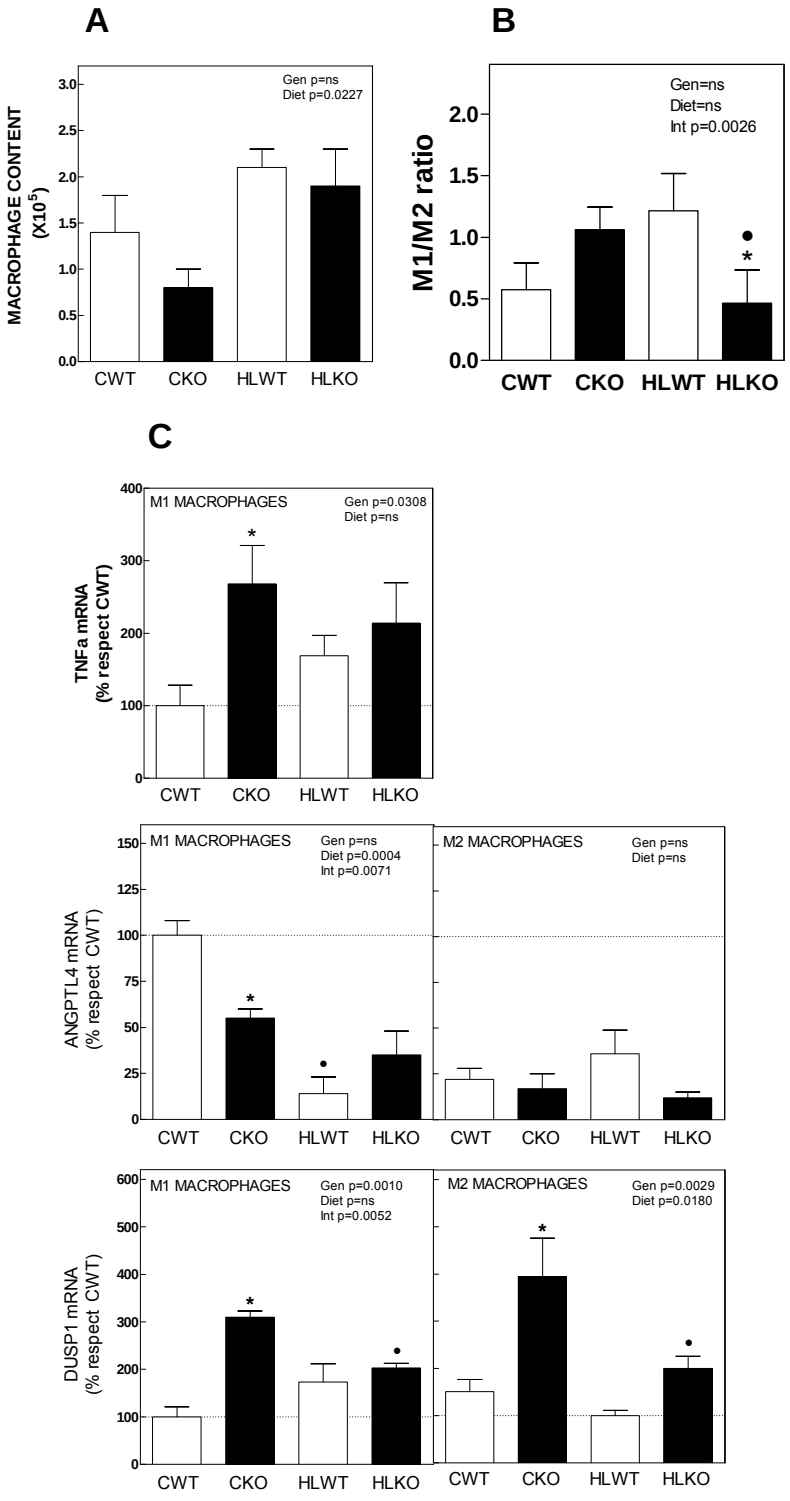

Figure 6. A) Total macrophage content, B) **p**roportion of M1 and M2 macrophages and C) TNFα, ANGPTL4 and DUSP1 mRNA expressions in M1 (left column) and M2 (right column) macrophages obtained from EWAT by FACS-sorting in WT mice or KO fed standard (CWT and CKO) or HL diet (HLWT and HLKO). Significativity of two-way ANOVA is depicted in each graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*) or between C and HL of the corresponding genotype (●) in each graph.

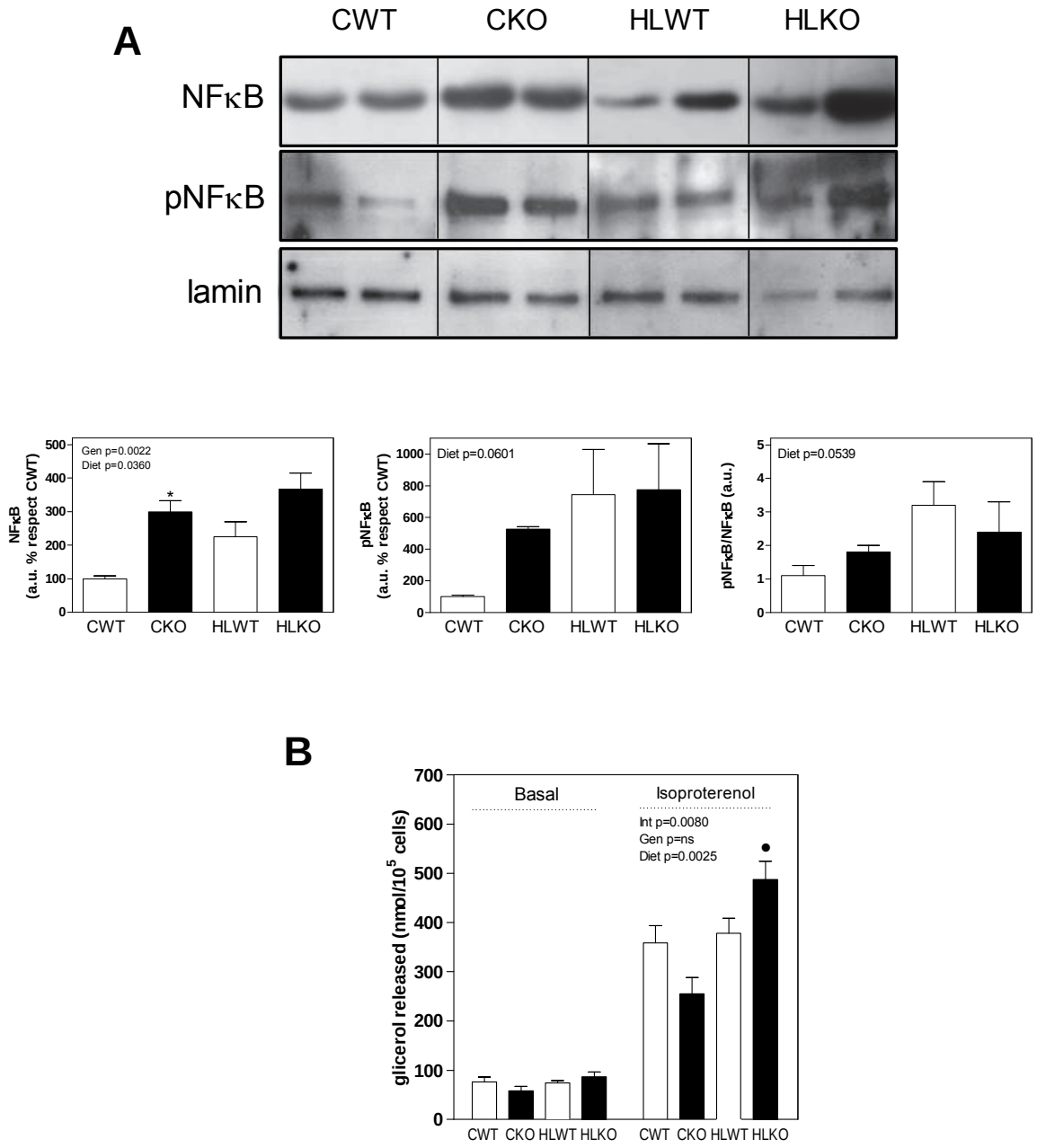

Figure 7. A) Western of total and phosphorylated NFκB and B) lipolysis activity in absence (basal) or presence of 1μM isoproterenol in adipocytes from WT and KO mice submitted during 12 weeks to a standard (CWT and CKO) or an HL diet (HLWT and HLKO). Significativity of two-way ANOVA is depicted in each graph. Symbols denote statistical significativity by Bonferroni post-test between WT and KO mice of the corresponding diet (*) or between C and HL of the corresponding genotype (●) in each graph.

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5. *New roles of Corticosteroid-Binding Globulin and opposite expression profiles in lung and liver*

Previamente se había publicado que los ratones deficientes en CBG presentaban mayor mortalidad a consecuencia de una inyección intraperitoneal de LPS que los WT. Así que nos planteamos utilizar otro modelo que cursara con inflamación aguda pero por distinta vía. El modelo experimental de pancreatitis aguda por hiperestimulación con ceruleína, un análogo de la colecistoquinina extraído de la rana arborícola australiana *Litoria caerulea*[221], provoca en el páncreas edema, necrosis y liberación del contenido vacuolar. Ello causa la aparición en plasma de enzimas pancreáticas como la lipasa y la amilasa en apenas 15 minutos y con un pico máximo de afectación a las 12 horas de iniciado el tratamiento. Y todo ello acompañado de la migración masiva de neutrófilos en los tejidos, incluido el propio páncreas. El pulmón es uno de los tejidos más afectados y dado que pulmón también resultó especialmente afectado a consecuencia de la inyección con LPS en ratones KO para CBG, decidimos averiguar si la deficiencia de CBG modificaba la gravedad y la progresión de la pancreatitis. Para ello colaboramos con el equipo del Dr. Daniel Closa (Institut d'Investigacions Biomèdiques Agustí Pi i Sunyer, CSIC), con amplia experiencia en el modelo experimental de pancreatitis aguda en roedores.

Los resultados evidenciaron que la falta de CBG no modificó la progresión ni la gravedad de la pancreatitis. Pero nos ofreció un resultado inesperado: las hembras KO para CBG perdían el dimorfismo sexual que caracteriza los niveles de corticosterona circulante, y presentaban niveles comparables a los machos KO y no mayores, como sí ocurría en las hembras WT. Y por otra parte, los niveles de corticosterona plasmática incrementaron a consecuencia de la inducción de pancreatitis únicamente en ratones KO.

En pulmón se detectó expresión de CBG, tanto mRNA como proteína y se puso de manifiesto que el patrón de expresión en hembras y machos era inverso al de plasma e hígado. Mientras que en hembras la CBG en hígado y plasma es más abundante que en machos, en paralelo con la corticosterona total, en pulmón resultó mayor la expresión en machos que en hembras WT. También se observó una alteración en la biodisponibilidad de los glucocorticoides a través de la reducción de la expresión de 11β-HSD2, enzima implicada en la inactivación de la corticosterona.

En conclusión, los datos presentados indicaron que, además de trasportar glucocorticoides, la CBG está involucrada en las diferencias de género observadas en la corticosterona total

circulante y desempeña un papel en la regulación local de la disponibilidad de glucocorticoides en pulmón ante daño inflamatorio pero su déficit no agrava la progresión de la pancreatitis aguda.

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RESEARCH ARTICLE

New Roles for Corticosteroid Binding Globulin and Opposite Expression Profiles in Lung and Liver

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Abstract

Corticosteroid-binding globulin (CBG) is the specific plasma transport glycoprotein for glucocorticoids. Circulating CBG is mainly synthesized in liver but, its synthesis has been located also in other organs as placenta, kidney and adipose tissue with unknown role. Using an experimental model of acute pancreatitis in *cbg*$^{-/-}$ mice we investigated whether changes in CBG affect the progression of the disease as well as the metabolism of glucocorticoids in the lung. Lack of CBG does not modify the progression of inflammation associated to pancreatitis but resulted in the loss of gender differences in corticosterone serum levels. In the lung, CBG expression and protein level were detected, and it is noteworthy that these showed a sexual dimorphism opposite to the liver, i.e. with higher levels in males. Reduced expression of 11β-HSD2, the enzyme involved in the deactivation of corticosterone, was also observed. Our results indicate that, in addition to glucocorticoids transporter, CBG is involved in the gender differences observed in corticosteroids circulating levels and plays a role in the local regulation of corticosteroids availability in organs like lung.

Introduction

Acute pancreatitis is a serious inflammatory process with significant morbidity and mortality. The most relevant complication during acute pancreatitis is the systemic inflammation that, in the severe forms of the disease, may lead to an acute respiratory distress syndrome [1]. This pulmonary dysfunction is characterized by an influx of inflammatory leukocytes and increases in pulmonary vascular permeability, being one of the most important factors contributing to death during the first week of the disease [2]. The mechanisms responsible for the involvement of distant organs are still unclear and different pathways have been suggested, including oxygen-derived free radicals and cytokines [3].

As occurs with other critical illnesses, such as sepsis, trauma and septic shock, the hypothalamic–pituitary–adrenal axis plays an important modulatory role in the control of the inflammatory process. Several reports suggested a link between an impaired adrenal secretion and the progression of systemic inflammation in acute pancreatitis [4]. In addition, corticosteroid insufficiency has been reported in patients with acute pancreatitis [5]. Nevertheless, the use of corticosteroid in the treatment of acute pancreatitis is still being debated and experimental studies suggest that although the prophylactic use of corticosteroids showed efficacy on some features of the disease, this effect was not observed with the therapeutic use [6].

In addition to glucocorticoids, changes in the levels of corticosteroid-binding globulin (CBG) have also been reported in patients with acute pancreatitis [7][8]. CBG is the specific high-affinity plasma transport glycoprotein for glucocorticoids. It is mainly synthesized in liver, although it could be also produced by the placenta, kidney and adipose tissue [9][10][11]. The main function of CBG seems to be glucocorticoids transport since under normal conditions 80–90% of circulating cortisol is bound with high affinity to CBG, while only 10–15% binds with low affinity to albumin and the remaining 5–10% is known as "free cortisol" [12].

It is accepted that CBG actively deliver glucocorticoid to inflamed tissue due to the action of elastase released by activated neutrophils. This protease cleaves CBG and disrupts the glucocorticoid-binding site, thus resulting in the release of the corresponding glucocorticoid in the areas of inflammation [13]. However, CBG has been located also in some intracellular compartments suggesting additional, and yet unknown, functions [14].

Herein, our study was designed to investigate, in an experimental model of acute pancreatitis in mice, whether changes in CBG could affect the progression of the disease as well as the metabolism of glucocorticoids in the lung. To do this, we compared the effects of pancreatitis in $cbg^{+/+}$ and in $cbg^{-/-}$ animals. Moreover, considering the remarkable differences in the metabolism of glucocorticoids between males and females, we did the experiments comparing the effects between the two genders.

Materials and Methods

Male and female C57BL/6 mice (25–30 g b.w.) $cbg^{+/+}$ and $cbg^{-/-}$ were used in all experiments. The colony of $cbg^{-/-}$ was established through crossing heterozygous breeding kindly provided by Dr. Willnow. The procedure followed for disruption of the CBG gene was described by Petersen et al. [15]. Animals were housed in a controlled environment, fed with standard laboratory pelleted formula (Teklad Global 2018, Harlan-Interfauna Ibérica, Sant Feliu de Codines, Spain) and tap water ad libitum. This study conformed to European Community for the use of experimental animals and the institutional committee of animal care and research (C.E.E.A. Universitat de Barcelona) approved it.

Animal model of acute pancreatitis

Mice received 10 hourly intraperitoneal injections of 50 μg/kg cerulein (Sigma, USA) or PBS as control and were sacrificed one hour after the last injection [16]. Samples of pancreas, liver and lung were obtained, immediately frozen and maintained at -80°C until processed. Lung samples were also obtained for histological study. Samples of blood were centrifuged to obtain serum. Taking into account that some parameters evaluated show important circadiary changes, the experiments were performed at the same hour, being the procedure started at 8 a.m. and animals sacrificed at 7 p.m.

Lipase

Plasma lipase was determined by using commercial turbidimetric assay kits from Randox (Antrim, U.K.), according to the supplier's specifications.

Myeloperoxidase

Neutrophilic infiltration was assessed by measuring myeloperoxidase (MPO) activity. MPO was measured photometrically with 3,3',5,5'-tetramethylbenzidine as a substrate. Tissue samples were homogenized with 0.5% hexadecyltrimethylammonium bromide in 50 mM phosphate buffer at pH 6.0. Homogenates were disrupted for 30 seconds using a Labsonic sonicator (Braun Biotech, Inc., Allentown, PA, USA) at 20% power and submitted to three cycles of snap freezing in dry ice and thawing before a final 30 second sonication. Samples were incubated at 60°C for 2 hours and then spun down at 4000 xg for 12 minutes. The supernatants were collected for MPO assay. Enzyme activity was assessed photometrically at 630 nm.

RNA isolation and RT-PCR

Total RNA from tissue samples were extracted using the TRI Reagent® Solution (Ambion, Inc, USA). The RNA was quantified by measurement of the absorbance at 260 and 280 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

cDNA was synthesized using the an MMLV reverse transcriptase (Promega, USA) and oligo-dT primers, reverse transcription was then performed from 2 μg RNA sample. The reaction was incubated at 72°C for 5 min and 42°C 60 min, and then stored at -80°C.

Subsequent PCR amplification was performed in ABI PRISM 7900 HT detection system (Applied Biosystems), and was carried out using 10 μl of amplification mixtures containing SYBR Green PCR Master Mix (Life Technologies), 8 ng of reverse-transcribed RNA and 300 nM of the correspondent mice primers: CBG forward: 5'-CCACCAAAGACACTCCCTTG-3' reverse: 5'-GCACATTCCCTTCATCCAGT-3'; 11β-HSD1 forward: 5'-CAAGGTCAA CGTGTCCATCA-3' reverse: 5'-TCCCAGAGATTTCCTTCATAGC-3'; 11β-HSD2 forward: 5'-CTCCAAGGCAGCAATAGCAC-3' reverse: 5'-CGTTTCTCCCAGAGGTTCAC-3'; and RPL32 forward: 5'-ACCAGTCAGACCGATATGTGAAAA-3' reverse: 5'-TGTTGTC AATGCCTCTGGGTTT-3'. Reactions were performed in duplicate and threshold cycle values were normalized to RPL32 gene expression. The specificity of the products was determined by melting curve analysis. The ratio of the relative expression of target genes to RPL32 was calculated by using the ΔC(t) formula.

Histological study

For histological studies, tissue samples were fixed in 10% neutral buffered formalin, paraplast-embedded, cut into 5 μm thick sections and stained with hematoxylin–eosin according to standard procedures. Sections were evaluated by light microscopy and the severity score was calculated based in a semiquantitative evaluation scale [17].

Immunohistochemistry

Briefly, the sections were deparaffinized, rehydrated and washed in PBS-Tween, and then they were treated with 0.3% hydrogen peroxide, blocked with 25% Rabbit serum and incubated overnight at 4°C with goat-antimouse CBG (LS-C39044, LifeSpan BioSciences, USA) diluted 1:100. After that, sections were sequentially incubated with biotinylated rabbit anti-goat (1:400, Vector Labs, USA) and avidin–biotin complex reaction (1:200, ABC Elite Kit, Vector

Laboratories) and developed with a diaminobenzidine hydrochloride chromogen (Sigma, USA). The slides were scanned and visualized using the Pannoramic Viewer 1.15.2 software.

Western blot of CBG

Serum samples (100μg per well) were separated by SDS/PAGE in a 10% gel and electrotransferred on to a PVDF membrane (Millipore, Billerica, MA, U.S.A.). After blocking nonspecific binding sites with 5% nonfat milk and in TBSTween 0.05% for 90 minutes at room temperature, the membranes were incubated overnight with a CBG goat anti-mouse polyclonal antibody (1:1,000; LifeSpan) and albumin as control (1:50.000; sc-46293 Santa Cruz Biothecnolgy, USA)

Protein fraction from samples of lung was isolated using the TRI Reagent® Solution (Ambion, Inc, USA). Protein concentration was quantified by QuantiPro™ BCA Assay Kit (Sigma, St. Louis, MO, USA) and 30 μg of total protein per well were separated by SDS/PAGE proceeding similarly to serum samples for CBG detection. For lung the control was made using a primary antibody against tubulin (dil 1:1000, DM1A, Abcam, U.K.).

Immunoreactive proteins were further detected by an anti-goat horseradish peroxidase-conjugated secondary antibody (1:20,000; sc-2922, Santa Cruz Biotechnology, USA) and using the Luminata™ Forte Western HRP Substrate (Millipore, Billerica, USA).

Total Corticosterone

Corticosterone in serum was measured by radioimmunoanalysis using a sheep anti-corticosterone antibody (AB1297, Millipore, USA) and we proceeded as described previously [18].

CBG binding capacity

To remove corticosterone from serum samples, 10 μl of serum samples were added to 0.5 ml of a charcoal-dextran suspension [0.05% dextran and 0.5% charcoal (Norit A, Sigma) in PBS with 0.1% of gelatin (PBSG) at pH 7.4] and incubated at room temperature for 30 minutes with occasional shaking. Samples were then centrifuged and the clear supernatants were further diluted 1/5 with PBSG buffer. These hormone-stripped samples were used for corticosterone binding assays. The final concentration of labelled corticosterone in the tubes was 15 nM in a total volume of 200μl. Then, 200 μl of a constant stirring charcoal-dextran suspension were added to each tube. These were shaken, left to stand for 10 min in an ice bath, and centrifuged for 10 min. Aliquots of 200 μl of the clear supernatants were added to 4 ml of a water-miscible scintillation cocktail (Ecolite, MP Biomedicals, USA) and counted in a standard scintillation counter. In all samples, individual unspecific binding (measured in a 500-fold excess of non-radioactive corticosterone) was measured and subtracted from the total radioactivity bound to diluted stripped serum.

Statistical analysis

The data were analyzed using the GraphPad software program version 5.0 and were expressed as the mean ± SEM. Statistical comparisons were made by two-way ANOVA analysis, evaluating the variables "treatment" (control, cerulein) and "sex" (male, female). Bonferroni was used as a post-test. The P values < 0.05 were considered significant.

Results

Induction of acute pancreatitis

Administration of cerulein induced an acute pancreatitis evidenced by the increased levels of circulating lipase. In the $cbg^{+/+}$ mice, this increase was higher in females than in males. By contrast, there no gender differences were observed in the $cbg^{-/-}$ mice. In males the increase of lipase circulating levels promoted by pancreatitis was higher in $cbg^{-/-}$ than $cbg^{+/+}$ mice, but in female the inverse effect was observed. (Fig 1A).

Inflammation in the pancreas, measured as MPO activity, showed an increase between four to five times in all cerulein treated mice compared to controls without differences among males and females. (Fig 1B). Similar profile was observed in the lung (Fig 1C) but in this case the increase observed in pancreatitis induced mice was between two and three times higher than controls. Histological study (Fig 2) of pancreas showed interstitial edema and infiltration of polymorphonuclear leukocytes (PMN) in all cerulein treated groups. Acinar necrosis was only promoted by cerulein in females. In lung cerulein induced alveolar thickening and polymorphonuclear leukocytes infiltration in all treated mice.

Corticosterone and CBG in serum and liver

Pancreatitis did not induce significant changes in $cbg^{+/+}$ animals, although females showed higher levels of circulating corticosterone than males. Interestingly, the $cbg^{-/-}$ mice showed lower levels of corticosterone in control animals and cerulein treatment induced recovery of the levels to the $cbg^{+/+}$ in both males and females (Fig 3A).

Similar results were found when measuring the CBG binding capacity in serum of $cbg^{+/+}$ animals; females showed higher levels than males and, in both genders, pancreatitis did not modify these levels. As expected, all $cbg^{-/-}$ mice showed negligible binding activity (Fig 3B).

Circulating CBG levels in $cbg^{+/+}$ animals revealed that females had higher levels than males. Pancreatitis did not induce changes in males but resulted in an increase in CBG in females. No detectable levels of CBG were found in $cbg^{-/-}$ animals (Fig 4A).

Finally, liver is the main source of circulating CBG and, as occurred with the serum levels, significant differences were found when comparing males and females in $cbg^{+/+}$ animals (Fig 4B). The higher levels of expression were observed in females and pancreatitis had no effect on this expression. A residual expression of CBG was detected in $cbg^{-/-}$ mice.

CBG and corticosterone metabolism in the lung

In the lung tissue, CBG expression in males $cbg^{+/+}$ was increased after induction of pancreatitis. By contrast, CBG expression in females $cbg^{+/+}$ after induction of acute pancreatitis remained unmodified (Fig 5A) resulting in lower levels than males $cbg^{+/+}$ with pancreatitis. As occurred in liver, residual expression of CBG was observed in the lung of $cbg^{-/-}$ animals. Similar pattern was found when analyzing by western blot the levels of CBG in tissue (Fig 5B). Finally, immunohistochemical analysis confirmed the induction of CBG in lung from male mice and the lack of induction in female (Fig 6). CBG expression was observed in the alveolar epithelial cells and macrophages. Although there was a residual RNA expression of CBG in $cbg^{-/-}$ mice, no staining was observed in the corresponding histological samples.

In order to evaluate the capability of corticosterone synthesis or degradation in lung, we determined the expression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2). No differences were observed in 11β-HSD1 expression in any condition, nor due to genotype, cerulein treatment or gender (Fig 7A).

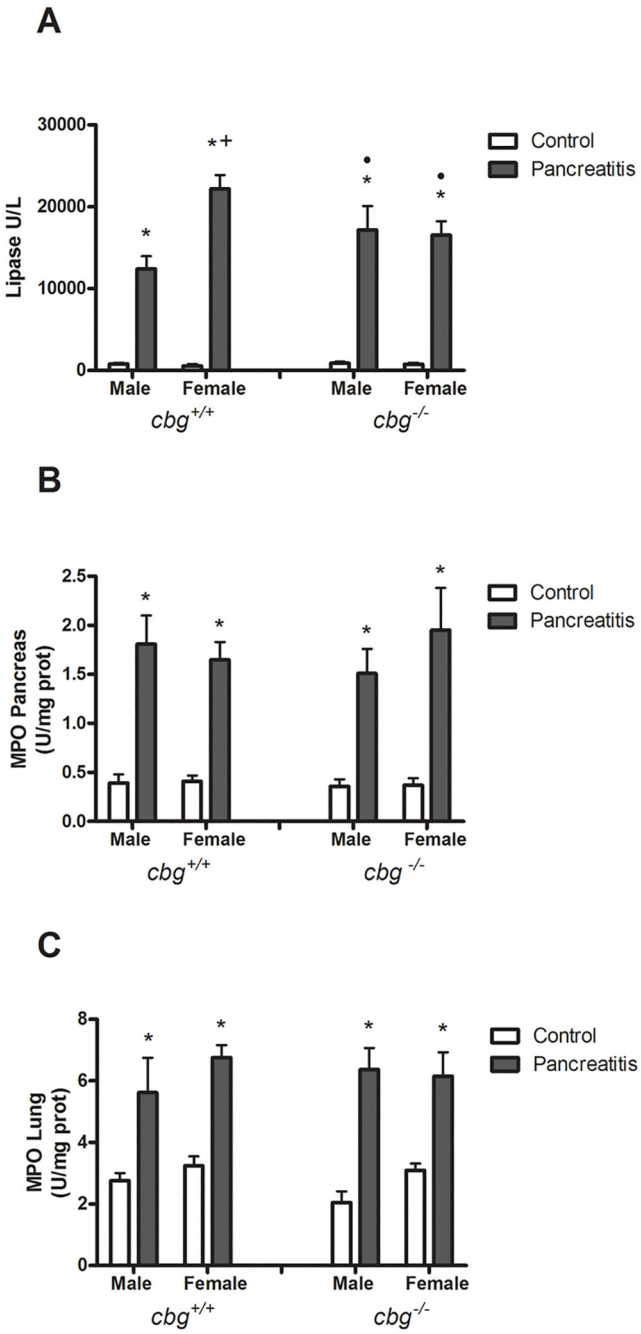

Fig 1. Induction of pancreatitis. (A) Levels of circulating lipase. Pancreatitis resulted in an increase in plasma lipase in all experimental groups. This increase was significantly higher in female $cbg^{+/+}$. (B) Mieloperoxidase activity in pancreas and (C) lung. Pancreatitis resulted in an increase in myeloperoxidase in all experimental groups. This increase resulted more marked in pancreas than in lung. Two Way Anova: circulating Lipase: Treatment P<0.0001, Sex P = 0.0063 Interaction P = 0.0040; Mieloperoxidase activity in pancreas: Treatment P<0.0001, Sex P = 0.7436, Interaction P = 0.7499; Mieloperoxidase activity in lung: Treatment P<0.0001, Sex P = 0.5030, Interaction P = 0.6427; Bonferroni Post-test: * P<0.05 control vs pancreatitis; + P<0.05 male vs female; • P<0.05 $cbg^{+/+}$ vs $cbg^{-/-}$.

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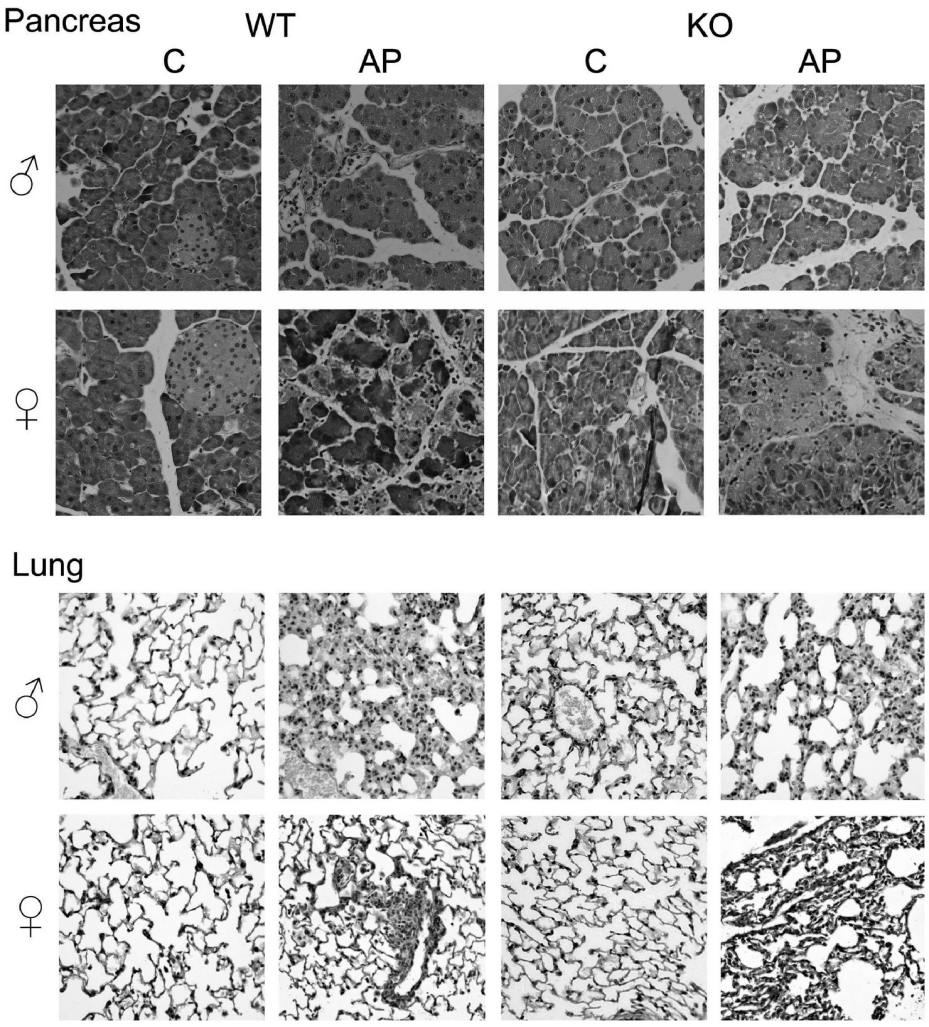

	Males				Females			
	$cbg^{+/+}$		$cbg^{-/-}$		$cbg^{+/+}$		$cbg^{-/-}$	
PANCREAS	Control	Pancreatitis	Control	Pancreatitis	Control	Pancreatitis	Control	Pancreatitis
Interstitial edema	+	++	+	++	+	++	+	++
Acinar necrosis	-	-	-	-	-	+	-	++
PMN infiltration	-	++	-	++	-	++	-	+++
LUNG								
Alveolar thickening	-	++	-	++	-	+	-	+
PMN infiltration	-	++	-	++	-	++	-	++

Fig 2. Injury and severity scores for pancreas and lung. Representatives H&E stained sections and severity score for each experimental group. Severity score was obtained using semiquantitative evaluation scale and the results were the mean of the lesions observed in each group. PMN = polymorphonuclear leukocytes;— = no lesion; + = mild lesion; ++ = moderate lesion; +++ = intense lesion.

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With respect to 11β-HSD2 expression (Fig 7B), gender differences were not found, nor in $cbg^{+/+}$ nor in $cbg^{-/-}$ mice. Pancreatitis, in males and females $cbg^{+/+}$ mice, significantly reduced the expression of 11β-HSD2. In the $cbg^{-/-}$ mice, control expression was lower than in $cbg^{+/+}$ and pancreatitis also resulted in a decreased expression although in this case only in females achieved significantly values.

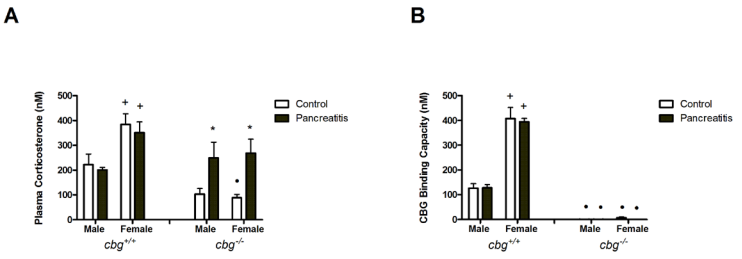

Fig 3. (A) Total corticosterone circulating in serum and (B) CBG binding capacity in serum. Female mice showed higher levels of corticosterone and CBG binding capacity than males in *cbg*$^{+/+}$ animals. These differences disappeared in *cbg*$^{-/-}$ animals. *cbg*$^{-/-}$ females showed significantly lower levels of corticosterone. Pancreatitis did not modify the levels of corticosterone in *cbg*$^{+/+}$ animals, while in *cbg*$^{-/-}$ mice induced a recovery to levels of *cbg*$^{+/+}$ animals. Two Way Anova: Total corticosterone: Treatment P = 0.0210, Sex P<0.0001, Interaction P = 0.0171; CBG binding capacity: Treatment P = 0.7538, Sex P<0.0001, Interaction P = 0.9624; Bonferroni Post-test: * P<0.05 control vs pancreatitis; + P<0.05 male vs female; • P<0.05 *cbg*$^{+/+}$ vs *cbg*$^{-/-}$.

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Since 11β-HSD1 catalyzes the conversion of 11-dehydrocorticosterone to the active corticosterone, whereas 11β-HSD2 catalyzes the opposite reaction, the ratio 11β-HSD1/11β-HSD2 indicates the changes in the local glucocorticoid metabolism (Fig 7C). Induction of pancreatitis increases the ratio 11β-HSD1/11β-HSD2 in both genders, indicating a higher level of local corticosterone activation. Similar effect was observed in the *cbg*$^{-/-}$ mice.

Discussion

The progression of the inflammatory response results from the balance of several pro- and anti-inflammatory mediators. Cytokines, free radicals, lipid mediators and activated enzymes promote the activation of inflammatory pathways, not only locally, but also in circulating cells and in distant organs [19]. These effects are counteracted by the release of anti-inflammatory cytokines as IL-10 [20] or proteins as pancreatitis associated protein [21]. However, glucocorticoids are known to play a major role in the control of the inflammatory response and corticosteroid insufficiency has been reported in patients with inflammatory diseases as acute pancreatitis [5][22].

It is known that the glucocorticoids ability to regulate the inflammatory response is strongly related with their availability on the site of inflammation. Tissue availability of glucocorticoids, namely the proportion of glucocorticoid able to bind to the receptor and carry out a response, depends of its synthesis at adrenal gland [23], its binding to the CBG [24], as well as its activation or inhibition catalyzed locally by the intracellular activity of enzymes 11β-HSD1 and 11β-HSD2 [25]. Elastase from activated neutrophils can cleave CBG and promote the release of bound glucocorticoids at the inflammation precise site [26]. An active role of CBG in glucocorticoid response is supported by observations in CBG deficient mice, that show a higher mortality in response to septic shock with lung particularly affected [15]. In acute pancreatitis the respiratory distress syndrome is the most relevant problem associated [1]. Therefore, we aimed to study the changes in blood and specifically lung glucocorticoid availability to cope with inflammation and the lung injury associated in CBG deficient mice with acute pancreatitis. Also we included males and females since the known sexual dimorphism of CBG could be an important modulator that would lead to different responses in each gender.

In general the induction of pancreatitis in mice resulted in higher levels of circulating lipase that were more pronounced in female *cbg*$^{+/+}$ animals. This difference was not observed in *cbg*$^{-/-}$ mice and the levels achieved were similar to that observed in male *cbg*$^{+/+}$ mice (Fig 1A). In pancreas and lung the induction of pancreatitis also increased the inflammatory response without

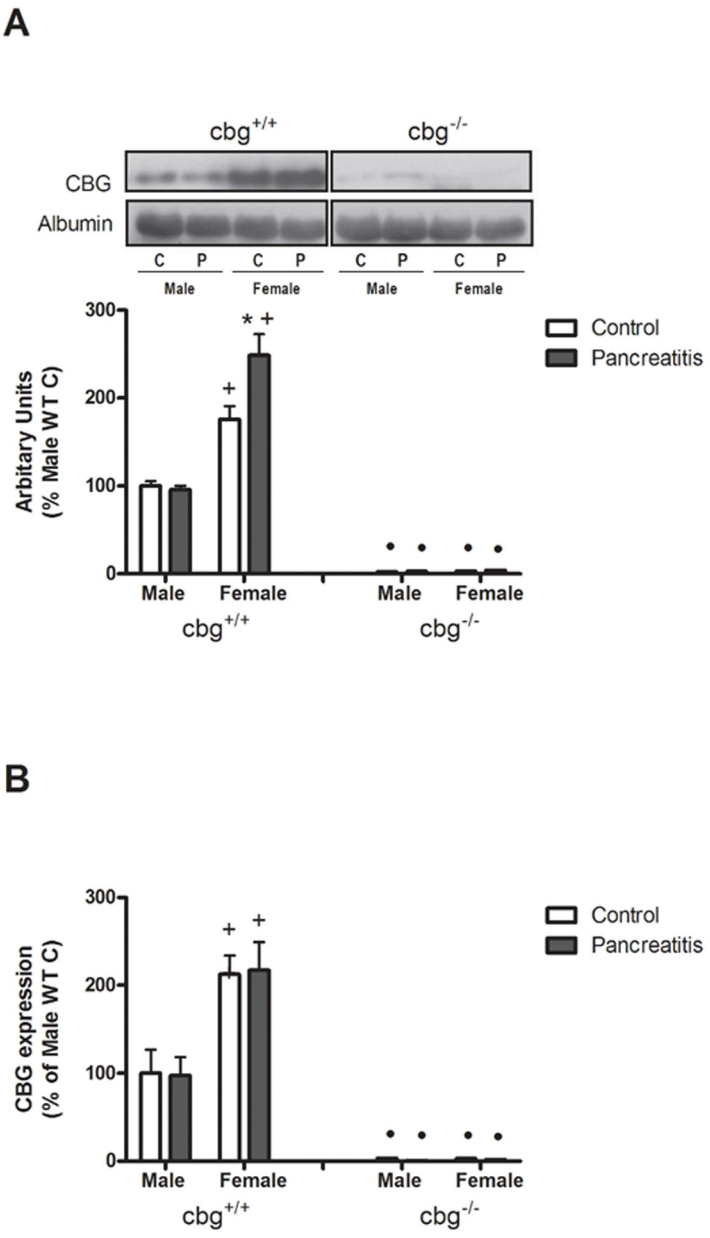

Fig 4. (A) Serum CBG protein levels measured by western blot and (B) liver CBG expression measured by RT-PCR. In both cases, females had higher levels than males and pancreatitis promoted an increase of CBG protein levels only in females. No CBG was detected in serum of *cbg*$^{-/-}$ mice and only a residual expression was detected in liver. C refers to control mice and P refers to pancreatitis mice. Two Way Anova: Serum CBG levels: Treatment P = 0.0155, Sex P<0.0001, Interaction P = 0.0007; liver CBG expression: Treatment P = 0.9681, Sex P<0.0001, Interaction P = 0.9972; Bonferroni Post-test: * P<0.05 control vs pancreatitis; + P<0.05 male vs female; • P<0.05 *cbg*$^{+/+}$ vs *cbg*$^{-/-}$.

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differences between genders or *cbg*$^{+/+}$ mice versus the *cbg*$^{-/-}$ (Fig 1B and 1C). The pancreatitis severity score determined by histological study (Fig 2) showed pancreatic acinar necrosis in female but not in male while a minor lung alveolar thickening was observed in female. Differences triggered by the genotype were only observed in the females with a slightly more damaged pancreas in *cbg*$^{-/-}$ compared to *cgb*$^{+/+}$ mice. The results clearly state that pancreatitis was

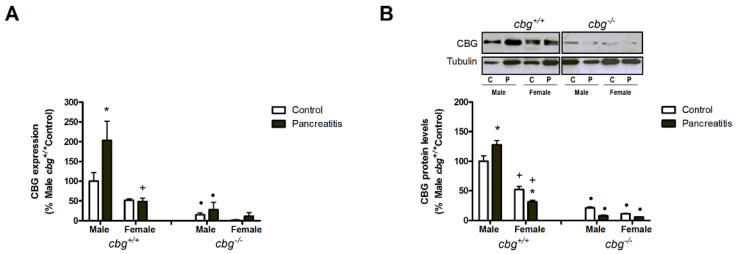

Fig 5. (A) Lung CBG expression measured by RT-PCR and (B) lung CBG protein levels measured by western blot. Females had significantly lower levels of CBG protein than males without differences in the expression levels. Only in mice males the both parameters were increased by induction of pancreatitis. A residual expression and levels of CGB were detected in *cbg*$^{-/-}$ mice. C refers to control mice and P refers to pancreatitis mice. Two way Anova: Lung CBG expression: Treatment P = 0.0182, Sex P<0.0001, Interaction P = 0.0188; lung CBG levels: Treatment P = 0.4544, Sex P<0.0001, Interaction P = 0.0009; Bonferroni Post test: * P<0.05 control vs pancreatitis; + P<0.05 male vs female; • P<0.05 *cbg*$^{+/+}$ vs *cbg*$^{-/-}$.

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induced but failed to show significant differences in the severity of the disease as a consequence of circulating CBG levels. Whereas serum lipase suggested more severity in females *cbg*$^{+/+}$ other parameters showed no differences between *cbg*$^{+/+}$ vs *cbg*$^{-/-}$ as pancreas and lung MPO activity or severity scores in the case of males. Finally, the histological analysis in females suggested more severity in cbg$^{-/-}$ mice. To clearly determine if there are differences between *cbg*$^{+/+}$ and *cbg*$^{-/-}$ mice in the severity of the pancreatitis induced by cerulein further studies are needed.

On the other hand, when analyzing the total serum corticosterone levels in the *cbg*$^{+/+}$ mice (Fig 3A) we observed that there was a clear difference between male and female, showing females the expected higher levels than males [27], however no changes due to pancreatitis. By contrast, *cbg*$^{-/-}$ control mice showed low levels of corticosterone without differences between male and female. Interestingly, pancreatitis significantly increased corticosterone in *cbg*$^{-/-}$ animals in a similar magnitude in both genders, until the levels of cbg$^{+/+}$ mice (Fig 3A). We were very careful inducing pancreatitis and obtaining samples at the same time of day in all animals to avoid errors due to circadian changes in circulating corticosterone, a factor that may cause confusion as it has been demonstrated by Richard et al. [28].

The low levels observed in circulating corticosterone in *cbg*$^{-/-}$ mice agree with those reported by Petersen et al [15]. It has been suggested that this carrier protein have specific roles in the regulation of circulating turnover, local delivery, and cellular signal transduction of steroid hormones [29]. The total corticosterone levels are regulated through hypothalamus-pituitary-adrenal (HPA) axis by a negative feed-back mechanism. It is accepted that the biologically active corticosterone is the free fraction circulating in blood while the corticosterone bound to CBG is unable to enter the cell and, therefore, is considered inactive. In our study, *cbg*$^{-/-}$ mice display increased free levels, i.e. active glucocorticoid. Despite that, it has been described that *cbg*$^{-/-}$ mice exhibit higher levels of ACTH, a sign of HPA activity [15]. Further, we have observed that the *cbg*$^{-/-}$ mice show an adrenal gland weight significantly larger than *cbg*$^{+/+}$ mice (unpublished results) indicating a major activity of the gland. These features suggest that the HPA axis is irresponsive to the negative feed-back exerted by free corticosterone in *cbg*$^{-/-}$ pointing to a role of CBG in the HPA axis control and regulating corticosterone synthesis.

In addition to the higher levels of total serum corticosterone in female (Fig 3A) there were also clear differences in circulating CBG (Fig 4A), and CBG binding capacity (Fig 3B) when comparing males and females, as expected, having the females higher values in all these parameters [27]. It is noteworthy that in *cbg*$^{-/-}$ mice, the gender difference in corticosterone levels was not observed in controls neither in pancreatitis induced animals. The role of CBG in the gender differences on circulating glucocorticoids have been observed recently in a model of emotional

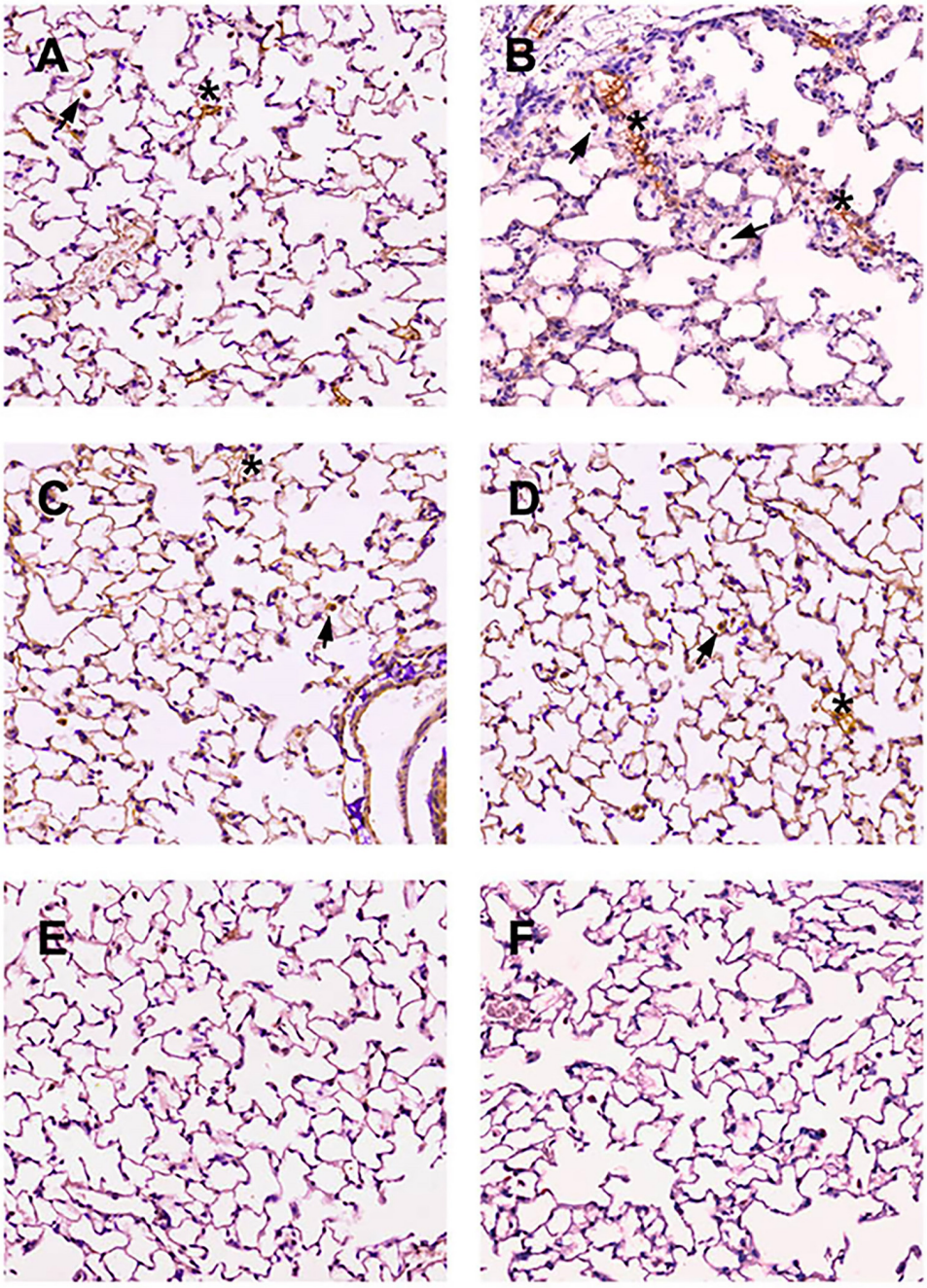

Fig 6. Immunohistochemical analysis of CBG in lung. A) male *cbg*$^{+/+}$ control, B) male *cbg*$^{+/+}$ pancreatitis, C) female *cbg*$^{+/+}$ control, D) female *cbg*$^{+/+}$ pancreatitis, E) mice *cbg*$^{-/-}$ control and F) negative control without primary antibody. In all *cbg*$^{+/+}$ groups there were stained alveolar epithelial cells indicated with an asterisk and some macrophages indicated with an arrow. There was an increase of positive cells in males after induction of pancreatitis. No differences were observed between controls and pancreatitis in females.

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stress [30] where was found that the sex differences normally described for stress reactivity disappear when estrogens are removed in ovariectomiced mice. Furthermore, the *cbg*$^{-/-}$ mice showed no differences between males and females in stress reactivity and neither in the absence of estrogens. Nevertheless, there are some differences in the response of *cbg*$^{-/-}$ animals to stress

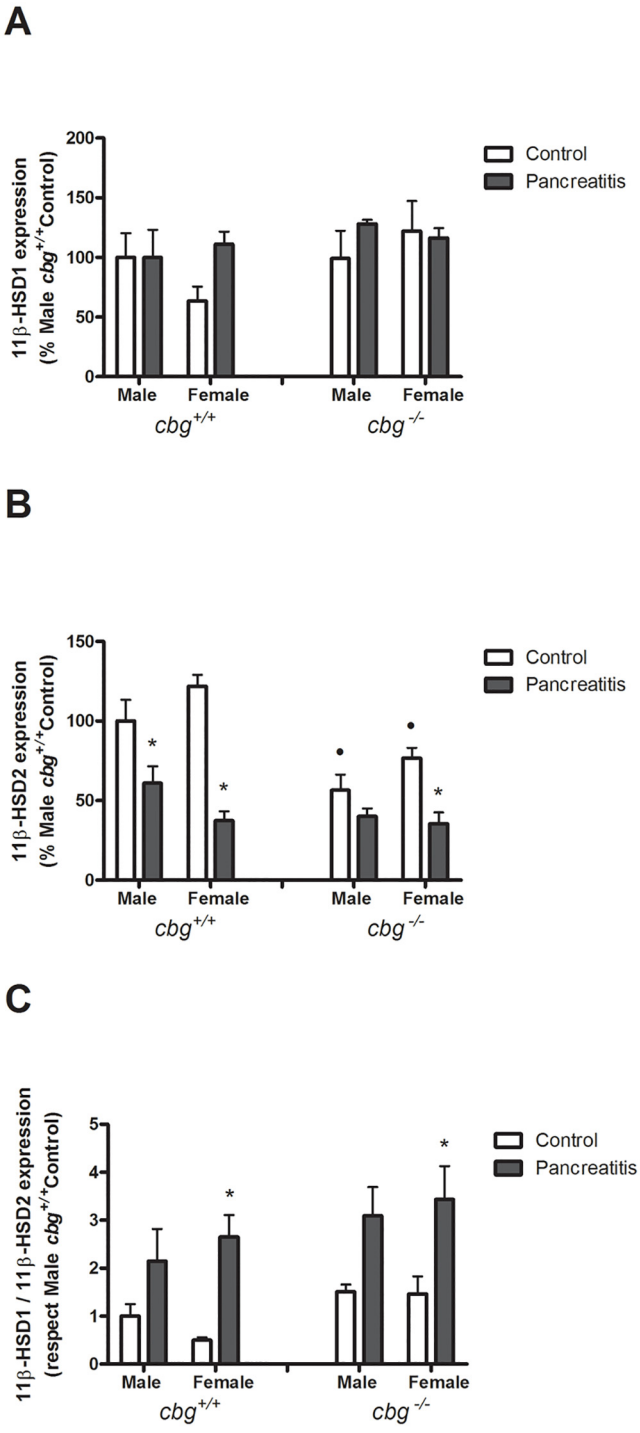

Fig 7. (A) Lung expression of 11β-HSD1, (B) of 11β-HSD2 and (C) the ratio 11β-HSD1/11β-HSD2 (C). The expression of 11β-HSD1 only showed a not significant increase induced by pancreatitis in *cbg*$^{+/+}$ female. In contrast, pancreatitis decreased in both genders the expression of 11β-HSD2. *Cbg*$^{-/-}$ mice showed lower expression of 11β-HSD2. The ratio 11β-HSD1/11β-HSD2 was increased by pancreatitis in all groups studied but only significantly in females for both genotypes. Two Way Anova: Lung expression of 11β-HSD1: Treatment P = 0.2238, Sex P = 3996, Interaction P = 5188; Lung expression of 11β-HSD2: Treatment P<0.0001, Sex P = 0.0012, Interaction P = 0.0057; ratio11β-HSD1/11β-HSD2: Treatment P<0.0001, Sex P = 0.1818, Interaction P = 0.7634; Bonferroni Post test: * P<0.05 control vs pancreatitis; + P<0.05 male vs female; • P<0.05 *cbg*$^{+/+}$ vs *cbg*$^{-/-}$.

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or to acute pancreatitis inflammation. While under the stress, *cbg*$^{-/-}$ animals failed to increase corticosterone levels [30], in our model they show the ability to increase its levels in response of inflammation. Therefore, CBG seems determinant to maintain the levels of circulating' glucocorticoids and their gender differences.

It is well known that CBG regulates the amount of free corticosterone released during inflammation [26] but our results indicate that this protein also plays a role in modulating the concentration of total circulating corticosterone. The highest levels of serum CBG detected in females probably induces a high buffering effect thus promoting the synthesis of more total corticosterone in order to achieve the concentrations of free hormone necessary for homeostasis. Although liver is the main site of synthesis for CBG which is secreted into the blood to transport corticosterone, some reports described the generation of this globulin in other organs as kidney, pancreas, placenta, hypothalamus and adipose tissue [9] [31] [10] [32][11] where its function remains unknown. The cDNA of CBG was cloned firstly from a cDNA library of liver and lung [33] and a CBG precursor has been described in pleural effusions from lung adenocarcinoma patients [34], but despite this, the significance of its presence in the lung has been little explored. Here, we evaluated the presence of CBG in the lung. RNA expression, western blot and immunohistochemistry analysis revealed that lung also generate CBG. Interestingly, the local expression of CBG in the lung (Fig 5) showed a completely opposite pattern than that observed in liver (Fig 4B), having males higher expression of CBG than females. This fact suggests a different role for CBG in these organs. While liver is the main source for circulating CBG, the role in the lung appears to be restricted to the control of local corticoids bioavailability.

Although the circulating levels of corticosterone reflect the general systemic anti-inflammatory response, the action of corticosterone also depends on the local activity of the enzymes 11β-HSD1 and 11β-HSD2. In general, it is accepted that in response to inflammation the levels of 11β-HSD1 are increased in order to potentiate the anti-inflammatory effects of glucocorticoids. Less information is available on 11β-HSD2. In vitro it is known that pro-inflammatory cytokines, as TNFα, upregulate 11β-HSD1 [35] and downregulate 11β-HSD2 [36]. Downregulation of 11β-HSD2 without changes in 11β-HSD1 has also been described in skin lesions of leprosy patients [37]. In our study, the main change observed in the lung was the decreased levels of the inactivating enzyme 11β-HSD2 elicited by pancreatitis without modification of 11β-HSD1 expression. Irrespective of gender or genotype, pancreatitis resulted in similar high levels of 11β-HSD1/11β-HSD2 ratio, evidencing the expected corticosterone activation status triggered by an inflammatory stimulus. Altogether suggests that in lung the anti-inflammatory actions of glucocorticoids would be determined by 11β-HSD2.

Interestingly, in control *cbg*$^{-/-}$ mice lung expression of 11β-HSD2 showed lower levels than *cbg*$^{+/+}$. This fact could be a mechanism to increase corticosterone levels into the tissue to counteract the impaired availability of tissue corticosterone observed in *cbg*$^{-/-}$ [15].

In conclusion, our results indicate that the role of CBG is not only restricted to act as a carrier of corticosteroids and a modulator of its tissue availability. It is also an important factor involved in the gender differences observed in corticosteroids levels. CBG is generated in the lung showing a pattern of expression opposite to the liver either in the response to the acute pancreatitis as in the sexual dimorphism. These differences between liver and lung suggest that CBG has a specific, and yet unexplored, role in the lung.

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Author Contributions

Conceived and designed the experiments: ME MG DC. Performed the experiments: JG AL SG-S LB. Analyzed the data: ME MG DC JG AL. Wrote the paper: ME MG DC.

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DISCUSIÓN

"¿Glucocorticoides? Tan poca dosis como sea posible siempre que sea necesario"

No hay duda de que los glucocorticoides (GCs) constituyen una de las familias de fármacos antiinflamatorios más utilizadas. Son reguladores críticos de una amplia variedad de procesos fundamentales, incluyendo la homeostasis metabólica, la proliferación celular, las respuestas inflamatorias e inmunes, el desarrollo y la reproducción. A concentraciones farmacológicas, los GCs muestran efectos antiinflamatorios potentes, pero a pesar de su excelente eficacia, el uso como agentes terapéuticos es a menudo restringido debido a dos grandes inconvenientes[222]. En primer lugar, el tratamiento a largo plazo con GCs suele ir acompañado de efectos secundarios graves, tales como resistencia a la insulina, osteoporosis, hipertensión, y atrofia muscular [223,224]. En segundo lugar, la aparición de resistencia a los GCs también limita su éxito terapeútico[222].

Todo ello ha llevado a potenciar diversas estrategias para mejorar el uso farmacológico de los GCs. Por un lado, la búsqueda de análogos que conserven la actividad antiinflamatoria de los GCs y eludan las acciones que conllevan efectos secundarios indeseables. Son los llamados *selective glucocorticoid receptor agonists* (SEGRA)[225,226]. Por otro, la caracterización de las acciones de los GCs relacionadas con la inflamación, incluidas las proinflamatorias, y los mecanismos de acción implicados, entre ellos el rol de la CBG.

En los últimos años, diversos estudios han evidenciado acciones proinflamatorias llevadas a cabo por los GCs. Se considera que antes de la llegada del estímulo inflamatorio, los GCs endógenos estimulan el sistema inmune para que puedan hacerle frente con garantías[10,17]. Todas estas funciones, que pueden parecer antagónicas respecto la actividad antiinflamatoria, se circunscriben en lo que se ha dado en llamar las *5Rs*, que resumen los efectos globales que pueden ejercer los GCs sobre el sistema inmune: *reinforce, ready, repress, resolve and restore.* No únicamente colaboran en reprimir y resolver la inflamación y restaurar la homeostasis, sino que también refuerzan y permiten al sistema inmune estar preparado para afrontar cualquier ataque[17].

La CBG ha sido implicada en la mediación de los efectos antiinflamatorios de los glucocorticoides. Su pertenencia a la familia de inhibidores de las serina proteasas la convierte en sustrato de la elastasa secretada por neutrófilos activados que se acumulan

en el espacio extracelular de zonas inflamadas[70].La elastasa hidroliza la CBG liberando un péptido de apenas 5 kDa lo que conlleva cambios conformacionales permanentes que reducen la afinidad de la CBG por el cortisol. En consecuencia, el cortisol es liberado en el preciso lugar donde se produce la inflamación. Este verano se han publicado por primera vez dos artículos en los que se distingue a través de ELISAs específicos, la CBG de alta afinidad, es decir intacta, y la de baja afinidad, es decir hidrolizada, en el plasma de humanos afectados por artritis reumatoide[227] y obesidad central con síndrome metabólico[83]. En ambos casos, se ha hallado menor concentración de CBG de baja afinidad que la obtenida en personas sanas, lo que ha llevado a los autores a hipotetizar que la falta de mediación de la CBG en la liberación de cortisol en la zona inflamada puede estar agravando ambas patologías. Por lo tanto, la CBG parece tener un papel en la administración dirigida de cortisol para los tejidos inflamados, facilitando su acción inmunomoduladora.

Nuestro objetivo general fue por tanto, evaluar la acción de los GCs en diversos modelos de patologías asociadas a la inflamación, donde juegan un papel importante, y evaluar si la falta de CBG modulaba la evolución de algunas de ellas. Así nació esta tesis.

El primer modelo que nos planteamos utilizar fue la aterosclerosis, que es un proceso inflamatorio que afecta a las arterias de diferentes lechos vasculares y se caracteriza por el engrosamiento de la capa íntima y media con pérdida de la elasticidad. Su lesión básica es la placa de ateroma compuesta fundamentalmente por *foam cells*, macrófagos repletos de ésteres de colesterol captado y almacenado al eliminar de la circulación las OxLDL[140]. Los macrófagos desempeñan por tanto un papel fundamental en el desarrollo y la progresión de la aterosclerosis y el conocimiento de los factores que regulan su metabolismo son cruciales para controlar el proceso. En modelos con exceso de GCs, como pacientes con síndrome de Cushing[228] aumenta el riesgo de sufrir aterosclerosis, así como a consecuencia del estrés[229]. En general, se asume que ello puede estar relacionado con las alteraciones metabólicas que provocan, desde la dislipemia a la hipertensión arterial. Sin embargo, en la mayoría de casos la aterosclerosis no cursa con cortisolemia elevada o es consecuencia de la administración exógena de cortisol, por lo que parte de la investigación se ha dirigido a evaluar la activación intracelular de cortisona mediante la enzima 11β-HSD1. Estudios en ratones KO para 11β-HSD1 han concluido que presentan un riesgo inferior que los WT de desarrollar aterosclerosis[233] mientras que en ratones KO para 11β-HSD2 el riesgo incrementa[234].
A pesar de estos datos clínicos, nos dimos cuenta de que pocos trabajos abordaban los efectos directos que ejercían los glucocorticoides en los macrófagos. La enzima 11β-

HSD1 se había identificado en macrófagos pero su función en la respuesta inflamatoria y concretamente en el proceso de formación de *foam cells* tampoco estaba aclarada.

Por todo ello, nos propusimos en primer lugar, analizar los efectos directos de la exposición simultánea a OxLDL, un potente agente proaterosclerótico, y glucocorticoides en la línea celular de macrófagos humanos THP-1. Ello supuso en primer lugar, la obtención de LDL y la puesta a punto de la metodología para su oxidación y validación de los efectos en las THP-1 que se llevó a cabo en el INIBIOLP de La Plata durante mi estancia en el grupo de investigación de la Dra. Marina Gonzalez. Finalmente, el trabajo realizado sirvió para determinar que con un grado de oxidación medio y en tan solo 24 horas de incubación, las OxLDL eran capaces de inducir la conversión de los macrófagos en *foam cells* y que en 24 horas se disparaba la expresión de proteínas implicadas tanto en la respuesta inflamatoria como metabólica.

Una vez establecidas las condiciones experimentales, decidimos incubar THP-1 expuestas a OxLDL de grado medio y durante 24 horas con dosis crecientes de cortisol, dentro del rango de fluctuación del ritmo circadiano (no a dosis farmacológicas). También nos resultaba interesante evaluar los efectos de la cortisona para determinar la importancia de la actividad 11β-HSD1 en los macrófagos activados, así que incubamos con cortisona y cortisona en presencia de BVT.2733, un inhibidor de la actividad de la enzima, para confirmar que realmente los efectos de la cortisona, si los hubiera, se producían a través de la actividad de la 11β-HSD1.

Los resultados obtenidos pusieron de relieve por primera vez según nuestro conocimiento que en macrófagos el cortisol frena el proceso de formación de *foam cells* de forma dosis dependiente. Ello ocurre a través de la disminución de la captación de OxLDL (inhibición de la expresión de CD36 y SRA1) así como la menor expresión de la enzima responsable de la esterificación del colesterol intracelular (ACAT) y de las proteínas implicadas en la salida de colesterol (ABCA1 y ABCG1). En un primer momento nos resultó algo paradójico: si disminuye la entrada pero también la salida, no es evidente que el resultado neto sea la menor acumulación. Probablemente, la disminución de la expresión de ABCA1 y ABCG1 sea consecuencia indirecta de la menor concentración de colesterol intracelular puesto que son inducidas en respuesta a su acumulación[232].

El único artículo que pudimos encontrar en el que evaluaban el efecto del cortisol sobre THP-1 expuestas a OxLDL[233] presentaba resultados antagónicos: mayor captación de colesterol, menor salida al exterior y aceleración de la formación de *foam cells*. La única diferencia en el planteamiento experimental fue que las THP-1 se preincubaban durante 16 horas con cortisol antes de la exposición a LDL y no simultáneamente como en nuestro caso. Su resultado era tan claro como el nuestro con lo que cabe especular,

utilizando el modelo de las 5Rs, que la incubación previa sin estímulo inflamatorio "*reinforce and ready*" a los macrófagos y provoca una fuerte respuesta posterior. Sin embargo, al incubar simultáneamente, el cortisol estaría en la línea de "*repress, resolve y restore*".

Ello vendría reforzado por el otro importante resultado observado: mientras que los macrófagos expuestos únicamente a OxLDL presentan un perfil de polarización intermedio entre M1 y M2 (aumento de citoquinas proinflamatorias y EMR1 y disminución de MMR por un lado pero aumento de SRA1 y CD163 por otro), la presencia de cortisol potencia el fenotipo antiinflamatorio de los macrófagos (aumento de la expresión de MMR, CD163 y disminución de EMR1 y citoquinas proinflamatorias) lo cual es consistente con su probada inducción de la polarización a fenotipo M2 en macrófagos[17].

El rol de la 11β-HSD1 en macrófagos activando cortisol intracelularmente quedó también demostrado. La cortisona fue capaz de mimetizar casi todos los efectos causados por el cortisol, aunque a mayor concentración, y BVT.2733 fue capaz de bloquearlos. A ello hay que añadir la profunda inducción de la expresión de la 11β-HSD1 en respuesta a la exposición al estímulo inflamatorio OxLDL, tal y como previamente se había descrito que ocurría al incubar con LPS[234], probablemente como mecanismo de seguridad frente a una respuesta inflamatoria descontrolada. Además también incrementó la expresión de las proteínas que permiten su actividad, la hexosa-6-P-dehidrogenasa (H6PDH) que provee de poder reductor y el transportador de glucosa-6-P (G6PT) que provee de substrato a la H6PDH transportando la gucosa-6-P del citosol al interior del retículo endoplasmático.

Nuestros resultados muestran que el cortisol tiene un efecto inhibidor sobre la expresión de 11β-HSD1 y un doble efecto sobre 11β-HSD2. Las concentraciones bajas no afectan o inhiben la expresión 11β-HSD2, pero la concentración más alta de cortisol, induce de manera muy marcada su expresión. Este perfil es coherente con una respuesta protectora, también, a los niveles excesivos de cortisol intracelular.

De todas formas, nuestro resultado mantenía en el aire la pregunta: ¿por qué entonces, en pacientes con síndrome de Cushing o tratados con glucocorticoides aumenta el riesgo de aterosclerosis? Aislar los efectos de una hormona en un modelo celular da información sobre mecanismos de acción pero no sobre lo que finalmente ocurre en un organismo vivo donde se globalizan las acciones en todos los tejidos y de todas las hormonas. Es evidente que la resistencia a la insulina, hipertensión y el aumento de TAG y NEFA que provocan los glucocorticoides son factores de riesgo. Pero éstos aparecen cuando su acción perdura en el tiempo. A corto plazo y dosis fisiológicas, es posible que resulten ateroprotectores[235].

Aunque no aparezca en la publicación, o precisamente porque no aparece, se puede deducir que no hallamos expresión de CBG en los macrófagos de la línea celular THP-1, a pesar de haberse identificado expresión de CBG en diversos tejidos tales como el riñón, útero, pulmón, hipófisis y el tejido adiposo blanco donde se ha planteado la hipótesis de que actúe modulando el exceso intratisular de glucocorticoides[4].

El siguiente paso pues fue saltar a un modelo *in vivo* de déficit de CBG y con una patología hondamente asociada al exceso de GCs, la obesidad. El síndrome de Cushing, que cursa con un exceso de cortisol circulante se caracteriza por la obesidad central que presentan los pacientes[43]; los ratones KO para 11β-HSD1 son resistentes a la obesidad inducida por la dieta[236] y su sobreexpresión en tejido adiposo causa síndrome metabólico[42]. Por otra parte, datos previos de nuestro grupo de investigación habían identificado expresión de CBG en tejido adiposo de rata[68] y en humanos, correlación inversa entre la CBG circulante y resistencia a la insulina y obesidad central[82].

El modelo de ratón KO para CBG había sido descrito en 2006 por el Dr. Thomas Willnow[72]. Para esta tesis se utilizaron ratones de la colonia propia que montamos a partir de ratones heterozigotos enviados por él desde Berlin. Él y su equipo, en su única publicación con este ratón, habían observado que una dieta con un 30% de contenido energético aportado por los lípidos no modificaba el peso corporal ni la ingesta después de 6 semanas. Así que nos propusimos evaluar qué ocurría si ofrecíamos una dieta con un 60% de contenido graso desde el punto de vista calórico durante 12 semanas que es un período más apropiado, según nuestra experiencia, para conseguir obesidad en ratones. Nos interesaba evaluar tanto los cambios en la biodisponibilidad de glucocorticoides como si la ausencia de CBG modificaba la progresión de la inflamación asociada a la obesidad. Hay que recordar que Willnow había concluido que los ratones KO para CBG presentan un déficit de respuesta a GCs.

En nuestras manos, los ratones KO para la CBG aumentaron la concentración sérica de corticosterona libre (2,5 veces más que en WT), pero disminuyeron los niveles de corticosterona total (tres a cuatro veces inferior) tal y como había descrito el equipo de Willnow. Según la hipótesis de la hormona libre, estaríamos ante un modelo de exceso de glucocorticoides. La involución del timo observada en nuestros ratones KO cuadraría puesto que es uno de los efectos de los GCs. Por el contrario, el aumento de peso de la glándula adrenal en ratones KO, es una característica asociada con la hiperactividad adrenal en respuesta a un eje HPA activo y a altos niveles de corticosterona circulantes que no observábamos.

Como le había ocurrido antes al equipo de Willnow, el peso corporal y la ingesta incrementaron en respuesta a la dieta HL pero no se vieron modificados a consecuencia de la falta de CBG. Sí que hubo una tendencia sostenida en el tiempo a incrementar la ingesta y el peso en los ratones KO alimentados con dieta estándar, de alto contenido en hidratos de carbono y bajo en grasa. ¿Por qué? Los glucocorticoides promueven la ingesta de proteína e hidratos de carbono principalmente[237]. La elección de una dieta con alto contenido en grasa es promovida por los glucocorticoides en presencia de insulina[238]. Datos aún no publicados de nuestro grupo de investigación y presentados en la tesis de Jose Gulfo indican que en ratones KO se produce resistencia central, en hipocampo, a la acción de la insulina. Esta resistencia a la insulina quizás extensiva a hipotálamo podría ser una posible explicación a la falta de respuesta de la ingesta a la dieta HL en ratones KO.

De nuevo identificamos CBG en el tejido adiposo blanco de ratón, nunca publicado anteriormente, por lo que el déficit de CBG no solo afectaba a través de cambios en la disponibilidad en sangre de glucocorticoides sino también por la propia ausencia en el tejido. La cantidad de tejido adiposo blanco en su conjunto tampoco pero sí su ubicación: en ratones KO hubo un desplazamiento significativo de la acumulación de lípidos en depósitos subcutáneos a depósitos viscerales y con un descenso del área de adipocitos subcutáneos y un aumento del área de adipocitos con ubicación epididimal en los ratones KO respecto los WT. Además, específicamente en tejido adiposo epididimal se produjo la alteración más significativa de la expresión de las enzimas 11-βHSD1 y 11-βHSD2: reducción de la isoforma 1 productora de corticosterona y aumento de la isoforma 2, que la desactiva lo cual parece constituir una defensa ante el exceso de GCs. Es de remarcar que a pesar de que la enzima 11-βHSD2 se encuentra principalmente en los tejidos diana de mineralocorticoides, una expresión menor, pero significativa se ha encontrado en hígado[239] y en tejido adiposo blanco[240–242] aunque hay poca bibliografía al respecto.

En los ratones KO tampoco observamos una mayor esteatosis hepática ni una alterada acumulación de glicógeno en hígado así como tampoco incremento de la expresión del gen diana de GCs PEPCK, lo cual es consistente con lo observado por el equipo de Willnow. Parece que el hígado resulta inmune mientras que el tejido adiposo, adrenal y timo aparecen como tejidos más sensibles a alteraciones en ratones deficientes para CBG. Con todos estos datos, nuestra primera impresión fue que el ratón KO para CBG resulta un modelo de exceso moderado de GCs, suficiente para hacer evidentes algunas alteraciones, insuficiente para manifestar otras propias de GCs crónicamente elevados. ¿Qué ocurría con la inflamación en tejido adiposo asociada a la obesidad? ¿Presentaba este modelo atenuación de la inflamación consecuencia del exceso de glucocorticoides, o

por el contrario la aumentaba como sugieren los estudios con tratamientos crónicos con GCs que provocan síndrome metabólico?

Uno de los resultados clave que nos empujó a estudiar el papel de la CBG, llevado a cabo por el equipo del Dr. Thomas Willnow, fue la respuesta a la inyección de LPS en ratones KO para la CBG[72]. Los ratones KO mostraron un aumento significativo de la incidencia de shock séptico agudo en comparación con los controles, y de la mortalidad dentro de las 48 horas después de la inyección. Ello fue explicado por los autores por la incapacidad de los GCs de generar una respuesta antiinflamatoria adecuada en ausencia de CBG[72]. Este era nuestro punto de partida. Decidimos estudiar tejido adiposo epididimal, uno de los viscerales y en el que habíamos descrito el incremento de área de los adipocitos.

La primera sorpresa fue comprobar que los ratones KO con dieta estándar mostraban en tejido adiposo una inflamación basal mayor que en los ratones WT. Presentaban mayor expresión de TNF-α, neutrófilos y los macrófagos ubicados en el tejido adiposo epididimal presentaban en mayor proporción un fenotipo M1 (comparable a la encontrada con dieta HL en ratones WT aunque el número global de macrófagos fuera inferior). Sin embargo, la diferencia entre los ratones WT y KO con dieta control fue mucho mayor con 7 semanas de edad que con 18 semanas (al finalizar el experimento). En realidad, lo que se observó es que los WT con el tiempo, van aumentando en tejido adiposo sus marcadores de inflamación para acabar igualándose a los ratones KO que apenas cambian. El aumento de la inflamación en tejido adiposo, especialmente epididimal, con la edad ha sido descrita recientemente[243] y, atendiendo a esta posibilidad, se podría aventurar que los ratones KO presentan una inflamación temprana o un envejecimiento "precoz" de tejido adiposo epididimal. Con una salvedad: la expresión de uno de los mediadores antiinflamatorios más importantes de los GCs, DUSP-1, estaba incrementada en tejido adiposo y en los macrófagos M1 obtenidos de tejido adiposo. Ello puede explicar la menor fosforilación de NF-kB en ratones KO a pesar de partir de mayor expresión de NF-kB total. DUSP-1, también llamada *mitogen-activated protein kinase phosphatase 1* (MKP-1), impide la transducción de señal en la vía de las MAPK a través de la defosforilación. Uno de los *end-points* de la vía es justamente NF-kB.

En segundo lugar, una semana de dieta hiperlipídica causó una mayor respuesta inflamatoria en los ratones KO (mayor infiltración de neutrófilos y expresión de marcadores proinflamatorios) que se mitigó con el tiempo. De hecho, después de 12 semanas de tratamiento, estas diferencias desaparecen. No hubo mayor infiltración de macrófagos, aunque el perfil de polarización respecto los hallados en tejido adiposo de

ratones WT fue claramente en mayor proporción antiinflamatorio (identificados por la presencia de MMR). De nuevo, con la dieta hiperlipídica también, DUSP-1 presentó mayor expresión en KO que en WT y de nuevo también, ello se tradujo en menor fosforilación de NF-kB en ratones KO.

¿Cómo explicar estos efectos? De nuevo, el modelo de las 5Rs parece encajar. Por una parte, observamos acciones proinflamatorias en los ratones KO con dieta estándar, es decir, sin estímulo inflamatorio o a los pocos días de iniciada la dieta ("*ready and reinforce*") mientras que a las 12 semanas de dieta, no se aprecian diferencias en la expresión de marcadores inflamatorios sino más bien características antiinflamatorias como el aumento de IL-10 circulante y más macrófagos de tipo M2 ("*resolve and restore*")[17]. Ahora bien, la resistencia a la insulina típica de glucocorticoides aparece en presencia de la dieta HL de manera que, en este modelo, coexisten ambos efectos, sin que la resistencia promueva mayor inflamación o viceversa. En la bibliografía, se han sugerido tres diferentes niveles de acción glucocorticoide en los macrófagos: niveles basales que mantendrían el sistema inactivo y sin estímulo, los niveles medios que facilitarían la acción inmune preparándola para la lesión y niveles altos, en que funcionan como inmunosupresores[244] para ayudar a restaurar la homeostasis. Por ello, se las funciones sobre el sistema inmune de los GCs naturales se han dado en llamar inmunoreguladoras, por su doble vertiente, contra a los efectos claramente antiinflamatorios de los glucocorticoides sintéticos administrados normalmente como fármacos[234]. Estos suelen ser agonistas específicos de los GCr, como la metilprednisolona, la dexametasona o la fluticasona, a través de los cuales se mediatizan las acciones antiinflamatorias, mientras que el cortisol o la corticosterona, los GCs naturales son capaces de interactuar a tanto con los MRr y los GCr. Se considera que los MRr son los mediadores de las acciones proinflamatorias[234].

Nuestra conclusión en este caso discrepaba de la de Willnow y su equipo al inyectar LPS, en la que deducía la falta de efecto de los glucocorticoides en los KO. El modelo de inflamación, sin embargo, era bien diferente: en la obesidad, leve y crónico. A consecuencia de LPS, intenso y agudo. Ello nos llevó a buscar un nuevo modelo de inflamación que cursara de forma aguda. Se planteó utilizar el modelo de la pancreatitis por varias razones: una de ellas, que en los ratones KO inyectados con LPS el pulmón fue uno de los órganos más afectados, con mayor expresión de IL-1β, al igual que ocurre en la pancreatitis. La complicación más relevante durante la pancreatitis aguda es la inflamación sistémica que, en las formas graves de la enfermedad, puede dar lugar a un síndrome de dificultad respiratoria aguda[245]. Esta disfunción pulmonar se caracteriza por una afluencia de leucocitos inflamatorios y un aumento de la permeabilidad vascular

pulmonar, siendo uno de los factores más importantes que contribuyen a la mortalidad durante la primera semana de la enfermedad[246]. El modelo de inducción de pancreatitis aguda mediante inyección de ceruleína, que es el que se suele utilizar en ratón, provoca en apenas 12 horas daño en páncreas que cursa con edema, necrosis y liberación del contenido vacuolar. Ello causa la aparición en plasma de enzimas pancreáticas como la lipasa y la amilasa acompañada de la migración masiva de neutrófilos en los tejidos, entre ellos el pulmón y también el propio páncreas[221]. Por otra parte, conocíamos el trabajo del Dr. Daniel Closa, con amplia experiencia en el modelo experimental de pancreatitis en roedores y le planteamos esta colaboración. Además, dado que disponíamos de suficientes ratones, incluimos machos y hembras en los grupos experimentales. Se trataba de comparar la gravedad de la pancreatitis en ausencia de CBG.

En general, la inducción y la gravedad de la pancreatitis se evalúan cuantificando la actividad de la lipasa pancreática en plasma, indicador del daño pancreático, y de mieloperoxidasa en tejidos, enzima asociado a infiltración tisular por neutrófilos y por tanto, indicador de la extensión de la inflamación. También por histología, se evalúa el daño pancreático (otorgando un valor en función de la intensidad del edema, la necrosis de los acinos y la infiltración de neutrófilos) y pulmonar (infiltración de neutrófilos y engrosamiento alveolar)[247]. En este caso, la evaluación la llevaron a cabo el equipo del Dr. Closa por su amplia experiencia.

Uno de los cambios más importantes que observamos y que resultó inesperado fue la pérdida del dimorfismo sexual que caracteriza los niveles totales de corticosterona circulante, que son mayores en hembras que en machos. La diferencia de género en los niveles de corticosterona no se observó ni en el grupo de ratones KO inyectados con salino ni en los inyectados con ceruleína y por tanto con pancreatitis inducida. Alrededor de la época en qué realizábamos las valoraciones del experimento se publicó un artículo con el segundo modelo obtenido de ratón deficiente en CBG por la Dra. Marie Pierre Moisan de Burdeos (Francia) en el que justamente observaban el mismo resultado de pérdida de dimorfismo sexual en la corticosterona circulante de los ratones KO[248].

Los cambios más importantes que observamos en ratones KO a consecuencia de la pancreatitis tampoco fueron los esperados: para nuestra sorpresa, no hubo diferencias destacables en la gravedad del daño pancreático o pulmonar a consecuencia del genotipo, a pesar de confirmar que la pancreatitis estaba correctamente inducida. Lo que sí observamos es que la pancreatitis aumentó significativamente el nivel de corticosterona en los ratones deficientes en CBG en una magnitud similar en ambos

sexos, hasta llegar a los niveles de los ratones WT, mientras que en los ratones WT la pancreatitis no alteró la corticosterona circulante.

El principal cambio observado en pulmón fue la disminución de la expresión de la enzima 11β-HSD2 provocada por la pancreatitis y sin modificación de la expresión de la enzima 11β-HSD1. Independientemente del género o del genotipo, la pancreatitis dio lugar a altos niveles de la relación 11β-HSD1/11β-HSD2, lo que evidencia el estado de activación de la corticosterona, provocado por un estímulo inflamatorio. Eso sugiere que la biodisponibilidad de los glucocorticoides en el pulmón viene determinada por la regulación de la enzima 11β-HSD2 más que de la 11β-HSD1. Otras situaciones en las que se ha descrito una reacción semejante es por ejemplo en una línea celular renal en respuesta a TNF-α *in vitro*, que regula negativamente la 11β-HSD2 a fin de incrementar la biodisponibilidad de glucocorticoides[249].

En pacientes humanos con pancreatitis aguda se ha descrito insuficiencia adrenal[250]. Sin embargo, el uso de corticosteroides en el tratamiento de la pancreatitis aguda no está indicado puesto que no se observa eficacia terapéutica[251]. Un interesante estudio de hace ya algunos años, evaluó los niveles de CBG, cortisol total y libre en pancreatitis aguda con necrosis pancreática seguida de o no de infección (que resulta mortal) [252]. La CBG en plasma resultó un parámetro predictor de la posterior infección, desarrollada durante los siguientes 6 días de iniciado el dolor abdominal agudo que precede el diagnóstico de pancreatitis. ¿Por qué no observamos ninguna diferencia en la gravedad de la pancreatitis en nuestro modelo? Una posibilidad es que nos precipitáramos. Observamos las diferencias a las 12 horas de inducida la pancreatitis, que según la bibliografía es el punto álgido de daño, pero quizás en nuestro caso hubiéramos visto diferencias a más largo plazo. En el estudio de Willnow con LPS, la mortalidad asociada empieza a manifestarse a las 48 horas. A pesar de que son distintos modelos de inflamación es posible que la ausencia de CBG provoque diferente respuesta a medio plazo.

Otra posible explicación más elaborada, sería que en realidad, el equipo del Dr. Willnow no estuviera observando la ausencia de acción antiinflamatoria de los GCs en los ratones con deficiencia en CBG sino lo contrario, es decir, un exceso de efecto de los GCs promovido antes de que llegara el potente estímulo proinflamatorio que supone el LPS, preparando y reforzando el sistema inmune para solucionar cualquier evento. Ello estaría de acuerdo con nuestra observación de que a la semana de ingerir una dieta hiperlipídica se produce una intensa respuesta proinflamatoria que después se mitiga con el tiempo.

La falta de respuesta sobre el peso corporal y la ingesta o la expresión de genes hepáticos controlados por los glucocorticoides, el resto de acciones que el equipo del Dr.

Willnow obtuvo y que apoya su hipótesis de la falta de acción glucocorticoide no concuerda con todo lo que hemos observado. Sí en lo que respecta al hígado, que también resultó insensible en general. Hay que constatar que en hígado se encuentra la maquinaria enzimática que elimina glucocorticoides, la enzima 5-α-reductasa cuya expresión no se vió afectada por la falta de CBG pero que puede ser suficiente para eliminar el exceso por otra parte moderado de GCs[253]. Y además, que al profundizar en los efectos metabólicos de la dieta HL en los ratones KO para CBG, hemos detectado algunos efectos típicos de los GCs, como el desplazamiento de lípidos desde depósitos subcutáneos a viscerales o la resistencia a la insulina. La otra teoría expuesta en la bibliografía sobre la posible función de la CBG en los tejidos de controlar la biodisponibilidad intratisular cobra mayor relevancia a la luz de nuestros resultados. Tanto en la obesidad como en la pancreatitis aguda, la falta de CBG altera la biodispobibilidad celular modificando específicamente la expresión de la 11β-HSD2, otro de los mecanismos disponibles para su regulación.

Por otra parte, nuestros resultados sobre la progresión de la inflamación en la obesidad junto con lo observado en el modelo celular THP-1 incubando cortisol junto a OxLDL y en los resultados de los autores que realizan el mismo experimento preincubando las células con cortisol, refuerzan la teoría de las 5Rs formulada recientemente para explicar la compleja capacidad inmunoreguladora de los glucocorticoides. En conjunto, creemos que nuestros resultados refuerzan la necesidad de evaluar con mayor precisión los niveles de cortisol en sangre, no solo los totales sino también los libres que suelen estar estimados en base a los metabolitos presentes en urina, sin tomar en cuenta los niveles de CBG circulante ni su capacidad de unión. Cada vez más frecuentemente, aparecen en la bibliografía estudios donde se analizan la presencia de polimorfismos en el gen de la CBG capaces de alterar su capacidad de unión y/o su afinidad por el cortisol[254] que pueden provocar, según nuestros resultados y los de los equipos del Dr. Willnow y la Dra. Moisan, alteraciones en la respuesta al estrés, inflamatoria o desajustes metabólicos de difícil interpretación si no se toma en cuenta la importancia de la CBG más allá de la de mera transportadora de GCs en sangre.

CONCLUSIONES

1. En la línea celular de macrófagos humanos THP1, las OxLDL con grado medio de peroxidación, provocan una respuesta inflamatoria a partir de las 12 horas de exposición, manteniendo inalterada la viabilidad celular con aumento de la expresión de TNF-α y la disminución del receptor MMR característicos de macrófagos M2 antiinflamatorios.

2. El inicio de la formación de *foam cells* se observa ya a las 24 horas de exposición a OxLDL de grado medio y acompañada de un incremento de la expresión de las proteínas responsables de la captación de las OxLDL (FAT/CD36 y SRA1) y de la esterificación del colesterol intracelular (ACAT), así como las proteínas implicadas en la salida de colesterol (ABC-A1, ABC-G1) y el receptor nuclear LXR directamente implicado en su regulación.

3. La respuesta inflamatoria de los macrófagos THP1 expuesto a OxLDL va acompañada al incremento de la biodisponibilidad del cortisol intracelular, como consecuencia del incremento da la expresión de la enzima 11βHSD1 y las proteínas que favorecen su actividad H6PDH, G6PT. De forma menos pronunciada incrementa también la enzima 11βHSD2 indicando una contra regulación al incremento de cortisol intracelular.

4. El efecto global del cortisol sobre las células THP1 expuesta a OxLDL es una disminución de la inflamación acompañada de la menor formación de *foam cells* consecuencia de la reducción de la captación de OxLDL y esterificación de colesterol intracelular; el cortisol también promueva la reducción de la expresión de ABC-A1, ABC-G1 involucradas en la salida de colesterol, posiblemente a consecuencia de la reducción del pool del colesterol intracelular.

5. La cortisona mimetiza los efectos del cortisol, aunque con menor potencia a través de la actividad de la 11β-HSD1, puesto que la presencia del inhibidor especifico BVT.2733 bloquea todos sus efectos.

6. El ratón deficiente en CBG presenta niveles de corticosterona total disminuidos y de hormonas libre aumentados que, teniendo en cuenta la reducción del peso del timo, indican que se trata de un modelo de exceso de glucocorticoides. En tejido adiposo blanco se reduce la presencia de 11 β-HSD1 mientras que en el tejido adiposo epididimal, incrementa la 11β-HSD2, posiblemente para contraregular el exceso de hormona libre circulante.

7. Ante una dieta hiperlipídica, los ratones deficientes en CBG presentan un peso e ingesta comparable a los ratones WT, en el tejido adiposo de los ratones KO se produce un desplazamiento de depósito de los lípidos del tejido adiposo subcutáneos a visceral, donde se produce un importante aumento de la expresión de la 11β-HSD2 y reducción de la 11β-HSD1 más pronunciado en los ratones WT. Este patrón se considera una respuesta protectora frente al estrés metabólico del tejido adiposo que estaría exacerbado en los ratones KO.

8. El tejido adiposo de los ratones deficientes en CBG alimentados con dieta estándar, presenta más signos de inflamación que el de ratones WT, como mayor expresión de TNF-α e IL-6, mayor infiltración de neutrófilos y mayor proporción de macrófagos M1 activados, pero también se da una elevada expresión del mediador de la acción antiinflamatoria de los glucocorticoides DUSP-1. Todo ello indicaría que esta potenciada la acción de los glucocorticoides preparando y potenciando el sistema inmune antes de una posible inflamación.

9. Ante la obesidad inducida por la dieta, en los ratones KO se da una respuesta temprana con mayor inflamación de acuerdo a lo observado con la dieta estándar, pero que se mitiga con el tiempo progresando hacia un estado más antiinflamatorio y caracterizado por mayor expresión de DUSP-1 y el aumento de macrófagos de tipo M2. En este caso, los glucorticoides en presencia del estímulo inflamatorio, promoverían la resolución potenciando la respuesta antiinflamatoria.

10. La gravedad de la pancreatitis, un modelo de inflamación aguda donde el pulmón se encuentra gravemente afectado, no se modifica debido a la ausencia de CBG. Una importante respuesta diferencial en ratones KO, es el aumento de los niveles circulantes de corticosterona respecto a los WT frente a la pancreatitis. Tanto en ratones WT como en KO se da un aumento de la biodisponibilidad de glucocorticoides en pulmón a tras de la disminución de la expresión de 11β-HSD2.

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