EFECTE DE LA PEROXIDACIÓ LIPÍDICA SOBRE LA INFLAMACIÓ I LA TROMBOSI

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Ha realitzat sota la meva direcció la tesi doctoral titulada **"Efectes de la peroxidació lipídica sobre la inflamació i la trombosi"** i està en condicions de ser presentada per a l'obtenció del grau de doctora.

I perquè així consti, signo aquest certificat.

Reus, 26 de maig de 2004

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JUSTIFICACIÓ

L'aterosclerosi es considera una malaltia inflamatòria crònica. En patogènesi tenen un paper important els lípids i la peroxidació. Els lípids són transportats pel plasma en partícules complexes anomenades lipoproteïnes. El tipus i la quantitat d'àcids grassos influeixen en la susceptibilitat a l'oxidació d'aquestes lipoproteïnes i podrien contribuir a la predisposició a desenvolupar una malaltia cardiovascular. Tots els components lipídics i proteics poden ser objecte d'oxidació i els productes que se'n deriven tenen diferents capacitats biològiques. És important conèixer els efectes citotòxics dels diferents productes oxidats i la capacitat de modular els processos vinculats als mecanismes inflamatoris, al progrés de la lesió ateroscleròtica i també als fenòmens trombòtics, ja que estan associats a les complicacions clíniques de l'aterosclerosi, que són la causa principal de morbi-mortalitat en les societats desenvolupades. En aquest sentit, serà d'especial interès l'estudi dels efectes dels components oxidats de les lipoproteïnes sobre el principal inductor dels processos trombògens, el factor tissular.

Els pacients diabètics constitueixen un grup d'alt risc cardiovascular i un bon model *in vivo* d'aterosclerosi, d'estrés oxidatiu i de nivells elevats de factor tissular. Recentment s'han introduït al nostre país les tiazolidinediones, fàrmacs hipoglucemiants que han demostrat tenir activitat antiateroscleròtica *in vivo* i antinflamatòria en estudis *in vitro* i *in vivo* encara que el mecanisme d'acció d'aquests fàrmacs segueix en estudi. El nostre interès s'ha centrat a estudiar el mecanisme antiinflamatori d'inhibició de l'expressió de la citocina proinflamatòria TNF-α, de les tiazolidinediones i el seu efecte sobre el CD36. La recerca d'un possible mediador fisiopatològic dels PPAR, receptors nuclears activats per aquests fàrmacs, també ha estat motiu d'estudi en el nostre treball perquè els PPAR es troben presents en elevades concentracions en plaques ateroscleròtiques, on es colocalitzen amb lípids oxidats.

INTRODUCCIÓ

1. L'ATEROSCLEROSI

Els trastorns del sistema vascular que fan referència a l'engruiximent i la rigidesa de la paret arterial s'anomenen genèricament arteriosclerosi. Així doncs, l'aterosclerosi és una forma localitzada d'arteriosclerosi que afecta les grans artèries.[1] Es tracta d'una malaltia progressiva caracteritzada per l'acumulació de lípids, elements fibrosos i diversos tipus cel·lulars al subendoteli vascular. L'engruiximent de la paret arterial provoca una reducció del pas de llum de l'artèria que dificulta o obstrueix totalment el flux sanguini[2] (fig. 1). Les manifestacions clíniques d'aquesta malaltia depenen de la regió vascular afectada.

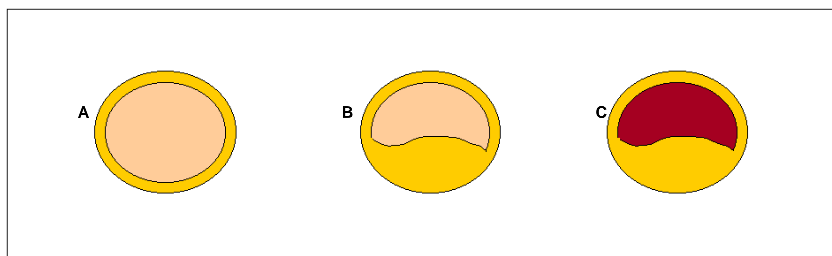


Figura 1. Esquema de la reducció del pas de llum d'una artèria. *a*) Artèria no patològica. *b*) Artèria amb engruiximent de la paret arterial a causa d'una lesió ateroscleròtica. *c*) Artèria amb trencament de la placa i formació d'un trombe que obstrueix totalment el pas del flux sanguini.

En general és la causa de l'infart agut de miocardi i de l'angina de pit quan es localitza a les artèries coronàries, dels accidents vasculars cerebrals quan es troba a les artèries que irriguen el sistema nerviós central o de les malalties isquèmiques de les extremitats inferiors quan es localitza a aquest nivell.[3]

1.1 Factors de risc

Durant els últims cinquanta anys s'han realitzat nombrosos estudis epidemiològics per tal de trobar els condicionants que predisposen a desenvolupar aquestes malalties perquè són la principal causa de mort en les societats occidentalitzades. Encara que aquests estudis han definit un gran nombre de factors de risc cardiovasculars, no expliquen la malaltia en la seva totalitat. Ens trobem, doncs, davant una malaltia d'etiologia complexa amb origen multifactorial. Els factors de risc cardiovasculars es poden classificar bàsicament en modificables (nivells elevats de lípids plasmàtics, hipertensió arterial, tabaquisme, diabetis, sobrepès/obesitat, sedentarisme, estrès i dieta aterògena) i no modificables (edat, sexe, antecedents familiars i factors genètics). La identificació d'aquests factors de risc ha proporcionat les bases científiques per a la prevenció

primària de les malalties cardiovasculars.[4] El tercer panell d'experts (ATPIII) del Programa Nacional d'Educació del Colesterol (NCEP) americà que engloba aquestes pautes i els factors de risc emergents posa especial èmfasi en el control dels nivells lipídics plasmàtics. En concret, l'objectiu principal del tractament es basa a reduir els nivells de colesterol LDL.[5] Hi ha força evidències observacionals i experimentals que indiquen clarament que les LDL són les lipoproteïnes aterogèniques més abundants i els seus nivells elevats són un important factor de risc. Més recentment s'ha suggerit que la causa de l'aterogènesi de l'LDL no seria només quantitativa sinó que també seria deguda a la composició qualitativa d'aquestes lipoproteïnes. Els components provenen en part dels lípids que ingerim amb la dieta. Per tant, un punt clau en la prevenció d'aquestes malalties serà un comportament nutricional adequat basat sobretot en la quantitat i tipus de greix.[5] Un altre factor de risc que cal destacar és la diabetes, ja que en individus que en pateixen hi ha més incidència d'aterosclerosi i d'infart de miocardi que en individus no diabètics.[6]

1.2 Patogènesi de l'aterosclerosi

L'anomenada resposta a la lesió és la hipòtesi acceptada com a fenomen desencadenant del procés ateroscleròtic[7-8] (fig. 2). Segons aquesta hipòtesi, inicialment diferents factors

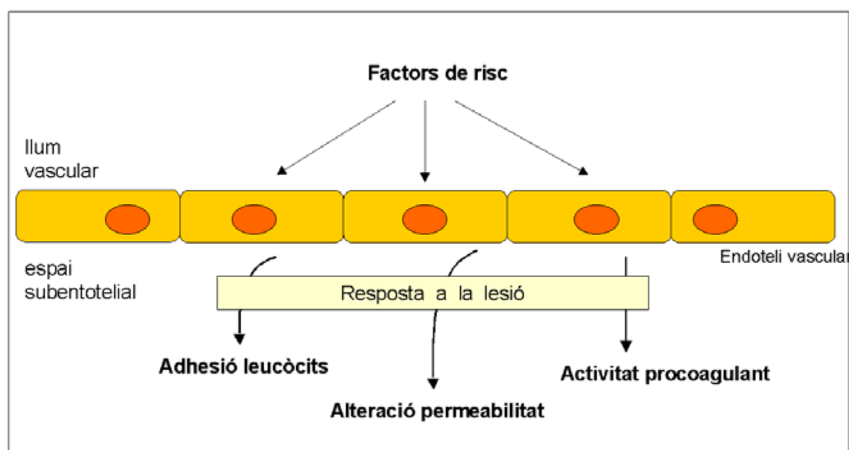


Figura 2. Esquema de la hipòtesi de la resposta a la lesió de l'endoteli vascular

de risc associats podrien alterar la fisiologia de les cèl·lules de l'endoteli vascular i provocar en un primer moment una resposta protectora per part de l'endoteli. Més tard s'indueix una reacció inflamatòria crònica específica seguida d'una fibroproliferació. El manteniment de la integritat i el funcionament correcte de l'endoteli és essencial per

preservar la fisiologia vascular. El conjunt d'alteracions que es generen reben el nom genèric de disfunció endotelial. Entre aquestes s'inclouen: *a*) un augment de la permeabilitat de les lipoproteïnes i la seva subsegüent modificació oxidativa, *b*) un augment de l'adhesió dels leucòcits circulants i *c*) una pèrdua local de l'equilibri funcional entre substàncies dels sistemes de coagulació i fibrinolítics. Aquestes manifestacions ocasionades per la disfunció endotelial es converteixen en un factor crític en la patogènesi de les malalties vasculars, ja que tenen un paper important en la iniciació, progressió i complicacions clíniques.[9]

1.2.1 Procés inflamatori crònic

L'endoteli, en resposta a la lesió, secreta molècules proinflamatòries (molècules d'adhesió, proteïnes quimiotàctiques, factors de creixement i citocines) que participen en l'entrada de tipus particulars de leucòcits circulants (monòcits i cèl·lules T) a l'interior de la paret vascular. Les selectines intervenen en el primer pas d'aquest procés, el rodament dels leucòcits al llarg de la superfície de l'endoteli, i les molècules d'adhesió i les proteïnes quimiotàctiques intervenen en l'adhesió dels monòcits i les cèl·lules T a l'endoteli i en la internalització cap a l'espai subendotelial. L'activació d'aquestes cèl·lules provocarà l'alliberament d'enzims hidrolítics, citocines, proteïnes quimiotàctiques i factors de creixement que mantindran la resposta inflamatòria[2] (fig. 3).

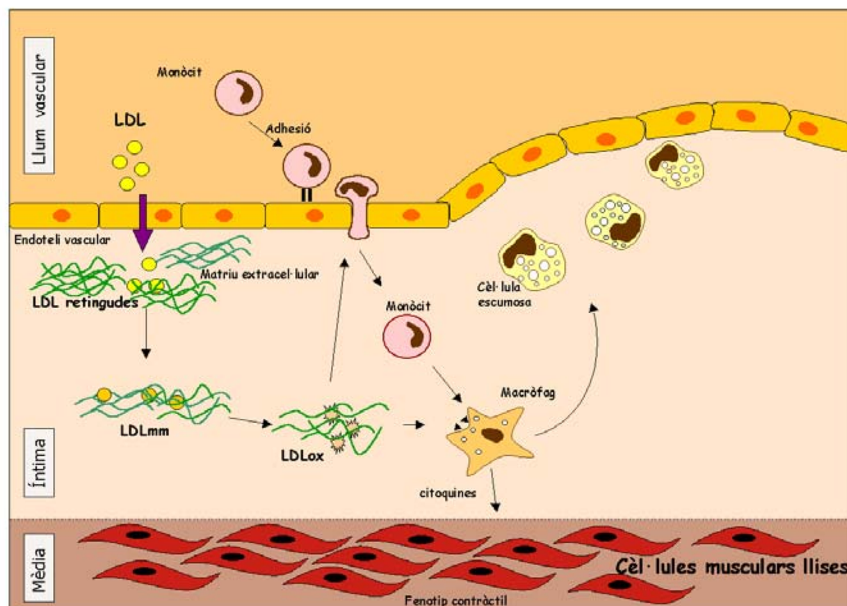


Figura 3. Monòcits circulants són retinguts i migren cap a l'espai subendotelial com a resposta al dany generat. Inicien i mantenen la resposta inflamatòria protectora.

Una altra alteració de l'endoteli, que participa en la continuïtat de la resposta inflamatòria, és la captació de lipoproteïnes a causa de l'augment de permeabilitat de l'endoteli provocat per forces hemodinàmiques. Les lipoproteïnes que superen la barrera endotelial queden retingudes a la matriu extracel·lular de l'espai subendotelial per interaccions entre l'apolipoproteïna B (apo B) constituent de l'LDL i els proteoglicans de la matriu extracel·lular (fig. 4). Les LDL retingudes poden tenir processos de modificació com oxidació, lipòlisi, proteòlisi i agregació, dels quals l'oxidació és la més significativa en la formació de la lesió inicial. El segrest i l'eliminació de les LDL modificades suposen importants accions dels macròfags (monòcits activats) en el seu paper protector inicial en la resposta inflamatòria, i minimitzen els efectes de les LDL modificades sobre les cèl·lules endotelials i les cèl·lules musculars llises. Aquesta acció dels macròfags provoca alliberar més mediadors de la inflamació (citocines com el factor de necrosi tumoral (TNF-α) i la interleucina-1 (IL-1) i factors de creixement com el factor estimulador de colònies de macròfags (M-CSF)), que al seu torn incrementen la unió de LDL a l'endoteli. D'aquesta manera es crea un cercle viciós en el qual la inflamació és mantinguda en l'artèria per la presència de lípids.[10] Si els agents que provoquen el dany no s'eliminen o no són neutralitzats per la resposta inflamatòria de l'endoteli, la inflamació progressa i la resposta protectora esdevé crònica i patològica.[11]

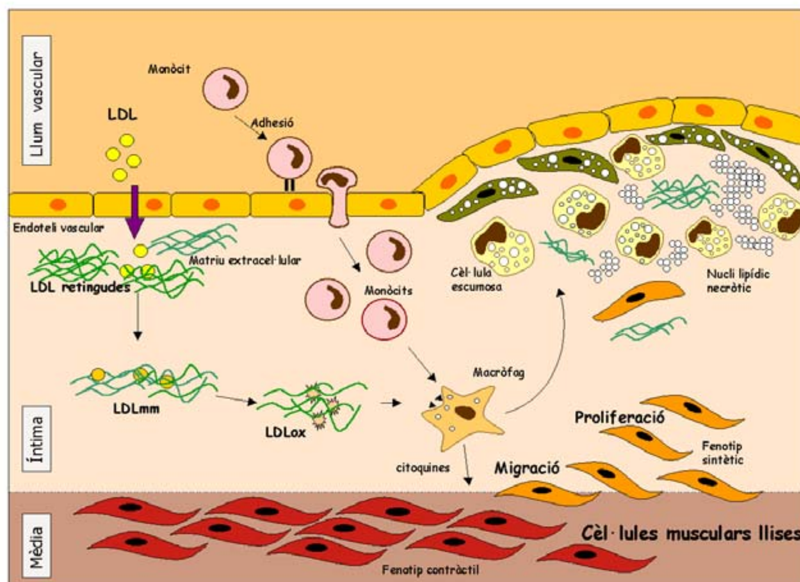


Figura 4. L'augment de permeabilitat de l'endoteli provoca que lipoproteïnes circulants travessin l'espai subendotelial on queden retingudes i poden modificar-se. Les accions dels monòcits per eliminar-les mantindran la resposta inflamatòria. En aquest procés els monòcits es diferenciaran a macròfags i provocaran la migració de cèl·lules musculars llises de la capa mèdia cap a l'íntima, es dipositaran sota les cèl·lules endotelials i crearan el cap fibrós de la lesió. Aquest cap fibrós englobarà un acúmul cel·lular i lipídic que es coneix com a nucli lipídic necròtic.

1.2.2 Hipòtesi oxidativa

L'acumulació de lipoproteïnes apo B (principalment LDL) riques en colesterol a l'íntima arterial i la subsegüent modificació sembla que són dues condicions necessàries i suficients perquè es desenvolupin lesions ateroscleròtiques. L'estructura i la composició de les lipoproteïnes són importants, ja que l'alteració de les propietats caracteritza les lipoproteïnes oxidades. A més, el grau de susceptibilitat a l'oxidació dependrà de la composició, que és variable per la dieta. Les partícules de LDL són emulsions esfèriques formades majoritàriament per associacions d'èsters de colesterol, colesterol lliure, triglicèrids, fosfolípids i proteïnes. Els lípids polars, colesterol lliure i fosfolípids, es concentren a la superfície de les LDL juntament amb molècules d'antioxidants com l'α-tocoferol i els lípids més hidrofòbics, triglicèrids i èsters de colesterol es localitzen al centre de les partícules. A la superfície de cada partícula s'hi troba associada mitjançant forces covalents una molècula d'apo B (fig. 5).

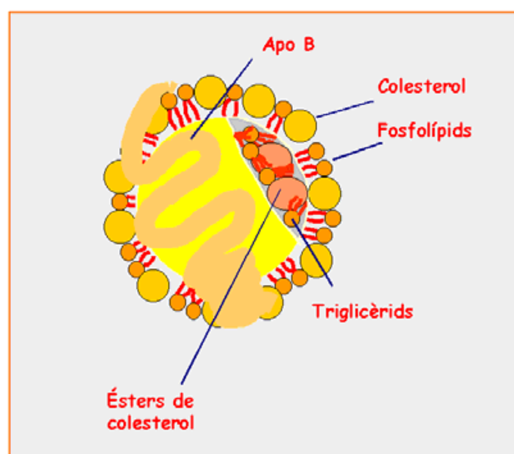


Figura 5. Esquema dels components d'una lipoproteïna de baixa densitat.

Cadascun d'aquests components pot ser degradat per reaccions hidrolítiques catalitzades per enzims de l'íntima arterial i oxidat per agents prooxidants quan aquestes partícules de LDL queden retingudes a la matriu extracel·lular del subendoteli vascular. El nostre organisme té diversos mecanismes per prevenir l'oxidació dels lípids circulants a la sang, però poden accedir amb dificultat al subendoteli vascular, mentre que la resposta inflamatòria iniciada a l'endoteli genera prooxidants. El desequilibri entre substàncies antioxidants i prooxidants al subendoteli vascular (capa íntima arterial)

afavoreix l'oxidació dels components de les LDL en aquest compartiment de la paret arterial. Els components més externs i d'entre ells les apolipoproteïnes i els fosfolípids seran els més accessibles per als enzims hidrolítics, els radicals lliures i les espècies reactives de l'oxigen i del nitrogen. La modificació provocarà un canvi en l'organització de la superfície de les partícules. L'apo B pot ser fragmentada en polipèptids i aquests poden formar complexos amb productes oxidats i generar nous epítops que afavoririen la formació d'autoanticossos.

D'altra banda els fosfolípids poden ser hidrolitzats per fosfolipases que generaran lisofosfatidilcolina i àcids grassos lliures a partir del fosfolípid majoritari. S'ha suggerit que aquests productes tant poden quedar a la partícula com formar complexos amb l'albúmina. D'aquesta manera els àcids grassos poliinsaturats, els àcids grassos lliures més susceptibles a la modificació oxidativa, podrien quedar en part separats dels antioxidants de la partícula, passarien a ser més accessibles pels agents prooxidants com els radicals lliures. Així es podria propagar tant l'oxidació d'aquests compostos lliures com l'oxidació cap a l'interior de la partícula. Les LDL oxidades són reconegudes i internalitzades pels receptors *scavenger* de la superfície dels macròfags. A mesura que aquestes cèl·lules i les cèl·lules musculars llises que han migrat des de la capa mèdia acumulen lípids oxidats, es generen les cèl·lules escumoses.[12]

1.2.3 Progressió de la lesió

Anitschkow l'any 1913 va evidenciar per primera vegada el paper del colesterol i la formació de les cèl·lules escumoses en la patologia i la morfogènesi de l'aterosclerosi.[13] Tots els estudis morfològics realitzats des d'aleshores han servit per establir l'evolució de la lesió ateroscleròtica. L'engruiximent difós inicial de l'íntima arterial progressa cap a lesions d'estria grassa, plaques ateroscleròtiques i lesions avançades complicades.[14]

L'acumulació de macròfags i la transformació d'aquests en cèl·lules escumoses que s'inicia en l'estat d'estria grassa es fa més evident a la placa ateroscleròtica. El centre de la placa va esdevenint necròtic i s'hi acumulen lípid extracel·lular i el contingut de les cèl·lules escumoses alliberat en la lisi.[15] Un procés que caracteritza el pas a una lesió més complicada és la migració de cèl·lules musculars llises (SMC) de la capa mèdia de la paret arterial passant per la làmina elàstica interna fins a dipositar-se en l'íntima o l'espai subendotelial[16] (fig. 4).

1.2.3.1 Cap fibrós

En aquest procés les SMC canvien de fenotip de contràctil a sintètic, perden la capacitat de contraure's, entren en un estat proliferatiu, migren cap a l'íntima on poden internalitzar lípids com els macròfags i esdevenir cèl·lules escumoses que juntament amb les proteïnes de matriu extracel·lular que sintetitzin i alliberin formaran el cap fibrós de la placa.[16] Citocines i factors de creixement secretats pels macròfags i limfòcits T són importants en els processos de migració i proliferació de les SMC i en el creixement i acumulació de la matriu extracel·lular que secreten.[2] D'altra banda, aquests factors alliberats per macròfags i limfòcits T contribuiran a l'estat inflamatori del cap fibrós de la placa.[17]

1.2.3.2 Nucli lipídic necròtic

El nucli lipídic necròtic es troba en lesions ateroscleròtiques avançades. Generalment és una regió acel·lular a la base de la placa, rica en lípids, que conté restes cel·lulars i freqüentment pot calcificar-se (fig. 4). La seva presència pot canviar les propietats estructurals i contràctils de l'artèria.[9] El lípid acumulat possiblement prové de dues fonts:
1) Es podria acumular lípid extracel·lular perque l'influx de lípid des de la llum de l'artèria sobrepassaria la capacitat de captació per part dels macròfags i les SMC en el seu intent d'eliminar l'acumulació de lípid de la lesió.[18]
2) Podria provenir de les cèl·lules escumoses atrapades a la lesió per la formació del cap fibrós que recobreix la placa. Aquestes cèl·lules eventualment poden morir, ja sigui per processos de mort cel·lular programada o bé per hipòxia o pels efectes citotòxics dels lípids oxidats o citocines secretades per altres cèl·lules de la lesió. En la formació del nucli lipídic necròtic no es coneix si predominen més els processos de necrosi o els d'apoptosi cel·lular. I si aquests fenòmens es produeixen, les cèl·lules escumoses poden alliberar tot el seu contingut lipídic i els enzims proteolítics a la matriu extracelular i formar un gran *pool* de lípids i restes cel·lulars. En estudis *in vitro* s'ha vist que elevades concentracions de LDL oxidada són citotòxiques per diferents tipus cel·lulars incloent-hi macròfags i SMC, però no hi ha gaires evidències del que passa a la lesió.[9]

1.2.3.3 Mort cel·lular

Estudis en humans i en models animals d'aterosclerosi mostren que la mort cel·lular programada té un paper important en la formació del nucli lipídic necròtic. L'apoptosi de

macròfags i SMC és el resultat d'interaccions cèl·lula-cèl·lula i de l'ambient ric en citocines de la paret arterial.[16] Molts senyals poden estar involucrats en la regulació d'aquesta apoptosi. El gen supressor de tumors p53, el factor de necrosi tumoral alfa (TNF-α) i l'interferó gamma (INF-γ) funcionen activant l'apoptosi mentre que el gen bcl-2 sol ser protector de l'apoptosi.[19] S'han detectat diferents localitzacions dels processos d'apoptosi de cèl·lules escumoses. Les derivades de macròfags principalment pateixen apoptosi en el nucli de les lesions i les derivades de SMC, a les àrees fibròtiques.[20]

1.2.4 Vulnerabilitat de la placa i trombosi

L'aterosclerosi coronària i les complicacions trombòtiques són la causa de morbi-mortalitat més important en les societats industrialitzades.[10] El 75% dels episodis cardiovasculars aguts que resulten d'un infart agut de miocardi o d'un accident vascular cerebral són deguts a trencaments de la placa i trombosi més que causats per una isquèmia resultat final d'un procés progressiu de reducció de pas de flux sanguini per l'artèria.[9] El risc d'episodis trombòtics està associat al grau de vulnerabilitat de la placa, que principalment ve donat per la composició, la qual li dóna les propietats físiques i químiques per respondre al microambient de la lesió.[10]

1.2.4.1 Trencament de la placa

El trencament de la placa exposa els lípids de la placa i el factor tissular (TF) als components de la sang i inicia la cascada de la coagulació sanguínia, l'adhesió de plaquetes i la trombosi (fig. 6). En general, el trencament de la placa associat amb infart agut de miocardi té lloc als extrems de les plaques i amb més freqüència en lesions formades per un gran nucli lipídic necròtic envoltat per una fina capa fibrosa.[16] Aquesta configuració és especialment inestable per la morfologia tova del nucli lipídic, ja que cedeix a les forces mecàniques que es generen a la superfície, concentra tota la pressió sobre la fina capa fibrosa i, quan aquesta no les pot suportar, la placa es trenca. L'estabilització de la placa davant d'aquestes forces mecàniques dependrà de l'organització de les fibres que formen la matriu extracel·lular, així com del balanç entre la síntesi i la degradació dels seus components, principalment fibres de col·lagen.[19] La mida del nucli lipídic necròtic és un altre punt important. Es considera que plaques que continguin nuclis lipídics necròtics que ocupin més del 40% del volum tenen un alt risc de trencar-se i desencadenar trombosi.[9]

Per tant, l'apoptosi de macròfags i SMC també influirà en l'estabilitat de la placa, ja que s'ha suggerit que els lípids del nucli necròtic afavoreixen la trombosi, junt amb la

composició lipídica d'aquest nucli. La teràpia hipolipemiant redueix el contingut en èsters de colesterol i fa augmentar la consistència del nucli de la placa.[9] Finalment, les metal·loproteïnases de matriu secretades per macròfags poden influir en l'estabilitat de la placa perquè poden degradar proteïnes que formen la matriu extracel·lular fent més fina la capa fibrosa.[9]

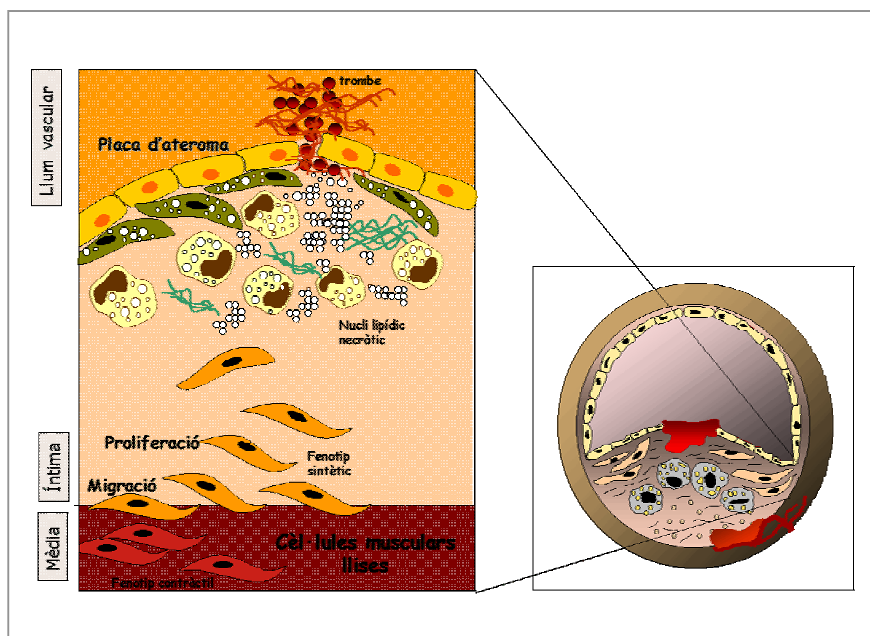


Figura 6. Representació d'una secció d'una placa vulnerable trencada que ha patit un procés trombòtic.

2. L'OXIDACIÓ I EL PROCÉS ATEROSCLERÒTIC

2.1 Evidències de la presència de lípids oxidats *in vivo*

La hipòtesi que l'oxidació de les lipoproteïnes riques en apo B contribueix a l'aterogènesi es fa palesa en diversos estudis que mostren evidències de l'existència *in vivo* de LDL almenys parcialment oxidada en lesions ateroscleròtiques humanes i fins i tot en plasma. Aquests treballs s'han basat en quatre tipus d'aproximacions:

1) Estudis comparatius de les propietats de lipoproteïnes riques en apo B aïllades del plasma i d'íntimes arterials humanes (normals i amb lesió) que demostren que les lipoproteïnes aïllades de lesions humanes presenten canvis en la composició característics de l'oxidació induïda per radicals lliures: diferències en la distribució d'àcids grassos (disminució important d'àcid araquidònic, àcid linoleic i triglicèrids), augment de la mobilitat electroforètica, fragmentació de l'apo B, augment del diàmetre de la partícula,

augment de peròxids i substàncies que reaccionen amb l'àcid tiobarbitúric (TBARS) i són més ràpidament internalitzades per macròfags *in vitro*.[21]

2) Estudis immunohistoquímics que busquen lípids oxidats demostren colocalització d'antígens d'apo B-100 intacta i oxidada i presència de productes de la peroxidació lipídica (com els aldehids: 4-hidroxinonenal i malondialdehid) units a residus de lisina de polipèptids en la matriu extracel·lular de l'íntima de lesions ateroscleròtiques humanes i animals.[22-24]

3) Estudis de paràmetres oxidatius en plasma demostren l'existència en plasma d'individus diabètics no controlats glicèmicament, en models animals de diabetis i en pacients amb alt risc d'infart de miocardi. A més, s'ha demostrat que hi ha autoanticossos circulants contra l'LDL oxidada en plasma d'individus normals i es troben nivells elevats d'aquests anticossos en plasma de pacients amb aterosclerosi carotídia avançada, malaltia coronària arterial, diabetis, malaltia vascular perifèrica, hipertensió i preeclàmpsia.[9]

4) L'extracció de productes oxidats de plaques ateroscleròtiques i l'anàlisi posterior mitjançant cromatografia líquida d'elevada resolució (HPLC) i cromatografia de gasos associada a espectrefotometria de masses (GC-MS) ha demostrat presència de F_2-isoprostans, lipoperòxids (hidroxioctadecanoics (HODE), oxo-octadecanoics (oxoODE), hidroxieicosatetranoics (HETE), dihidroxieicosatetraenoics (diHETE) i oxoeicosa-tetraenoic (oxoEET)), oxisterols i fosfolípids fragmentats *in vivo*.[25-32]

Tots aquests fets corroboren l'existència de formes de LDL parcialment oxidades en lesions ateroscleròtiques malgrat el contingut en antioxidants de les partícules de LDL. També indiquen que els processos de modificació oxidativa s'inicien quan les partícules queden atrapades per components de la matriu extracel·lular de l'íntima, els provoquen canvis en l'estructura i augmenta el temps d'estada en aquest microambient.

2.2 Components de l'LDL oxidada

L'oxidació de l'LDL és un procés gradual, per tant, la composició de la partícula és complexa i variable. Terminològicament s'ha establert que quan parlem de fases inicials d'oxidació de l'LDL ens referim a LDL mínimament modificades i quan ja ens trobem en la fase final del procés, utilitzem el terme LDL extensament oxidades.

Els primers compostos oxidats que es formen provenen de la modificació dels àcids grassos insaturats. Aparentment els productes de la peroxidació lipídica d'aquests àcids grassos i els èsters tenen un paper molt important en l'aterogènesi i els derivats oxidats

del colesterol, els oxisterols, es produirien amb menys facilitat i més tard. L'LDL és molt rica en àcids grassos i aproximadament la meitat són poliinsaturats (PUFA). L'àcid linoleic és el majoritari i junt amb l'àcid araquidònic representen el 98% del contingut total en PUFA de l'LDL[33] i els nivells provenen íntegrament de la dieta. Es troben formant part sobretot dels fosfolípids de la superfície i també en els èsters de colesterol de l'interior de la partícula. El grau d'oxidació afecta molt significativament la composició en àcids grassos de l'LDL. L'LDL extensament oxidada presenta una reduccció aproximada del 90% del contingut d'àcid linoleic i araquidònic tant de la fracció de fosfolípids com de la d'èsters de colesterol.[34] Els productes inicials de la peroxidació lipídica d'aquests PUFA són els hidroperòxids lipídics 9-HPODE i 13-HPODE, altament inestables, que generen posteriorment: 1) per processos de descomposició, els isoprostans si deriven de l'àcid araquidònic o els fitoprostans si deriven de l'àcid linoleic o 2) els corresponents hidroxiàcids: 9-HODE i 13-HODE que són els compostos majoritaris de les LDL mínimament modificades.

Els HODE segueixen reaccionant per donar lloc a productes finals de la peroxidació lipídica més estables com els aldehids, principals productes oxidats de les LDL extensament oxidades. Aquests poden difondre's per la partícula i fins i tot escapar-se i provocar efectes fora d'on s'ha originat el dany oxidatiu, ja que poden actuar com a missatgers secundaris citotòxics.[35] Els aldehids es classifiquen en polars, hidroxialdehids i apolars, entre altres, segons la composició química. Dels hidroxialdehids el més estudiat és el 4-hidroxi-2-nonenal (4-HNE). Té una elevada reactivitat amb diverses biomolècules. Pot unir-se a proteïnes, DNA i fosfolípids i s'ha suggerit que els productes generats poden tenir un paper important com a reguladors de la disfunció vascular. D'entre els aldehids apolars destaquen l'hexanal que és l'aldehid majoritari de l'LDL oxidada, i el 2,4-decadienal, que és el que produeix efectes biològics més potents.

D'altra banda, la dieta podria ser una font de productes oxidats. S'ha demostrat que processos tèrmics en ambients amb oxigen causen formació de productes derivats de la peroxidació lipídica en aliments rics en colesterol. En concret s'ha detectat presència d'àcid linoleic conjugat i oxisterols en ous, productes càrnics i làctics sotmesos a aquests processos o a emmagatzemaments perllongats.[36-38] I s'ha estimat que aproximadament un 1% del colesterol que es consumeix en una dieta occidental està oxidat.[37] Aquests productes oxidats ingerits amb la dieta poden ser absorbits i transportats als quilomicrons i les VLDL[39-41] i s'ha suggerit que posteriorment podrien ser transferits a les LDL.[42]

2.3 Efectes de l'LDL oxidada

Nombrosos estudis s'han centrat en els efectes de l'LDL oxidada en l'expressió de gens que regulen els processos de desenvolupament de la lesió. D'una manera generalitzada, sembla que l'activitat biològica de l'LDL varia amb el grau d'oxidació. En estadis de modificació mínima indueix l'expressió de mediadors de la inflamació crònica i en estats d'oxidació extensa inhibeix aquests mediadors i n'augmenta la capacitat citotòxica[43] (fig. 7). Els resultats sobre diversos gens mostren una discrepància important que podria atribuir-se a diferències en la composició de les LDL i les cèl·lules utilitzades. L'oxidació de l'LDL és un procés gradual i dependent de la partícula inicial, que pot variar en funció de la dieta; per tant, diversos processos d'oxidació *in vitro* poden donar LDL oxidades molt diferents en composició. I si tenim en compte que tots els productes derivats de la peroxidació lipídica esmentats en l'apartat anterior han demostrat efectes rellevants *in vitro*, és complicat definir els efectes biològics de l'LDL oxidada.

Cal concretar la caracterització composicional de la partícula utilitzada i estudiar els diferents compostos per separat per poder establir correctament la contribució de cadascun en els efectes observats. I el més important és poder saber la rellevància que això pugui tenir *in vivo*. Molts dels compostos formats es descomponen ràpidament i en formen d'altres amb qualitats diferents. És clau, doncs, hipotetitzar el potencial d'aquests productes en el nucli lipídic necròtic de la placa d'ateroma on es poden trobar lliures i en concentracions més elevades.

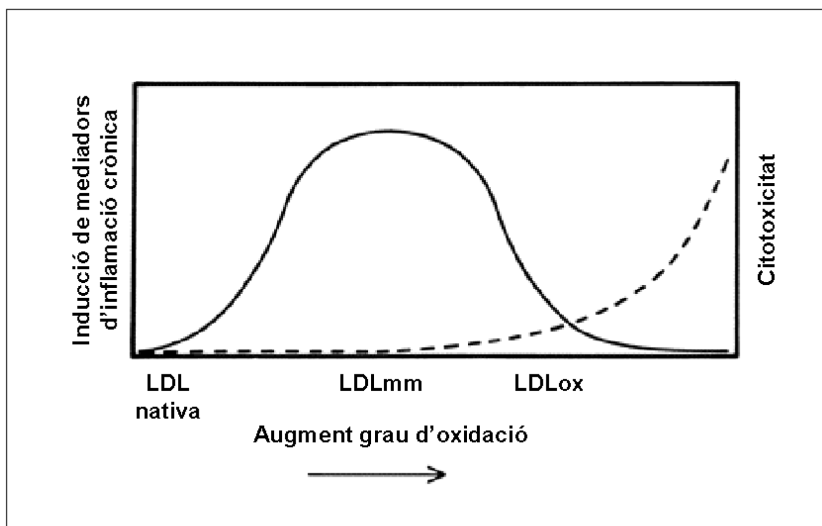


Figura 7. Figura extreta de Berliner et al. que resumeix l'activitat biològica de l'LDL segons el grau d'oxidació.

2.3.1 Oxidació i resposta inflamatòria

L'LDL oxidada produeix un estímul directe sobre les citocines proinflamatòries secretades pels macròfags o modula la resposta inflamatòria iniciada per algun altre estímul? La majoria d'estudis sobre els efectes de l'LDL oxidada en la secreció de citocines proinflamatòries pels macròfags inclouen un tractament amb lipopolisacàrid (LPS). L'LPS és el component principal de la membrana externa dels bacteris gramnegatius[44] i indueix l'expressió de gens proinflamatoris que intervenen en la resposta a infeccions bacterianes en cèl·lules endotelials i monòcits.

El resultat d'un assaig per *microarray* d'aproximadament 16.000 cDNA humans confirma que la captació prèvia de LDL oxidada modula l'expressió de gens induïts per LPS en cèl·lules escumoses. De 127 gens l'expressió dels quals variava ≥4 vegades per efecte del LPS, 57 eren modificats per l'LDL oxidada, entre els quals es troben els gens que codifiquen per citocines proinflamatòries com el TNF-α. La majoria dels efectes de l'LDL oxidada eren disminuir l'efecte del LPS excepte en les citocines proinflamatòries, en què n'incrementava l'expressió.[45] La gran heterogeneïtat de la partícula de LDL oxidada i el gran nombre de gens involucrats en la resposta inflamatòria en dificulten l'estudi. Delimitar els efectes a un gen en concret pot facilitar l'observació dels efectes i posteriorment poder globalitzar-los. El TNFα és un dels principals mediadors de la fase aguda de la resposta inflamàtoria[46] i la secreció per part dels macròfags és important en l'evolució de l'aterosclerosi.[2]

Estudis en macròfags mostren que l'LDL oxidada disminueix l'expressió gènica del TNFα induïda pel LPS.[47-48] Aquesta acció antiinflamatòria de l'LDL extensament oxidada s'atribueix a la part lipídica de la partícula. Diversos components oxidats de forma individual han mostrat aquesta capacitat antiinflamatòria. L'àcid linoleic conjugat[49] i els oxisterols 7β-hidroxicolesterol, 27-hidroxicolesterol i 25-hidroxicolesterol[50] disminueixen la inducció dels nivells de mRNA i la secreció del TNFα . Aquest efecte s'explicaria a través d'una regulació transcripcional. Tant l'LDL oxidada com els oxisterols esmentats disminueixen l'augment d'unió dels factors de transcripció AP-1 i NF-κB produït pel LPS al promotor del TNFα.[47] I sembla que en el cas de NF-κB l'efecte es produiria en l'activació del complex.[48] A més, se sap que el 25-hidroxicolesterol augmenta la unió dels factors de transcripció Sp-1 i Sp-3 al promotor del TNF-α. Sp-3 és un repressor transcripcional i s'ha suggerit que Sp-1 i Sp-3 podrien competir amb NF-κB per unir-se al promotor del TNFα o interaccionar amb NF-κB ja unit a DNA i d'aquesta manera contribuir a disminuir l'expressió del TNFα induïda pel LPS.[50]

D'altra banda, el receptor nuclear PPARγ també podria estar implicat en la inhibició de l'activació d'AP-1 i NF-κB induïda pel LPS i en la inhibició de la inducció del TNFα pel LPS en macròfags.[51] Però falten més estudis que ho evidenciïn, ja que s'ha observat el mateix efecte inhibidor de TNFα en cèl·lules en les quals no s'expressa PPARγ.[52] L'LDL oxidada podria seguir el mecanisme dependent de PPARγ, ja que s'ha descrit que activa aquest receptor, però no és el cas dels oxisterols.[51] En canvi, l'activitat antiinflamatòria dels oxisterols podria estar mediada, en part, per uns altres receptors nuclears, els LXR. El mecanisme pel qual disminuiria el TNFα a través de LXR no està definit, però no s'observa reducció d'aquesta citocina en models animals de dermatitis de contacte al·lèrgica i irritant deficients en LXR-β.[52] Per tot això s'ha suggerit que a la inhibició de la secreció del TNFα induïda pel LPS en macròfags hi contribuirien diversos mecanismes.[50] D'altra banda, l'aldehid 4-HNE disminueix els nivells de proteïna TNFα i l'activitat transcripcional induïda pel LPS, i impedeix activar NF-κB.[53] També s'ha demostrat en macròfags humans que tant l'LDL oxidada[34,54] com la HDL oxidada[54] disminueixen la secreció basal del TNFα. Aquest efecte també ha estat atribuït a la part lipídica d'aquestes partícules. Els hidroperòxids lipídics 9-HPODE, 13-HODE i 13-HPODE i els aldehids 2-octenal, 2-nonenal, hexanal, 2,4-heptadienal, decanal, 2-hexenal i 2,4-decadienal disminueixen la secreció basal de TNFα[54] i els aldehids 2,4-decadienal, hexanal i 4-HNE redueixen els nivells basals de mRNA de TNFα.[55] En aquest cas no hi ha estudis sobre el mecanisme d'acció implicat.

2.3.2 Oxidació i citotoxicitat cel·lular

L'LDL oxidada promou citotoxicitat en diferents tipus cel·lulars, per exemple, cèl·lules endotelials,[56] SMC[57] i macròfags.[58] En estat no patològic hi ha un recanvi cel·lular constant per mantenir la integritat i el bon funcionament de la paret vascular. La toxicitat causada per l'acumulació de l'LDL oxidada al subendoteli vascular trenca l'equilibri entre els processos de proliferació i mort cel·lular i fa desenvolupar la lesió ateromatosa. En aquest efecte podria intervenir un fenomen passiu i espontani de lisi d'organel·les i membranes com necrosi o bé un procés actiu de mort cel·lular com la mort cel·lular programada o apoptosi. Encara que el mecanisme pel qual es produiria aquesta mort cel·lular no es coneix, aquest efecte s'ha atribuït a la part lipídica de l'LDL oxidada i en particular, als oxisterols en SMC.[59] Com que la contribució d'aquests productes no és suficient per explicar la toxicitat de l'LDL oxidada, s'ha suggerit que altres productes derivats de la peroxidació lipídica podrien actuar sinèrgicament amb els oxisterols i

augmentar-ne el potencial citotòxic. Els hidroperòxids de l'àcid linoleic i els aldehids també han mostrat citotoxicitat *in vitro* en cèl·lules endotelials[60] i macròfags[55,61] respectivament. Ara bé, *in vivo* no hi ha evidències directes que aquests productes contribueixin a l'aterogènesi.

L'apoptosi de VSMC està fortament regulada pel protooncogèn dominant c-myc i pel gen supressor de tumors p53.[62] Ambdós gens regulen tant l'apoptosi com la proliferació cel·lular. L'expressió de p53 està associada a una aturada de la proliferació cel·lular i a un augment d'apoptosi. El gen c-myc contribueix en un o altre procés en funció dels nivells d'expressió.[63] S'ha demostrat que una sobreexpressió de c-myc pot augmentar l'expressió del gen del p53[64], ja que el promotor conté llocs consens d'unió de c-Myc i c-Myc pot transactivar p53.[65] Pel que fa a la regulació d'aquests gens per l'oxidació, l'LDL oxidada augmenta els nivells de mRNA i proteïna de p53[66] i els hidroperòxids lipídics 9-HPODE i 13-HPODE augmenten els nivells de mRNA de c-myc.[67] En canvi, l'aldehid 4-HNE bloqueja l'expressió de c-myc en diversos tipus cel·lulars.[68]

2.3.3 Oxidació i trombosi

El TF és considerat el principal mediador dels mecanismes trombogènics associats al trencament de la placa.[69] Es localitza en SMC, cèl·lules escumoses, a l'endoteli i al nucli lipídic necròtic de plaques ateroscleròtiques[70-73], on colocalitza amb l'LDL oxidada.[74] A més, els pacients amb diabetis, una malaltia associada a un estat oxidatiu elevat, presenten nivells plasmàtics elevats de TF.[75]

Els efectes de la modificació de l'LDL sobre l'expressió del TF han estat sovint motiu d'estudi. S'ha suggerit que l'efecte de l'LDL oxidada sobre el TF en SMC seguiria un patró de regulació diferent que en cèl·lules endotelials i macròfags. Diversos graus de modificació oxidativa de l'LDL augmenten l'activitat i els nivells de mRNA en cèl·lules endotelials.[76-80] Els resultats més discrepants s'han observat en macròfags en què l'LDL oxidada no altera, augmenta o inhibeix l'activitat i l'expressió del TF[81-83] i potencia la inducció del TF causada pel LPS.[84]

D'altra banda, s'ha vist que agents antioxidants regulen de manera posttranscripcional l'expressió de TF induïda per LPS en macròfags.[85] En canvi, el paper de l'LDL oxidada en el TF de SMC està més definit. L'LDL oxidada en aquest tipus cel·lular augmenta els nivells de mRNA del TF i ve donat per un control transcripcional.[86-87] En concret hi estan implicats dos factors de transcripció: Egr-1 i Sp-1. L'activitat d'unió d'aquests dos factors de transcripció al promotor del TF augmenta en SMC en presència de LDL oxidada.[86]

Aquesta acció transcripcional consisteix en el fet que la regulació del TF sigui específica del tipus cel·lular. Els resultats d'una anàlisi d'expressió diferencial mitjançant *microarray* d'uns 36.000 gens humans en SMC tractats amb LDL oxidada, mostren una forta capacitat d'induir gens involucrats en la transcripció.[88] Aquesta regulació del TF en SMC s'ha atribuït a la fracció lipídica de l'LDL. S'ha demostrat que una part de l'augment d'activitat TF vindria donat per hidroperòxids del colesterol de l'LDL oxidada com el 7β–hidroperoxicolesterol, mentre que els oxisterols no hidroperòxids no tindrien efecte. S'ha suggerit que la resta de l'efecte seria induït per lípids menys polars continguts en la LDL oxidada encara no identificats.[87]

3. PPAR I EL PROCÉS ATEROSCLERÒTIC

Els factors de transcripció representen dianes amb un potencial terapèutic molt elevat, ja que s'expressen en molts teixits i regulen un gran nombre de gens. La intervenció, doncs, sobre un d'ells genera una cascada d'amplificació del senyal. En el cas que ens ocupa, l'oxidació lipídica en dos processos claus de l'evolució de l'aterosclerosi (la inflamació i la trombosi), hi tindran un paper destacat aquells que regulen l'homeòstasi dels lípids, la biologia de la paret vascular i la resposta inflamatòria. Els components de la família dels PPAR semblen uns bons candidats, d'una banda, perquè són sensors lipídics i, d'una altra, perquè són dianes de fàrmacs que mostren efectes beneficiosos en el tractament de la malaltia. En concret, els efectes antiateroscleròtics són deguts al potencial hipolipemiant, antidiabètic, antiinflamatori i antitrombòtic (fig. 8).

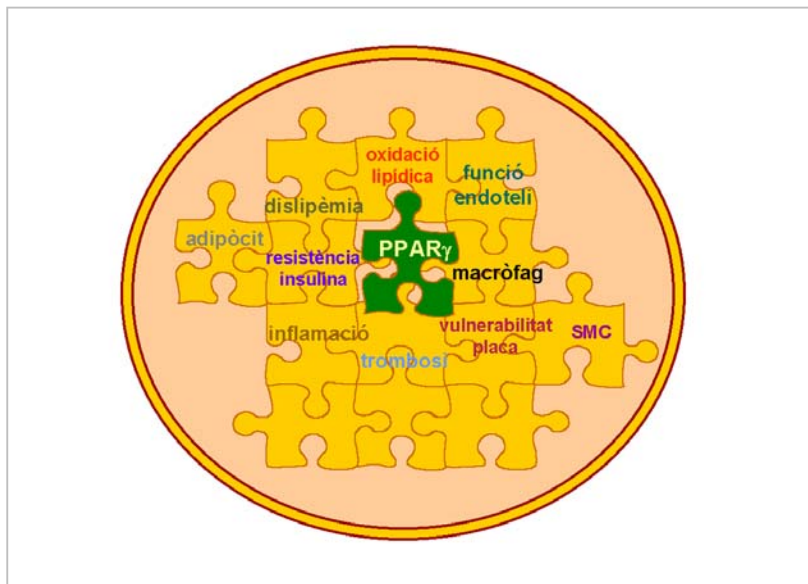


Figura. 8. Les evidències observacionals fan pensar en el receptor nuclear PPARγ com una peça clau en el complicat trencaclosques de l'aterosclerosi.

3.1 PPARγ

El PPARγ és un dels tres subtipus de PPAR que constitueixen una subfamília de la superfamília de receptors hormonals nuclears. Per actuar com a factors de transcripció, els PPAR requereixen activació per lligand i formar heterodímers amb RXRα, un altre receptor nuclear.[89] Cal tenir en compte que el PPAR també pot actuar com a homodímer en funció de la proporció entre PPAR i RXR en una determinada cèl·lula.[90] El PPAR reconeix llocs de resposta a PPAR (PPRE) situats en els promotors dels seus gens diana i en modula l'activitat transcripcional (fig. 9), generalment augmentant-la però en el cas de les citocines proinflamatòries, la inhibeix. Aquests elements estan formats per una repetició directa imperfecta de la seqüència hexàmera AGGTCA, separada normalment per un (DR-1) o en alguns casos per dos nucleòtids (DR-2).[91-94] Recentment s'han descrit PPRE que difereixen més de la seqüència consens esmentada. En el promotor del gen de la colesteroil èster hidrolasa (CEH) es troben tres llocs PPRE funcionals formats per una sola de les repeticions hexàmeres, dos d'ells en la cadena complementària i en ambdues direccions.[95] En el promotor del gen del factor de creixement d'hepatòcit (HGF), el lloc PPRE funcional consta de dues repeticions imperfectes invertides i separades per dos nucleòtids.[96]

El PPARγ s'expressa majoritàriament en teixit adipós i està implicat sobretot en la regulació de la diferenciació cel·lular.[97] També s'expressa en cultius primaris de cèl·lules vasculars: cèl·lules endotelials,[98] SMC[99] i macròfags.[100]

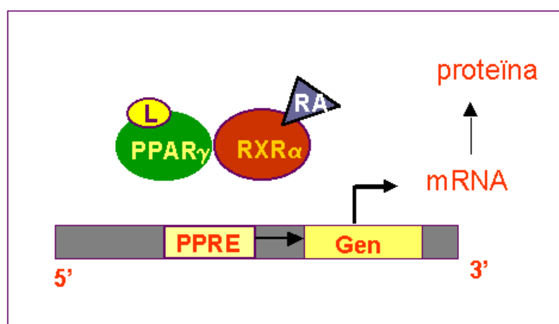


Figura 9. Mecanisme d'acció de PPARγ per activar la transcripció dels gens diana.

3.2 PPARγ i la retenció i migració de monòcits circulants

Evidències recents suggereixen que els PPARs podrien afectar l'adhesió dels monòcits circulants a l'endoteli vascular i la subsegüent internalització cap a l'espai subendotelial a

través de diferents mecanismes (fig. 10). El PPARγ podria reduir la secreció de molècules proinflamatòries secretades per l'endoteli en resposta a la lesió.
S'ha observat que els lligands farmacològics d'aquest receptor nuclear disminueixen l'expressió de molècules d'adhesió com la molècula d'adhesió de cèl·lules vasculars 1 (VCAM-1) i la molècula d'adhesió intracel·lular 1 (ICAM-1),[101-102] de proteïnes quimiotàctiques com la proteïna quimiotàctica de monòcits 1 (MCP-1) en cèl·lules endotelials[103] i en individus obesos[104], i del receptor de proteïnes quimiotàctiques 2 (CCR2) en la superfície de monòcits que podria reduir la internalització de monòcits mediada per MCP-1.[105-106] En conjunt, les accions dels PPARs sobre el control de la captació de monòcits en resposta a una inflamació local de la paret arterial podria modular la formació inicial de la placa ateroscleròtica.[107]

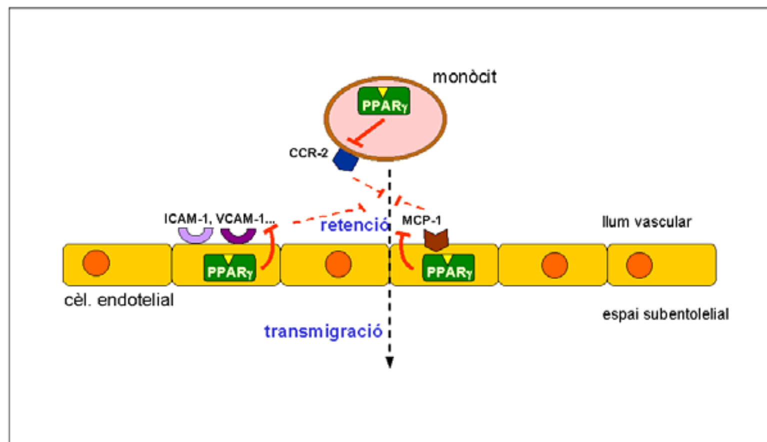


Figura 10. Efecte de PPARγ sobre la retenció i transmigració dels monòcits circulants.

3.3 PPARγ i l'oxidació lipídica

L'expressió de PPARγ és elevada en el subendoteli vascular i en el nucli lipídic necròtic de plaques ateroscleròtiques humanes, on colocalitza amb lípids oxidats.[100,108] Diversos estudis *in vitro* mostren que el PPARγ és activat per derivats oxidats dels PUFA. L'àcid linoleic conjugat és un activador amb baixa afinitat perquè se'n requereixen concentracions elevades (μM-mM).[109] En canvi, els hidroperòxids 9-HODE i 13-HODE ja són activadors amb afinitat mitjana (rang μM)[110] i els oxo-octadecanoics 13-oxoODE i 9-oxoODE junt amb el fosfolípid oxidat hexadecil acelaoil fosfatidilcolina (azPC)[32] són activadors d'elevada afinitat (<1μM). A més, l'hidroxialdehid 4-HNE mostra sinergisme

amb els lligands PPARγ en el control del creixement i la diferenciació de cèl·lules tumorals humanes.[111] Tot i el coneixement actual encara falta per descobrir el lligand endogen de PPARγ. Els millors candidats sembla que es troben entre els derivats oxidats dels fosfolípids, ja que de tots els compostos testats són els més potents.

3.4 PPARγ i l'homeòstasi dels lípids en el macròfag

La relació entre la captació de lípid i l'eflux de colesterol en el macròfag influeix en el desenvolupament i la progressió de les lesions ateroscleròtiques a causa de la importància de les cèl·lules escumoses en l'aterogènesi.[8] Nagy et al. i Tontonoz et al. van suggerir la implicació de PPARγ en la captació de LDLoxidada per part del macròfag a través del receptor *scavenger* CD36.[110,112] Aquesta primera evidència va fer pensar que el PPARγ promovia l'acumulació de lípid en el macròfag.[110] D'altra banda, s'ha demostrat un augment intracel·lular de colesterol en absència de LDL modificada que s'explicaria per un increment de l'expressió de dos gens implicats en el metabolisme del colesterol: 3-hidroxi-3-metilglutaril coenzima A (HMG-CoA) sintasa i la HMG-CoA reductasa a través de PPARγ.[113] No obstant això, estudis recents demostren que no hi ha acumulació neta de colesterol.[114]

D'una banda, l'activació de PPARγ exerceix efectes oposats sobre els diferents receptors *scavenger:* mentre que augmenta l'expressió del receptor CD36, disminueix la de SR-A.[115] A més, redueix la captació de LDL glicades i de les lipoproteïnes riques en triglicèrids disminuint la secreció i l'activitat de l'enzim lipoproteïna lipasa (LPL) [116-117] i suprimint l'expressió del receptor de l'apo B48[118] respectivament. D'una altra, també s'ha observat que l'activació de PPARγ redueix l'acumulació d'èsters de colesterol en el macròfag.[114] I a més els activadors de PPARγ també controlen el transport revers de colesterol, l'anomenat eflux de colesterol. L'efecte potenciador de l'eliminació de colesterol en què intervé l'apolipoproteïna AI per donar lloc a la formació de partícules HDL es provoca augmentant l'expressió del transportador ABCA-1,[119-120] i del receptor *scavenger* CLA-1/SR-BI[121] del macròfag.

Per parlar, doncs, dels efectes sobre els nivells de lípid en el macròfag cal que considerem el balanç dinàmic entre l'entrada i l'acumulació de lípid amb l'eflux de colesterol en aquestes cèl·lules. El resultat net serà un factor molt important en la progressió de l'aterosclerosi[107] (fig. 11).

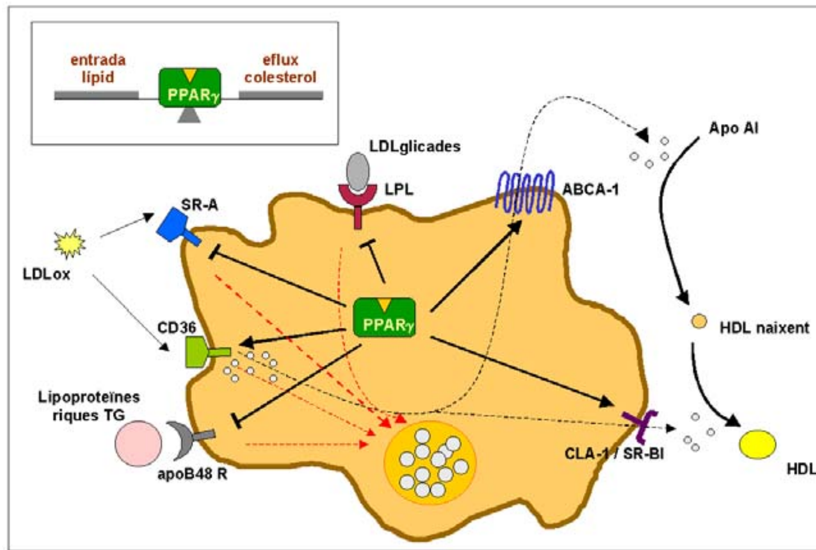


Figura 11. Efectes del PPARγ sobre l'homeòstasi dels lípids en el macròfag.

3.5 PPARγ i la resposta inflamatòria del macròfag

S'ha proposat la implicació del PPARγ en la resposta inflamatòria del macròfag, ja que els activadors mostren efectes antiinflamatoris en estudis *in vitro* i *in vivo*.[122-126] A més, s'ha observat que el PPARγ pot interferir en diferents passos de la resposta inflamatòria que inhibiran l'expressió de molècules proinflamatòries secretades pels macròfags com el TNFα, l'IL-6 i l'IL-1β. L'efecte sobre la resposta inflamatòria seria degut a mecanismes indirectes, ja que no s'han descrit PPRE en gens implicats en la resposta inflamatòria com en el gen del TNFα.[127] D'una banda, s'ha postulat que l'efecte podria ser el resultat d'una competició del PPARγ activat per cofactors (per ex. CBP/p300)[128] que compartirien altres factors de transcripció com NF-κB i AP-1, els quals regulen la transcripció del gen del TNFα.[47,129] D'una altra, el PPARγ activat podria unir-se directament a subunitats d'aquests factors de transcripció. Ambdues accions bloquejarien l'efecte activador de NF-κB i AP-1 sobre TNFα, que podria generar una inhibició de l'expressió de la citocina proinflamatòria (fig. 12). Patel et al. van publicar la primera evidència de la implicació de PPARγ en l'efecte d'un lligand de PPARγ, la rosiglitazona, sobre la reducció de la secreció d'IL-6 en macròfags utilitzant HMDM (macròfags derivats de monòcits humans aïllats de sang perifèrica) *knockout* per PPARγ obtinguts per tractament *antisense*.[130] Actualment existeix controvèrsia en aquest tema, ja que Chawla et al. havien publicat prèviament un treball en el qual demostraven que els efectes inhibidors dels activadors

de PPARγ en la producció de citocines i en la inflamació podrien ser independents del receptor en macròfags murins diferenciats a partir de cèl·lules mare embrionàries deficients en PPARγ.[131] A més, en aquest estudi van utilitzar dosis d'activadors de PPARγ molt elevades, superiors a les requerides per unir i activar el receptor. A aquests nivells es poden activar altres subtipus PPAR i fins i tot s'ha descrit que els activadors de PPARγ a dosis elevades poden exercir efectes antiinflamatoris independents de receptor i modificar covalentment el domini d'unió a DNA de NF-κB o l'activació de la seva quinasa IKK,[132-134] ambdues accions bloquejarien l'efecte activador de NF-κB sobre TNFα.

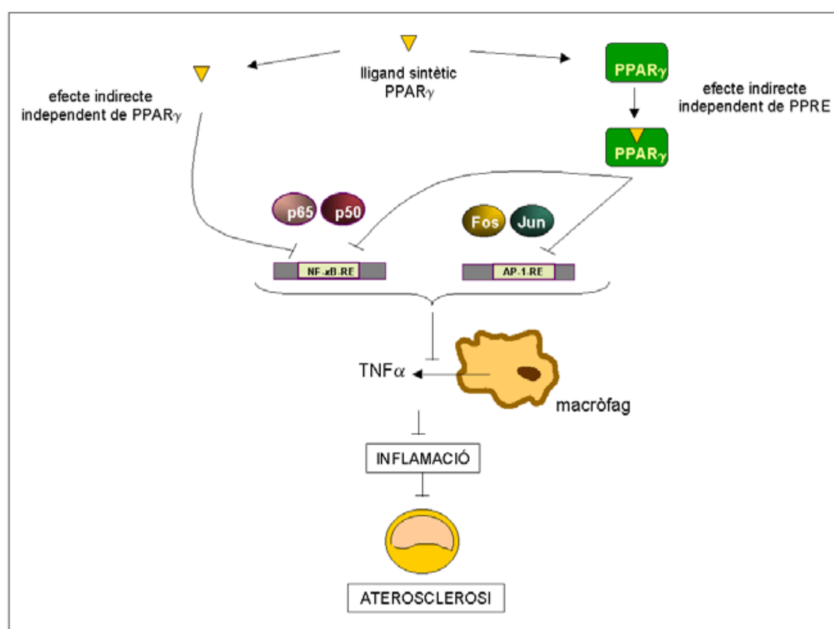


Figura 12. Mecanismes indirectes d'acció antiinflamatòria postulats pels lligands de PPARγ.

4. PREVENCIÓ I TRACTAMENT DE L'ATEROSCLEROSI

La principal recomanació, com en tota malaltia, és la prevenció tant en població general com en població de risc. En aquest s'hauran d'establir uns hàbits de vida saludables entre els quals destaquem la ingesta d'una dieta cardiosaludable, el control del pes corporal, un augment de l'activitat física i deixar de fumar. Totes les recomanacions de les societats científiques sobre el control del colesterol basen les seves pautes a reduir els nivells de colesterol LDL.[5,135-137] Així doncs, la reducció alentiria la formació de plaques ateroscleròtiques i reduiria la susceptibilitat a l'oxidació de les LDL abans de la malaltia. Un cop la malaltia s'hagués establert, estabilitzaria les plaques i previndria

síndromes coronaris aguts. També es posa especial èmfasi en el tractament de la diabetis.[5] Aquests pacients tenen un elevat estrès oxidatiu que els condiciona com a grup d'alt risc cardiovascular.[138-140]

4.1 Recomanacions dietètiques

La manera més saludable d'influir sobre els nivells de colesterol LDL i la seva susceptibilitat a l'oxidació és controlant de la ingesta de lípids. Aquest control estarà basat sobretot en la quantitat i el tipus de greix. Es recomana reduir la ingesta total de greix, reduir el greix saturat i augmentar la ingesta de fruita i verdura. Segons les últimes pautes de l'ATP III, també s'inclou la ingesta de fibra soluble, proteïnes de la soja i esterols.[5] Bàsicament el perfil general de dieta recomanable s'ajusta a la dieta mediterrània. La característica més important és que es tracta d'una dieta molt variada amb un alt contingut en agents antioxidants però el fet més destacable rau en el contingut en greix. Encara que podria considerar-se elevat, la major proporció ve donada per la ingesta d'àcids grassos monoinsaturats, essencialment àcid oleic, provinent sobretot de l'oli d'oliva. Aquest tipus de greix és més resistent a l'oxidació i està associat a una menor incidència de malaltia cardiovascular.[141]

4.2 Fàrmacs

Malgrat que en molts casos l'aplicació de mesures dietètiques millora les variables esmentades, en general es requereix un tractament farmacològic. Per assolir el primer objectiu terapèutic, reduir els nivells de colesterol LDL, les estatines són els fàrmacs de primera línia.[5] Per al segon objectiu, el control de la diabetis, les tiazolidinediones són fàrmacs d'implantació recent al nostre país que han aportat millores terapèutiques importants, no només pel que fa a la resistència a la insulina i al control de la glucèmia sinó també pel potencial paper protector en el desenvolupament de la patologia cardiovascular ateroscleròtica.

4.2.1 Estatines

Les estatines o inhibidors específics de l'enzim 3-OH-3-metil-glutaril-CoA (HMG-CoA) reductasa de la via de síntesi del colesterol són els fàrmacs hipolipemiants més utilitzats en el tractament de l'aterosclerosi. Nombrosos estudis d'intervenció clínica avalen els beneficis de la teràpia amb estatines per reduir el risc d'esdeveniments cardiovasculars tant en prevenció primària (WOSCOPS,[142] AFCAPS/TexCAPS,[143] HPS[144]) com en

prevenció secundària (4S,[145] CARE,[146] LIPID,[147] MIRACL,[148] LIPS,[149] HPS[144]). No obstant això, s'ha observat que els efectes clínics són més potents que la reducció lipídica i l'alentiment de la progressió de la lesió que produeixen.[150] Aquest fet ha dut descobrir el que s'anomenen efectes pleotròpics de les estatines i que són independents de la seva activitat hipolipemiant (fig.13). Aquests efectes estarien involucrats en el desenvolupament de la placa d'ateroma. Les estatines tenen efectes antiinflamatoris, antioxidants i antitrombòtics i poden millorar la funció endotelial, l'estabilitat de la placa i inhibir la proliferació de les SMC.[151-153] La simvastatina i la fluvastatina, dues estatines lipofíliques, redueixen l'oxidació de les LDL en individus hiperlipèmics.[154-155] A més, la simvastatina redueix força la producció global d'aldehids en aquestes partícules *in vitro*. Concretament, la major reducció s'observa en els nivells d'hexanal i 2,4-decadienal.[154] D'altra banda, tant la fluvastatina com la simvastatina disminueixen l'activitat del TF basal i la induïda per LPS i LDL acetilada en macròfags *in vitro*.[156]

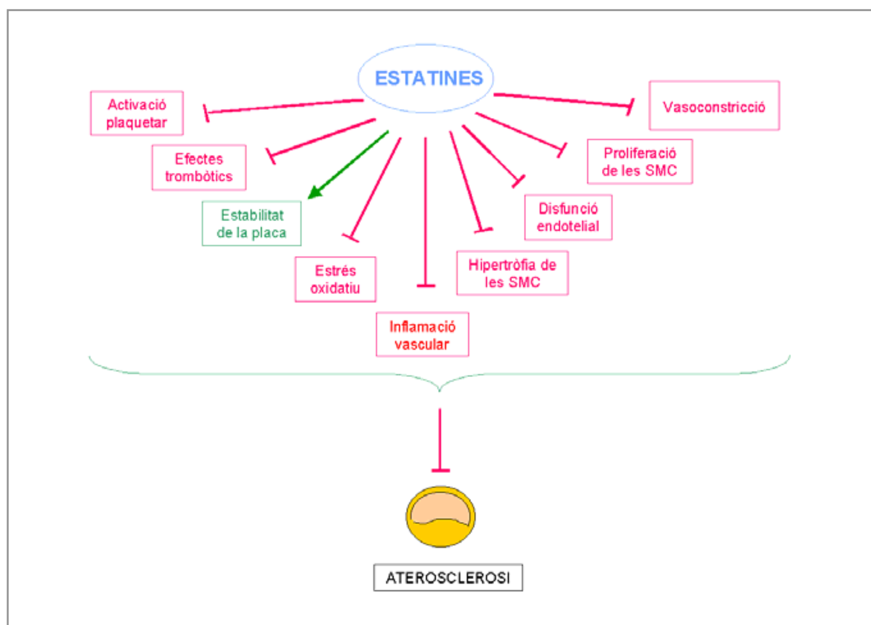


Figura 13. Efectes pleotròpics de les estatines.

4.2.2 Tiazolidinediones

Les benzil-tiazolidinediones o glitazones són una família de nous fàrmacs que han demostrat eficàcia en el control de la glucèmia i en la reducció de la resistència a la insulina en pacients amb diabetis *mellitus* tipus 2.[157-161] Aquests fàrmacs també presenten potencials efectes beneficiosos sobre el risc de malaltia cardiovascular en aquests pacients.[162-163] Les més potents són la rosiglitazona i la darglitazona.[159] Actualment només es tenen dades clíniques de dos anys de tractament amb aquests fàrmacs i s'esperen resultats d'assajos clínics a més llarg termini que evidenciïn aquest fet i n'avaluïn l'efecte sobre la placa d'ateroma. A més, s'han establert un seguit d'efectes pleotròpics[164] el mecanisme dels quals no es coneix però se suggereix que estan associats a la interacció d'aquests fàrmacs amb el PPARγ.[165] La rosiglitazona actua com un agonista total del PPARγ pel qual presenta una afinitat elevada.[166] En el nostre cas, ens són d'especial interès els seus efectes sobre l'aterosclerosi. Les dades demostren un efecte global antiateroscleròtic sobretot basat en l'acció antiinflamatòria que es manifesta en la inhibició dels nivells de citocines proinflamatòries com el TNFα en sèrum de pacients diabètics tipus 2 amb malaltia coronària tractats amb rosiglitazona[126] i en macròfags d'estudis *in vitro.*[122] Altres accions beneficioses de les tiazolidinediones sobre la biologia vascular se centren en el metabolisme del colesterol en el macròfag. Recentment s'ha suggerit que a través de la modulació oposada que exerceixen sobre els diferents receptors *scavenger* dels macròfags[115] i potenciant l'augment de l'eflux de colesterol del macròfag,[119-121] no afavoririen l'acumulació de colesterol en aquestes cèl·lules i podrien influir en la progressió de la lesió ateroscleròtica.[107] Aquesta hipòtesi contradiu estudis anteriors que suggerien que l'acció d'aquests fàrmacs potenciaria l'acumulació de colesterol, la formació de cèl·lules escumoses i l'evolució de la lesió.[110]

Encara són necessaris més estudis per determinar quin és el paper del PPARγ en la progressió de l'aterosclerosi. Es postula que l'acció antiinflamatòria de les tiazolidinediones seria deguda a efectes indirectes de PPARγ o fins i tot independents de receptor en el cas de dosis elevades de fàrmac. S'ha suggerit que les tiazolidinediones *per se* podrien modificar covalentment el domini d'unió al DNA d'aquests factors de transcripció o bé inhibir-ne l'activitat transcripcional.[132-134]

HYPOTHESIS AND OBJECTIVES

Hypothesis

Apolar aldehydes, the end products of PUFA peroxidation present in LDL, might accumulate in the lipidic necrotic core of atherosclerotic plaques and contribute to the generated damage. At this stage, aldehydes might act as gene expression modulators by inhibiting the inflammatory response and leading to the thrombogenic processes associated to plaque rupture.

General objective

This study tries to deepen the knowledge of the role of lipid peroxidation on inflammation and thrombosis.

The specific objectives of this study were to investigate:

1. The cytotoxic effects of lipid peroxidation:
    - 1.1. Aldehyde effect on cellular integrity
    - 1.2. Aldehyde cytotoxicity mechanism
2. The implication of lipid peroxidation on thrombosis development through the study of human TF gene expression on response to aldehydes in human smooth muscle cells:
    - 2.1. Aldehyde effect on TF gene expression
    - 2.2. Regulatory mechanism involved
    - 2.3. The effect on this mechanism of a hypolipidemic drug with antioxidant properties
3. Implication of the nuclear receptor PPARγ on the inflammatory response
    - 3.1. Study of the human CD36 gene
    - 3.2. Study of the human TNFα gene
        - 3.2.1 Identification of PPARγ response elements in the promoter
    - 3.3. Aldehyde effect on PPARγ

ABSTRACT AND GENERAL DISCUSSION

The results of this thesis are presented as five studies in the Article format. They are divided in two sections: results and annexes. The results section includes studies 1 and 2, which have already been published in *Atherosclerosis* and in the *Journal of Cellular Physiology* respectively. The Annex section includes study 3, which consists of a short communication published in Clinical Chemistry Laboratory Medicine and a manuscript submitted to *Biochemical Journal*. Also included in this section is, study 4, which is a manuscript in preparation, and study 5, which is a paper published in the *European Journal of Clinical Investigation* in collaboration with another research group.
The work presented in this thesis has been done in the field of atherosclerosis. Results show new knowledge of the lipid peroxidation role in the pathogenesis of atherosclerosis emphasized in the action on inflammation and thrombosis, which are key processes in the beginning, the evolution and the complications of the disease.
In summary, the obtained results support the theory that lipid oxidative modification plays an important role in the progression of atherothrombotic disease.

Studies 1 and 2 show the effects in HVSMC of two representative apolar aldehydes: hexanal and 2,4-decadienal, compounds derived from the oxidation of main PUFAs in LDL. In study 1, we analyzed aldehyde cytotoxicity and in study 2, their prothrombotic potential based on TF expression. Results show that from both aldehydes studied, 2,4-decadienal contributes to the well-known toxicity of oxidized LDL in SMC associated to its lipidic part and partially explained by the action of oxysterols.[59] The expression of the oncosuppressor gene p53 and the overexpression of the c-myc gene are associated to up-regulation of apoptosis.[62] Our data show that both apolar aldehydes tested reduce p53 and c-myc mRNA levels and do not induce the DNA laddering characteristic of apoptosis, suggesting that programmed active cell death is not induced by these peroxidation products in HVSMC. The protooncogen c-myc mediates both cell death and proliferation depending on the levels of expression.[63] An overexpression of c-myc can increase the expression of the proapoptotic p53 gene.[64] This is due to the fact that p53 promoter contains a c-Myc consensus DNA binding element and could be transactivated by c-Myc.[65] Our results suggest that the observed decrease in p53 gene expression may be explained by the decrease in c-myc gene expression. Our results also show that the downregulation of c-myc expression produced by aldehydes was associated with cell growth arrest. A relationship between regulation of c-myc expression and SMC growth inhibition has previously been reported.[167] In conclusion, our results suggest that the well-

known cytotoxic effect of aldehydes would be promoted by cell necrosis instead of an apoptotic mechanism. Therefore, these oxidative products may contribute to atherosclerotic plaque instability.

In study 2, we detected the presence of aldehydes in human artery wall fragments. We observed an increase of the apolar fraction of aldehydes in pathological tissue. Previous reports support the fact that oxidized LDL and their oxidative compounds are present *in vivo*.[21-32] Thus, it is important to study their biological activity in order to establish their potential implication in atherosclerotic plaque evolution. We evaluated the prothrombotic effects of these two aldehydes. Hexanal and 2,4-decadienal enhance TF mRNA and protein levels. TF is thought to be the major mediator of the thrombogenic mechanism associated to plaque rupture.[69] Previously, reports in different cell types on oxidized LDL effects in TF expression showed discrepancies in the results. An increase of TF expression was reported in endothelial cells[76-80] whereas induction, no effect or inhibition of TF expression was described in macrophage cells.[81-83] It has also been established that oxidized LDL induces TF activity in SMC and that this effect was associated to the lipidic part of the lipoprotein particle.[86-87] Two lipidic fractions of the particle are particularly responsible for this action. One was identified as the hydroperoxide lipids of cholesterol present in it and the other one, which is formed by less polar lipids, remains unknown.[87] Our data suggest that this other fraction may be represented by apolar aldehydes due to the observed actions on TF expression of these oxidative products which are present in oxidized LDL and they are less polar than the above mentioned lipidic products. It is important to emphasize the observed action of hexanal, which is a less studied aldehyde and has usually been considered biologically neutral. However, hexanal is quantitatively very important in *in vitro* oxidized LDL. In our study, hexanal showed higher prothrombotic potential than 2,4-decadienal based on their effects on TF mRNA and protein levels. These results support the previously demonstrated less polar property of TF inducers. Our data suggest that the mechanism of action of TF induction by aldehydes in SMC may be through the activation of the AP-1 transcription factor formed by the complex cFos-cJun. Results showed that both aldehydes studied increase the mRNA levels of the early response gene c-fos and AP-1 binding activity to the promoter of the TF gene. These data correlate with the results from an array assay that showed that oxidized LDL has the ability to induce a group of genes involved in transcription in SMC.[88] On the other hand, the antioxidant ability of two lipophilic statins (simvastatin and fluvastatin) used in lipid lowering treatment in atherosclerosis has been

previously described.[154-155] From them, simvastatin reduces aldehyde production *in vitro* and *in vivo*.[154] Indeed both statins modulate gene expression and prevent TF induction by LPS and acetylated LDL in macrophages.[156] In this study, we introduced pretreatment of cells with simvastatin, which produced attenuation of c-Fos protein expression induced by apolar aldehydes, probably by interference with intracellular signalling. Our findings from studies 1 and 2 suggest that the studied aldehydes may activate TF through *de novo* synthesis of c-Fos and they add more data about the benefits of the pleiotropic effects of statins in the pathogenesis of atherosclerosis.

These aldehydes may be retained in the core of oxidized LDL particles due to their polarity characteristics. In the lipidic necrotic core of the lesion, the amount of these products may be higher as a result of cellular apoptosis and they may exert their biological effects. If these aldehydes *in vivo* showed similar effects to our findings, they may enhance the damage in the artery wall. Processes of cell death and thrombosis may dominate this environment since at this level the inflammatory response might not be able to protect the artery segment affected. This hypothesis is in accordance with Berliner et al., who suggested that products formed in mildly LDL oxidation have important inflammatory effects whereas those generated as a result of an extended LDL oxidation will contribute to cytotoxicity.[43] Moreover, previous reports of the group describe that the studied aldehydes have antinflammatory effects decreasing the mRNA levels of the proinflammatory cytokine TNFα and its secretion in macrophages.[54-55] The mechanism by which aldehydes produce this action is still unknown and it was our intention to shed some light on it in studies 3 and 4. Thiazolidinediones, drugs used in the treatment of type 2 diabetes *mellitus*, show beneficial effects for the vascular wall independent of their hypoglycaemic action.[164] *In vitro*, these drugs have antiinflammatory potential in macrophages inhibiting TNFα.[122] At the moment, the mechanism underlying this effect is not well-established, although there exist several hypotheses most of which link to the fact that these drugs are high affinity agonists of PPARγ.[165] Rosiglitazone maleate or BRL is one of the most potent PPARγ agonists.[166] It has been postulated that this action could be receptor independent. On the one hand, it has been suggested that PPARγ agonists *per se* (independently of their receptor, PPARγ) at high doses may inhibit the expression of proinflammatory cytokines such as TNFα.[132-134] On the other hand, it has also been suggested that there could be an indirect effect of PPARγ.[127-128,131] Both hypotheses

converge in that the observed antiinflammatory effect will be by result of a negative interference in the NF-κB, AP-1 and STAT-1 pathway.

PPARγ expression has been detected in vascular cells (endothelial cells[98], SMC[99] and macrophages[100]) and in atheroma plaques, where it colocalizes with oxidized lipids.[100,108] The endogenous PPARγ ligand is also still unknown. The oxidative products derived from phospholipids seem to be the best candidates since, from all the studied compounds, they are the ones which present a higher affinity for PPARγ. Our hypothesis was that PPARγ may produce a direct effect on TNFα and we proposed aldehydes as candidates to be endogenous ligands of PPARγ.

In study 3, we focused on the analysis of the human TNFα promoter sequence in order to find putative PPARγ binding elements (PPRE) and study 4 shows our present data on the research of aldehydes as candidates to be PPARγ ligands. Study 3 describes two regions of the human TNFα promoter similar to the PPRE sequence. The structure of the consensus element consists of two direct imperfect repeats of the hexamer AGGTCA with one base spacing (DR-1) or in some cases two base spacing (DR-2).[91-94] In the last few years, the research of many groups on the finding of putative PPRE in genes involved in lipid homeostasis, has lead to the description of important variations in the described consensus sequence and in the PPARγ mechanism of action. A high number of base changes in the described repeats have been reported but the constitutively most different with respect to the consensus sequence were found in the promoters of the cholesteryl ester hydrolase (CEH) and the hepatocyte growth factor (HGF) genes. In the CEH gene, there are three functional PPRE half-sites, two of them in the complementary strand and in both sense and antisense directions.[95] Functional PPRE in HGF is composed of an imperfect inverted repeat of the AGGTCA motif with two spacers.[96] Apart from this, PPARγ, in general, acts as a transcription factor heterodimerized to RXRα, another nuclear receptor,[89] but depending of the relation between PPARγ and RXRα content in the cell, PPARγ may act as a homodimer.[90] Considering the elements that we described in the human TNFα promoter, PPRE1 has a DR-3 structure (3 base spacing the two repeats) whereas PPRE2 seem to be a degenerated DR-1 since the base spacing the two repeats is lacking. In both cases, the elements are formed by imperfect direct repeats (1 base differs in each hexamer from the consensus sequence) and both elements are located in the complementary strand. We could wonder: How is it possible that a protein

with well defined structural characteristics as PPARγ can bind to such a wide variety of regions? It may be explained by modifications in its DNA coupling. Transcription factors bind to two consecutive major grooves of DNA and the distance between them is known and fixed. Thus, it is reasonably to think that DNA binding proteins should adopt the conformation necessary to fit in this distance. However, it has been reported that in certain occasions the DNA double helix may undergo slight coiling changes. This could be the explanation by which having one base less or three bases more in the sequence, as our case, could be reverted by a DNA supercoiling or with a DNA uncoiling, leading to the correct orientation and distance of both hexameric repeats to allow PPARγ binding. Indeed, the molecules that are described as possible ligands have important structural differences between them. This could be explained by the big ligand pocket that has PPARγ and the ability of it to couple to different structures.[168-170] All this variety may explain that PPARγ activation leads to the widely described actions attributed to PPARγ and one could even speculate that the response of different PPRE elements could depend on the different ligand bound or even the fact that PPARγ acts as a heterodimer or as a homodimer. Our data show that rosiglitazone clearly induces PPARγ binding activity to PPRE2 whereas it is more difficult to establish an increase to PPRE1 but it seems that there exists a tendency. However, it is evident that in both cases PPARγ binds to the studied elements since both shifts are affected by competition assays with DR-1. On the other hand, we don't know how the elements will respond to other ligands or to different conditions of the cell. The transfection studies gave us data about the human TNFα transcriptional activity. PPARγ expression decreased the basal transcriptional activity as well as the induced by phorbol esters in the presence and in the absence of rosiglitazone and this effect was lower when the possible PPRE elements included mutated bases in its sequence. These results support our hypothesis about a PPARγ direct action on TNFα expression. The remaining inhibitory effect observed in the presence of both mutated sites, could be explained by the indirect PPARγ mechanism suggested by other authors. The results obtained as a result of blocking the described indirect pathway confirm the existence of a PPARγ direct action on the inflammatory response. Our results also discard the action of rosiglitazone *per se* and independent of its receptor because all the experiments were performed at low doses of the drug (1μM). At this concentration, it has been demonstrated that there is no activation of the other PPAR isoforms[171] and we show that the drug was even able to reduce the endogenous TNFα of macrophages. On the other hand, PPRE1 seems to be a very important region

in the regulation of TNFα basal trancriptional activity because the introduction of mutations on its sequence produces high changes in this activity. In conclusion, data from study 3 suggest the existence of a new signaling pathway that contributes to the antiinflammatory action of PPARγ ligands.

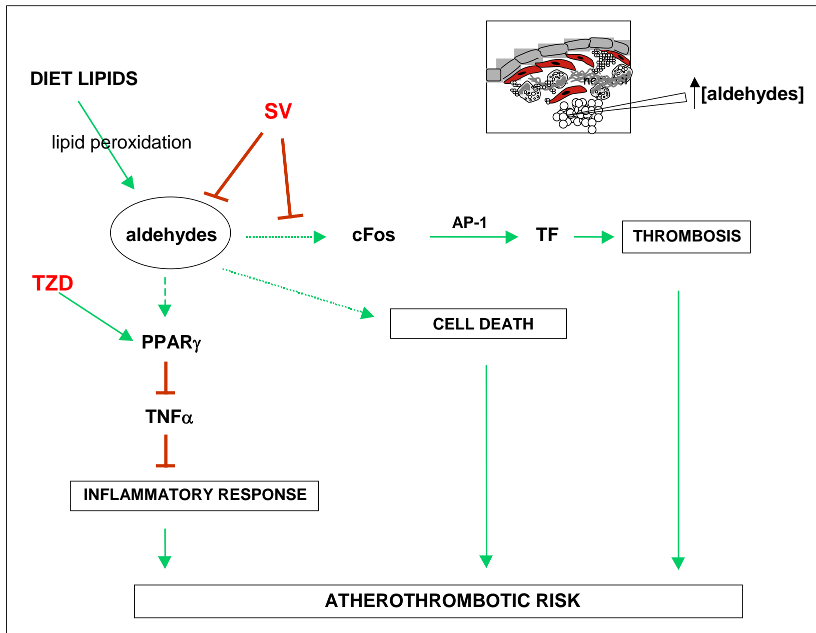
This study implied the setting up of many molecular biology techniques such as site-directed mutagenesis. The short communication published in Clin Chem Lab Med 2004 describes an improvement of the method based in the commercial kit QuickChange Site-Directed Mutagenesis kit developed by Stratagene. This kit is widely used to introduce up to four nucleotide changes in specific DNA regions. To introduce a high number of mutations it is necessary to perform sequential mutagenesis experiments. The approach to the methodology that we present in the paper is a faster and cheaper way of introducing up to eight consecutive mutations in a DNA sequence in a single step using only one pair of primers. Thus, reduces the possibility of random mutation in the rest of the target sequence.

Once we had seen the possibility that PPARγ directly acts in the regulatory mechanism of TNFα, we needed the rest of our hypothesis to establish whether aldehydes regulate TNFα through PPARγ. In study 4, we looked into the possibility that aldehydes may act as PPARγ activators. This paper is in preparation, as we are still running some experiments. We started the work using the aldehyde 2,4-decadienal because it is the aldehyde that shows the highest inhibition of TNFα. 2,4-decadienal is able to produce changes in the nuclear levels of PPARγ protein without altering PPARγ steady state mRNA levels in macrophages. These data suggest that 2,4-decadienal may be involved in the translocation of PPARγ to the nucleus, which may caused effects on PPARγ activation non related to its synthesis. 2,4-decadienal also enhances PPARγ mediated transcriptional activity dose dependently. We also studied the effect of 2,4-decadienal on the main mechanism mediated by PPARγ, the differentiation of adipocytes. We observed that the aldehyde, *per se*, was able to induce the adipogenesis of 3T3-L1 cells and it seems to act synergistically to thiazolidinediones in this action. Our data, suggest that 2,4-decadienal may be an endogenous mediator of PPARγ in the atheromatous plaque and they open the possibility to follow studying on this way.

Study 5 is the result of a collaboration with a research group from the Wallenberg Laboratory in cardiovascular research, Göteborg University and the AstraZeneca

company, both from Sweden. AstraZeneca was interested in the effects of a new thiazolidinedione, darglitazone. As it has been reported, thiazolidinediones have shown beneficial effects on the vascular wall, independent of their hypoglycemic action.[164] Although existing data showed a global antiatherosclerotic action, it has been suggested that these drugs may produce a proatherosclerotic effect in macrophages.[110] As a result of the induction of CD36 expression that they produce, macrophages begin to accumulate lipid and to differentiate to foam cells. The influx of these cells to the lesion may lead the progression of it. Our data suggest that this effect may not be produced in insulin resistance and in type two diabetic patients treated with these drugs. These patients have high levels of circulating free fatty acids (NEFA) and results from study 5 reveal that darglitazone in the presence of NEFA neither alters CD36 expression in human monocytes and macrophages nor modifies macrophage lipid content. These data about the lipidic balance contradict evidencies of other research groups that suggest a proatherosclerotic effect of PPARγ caused by an increase of lipid accumulation in macrophages. Other recent studies, have already shown that PPARγ activators do not produce cholesterol accumulation in macrophages since they regulate lipid entrance through the modulation of the expression of *scavenger* receptors as well as cholesterol efflux increasing the expression of the ABCA1 transporter.[119-120] More recently, Argman et al., demonstrated that PPARγ activators reduce the accumulation of cholesterol esters in macrophages.[114] Our study offers more physiological data coincubating cells with NEFAs and darglitazone and shows that darglitazone does not cause any changes in the triglyceride content of macrophages. Thus, the net balance between the cholesterol entrance and efflux in the macrophage seem to be negative in response to PPARγ activators. This fact suggests that again these drugs may exert benefits at vascular level decreasing the progression of the lesion.

The following figure summarizes the implications on the atherothrombotic risk of the results obtained in the studies presented in this thesis:



SV: simvastatin, AP-1: activated factor 1, TF: tissue factor, TZD: thiazolidinedione, PPARγ: peroxisomal proliferator activated receptor gamma, TNFα: tumor necrosis factor alpha.

CONCLUSIONS AND PERSPECTIVES

Study 1 and 2

- 2,4-decadienal shows cytotoxic effects on human vascular smooth muscle cells that could be explained by cell necrosis.
  These data suggest that 2,4-decadienal may contribute to increase the lipid and cell debris core of advanced atherosclerotic plaques and to the reduction of the lesion cap, through cell necrosis of smooth muscle cells from the lipid-rich core and from the fibrous cap of the lesion. These actions may contribute to atheromatous plaque instability leading to a more vulnerable plaque.

- The studied aldehydes have prothrombotic properties in human smooth muscle cells. They increase TF expression activating its transcription factors.
  These data suggest that the studied aldehydes may contribute to the thrombogenic processes associated with atherosclerotic plaque rupture.

Taken together, both studies support the theory that lipid modification and its derived products have an important role in the progression of atherothrombotic disease and in its associated complications.

Study 3 and 4

- PPARγ has a direct implication in the regulation of the inflammatory response inhibiting TNF-α expression since we have identified two PPRE-like elements in the human TNF-α promoter.
- The proposed approach to the widely used site-directed mutagenesis methodology, allows a faster and cheaper way of introducing up to eight consecutive mutations and reduces the possibility of potential random mutations in the target sequence.
- 2,4-decadienal induces the transcriptional activity mediated by PPARγ and also enhances adipocyte differentiation, which is the main mechanism regulated by PPARγ.

These results demonstrate that aldehydes may regulate gene expression and they also define a mechanism by which lipid peroxidation regulate the inflammatory response. Furthermore, it opens a new antiinflammatory target pathway for other PPARγ ligand drugs. Moreover, 2,4-decadienal may be a potential candidate to PPARγ endogenous ligand.

Study 5

- Darglitazone, in the presence of physiological and supraphysiological concentrations of NEFA,:
    1. does not activate the expression of the scavenger receptor CD36 on the surface of human monocyte and macrophages, and
    2. it does not produce alterations in the triglyceride content of human macrophages

- Darglitazone does not increase cholesterol accumulation in human macrophages.

These data suggest that treatment with PPARγ activating drugs would reduce lipid net accumulation in macrophages of type 2 diabetic patients.

The observed effects would reduce the formation of foam cells and their influx to the lesion, which would decrease the progression of the atherosclerotic lesion and plaque vulnerability.

The results of the studies in this thesis have motivated us to continue this line of investigation. We have answered the questions posed by our hypotheses and many possibilities have opened up for future investigation. The immediate objectives derive from studies 3 and 4:

1. To study the possibility of a direct interaction between 2,4-decadienal and PPARγ.
2. To study the functionality of the putative PPRE elements described in the human TNF-α promoter, in response to 2,4-decadienal.
3. To evaluate the antiinflammatory effect of PPARγ activating drugs treatment in type 2 diabetic patients.
    - 3.1. To analize the relationship between the inflammatory status and the oxidative stress with the severity of the disease.
    - 3.2. To evaluate the activation status of PPARγ in cells from peripheral blood of these patients.
4. To investigate the potential of 2,4-decadienal on other processes mediated by PPARγ, such as proliferation of tumoral cells, differentiation of adipocytes and glucose metabolism.

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TREBALLS

ESTUDI 1

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ATHEROSCLEROSIS

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Cytotoxic effects of the lipid peroxidation product 2,4-decadienal in vascular smooth muscle cells

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Abstract

It is well known that oxidized LDL can be cytotoxic to smooth muscle cells (SMC) and then contribute to the progression of atherosclerosis. Nevertheless, which oxidized compound and which mechanism are involved in cell death is still under study. In this work we have studied the role of two representative apolar aldehydes (hexanal and 2,4-decadienal (2,4-DDE)), derived from polyunsaturated fatty acids oxidation, on human SMC cytotoxicity. Cell cytotoxicity was assessed by means of lactate deshydrogenase (LDH) release, cell morphology and DNA fragmentation. Results showed that hexanal up to 50 μM for 24 h was not cytotoxic to cells. However, 2,4-DDE at 50 μM for 24 h induced a 48% LDH leakage. The observed cytotoxic effect of 2,4-DDE was not due to a programmed cell death as no DNA ladder was detected. After aldehydes exposition a decreased expression of p53 and c-myc mRNA, genes involved in cell death regulation, was also demonstrated by RT-PCR. These observations suggest that 2,4-DDE may be the molecular cause of lipid oxidation cytotoxicity to human vascular SMC. By inducing cell necrosis in advanced stages, lipid oxidation may contribute to the cell debris core which is growing in the atherosclerotic lesion leading to a weakened and unstable plaque.
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Keywords: Atherosclerosis; Lipid peroxidation; Cytotoxicity; Human vascular smooth muscle cells

1. Introduction

Experimental evidence supports the fact that oxidation of LDL is implicated in the progression of atherosclerotic lesions [1]. Oxidized LDL (ox-LDL) has been found to have both a positive and negative effect on gene expression and can be toxic for a variety of cell types, including smooth muscle cells (SMC) [2–4]. LDL oxidation represents a gradual process that leads to the formation of different products, several of which could play a role in its cytotoxicity, such as lipid hydroperoxides, aldehydes, oxysterols and lysophosphatidilcholine [5,6].

Of those, lipid hydroperoxides derived from the oxidation of polyunsaturated fatty acids (PUFA) are the first products formed and they have been shown to have a high proinflammatory effect. In contrast, aldehydes, which are final products derived from PUFA oxidation, have been implicated in cytotoxic actions [7]. We have previously reported that the inhibitory effect of extensively oxidized lipoproteins on TNF-α expression in macrophages was due, at least in part, to the apolar aldehydes present in the oxidized lipoprotein particle. From all the aldehydic compounds formed during LDL oxidation, we have found that hexanal is the major molecule formed and 2,4-decadienal (2,4-DDE) has a high biological activity by inhibiting cytokine expression and being cytotoxic in macrophages [8,9].

On the other hand, toxicity to SMC is presumed to be an important atherogenic process, since these cells are lost from deep to mid-intimal locations during development of the lipid-rich core region in atherosclerotic plaques [10]. Different studies have demonstrated that ox-LDL is cytotoxic to SMC and this effect can be mediated by an active death process [11–13]. The tumor suppressive gene p53 and the protooncogene c-myc are expressed in vascular cells playing a role in apoptosis

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regulation during lesion development [14,15]. Hughes et al. showed that ox-LDL cytotoxicity on SMC is attributed to its lipidic extract [16], particularly to oxysterols, although the contribution of other products of oxidation is needed to explain ox-LDL toxicity. Moreover, the exact molecular bases of this mechanism are not known and no data are available on the involvement of apolar aldehydes in this process.

The objective of the present study was to focus on the cytotoxic effects of two apolar aldehydes (2,4-DDE and hexanal) and their effects on p53 and c-myc gene expression in human vascular SMC (HVSMC).

2. Material and methods

2.1. Cell culture

HVSMC from human uterine artery were a kind gift from Dr Eva Hurt-Camejo, Göteborg University (Sweden). Cells were grown in Waymouth medium supplemented with 10% (v/v) foetal bovine serum (FBS), 2% (v/v) sodium pyruvate, 1% (v/v) L-glutamine and antibiotics (Invitrogen, USA). Cells were used at passages 3–6 and all experiments were done in the absence of FBS and at 70–80% confluence.

2.2. Cellular cytotoxicity

Lactate deshydrogenase (LDH) activity was assayed by an enzymatic method (Roche Molecular Biochemicals) in the culture medium and in cell lysates after treatment with 1% triton to assess the cytotoxic effects of the studied aldehydes. HVSMC were incubated with 2,4-DDE and hexanal in the 0–50 μM range for 0.5–24 h. Hexanal and 2,4-DDE (Aldrich, Spain) were of the highest grade of purity available (98 and 85%, respectively) [17]. These percentages were taken into consideration in solution preparations. Each aldehyde was dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Results are expressed as the percentage of LDH leakage, which was calculated as LDH (U/l) in the media divided by LDH in the media plus cell-associated LDH.

Cells were observed under phase contrast microscopy in search of any morphological changes.

2.3. Analysis of mRNA levels by RT-PCR

HVSMC were incubated in the presence of 2,4-DDE and hexanal (0.5, 5 and 25 μM) for up to 2 h. Total cellular RNA was isolated from the cells by the Ultraspec RNA isolation reagent (Biotecx Lab., Inc., USA). A semiquantitative RT-PCR method was used to determine c-myc and p53 mRNA using the SuperScript one-step RT-PCR system (Invitrogen). β-Actin was used as a constitutive expression gene to normalise the results of the genes of interest. Results are expressed as the percentage of variation of the net intensity of each condition with respect to the untreated cells after normalisation to β-actin results.

The sequences of primers (Invitrogen) were as follows: *c-myc* primer minus 5′-CCTGTTGGTGAAGCTAACGTTGA-3′, primer plus 5′-CACTGGAACTTACAACACCCGAG-3′; *p53* primer minus 5′-TTCCTCGTGCGCCGGTCTC-3′, primer plus 5′-AACCTACCAGGGCAGCTACG-3′; and *β-actin* primer minus 5′-TACAGGTCTTTGCGGATGTCC-3′, primer plus 5′-GCTACAGCTTCACCACCACG-3′.

2.4. Cell growth

Cell growth was determined using the vital dye Hoechst 33342 (Sigma) which is permeable through cell membranes and binds specifically to A–T base pairs. Differences in fluorescence values could be related to cell number. HVSMC were incubated with 1 μM of 2,4-DDE and hexanal for up to 3 days. Culture medium was removed and substituted for PBS. Hoechst 33342 dissolved in PBS was added to a final concentration of 1.25 μg/ml for 5 h at 37 °C. Cells incubated with vehicle alone were designated as untreated cells and 10% FBS was used as a positive control of cell growth induction. Results are expressed as the fluorescence intensity measured with a Fluoroskan II (Labsystems).

2.5. DNA fragmentation

To evaluate the existence of a DNA ladder, HVSMC were treated with 50 μM hexanal or 2,4-DDE and after 24 h DNA was extracted from cells using the apoptotic DNA ladder kit (Roche Molecular Biochemicals). DNA samples were resolved following the manufacturer's protocol. DNA extracted from U937 apoptotic cells (treated with 4 μg/ml camptothecin for 3 h) provided in the kit was evaluated with the samples as a positive control of apoptosis.

3. Experimental results

3.1. Valuation of the cytotoxic capacity of aldehydes

Fig. 1 shows the cytotoxic effects of hexanal and 2,4-DDE in HVSMC at a concentration range (0–50 μM) and different exposition time (0.5–24 h) expressed as percentage of LDH leakage in the culture medium. Concentrations of hexanal up to 50 μM did not produce any significant changes of LDH leakage (Fig. 1A). However, 50 μM of 2,4-DDE at 6 h starts to produce elevations in LDH leakage (9%) arriving at very high levels (48%) at 24 h (Fig. 1B).

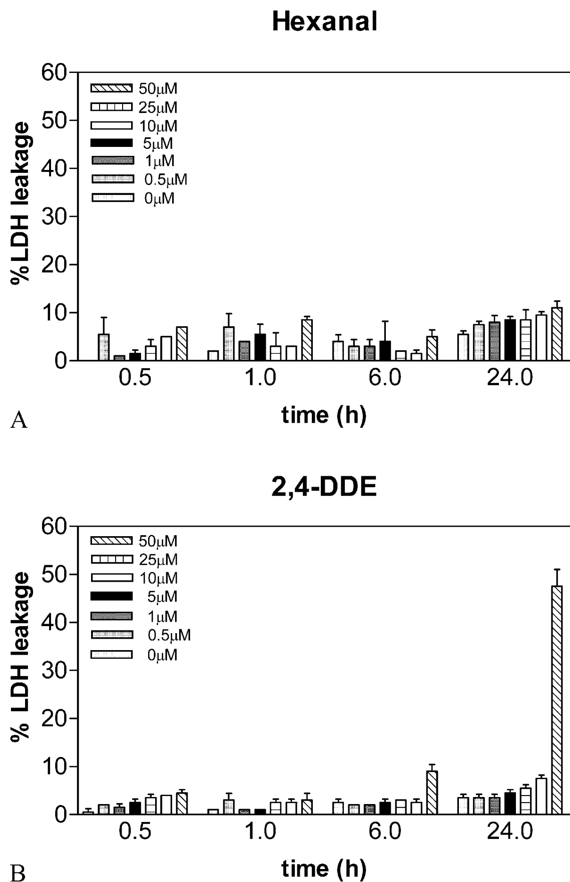

Fig. 1. Cytotoxicity of apolar aldehydes. HVSMC were incubated with 0–50 μM hexanal (A) or 2,4-DDE (B) for 0.5–24 h. Results shown are the percentage of LDH leakage into the medium ±S.E.M. for duplicate wells from one representative experiment.

Neither cellular density nor morphological observable differences under phase contrast microscopy were detected with hexanal. A concentration of 50 μM 2,4-DDE for 24 h was required to observe cellular morphological changes: reduction of cell viability and adoption of a rounded phenotype instead of the typical spindle shape (Fig. 2).

3.2. Effects of aldehydes on c-myc and p53 genes

The effects of hexanal and 2,4-DDE on c-myc and p53 mRNA levels were studied at 2 h by RT-PCR in the non-cytotoxic range of aldehydes concentration (0.5–25 μM). Results show that the apolar aldehydes studied (2,4-DDE and hexanal) decrease both genes in a similar pattern. Fig. 3A shows that concentrations of 0.5 and 5 μM 2,4-DDE produced a 50 and 65% reduction of c-myc and p53 transcription, respectively. On the other hand, hexanal incubations caused maximal reductions of c-myc (61%) and p53 (48%) at 0.5 μM (Fig. 3B).

3.3. Effects of aldehydes on cell growth

2,4-DDE and hexanal at 1 μM do not produce changes in HVSMC growth compared to untreated cells. In parallel, 10% of FBS was used as a positive control to confirm an increase in cell number (Fig. 4).

3.4. Effects of aldehydes on DNA ladder

Due to the fact that 2,4-DDE showed an important cytotoxic capacity, we have studied its effects on nucleosome laddering. DNA extracted from cells incubated 24 h at 50 μM 2,4-DDE did not present the characteristic ladder pattern of an apoptotic state (Fig. 5).

4. Discussion

The present report shows that 2,4-DDE, but not hexanal, aldehydes derived from extensively LDL oxidation, contribute to the cytotoxic effect described for ox-LDL in SMC.

2,4-DDE is an oxidative compound derived from the oxidation of two PUFAs, linoleic acid and arachidonic acid [18] and its level is provided entirely from the lipid intake. Its worth noting that their precursors represent 98% of the total PUFAs in LDL [19]. Although 2,4-DDE is not the major oxidative product in ox-LDL, it has a very high biological action and it has been involved in the modulation of atherogenic processes [8,9].

Our experiments demonstrate that 2,4-DDE at 50 μM and 24 h of exposition produced cell death detectable by increasing LDH leakage, cellular destructuration and morphological alterations in HVSMC. LDH and morphological data also defined a range of between 0 and 25 μM for 30 min to 6 h at which the studied aldehydes were not cytotoxic. Previous reports have shown the cytotoxic effects of cholesterol oxidative components of ox-LDL, such as oxysterols in SMC [16]. However, the contribution of oxysterols is insufficient to account for the toxicity of ox-LDL, suggesting that other products of oxidation present in ox-LDL could act synergistically with oxysterols to increase its cytotoxicity. This hypothesis is in agreement with our findings about cytotoxic effects of aldehydes. Other authors have previously demonstrated the cytotoxic effects of other types of aldehydes. Most published data are about 4-hydroxynonenal (4-HNE) and malondialdehyde, very reactive aldehydes that bind to proteins which impair their function [20]. Although the mechanism of action of 2,4-DDE is still unknown, it is more potent than these other aldehydes probably due to the differences in their polarity and chain length.

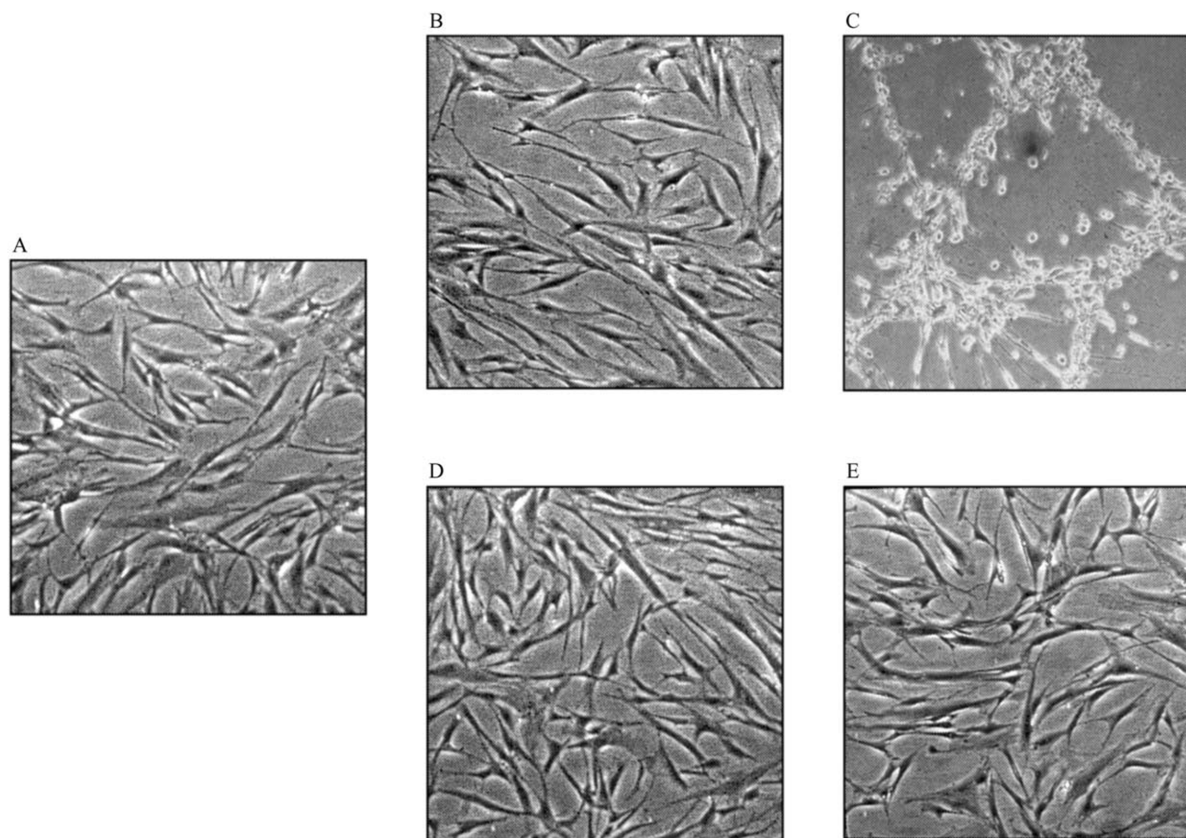

Fig. 2. Phase contrast microscopy photographs (× 100) showing the morphology of HVSMC incubated 24 h with vehicle alone (A), 5 μM 2,4-DDE (B), 50 μM 2,4-DDE (C), 5 μM hexanal (D), 50 μM hexanal (E).

The expression of the oncosuppressor gene p53 and the overexpression of the c-myc gene are associated to up-regulation of apoptosis. Bennet et al. reported that p53 mediates induction of apoptosis by deprivation of growth factors in VSMC transfected with c-myc [14]. Both genes have also been implicated in SMC proliferation control in plaques [21] and also in restenotic lesions [22]. We analyzed the effect of aldehydes at non-toxic concentrations (0–25 μM, 2 h) on p53 and c-myc expression. Our data show that both apolar aldehydes tested reduce the proapoptotic genes p53 and c-myc mRNA levels. Moreover, the DNA laddering characteristic of apoptosis was not induced by aldehydes, even at cytotoxic concentrations, suggesting that programmed active cell death is not induced by these peroxidation products in HVSMC. Previous studies have demonstrated that linoleic acid and its oxidative metabolites, the hydroxyperoxydecadienoic acids at 1 μM induce c-myc mRNA levels [23] whereas, with aldehydes (5 μM), such as the ones used in our study and the hydroxyaldehyde 4-HNE, are able to block c-myc expression in several cell types [24], suggesting a different role for early lipid peroxidation derived products and those produced after a more extended oxidation period. Aldehydes at low concentrations seem to be more related to effects on the expression of early genes whereas cytotoxic effects are attributed to high concentrations. According to Uchida et al., 4-HNE accumulates in membranes at concentrations up to 5 mM in response to oxidative insults [20]. However, there is no data on 2,4-DDE levels in vivo. Published data of apolar aldehyde concentrations in extensive ox-LDL (50 μg protein/ml) give a level of 1 μM for 2,4-DDE [8]. From these data we hypothesized that the levels used in our study could be present in atherosclerotic plaques due to the high accumulation of ox-LDL in the lesion. Moreover, in the atherosclerotic lesion, there may be other lipidic components apart from LDL that could be oxidized and could lead to 2,4-DDE, such as PUFAs from cell membranes or from other lipoproteins.

The protooncogene c-myc mediates both cell death and proliferation depending on the levels of expression [25]. An overexpression of c-myc can increase the expression of the proapoptotic p53 gene [14] due to the p53 promoter containing a c-Myc consensus DNA binding and could be transactivated by c-Myc [26]. Our results suggest that the observed decrease in p53 gene expression may be explained by the decrease in c-myc gene expression.

Our results also show that the downregulation of c-myc expression produced by aldehydes was associated with cell growth arrest. A relationship between down-

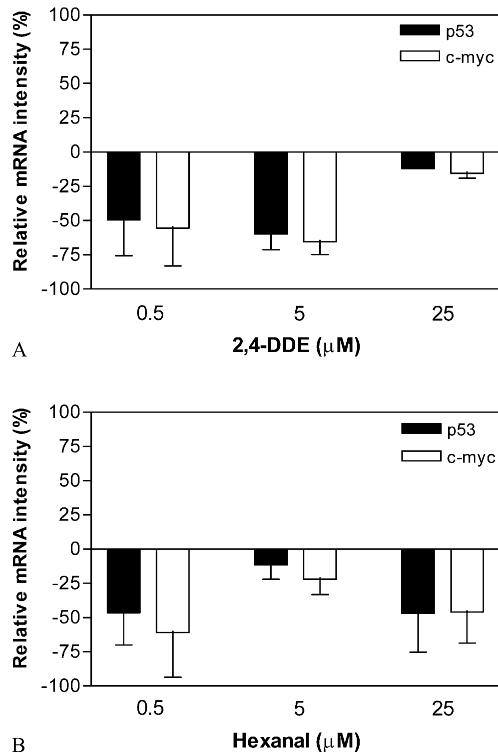

Fig. 3. 2,4-DDE and hexanal down-regulate p53 and c-myc mRNA levels. HVSMC were incubated with 2,4-DDE (A) or hexanal (B) at concentrations of 0.5, 5 and 25 μM for 2 h. Each commercial pure aldehyde (Aldrich, Spain) was dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Cells incubated with vehicle alone were designated as untreated cells. mRNA levels were quantified as described in Section 2 and the data represent the percentage of the relative mRNA intensity with respect to untreated cells. The data are shown as the mean ± S.E.M. of three different experiments.

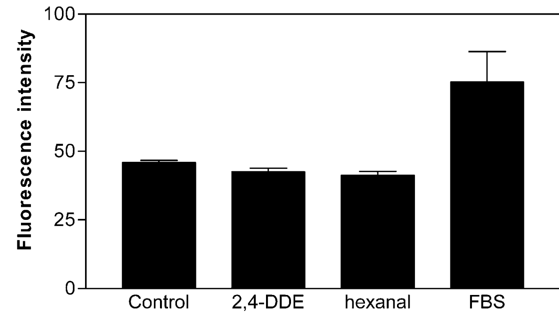

Fig. 4. 2,4-DDE and hexanal arrest HVSMC growth. HVSMC were incubated with 1 μM of 2,4-DDE and hexanal for up to 3 days. Culture medium was substituted for PBS and the dye Hoechst 33342 was added to a final concentration of 1.25 μg/ml for 5 h at 37 °C. Cells incubated with vehicle alone were designated as control cells and 10% FBS was used as a positive control of cell growth induction. Results are expressed as the fluorescence intensity of wells.

regulation of c-myc expression and SMC growth inhibition has been previously demonstrated [27]. However, contradictory results have been published about the effects of 4-HNE on cell growth [24,28].

It has been suggested that apoptosis may be the main regulator of cell number in the vessel wall [29]. The local wall environment created during development of atherosclerosis may imbalance, as the present report reflects, the relation between proliferation and cell death in the artery. Our results suggest that the well-known cytotoxic effect of aldehydes would be promoted by cell necrosis instead of an apoptotic mechanism. However, our results do not describe the molecular mechanisms involved in this process. Therefore, this necrosis induced by oxidation may enhance its cellular damage, multiplying the oxidative effects. On the other hand, we previously reported that aldehydes modulate the inflammatory response by inhibiting TNF-α mRNA expression in macrophages [8,9]. These finding, together with the present results, are in accordance with Berliner et al. who hypothesized that products formed in mildly LDL oxidation have important inflammatory effects whereas those generated as a result of an extended LDL oxidation, such as 2,4-DDE, will contribute to cytotoxicity [5]. Finally, we hypothesize that the extended SMC necrosis in the nucleus and in the cap of the lesion induced by products derived from advanced LDL oxidation, such as 2,4-DDE, may contribute to the increase in the lipid and cell debris core of advanced atherosclerotic plaques and to the reduction of the lesion cap, contributing to its instability. Therapies aimed at blocking the formation of these compounds, such as statins, as observed by our group [30], could play a role in the plaque stabilization by this pathway.

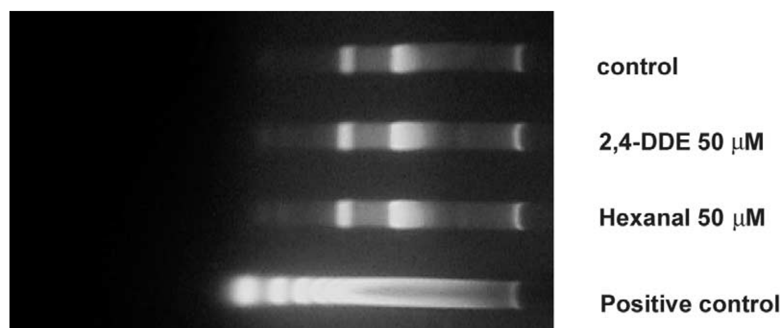

Fig. 5. 2,4-DDE and hexanal do not induce DNA laddering in HVSMC. DNA was extracted from cells incubated with 2,4-DDE or hexanal at 50 μM for 24 h and analyzed by agarose gel electrophoresis.

Acknowledgements

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ESTUDI 2

Aldehydes Mediate Tissue Factor Induction: A Possible Mechanism Linking Lipid Peroxidation to Thrombotic Events

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Tissue factor (TF), which is expressed in atherosclerotic plaques and colocalizes with oxidized lipids, initiates the thrombogenic process. We have analyzed the effect of aldehydes derived from peroxidation of polyunsaturated fatty acids on TF expression in human vascular smooth muscle cells (HVSMC). Our results demonstrate that hexanal and 2,4-decadienal (2,4-DDE), two apolar aldehydes, increase TF expression. Exposure of HVSMC to hexanal for 2 h led to TF protein levels up to seven times higher than untreated cells whereas 2,4-DDE for 30 min led to them being up to 2.2 times higher. This induction of TF antigen by aldehydes correlates with an increase in TF mRNA levels. Electrophoretic mobility shift assays (EMSAs) showed that the binding activity of the transcription factor AP-1 (c-Fos/c-Jun) to TF promoter was elevated in response to these oxidation products. This enhancement was associated to an increase of c-fos transcriptional activity, which was reversible by pretreatment with simvastatin. We conclude that the induction of TF by aldehydes might contribute to the severity of atherogenesis. J. Cell. Physiol. 198: 230–236, 2004. © 2003 Wiley-Liss, Inc.

Tissue factor (TF) is a glycoprotein that has been localized in smooth muscle cells (SMC), foam cells, in the endothelium and in the acellular lipid-rich core of atherosclerotic plaques (Drake et al., 1989; Wilcox et al., 1989; Moreno et al., 1996; Thiruvikraman et al., 1996) and it colocalizes with oxidized LDL (oxLDL) (Lewis et al., 1995). Atherosclerotic lesions with a thin fibrous cap overlying a prominent lipid-rich core are more susceptible to rupture. Plaque rupture exposes TF to flowing blood, leading it to complex with the coagulation factor VII and initiating the thrombogenic mechanisms associated with lesion disrupture (Tremoli et al., 1999).

A wide range of studies have focused on the effects of modified LDL on TF expression. Nevertheless, a clear effect has not been demonstrated because increase, no effect, or even inhibition of TF expression have been reported (Drake et al., 1991; Weis et al., 1991; Brand et al., 1994; Kaneko et al., 1994; Schlichting et al., 1994; Lewis et al., 1995; van den Eijnden et al., 1999; Penn et al., 2000). The observed discrepancy might be related to differences between oxLDL used or the cell types used. When LDL is oxidized it becomes modified and many new molecules derived from lipid oxidation are formed. These include oxysterols, lysophosphatidylcholine, hydroperoxides, and the final products derived from polyunsaturated fatty acid (PUFA) oxidation, the aldehydes (Esterbauer and Ramos, 1995). These lipid oxidation derived products have important biological functions modifying atherogenic processes. Some of these actions seem to modulate gene expression (Berliner et al., 1995). The expression of the TF gene is regulated by several transcription factors such as SP-1, Egr-1, NF-κB, and AP-1 (Oeth et al., 1997). The AP-1 family of transcription factors is divided into the fos related genes and the jun family that bind to DNA as homodimers or heterodimers (Oeth et al., 1997). It has been shown that the hydroxyaldehyde 4-HNE induces the proliferation of vascular smooth muscle cells (VSMC) through the regulation of the early response genes *c-fos*, *c-jun*, and *c-myc* (Barrera et al., 1994; Parola et al., 1998; Ruef et al., 1998; Page et al., 1999; Watanabe et al., 2001).

We hypothesized that aldehydes could be involved in the final processes that complicate the arteriosclerotic lesion, rupture, and thrombotic mechanisms due to highly oxidized lipids that might progressively be accumulated in the big lipid core of mature plaques.

In the present report, we detected the presence of aldehydes in the human artery wall and we studied the effect of apolar aldehydes on TF expression in HVSMC.

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Hexanal, as the mainly apolar aldehyde produced in LDL oxidation, and 2,4-DDE, due to its high biological activity, were chosen as representative apolar aldehydes.

MATERIALS AND METHODS

Analysis of aldehydes ex vivo in human artery wall

Atherosclerotic lesion tissue was obtained postmortem from a human aorta thoracica. A proximal piece of the same artery was considered as non-pathological sample. Material was immediately placed in PBS containing antioxidants (1 mM EDTA, 0.1 mM BHT) and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) and kept at −70°C until the aldehyde analysis. The tissue was carefully blotted dry and weighed. Approximately 1 g of tissue was cut into small pieces and homogenized at 4°C using a Polytron (92/Polytron PTA 10s) in 2 ml of aldehyde derivatization 2,4-dinitrophenylhydrazin (DNPH) buffer. Aldehydes were extracted from the resulting homogenate and evaluated by thin layer chromatography to separate the different types present (Esterbauer and Cheeseman, 1990). The zone III band was analyzed by the Kodak Digital Science 1D program.

Cell culture and experiments design

HVSMC from a human uterine artery were a kind gift from Dr. Eva Hurt-Camejo, Göteborg University. Cells were grown in Waymouth medium supplemented with 10% (v/v) FBS, 2% (v/v) sodium pyruvate, 1% (v/v) L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and passaged before reaching confluence. Cultures were maintained in a humidified incubator at 37°C and 5% CO_2. Cells were used at passages 3–6, at 70–80% confluence and they exhibited typical morphological characteristics of VSMC on phase-contrast microscopy: spindle shaped with a hill-and-valley pattern. In all the experiments, cells were left for 24 h in serum-free medium before the addition of aldehydes in order to obtain quiescent cells and to avoid a specific binding of aldehydes to serum proteins. 2,4-DDE and hexanal have been used at 5 μM (i.e., a concentration at which aldehydes had shown effects on gene expression and within the range detected in oxLDL) (Cabré et al., in press; Girona et al., 1997) for up to 2 h. After incubations, either total, nuclear protein extracts or total mRNA were obtained from cells for Western blot, electrophoretic mobility shift assays (EMSAs), or RT-PCR studies, respectively. Cytotoxicity was evaluated by means of LDH leakage in the medium and cells were also visualized under phase-contrast microscopy to assess morphological changes.

Each commercial pure aldehyde (Aldrich, Madrid, Spain) was dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Cells incubated with vehicle alone were designated as untreated cells.

Western blot analysis

Total protein extracts were obtained as previously described (Vallvé et al., 2002). Protein concentrations were determined by Bradford (1976) using BSA as standard and stored at −70°C. TF protein was determined in cell lysates from HVSMC treated with 2,4-DDE and hexanal (5 μM) for 30 min and 2 h. c-Fos protein was also determined in cell lysates from HVSMC in the same conditions but previously treated or untreated with simvastatin (1 μM) for 4 h. Samples were subjected to SDS–PAGE on gradient 8–18% acrylamide gels and electroblotted to nitrocellulose membranes (Amersham Biosciences, Europe GmbH, Freiberg, Germany). Unspecific binding was blocked with 5% (w/v) non-fat milk in TBS. The membranes were first incubated with 4503 mouse monoclonal anti-human TF antibody (American diagnostica, Stamford, USA) or with sc-52x rabbit polyclonal anti-human cFos antibody (SantaCruz Biotechnologies, Inc., Santa Cruz, CA) and then with an anti-mouse or anti-rabbit IgG antibody conjugated to horseradish peroxidase (Dako, Glostrup, Denmark). The blots were developed with the ECL+Plus (Amersham-Pharmacia Biotech). The bands were analyzed by the Kodak Digital Science 1D program. The protein sizes were confirmed by comparison with molecular weight standards (Bio-Rad, Hercules, USA).

RT-PCR analysis

The HVSMC were incubated in 12-well dishes in the presence of 2,4-DDE and hexanal for 30 min, 1, and 2 h at 5 μM of each aldehyde. Total cellular RNA was isolated from the cells using Ultraspec RNA isolation reagent (Biotecx Lab., Inc., Houston, USA) following the manufacturer's protocol. A semiquantitative RT-PCR method was used to determine TF, c-fos, and c-jun mRNA using the SuperScript one-step RT-PCR system (Life Technologies, Barcelona, Spain). β-actin was used as a constitutive expression gene to normalize the results of the genes of interest. RT-PCR products were analyzed by electrophoresis through a 2% agarose gel containing 0.5 μg/ml ethidium bromide in TAE buffer (Biorad). The bands were visualized using a UV transilluminator and their net intensity was analyzed by the Kodak Digital Science 1D program. Results are expressed as the percentage of variation of the net intensity of each condition and time point with respect to the corresponding untreated cells after normalization to β-actin results.

The sequences of RT-PCR primers (Life Technologies) were as follows: TF primer minus 5′-CAGTGCAATATAGCATTTGCAGTAGC-3′, primer plus 5′-CTACTGTTTCAGTGTTCAAGCAGTGA-3′ (Iochmann et al., 1999); *c-fos* primer minus 5′-CTCCTGTCATGGTCTTCACAACG-3′, primer plus 5′-GGCTTCAACGCAGACTACGAGG-3′ (Lane et al., 1998); *c-jun* primer minus 5′-CTTCAGCCACACTCAGTGCAA-3′, primer plus 5′-GAGCATTACCTCATCCCGTGA-3′ and β-actin primer minus 5′-TACAGGTCTTTGCGGATGTCC-3′, primer plus 5′-GCTACAGCTTCACCACCACG-3′.

Non-radioactive electrophoretic mobility shift assay

Nuclear protein extracts were prepared essentially as previously described (Ohlsson et al., 1996). DNA-binding c-Fos/c-Jun activity to AP-1 site was determined in nuclear protein extracts from HVSMC treated with aldehydes at 30 min, 1, and 2 h by EMSA. A digoxigenin 3′ end labeling kit (Roche, Diagnostics GmbH, Manheim, Germany) was used to label the 3′ end of the double-stranded oligonucleotide with the TF AP-1 site. The protein–DNA binding was carried out in a volume of 20 μl containing 20 mM HEPES, pH 7.6,

1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.2%(w/v) Tween 20, 30 mM KCl, 2 μg poly [dI-dC] poly [dI-dC], and 0.1 μg poly L-lysine. Each reaction also contains 0.8 ng of labeled oligonucleotide and 4 μg of nuclear protein extracts and were developed at room temperature for 15 min. Before loading the samples onto the gel 8% glycerol was added. The samples were resolved on 5% polyacrilamide gel containing 0.25× TBE and glycerol to separate DNA–protein complexes from the unbound DNA probe, electroblotted to nylon membranes positively charged and crosslinked to those membranes. Bands were visualized following the instructions of the DIG Gel Shift kit (Roche Molecular Biochemicals).

The TF gene promoter sequence from −216 to −198, containing the proximal TF AP-1 site (Oeth et al., 1997), underlined, 5′-CTGGGGTGAGTCATCCCTT-3′ (Life Technologies), was used for the EMSA studies. The two single-stranded oligonucleotides were annealed overnight.

Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 9.0) and the unpaired Student's *t* test was used for comparison of means. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Analysis of aldehydes ex vivo

Total aldehydes were analyzed in human artery wall fragments by TLC with the purpose of finding evidence of their presence in human atherosclerotic plaques. The artery wall studies showed an increased presence of apolar aldehydes in the arteriosclerotic lesion compared with the normal wall (Fig. 1). Compared to normal artery a 3.4 fold increase of total intensity was observed in zone III (apolar aldehydes) of pathological tissue.

Apolar aldehydes induce TF expression in HVSMC

We investigated the effect of 2,4-DDE and hexanal on TF expression by Western-blot in order to evaluate the action of these aldehydes at the protein level (Fig. 2). shows that both apolar aldehydes at 5 μM consistently increased the 47 kDa fully glycosylated mature form of TF and did not produce an accumulation of the 33 kDa less mature form. 2,4-DDE enhances TF protein at 30 min (125%) whereas hexanal does at 2 h (600%).

We next examined whether apolar aldehydes induced TF mRNA. Our data revealed that 2,4-DDE incubations produced an increase in TF mRNA levels between 30 min (14%) and 1 h (26%), which later returned to baseline, and that hexanal incubations increase TF mRNA levels between 1 h (24%) and 2 h (103%) (Fig. 3).

AP-1 mediates apolar aldehydes overexpression of TF

To demonstrate the mechanism by which apolar aldehydes modulate TF gene expression in HVSMC, we examined the binding of nuclear protein extracts to an oligonucleotide that contains the proximal TF AP-1 site sequence. EMSA studies demonstrated that 2,4-DDE treatment increases AP-1 binding activity to TF promoter (Fig. 4), whereas hexanal did not modify this binding activity (data not shown). Competition studies demonstrated specificity of the complex formation to the TF AP-1 site. To determine the presence of c-Fos in the complex bound to AP-1 site, supershift experiments were performed using an antibody specific to c-Fos. The data show that the antibody completely abolished the protein–DNA complex formation (Fig. 4).

Involvement of c-Fos expression in TF upregulation by apolar aldehydes

In further studies, we investigated the time course for apolar aldehydes to increase the components of the AP-1 complex (c-fos and c-jun) at the mRNA level in HVSMC. For these experiments, 2,4-DDE and hexanal (5 μM) was added to monolayers for the different times, and mRNA levels of c-fos and c-jun were analyzed at 30 min, 1, and 2 h by RT-PCR. The data showed that the maximum increase in c-fos mRNA levels by 2,4-DDE and hexanal were observed at 30 min (32%) and 2 h (57%), respectively (Fig. 5). However, 2,4-DDE and hexanal did not affect the expression of the *c-jun* gene under the conditions tested (data not shown).

To study the effect of apolar aldehydes on c-fos expression we determined its protein levels in the presence of

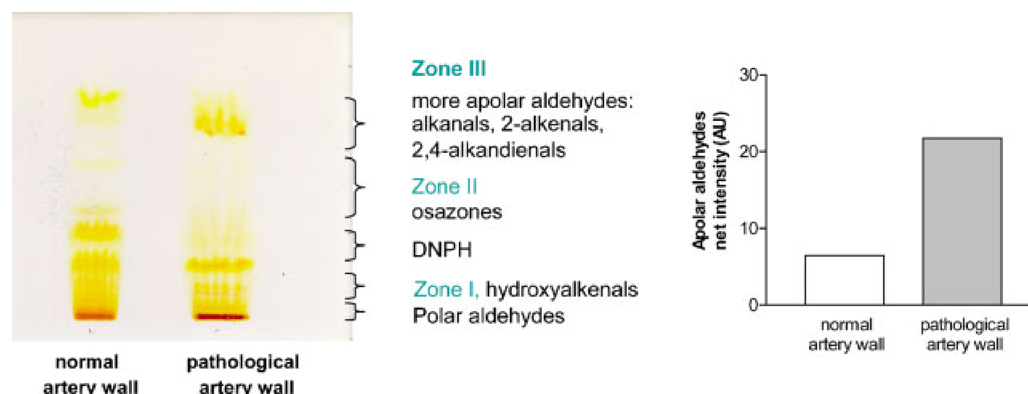

Fig. 1. Analysis of aldehydes in arterial wall. Non-pathological and atherosclerotic tissues were derivatized with 2,4-dinitrophenylhydrazin (DNPH). The hydrazone derivatives were extracted in dichloromethane and subjected to thin layer chromatography to separate the different types of aldehydes present by their polarity. 2,4-DDE and hexanal as apolar aldehydes belong to zone III. Bar graph values represent total net intensity of zone III in normal and pathological artery walls expressed as arbitrary units. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

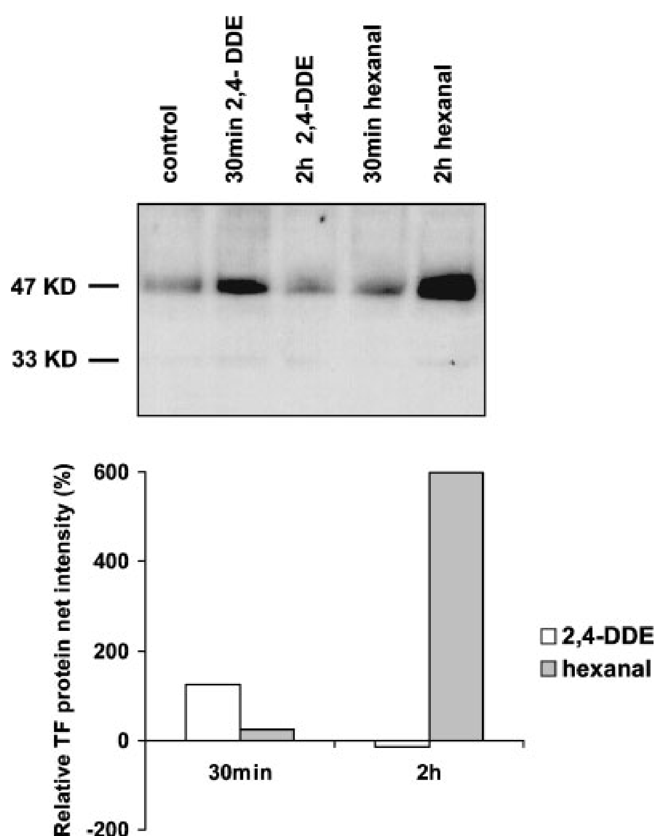

Fig. 2. 2,4-DDE and hexanal enhance TF expression in HVSMC. Cell lysates from HVSMC treated with 2,4-DDE or hexanal at 5 μM for 30 min and 2 h were analyzed by Western blot using a monoclonal antihuman TF antibody. Mature fully glycosylated TF (47 kDa) and unglycosylated form of the protein (33 kDa) are indicated by arrows. Bar graph values represent the percentage of variation of each condition with respect to untreated cells. The figure shown is representative of three independent experiments.

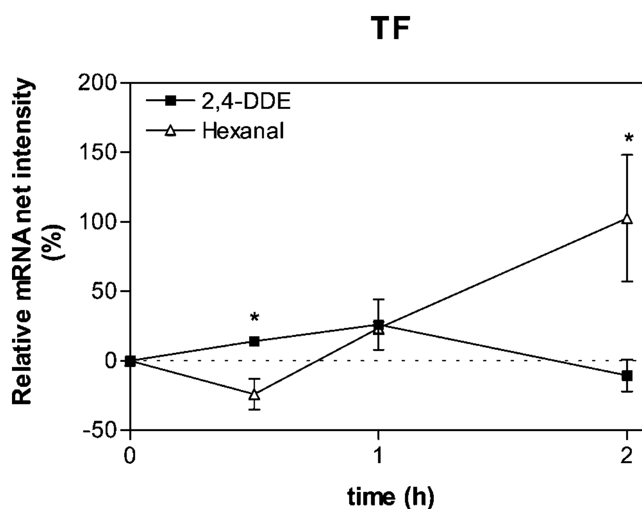

Fig. 3. 2,4-DDE and hexanal enhance TF mRNA in HVSMC. Cells were incubated with 2,4-DDE or hexanal at 5 μM for 30 min, 1, and 2 h. RNA levels were quantified as described in Materials and Methods and the data represent the percentage of the relative mRNA net intensity with respect to untreated cells at each time point. Cells incubated with vehicle alone were designated as untreated cells. Data are expressed as mean ± SEM of three different experiments.

simvastatin, which reduces aldehyde production in vivo. HVSMC incubated with both apolar aldehydes and previously pretreated for 4 h with simvastatin (1 μM) showed reduced levels of c-Fos protein with respect to aldehydes alone (83 and 62% reduction for 2,4-DDE and hexanal, respectively) (Fig. 6).

DISCUSSION

The present report shows that apolar aldehydes significantly increase TF mRNA and protein levels in HVSMC, suggesting a regulation of the transcriptional activity of the TF gene by these compounds. Apolar aldehydes upregulate the constitutive binding of AP-1 complexes to the TF promoter due to synthesis de novo of c-fos. The effect of apolar aldehydes on c-Fos protein is prevented by simvastatin.

Progression of advanced atherosclerotic lesions and induction of thrombotic events are related to plaque vulnerability (Lusis, 2000). Rupture frequently occurs in lesions with a thin fibrous cap overlying a necrotic lipid core and lipid-laden cells. TF is thought to be the major mediator of the thrombogenic mechanism associated to plaque rupture (Tremoli et al., 1999). More of the lipidic compounds present in the lesion, such as hydroperoxides, oxysterols, lysophosphatidylcholine, and aldehydes derive from lipid peroxidation (Esterbauer and Ramos, 1995). oxLDL and 7β-hydroperoxycholesterol induce TF expression in SMC (Penn et al., 2000) but in other cell types either no effect or inhibition of TF expression by oxLDL has been reported (Brand et al., 1994; Schlichting et al., 1994; van den Eijnden et al., 1999). The observed discrepancy in the results in TF expression might be related to differences in the composition in oxidized derived products in modified LDL and the different cell types used in these studies. In our laboratory, we have observed that the composition in aldehydes of modified LDL varied in relation to the method used to modify the particle (unpublished data). Then the exact composition of modified LDL might be of interest in order to elucidate its effects. In this study, we have observed a large amount of apolar aldehydes in human atherosclerotic lesions compared to normal arteries. We have previously reported the presence of these products in oxLDL (Vallvé et al., 2002) and their amount differs depending on the method used to oxidize the lipoprotein in vitro (Esterbauer et al., 1987). It has been previously reported that 2,4-DDE has an important biological activity usually associated with cytotoxicity at high concentrations (Esterbauer, 1993). Surprisingly, hexanal, which in composition is the major apolar aldehyde of oxLDL, has been less studied and in general has been considered biologically neutral. Our results show that these two apolar aldehydes, final products of LDL oxidation, probably accumulated in the most advanced stages of the lesion, also account for TF expression upregulating its mRNA and protein. The time course experiments show that TF mRNA increased by 2,4-DDE from 30 min to 1 h. This kinetic is similar to that found in human aortic SMC after incubation with PDGF (Schecter et al., 1997). Hexanal needed more time to produce an increase in TF mRNA levels, starting at 1 h

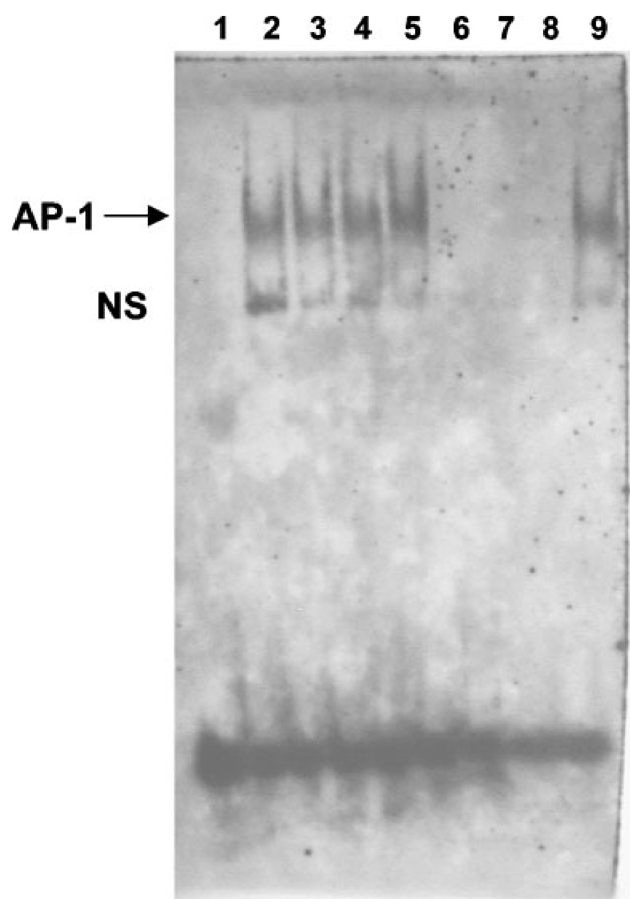

Fig. 4. 2,4-DDE enhances c-Fos binding activity into the AP-1 TF promoter site. Nuclear protein extracts were assayed for AP-1 binding activity using non-radioactive EMSA. The position of the specific band is indicated by an arrow. Lanes 2–5 showed the AP-1 complex bound to the labeled probe in HVSMC nuclear extracts from untreated cells and 2,4-DDE treated cells for 30 min, 1, and 2 h, respectively. To identify the protein present in the complex, anti-c-Fos antibody was added to the reaction 30 min before labeled probe to identify the protein present in the complex (lane 6). To demonstrate the specificity of protein binding, a non-specific nuclear protein was used instead of nuclear extracts (lane 1) or 50 and 200-fold excess of a cold specific competitor oligonucleotide containing TF AP-1 site (lanes 7–8) or a 200-fold excess of a cold non-specific competitor containing Oct2A site (lane 9). NS, non-specific binding. The figure shows a representative experiment.

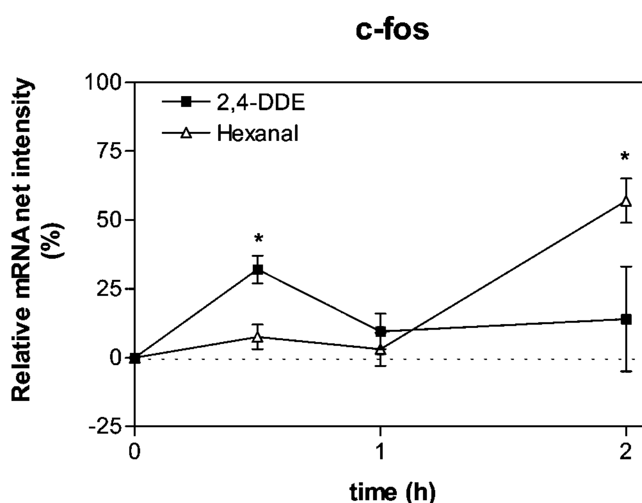

Fig. 5. Hexanal and 2,4-DDE up-regulate c-fos mRNA levels. HVSMC were incubated with hexanal or 2,4-DDE at 5 μM for 30 min, 1, and 2 h. Each commercial pure aldehyde (Aldrich) was dissolved in ethanol and the final concentration of ethanol in the cell culture media was less than 0.5%. Cells incubated with vehicle alone were designated as untreated cells. RNA levels were quantified as described in Materials and Methods and the data represent the percentage of the relative mRNA net intensity with respect to untreated cells. The data are shown as the mean ± SEM of three different experiments.

and increasing to 2 h. It has been proposed that ROS, which were generated during lipid peroxidation, act as second messengers for gene activation involving NF-κB (Satriano et al., 1993). In this way, it has been speculated that superoxide anion and nitric oxide could participate in TF induction through transactivating NF-κB and AP-1 respectively (Polack et al., 1997). 4-HNE (Ruef et al., 1998; Watanabe et al., 2001) as well as linoleic acid and its oxidative metabolites, the hydroxyperoxydecadienoic acids (HPODEs) (Rao et al., 1995), are potent mitogens in VSMC increasing c-fos and c-jun mRNA. Two AP-1 binding sites at −223 and −210 bp have been described in TF promoter, namely, proximal and distal AP-1 sites, respectively (Oeth et al., 1997). Different members of the AP-1 family regulate TF gene expression. In this work, we have used the proximal site which has high affinity for AP-1 and bound specifically c-Fos/c-Jun heterocomplexes (Oeth et al., 1997). Although these complexes are constitutively bound to the TF promoter they are not related to TF basal transcriptional activity. However, AP-1 sites are involved in TF gene transcriptional activation in stimulated cells (Oeth et al., 1997). EMSA data show that hexanal and 2,4-DDE conditioned cells have an enhanced AP-1 binding activity to TF promoter. No supershift, but a complete disappearance of the band has been shown in EMSA experiments when the c-Fos antibody was added in the assay as previously described (Oeth et al., 1997). Our data demonstrate that the apolar aldehydes studied, hexanal and 2,4-DDE, are able to induce c-fos expression in HVSMC and the main effect of the study was observed with 2,4-DDE. Although in this work c-jun expression is not increased by apolar aldehydes, preexisting c-Jun may be enough to form the heterodimer. It has been shown that c-fos and c-jun are constitutively expressed in human monocytes (Oeth et al., 1997). These observations suggest that AP-1 modulates TF transcription via de novo synthesis of c-fos, according to the increase observed in c-fos mRNA levels and the results from the supershift.

Our group has demonstrated previously that simvastatin reduces the production of aldehydes in vitro and in vivo (Girona et al., 1999). Furthermore, simvastatin treatment alone modulates gene expression (Maeda et al., 2003) and in combination with TF inducers, such as LPS or acetylated-LDL, influences their effects, preventing TF induction (Colli et al., 1997). In this study, we introduced pretreatment of cells with simvastatin, which produced an attenuation of c-Fos protein expression induced by apolar aldehydes, probably by interference with intracellular signaling.

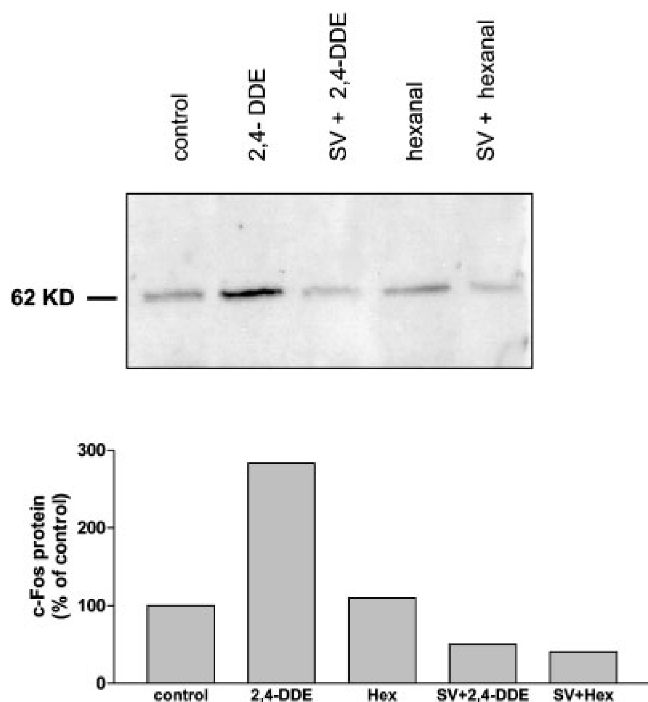

Fig. 6. Simvastatin treatment prevents c-Fos protein induction by apolar aldehydes. HVSMC pretreated with simvastatin (1 μM) for 4 h were incubated with 2,4-DDE and hexanal (5 μM) for 30 min and 2 h. Cell lysates were analyzed by Western blot using a polyclonal anti-human c-Fos antibody. Bar graph values represent the percentage of control. The figure shows a representative experiment.

Our findings could have important pathophysiological implications, because TF is expressed in atherosclerotic plaques associated to different cell types and because oxidized lipoproteins compounds, such as aldehydes, have been detected in atherosclerotic lesions. If these aldehydes in vivo showed similar effects to our findings they may contribute to the development of atherosclerotic lesion and plaque rupture associated with thrombotic events, due to their prothrombotic properties observed. Furthermore, this supports the theory that modified lipids have an important role in the progression of atherothrombotic disease.

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ANNEXOS

ESTUDI 3

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Short Communication

Generation of eight adjacent mutations in a single step using a site-directed mutagenesis kit

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Abstract

Studies that characterize transcriptional regulatory elements involve the site-directed mutagenesis methodology to generate deletions, insertions and point mutations. Commercial PCR-based system kits are widely used to introduce up to four base changes in a target sequence. Sequential mutagenesis experiments allow more than four bases to be altered. In this work, we show that the QuikChange site-directed mutagenesis kit developed by Stratagene, with an optimum primer design, can change eight adjacent bases. This approach allows us to study the effect of DNA sequence changes on functionality of specific sequences from gene target regions, including promoters, exons and introns. As a result of this methodology, a faster and cheaper way of introducing this number of mutations is achieved in a single step with only one pair of primers, thus reducing the possibility of potential random mutation in the rest of the target sequence.

Keywords: oligonucleotides; PCR; regulatory elements; site-directed mutagenesis.

The characterization of transcriptional regulatory elements involves modification of the native sequence, either by deletions or by single or multiple point mutations (1). Commercial site-directed mutagenesis kits are available for this purpose. The QuikChange site-directed mutagenesis kit developed by Stratagene (La Jolla, CA, USA) is a widely used PCR-based system that eliminates the need to subclone the amplified DNA (2). It generates deletions, insertions and point mutations in a target sequence using the high fidelity *Pfu* DNA polymerase and two complementary primers containing the desired alterations. This protocol is well established for the introduction of up to four base changes in a target sequence (3). Sometimes it is necessary to alter more than four bases to completely abolish a transcription factor binding site or coding regions in study. On these occasions, sequential mutagenesis experiments would achieve that goal, although a single step would simplify the methodology, save time and reduce the possibility of potential random mutations in the rest of the target sequence.

In this work, we performed a substitution of eight adjacent bases in a single step using the QuikChange site-directed mutagenesis kit (Stratagene) which we used to mutate an insert cloned into luciferase reporter vector pGL2-basic (Promega, Madison, WI, USA) (total length 6.7 kb). Sense and antisense primers were designed to introduce eight adjacent base changes. Furthermore, the mutations generated a NruI site that was used subsequently for selection of positive clones. The sense primer sequence was: 5′-CAGCTTTCTGAAGCCCC TCCtcgcgaATGTTCTAT CTTTTTCCTGCATC-3′ (Invitrogen, Life Technologies, Barcelona, Spain). The antisense primer was complementary to the sequence of the sense primer. The mutated bases are underlined in the sequence and the recognition NruI site bases are shown in lower case. The PCR mutagenesis reactions were carried out in a volume of 50 μl which included 15 ng of dsDNA plasmid template, 180 ng of each primer, 500 μM dNTPs, 2.5 U of *PfuTurbo* DNA polymerase, 10 mM of KCl, 10 mM of $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100 and 0.1 mg/ml nuclease-free bovine serum albumin (Stratagene). The reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA) programmed for an initial denaturation step of 30 s at 95°C followed by 16 cycles at 95°C for 30 s, 55°C for 1 min and 68°C for 14 min.

To check whether the changes had been successful, plasmid DNA (300 ng) from four putative positive clones (for the internalization of the plasmid) were digested with 5 U of NruI for 3 h at 37°C. The digestions were then subjected to 0.9% agarose gel electrophoresis and the DNA fragments were visualized with ethidium bromide staining. Non-mutated clones lack NruI sites, so only mutated clones carrying mutations were linearized with the NruI enzyme.

The insert sequence fidelity of two of the positive clones for enzymatic restriction were verified by automatic sequencing with Cy5 5′ end labeled primers (Proligo LLC, Boulder, CO, USA) and the thermo

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sequenase fluorescent labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences Europe GmbH, Freiburg, Germany) under conditions recommended by the manufacturer and were then loaded onto a Pharmacia LKB ALF DNA sequencer (Amersham Biosciences). DNA sequences were visualized with the ALFwin software (Amersham Biosciences) and then aligned to the original sequence.

The main issue in site-directed mutagenesis assays is the primer design. The mutated bases have to be in the middle of the primer and the length of the matching sequence on both sides should be sufficient to ensure enough primer annealing for the DNA polymerase action. We designed a pair of complementary primers of 49 bases long, which resulted in 20 and 21 correct bases on each side of the eight central mutated bases. The mutated bases contained a recognition site to the restriction enzyme NruI. This site was not present in our construct and gave us an easy way of determining the incorporation of at least six of the eight changed bases. We analyzed four putative mutated clones by enzymatic restriction. Figure 1 shows that three of them were positive for the enzyme cleavage.

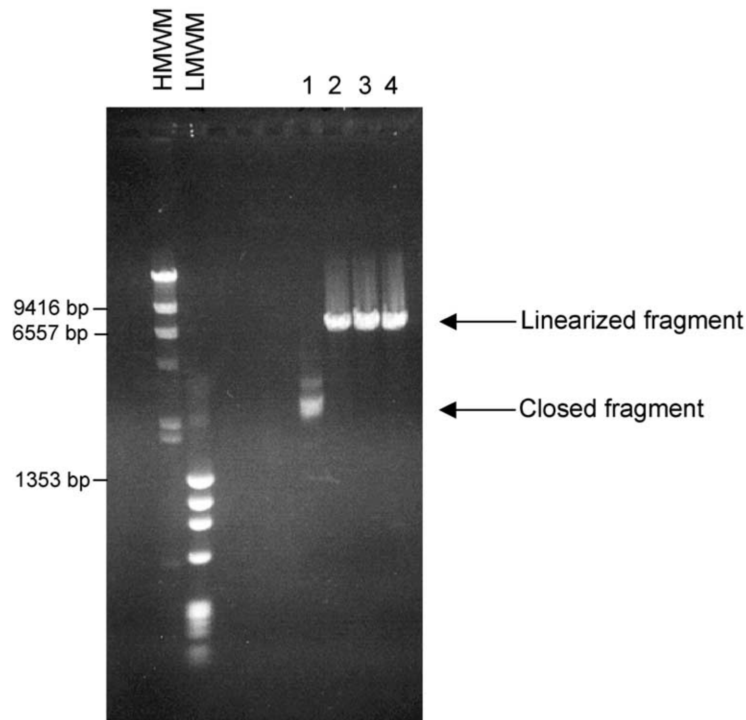

Figure 1 Enzymatic restriction of DNA plasmid clones resulting from the mutagenesis assay. Plasmid DNA was digested with 5 U of NruI for 3 h at 37°C. The digested products were subjected to 0.9% agarose gel electrophoresis and visualized with ethidium bromide staining. Four putative clones were analyzed: (1) a non-mutated clone, (2–4) positive mutated clones. Cleaved and non-cleaved fragments are indicated by arrows. HMWM, high molecular weight marker (Lambda *Hind* III, Abgene); LMWM, low molecular weight marker (174 RF DNA *Hae* III, Abgene).

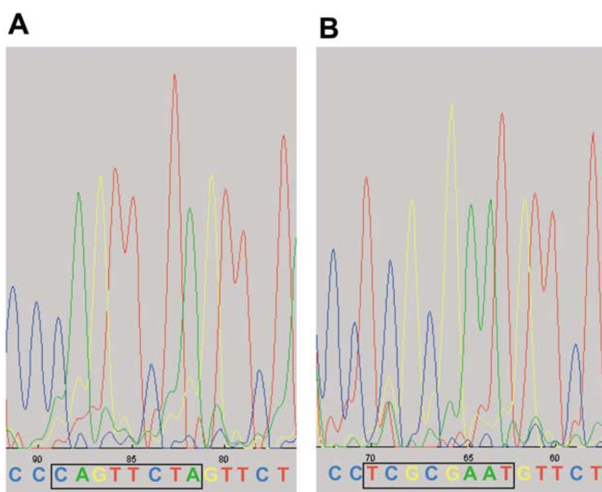

Figure 2 Gene sequence analysis. Gene sequences were analyzed by automatic sequencing as described in the text. A represents the wild-type sequence and B the mutated sequence. The wild-type and mutated base changes are framed. Results are representative of two experiments analyzed in duplicate.

Afterwards, in order to verify that the whole expected bases were correctly changed and no other mutations were added in our gene sequence, we sequenced twice the whole insert sequence of two positive clones. No random mutations were found along the analyzed mutated region. Figure 2 shows part of the sequencing results including the target zone. In Figure 2A we can observe the wild-type sequence and in Figure 2B the mutated bases (incorporated).

In summary, the QuikChange site-directed mutagenesis kit, with an optimum primer design, allows the change of eight adjacent bases. This approach allows us to study the effect of DNA sequence chang-

es on functionality of specific sequences from gene target regions, including promoters, exons and introns. As a result of this methodology, a faster and cheaper way of introducing this number of mutations is achieved in a single step with only one pair of primers.

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Identification of two peroxisomal proliferator response like elements in the human TNF-α promoter

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ABSTRACT

Peroxisome proliferator activated receptor (PPAR) ligands ameliorate the profile of chronic inflammatory associated diseases such as atherosclerosis, insulin resistance or rheumatoid arthritis. The antiinflammatory action of these drugs is still not completely understood. It has been attributed in part to a decrease on the TNF-α expression via repression of several transcription factors.

In this work we identify two PPAR response-like elements (PPRE) in the human TNF-α promoter with high similarity to the previously described consensus sequence. Transfection studies showed that TNF-α promoter activity was decreased by expression of PPAR-γ and addition of its ligands. The observed reduction is ligand dose dependent. Electrophoretic mobility shift assays using nuclear extracts of the human THP-1 cell line revealed that PPAR-γ binds to these regions. These data link PPAR with the modulation of inflammatory responses providing a new mechanism of action of PPAR activators that could benefit the treatment of related inflammatory diseases.

INTRODUCTION

Tumor necrosis factor alpha (TNF-α) is one of the main mediators of the acute phase inflammatory response, which has an important role in the pathogenesis of chronic inflammatory diseases such as atherosclerosis [1]. In addition to a proinflammatory function, TNF-α has a variety of effects on lipid metabolism and adipocyte function. TNF-α is a potent inhibitor of adipocyte differentiation [2] and stimulates lypolisis which increases insulin resistance [3-5]. Elevated TNF-α levels have been detected in humans and in several rodent models of obesity and type 2 diabetes [6-7]. As a result of its actions, TNF-α may enhance the insulin resistance syndrome, leading to diabetes and higher risk of coronary heart disease.

Expression of TNF-α is mainly regulated by the transcription factors NF-κB and AP-1 [8-9]. In addition, peroxisome proliferator activated receptor-γ (PPAR-γ) ligands appear to antagonize TNF-α mediated effects [3-5]. A reduction of TNF-α expression has been observed in several cell and animal models of insulin resistance and inflammation upon treatment with these drugs [10-12]. The net effect of PPAR-γ ligands indicates that their antiatherogenic potential [13-16] is due to the beneficial antiinflammatory and metabolic actions observed in both mice and humans, including amelioration of insulin resistance and lipid profiles. PPAR-γ is one of the three PPAR isoforms, which together constitute a distinct subfamily of the nuclear receptor superfamily [17] and which are all activated by naturally occurring fatty acids or fatty acid derivates [18-20]. PPAR-γ heterodimerizes with retinoid X receptors (RXR) and alters the transcription of numerous target genes after binding to specific response elements or PPREs, which are found in several genes involved in lipid metabolism [21].

So far, no evidence of a direct regulation of TNF-α by PPAR-γ has been reported. The effect of PPAR-γ ligands on TNF-α expression has been attributed to transrepression of AP-1, NF-κB and STAT-1 activity [22-25]. There have been several roles postulated for this PPAR-γ mediated transrepression. Activated PPARγ itself could compete with AP-1 and NF-κB coregulators [26] or could bind directly with these transcription factors. Moreover, PPAR-γ ligands at high doses inhibit cytokine expression independently of PPAR-γ [27]. All these studies point towards an indirect effect of PPAR on cytokine expression probably linked to the fact that no consensus PPRE has been found in the promoter regions of the TNF-α gene [28].

However, studies aimed at elucidating the possible direct implication of PPAR on inflammatory inhibition have not always used the appropriate model systems and have been performed on PPAR-α null mice [29] on mice cells [27] or they have been based on the rat TNF-α promoter sequence [28]. Similar as observed for other PPAR target genes such as Apo A-I, the TNF-α promoter sequence is not phylogenetically

conserved [30-31]. In the present work, we identified two PPRE-like elements in the human TNF-α gene. Furthermore, we show that PPAR-γ represses the activity of the TNF-α promoter through directly binding to both elements.

EXPERIMENTAL

Cell culture

Monocytic leukemia THP-1 cells were cultured in RPMI 1640 supplemented with 10% foetal bovine serum (myoclone super plus FBS USA), 100U/ml penicillin, 100μg/ml streptomicyn (Invitrogen, Life Technologies, Barcelona, Spain) and 50μM 2-mercaptoethanol (Sigma-Aldrich, Madrid, Spain). Cells were differentiated to macrophages by the addition of phorbol 12-myristate 13-acetate (PMA), solubilized in DMSO (Sigma-Aldrich) at a concentration of 50ng/ml to suspension cells. MEF embryo fibroblasts were cultured in DMEM supplemented with 10% FBS and 100U/ml penicillin, 100μg/ml streptomicyn (Invitrogen).

Cells were incubated with 1μM rosiglitazone dissolved in DMSO or with vehicle alone. THP-1 cells were used to obtain nuclear protein extracts and total RNA and MEF cells were used for the transfection studies.

TNF-α real-time RT-PCR

Total cellular RNA was isolated from the cells using Ultraspec RNA isolation reagent according to the manufacturer's instructions (Biotecx Laboratories, Inc., Houston, USA). The purity and concentration of RNA were estimated by the relative absorbance at 260nm/280nm as determined by spectrometry. One μg of total RNA was reverse transcribed to cDNA using random hexamers and MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, USA) following the manufacturer's instructions. TNF-α mRNA expression was quantified by real-time PCR with the use of an ABI Prism 5700 Sequence Detector System (Applied Biosystems) combined with dual-label fluorogenic detection system (TaqMan) based on the 5' nuclease assay. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) was used as endogenous control. TaqMan primers and probes for TNF-α and GADPH were obtained from validated and Pre-Developed Assay Reagents (Applied Biosystems) and were used in real time PCR amplifications. These reactions were performed in triplicate using 10μl of cDNA in a 25μl volume reaction. Standard curves were constructed for each gene to confirm equal amplifications efficiencies. The sample without rosiglitazone treatment was defined as the calibrator in this experiment, and therefore, the amount of TNF-α transcripts in the other sample was a dimensionless number relative to the levels in the calibrator sample. Because of amplification efficiencies of TNF-α and GADPH were equal, TNF-α mRNA expression was calculated as $2^{-\Delta\Delta Ct}$ according to the manufacturer's instructions [32].

Plasmids construction

The TNF-α promoter fragment –993 to +110 cloned into the SacI and HindIII restriction sites located upstream of the luciferase gene in the pGL2- Basic plasmid (pTNF-wt) was kindly supplied by Dr David A. Joyce and Dr Jay Steer from the Departments of Pharmacology and Biochemistry, University of Western Australia. Mutant constructs pTNF-PPRE(-1) and pTNF-PPRE(-2) were generated by the introduction of 8 bp substitutions on PPRE-like sites of the construct pTNF-wt in one step using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, USA) and the pTNF-PPRE(-1/-2) was produced by introducing the same substitutions to generate pTNF-PPRE(-2) using pTNF-PPRE(-1) as target. DNA sequence fidelity and the introduced mutations were veriffied by sequencing. Human PPAR-γ (pCMV-hPPAR-γ) and mouse RXR-α (pCMV-mRXR-α) expression plasmids were described previously [33].

Transient transfection and gene reporter assays

Transient transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 1μg pTNF-wt, 100ng pCMV-hPPAR-γ and 10μl LipofectAMINE 2000 were gently mixed in 200μl DMEM (Invitrogen) and incubated for 30min at room temperature. In some experiments a 100-fold excess of oligonucleotides containing AP-1 and NF-κB binding sites were added in respect to the amount of TNF-α promoter. After 4h, transfected cells were incubated with rosiglitazone or its vehicle alone (DMSO) for 20h. Luciferase activities were measured in cell lysates in a luminometer (MicroLumat LB96P, Berthold, Australia) using the Luciferase Reporter Assay System (Promega, Madison, USA) according to the manufacturer's protocol. Total protein content of total lysates were measured by Bradford to normalize the transfection results.

Preparation of nuclear protein extracts

Nuclear protein extracts were prepared essentially as described previously [34]. Protein concentration of all the preparations were determined by Bradford [35] using BSA (Bio-Rad, Hercules, USA) as standard and stored at -70ºC.

***In vitro* transcription/translation**

In vitro transcription/translation of hPPAR-γ and mRXR-α (from the expression vectors pCMV-hPPAR-γ and pCMV-mRXR-α respectively) were performed using the TNT quick coupled transcription/translation system (Promega) according to the manufacturer's recommendations. Translation products were verified by western-blot. Briefly, proteins were subjected to an SDS-PAGE electrophoresis, electroblotted to nitrocellulose membranes (Amersham Biosciences Europe GmbH, Freiburg, Germany) and blocked overnight at 4ºC with 5% (w/v) skimmed milk in TBS to avoid unspecific binding. The membranes were incubated for 1h at room temperature with mouse IgG anti-PPAR-γ or rabbit IgG anti-RXR-α (SantaCruz Biotechnology, Inc., Stamford, USA) and then incubated for 30min at room temperature with goat anti-mouse or goat anti-rabbit IgGs conjugated to horseradish peroxidase (Dako, Glostrup, Denmark) respectively. The blots were developed with the ECL+Plus reagent (Amersham Biosciences) according to manufacturer's protocol. The protein sizes were confirmed by comparision with molecular weight standards (Bio-Rad).

Non-radioactive electrophoretic mobility shift assay

Nuclear extracts from THP-1 and *in vitro* translated proteins were used in these analyses. A digoxigenin 3' end labelling kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to label the 3' end of the double-stranded oligonucleotides. Gel retardation assays were carried out according to the instructions of the DIG Gel Shift kit (Roche Diagnostics). The samples were resolved on 5% polyacrilamide gel containing 0.25 x TBE and glycerol to separate DNA-protein complexes from unbound DNA probe and electroblotted to nylon (+) membranes, crosslinked to that membrane and the bands were developed following the manufacturer's protocol. Doubled-stranded oligonucleotides containing the following sequences were used for shift and competition analysis: TNF-PPRE1, 5'-GAGCCTCCAG-GACCTCCAGGTATGG-3'; TNF-PPRE2, 5'-AG-CCCCTCCCAGTTCTAGTTCTATC-3'; TNF-$PPRE1_{mut}$, 5'-GAGCCTCCAGGAATTCCAGGTAT-GG-3'; TNF-$PPRE2_{mut}$, 5'-AGCCCCTTTAAG-CTTGGGCTCTATC-3' and consensus PPRE (DR-1), 5'-AGCTTCAGGTCAGAGGTCAGAGAGCT-3'.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS 9.0) and the unpaired Student's *t* test was used to compare differences between groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

PPAR-γ agonist decreases endogenous TNF-α expression in macrophages

We examined endogenous TNF-α mRNA expression in human macrophages by real time RT-PCR. Results show that 1μM rosiglitazone is able to significantly decrease (50% reduction) the basal levels of endogenous TNF-α mRNA in macrophages (Figure 1). This result is consistent with previous studies [28, 36], confirming the inhibitory effect of PPAR-γ agonist on TNF-α expression.

The human TNF-α gene contains two PPRE-like sites

We also examined the promoter sequence of the human TNF-α gene cloned by Takashiba et al. [37] for possible PPRE-like sites. The results of the screening of both strands revealed that the complementary strand contains two potential binding sites for the nuclear hormone receptors similar to the consensus sequence proposed by Palmer et al. [38]. These two potential binding sites contain two direct repeat motifs separated by 0 and 3 bases respectively. The sequence of these two PPRE-like sites correspond to TCCAGGACCTCCAGG and CCCAGTTCTAGT and they are localized at -807 to -793 and -415 to -404 respectively (Figure 2). These sites are not conserved in rodents (data not shown).

PPAR-γ agonist decreases the transcriptional activity of the TNF-α promoter in MEF cells

To determine whether PPAR-γ is involved on TNF-α expression, we performed transient transfection analysis. Our results showed that the addition of a synthetic PPAR-γ agonist rosiglitazone (BRL 49653) decreases pTNF-wt activity by two- to four-fold. Cotransfection with PPAR-γ expression plasmid resulted in a similar reduction of pTNF-wt transcription. With cotransfection and stimulation with PPAR-γ agonist, an additive effect was achieved (Figure 3A).

To further analyze the action of PPAR-γ agonist on the regulation of TNF-α gene, a dose response experiment was performed. MEF cells were cotransfected with the pTNF-wt and with a expression plasmid of PPAR-γ and stimulated with different concentrations of PPAR-γ agonist, in a range between 0.1-10μM of rosiglitazone (Figure 3B). The response of the TNF-α promoter to PPAR-γ agonists is clearly dose-dependent.

The PPRE-like sequences in the human TNF-α promoter are functional

In order to determine whether the potential PPRE-like sequences have a role in the inhibitory effect of PPAR-γ agonist on TNF-α transcriptional activity, we generated 3 constructions of the TNF-α promoter in which the PPRE-like sites were mutated. pTNF-PPRE(-1) contained the PPRE1-like site mutated; pTNF-PPRE(-2), the PPRE2-like site mutated and pTNF-PPRE(-1/-2) with both sites mutated (Figure 4). MEF cells were cotransfected with these constructions and with a human PPAR-γ expression vector (pCMV-hPPAR-γ). Next, cells were exposed to a PPAR-γ agonist and the luciferase activity was assayed. As shown in Figure 4, mutation of the PPRE-like sites reduced the inhibitory action of PPAR-γ agonist on TNF-α transcriptional activity. This effect was observed with pTNF-PPRE(-1) and pTNF-PPRE(-2) indicating that both PPRE-like sites are functional. The presence of both mutated sites did not produce a synergistic effect on TNF-α transcriptional activity.

Finally, in order to test our hypothesis that the inhibitory effect observed is due to a direct action of PPAR-γ, we blocked the indirect pathway which involves NF-κB and AP-1 as described above. Therefore “decoy” oligonucleotides containing NF-κB and AP-1 recognition binding sites were added in a 100-fold excess in respect to the amount of TNF-α transfected promoter to the transfection experiments. In the absence of these active transcription factors, the basal transcriptional activity was significantly lower, but interestingly the

inhibitory effect of the PPAR-γ agonist was conserved (Figure 5A). In the presence of mutations in both PPRE sites (pTNF-PPRE(-1/-2)), the PPAR-γ agonist fully lost its ability to reduce the transcriptional activity of TNF-α (Figure 5B).

PPAR-γ binding to PPRE sites on human TNF-α gene

Gel mobility shift assays were then performed to analyze whether PPAR-γ bound directly to the potential PPRE-like sites (Figure 6). Nuclear extracts from macrophage-like THP-1 cells showed a shifted band, corresponding to the PPAR-γ/RXR-α heterodimer. Cells treated with a PPAR-γ agonist showed an increase in the retarded complex, a phenomenon especially clear with PPRE2-like site. The specificity of the binding was confirmed by the disappearance of the band by the addition of a 200-fold excess of cold oligonucleotides and with the absence of competition with a 60- and a 125-fold excess of mutant competitors. In addition, the purified transcription factor Oct 2A was not able to produce any shifted band on these PPRE-like elements, precluding non-specific binding. To further characterize the protein complex binding to the TNF-α promoter oligos, we examined the competition of the binding with DR-1, the sequence described as the consensus binding site for PPAR/RXR. DR-1 was able to efficiently compete for the proteins that produce the shift. These results suggest that putative PPRE-like sites of TNF-α promoter most likely bind the PPAR-γ/RXR-α heterodimer. Furthermore, PPAR-γ and RXR-α synthesized in an *in vitro* translation/transcription system were able to bind as heterodimers to the PPRE2-like site of the TNF-α promoter, confirming our results.

DISCUSSION

It has been previously reported that PPAR ligands have an antiinflammatory effect by reducing cytokine production, which is reflected by decreased TNF-α mRNA and protein levels [10]. In addition, it has been proposed that thiazolidinediones, could reduce cytokine production in monocytes/macrophages [39]. At the moment, the mechanism underlying the antiinflammatory effects of PPAR ligands is not well-established. This report demonstrates that PPAR-γ inhibits TNF-α expression directly. PPAR-γ belongs to a large family of nuclear receptors that regulate gene expression through binding to cis-acting sequences in their respective target genes. PPAR heterodimerizes with RXR to modulate gene transcription. The specific DNA sequences bound by the receptors, known as PPAR response elements (PPRE), are, typically, direct repeats of AGGTCA with 1 base spacing (DR-1 consensus sequence) [17]. In our study we have examined the complete sequence of the human TNF-α promoter and identified two potential PPRE-like sites, which resemble consensus half site repeats. The PPRE1-like sequence is a DR-3, whereas the PPRE2-like sequence seems to be a degenerated DR-1 because the spacing base is lacking. Over recent years many reports described the identification of PPRE-like sites in a wide range of genes leading to the re-definition of the exact sequence requirements for the PPAR/RXR binding. Several novel variant PPREs are described, which in most cases represent imperfect direct repeats or response elements with variations in the number of spacing bases between the two half sites. PPREs described in the human cholesteryl ester hydrolase (CEH) [40] and in the hepatocyte growth factor (HGF) (41) gene promoters are most different with respect to the consensus sequence. In CEH, there are three PPRE half-sites, two of them in the complementary strand and in both sense and antisense directions, whereas the PPRE in HGF is composed of an imperfect inverted repeat of the AGGTCA motif with two spacers, and all of them resulted in functional sites.

In accordance with the transfection analysis, PPAR-γ reduced basal TNF-α transcriptional activity in the presence and absence of activator

and this inhibitory action was reduced by mutation of either of the PPRE-like sites. Previous reports have suggested that the PPAR-γ agonist may play an indirect role on TNF-α inhibition by negatively interfering with the transcriptional regulation of TNF-α by AP-1 and NF-κB [22-25]. It has been hypothesized that this action could be receptor independent [27] and explained by a direct effect of the PPAR-γ agonist modifying covalently the NF-κB DNA binding domain or its activation kinase IKK [23-25]. Moreover, it has also been postulated that this effect could be the result of cofactor (i.e. CBP/p300) squelching for NF-κB and AP-1 by activated PPAR-γ [26]. Our results show that at low doses the antiinflammatory action of rosiglitazone is PPAR-γ dependent. To evaluate the contribution of such a direct mechanism we blocked the binding activity of NF-κB and AP-1 to TNF-α promoter by response element "decoy" oligonucleotides. These transfection experiments which eliminated most indirect effects through NF-κB and AP-1 confirmed the functionality of both PPRE-like elements as well as the existence of a direct effect of PPAR-γ on TNF-α expression.

PPAR-γ binding to the potential PPRE-like sites was confirmed by gel retardation assays in which a consensus DR-1 PPRE element was able to compete for the protein that binds to both TNF-PPRE-like sites. Moreover, PPAR-γ ligand enhanced binding of the PPAR-γ/RXR-α heterodimer to the PPRE2 site. Rocchi et al. and Kodera et al. reported that different ligands conferred distinct changes in the global PPAR-γ coactivator complex structure [42-43] which could explain the diversity between the functional PPRE described.

Comparative studies showed that the two PPRE-like sites, identified in the human TNF-α promoter were not conserved in rodents. This observation, together with the fact that previous studies have been performed in rodents or used shorter fragments of the human TNF-α promoter, could explain why this has not been noticed previously and could be related to the different susceptibility to atherosclerosis in these species with respect to humans.

Previous studies have also emphasized that it is necessary to be careful when assessing ligand related function when PPAR-γ ligands are used at concentrations far exceeding those required to bind and activate the receptor [27]. For this reason it is interesting to emphasize that in all the experiments we used rather low levels of rosiglitazone (1μM) compared to previous studies of such PPAR-γ ligands on TNF-α expression. At such relatively low concentrations of rosiglitazone, no activation of other receptors is induced [44], yet nevertheless such levels were able to inhibit endogenous TNF-α transcription in human macrophages.

In conclusion the present study highligths the existence of a multiple signalling pathway affecting TNF-α transcription that contributes to the antiinflammatory action of PPARs ligands. In addition to interfering with AP-1 and NF-κB signalling, the mechanism described here involves a direct interaction between PPAR-γ and the TNF-α promoter through two PPRE-like elements that modulate TNF-α expression by PPAR-γ agonists *in vivo*. Therefore the effects exhibited by PPAR-γ agonists could be the results of multiple combined actions and different regulatory pathways that converge on the same final effect, reduction of TNF-α expression. This is in accordance with the breadth of actions described for PPAR-γ drugs. Although further experiments are needed to confirm this hypothesis *in vivo*, this report indicates a new way for the modulation of TNF-α expression by other PPAR activators used in atherosclerosis treatment, such as fibrates.

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FIGURES

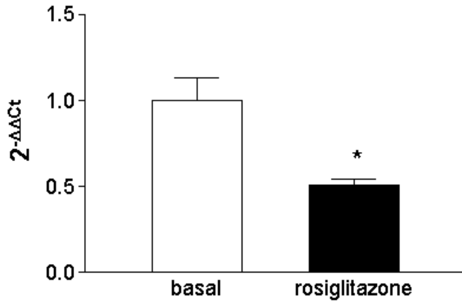

Figure 1

Rosiglitazone decreases endogenous TNF-α. Human THP-1-derived macrophages were treated with either vehicle (DMSO) or rosiglitazone (1μM). After 24h, the cells were harvested for total RNA extraction. Real time RT-PCR was performed for the human TNF-α mRNA, which was normalized to the GADPH mRNA. Results from DMSO treated cells were considered as basal levels of human TNF-α. Results, expressed as $2^{-\Delta\Delta Ct}$, are the average of triplicate determinations.

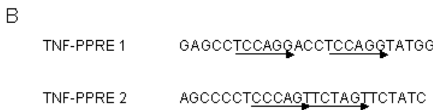

Figure 2

Upstream sequence of the human TNF-α gene. The two potential PPRE-like elements were underlined (A). Both PPRE-like elements are shown in B.

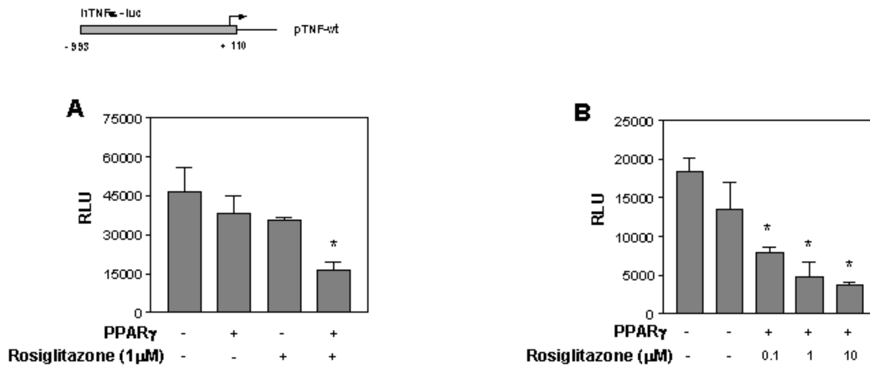

Figure 3

Rosiglitazone decreases the transcription of pTNF-wt. MEF cells were transiently transfected with a plasmid containing a luciferase reporter fused to the -993 to +110 promoter sequence of the human TNF-α gene (pTNF-wt) (1μg) or cotransfected with pTNF-wt and a human PPAR-γ expression vector (pCMV-hPPAR-γ) (100ng) and incubated with (A) either vehicle or rosiglitazone 1μM for 20h or (B) either vehicle and increasing concentrations of rosiglitazone (0.1-10μM) for 20h. Cells were then harvested and luciferase assays were performed. The results are expressed as the mean of relative luciferase units ± SEM (n=3).

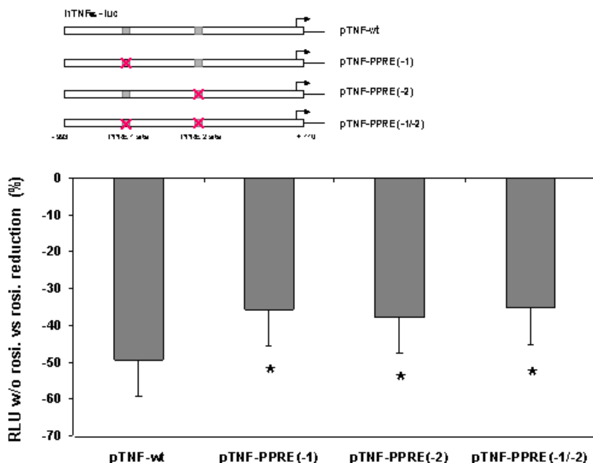

Figure 4

Mutations in PPRE-like sequences resulted in a reduction of functionality. Mutations were induced in either PPRE-like site in the pTNF-wt plasmid to generate plasmids containing either PPRE1 (pTNF-PPRE(-1)), PPRE2 (pTNF-PPRE(-2)) or both sites mutated (pTNF-PPRE(-1/-2)). MEF cells were transiently cotransfected with pTNF-wt (1μg), pTNF-PPRE(-1) (1μg), pTNF-PPRE(-2) (1μg) or pTNF-PPRE(-1/-2) (1μg) and pCMV-hPPAR-γ (100ng) and incubated with either vehicle or rosiglitazone (1μM) for 20h. Cells were then harvested and luciferase assays were performed. Luciferase activities are shown as mean ± SEM (n=3). Results are expressed as the percentage of reduction of TNF-α transcriptional activity by rosiglitazone.

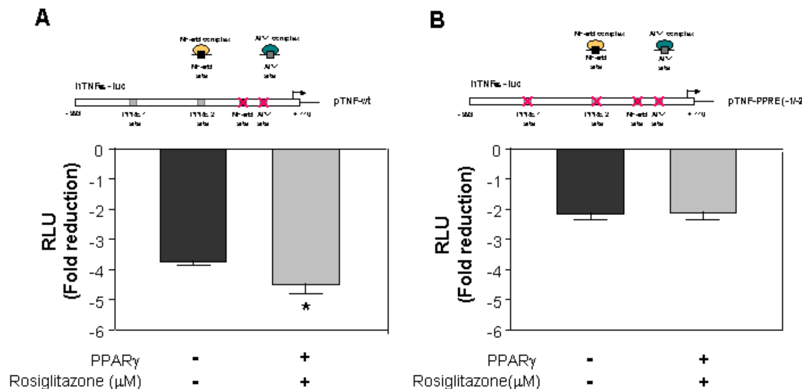

Figure 5

PPAR-γ directly activates TNF-α transcription. MEF cells were transient cotransfected with pTNF-wt (1μg) (A) or pTNF-PPRE(-1/-2) (1μg) (B) and pCMV-hPPAR-γ (100ng) and with a 100-fold excess of oligonucleotides containing the recognition elements for AP-1 and NF-κB with respect to the amount of the TNF-α promoter and then treated with vehicle or rosiglitazone (1μM) for 20h. Then cells were harvested and luciferase assays were performed. Luciferase activities are shown as mean ± SEM (n=3). Results are expressed as n-fold reduction relative to the basal TNF-α transcriptional activity.

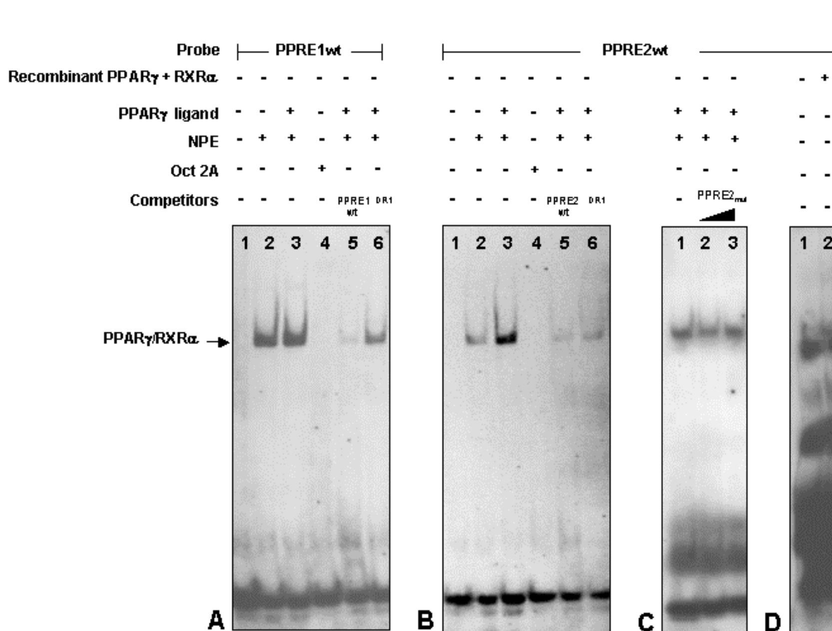

Figure 6

PPAR-γ binding analysis to the TNF-α PPRE-like sites. Nuclear protein extracts (NPE) from human THP-1 monocytes derived macrophages treated with vehicle or rosiglitazone (1μM) were assayed for binding to labelled doubled-stranded oligonucleotides by EMSA. Labelled $PPRE1_{wt}$ (A) or $PPRE2_{wt}$ (B) oligonucleotide probe was incubated with NPE treated (lanes 3,5-6) or untreated with PPAR-γ ligand (lane 2), with the purified Oct2A (lane 4) for testing binding specificity or with any transcription factor at all. The competition experiments were performed using a 200-fold excess or 125-fold excess of unlabelled oligonucleotides of $PPRE1_{wt}$ or DR-1 probe respectively (lanes 5-6). Competition experiments of labelled $PPRE2_{wt}$ using 60- or 125-fold excess of unlabelled oligonucleotide of $PPRE2_{wt}$ probe is shown in panel (C) (lanes 2-3). (D) Labelled $PPRE2_{wt}$ oligonucleotide probe was incubated with hPPAR-γ and mRXR-α subunits synthesized by *in vitro* transcription/translation (lane 2). The arrow indicates PPARγ/RXRα bound complex. The figures show representative experiments.

ESTUDI 4

EFFECTS OF 2,4-DECADIENAL ON PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ (PPARγ) ACTIVATION

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Key words: aldehyde, atherosclerosis, PPARgamma, thiazolidinedione, adipocyte differentiation

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ABSTRACT

Peroxisome proliferator activated receptor γ (PPARγ) synthetic agonists have shown net antiatherosclerotic effects in atherosclerotic animal models and in clinical studies with patients with high aterogenic risk factors. One contribution to this action may be their antiinflammatory activity since atherosclerosis is considered a chronic inflammatory disease. Extensively oxidized low density lipoprotein (oxLDL) inhibits macrophage cytokine secretion and has been pointed as a source of PPARγ biological ligands. In this work we have studied the effects of 2,4-decadienal, an oxidative end product which accumulates in oxLDL, on PPARγ activity. Results showed a 2,4-decadienal dose-dependent activation of transcriptional activity mediated by PPARγ. This action correlates with an increase in PPARγ protein levels in nuclear protein extracts from human monocytes derived macrophages incubated with 2,4-decadienal. Protein increases following a bimodal pattern (4.7- and 3.6-fold increase after 30min and 24h of 2,4-decadienal treatment respectively). However no variations were observed in the steady state PPARγ mRNA levels under the same conditions. Indeed, 2,4-decadienal induces 3T3-L1 adipocyte differentiation. These data suggest that 2,4-decadienal may be an endogenous PPARγ ligand in the atherosclerotic plaque.

INTRODUCTION

Atheroclerosis is a progressive disease characterized by a lipid accumulation in the subendothelial artery wall (1). There is considerable evidence to suggest that lipid peroxidation has an important role in the pathogenesis of atherosclerosis (2). Oxidized LDL and some of their oxidized lipidic compounds have been localized in atherosclerotic lesions (3-9). A recent interesting finding is the possible role of PPARγ in atherosclerosis. Thiazolidinediones, drugs used in the treatment of non insulin dependent diabetes mellitus, are high affinity PPARγ ligands (10) and have shown antiatherosclerotic effects in LDL receptor $^{-/-}$ and apo E $^{-/-}$ mice (11-14) and in limited human studies (15-16). PPARγ is a ligand dependent nuclear receptor (17) expressed in primary cultures of vascular cells (endothelial cells (18), smooth muscle cells (19) and macrophages (20-21)) and overexpressed in atherosclerotic plaques where colocalizes with oxidized lipids (20-21). Since it was cloned, many groups have focused their research ahead of the discovery of its endogenous ligand. To date a large list of lipidic compounds have been proposed to be its natural ligand (22-23) which have lead to suggest that PPARγ as other PPAR family members acts as a general lipid sensor. All of these putative PPARγ ligands have shown their PPARγ potential activation *in vitro* with different grade affinity, however consistent evidence about their efficiency at physiological status is lacking. Bishop et al. recently reviewed the variety existing endogenous PPARγ ligands pathways using Dale's criteria for endogenous mediator classification. And they speculate with the possibility that we still do not know the true biological ligand (23). It has also been suggested that there could exist tissue specific ligands which may explain their different described actions. Regarding this point, in our field of study lipid peroxidation products accumulated in the lipidic necrotic core of atherosclerotic plaques are serious tempting candidates. Recent findings that demonstrate an association between the degree of LDL oxidation and PPARγ activation support this fact (24-25). Indeed constituent products of oxidized LDL derived from phospholipid modification have been firmly hypothesized as potential PPARγ mediators (24,26). Our group has previously reported PPARγ-like actions mediated by aldehydes (27-28). Aldehydes are the final products of the oxidation of PUFAs and accumulate in extensively oxidized LDL (29). PUFAs are the main fatty acids of LDL and they are esterified to cholesterol or forming part of phospholipid structure in the core and the surface of the particle respectively (30). Aldehydes at high doses have shown citotoxicity and at low doses have potent actions on gene expression, specially 2,4-decadienal. This apolar aldehyde derives from the oxidation of the main

PUFAs present in LDL: linoleic and arachidonic acid (30-31). 2,4-decadienal precursors, fatty acids and intermediate oxidative compounds: HODEs and HETEs are considered PPARγ activators (23-24).

We hypothesized that aldehydes may be PPARγ mediators in atherosclerotic plaques. In the present study we have tested the potential role of 2,4-decadienal as a PPARγ ligand.

EXPERIMENTAL PROCEDURES

Cell culture

Monocytic leukemia THP-1 cells were cultured in RPMI 1640 supplemented with 10% foetal bovine serum, 100U/ml penicillin, 100μg/ml streptomicyn (Invitrogen) and 50μM 2-mercaptoethanol (Sigma). Cells were differentiated to macrophages by addition of phorbol 12-myristate 13-acetate (PMA), solubilized in DMSO (Sigma) at a concentration of 50ng/ml to suspension cells. MEF embryo fibroblasts were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomicyn (Invitrogen).

Mouse 3T3-L1 preadipocytes were cultured in DMEM supplemented with 10% FBS, 100U/ml penicillin and 100μg/ml streptomicyn (Invitrogen). Adipocyte differentiation was induced 2 days after the cells reached confluence by changing the medium to one containing 0.5mM dexamethasone, 5μg/ml insulin and 1μM 3-isobutyl-1-methylxanthine (SIGMA). After 3 days, medium was replaced to one containing 10μg/ml insulin and DMSO or 2,4-decadienal (Fluka) or rosiglitazone (BRL) or either 2,4-decadienal and rosiglitazone. Medium was refed every 2 days.

Cells were incubated with 2,4-DDE 0.5-5μM (i.e., a non toxic concentration range at which modulation of gene expression has been observed and within the range detected in oxidized LDL) (28-29) for up to 24h dissolved in DMSO or with vehicle alone. BRL at 1μM was used as a positive control. THP-1 cells were used to obtain nuclear protein extracts and total RNA, MEF cells were used for the transfection studies and 3T3-L1 cells were used for the differentiation assay.

Preparation of nuclear protein extracts and Western blot analysis

Nuclear protein extracts were prepared essentially as described previously (32). Modifications include lysis of plasmatic membranes by repeated aspirations through a 26-gauge needle rather than using a Douncer homogenizer. Protein concentration of all the preparations were determined by Bradford (33) using BSA as standard and stored at -70ºC. PPARγ protein was determined in nuclear protein extracts from THP-1 macrophages treated with 2,4-decadienal (1μM) for 0-24h or with vehicle alone as a negative control.

All proteins were subjected to an SDS-PAGE electrophoresis, electroblotted to nitrocellulose membranes (Amersham-Pharmacia Biotech) and blocked overnight at 4ºC with 5% (w/v) skimmed milk in TBS to avoid unspecific binding. The membranes were incubated for 1h at room temperature with mouse IgG anti-PPARγ (SantaCruz Biotechnology, Inc) and next incubated for 30min at room temperature with goat anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences). Blots were developed with the SuperSignal west femto maximum sensitivity substrate (Pierce) according to manufacturer's protocol. Densitometric analysis was performed by using the software program Kodak Digital Science 1D (Invitrogen). The protein sizes were confirmed by comparision with molecular weight standards (Invitrogen).

Plasmids

Human PPARγ (h PPARγ) expression vector and the reporter plasmid J_3-tk-Luc which contains three copies of the PPAR response element (PPRE) followed by a thymidine kinase gene promoter in front of luciferase cDNA were described previously (34).

Transient transfection and gene reporter assays

Transient transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 1μg J_3-tk-Luc containing consensus DR-1 x3, 100ng hPPAR-γ expression plasmid and 5μl LipofectAMINE 2000 were gently mixed in 250μl DMEM (Invitrogen) and incubated for 20min at room temperature. After 4h, transfected cells were incubated with 2,4-DDE for 2h, and after medium replacement cells were leaved until luciferase experiments were done or incubated with BRL or their vehicle alone (DMSO) for 24h. Luciferase activities were measured in cell lysates in a luminometer (Berthold) using the Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Total protein content of total lysates were measured by Bradford to normalize the transfection results.

PPAR-γ real-time RT-PCR

THP-1 macrophages were incubated in the presence of 2,4-decadienal (1μM) for 30min and 24h or BRL (1μM) for 24h and a negative control was obtained at each time point with vehicle alone. Total cellular RNA was isolated from the cells using PerfectRNA mini kit according to manufacturer's recommendations (Eppendorf). Reverse transcription reactions were performed using 1μg total RNA, 1X TaqMan RT buffer, 5.5mM $MgCl_2$, 500μM of each dNTP, 2.5μM random hexamers, 40U RNase inhibitor, and 125U MultiScribe Reverse Transcriptase (PE Applied Biosystem, Foster City, CA). Reactions were carried out at 25ºC for 10min, 48ºC for 30min and 95.5ºC for 5min. Real time PCR detection of PPARγ expression was performed in triplicate using 10μl first strand cDNA for each sample, the PPARγ Assay-on-demand Kit and the Pre-Developed TaqMan Assay Reagents Target Kit for human GADPH and the ABI PRISM 5700 (Applied Biosystems, Foster City, CA). Due to amplification efficiencies of PPARγ and GADPH were equal, the ratio between them could be calculated as ΔC_t=TNF-α C_t - GADPH C_t and results are expressed as $2^{-\Delta\Delta Ct}$, where C_t= cycle number at which the fluorescence signal exceeds background and $\Delta\Delta C_t = \Delta C_t$ BRL - ΔC_t control.

Oil Red O staining

On day 11 after differentiation induction, cells were washed with phosphate buffered saline (Invitrogen), fixed with 10% formaldehyde (Sigma) and stained with an Oil Red O-isopropyl alcohol solution (Sigma) which allowed to visualize under microscopy lipid droplets stained in red.

RESULTS

Effects of 2,4-decadienal on PPARγ protein and mRNA levels

To analyze the effect of 2,4-decadienal on PPARγ activation we first tested whether it produces changes at protein level. Western-blot assays revealed that 2,4-decadienal at 5μM increases PPARγ protein levels in a bimodal pattern respect to vehicle alone in nuclear protein extracts from human THP-1 monocytes derived macrophages. Peaks were observed at 30min and 24h after aldehyde treatment and corresponded to a 4.7- and 3.6-fold induction of protein levels respectively (Fig. 1A). These results indicate that 2,4-decadienal can activate PPARγ since inactive PPARγ form resides in the cytoplasm complexed to its corepressors and as a result of ligand induction translocates to the nucleus in an active form.

Subsequent experiments were set to determine potential effect of 2,4-decadienal at PPARγ mRNA level. No evident alterations were found in PPARγ steady state mRNA levels by effect of 1μM 2,4-decadienal incubations using real time RT-PCR analysis (Fig. 1B).

Effects of 2,4-decadienal on PPARγ dependent transcriptional activity

To assess the ability of 2,4-decadienal on activating a PPRE element, we transient cotransfected MEF cells with a hPPARγ expression vector and with a vector containing 3

consecutive consensus PPRE elements upstream luciferase gene. Results showed that 2,4-decadienal enhances PPARγ dependent transcriptional activity in MEF cells. This effect was dose dependent and at 2μM (2.1-fold induction) resembles to the effect of the synthetic PPARγ ligand, rosiglitazone (BRL) (1μM) (2.6-fold induction) (Fig. 2).

Effects of 2,4-decadienal on adipocyte differentiation

Finally, we tested the effect of 2,4-decadienal on a mechanism mediated by PPARγ. Because PPARγ has shown a pivotal role in adipocyte differentiation, we analized lipid accumulation in response to 2,4-decadienal in 3T3-L1 cells. After the induction of differentiation to adipocytes by treating cells with the induction medium for 72h, addition of 2,4-decadienal enhanced cellular lipid droplets accumulation dose-dependently compared to vehicle. Microscopic visualization of cell preparations at 11 days (Fig. 3) show that 2,4-decadienal up to 10μM produces strong lipid droplets storage in cells. Furthermore, 2,4-decadienal seems to act synergistically to rosiglitazone on promoting adipocyte differentiation.

DISCUSSION

PPARγ is a ligand dependent nuclear receptor which activation has been involved in lipid homeostasis, inflammation, cellular differentiation and proliferation (35). Since the last few years there is increasing interest on its potential role in diverse pathological conditions such as atherosclerosis. Oxidized LDL has been pointed as an important source of PPARγ ligands in the vasculature since recently the entirely particle and some of its oxidized lipidic constituents showed PPARγ ligand activities (24,36). Indeed PPARγ is overexpressed in atherosclerotic lesions where colocalizes with oxidized lipids (20-21). *In vitro* studies showed that increasing degree of oxidation of LDL enhanced PPARγ mRNA levels and PPARγ dependent transcriptional activity (24-25). It may be suggested from these previous results that oxidized lipidic compounds that accumulate in late stages of LDL oxidation are better PPARγ candidates than the ones produced in early stages of lipid peroxidation. These would be final oxidative products such as oxysterols and aldehydes. Oxysterols as well as cholesterol, its precursor, were reported not to have any effect on PPARγ activation (24). However, little and contradictory data exists about aldehydes action. According to Nagy et al. the hydroxyaldehyde 4-HNE was inactive on terms of activation of PPARγ dependent transcription (24) whereas Pizzimenti et al. described synergistic effects of 4-HNE and PPARγ ligands in controlling human cell growth and differentiation (37). Its worthnoting that dispared concentrations were used in these experiments. While in the first described experiments, 4-HNE was used at a very high dose which has been demonstated to be toxic for human macrophages (27), the synergistic effect was shown at a low dose at which diverse aldehydes have produced gene expression modulation in several cell types (27-28). Furthermore 4-HNE precursors: linoleic acid, arachidonic acid and some of their oxidative products: 9-HPODE, 9-HODE, 13-HPODE, 13-HODE, 9-oxoODE,13-oxoODE and 15-HETE were all considered putative PPARγ ligands (23). Crystallographic studies of PPARγ revealed a large hydrophobic ligand cavity which suggests that the wide variety of known ligands may be accommodated in its pocket (38), but physiological potential of these ligands is still lacking. These had lead to suggest by diverse authors that PPARγ may act as a general lipid sensor. Recently, Bishop et al. reviewed the variety existing endogenous PPARγ ligands pathways and they speculate with the possibility that there could exist tissue specific ligands which may explain their different described actions (23). And also it has been demonstrated that different ligands confered changes in the global PPARγ complex coactivator

structure that may result in different gene target activation which would support the above hypothesis (39). In the present study we have focused the attention on the apolar aldehyde 2,4-decadienal and its ability to activate PPARγ. There are several evidence that support its potential as a PPARγ mediator in the pathological environment of atherosclerotic lesions: 1) it derives from the same precursors as 4-HNE mentioned above, the main PUFAs present in LDL and it has been suggested that aproximately 30% of plaque fatty acids are oxidized (40); 2) indeed oxidative metabolites above mentioned showed increased PPARγ activation associated to its degree of oxidation; 3) 2,4-decadienal belongs to the apolar fraction of aldehydes which have been detected in human lesions in higher amounts compared to non pathological areas (41); 4) its levels accumulates in *in vitro* extensively oxidized LDL where approximately 90% of linoleic and arachidonic acid are depleted (25); 5) reactive aldehydes are elevated in patients with hyperlipidemia and inflammatory diseases (42-44); 6) aldehydes have shown a high reactivity to bind to proteins and DNA (45); 7) other aldehydes such as 4-HNE have shown nuclear action (46); 8) 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) one of the two most active natural PPARγ ligands described shares half of its structure with 2,4-decadienal (Fig. 4); 9) our group have previously reported PPARγ-like actions directed by non toxic concentrations of 2,4-decadienal: cell growth arrest and inhibition of c-myc mRNA levels in human vascular smooth muscle cells, (28) and inhibition of TNFα mRNA levels and protein secretion in human macrophages (27); 10) non-steroidal anti-inflammatory drugs and PPARγ activation by clofibrate decreases ALDH3 expression (47-48), the enzyme that metabolises 2,4-decadienal (49).

In the present study, we sought to investigate 2,4-decadienal action on PPARγ activation. We detected that 2,4-decadienal increases nuclear PPARγ protein levels in human THP-1 macrophages without affecting PPARγ steady state mRNA levels. This indicates that 2,4-decadienal is involved in translocation of the nuclear receptor to cell nucleus which may cause effects on PPARγ activity non related to its synthesis. Thus, we focused on PPARγ activation by 2,4-decadienal. Transfection studies showed 2,4-decadienal ability of inducing PPARγ mediated transcriptional activity through PPRE elements. This action suggests that 2,4-decadienal may modulate PPARγ target gene regulation which may affect mechanisms in which PPARγ has shown a pivotal role such as lipid homeostasis, inflammation, cellular differentiation and proliferation. Further, in order to study this hypothesis, we studied 2,4-decadienal effect on 3T3-L1 adipocyte differentiation. Oil Red O staining results supported the potential role of 2,4-decadienal as a PPARγ mediator due to that aldehyde addition stimulated stronger lipid droplet accumulation characteristic of adipocyte differentiation. This observation was comparable to that caused by the synthetic PPARγ ligand rosiglitazone, the most potent adipogenic promoter and both compounds seemed to act synergistically on driving adipocyte differentiation.

The two most potent endogenous ligands described are 15d-PGJ_2 (50-51) and the hexadecyl azelaoyl phosphatidilcholine (azPC) (36). The role of 15d-PGJ_2 as a PPARγ ligand has been the subject of a recent review (52). Neither detectable levels of 15d-PGJ_2 in synovial fluid of patients with arthritis, nor alteration of 15d-PGJ_2 levels in urine of volunteers after acute inflammation induction or of diabetic patients with or without concurrent obesity were observed (53), pathologies at which the role of 15d-PGJ_2 as a PPARγ ligand had been involved. However, 15d-PGJ_2 biosynthesis data in macrophages cells is still lacking (54) although an immunoassay detected its presence in foam cells cytoplasm but failed in the aortic wall (55). Further, Bell-Parikh et al. demonstrated that 15d-PGJ_2 is not the endogenous mediator of PPARγ dependent adipocyte maturation *in vitro* (53).

azPC is the last PPARγ ligand discovered. It is an oxidized phospholipid present in oxidized LDL (36) and fragmented phospholipids have been found in human plasma and atherosclerotic lesions (5). However, oxidized phospholipids transiently appeared during LDL oxidation. These products are more abundant in early stages of oxidation. And it has been hypothesized that at last stages final oxidative products accumulates in oxidized LDL such as MDA, 4-HNE, oxysterols and lysophosphatidilcholine (5) which will give more potential in atherosclerotic plaques to our proposed mediator.

Thus, considering the list of evidence that support the potential of 2,4-decadienal and the results of the present study, our hypothesis is that the studied aldehyde may be a firmly candidate to PPARγ mediator in pathological conditions. Further experiments, based on PPARγ interactions assays and on the involvement of PPARγ on the mechanism of action of 2,4-decadienal on TNFα gene regulation and on other PPARγ controled processes are needed to confirm 2,4-decadienal potential action *in vivo*.

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Figures

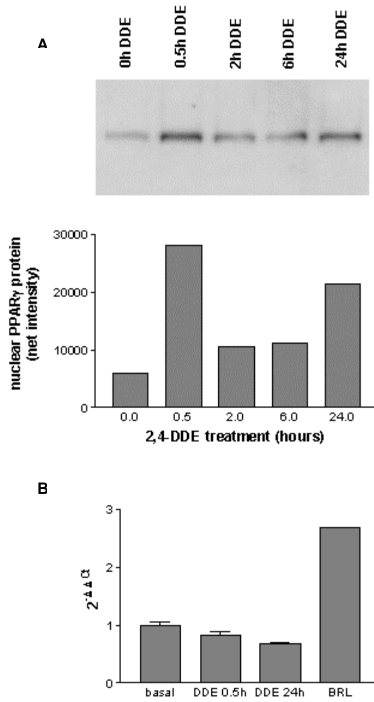


Figure 1

2,4-decadienal increases PPARγ translocation to the nucleus of human macrophages without altering its mRNA levels. (A) Nuclear protein extracts from human macrophages treated with 2,4-decadienal at 1μM for 0-24h or with vehicle alone were analyzed by Western blot using an antibody against PPARγ. Bar graph values represent total net intensity of the Western blot bands expressed as rbitrary units. (B) Total RNA was isolated from human macrophages treated with 2,4-decadienal at 1μM for 30min or 24h or with BRL at 1μM for 24h or with vehicle alone at each time point. Real time RT-PCR of PPARγ was performed as indicated in material in methods. Results are expressed as $2^{-\Delta\Delta Ct}$.

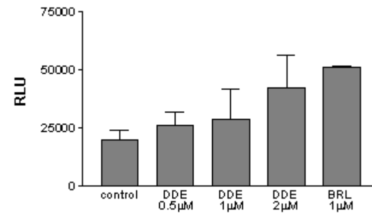


Figure 2

2,4-decadienal increases gene transcription directed by PPARγ. MEF cells were transiently transfected with a hPPARγ expression vector and with a vector containing 3 consecutive PPRE elements (J_3-tk-Luc) and incubated with 2,4-decadienal at 0-2μM for 2 h or with rosiglitazone (BRL) at 1μM for 20h. Then cells were harvested and luciferase assay were performed. Results are expressed as relative luciferase units.

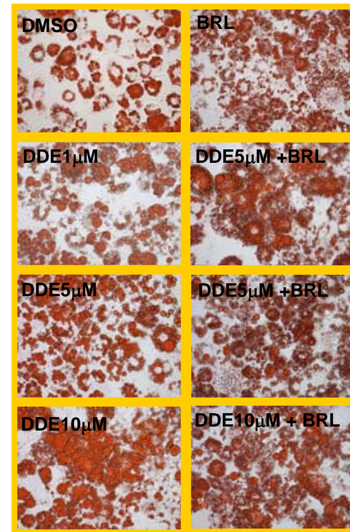
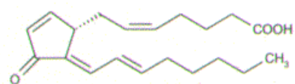


Figure 3

2,4-decadienal promotes adipogenesis. 3T3-L1 were differentiated to adipocytes in media containing DMSO (vehicle), 1-10μM 2,4-decadienal, 1μM rosiglitazone or 2,4-decadienal and rosiglitazone. After 11 days, cells were fixed, stained with oil red O and photographed. Pictures were microscopic views at 20x magnification.



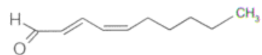


Figure 4

Structure of 15d-PGJ_2, one of the most active endogenous PPARγ ligand described, and 2,4-decadienal, an apolar aldehyde present in oxLDL.

ESTUDI 5

European Journal of Clinical Investigation (2003) **33**, 464–471

Fatty acids modulate the effect of darglitazone on macrophage CD36 expression

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Abstract

Background Scavenger receptor-mediated uptake of cholesterol by macrophages in the arterial wall is believed to be proatherogenic. Thiazolidinediones are peroxisome proliferator-activated receptor γ (PPARγ)-agonists, which are used in the treatment of type II diabetes. They reduce atherogenesis in LDL receptor deficient and ApoE knockout mice, but up-regulate CD36, which may contribute to foam cell formation. The dyslipidaemia in type II diabetes is characterized by high levels of nonesterified fatty acids. Therefore we tested the effect of fatty acids and how fatty acids and the thiazolidinedione darglitazone interact in their effect on CD36 expression in human monocytes and macrophages.

Materials and methods Flow cytometry and reverse transcription-polymerase chain reaction were used to study CD36 expression. Cellular lipids were analyzed with high performance liquid chromatography.

Results Darglitazone increased CD36 mRNA and protein expression in human macrophage cells. In the presence of 5% human serum, darglitazone increased the accumulation of triglycerides, but did not affect cholesterol ester levels. In the presence of albumin-bound oleic or linoleic acid, darglitazone did not increase CD36 mRNA, cell-surface CD36 protein or triglyceride content. Fatty acids per se increased CD36 mRNA and protein.

Discussion The increase in CD36 in macrophages suggests a role for fatty acids in the regulation of foam cell formation. The results also suggest that the potentially proatherogenic CD36 up-regulating effect of thiazolidinediones in macrophages might not be present when the cells have access to physiological levels of albumin-bound fatty acids.

Keywords Atherosclerosis, CD36, fatty acid, human macrophage, scavenger receptor, thiazolidinedione.

Eur J Clin Invest 2003; 33 (6): 464–471

Introduction

Deposition of cholesterol in the arterial wall is a key element in the development of atherosclerosis. A large part of the cholesterol in atherosclerotic plaques is present within the macrophage-derived foam cells. The uptake of cholesterol, as part of modified lipoproteins, is mediated via a family of proteins called scavenger receptors. Of macrophage scavenger receptors characterized today, CD36 and scavenger receptor class A (SR-A) types I and II are considered the most important for foam cell formation [1]. Both CD36 and SR-A are expressed in atherosclerotic plaques [2,3] and deficiency of CD36 leads to decreased atherosclerosis in ApoE knockout mice [4].

CD36 is a 88-kDa membrane-bound glycoprotein that is expressed on several cell types, including monocytes and macrophages (for review, see [5]). This receptor binds a wide spectrum of ligands, including native or modified lipoproteins [6,7] and isolated phospholipids [8] and it has been postulated to be a fatty acid translocase involved in fatty acid uptake in cells with an active lipid metabolism,

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such as cardiac and skeletal muscle, and adipocytes [9]. The expression of CD36 is influenced by various cytokines [10] but also, which seems very important because of its function as a fatty acid translocase, by cellular or extracellular lipids [11–13]. Transcription of the CD36 gene is regulated by peroxisome proliferator-activated receptor γ (PPARγ) [14], a transcription factor that modulates the expression of genes that control how fatty acids are converted to and stored as triglycerides and other esters in the cell (for review, see [15]). As well PPARγ is involved in cholesterol handling in macrophages [16,17].

Thiazolidinediones (TZDs) are a relatively new class of drugs used in the treatment of type II diabetes and have PPARγ as their target [18]. Type II diabetes is associated with a marked increase in the risk of coronary heart disease [19] and any proatherogenic effect of these drugs would be undesirable. Based on findings on gene-manipulated mice and limited human studies these drugs are considered antiatherogenic [20–22]. However, these findings seem to be in conflict with the increased CD36 expression that the TZDs induce in macrophages, which has been suggested to be potentially proatherogenic [14,20,23]. Patients with type II diabetes also have lipid abnormalities, such as increased levels of large VLDL and small, dense LDL and low levels of HDL [24]. Additionally they frequently have high levels of postprandial and fasting albumin-bound non-esterified fatty acids (NEFAs) [25]. The possible impact of increased fatty acid supply to macrophages that may contribute to atheroma formation has not been thoroughly investigated despite strong evidence that NEFAs can mediate potentially atherogenic actions in endothelial and smooth muscle cells [26,27].

The aim of this study was to determine the impact of albumin-bound fatty acids and the TZD derivate darglitazone and their interaction on the expression of scavenger receptor CD36 in human monocyte-derived macrophages.

Materials and methods

Macrophage cell culture

Mononuclear cells were isolated through a Ficoll–Hypaque discontinuous gradient [28] from buffy coats obtained from the local blood bank (Sahlgrenska University Hospital, Göteborg, Sweden). The cells were resuspended in RPMI1640 medium supplemented with 2 mmol L^{-1} of glutamine, 2 mmol L^{-1} of nonessential amino acids, 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin, and 20 mmol L^{-1} of sodium pyruvate (BioWhittaker, Verviers, Belgium). Monocytes were obtained by allowing cells to adhere to plastic for 60 min at 37 °C. Non-adhered cells were removed by washing with PBS and the adhered monocytes were used immediately for experiments (freshly isolated monocytes) or left to differentiate into macrophages in supplemented RPMI1640 containing 20% heat-inactivated human serum for 8 days. Macrophages then constituted approximately 95% of the cell population, as judged by flow cytometry (data not shown). For experiments the cells were incubated for 4 or 24 h with supplemented RPMI1640 (pH 7·4) with 1 μmol L^{-1} of α-tocopherol in the presence of 100 or 300 μmol L^{-1} of various fatty acids (linoleic, oleic and palmitic acid) (Sigma Aldrich, Stockholm, Sweden) bound to 100 μmol L^{-1} of BSA. Darglitazone (AstraZeneca, Mölndal, Sweden) was added directly to this medium from a 2·3-mmol L^{-1} solution (in ethanol) to a final concentration of 4 μmol L^{-1}. In some experiments albumin and α-tocopherol were exchanged to 5% heat-inactivated human serum, as indicated. In all experiments, ethanol was added to the control medium at a concentration comparable to that in medium containing darglitazone and/or fatty acids. The viability of cells was > 95% in all incubations, as determined by Trypan blue exclusion.

Isolation and analysis of RNA

Total RNA was isolated from monocytes and macrophages using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure according to Chomczynski and Sacchi [29]. The expression of CD36 mRNA was related to β-actin mRNA expression by semiquantitative RT-PCR as previously described [30]. Specific primers had the following sequences: 5′-TGGGTCAGAAGGATTCCTATGT-3′, 5′-CAGCCTGGATAGCAACGTACA-3′ for β-actin and 5′-GAGAACTGTTATGGGGCTAT-3′, 5′-TTCAACTGGAGAGGCAAAGG-3′ for CD36 [13] (Gibco BRL). All incubations were performed in a Biometra TRIO Thermoblock (Biometra, Göttingen, Germany). The PCR products were separated and analyzed on an ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA) and the fluorescent signals were analyzed with Gene Scan 2·1 (Applied Biosystems).

Immunofluorescent flow cytometry

Cell-surface expression of CD36 was assessed with immunofluorescent flow cytometry. The analysis was carried out by using a mouse monoclonal antibody against CD36 (Clone FA6-152). After incubation with the indicated substances, macrophages were washed with PBS and fixed in 4% formaldehyde in phosphate buffer. Cells were gently removed from the dish with a rubber policeman, washed and incubated with FITC-conjugated antihuman CD36 antibody (Immunotech, Marseille, France) or FITC-conjugated control mouse IgG (Becton Dickinson, San Jose, CA) in the presence of 0·1% BSA. Incubations were performed at saturating antibody concentrations. The cells were analyzed using a FACScan flow cytometer (Becton Dickinson). Forward- and side-angle light scatter were used to distinguish macrophages from lymphocytes and cell debris. A population of 5000 gated cells was analyzed in each experiment and median fluorescence intensity was measured using CellQuest software (Becton Dickinson). The arbitrary units of fluorescence were then converted to molecules of equivalent fluorochrome (MEF) values by

construction of a calibration curve with DAKO Fluorospheres (DAKO, Glostrup, Denmark) according to the manufacturer's protocol.

Extraction and analysis of lipids

Lipids were extracted from cells with hexane-isopropanol (1 : 2) containing 10 µmol L^{-1} of butylated hydroxytoluene and purified with chloroform-methanol extractions according to Folch [31]. The lipids were fractionated and analyzed with HPLC using a Gynkotec 14480 ternary system (Gynkotec, Munich, Germany) and an evaporate light-scattering mass detector, PL-ELS 1000 (Polymer Laboratories, Church Stretton, Shropshire, UK). The chromatographic conditions were essentially those described by Homan and Anderson, but with n-heptane:tetrahydrofurane (99 : 1, v/v) as the first solvent mixture [32]. Cellular proteins were dissolved with 0·2 mol L^{-1} of NaOH and analyzed using the Bradford method with BSA as the standard [33].

Statistical analyses

Data from semiquantitative RT-PCR and flow cytometry were analyzed with Wilcoxon signed ranks test and data from lipid analyses with Student's two-tailed *t*-test, using SPSS 10·0 software package for Windows. $P < 0·05$ was considered statistically significant.

Results

The effect of darglitazone on CD36 mRNA expression was evaluated both in freshly isolated monocytes and in macrophages differentiated in culture for 8 days. The cells were incubated with darglitazone at a concentration of 4 µmol L^{-1} for 4 and 24 h. When added directly to the NEFA-free medium containing albumin, darglitazone increased CD36 mRNA levels in both monocytes and macrophages, as shown in Fig. 1. To investigate if the enhancement in CD36 mRNA expression caused by darglitazone was associated with an increase in CD36 protein, monocytes and macrophages were incubated with 4 µmol L^{-1} darglitazone for 4 and 24 h. We studied the surface expression of CD36 with the immunofluorescent flow cytometry technique. Experiments were performed with cells from six different donors. The cell-surface CD36 expression was increased approximately 30% and 20% after 4 and 24 h, respectively ($P < 0·05$), as shown in Figs 2 and 3.

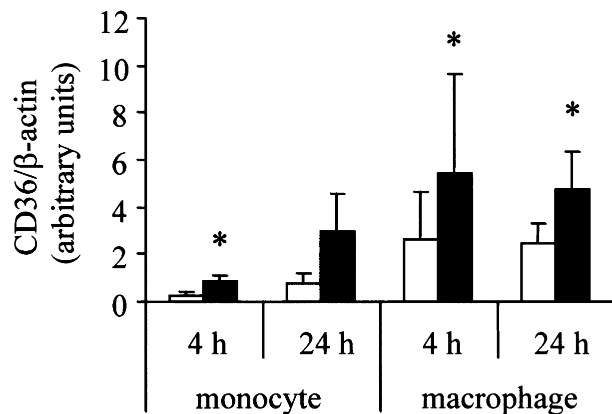

Figure 1 Effect of darglitazone on monocyte and macrophage CD36 mRNA levels. Freshly isolated monocytes and differentiated macrophages were incubated with (■) and without (control; □) 4 µmol L^{-1} of darglitazone for 4 and 24 h. RNA was isolated and analyzed with semiquantitative RT-PCR. The results are shown as CD36 mRNA:β-actin ratios. Data are presented as means ± SEM of six different donors for each cell type. $^{*}P < 0·05$ vs. control for the corresponding time and cell type, as analyzed with Wilcoxon signed ranks test.

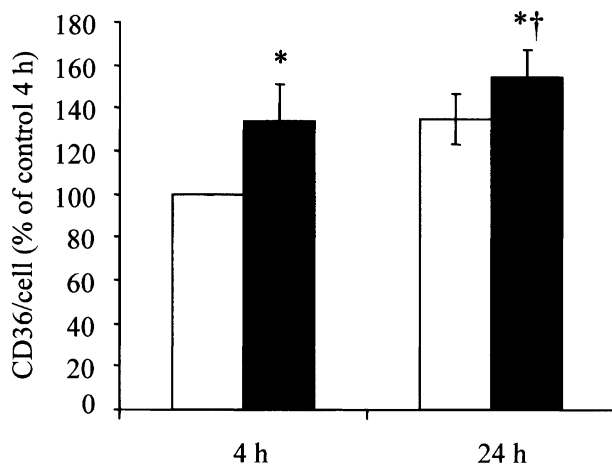

Figure 2 Effect of darglitazone on CD36 protein expression. Differentiated macrophages were incubated for 4 and 24 h with (■) and without (□) 4 µmol L^{-1} of darglitazone. Cells were stained with FITC-conjugated antihuman CD36 antibody and analyzed with flow cytometry. The results were calculated as median fluorescence intensity and expressed as percentage of control 4 h. Data are presented as means ± SEM of six different donors. $^{*}P < 0·05$ vs. control 4 h, $^{\dagger}P < 0·05$ vs. control 24 h.

To determine if physiological concentrations of albumin-bound fatty acids could influence the expression of CD36 mRNA, differentiated macrophages were incubated for 4 h with oleic (C18 : 1), linoleic (C18 : 2) and palmitic (C16 : 0) fatty acids in two different concentrations, 100 and 300 µmol L^{-1}, together with albumin. α-Tocopherol was added as an antioxidant. The control cells also received albumin and α-tocopherol. All three fatty acids increased the expression of CD36 mRNA in human macrophages after 4 h of incubation (Fig. 4). Of these three, oleic acid seemed to be the most potent inducer of CD36 mRNA. We also performed flow cytometric analyses on macrophages that were incubated for 4 h with albumin-bound fatty acids. When cells from six different donors were analyzed, linoleic

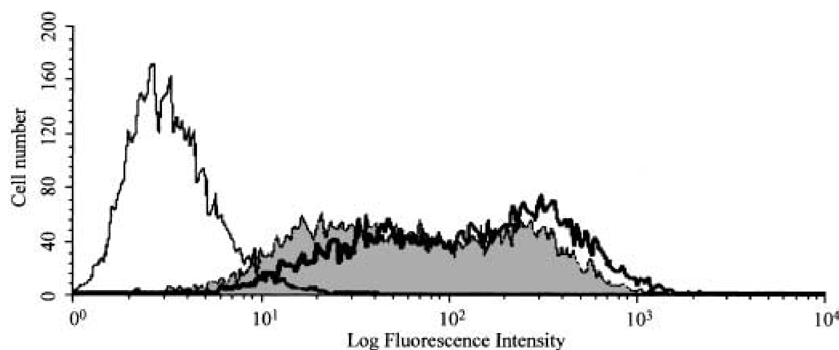

Figure 3 Flow cytometry profile of macrophages treated with (solid black line) and without (control; filled grey) 4 µmol L^{-1} of darglitazone for 4 h. Cells were stained with FITC-conjugated antihuman CD36 antibody. Shown to the left are cells stained with FITC-conjugated control mouse IgG as negative control (thin black line). All cells were from the same donor.

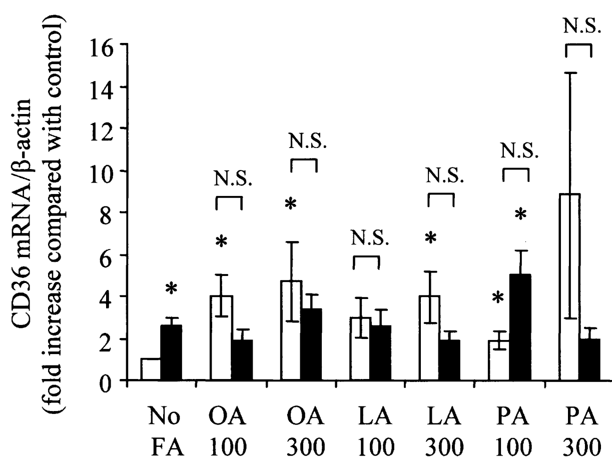

Figure 4 Effects of fatty acids and darglitazone on macrophage CD36 mRNA levels. Differentiated macrophages were incubated with oleic acid at concentrations of 100 (OA100) and 300 µmol L^{-1} (OA300), linoleic acid at 100 (LA100) and 300 µmol L^{-1} (LA300), and palmitic acid at 100 (PA100) and 300 µmol L^{-1} (PA300), both with (■) and without (□) 4 µmol L^{-1} of darglitazone, for 4 h. Albumin and α-tocopherol were present in all incubations. RNA was isolated and analyzed with semiquantitative RT-PCR. The results are shown as CD36 mRNA:β-actin ratios relative to the control (cells incubated without fatty acids (no FA) or darglitazone), in which the level of expression was set at 1·0. Data are presented as means ± SEM of six different donors. $^*P < 0·05$ vs. control.

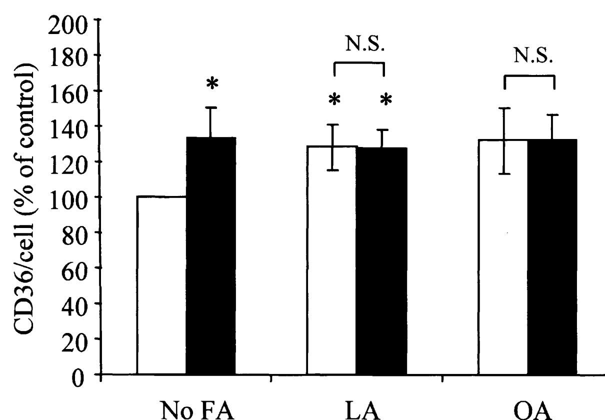

Figure 5 Effects of darglitazone and fatty acids on CD36 cell-surface expression. Differentiated macrophages were incubated for 4 h with (■) and without (□) darglitazone (4 µmol L^{-1}) and/or linoleic (LA) or oleic (OA) acid (300 µmol L^{-1}) in the presence of albumin and α-tocopherol. Cells were stained with FITC-conjugated antihuman CD36 antibody and analyzed with flow cytometry. The results were calculated as median fluorescence intensity and expressed as percentage of control cells incubated without fatty acids (no FA) or darglitazone. Data are presented as means ± SEM of six different donors. $^*P < 0·05$ vs. control.

acid increased cell-surface expression of CD36 protein by approximately 30% ($P < 0·05$) (Fig. 5). In the case of oleic acid, all but one donor responded with an up-regulation of CD36. After 24 h of incubation, both oleic and linoleic acid increased CD36 mRNA ($P < 0·05$ for both NEFAs) and cell-surface protein ($P < 0·05$ for both NEFAs) (data not shown).

The function of CD36 as a NEFA translocase and the fact that macrophages *in vivo* are always exposed to substantial concentrations of albumin-bound fatty acids prompted us to evaluate the actions of darglitazone in the presence and absence of fatty acids. In all media, 100 µmol L^{-1} fatty acid-free BSA and 1 µmol L^{-1} α-tocopherol were present. When the medium contained no fatty acids, we could detect the described increase in CD36 mRNA expression. However, when physiological levels (100 or 300 µmol L^{-1}) of fatty acids like oleic (C18 : 1), linoleic (C18 : 2) or palmitic (C16 : 0) acid were present in the culture medium the up-regulation of CD36 was no longer observed (Fig. 4). Thus, there was no additive effect of this drug and fatty acids on CD36 expression. As analyzed with flow cytometry, darglitazone showed no effect on CD36 cell-surface expression in the presence of either oleic or linoleic acid (Fig. 5).

During experiments we observed an increased accumulation of lipid-like droplets in the cytoplasm of macrophages treated with darglitazone in the presence of 5% human serum (data not shown). Therefore the lipid content of the cells was analyzed. Lipid analyses of macrophages from three different donors, which were incubated with and without 4 µmol L^{-1} of darglitazone for 4 and 24 h are shown in Table 1. There was a significant increase in the triglyceride content of cells treated with darglitazone compared with control cells. However, no significant changes were seen in the levels of cholesterol ester, free cholesterol or individual

Table 1 Lipid content in macrophages incubated with and without 4 μmol L^{-1} of darglitazone for 4 and 24 h in medium containing 5% human serum

	Lipid content (μg mg^{-1} of protein)			
	Control		Darglitazone	
	4 h	24 h	4 h	24 h
Cholesterol ester	5·7 ± 0·7	18 ± 11	8·9 ± 3·5	9·3 ± 2·6
Triglyceride	243 ± 36	251 ± 42	424 ± 51*	354 ± 46*
Cholesterol	31 ± 5·7	44 ± 9·0	61 ± 20	62 ± 21
Phosphatidylethanolamine	44 ± 8·9	68 ± 15	101 ± 31	107 ± 36
Phosphatidylserine	38 ± 7·5	54 ± 12	83 ± 30	75 ± 24
Phosphatidylcholine	47 ± 8·6	70 ± 13	107 ± 33	109 ± 38
Sphingomyeline 1	6·8 ± 1·1	9·3 ± 1·1	14 ± 5·1	14 ± 5·5
Sphingomyeline 2	14 ± 2·2	19 ± 3·5	34 ± 14	29 ± 13
Diacylglycerol	1·9 ± 1·0	6·9 ± 1·8	5·7 ± 1·5	5·8 ± 1·4

*$P < 0{\cdot}05$ vs. control.
Means ± SEM of three different donors are shown.

phospholipids. Such morphological and biochemical evidence confirms the well-established action of PPARγ-agonists to increase triglyceride synthesis in macrophages. It also indicates that in spite of a superficial morphological resemblance to arterial foam cells, the drug-induced changes are caused by triglyceride-rich inclusions with no enrichment of cholesterol or cholesterol ester.

We also evaluated the lipid content of cells that were incubated with darglitazone in the presence of fatty acids. Fatty acids alone increased the triglyceride content in the cells (oleic acid by 200%, NS and linoleic acid by 136%, $P < 0{\cdot}05$) compared with untreated cells. As with CD36 mRNA and protein there was no response in cellular lipid composition to darglitazone treatment in the presence of fatty acids. Shown in Fig. 6 is the triglyceride content of macrophages incubated with darglitazone in the presence and absence of fatty acids.

Discussion

In the present study we explored the regulation of CD36 by albumin-bound NEFAs and the PPARγ-agonist darglitazone. Both fatty acids and darglitazone increased the expression of CD36 in human monocyte-derived macrophages. However, when the effect of darglitazone was studied in the presence of physiological concentrations of fatty acids, darglitazone did not increase the expression of CD36 mRNA above the expression that was induced by the NEFAs. Thus the significance of effects of thiazolidinediones in the absence of physiological concentrations of an important CD36-ligand like albumin-bound NEFA must be re-evaluated [14,20,21].

In our experiments, linoleic and oleic acids, two of the most abundant NEFAs in plasma [34], increased CD36 mRNA and cell-surface expression in human monocyte-derived macrophages. In an earlier study, Pietsch *et al.* found that long chain fatty acids C22 : 6n-3 and C20 : 5n-3 decrease CD36 in leukaemia cell line U937 [35]. This effect was attributable to n-3 fatty acids, but not to n-6 fatty acids such as linoleic acid. The influence of fatty acids on CD36 has also been studied in other cell types. Infusion of intra-lipid plus heparin, which causes a dramatic increase in circulating NEFAs, resulted in an up-regulation of CD36 in subcutaneous adipose tissue in healthy men [36] and oleic and palmitic acids increase CD36 mRNA in neonatal rat cardiac myocytes [37]. All three of the fatty acids used in our experiments have been shown to bind to PPARγ *in vitro*, although with lower affinity than to the PPARα nuclear receptor [38]. Peroxisome proliferator-activated receptor α-activation by agonists has been shown to regulate the fatty acid translocase function of CD36 in the liver [39], but this has not been documented in monocytic cells. The oxidized metabolites of linoleic acid, 9-hydroxyoctadecadienoic and 13-hydroxyoctadecadienoic acid are regarded as stronger activators of PPARγ than linoleic acid itself [14]. To prevent the possibility of nonenzymatic oxidation of NEFAs α-tocopherol was added to the medium in our experiments, but we cannot exclude some intracellular oxidation of fatty acids over time by oxygenases. However, as oleic acid, which is not a substrate for lipo- or cycloxygenases, has a similar effect to that of linoleic acid, we believe that the effect we see is mediated directly by the NEFAs. As NEFAs in the circulation are bound to albumin, we have performed all incubations with fatty acids in the presence of BSA. In order to correct for any inhibitory effects of albumin [40], the same concentration of albumin was added to all incubations, including the control. Our results indicate that increased levels of circulating NEFAs, as in type II diabetes, may influence CD36 expression in arterial resident macrophages leading to an enhanced foam cell formation.

One of the most important functions of CD36 may be to facilitate uptake of NEFAs from their physiological circulating form as albumin complexes. In many of the published experiments about regulation of CD36 these important ligands have been missing, including those in which TZDs have been shown to up-regulate this receptor

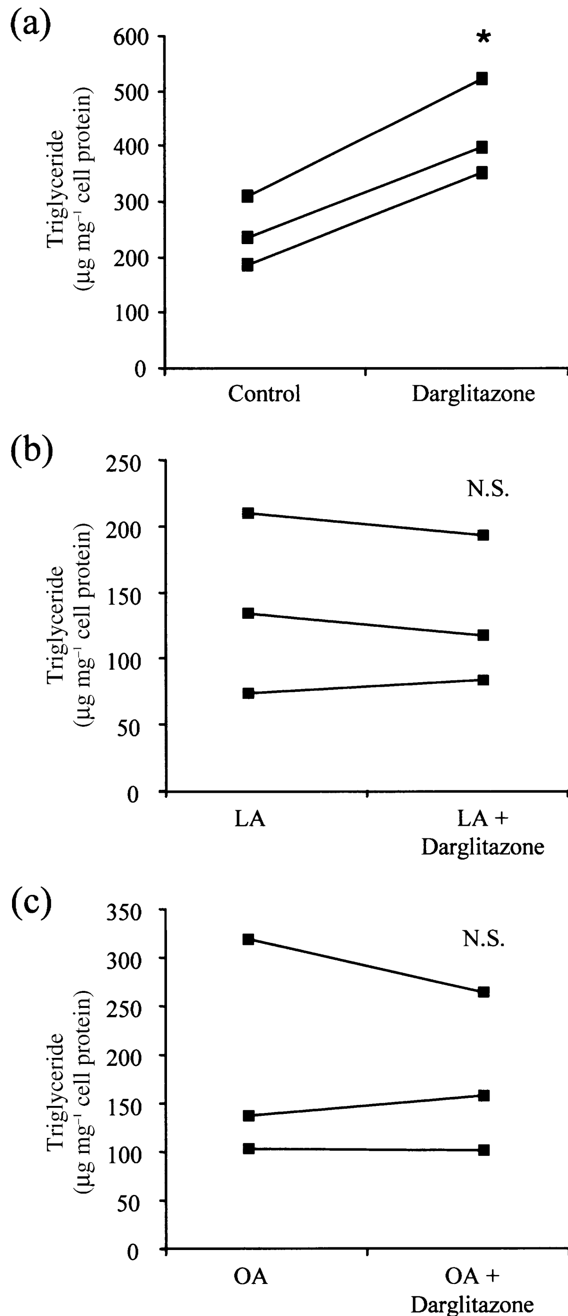

Figure 6 Effect of darglitazone on triglyceride content in the presence and absence of fatty acids. Differentiated macrophages were incubated with darglitazone (4 µmol L^{-1}) in the absence of fatty acids (with 5% human serum in the medium) (panel A) and in the presence of linoleic (LA; panel B) and oleic (OA; panel C) acid at 300 µmol L^{-1} for 4 h. Cellular lipids were analyzed with HPLC and are expressed as the amount of triglyceride per mg of total cellular protein. In the case of B and C, albumin and α-tocopherol were present in all incubations. Shown are data from three donors in each panel. $^*P < 0{\cdot}05$ vs. control.

[14,20,23]. There can be at least two nonexcluding explanations for our findings indicating that darglitazone induces no up-regulation of CD36 when physiological levels of albumin-bound NEFAs are present. One is that the uptake of NEFAs causes increased intracellular concentration of the natural ligands for PPARγ, which may compete for some of the actions of darglitazone such as up-regulation of CD36 [14]. This is most probably achieved by occupation of the ligand-binding domain of PPARγ or perhaps by differential binding of coactivators to the PPARγ-ligand-complex [41]. Our observations regarding the effect of darglitazone on triglyceride content in the presence of fatty acids further support this hypothesis. An alternative explanation is that the cellular capacity of CD36 synthesis reaches its maximum after stimulation with fatty acids and therefore darglitazone cannot further stimulate this process. Moreover, the observed effect does not seem to be a result of a cytotoxic effect, as there was no effect of NEFAs or darglitazone on cell viability at the concentrations used. *In vivo*, it is reasonable to believe that the surroundings of resident macrophages in the arterial intima are rich in NEFAs. Albumin-bound NEFAs are present in circulation, and in addition lipoprotein lipase, the enzyme that hydrolyzes fatty acids from triglycerides in lipoproteins, has been found in atherosclerotic plaques and may continuously generate more albumin-NEFA complexes [42]. Therefore, we believe that in cell culture experiments designed to evaluate the possible role of PPAR-agonists in atherogenic responses albumin-NEFAs should be included and that the proposed proatherogenic effect of PPARγ-agonists, CD36 up-regulation, needs to be re-evaluated.

Darglitazone is a potent PPARγ-specific TZD with qualities like other drugs in this class [43]. Previously, PPARγ-agonists have been shown to up-regulate CD36; for example, *in vitro* troglitazone increases CD36 in the human monocytic cell line THP-1 [14], as well as in mouse peritoneal macrophages [20] and embryonic stem cell macrophages [23]. *In vivo*, troglitazone increases CD36 expression in arterial tissue in ApoE knockout mice [20] and rosiglitazone in LDL-receptor deficient mice [21]. These observations in mouse models might seem contradictory to our findings, which indicate that darglitazone has no effect on CD36 in the presence of fatty acids. However, the increased expression of CD36 in these studies was shown in whole tissue extracts and not specifically in macrophages or foam cells [20,21]. Furthermore, Li *et al.* found the effect of glitazones only in male mice and the increased CD36 expression was shown to be tissue specific, as found in the arterial wall but not in the myocardium [21]. A possible difference between various glitazones should also be considered. To explore this matter further an animal model for insulin resistance with high plasma NEFAs should be used.

Earlier reports have suggested a role for CD36 in foam cell formation [14]. In this study we demonstrate that darglitazone can increase lipid accumulation in macrophages in the presence of 5% human serum. However, the accumulated lipids were mainly triglycerides and not cholesterol esters, as in foam cells in atheroma [44]. The accumulation of triglycerides, however, is not unexpected, as PPARγ is

known to be adipogenic [45]. The lack of cholesterol accumulation in TZD-stimulated cells has recently been found in other studies [17,23], with the explanation that PPARγ regulates not only the uptake of cholesterol but also the efflux through ABCA1 [17,46].

In conclusion, the increase in CD36 in macrophages suggests a role for fatty acids in the regulation of foam cell formation. Darglitazone behaved like other PPARγ-activators regarding the increased expression of CD36 in the absence of a source of albumin-bound NEFAs, but in the presence of physiological concentrations of NEFAs this substance did not further increase the expression of CD36. Today, TZDs are considered to be substances with mainly antiatherogenic effects [20–22], with the exception of the possible proatherogenic effect of increased CD36 expression in macrophages. Our findings suggest that this apparent paradox might not exist when physiological or supra-physiological levels of albumin-bound NEFAs are present, as in the individuals with insulin resistance and type II diabetes who are being treated with these drugs.

Acknowledgements

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