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POTENCIALS DIANES FARMACOLÒGIQUES PEL TRACTAMENT DE LA RESISTÈNCIA A LA INSULINA I LA HIPERTRÒFIA CARDÍACA

Tesi doctoral que presenta Anna Planavila i Porta, dirigida pel doctor Manuel Vázquez Carrera, per a l'obtenció del grau de doctor en Farmàcia.

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ÍNDEX

INTRODUCCIÓ

Els àcids grassos són essencials per a la vida. Algunes de les seves principals funcions són l'emmagatzemament i la generació d'energia, la síntesi de fosfolípids (molècules necessàries per tal de mantenir l'estructura, la integritat i la funció de la membrana plasmàtica), i la síntesi de potents mediadors biològics com les prostaglandines. A més, els àcids grassos poden modificar l'expressió de molts gens, provocant canvis en el metabolisme, el creixement i la diferenciació cel·lular (Cabrero i col., 2003a). Tenint en compte la seva importància, és lògic pensar que les alteracions del metabolisme dels àcids grassos poden estar implicades en la fisiopatologia de nombroses malalties. Entre elles, cal destacar patologies com la resistència a la insulina, la diabetis mellitus de tipus 2 (DM2) i la hipertròfia cardíaca. De fet, alguns autors parlen de malalties lipotòxiques per fer referència a aquestes patologies que es produeixen quan els àcids grassos s'escapen de les seves vies d'oxidació habitual (β-oxidació) per entrar en vies metabòliques no oxidatives que són potencialment tòxiques (Unger i col., 2001).

La hipertròfia cardíaca és una patologia que es caracteritza perquè es produeix un desplaçament en la utilització dels substractes energètics des dels àcids grassos a la glucosa, situació característica de l'estat fetal. Aquest fet provoca una disminució de la capacitat oxidativa de lípids per part del cor que acaba provocant l'acumulació intracel·lular d'àcids grassos al miòcit. Actualment es desconeix si aquestes alteracions que es produeixen en el metabolisme dels àcids grassos són la causa o la conseqüència de la hipertròfia cardíaca (Van Bilsen i col., 1998).

D'altra banda, l'aparició de la resistència a la insulina al múscul esquelètic precedeix a la instauració de la DM2. En aquest cas es produeix una més gran utilització d'àcids grassos en detriment de la glucosa. Aquest fet s'acompanya d'una acumulació intracel·lular d'àcids grassos en forma de triglicèrids al múscul esquelètic, que és el factor que més directament es relaciona amb l'aparició de la resistència a la insulina (Kelley i Mandarino, 2000). Aquestes dades suggereixen la presència d'alteracions en el metabolisme dels àcids grassos durant el desenvolupament d'aquesta patologia (McGarry, 1992).

Els resultats presentats en aquest treball pretenen aportar noves dades que permetin conèixer els mecanismes implicats en l'aparició d'aquestes dues patologies, així com noves possibilitats terapèutiques per al seu tractament.

I. HIPERTRÒFIA CARDÍACA

El cor és el primer òrgan que es forma i que funciona en l'embrió, i tots els esdeveniments que es produeixen en la vida de l'organisme depenen de la capacitat que aquest té per cobrir les demandes d'oxigen i nutrients. El cor adult és susceptible de patir anormalitats que alteren el seu creixement i la seva funció contràctil. Tant la hipertensió arterial, com la isquèmia o la insuficiència valvular poden provocar hipertròfia cardíaca. Encara que inicialment el desenvolupament d'aquest procés pot ser beneficiós, normalitzant l'estrès sobre la paret i mantenint una funció cardíaca normal, el seu manteniment durant temps prolongats és una de les principals causes d'insuficiència cardíaca i de mort súbita (Levy i col., 1990). A més, la hipertròfia cardíaca és un factor de risc independent de la malaltia cardiovascular, ja que incrementa la mortalitat vascular més de dues vegades (Levy i col., 1990; Dekkers i col., 2002).

Així doncs, podríem definir la hipertròfia cardíaca com una resposta adaptativa del cor a increments en la pressió sanguínia i a desequilibris hormonals per tal d'incrementar la funció cardíaca i adaptar el cor a les noves condicions de treball i que es caracteritza per un increment de la massa ventricular esquerra del cor amb o sense increment en el gruix de la paret (hipertròfia concèntrica o excèntrica) (Lips i col., 2003).

El tamany del cor és modulat al llarg de la vida. Durant el desenvolupament fetal el creixement del cor és degut a hiperplàsia de les cèl·lules musculars cardíaques (o cardiomiòcits). Després del naixement les cèl·lules musculars cardíaques deixen de poder dividir-se irreversiblement ja que es troben totalment diferenciades i el creixement del cor o hipertròfia passa per un increment en el tamany dels cardiomiòcits amb absència de divisió cel·lular. El creixement postnatal normal del cor, també anomenat hipertròfia fisiològica, típica dels atletes d'èlit, permet mantenir l'increment de les demandes metabòliques del cor i és molecularment diferent del fenomen d'hipertròfia patològica que apareix com a resposta a senyals d'estrès o de dany. A diferència de la hipertròfia fisiològica, aquesta darrera es caracteritza per presentar una accelerada transició cap a la insuficiència cardíaca (Olson, 2004).

Els dos principals estímuls que provoquen una resposta hipertròfica patològica són l'estrès mecànic i factors neurals/humorals (Ritter i Neyses, 2003). Aquesta estimulació, activa vies de senyalització intracel·lulars que tenen com a resultat final l'alteració en l'expressió de gens i en la síntesi de proteïnes. Aquests canvis que es produeixen tant a nivell transcripcional com traduccional són els responsables de l'aparició de la hipertròfia cardíaca que té com a trets principals l'increment del tamany dels cardiomiòcits, la re-expressió de gens cardíacs fetals i la reorganització del sarcòmer. També es produeixen canvis en el metabolisme dels àcids grassos i de la glucosa de manera que hi ha un desplaçament de la font d'energia des dels àcids grassos cap a la glucosa, situació característica del metabolisme cardíac en estat fetal (Van Bilsen i col., 1998).

Finalment, els senyals d'estrès que provoquen hipertròfia cardíaca poden acabar desencadenant insuficiència cardíaca, procés en el qual el cor és incapaç de bombejar suficient quantitat de sang per mantenir les demandes energètiques de l'organisme. Aquest procés es caracteritza per una resposta apoptòtica massiva per part del cor. Així doncs el balanç entre la supervivència cel·lular i les vies apoptòtiques sembla ser el més gran determinant en la transició d'hipertròfia cardíaca a insuficiència cardíaca (Olson, 2004).

I.1. VIES DE SENYALITZACIÓ IMPLICADES EN L'APARICIÓ DE LA HIPERTRÒFIA CARDÍACA

La resposta hipertròfica s'inicia després de l'aparició d'estrès mecànic, provocat per increments de la pressió sanguínia, sobre les parets del cor. Aquest estrès mecànic afecta a totes les cèl·lules cardíaques tant cardiomiòcits com fibroblasts i cèl·lules vasculars (Figura 1). Totes elles responen a l'estímul activant vies de senyalització intracel·lulars que acaben provocant directament hipertròfia, i també alliberen factors autocrins i paracrins com l'angiotensina II, les catecolamines o interleucines. Tots aquests estímuls mecànics i neurohumorals acaben generant la resposta hipertròfica (Tarone i Lembo, 2003).

Existeixen un gran nombre de vies de senyalització intracel·lulars implicades en l'aparició de la hipertròfia cardíaca. Cada una de les molècules implicades no actuen independentment, sinó que una mateixa proteïna pot participar en diferents vies, de manera que hi ha una intercomunicació entre elles. Aquest fet afegeix un grau més de complexitat

que explica perquè els mecanismes implicats en l'aparició de la hipertròfia cardíaca encara no estan del tot clars.

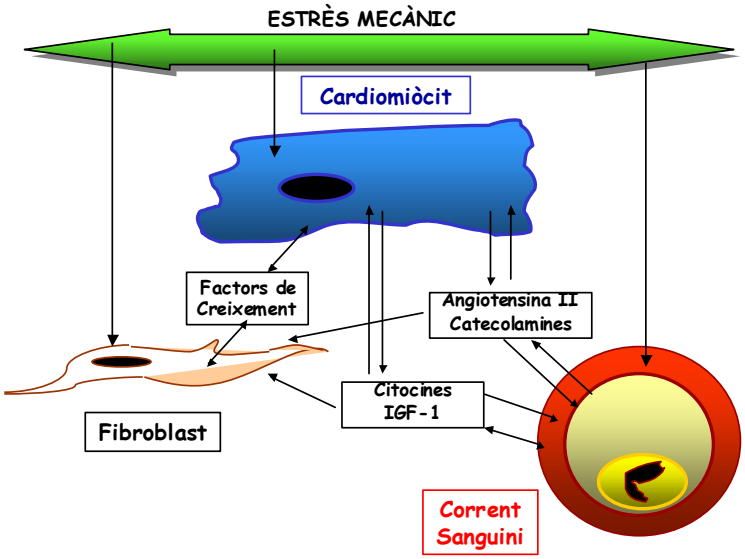

Figura 1. Factors neurohumorals i mecànics implicats en l'aparició de la hipertròfia cardíaca

I.1.1. RECEPTORS ACOBLATS A PROTEÏNA G

Els receptors acoblats a proteïna G o GPCRs (*G-protein coupled receptors*) són una família de receptors de membrana que juguen un paper molt important en la regulació de la funció cardíaca en resposta als constants canvis del medi (Rockman i col., 2002). Estan formats per una estructura de set hèlix transmembrana amb dominis intracel·lulars acoblats a proteïnes G específiques formades per 3 subunitats: α, β, γ. Quan el complex de proteïna G es troba inactiu, el guanosin difosfat (GDP) es troba unit a la subunitat α, mentre que el dímer $\beta\gamma$ actua estabilitzant la conformació. Després de la unió d'un lligand, es produeix un canvi conformacional a la proteïna G que disminueix l'afinitat del GDP a la subunitat α. L'estat actiu de Gα s'inicia quan el GDP s'intercanvia per GTP (Guanosin trifosfat), fet que provoca la dissociació del complex Gα de la resta del complex. Tant Gα com G$\beta\gamma$ són capaços d'activar de manera independent diferents vies de senyalització intracel·lular. Existeixen tres

tipus de proteïna Gα: Gαs, Gαq i Gαi els quals també activen diferents respostes a nivell cel·lular (Jalili i col, 1999).

Dins d'aquesta família de receptors acoblats a proteïna G s'hi inclouen els receptors adrenèrgics (tant els α-adrenèrgics com els β-adrenèrgics), els receptors de l'angiotensina i els de l'endotelina-1 (ET-1). Els receptors adrenèrgics responen a l'acció de les catecolamines endògenes (adrenalina i noradrenalina) que són alliberades pel sistema nerviós simpàtic. Els dos tipus de receptors adrenèrgics responen a les mateixes catecolamines però produeixen respostes diferents a nivell intracel·lular depenent del subtipus de proteïna Gα (s, q, i) que tinguin acoblada. Per tant la quantitat i el tipus de receptor predominant en un teixit determinen el tipus de resposta que es produirà (Brodde i Michel, 1999). Al cor els receptors β-adrenèrgics són el subtipus majoritari en front als α-adrenèrgics, la relació és aproximadament 10:1 en el cas dels humans (Rockman i col., 2002). Les alteracions en les vies de senyalització dels receptors α i β adrenèrgics estan associades a l'aparició de la hipertròfia cardíaca.

I.1.1.1. Senyalització a través de Gq

Els receptors α-adrenèrgics, el receptor de l'angiotensina i el receptor de l'endotelina-1 tenen acoblada la proteïna Gq, i s'ha demostrat que la seva estimulació a través d'agonistes específics (com la fenilefrina (FE) en el cas dels receptors α_1-adrenèrgics) és suficient per provocar hipertròfia cardíaca (Nicol i col., 2000).

Com ja hem dit, un cop el GPCR és activat per algun dels seus lligands, la proteïna G es dissocia en Gα i Gβγ. Cada una de les subunitats és capaç d'activar per si sola vies de senyalització diferents. En concret Gαq activa la fosfolipasa C (PLC) que és la responsable de la hidròlisi del fosfatidininositol bifosfat (PIP_2) en diacilglicerol (DAG) i inositol 1,4,5-trifosfat (IP_3) (Figura 2). Aquest darrer, s'uneix a la membrana del reticle sarcoplasmàtic i provoca la sortida de Ca^{2+} al citosol. Aquest Ca^{2+} alliberat, s'uneix a la proteïna cinasa C (PKC) provocant la seva translocació a la membrana plasmàtica. Un cop allà, la PKC és activada pels DAG prèviament alliberats i un cop activa és capaç de fosforilar factors de transcripció que controlen l'expressió de gens d'hipertròfia com c-jun i fos (Shubeita i col, 1992).

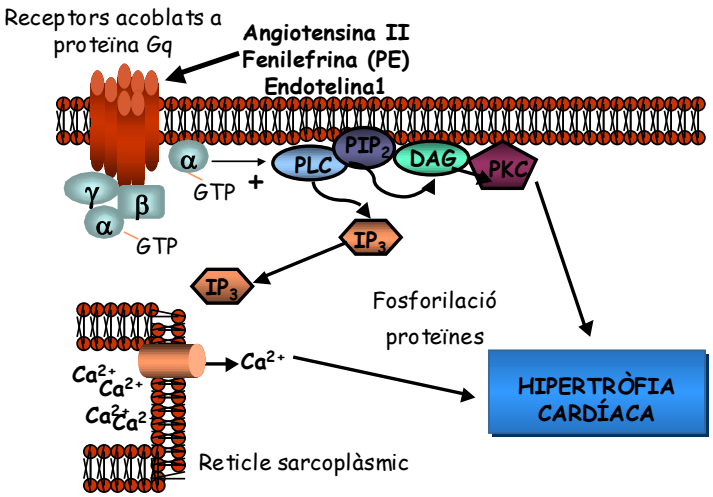

Figura 2. Receptors acoblats a proteïna Gq

I.1.1.2. Senyalització a través de Gs i de Gi

Com ja s'ha comentat, els receptors β-adrenèrgics són el subtipus majoritari en cor i per tant són els principals reguladors de la funció cardíaca en resposta a les catecolamines. Existeixen dos subtipus diferents de receptors β-adrenèrgics: β1 i β2. Al cor el subtipus de receptor més abundant és el β1 que representa un 75-80 % del total de receptors β (Rockman i col., 2002). Ambdós subtipus tenen acoblada la proteïna Gs, tot i que els receptors β2 també poden acoblar-se a la proteïna Gi (Daaka i col., 1997). La proteïna Gαs activa a la adenilat ciclasa, responsable d'incrementar els nivells d'AMPc que finalment activen a la proteïna cinasa A (PKA). La PKA fosforila canals de Ca^{2+} de tipus L i el fosfolambà (PLB) responsables de la regulació del fluxe de Ca^{2+} a l'interior de la cèl·lula (Rockman i col., 2002). Pel contrari, quan els receptors β2 s'acoblen a Gαi es produeix la inactivació de l'adenilat ciclasa (AC) que finalment, degut a la inactivació de la PKA, provoca la reducció del fluxe de calci intracel·lular.

El calci controla tant el creixement del cor com la funció contràctil. Anormalitats en el cicle del calci estan implicades en l'aparició de diferents tipus de patologies. Durant cada

batec del cor, el calci entra al cardiomiòcit a través dels canals de calci de tipus L (Figura 3). L'increment intracel·lular de calci, provoca l'alliberació de calci del reticle sarcoplasmàtic a través del receptor de la rianodina (RyR), incrementant el calci lliure intracel·lular fins a una concentració deu vegades superior (Bers, 2002). La unió del calci a la troponina C de l'aparell contràctil, inicia la contracció muscular (sístole). El retorn del calci al reticle sarcoplasmàtic a través de la Ca^{2+}-ATPasa del reticle sarcoplasmàtic (SERCA) provoca la relaxació cardíaca (diàstole). L'habilitat de SERCA per bombejar el calci a l'interior del reticle sarcoplasmàtic depèn de la seva interacció amb el fosfolambà, una proteïna que es troba a la membrana del reticle sarcoplasmàtic (Schmidt i col., 2001). En estat no fosforilat, el fosfolambà inhibeix l'entrada de calci a través de SERCA. La PKA fosforila el fosfolambà, permetent així l'entrada de calci a través de SERCA i per tant la relaxació cardíaca. La PKA també fosforila RyR provocant la dissociació de la proteïna FKBP (*FK-binding protein*), que estabilitza el canal, incrementant així la sensibilitat al calci (Marx i col., 2000).

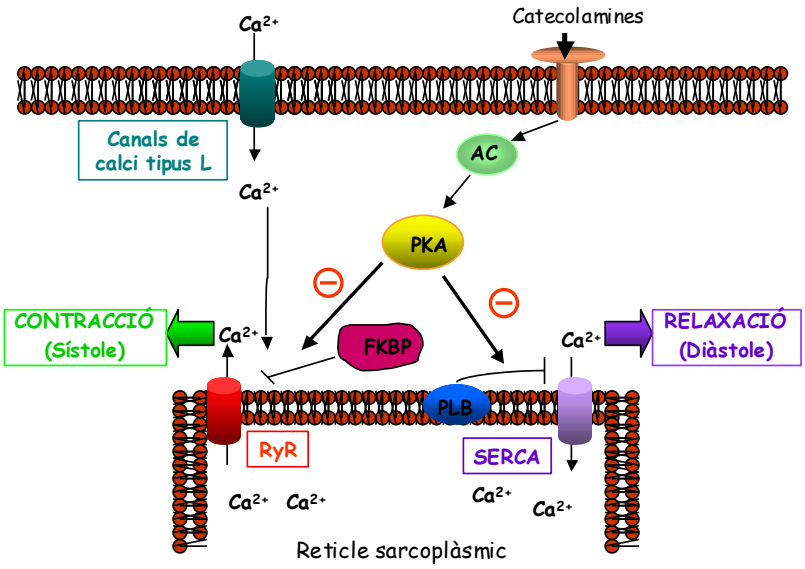

Figura 3. Senyalització del calci durant la contracció cardíaca.

Quan el cor pateix un dany, es produeix l'activació de la PKA i en conseqüència la fosforilació del fosfolambà. Aquest fet provoca un augment de la funció contràctil degut a que s'incrementa l'activitat de SERCA i per tant s'acumula més Ca^{2+} al reticle sarcoplasmàtic. Així doncs, en un principi l'activació dels receptors β-adrenèrgics permet incrementar la freqüència cardíaca, però l'activació simpàtica crònica té efectes fisiològics

adversos que poden iniciar o accelerar la patologia cardiovascular (Rockman i col., 2002). Increments crònics de catecolamines acabaran desencadenant insuficiència cardíaca la qual es caracteritza per una disminució de la funció dels receptors β-adrenèrgics deguda tant a la reducció en el nombre de receptors com en la seva funcionalitat, mecanisme conegut amb el nom de desensibilització (Bristow i col., 1982).

I.1.1.3. Senyalització a través de Gβγ

Existeixen diferents mecanismes de desensibilització: fosforilació del receptor per part de PKC o PKA, disminució de la síntesi o increment de la degradació del mRNA o aquells que són mediats per la subunitat Gβγ dissociada després de l'estimulació per part de l'agonista (Barki-Harrington i col., 2004).

Com a conseqüència de l'activació de Gβγ les cinases dels GPCRs o GRK fosforilen el receptor permetent així la unió de la β-arrestina. Aquesta proteïna permet d'una banda la internalització o endocitosi del GPCR (desensibilització) però també permet la unió de diferents elements que activaran vies de senyalització intracel·lular com la de les MAPK (*Mitogen-Activated Protein Kinases*), la calcineurina o la fosfatidininositol 3-cinasa (PI3K) que acaben provocant hipertròfia cardíaca (Rockman i col., 2002).

I.1.2. PI3K/Akt/GSK3β

Com ja s'ha descrit, la subunitat Gβγ que s'allibera després de l'activació del receptor GPCR és capaç d'activar a la fosfatidininositol 3-cinasa (PI3K), enzim que presenta tant activitat lípid-cinasa com proteïna-cinasa, i que està implicat en moltes funcions cel·lulars, particularment en el creixement, la supervivència i la proliferació cel·lular (Cantley LC, 2002). Els receptors GPCRs són capaços d'activar-la, però també pot activar-se a través dels receptors tirosina cinasa (RTKs) i la proteïna monomèrica Ras (Frey i Olson, 2003).

Existeixen 3 subtipus de PI3K, però en cor només s'expressa la classe I, que a la vegada també es divideix en dues isoformes diferents: I_A i I_B. La PI3K-I_A és la que s'activa en resposta a estímuls fisiològics com l'exercici, que actuen a través de l'activació dels receptors tirosina cinasa i que acaben donant lloc a l'aparició de la hipertròfia adaptativa o fisiològica.

Pel contrari, la PI3K-I_B s'activa davant l'estimulació dels GPCRs i d'increments en la pressió mecànica que acabaran generant hipertròfia patològica o maladaptativa (Oudit i col., 2004). Sembla ser que ambdós subtipus segueixen vies de senyalització similars però es desconeix quines són les diferències que acaben provocant respostes fenotípiques tan diferents (Oudit i col., 2004).

L'activitat lípid-cinasa de la PI3K catalitza l'addició de grups fosfat a la posició 3 de l'anell del fosfatidininositol (PtdIns) per tal de generar PtdIns(4)P, PIP_2 i IP_3 (Prasad i col, 2003). D'altra banda es desconeix quin és el paper de l'activitat proteïna cinasa en la regulació de la transducció de senyals a través de la PI3K. Un dels productes generats per la PI3K, l'IP_3, és capaç de segrestar la proteïna cinasa B (PKB o Akt) a la membrana plasmàtica on serà fosforilada i com a conseqüència activada, per una cinasa dependent de PtdIns, la PDK1 (Vlahos i col, 2003). La PDK1 també és capaç d'activar per fosforilació altres proteïnes com la PKC o la cinasa p70S6 (p70S6K), també implicades en l'aparició de la hipertròfia cardíaca (Toker i Newton, 2000).

L'Akt és una proteïna serina/treonina cinasa que controla diferents tipus de respostes cel·lulars com la inhibició de l'apoptosi o la regulació de la proliferació cel·lular, el metabolisme i la hipertròfia (Hanada i col., 2004). És capaç de regular una gran quantitat de substractes entre els quals s'hi inclouen la glicògen sintasa cinasa-3β (GSK-3β), la sintasa d'òxid nítric endotelial (eNOS), c-Raf, diferents efectors antiapoptòtics dels compartiments citoplasmàtics, mitocondrials i nuclears i mTor (*mammalian target of rapamycin*) (Oudit i col, 2004). mTor activa la cinasa p70S6 i eIF4E, que provoquen un increment en la síntesi proteica, un dels trets principals de la hipertròfia cardíaca (Schmelzle i Hall, 2000).

Un altre dels substractes de l'Akt, la GSK-3β, és una serina/treonina cinasa que inicialment es va descriure com a inhibidora de la síntesi de glicògen a través de la fosforilació de la glicògen sintasa (Embi i col., 1980) però s'ha vist que regula moltes funcions cel·lulars. La seva activitat és controlada per múltiples mecanismes, entre ells la fosforilació que condueix a la inactivació de la proteïna. L'Akt fosforila a la GSK-3β en la posició serina 9, inactivant-la. Com que l'Akt és substracte de la PI3K, els estímuls que la activin inhibiran la GSK-3β a través d'Akt (Hardt i Sadoshima., 2002). Morisco i col. (2000) van demostrar que l'estimulació β-adrenèrgica activava Akt i inhibia GSK-3β en

cardiomiòcits. Altres estímuls hipertròfics com els receptors acoblats a Gq i el receptor Fas també inhibeixen la GSK-3β (Haq i col., 2000; Badorff i col., 2002). Aquesta inhibició impedeix a la GSK-3β exercir els seus efectes inhibidors de la hipertròfia i per tant aquesta s'acaba desencadenant.

La GSK-3β exerceix els seus efectes antihipertròfics a través de diferents mecanismes. Un d'ells és la fosforilació de diferents factors de transcripció hipertròfics com NFAT (*Nuclear Factor of Activated T-cells*) i GATA4 (Hoeflich i col., 2000) causant la seva sortida del nucli i provocant, per tant, un descens de la transcripció nuclear. Pel contrari, quan hi ha un estímul hipertròfic es redueix la fosforilació d'aquests factors de transcripció de manera que s'incrementa la seva localització nuclear i s'estimula la hipertròfia (Morisco i col., 2001). A part de la transcripció, GSK-3β també és capaç de regular la traducció proteica i l'organització del citoesquelet (Hardt i Sadoshima, 2004).

I.1.3. CALCINEURINA-NFAT

La calcineurina és una serina-treonina fosfatasa que s'activa quan hi ha increments en la concentració de calci al citoplasma, promovent l'associació de la calmodulina amb la calcineurina i la conseqüent activació de l'enzim (Olson i Williams, 2000). La calcineurina desfosforila factors de transcripció de la família NFAT, provocant la seva translocació al nucli i l'activació de la transcripció de gens com la interleucina-2 (Crabtree, 1999). Molkentin i col (1998) van demostrar que l'activació de la via calcineurina-NFAT és suficient perquè es desenvolupi hipertròfia cardíaca. Estímuls hipertròfics com els α i β-adrenèrgics o l'exercici s'ha vist que incrementen l'activitat de la calcineurina (Vega i col., 2003).

Sembla ser que tant l'estimulació de la calcineurina com la inhibició de la GSK-3β són fonamentals per induir hipertròfia cardíaca encara que la via predominant dependria del tipus d'estímul hipertròfic. Per exemple, en el cas d'estímuls β-adrenèrgics la inhibició de la GSK-3β sembla ser principal enfront a l'estimulació a través de la calcineurina, mentre que en el cas dels receptors α-adrenèrgics tots dos mecanismes tindrien la mateixa importància (Hardt i Sadoshima, 2002).

I.1.4. VIA DE LES MAPK

La via de les MAPK (*Mitogen Activated Protein Kinase*) representa un altre complex sistema que participa en el creixement hipertròfic. Proporciona un important vincle d'unió entre els estímuls externs i el nucli a través de la fosforilació de múltiples factors de transcripció. La cascada de les MAPK s'organitza en tres nivells: les MAPKs són fosforilades i activades per les MAPKKs (*Mitogen Activated Protein Kinase Kinase*) que a la vegada són fosforilades i activades per les MAPKKKs (*Mitogen Activated Protein Kinase Kinase Kinase*). Les MAPKKKs s'activen per interacció amb les proteïnes petites unides a GTP (*Small GTPases*) com Ras i/o altres proteïnes cinases que connecten els receptors de superfície amb aquesta via de senyalització (Garrington i Johnson, 1999).

Existeixen tres subfamílies de MAPKs: ERKs (*Extracellulary Responsive Kinases*), JNKs (*c-Jun N-terminal Kinases*) i p38 MAPKs. La subfamília ERK està més especialitzada en transduir senyals quan es produeix una estimulació per factors de creixement o agents mitògens, mentre que JNK i p38 MAPKs s'activen preferentment en resposta a senyals d'estrès cel·lular o de dany (Garrington i Johnson, 1999). Els GPCRs activen les tres branques de les MAPKs. Malgrat que en cardiomiòcits tots tres tipus de MAPKs exerceixen un efecte regulatori prohipertròfic, estudis recents en cor adult sembla que atorgarien només a ERK un paper prohipertròfic mentre que JNK i p38 actuarien com a antagonistes del creixement hipertròfic (Molkentin, 2004).

I.1.5. NF-κB (*Nuclear Factor κB*)

El factor nuclear κB (NF-κB) és un factor de transcripció sensible a l'estrès oxidatiu que regula la transcripció de gens implicats en la resposta imflamatòria, l'apoptosi i el creixement cel·lular. Es tracta d'un heterodímer format principalment per dues subunitats, p65 i p50 (Figura 4). En situació normal, NF-κB es troba al citoplasma de la cèl·lula, on es mantè inactiu degut a la unió de la subunitat p65 amb proteïnes repressores com les IκB (α i β) (Evans i col, 2002). Existeixen moltes vies que activen NF-κB, la majoria de les quals convergeixen en l'activació de la IKK (*IκB Kinase complex*), responsable de la fosforilació de les IκBs. La fosforilació i posterior degradació de IκB pel proteosoma permet que NF-κB quedi lliure i pugui translocar-se al nucli on activarà la transcripció de gens com la MCP-1

(*Monocyte Chemoattractant Protein 1*) que desenvolupa un paper fonamental en la resposta inflamatòria i l'apoptosi (Hayden i Ghosh, 2004).

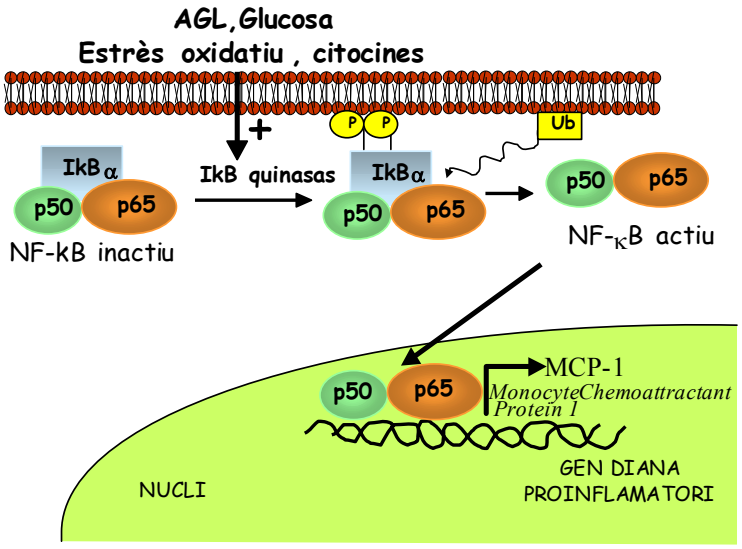

Figura 4. Modulació de l'activitat NF-κB

Estudis recents han demostrat que l'activació de NF-κB en cultiu primari de cardiomiòcits neonatals és fonamental perquè es desenvolupi hipertròfia cardíaca, ja que si aquest no s'activa, la resposta hipertròfica no es produeix (Purcell i col, 2001; Hirotani i col., 2002; Higuchi i col., 2002; Gupta i col., 2002). Altres estudis realitzats *in vivo* han arribat a la mateixa conclusió (Li i col., 2004). Estímuls com els agonistes dels GPCRs o citocines com el TNFα (*Tumor necrosis factor-α*), entre d'altres, són capaços d'activar NF-κB a través de diferents vies de senyalització, algunes de les quals encara no estan del tot clares.

Com ja s'ha comentat, l'estimulació dels GPCRs provoca l'activació de la PKC i la mobilització del calci intracel·lular. D'una banda, un dels subtipus de PKC, la PKCβ, és capaç de fosforilar i activar la IKK provocant per tant l'activació de NF-κB (Moscat i col., 2003) i d'altra banda l'increment de calci, es capaç de generar espècies reactives a l'oxigen (ROS, *Reactive Oxygen Species*). La producció de ROS activa la MAP cinasa ASK1 (*Apoptosis Signal-regulating Kinase 1*), que activa NF-κB mitjançant vies de senyalització com JNK i p38, i a més, inhibeix les IκB (Hirotani i col., 2002). El resultat final d'aquests dos processos és l'activació de NF-κB i en conseqüència l'aparició de la hipertròfia cardíaca.

D'altra banda, citocines com el TNFα estan implicades en els processos inflamatoris posteriors a l'aparició de la insuficiència cardíaca. En un principi, l'alliberament d'aquestes citocines s'associava al sistema immune, però més recentment s'ha observat que el TNFα també pot ser alliberat pels cardiomiòcits (Mann, 2002). De fet, TNFα és capaç d'induir hipertròfia cardíaca (Nakamura i col., 1998) i a més s'ha demostrat que és el responsable de l'aparició de la hipertròfia cardíaca que es manifesta en els pacients que han patit un transplantament (Stetson i col., 2001). La unió de TNFα al seu receptor TNFR provoca l'activació de NF-κB a través del reclutament de IKK al TNFR, on s'activarà i fosforilarà a IκB (Hayden i Ghosh, 2004). A part d'aquest mecanisme, sembla ser que TNFα també és capaç d'induir hipertròfia cardíaca a través de la generació de ROS i la conseqüent activació de NF-κB (Higuchi i col., 2002). Altres citocines com IL1β i toxines bacterianes com el lipopolisacàrid (LPS), després de la interacció amb els seus receptors de membrana, receptor de IL-1β i CD14/TLR4, respectivament, també són capaces d'activar NF-κB (Takano i col., 2000).

Finalment, tant els GPCRs com el TNFα activen la via de senyalització PI3K-Akt-GSK3β que també és responsable d'incrementar l'activitat NF-κB. Davant d'un estímul hipertròfic es produeix la fosforilació de Akt, la qual és capaç d'interactuar i fosforilar la IKK provocant així l'activació de la via de senyalització de NF-κB (Hanada i col., 2004). També la inactivació de GSK3β evita la fosforilació de p105 (precursor de la subunitat p50), fenòmen que fa que aquesta es transformi en p50 de manera constitutiva i per tant hi hagi més NF-κB disponible (Demarchi i col., 2003). Existeixen també altres evidències que indicarien que tant Akt com GSK3β poden fosforilar directament NF-κB, fet que permetria millorar l'eficiència d'unió al DNA de p65 i la unió de coactivadors com CBP/p300 que facilitarien l'inici de la transcripció (Hoeflich i col., 2000). Afegint un grau més de complexitat, Meng i col. (2002) van demostrar que també es pot produir el fenòmen contrari, que NF-κB activi Akt en cèl.lules neuronals. Així doncs, les vies de senyalització de GSK3β i de NF-κB poden interaccionar en diferents nivells davant d'un estímul hipertròfic, però seran necessaris més estudis per determinar quin dels mecanismes és el més rellevant per tal que es desenvolupi la hipertròfia cardíaca.

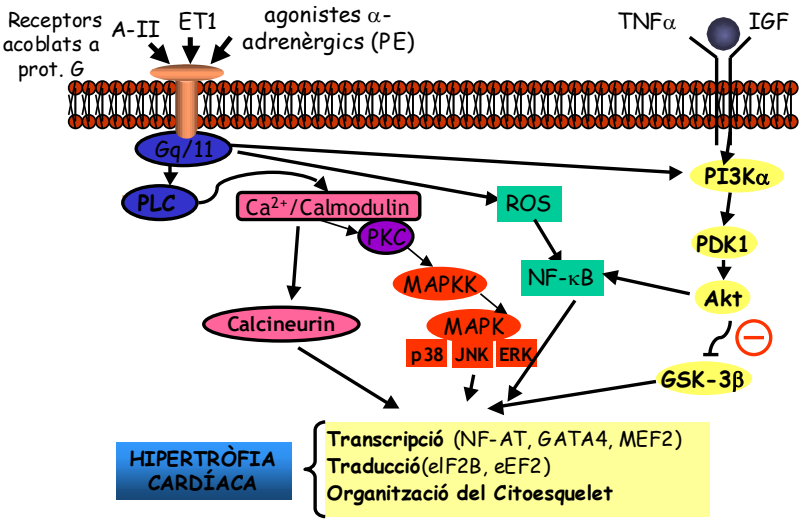

Figura 5. Vies de senyalització implicades en el desenvolupament de la hipertròfia cardíaca.

I.1.6. PPAR I METABOLISME DELS ÀCIDS GRASSOS

El cor obté l'energia a través de l'oxidació de diferents substractes, principalment àcids grassos i glucosa, en un 65 % i 30 %, respectivament (Van Bilsen i col., 1998). Malgrat això, a diferència d'altres teixits com el cervell, el cor és capaç d'adaptar el seu metabolisme a la disponibilitat de substracte energètic per tal de mantenir una funció contràctil continua. Així doncs, durant el desenvolupament i diferents situacions fisiològiques i fisiopatològiques el cor regula aquest procés en funció de la disponibilitat de substracte energètic. Per exemple durant el període fetal, en el qual el cor funciona en condicions relatives d'hipòxia, la font d'energia prové principalment del catabolisme de la glucosa i del lactat. Després del naixement, el cor passa a utilitzar els àcids grassos com a principal font d'energia, coincidint amb el període de lactància (Barger i Kelly, 2000). Durant el desenvolupament de la hipertròfia cardíaca es produeix una reversió cap a l'estat fetal, ja que es produeix una forta disminució en l'oxidació dels àcids grassos i un canvi cap a la utilització de glucosa com a substracte energètic. Com es comentarà més endavant, alguns autors suggereixen també que aquest canvi en la capacitat oxidativa del cor, més que el resultat de la hipertròfia, podria estar implicat en la seva aparició (Van Bilsen i col., 1998).

La capacitat de produir energia a través de la utilització d'àcids grassos al cor adult està controlada en part pels nivells d'expressió de gens que codifiquen pels enzims del cicle de la β-oxidació mitocondrial. En el cor, la capacitat d'emmagatzemar lípids és limitada i, per tant, en condicions normals, la majoria dels àcids grassos que entren als miòcits són oxidats. Per tant, existeix un alt acoblament entre la captació d'àcids grassos i la seva oxidació majoritària en la mitocondria, i en menor grau en els peroxisomes. L'expressió cardíaca dels gens que codifiquen pels enzims que catalitzen aquestes rutes d'oxidació d'àcids grassos i de glucosa està regulada transcripcionalment per una família de factors de transcripció anomenats receptors activats per proliferadors peroxisòmics (*Peroxisome Proliferator-Activated Receptor,* PPARs) (Barger i Kelly, 2000). A més els PPARs també són capaços de modular l'activitat del factor NF-κB (Delerive i col., 1999b; Daynes i Jones, 2002), que com ja s'ha comentat és fonamental perquè es desenvolupi la hipertròfia cardíaca.

Els PPARs són receptors nuclears que es comporten com a factors de transcripció depenents de lligand, regulant la transcripció dels seus gens diana (Desvergne i Wahli, 1999; Kersten i col., 2000). Van ser descoberts al 1990 per Isseman i Green i formen una subfamília dins la gran superfamília dels receptors nuclears hormonals. Participen en el metabolisme energètic de la cèl·lula regulant l'expressió de diferents gens relacionats amb el metabolisme i són activats per diferents metabòlits d'àcids grassos, així com per diversos compostos utilitzats en el tractament de malalties metabòliques. Per tant, tenen un paper clau en la traducció d'estímuls nutricionals, farmacològics i metabòlics en canvis en l'expressió gènica.

I.1.6.1. Estructura dels PPARs

Els PPARs, igual que altres receptors nuclears, presenten una estructura que es caracteritza per quatre dominis independents però que interactuen entre ells: una regió NH_2 terminal (regió A/B) variable, una regió conservada d'unió al DNA (DBD, *DNA-binding domain,* o regió C), el domini *hinge* (regió D), que fa de pont d'unió entre les regions C i E, i una regió conservada d'unió a lligand (LBD, *Ligand-binding domain* o regió E). A més, aquests receptors contenen regions necessàries per a l'activació transcripcional (AF1 i AF2, *Activation function-1, 2*) (Aranda i Pascual, 2001).

El DBD i el LBD són les dues regions més conservades en les diferents isoformes de PPAR. El DBD consisteix en dos dits de zinc que uneixen específicament el PPAR al seu

element de resposta PPRE (*Peroxisome proliferator response element*) situat a la regió promotora dels seus gens diana. El LBD està format per 13 α-hèlix organitzades de manera que generen una cavitat central de caràcter hidrofòbic que és on s'uneix el lligand. Aquesta cavitat en el cas dels PPARs, és més gran que en altres receptors nuclears fet que els permet interactuar amb un ampli rang de lligands naturals o sintètics amb estructures diferents. En l'extrem carboxi terminal de la regió LBD, es localitza una regió ampliament conservada denominada funció d'activació-2 (AF2) responsable de l'activació del receptor dependent de lligand i de la interacció amb els coactivadors. A l'extrem N-terminal de la regió A/B hi trobem un domini anomenat funció d'activació-1 (AF1) que té com a funció l'activació constitutiva independent de lligand (Berger i Moller, 2002).

I.1.6.2. Subtipus de PPAR i perfil d'expressió tissular

Existeixen tres subtipus de PPAR: PPARα (NR1C1, segons el sistema unificat de nomenclatura per a la superfamília de receptors nuclears), PPARβ/δ (NR1C2) i PPARγ (NR1C3) (Auwerx i col., 1999), que han estat clonats en diferents espècies (Issemann i Green, 1990; Dreyer i col., 1992; Kliewer i col., 1994), cada un dels quals és codificat per un gen independent. Aquests tres subtipus es caracteritzen per la seva distribució tissular i activitat fisiològica.

PPARα és el primer membre de la família que es va identificar i es caracteritza perquè s'expressa en teixits amb una gran capacitat de catabolitzar àcids grassos, com són el fetge, el ronyó, el múscul esquelètic, el teixit adipós marró, l'intestí, la glandula adrenal i el cor (Braissant i col., 1996). També s'expressa en cèl·lules de la paret arterial com són macròfags derivats de monòcits, cèl·lules de la musculatura llisa i cèl·lules endotelials. Tant en múscul esquelètic com en cor, el subtipus PPARα és una de les formes majoritàries, encara que els seus nivells d'expressió són inferiors als de PPARβ/δ, tal i com van demostrar Escher i col. (2001) en rates adultes. Aquest subtipus juga un paper fonamental en el desenvolupament de la hipertròfia cardíaca (Liang i col., 2003).

PPARβ/δ es troba abundantment distribuït en gairebé tots els teixits i en molts d'ells en quantitats superiors a la dels altres dos subtipus de PPAR, com és el cas del cor o el múscul esquelètic, on és la forma majoritària. Malgrat això, es coneix poc sobre la seva

regulació i les seves implicacions fisiològiques. Estudis recents però, sembla que atorgarien a PPARβ/δ un paper similar a PPARα, promovent l'oxidació i la utilització d'àcids grassos, encara que ambdós subtipus no sembla que tinguin funcions redundants ja que PPARβ/δ no sempre pot compensar a PPARα (Huss i Kelly, 2004). Recentment, Cheng i col. (2004) han demostrat que PPARβ/δ també estaria implicat en l'aparició de cardiomiopaties.

PPARγ s'expressa majoritàriament en el teixit adipós blanc i en el marró (Tontonoz i col., 1994). L'activació de PPARγ promou l'emmagatzemament de lípids donat els seus efectes en la diferenciació adipocitària i la funció al teixit adipós blanc (Desvergne i Wahli, 1999). PPARγ també s'expressa en nivells molt inferiors en altres teixits com són la paret vascular, el múscul esquelètic, les cèl.lules β pancreàtiques i el cor. La seva funció en aquests teixits és força controvertida, però estudis recents semblen indicar que PPARγ també podria jugar un paper important en la hipertròfia cardíaca tot i la seva reduïda expressió (Yamamoto i col., 2001; Asakawa i col., 2002).

I.1.6.3. Activadors de PPAR

Donada la gran importància dels PPARs en el metabolisme lipídic, la recerca dels seus activadors endògens es va centrar en els àcids grassos i els eicosanoides, els quals van ser identificats com els lligands naturals dels PPARs (Gottlicher i col., 1992). De fet, els àcids grassos activen els tres subtipus de PPARs, encara que amb afinitats i especificitats diferents. Per exemple, PPARα és activat per un ampli rang d'àcids grassos saturats i insaturats, com l'àcid palmític, l'àcid oleic, l'àcid linoleic i l'àcid araquidònic i amb afinitats de l'ordre de micromolar, fet que fa que es consideri que una de les seves funcions principals és la d'actuar com a sensor dels nivells d'àcids grassos lliures en els teixits on s'expressa. PPARγ té clarament preferència pels àcids grassos poliinsaturats, amb afinitats també del rang micromolar, mentre que PPARβ és capaç d'interactuar tant amb àcids grassos saturats com insaturats, però amb una selectivitat pel lligand intermitja entre PPARγ i PPARα. (Berger i Moller, 2002).

Els eicosanoides són un tipus de metabòlits d'àcids grassos derivats principalment de l'àcid araquidònic. En concret, la via de la lipooxigenasa, dóna lloc a la formació de leucotriens (LTs) i àcids hidroxieicosatetraènics (HETEs), mentre que la via de la

ciclooxigenasa origina les prostaglandines (PGs). Alguns d'aquests eicosanoides són lligands dels diferents PPARs (Devchand i col., 1998). La 15-deoxi-Δ12,14-PGJ2 (derivada de la PGD2) és lligand específic de PPARγ (Forman i col., 1995) i el 8(S)-HETE (compost associat amb la inflamació induïda per éster de forbol) i el leucotrié B4 (mediador quimiotàctic de la inflamació) són lligands de PPARα (Devchand i col., 1996). També els metabòlits oxidats de l'àcid linoleic, presents a les LDL, com l'àcid 9-hidroxi-octadecadienoic (9-HODE) i el 13-HODE s'han identificat com a lligands de PPARγ. Diferents eicosanoides, entre els que s'inclouen la PGA1 i el PGD2, s'ha demostrat que activen PPARβ/δ (Yu i col., 1995). La carbaprostaciclina (una PG semisintètica) és també un agonista PPARβ/δ en el rang micromolar (Forman i col., 1997).

Actualment, s'utilitzen dos tipus de lligands sintètics dels PPARs en el tractament dels desordres metabòlics: els fibrats i les tiazolidindiones. Els **fibrats** són els proliferadors peroxisòmics clàssics en rossegadors, que actuen com a lligands de la isoforma PPARα i que s'utilitzen en el tractament de la hiperlipidèmia. El clofibrat fou el primer representant del grup, però va anar sent substituït posteriorment per altres derivats fíbrics de major potència i amb menors efectes secundaris. El clofibrat i el fenofibrat activen PPARα amb una selectivitat deu vegades major que PPARγ (Wilson i col., 2000). El bezafibrat actua com a agonista amb una potència similar en les tres isoformes de PPAR. El Wy-15643 (àcid 4-cloro-6-(2,3-xilidin)-2-pirimidiniltio acètic) és un agonista potent del PPARα murí i un agonista dèbil del PPARγ. Agonistes potents del PPARα, que produeixin una important disminució dels nivells de triglicèrids plasmàtics són l'ureïdofibrat-5 (UF-5) i l'àcid ureïdo tioisobutíric (GW9578) (Minnich i col., 2001).

Les **tiazolidinediones** són activadors específics de PPARγ i gràcies a les seves propietats de millora de la sensibilitat a la insulina s'utilitzen de manera molt satisfactòria per al tractament de la diabetis de tipus 2. Formen part d'aquest grup de compostos, la rosiglitazona, la ciglitazona, la pioglitazona i la troglitazona (Figura 6). Aquests compostos actuen selectivament sobre PPARγ amb una elevada afinitat. La seva potència es correlaciona amb el seu efecte hipoglicèmic i hipolipemiant *in vivo* (Berger i col., 1996; Willson, 1996). Dins d'aquest grup, la rosiglitazona és l'agonista PPARγ més potent i selectiu, mentre que la troglitazona presenta una afinitat moderada (Murphy i Holder, 2000).

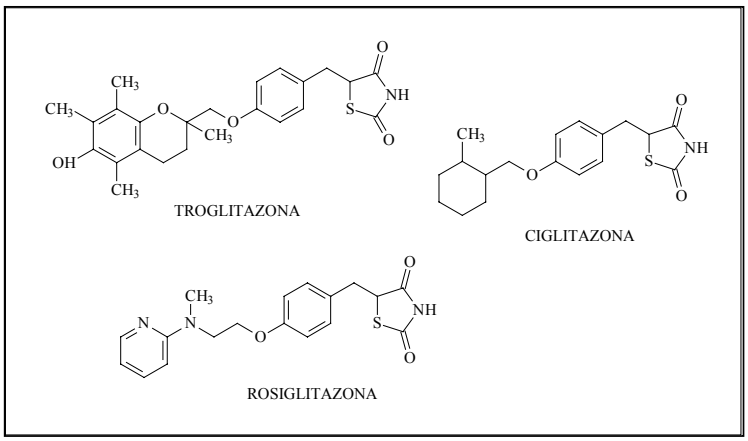

Figura 6. Estructura molecular de les tiazolidinediones

Finalment per tal de definir el paper fisiològic de PPARβ/δ, s'han desenvolupat compostos capaços d'activar-lo de forma selectiva. Entre ells hi trobem el L-165041 amb una selectivitat 30 vegades més gran sobre PPARβ/δ que sobre PPARγ i que és inactiu sobre PPARα murí, i el GW501516 més potent i selectiu per PPARβ/δ (Oliver i col., 2001).

I.1.6.4. Mecanismes d'acció dels PPARs

Els PPARs actuen a través de dos mecanismes diferents, la **trans-activació** i la **trans-repressió**. En el primer d'ells, actuen com a factors de transcripció dependents de lligand, regulant l'expressió de diferents gens mitjançant la unió a seqüències específiques, situades a la regió promotora dels seus gens diana (Figura 7).

Per a la seva unió al DNA és indispensable que formin heterodímers amb el receptor nuclear RXR. Els heterodímers formats són del tipus permissiu amb el receptor RXR, ja que poden ser activats tant per lligands PPAR com per lligands RXR específics, com és l'àcid 9-*cis* retinoic (Kliewer i col., 1992). La màxima activació transcripcional es produeix quan els dos receptors es troben activats de forma simultània (Keller i col., 1993). Aquestes seqüències específiques de DNA d'unió dels PPARs estan formades per repeticions directes de dos motius AGGTCA espaiats per un únic nucleòtid (DR-1, *Direct repeat 1*) (Kliewer i col., 1992) i reben el nom d'elements de resposta a PPAR (PPRE o *Peroxisome Proliferator Response Element*). Estudis posteriors han demostrat que d'altres membres de la superfamília

de receptors nuclears eren també capaços de reconèixer i unir-se a DR-1. Així amb la finalitat de garantir l'especificitat d'unió de l'heterodímer PPAR-RXR al DR-1, s'han establert nous matisos que defineixen el PPRE com una seqüència d'aproximadament 17 parells de bases, que conté una regió en 5' flanquejant, un nucli DR-1 imperfecte i el nucleòtid adenina com a espaiador entre els dos hexàmers, donant lloc a la següent seqüència *consensus* PPRE: 5'-AACT AGGNCA A AGGTCA-3' (Ijpenberg i col., 1997; Juge-Aubry i col., 1997; Osada i col., 1997).

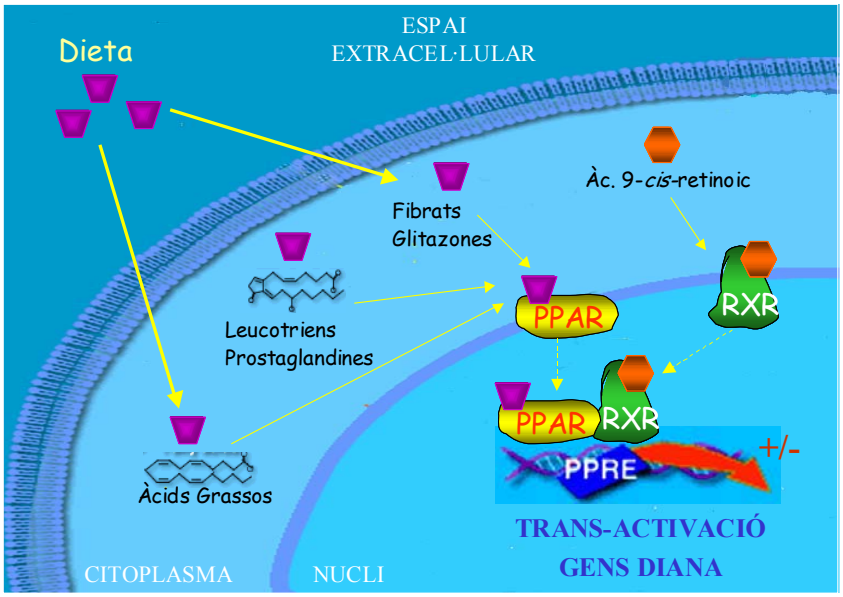

Figura 7. Mecanisme de trans-activació dels PPARs

Com la majoria de receptors nuclears, els PPARs poden interaccionar amb altres proteïnes anomenades **coactivadores** o **corepressores** en funció de la seva capacitat d'estimular o inhibir respectivament, l'activitat del receptor (McKenna i col., 1999). La repressió de l'activitat transcripcional normalment es produeix en absència de lligand o bé per la presència d'un antagonista. D'aquesta manera el receptor nuclear adopta una conformació que afavoreix la seva unió amb els corepressors. Entre els corepressors dels PPARs hi trobem, la proteïna RIP-140 (*receptor-interacting protein-140*), SMRT (*silencing mediator of retinoid and thyroid receptors*) i NCoR (*nuclear receptor co-repressor*) (Miyata i col., 1998; Robinson i col., 1999; Dowell i col., 1999). L'estimulació de l'activitat transcripcional és dependent de lligand o bé requereix la fosforilació de receptors nuclears (o proteïnes associades). Aquestes dues situacions produeixen canvis conformacionals en aquests factors de transcripció i afavoreixen la unió dels coactivadors. Alguns d'aquests

coactivadors com CBP/p300 (*CREBP-binding protein*) i el SRC-1 (*Steroid Receptor Coactivator-1*) presenten activitat histona acetilasa, capaç de modificar l'estructura de la cromatina (descondensació) fet que afavoreix que el DNA sigui més accessible a l'RNA-polimerasa i s'iniciï la transcripció gènica (Robyr i col., 2000). D'altres coactivadors coneguts són la PGC-1 (*PPARγ coactivator-1*) o el ARA-70.

Existeixen un gran nombre de gens amb un element de resposta a PPAR, que codifiquen per proteïnes que participen en el transport i el metabolisme dels àcids grassos i de la glucosa. Entre ells hi trobem proteïnes implicades en el transport de lípids (apolipoproteïnes A-I, A-II i C-III), en la captació de lípids (la lipoproteïna lipasa, LPL, responsable de la hidròlisi de triglicèrids; la translocasa d'àcids grassos o FAT/CD36, que facilita la captació d'àcids grassos; la proteïna transportadora d'àcids grassos FATP) i en el metabolisme (l'acil-CoA oxidasa, ACO, enzim limitant de la β-oxidació peroxisomal d'àcids grassos; l'acil-CoA sintetasa, ACS, que activa els àcids grassos a la seva forma de derivats CoA; la carnitina-palmitoiltransferasa-I, CPT-I, enzim limitant de l'entrada d'àcids grassos dins la mitocòndria i l'acil-CoA deshidrogenasa de cadena intermitja, MCAD, i de cadena llarga, LCAD, implicades en la β-oxidació mitocondrial).

També s'han descrit PPREs en els gens que codifiquen per les proteïnes desacobladores (UCP1, UCP2 i UCP3), responsables de desacoblar la fosforilació oxidativa de la respiració mitocondrial (Sears i col., 1996; Acín i col., 1999, Barbera i col., 2001) i en els gens que codifiquen per proteïnes implicades en la homeòstasi de la glucosa com la fosfoenolpiruvat-cinasa (PEPCK) implicada en la gluconeogènesi o el transportador de la glucosa GLUT-2.

A més, existeixen molts altres gens que també estan regulats per activadors de PPAR, encara que de moment no s'ha identificat cap PPRE en ells. Cal destacar el gen del TNF-α (Hofmann i col., 1994) i alguns components de la cascada de senyalització de la insulina i del transport de la glucosa, com el receptor de insulina, la subunitat p85α de la PI3-cinasa i el GLUT-4 (Young i col., 1995).

A part d'aquests efectes directes sobre l'activitat transcripcional, els PPARs també poden interferir les vies d'altres factors de transcripció d'una forma indirecta, sense unir-se al DNA, mitjançant un mecanisme que rep el nom de **trans-repressió**. A través d'aquest mecanisme independent de la unió al DNA es suprimeix l'activitat de diversos factors de transcripció com NF-κB, STAT (*Signal Transducer and Activator of Transcription*) i AP-1 (*Activator Protein-1*) (Delerive i col., 1999b; Zhou i Waxman, 1999). La majoria dels efectes antiinflamatoris dels PPAR s'explicarien probablement a través d'aquest mecanisme (Daynes i col., 2002). A més recentment, s'ha demostrat que la inhibició de l'activitat NF-κB és un mecanisme a través del qual els agonistes PPARγ milloren la sensibilitat a la insulina *in vivo* i que el NF-κB adipocitari és una diana terapèutica potencial per a la obesitat i la diabetis mellitus de tipus 2 (Ruan i col., 2003).

Existeixen tres mecanismes principals de trans-repressió a través dels quals els complexes PPAR-RXR activats per lligand poden regular negativament les activitats d'altres factors de transcripció (Figura 8):

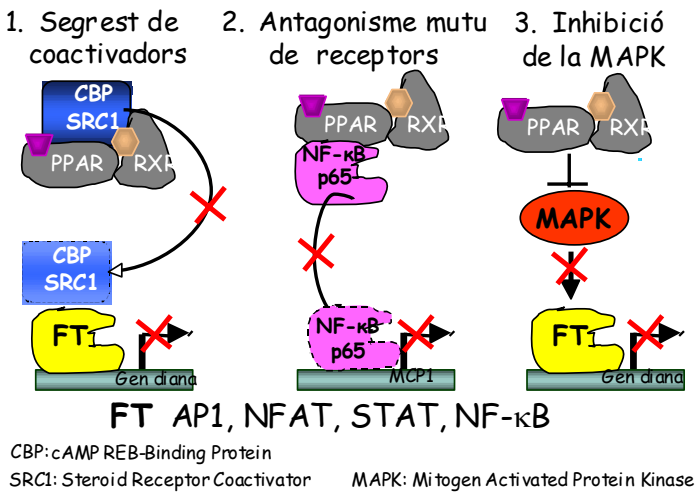

Figura 8. Mecanismes de trans-repressió dels PPARs

En el primer, la trans-repressió és conseqüència d'una competició per quantitats limitades de coactivadors compartits. Sota aquestes condicions en les quals els nivells de coactivadors específics són el factor limitant, l'activació de PPAR suprimeix l'activitat dels altres factors de transcripció que utilitzen el mateix coactivador (Li i col., 2000; Kamei i col.,

1996). En el segon mecanisme es creu que els heterodímers PPAR-RXR activats actuen a través d'interaccions físiques amb altres factors de transcripció (per exemple AP-1, NF-κB, NFAT o STAT). Aquesta associació impedeix la unió del factor de transcripció al seu element de resposta i per tant s'inhibeix la seva capacitat per induir la transcripció gènica (Delerive i col., 1999a). L'últim mecanisme de trans-repressió consisteix en la inhibició de la fosforilació i en l'activació de certs membres de la cascada MAPK, pels heterodímers PPAR-RXR, evitant així que les MAPK puguin activar altres factors de transcripció (Desreumaux i col., 2001).

I.1.6.5. PPARs i hipertròfia cardíaca

En els darrers anys, s'han publicat tota una sèrie d'estudis que relacionen la hipertròfia cardíaca amb els PPARs (Liang i col, 2003; Irukayama-Tomobe i col, 2004; Yamamoto i col., 2001; Asakawa i col., 2002), encara que el mecanisme a través del qual la regulen no està del tot clar. Actualment existeixen dues hipòtesis. La primera d'elles definiria les alteracions en els lípids i el metabolisme energètic com un fenomen secundari provocat per la hipertròfia cardíaca, mentre que la segona possibilitat es basaria en que les anormalitats en el metabolisme lipídic precedeixin i contribueixin de manera directa o indirecta al desenvolupament de la hipertròfia cardíaca. Aquesta darrera hipòtesi es fonamenta en diverses evidències. Per exemple, s'ha demostrat que l'expressió de certs enzims implicats en la glucòlisi, com la fosfofructocinasa, es troben augmentats abans de que aparegui la hipertròfia cardíaca (Taegtmeyer i Overturf, 1988). Una altra dada que reforça aquesta hipòtesi és que alguns fàrmacs que s'utilitzaven per tractar la diabetis mellitus de tipus 2, com l'etomoxir, que inhibeix la CPT-I i per tant, l'oxidació dels àcids grassos a la mitocòndria, van haver de ser retirats del mercat perquè provocaven hipertròfia cardíaca. També s'ha observat que l'administració a dosis molt altes de tiazolidinediones provoquen hipertròfia cardíaca en animals de laboratori per mecanismes encara desconeguts (Ghazzi i col., 1997; Cabrero i col., 2003b). Finalment també s'ha descrit que defectes genètics en l'espècie humana que afecten enzims implicats en l'oxidació dels àcids grassos freqüentment generen cardiomiopaties hipertròfiques (Jamshidi i col., 2002).

Al cor, l'activació de **PPARα** incrementa l'expressió de gens implicats en els tres passos claus de la via d'utilització d'àcids grassos a la cèl·lula (Figura 9): el transport dels àcids grassos a l'interior de la cèl·lula i la seva esterificació (FATP, FAT/CD36 i ACS), el

seu transport a l'interior de la mitocòndria (M-CPT-I) i la β-oxidació mitocondrial (MCAD) i peroxisòmica (ACO). A part de regular tots aquests gens, PPARα també és capaç de regular enzims implicats en les vies d'oxidació de la glucosa. Diversos estudis han demostrat que l'entrada d'àcids grassos i la seva oxidació es troba incrementada en ratolins que sobreexpressen PPARα al cor, mentre que la utilització de glucosa es troba disminuïda de manera recíproca (Finck i col., 2002; Hopkins i col., 2003). Aquesta disminució en l'oxidació de la glucosa s'explica, en part, per l'efecte inhibitori que provoca l'augment de les relacions acetil-CoA/CoASH, ATP/ADP i $NADH/NAD^+$ durant l'oxidació d'àcids grassos, sobre l'activitat del complex de la piruvat deshidrogenasa (PDH), responsable de l'entrada de glucosa a l'interior de la mitocòndria per a la seva oxidació. Malgrat això, el fet que la reducció en l'oxidació de la glucosa sigui recíproca en aquests ratolins, fa pensar que puguin haver-hi efectes a nivell de la regulació gènica. De fet, aquests ratolins presenten una inducció en l'expressió de la PDK-4, responsable de la inhibició de la PDH i una reducció dels nivells del transportador de glucosa GLUT4, i de l'enzim glucolític fosfofructocinasa. En conjunt, aquests resultats semblen indicar que PPARα regula l'expressió dels gens implicats en les vies d'utilització dels àcids grassos i de la glucosa per tal de mantenir els requeriments energètics del cor i mantenir el balanç lipídic net (Huss i Kelly, 2004).

Barger i col. (2000), van demostrar que durant el creixement hipertròfic, es redueix l'expressió nuclear de PPARα , de manera que el cor utilitza de nou glucosa i lactat com a substractes energètics. Aquesta reducció en els nivells de PPARα provoca una disminució dels enzims implicats de l'oxidació dels àcids grassos, com la M-CPT-I o la MCAD, i en la PDK-4, de manera que s'afavoreix l'oxidació de la glucosa. En un primer moment, la reducció de PPARα es considera una resposta adaptativa del cor ja que aquest canvi redueix els requeriments d'oxigen per produir ATP (Frey i Olson, 2002). De totes maneres, aquest canvi a la llarga resulta perjudicial ja que l'oxidació de la glucosa genera menys ATP, i a més, es produeix l'acumulació intracel·lular de lípids que resulta tòxica per a la cèl·lula cardíaca (Barger i Kelly, 2000). Aquesta desactivació de PPARα es produeix per diferents vies. En un primer moment, PPARα s'inactiva ràpidament per fosforilació de les MAPK (Barger i col., 2000). En estadis més avançats del creixement hipertròfic, repressors transcripcionals dels enzims de l'oxidació d'àcids grassos com el COUP-TF/ARP-1, s'indueixen inhibint l'activació d'aquests gens per PPARα (Kanda i col., 2000).

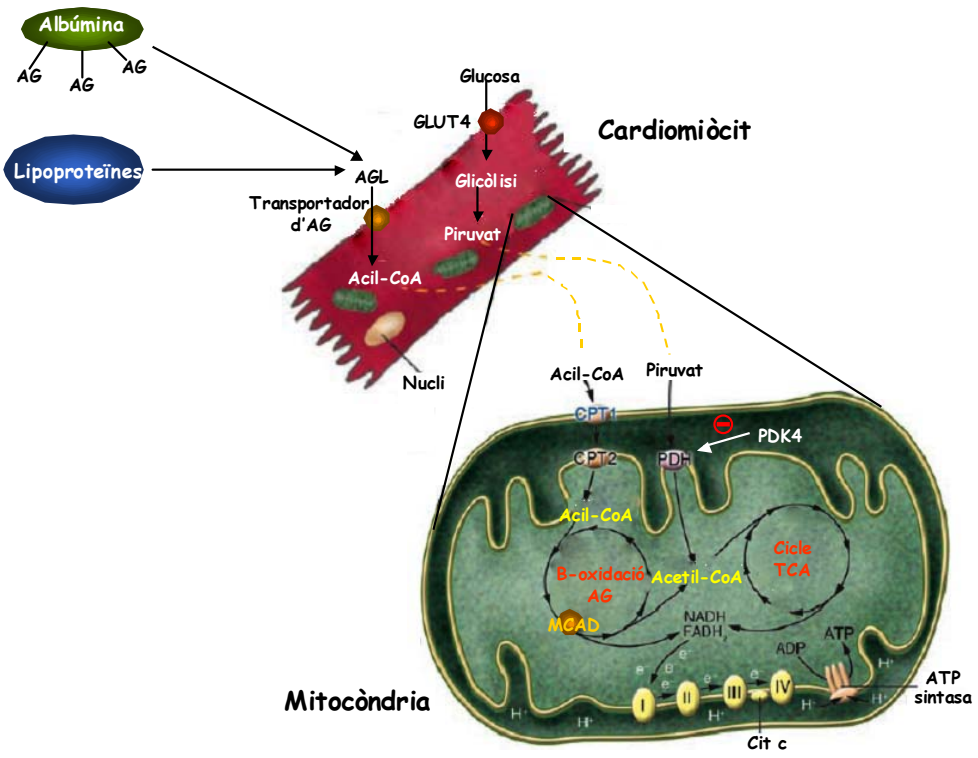

Figura 9. Metabolisme dels àcids grassos i la glucosa.

Com ja s'ha comentat, al cor la isoforma **PPARβ/δ** és la més abundant, tot i que la seva funció és força desconeguda. Recentment però, han aparegut una sèrie d'estudis que intenten determinar el paper d'aquest subtipus en el metabolime cardíac. Per exemple, s'ha demostrat que la utilització dels lligands selectius PPARβ/δ indueix l'expressió dels enzims mitocondrials que participen en l'oxidació dels àcids grassos i incrementa l'oxidació de palmitat en cardiomiòcits amb la mateixa efectivitat que els activadors de PPARα (Gilde i col., 2003; Cheng i col., 2004). D'altra banda, altres autors han demostrat que l'activació de PPARβ/δ reestableix l'expressió dels enzims d'oxidació d'àcids grassos en cardiomiòcits PPARα$^{-/-}$ on l'expressió d'aquests gens es toba reduïda a nivells basals (DeLuca i col., 2000). Malgrat que aquests resultats semblen indicar que PPARβ/δ tindria una funció similar a la de PPARα en la regulació del metabolisme lipídic, sembla que PPARβ/δ en determinades circumstàncies no és capaç de compensar a PPARα. Per exemple, els ratolins PPARα$^{-/-}$ que són fenotípicament normals, quan es sotmeten a situacions d'estrès, o amb l'envelliment, acumulen lípids a nivell cardíac (Leone i col., 1999). A més, Cheng i col. (2004) han demostrat recentment que la deficiència crònica de PPARβ/δ provoca l'aparició d'hipertròfia

cardíaca, suggerint que aquest subtipus és essencial per a l'expressió constitutiva dels enzims d'oxidació d'àcids grassos i necessari per tal de mantenir el balanç energètic i la funció cardíaca normal.

Finalment, el paper de la isoforma **PPARγ** al cor és força controvertit. En general, s'accepta que PPARγ pot modular el metabolisme cardíac a través dels efectes que exerceix en altres teixits. Per exemple, els canvis que PPARγ provoca en els nivells circulants d'àcids grassos degut als seus efectes sobre les reserves de lípids, poden modular l'activitat de PPARα i de PPARβ i també afecten a la sensibilitat a la insulina al cor. A més, el teixit adipós també secreta factors com el TNFα, la leptina i l'adiponectina, que afecten a la sensibilitat a la insulina i al metabolisme dels altres teixits. La regulació directa del metabolisme cardíac per PPARγ està subjecta a debat degut als nivells d'expressió tan baixos d'aquesta isoforma en cor i a que els activadors de PPARγ no afecten a l'expressió dels gens implicats en el metabolisme lipídic cardíac (Gilde i col., 2003). Malgrat això, l'activació de PPARγ inhibeix la inducció dels marcadors d'hipertròfia en cardiomiòcits, suggerint que PPARγ és funcional en aquestes cèl.lules (Yamamoto i col., 2001). Seran necessaris més estudis per tal de determinar si aquests efectes sobre el cor són deguts a mecanismes directes o indirectes d'aquesta isoforma.

D'altra banda, també és possible que els PPARs interfereixin amb altres factors de transcripció implicats en l'aparició de la hipertròfia, com NF-κB. Com ja s'ha comentat, aquest factor de transcripció és fonamental per al desenvolupament d'aquest procés, ja que si s'inhibeix la seva activació, la hipertròfia no es produeix (Purcell i col., 2001; Hirotani i col., 2002; Higuchi i col., 2002; Gupta i col., 2002). S'ha demostrat que PPARγ interacciona directament amb la subunitat p65 de NF-κB, inhibint l'activitat d'aquest mitjançant un mecanisme de trans-repressió (Chung i col., 2000).

Finalment, com ja s'ha comentat, existeixen diferents tipus de coactivadors que interactuen amb els PPARs i alguns d'ells poden controlar el metabolisme cardiac actuant com a transductors d'estímuls fisiològics a través dels PPARs. La majoria de coactivadors, s'expressen de manera ubiqua i no sembla que contribueixin específicament a la senyalització dels PPARs. Alguns d'ells però, com la PGC-1, presenten patrons d'expressió diferents en funció del teixit. Existeixen tres isoformes d'aquesta proteïna: PGC-1α, PGC-1β i el

coactivador relacionat amb PGC-1 (PRC). La primera d'elles es va identificar per primer cop com a coactivador de PPARγ, encara que estudis posteriors han demostrat que també és capaç d'activar a PPARα i a PPARβ (Dressel i col., 2003). PGC-1α s'expressa selectivament en teixits amb gran capacitat oxidativa com el cor, el múscul esquelètic, el teixit adipós i el fetge (Puigserver i col., 1998). Al cor, l'expressió de PGC-1α s'incrementa després del naixement coincidint amb el canvi en el substracte energètic de la glucosa als àcids grassos (Lehman i col., 2000). Davant d'estímuls com l'exposició al fred o l'exercici, s'activen vies de senyalització com p38 MAPK, β-AR (Receptor β-adrenèrgic)/AMPc o calmodulina que augmenten l'activitat i el patró d'expressió d'aquest coactivador, promovent per tant el metabolisme oxidatiu. D'altra banda, la sobreexpressió d'aquest coactivador en cardiomiòcits activa l'expressió dels gens implicats en el transport i l'oxidació dels àcids grassos (Lehman i col., 2000). Estudis recents han demostrat la interacció física entre PPARβ i PGC-1α (Wang i col., 2003) i també que els activadors d'aquest subtipus de PPAR estimulen els nivells de RNAm de la PGC-1α tant *in vivo* com *in vitro* (Tanaka i col., 2003). Finalment, altres estudis han demostrat que aquest coactivador també incrementa la biogènesi mitocondrial mitjançant la coactivació d'altres factors de transcripció com NRF-1 i 2 (*Nuclear Respiratory Factor-1/2*) i ERRα (*Estrogen-Related Receptorα*) (Huss i Kelly, 2004). Tots aquests resultats semblen indicar que PGC-1α podria jugar un paper important en la regulació del metabolisme cardíac (Figura 10).

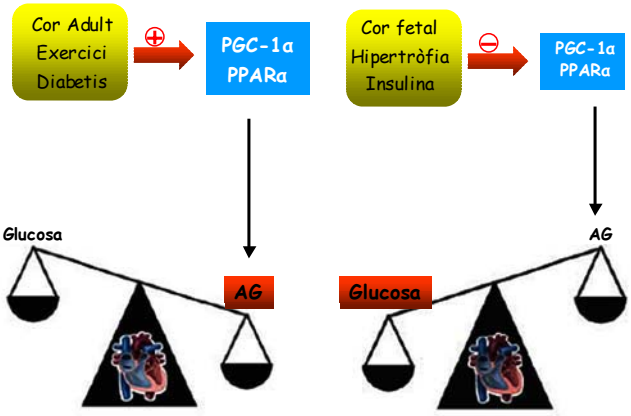

Figura 10. Selecció del substracte energètic durant el metabolisme cardíac.

I.2. TRACTAMENT FARMACOLÒGIC DE LA HIPERTRÒFIA CARDÍACA

La prevalència de la hipertròfia cardíaca s'incrementa gradualment amb l'edat i és més freqüent en els homes, degut possiblement a que hi juguen un paper important factors genètics i hormonals (Schirmer i col., 1999). Malgrat que en els darrers anys s'ha progressat notablement en el coneixement dels mecanismes implicats en la seva aparició, són molt menys coneguts els mecanismes potencials que poden inhibir o fins i tot revertir el desenvolupament d'aquest procés. Diversos agents terapèutics han demostrat ser parcialment efectius en la reversió d'aquest fenòmen, encara que cap d'ells exerceix els seus efectes directament sobre el cor, sinó que es fonamenten en el tractament de la hipertensió per tal de tractar la hipertròfia.

I.2.1. FÀRMACS UTILITZATS EN L'ACTUALITAT

Com s'acaba de comentar, el tractament farmacològic més utilitzat i efectiu per tal de prevenir la hipertròfia cardíaca és la teràpia antihipertensiva, principalment els diurètics i els inhibidors de l'enzim conversor de l'angiotensina (IECA). Malgrat això, cap d'ells és capaç d'aconseguir una reversió més gran del 13% (en el cas dels IECAs) en tractaments de 25 setmanes a dosis que es consideren antihipertensives. Aquesta efectivitat tan baixa, en el cas dels diurètics (en els quals la reducció de la massa ventricular esquerra és d'un 7%) és deguda a que aquests fàrmacs no actuen sobre factors neurohumorals que són claus en el desenvolupament de la hipertròfia cardíaca. Pel que fa als IECAs els resultats són una mica millors però no són els esperats degut d'una banda a que l'antagonisme del sistema renina-angiotensina és incomplet, a l'existència de vies alternatives de síntesi de neurohormones i finalment a que els IECAs no antagonitzen altres sistemes biològics que provoquen hipertròfia com el TNFα o l'endotelina-1 (Pokharel i col., 2003).

I.2.2. NOVES ESTRATÈGIES TERAPÈUTIQUES

En conjunt, totes aquestes dades reflecteixen com el tractament actual de la hipertròfia cardíaca és efectiu però està lluny d'arribar a ser òptim. Sembla que les noves estratègies terapèutiques per tractar aquest fenomen han d'anar dirigides a antagonitzar directament en el cor etapes clau de les vies de senyalització que provoquen la hipertròfia.

I.2.2.1 Activadors de PPAR

Tenint en compte que una utilització adequada dels substractes energètics per part del cor és fonamental pel correcte funcionament del miocardi i que alteracions en la utilització d'aquests substractes poden jugar un paper fonamental en el desenvolupament de la hipertròfia cardíaca, els PPARs es perfilen com a dianes potencials per tal d'inhibir aquest procés. De fet, recentment s'ha demostrat que els agonistes PPARα, com els fibrats, són capaços d'evitar la hipertròfia cardíaca (Liang i col., 2003). Però no només aquest subtipus és capaç d'inhibir-la, altres estudis han demostrat que els activadors de PPARγ també inhibeixen aquest procés, tant *in vivo* com *in vitro* (Asakawa i col., 2002; Yamamoto i col., 2001). Així doncs, tant l'activació de PPARα com de PPARγ inhibeixen la hipertròfia, sembla ser que mitjançant un mecanisme de trans-repressió (Irukayama-Tomobe i col., 2004). A més, tant activadors de PPARα com de PPARγ inhibeixen l'activació de NF-κB, fenòmen que també podria explicar els efectes antihipertròfics dels PPARs. Finalment, estudis realitzats en el nostre laboratori han demostrat que l'activació de PPARβ/δ també inhibeix la hipertròfia cardíaca a través d'un mecanisme que sembla reduir l'activitat de NF-κB durant aquest procés (Planavila i col., 2005).

I.2.2.2. Inhibidors de NF-κB

Com ja s'ha comentat anteriorment, l'activació de NF-κB és fonamental perquè es desenvolupi la hipertròfia cardíaca (Purcell i col., 2001). De fet, NF-κB s'activa en resposta a diferents estímuls fisiològics i patològics que desencadenen vies de senyalització intracel·lulars molt diverses. A més, la inhibició d'aquest factor de trancripció és suficient per atenuar o evitar el desenvolupament d'un procés tan complex com la hipertròfia cardíaca, el que suggereix que NF-κB pot ser clau en altres vies de senyalització que provoquen hipertròfia. Per aquesta raó, NF-κB pot esdevenir una diana terapèutica important en el tractament de la hipertròfia cardíaca.

A. Estatines

Les estatines són fàrmacs que s'utilitzen en el tractament de les dislipèmies ja que actuen principalment reduint el colesterol-LDL, produint millores importants dels episodis

coronaris. Aquesta reducció dels nivells de colesterol es deu al seu efecte inhibidor de la 3-Hidroxi-3-metilglutaril coenzima A reductasa (HMG-CoA Rd). Aquest enzim catalitza el pas limitant de la biosíntesi de colesterol i, per tant, la seva inhibició produeix una disminució dels nivells cel·lulars de colesterol. Aquesta reducció intracel·lular de colesterol activa a la proteïna SREBP-2 (*Sterol Regulatory Element Binding Proteins*); i com a conseqüència, es produeix un increment en l'expressió del receptor de les LDL a les membranes plasmàtiques, incrementant-se la captació cel·lular de les LDL circulants.

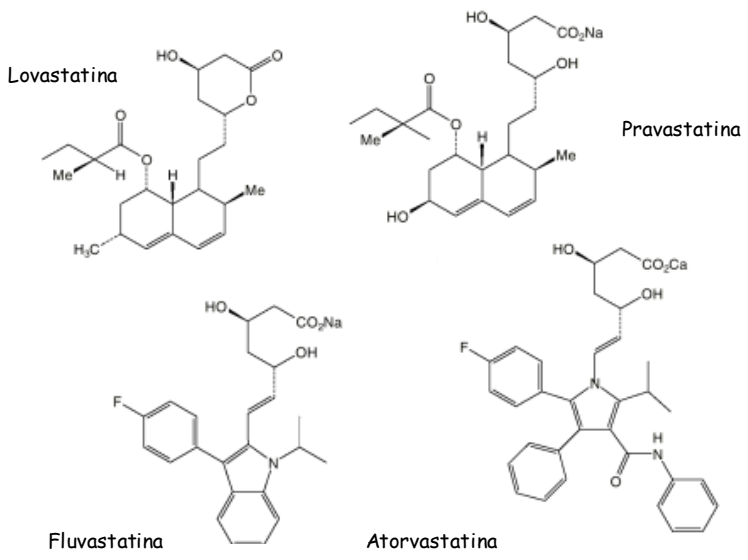

Figura 11. Estructura molecular de les estatines

Totes les estatines funcionen de manera similar, unint-se al centre actiu de la HMG-CoA Rd, inhibint l'enzim. La rosuvastatina és l'inhibidor més potent seguit per l'atorvastatina (Istvan i Deisenhofer, 2001). Les estatines s'han classificat en dos subtipus: (1) naturals (derivades de la fermenació de diferents fongs) i (2) sintètiques. Entre les estatines naturals hi trobem la lovastatina, pravastatina i simvastatina, amb estructures molt semblants (Figura 11). Pel contrari, les estructures de les sintètiques, atorvastatina, fluvastatina i cerivastatina, són molt diferents. Malgrat aquestes diferències estructurals, totes elles comparteixen un grup farmacòfor que és molt semblant estructuralment al de la HMG.

A part d'aquests efectes beneficiosos sobre els lípids plasmàtics, les estatines exerceixen altres efectes pleiotròpics, la majoria dels quals estan mediats per la seva capacitat

de bloquejar la síntesi d'intermediaris isoprenoids (Figura 12). Aquestes substàncies, són compostos lipídics imprescindibles per a les modificacions post-traduccionals d'una gran varietat de proteïnes, entre les quals destaquen les proteïnes petites d'unió a GTP, Rho, Ras i Rac1. La isoprenilació d'aquestes proteïnes determina la seva correcta localització a la membrana i la seva funció (James, 2003).

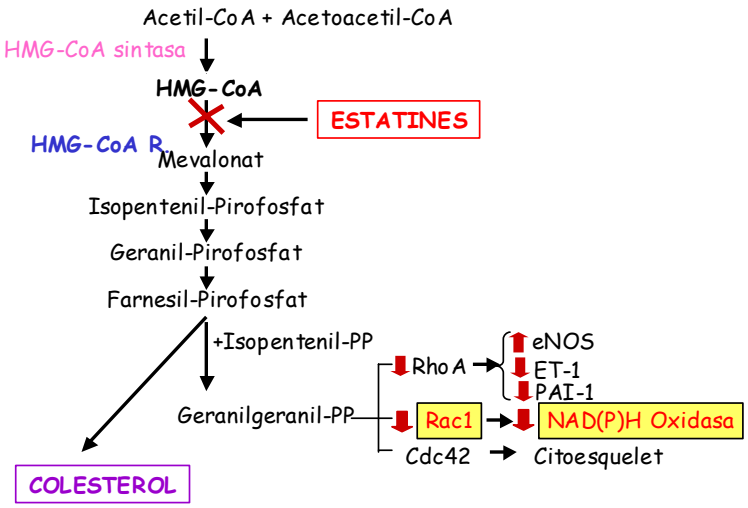

Figura 12. Efectes pleiotròpics de les estatines.

Les proteïnes petites d'unió a GTP juguen un paper important en l'organització del citoesquelet i regulen diversos processos com el creixement, la divisió i la supervivència cel·lular. Diversos estudis, les han relacionat amb l'aparició de la hipertròfia cardíaca. En concret, Rac-1 s'activa ràpidament en miòcits després d'estímuls hipertròfics com la fenilefrina i l'endotelina-1, provocant canvis morfològics i transcripcionals que provoquen hipertròfia (Clerck i col., 2001). Aquesta proteïna regula la transcripció gènica a través de dues vies diferents. D'una banda és capaç de regular a les MAPK, JNK i i p38 (Coso i col., 1995; Mackay i Hall, 1998) i d'altra banda activa a NF-κB (Sulciner i col., 1996). La Rac-1 també està present en el complex multienzimàtic de la NADPH oxidasa. Diversos estudis suggereixen que en cardiomiòcits la NADPH oxidasa és una font important de generació de ROS durant el desenvolupament de la hipertròfia cardíaca (MacCarthy i col., 2001; Bendall i col., 2002). La translocació de les diferents subunitats d'aquest complex a la membrana plasmàtica és un prerequisit per a la seva activació i la producció de ROS. Segons això, la

isoprenilació de Rac1 és essencial perquè s'activi l'enzim i es generin ROS. D'altra banda, Hirotani i col. (2002) van demostrar que la producció de ROS activa la MAP cinasa ASK1 (*Apoptosis Signal-regulating Kinase 1*), que activa a la vegada NF-κB mitjançant vies de senyalització com JNK i p38. Finalment, Higuchi i col. (2003) en un intent d'integrar totes aquestes dades han demostrat que Rac1 indueix hipertròfia cardíaca a través de l'activació d'ASK1 i NF-κB.

Recentment, s'ha demostrat que les estatines inhibeixen la hipertròfia cardíaca a través dels seus efectes pleiotròpics, bloquejant la isoprenilació i l'activació tant de RhoA com de Rac1. Takemoto i col (2001) han demostrat que la inhibició de Rac1 evita la generació de ROS. Aquest efecte podria ser el responsable de la inhibició de NF-κB i de la reversió de la hipertròfia cardíaca. Estudis previs en altres tipus cel·lulars, ja havien demostrat que les estatines inhibien l'activació de NF-κB (Ortego i col., 1999). Malgrat això, no es descarta que existeixin altres mecanismes que contribueixin als efectes antihipertròfics d'aquests fàrmacs.

B. Triflusal

Els salicilats pertanyen al grup de fàrmacs antiinflamatoris no esteroideus (AINEs), que es caracteritzen per ser potents inhibidors del procés inflamatori. El seu mecanisme d'acció consisteix en el bloqueig de l'enzim ciclooxigenasa (COX), inhibint així, la síntesi de prostaglandines (Vane, 1971; Ferreira i col., 1971). Aquests fàrmacs s'han utlitzat clàssicament com a analgèsics i antiinflamatoris, encara que en els darrers anys diverses dades suggereixen que podrien jugar un paper important com a protectors contra determinats càncers i en la malaltia de l'Alzheimer. D'altra banda, també s'ha demostrat que aquests fàrmacs inhibeixen el factor de transcripció NF-κB (Kopp i Ghosh, 1994).

El triflusal (àcid 2-acetoxi-4-tri-fluorometilbenzoic) és un AINE estructuralment relacionat amb el grup dels salicilats que fa més de 25 anys que s'utilitza clínicament com a antiagregant plaquetari (Figura 13). Presenta un bon perfil terapèutic en el tractament de diverses alteracions cardiovasculars amb una menor incidència d'efectes secundaris, especialment complicacions hemorràgiques, en comparació amb l'àcid acetilsalicílic (AAS). El principal mecanisme d'acció reconegut del triflusal, així com del seu metabòlit actiu,

l'àcid 3-hidroxi-4-trifluorometilbenzoic (HTB), consisteix en la reducció de la producció de tromboxà A_2 plaquetari, a través de la inhibició de l'enzim ciclooxigenasa. En comparació amb l'AAS, aquesta inhibició es mantè durant més temps degut a l'elevada vida mitja del fàrmac (McNeely i Goa, 1998).

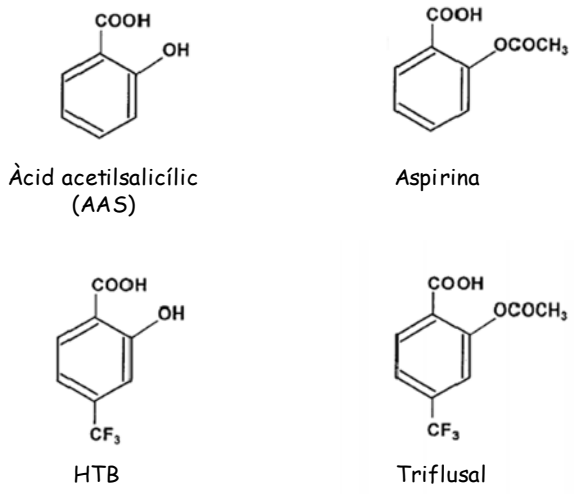

Figura 13. Estructura molecular d'AAS, Aspirina, HTB i Triflusal.

Independentment de l'activitat del triflusal sobre la ciclooxigenasa, aquest fàrmac i el seu metabòlit actiu HTB són també capaços d'inhibir *in vitro* i *in vivo* l'activitat transcripcional del factor NF-κB de forma més efectiva que l'AAS, a concentracions compatibles amb la seva utilització terapèutica (Bayón i col., 1999). Com ja s'ha comentat, l'activació de NF-κB juga un paper clau en el desenvolupament de la hipertròfia cardíaca. Fàrmacs capaços d'inhibir-lo podrien revertir aquest procés. Així doncs, el triflusal a través de la inhibició de NF-κB, podria convertir-se en una bona opció terapèutica per tractar la hipertròfia cardíaca.

II. RESISTÈNCIA A LA INSULINA

La diabetis mellitus de tipus 2 afecta a més de 100 milions de persones en tot el món, amb una major prevalència en les societats occidentals a causa dels estils de vida sedentaris i a les dietes riques en greixos. Es caracteritza per un desordre en la secreció d'insulina per part de les cèl·lules β del pàncrees i per l'aparició de resistència a la insulina en teixits com el múscul esquelètic, el fetge i el teixit adipós. La resistència a la insulina es pot definir com la incapacitat de l'organisme per respondre eficientment a la insulina, provocant que el múscul esquelètic i el teixit adipós captin menys glucosa. Aquesta situació produeix un estat d'hiperglicèmia que no es veu compensat per la hipersecreció d'insulina per part de les cèl·lules β del pàncrees. En concret, l'aparició d'aquest fenòmen al múscul esquelètic es considera un pas previ a l'aparició de la diabetis mellitus de tipus 2 (McGarry i col., 2002).

D'altra banda, a mesura que l'epidèmia de l'obesitat ha anat avançant en els països industrialitzats s'ha constatat l'estreta associació existent entre l'acumulació de greix abdominal visceral i el desenvolupament de la resistència a la insulina (el 80% dels individus amb DM2 són obesos), d'una banda, i un nou desordre clínic heterogeni, de l'altra, identificat amb un major risc de malaltia ateroscleròtica i que es denomina síndrome metabòlic. Aquest desordre clínic fa referència a un grup de factors de risc d'aterosclerosi, entre els que s'inclouen les dislipèmies, la resistència a la insulina, l'obesitat i la hipertensió (Reaven, 1988).

Segons això, la resistència a la insulina està estretament relacionada amb l'obesitat, la diabetis i el síndrome metabòlic i per aquest motiu té una gran importància determinar els mecanismes implicats en la seva aparició. Encara que la causa específica que la provoca no es coneix, el metabolisme lipídic i en concret l'increment en la disponibilitat de lípids hi estan fortament implicats.

II.1. MECANISMES IMPLICATS

L'organisme ha d'adaptar continuament el seu metabolisme en funció dels nutrients que té disponibles per tal de mantenir les seves funcions energètiques. Al múscul el principal substracte energètic és la glucosa, encara que també pot utilitzar els àcids grassos com a font d'energia. De fet als anys 60, Randle i col·laboradors (1965) ja van descriure que increments

en els nivells d'àcids grassos lliures en plasma, reduïen l'oxidació de glucosa al múscul esquelètic afavorint el catabolisme dels àcids grassos. Aquest augment de lípids circulants a la llarga acaba generant un acúmul intracel·lular d'àcids grassos a l'interior del múscul esquelètic que és el factor clau que més directament es relaciona amb l'aparició de la resistència a la insulina (Kelley i Mandarino, 2000).

Aquests àcids grassos són alliberats pel teixit adipós que és la major reserva energètica de l'organisme. Les seves cèl·lules, els adipòcits, acumulen energia en forma de triglicèrids en períodes d'afluència nutricional i els alliberen en forma d'àcids grassos lliures quan hi ha privació en l'aport de nutrients; d'aquesta forma, l'energia és transferida des de les reserves del teixit adipós fins a la resta de cèl·lules de l'organisme per al seu ús metabòlic. La mida dels adipòcits augmenta a mesura que aquests acumulen greixos, fet que converteix el teixit adipós en un teixit capaç de secretar substàncies, a banda dels àcids grassos lliures, que també poden modificar la sensibilitat a la insulina de l'organisme. Entre elles hi trobem adipocitocines com el TNFα i la resistina que indueixen resistència a la insulina (Hotamisligil i Spiegelman, 1994; Steppan i col., 2001), i d'altres com l'adiponectina que n'augmenten la sensibilitat (Matsuda i col., 2002). Tant els àcids grassos com aquestes citocines alliberades pel teixit adipós, poden interferir en la via de senyalització intracel·lular de la insulina.

La insulina és una hormona molt important sintetitzada per les cèl·lules β del pàncrees, que estimula la captació de glucosa al múscul esquelètic, al teixit adipós i al fetge, i inhibeix la lipòlisi al teixit adipós. El primer pas de la via de senyalització de la insulina consisteix en la unió al seu receptor situat a la membrana plasmàtica. Es tracta d'un receptor heterotetramèric format per dues subunitats α extracel·lulars i dues subunitats β intracel·lulars. La unió de la insulina a les subunitats α provoca l'autofosforilació del receptor i la fosforilació dels IRS (*Insulin Receptor Substrates*), entre els que s'inclou l'IRS-1 (Virkamaki i col., 1999). El resultat final de tot aquest procés és l'augment en la captació de glucosa per part de la cèl·lula.

La insulina estimula també a la proteïna Akt o proteïna cinasa B (PKB). Aquesta cinasa, està implicada en moltes funcions cel·lulars i a part d'actuar com a cinasa, també pot formar complexes amb altres proteïnes que no són pròpiament substractes però que actuen com a moduladors de la seva activitat i funció. L'activació de l'Akt és un component clau de

la resposta a la insulina ja que controla diferents aspectes del metabolisme de la glucosa. Un d'ells és la inhibició de la GSK3β per fosforilació de l'Akt, que activa la síntesi de glicògen (Halse i col., 1999). D'altra banda, l'activació de l'Akt també provoca la translocació del transportador de la glucosa GLUT4 de les vesícules intracel·lulars a la membrana plasmàtica (Hunter i col., 1998). El resultat final, és doncs, un increment de la captació i utilització de glucosa per part del múscul. Estudis recents, han demostrat que ratolins *db/db,* un model d'obesitat, resistència a la insulina i diabetis de tipus 2, presenten un descens en l'activitat d'aquesta cinasa (Shao i col., 2000).

A part de l'activació de l'Akt, el control de la seva degradació també pot resultar molt important en les vies de senyalització de la insulina. En aquest sentit cal dir que l'Akt es pot unir a les Hsp (*Heat Shock proteins*), que són xaperones que la protegeixen de la seva degradació quan la cèl·lula es troba exposada a una situació d'estrès ambiental. Tant la Hsp27 com la Hsp90 formen complexes amb l'Akt incrementant la seva activitat (Konishi i col., 1997; Sato i col., 2000). La formació del complex Akt-Hsp90 facilita l'activitat cinasa ja que evita la desfosforilació i la degradació de l'Akt (Brazil i col., 2002). Altres factors, a part de les Hsp, també participen en la regulació d'aquesta cinasa. Martín i col. (2002) van demostrar que tant els ROS com les ceramides estan implicats en la degradació d'aquesta proteïna.

D'altra banda, diverses evidències relacionen l'Akt amb el metabolisme dels àcids grassos i de la glucosa (Figura 14). Quan s'activa la via de senyalització de la insulina, s'afavoreix la captació de glucosa per part de la cèl·lula a través del receptor de membrana GLUT4. Un cop al citoplasma, la glucosa és transformada en piruvat pel procés de la glucòlisi. El piruvat es pot transformar en lactat anaeròbicament, o bé pot entrar a l'interior de la mitocòndria a través de la piruvat deshidrogenasa, on es transformarà en acetil-CoA. L'acetil-CoA es converteix a través de l'enzim acetil-CoA Carboxilasa (ACC) en malonil-CoA, un conegut inhibidor de la M-CPT-I, responsable de l'entrada dels àcids grassos a l'interior de la mitocòndria per a la seva oxidació. La regulació d'aquests enzims sembla clau pel desenvolupament d'aquesta patologia ja que ratolins deficients en ACC al múscul esquelètic tenen incrementada la seva capacitat oxidativa i estan protegits contra la diabetis (bu-Elheiga i col., 2003); mentre que inhibicions prolongades de la M-CPT-I, provoquen l'acumulació intracel·lular de lípids i l'aparició de resistència a la insulina (Dobbins i col., 2001).

Finalment, l'ACC s'inactiva per fosforilació d'una altra proteïna, l'AMPK (*AMP-activated protein kinase*), una serina-treonina cinasa que s'activa al múscul esquelètic quan es produeixen increments en la despesa energètica (durant l'exercici) i en situacions de dèficit energètic, on la relació intracel·lular AMP:ATP es troba augmentada (Ruderman i col., 1999). Quan s'activa, fosforila i inhibeix a l'ACC i també activa per fosforilació a la malonil-CoA decarboxilasa (MCD), provocant la reducció de la concentració de malonil-CoA i un increment en l'oxidació dels àcids grassos (Park i col., 2002). Estudis recents, han demostrat que l'activació de l'Akt pot provocar una disminució de l'activitat de l'AMPK (Kovacic i col., 2003), relacionant-la així amb la via de senyalització de la insulina.

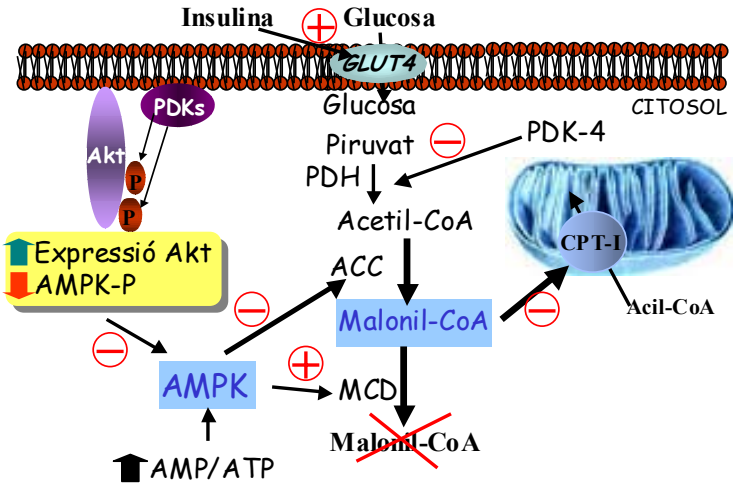

Figura 14. Relació entre Akt i el metabolisme de la glucosa

Com ja s'ha comentat, l'acúmul de triglicèrids al múscul és el factor clau que més es relaciona amb l'aparició de la resistència a la insulina i actualment es considera com un dels marcadors més importants per determinar el grau d'afectació d'aquesta patologia en tot l'organisme. Com a conseqüència d'aquest acúmul d'acids grassos, també augmenta el nombre d'espècies lipídiques capaces d'actuar com a segons missatgers, com els acil-CoAs de cadena llarga, el diacilglicerol (DAG) i les ceramides. Recentment s'han descrit un gran nombre de defectes en la via de senyalització de la insulina en models animals d'obesitat, diabetis i resistència a la insulina. S'ha proposat que siguin aquests segons missatgers lipídics

els responsables d'atenuar la via de senyalització de la insulina i per tant els responsables de l'aparició d'aquestes patologies (Schmitz-Peiffer, 2000).

Al múscul, els àcids grassos lliures, principalment palmitat, oleat i linoleat, són activats a acil-CoAs de cadena llarga després del seu transport a l'interior de a mitocòndria a través de la M-CPT-I. Un cop activats, poden oxidar-se a través de la β-oxidació mitcondrial o bé, poden actuar com a substractes per a la síntesi de triglicèrids, fosfolípids i segons missatgers com el DAG o les ceramides (Figura 15).

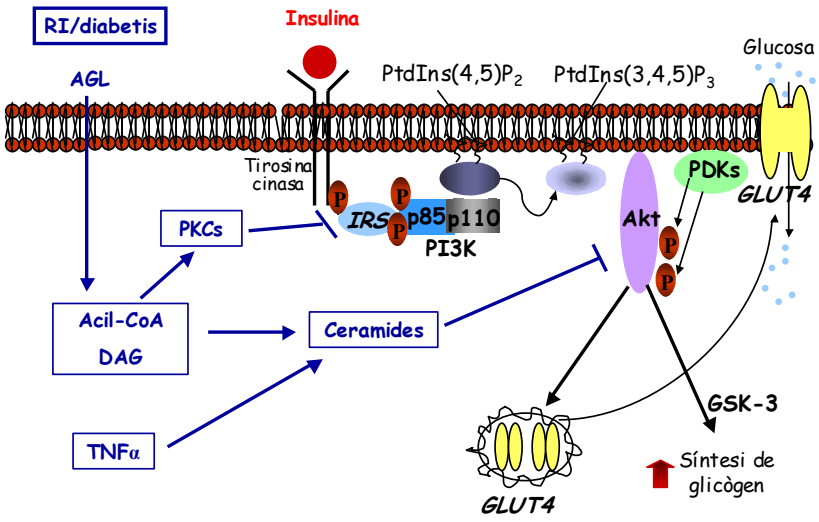

Figura 15. Efectes dels segons missatgers lipídics sobre la via de senyalització de la insulina

Els acil-CoAs de cadena llarga, per si mateixos, ja són molècules que participen en vies de senyalització que afecten a molts processos cel·lulars i s'ha suggerit que podrien atenuar l'acció de la insulina al múscul a través de diferents mecanismes. Thompson i Cooney (2000) van demostrar que aquestes substàncies poden inhibir l'hexoquinasa, enzim implicat en el metabolisme de la glucosa, contribuint així a la reducció del fluxe de glucosa a la cèl·lula. També poden interferir en aquest procés a través de l'activació de la proteïna cinasa C (PKC), que també està implicada en les alteracions en la via de senyalització de la insulina (Yaney i col., 2000).

El segon dels intermediaris lipídics citats és el DAG. Es tracta d'una molècula que s'acumula al múscul esquelètic en situacions de resistència a la insulina. Es genera tant com a intermediari en la síntesi de triglicèrids i fosfolípids, com per trencament de fosfolípids. La seva funció principal és la d'actuar com a segon missatger i sembla que és capaç d'atenuar la via de senyalització de la insulina a través de la seva capacitat per activar a la PKC (Schmitz-Peiffer, 2000).

L'activació de la PKC redueix la fosforilació normal de l'IRS-1, disminuint així la seva capacitat d'activar a la PI3K i atenuant l'acció de la insulina (Lewis i col., 2002). D'altra banda, un dels subtipus de la PKC, la PKCβ també és capaç d'activar al factor NF-κB a través de l'activació d'IKKβ que pot activar la producció de TNFα, que també està implicat en l'aparició de la resistència a la insulina. Finalment, l'activació de la PKC pels àcids grassos també està implicada en la generació de ROS, els quals també poden activar NF-κB (Barnes i Karin, 1997). Aquestes dades reflexen el paper clau que sembla que juga NF-κB en l'aparició de la resistència a la insulina.

El darrer dels missatgers lipídics anomenats, són les ceramides. Aquestes substàncies es poden formar a partir de l'esfingomielina, un fosfolípid que forma part de la membrana cel·lular, mitjançant l'acció de l'esfingomielinasa i també poden ser sintetitzades de nou a partir de palmitoil-CoA i serina (Figura 16). El TNFα, implicat en l'aparició de la resistència a la insulina, és capaç d'activar a l'esfingomielinasa, incrementant per tant el contingut intracel·lular de ceramides (Peraldi i col., 1996). Aquests missatgers lipídics, poden modificar l'activitat de cinases, fosfatases i factors de transcripció, de manera que juguen un paper molt important en la regulació de la diferenciació, proliferació i apoptosi cel·lular i també es troben implicats en l'aparició de la resistència a la insulina (Cutler i Mattson, 2001). Les ceramides, redueixen la fosforilació de l'IRS-1 *in vitro* (Kanety i col., 1996) i també inhibeixen directament la fosforilació de l'Akt, reduint la translocació de GLUT4 i l'entrada de glucosa a la cèl·lula (Summers i col., 1998). Diversos estudis han demostrat que una major disponibilitat de palmitat incrementa els nivells intracel·lulars de ceramides (Shimabukuro i col., 1998). D'altra banda, els nivells intracel·lulars d'aquests derivats lipídics es troben augmentats en el múscul de rates Zucker, un model d'obesitat i diabetis (Turinsky i col., 1990). Totes aquestes dades semblen indicar que les ceramides tenen un paper clau en l'aparició de la resistència a la insulina al múscul esquelètic.

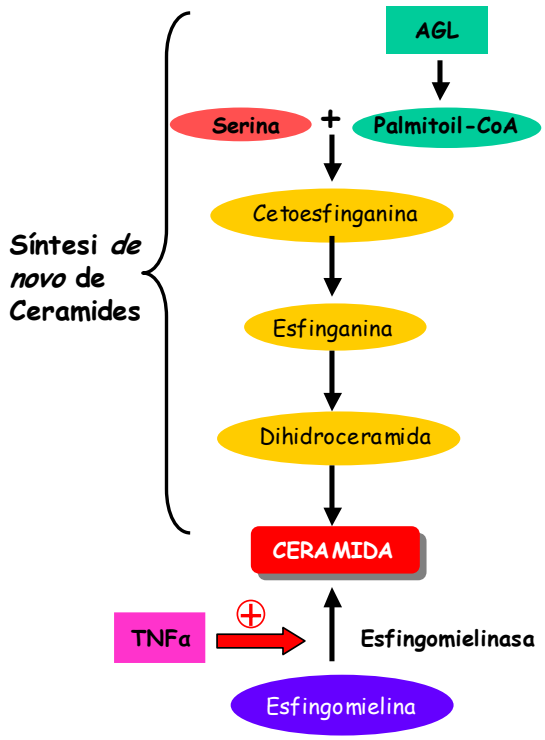

Figura 16. Síntesi de ceramides.

Finalment, cal destacar que els àcids grassos lliures i els seus derivats, també poden unir-se i activar específicament diferents tipus de factors de transcripció com són els PPARs (Gottlicher i col., 1992), provocant canvis en l'expressió dels gens implicats en el metabolisme dels àcids grassos i de la glucosa. PPARα i PPARβ/δ són els subtipus de PPARs majoritaris al múscul esquelètic i estudis recents indicarien que ambdós tenen funcions redundants, a diferència del que succeïa en cor, incrementant l'oxidació dels àcids grassos (Muoio i col., 2002). Els activadors de PPARα, com els fibrats, milloren la resistència a la insulina però sembla que a través dels seus efectes sobre el metabolisme hepàtic (Guerre-Millo i col., 2000). Pel que fa a PPARβ/δ, estudis recents han demostrat que la seva activació a través d'agonistes específics incrementa el catabolisme dels àcids grassos al múscul i es perfila com una possible nova diana terapèutica per al tractament de la resistència a la insulina (Dressel i col., 2003; Tanaka i col., 2003). Finalment, el darrer subtipus de PPAR, PPARγ, s'expressa molt poc en múscul; malgrat això, els activadors de

PPARγ, les tiazolidinediones, són els fàrmacs que principalment s'utilitzen com a agents antidiabètics.

II.2. EFECTE ANTIDIABÈTIC DE LES TIAZOLIDINEDIONES

PPARγ és fonamental pel desenvolupament de l'adipogènesi, el procés de formació del texit adipós (Rosen i col., 2000). La seva expressió és molt abundant al teixit adipós blanc tant en animals de laboratori com en l'espècie humana. Diversos estudis han demostrat que l'addició a preadipòcits humans d'agonistes PPARγ, com les TZDs, indueix la seva diferenciació a adipòcits (Rosen i col., 2002). L'activació de PPARγ a part de promoure l'adipogènesi, que augmenta el nombre d'adipòcits, també estimula l'acumulació d'àcids grassos als adipòcits madurs actuant en diferents nivells: estimula la lipoproteïna lipasa (LPL), responsable del transport dels triglicèrids des de les lipoproteïnes als adipòcits; la aP2 que s'encarrega del transport intracel·lular dels àcids grassos; l'acil-CoA sintasa (ACS) que activa als àcids grassos i també la CD36, responsable de l'entrada dels lípids a l'adipòcit. També sembla que estimula el transportador de glucosa GLUT4, que podria contribuir a incrementar la síntesi d'àcids grassos a partir de la glucosa. Així doncs, l'activació de PPARγ incrementa l'expressió de gens que promouen l'acumulació d'àcids grassos als adipòcits i reprimeix els gens que indueixen lipòlisi (Auwerx, 1999). Aquest fet provoca un desplaçament dels àcids grassos lliures circulants cap al teixit adipós, alliberant, així als altres teixits dels seus efectes tòxics. Donat que l'augment dels àcids grassos lliures circulants és un dels factors que més estretament es relaciona amb l'aparició de la resistència a la insulina al múscul, la reducció produïda pel tractament amb TZDs es considera una de les accions que més contribueixen a l'efecte antidiabètic d'aquests fàrmacs.

Una altra possibilitat que també podria explicar l'increment de la sensibilitat a la insulina provocada per aquests fàrmacs és l'acció que té PPARγ sobre les hormones secretades pels adipòcits o adipocitocines, entre les que s'inclouen: el TNFα, la resistina i la 11β-hidroxiesteroide deshidrogenasa 1, totes elles substàncies que indueixen resistència a la insulina, i l'adiponectina, que n'augmenta la sensibilitat. L'obesitat, especialment l'obesitat visceral, contribueix a l'aparició de resistència a la insulina alterant els nivells d'aquestes citocines. L'augment dels nivells de TNFα produeix resistència a la insulina i les TZDs en redueixen l'expressió (Peraldi i col., 1997). D'altra banda, les TZD incrementen l'expressió

de l'adiponectina, fet que suggereix que aquesta adipocitocina pot ser clau per mediar els efectes sensibilitzants a l'acció de la insulina de les TZDs (Pajvani i col., 2004). La resistina, s'ha descrit com un inductor de la resistència a la insulina al múscul i està regulada negativament per les TZDs (Steppan i col., 2001). Finalment, la sobreexpressió al teixit adipós de ratolins de la 11β-hidroxiesteroide deshidrogenasa 1, l'enzim que produeix cortisol localment en aquest teixit, desencadena l'aparició de síndrome metabòlic (Masuzaki i col., 2001). Les TZDs també redueixen l'expressió d'aquest enzim (Berger i col., 2001). Actualment s'accepta, que les TZDs milloren la sensibilitat a la insulina, almenys en part, gràcies a la seva capacitat per incrementar l'adipogènesi. L'increment d'aquest procés generaria nous adipòcits més petits que són més sensibles a l'acció de la insulina i disminuiria el nombre d'adipòcits grans, responsables de la producció d'aquestes adipocitocines i dels àcids grassos lliures (Okuno i col., 1998).

De fet, el teixit adipós és necessari per un correcte control de la glicèmia, ja que la lipodistròfia també s'associa amb la presència de resistència a la insulina severa (Moitra i col., 1998). Segons això, el teixit adipós, a través de PPARγ, controla l'homeòstasi metabòlica a través de la inducció de l'adipogènesi que regula la correcta secreció d'adipocitocines i que a la vegada incrementa la capacitat per captar més àcids grassos lliures del plasma alliberant així, la resta de teixits d'un possible excés dels mateixos.

Finalment, s'ha observat que el tractament d'animals obesos i diabètics amb TZDs indueix la proliferació adipocitària i un augment de pes, efectes que s'expliquen per la capacitat que tenen aquests fàrmacs per estimular l'adipogènesi. Aquest fet, pot semblar contradictori, ja que l'increment dels dipòsits de teixit adipós és un dels factors que més es correlaciona amb l'aparició de la diabetis mellitus de tipus 2. En humans, també s'han observat guanys de pes més modestos però que principalment es localitzen a nivell del teixit adipós subcutani i no del teixit adipós visceral, que és el que es relaciona amb la resistència a la insulina (Ferré, 2004).

Tots aquests resultats indiquen que el principal mecanisme antidiabètic de les tiazolidinediones està mitjançat pel PPARγ en els adipòcits, però cal tenir en compte que el teixit més important que controla la disponibilitat de glucosa per l'organisme és el múscul esquelètic (Figura 17). Tot i que PPARγ s'expressa en petites quantitats en el múscul

esquelètic, hi ha diferents evidències d'un efecte directe dels agonistes d'aquest receptor en aquest teixit. D'aquesta manera, diferents estudis en models animals de lipoatròfia indiquen que les TZDs no necessiten una gran quantitat de teixit adipós per millorar la resistència a la insulina i, per tant, que és probable que hi hagi una acció directa d'aquests fàrmacs en altres teixits, principalment en el múscul esquelètic (Kahn i col., 2000). Way i col (2001) van demostrar que el tractament amb TZDs produeix una disminució en l'expressió de la PDK-4 en el múscul esquelètic. Donat que aquest enzim inhibeix a la piruvat deshidrogenasa, necessària per al metabolisme oxidatiu de la glucosa, aquest podria ser un possible mecanisme que expliqués l'increment en l'ús de glucosa pel múscul. Altres estudis també han demostrat com el tractament amb troglitazona indueix la translocació de GLUT4 a la membrana, afavorint així, la utilització de glucosa (Yonemitsu i col., 2001).

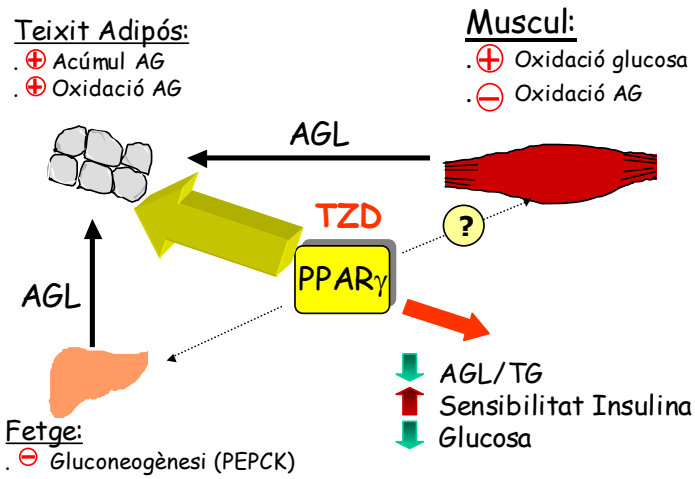

Figura 17. Mecanismes d'acció implicats en la millora de la sensibilitat a la insulina produïda per les TZDs.

En general però, es considera que aquests efectes sobre el múscul són un efecte secundari derivat de la reducció que es produeix en els nivells de lípids circulants després del tractament amb TZDs. Malgrat això, existeixen altres evidències que suggereixen un efecte directe de les TZDs sobre l'acció de la insulina, independenment dels nivells lipídics plasmàtics. Per exemple, en ratolins amb lipodistròfia, la troglitazona també millora la sensibilitat a la insulina (Burant i col., 1997) i a més, estudis recents en múscul aïllat han demostrat com les TZDs tenen efectes directes sobre el metabolisme lipídic provocant

l'alliberació de lactat i la inhibició de l'oxidació de palmitat per un mecanisme independent de PPARγ (Brunmair i col., 2001). També cal destacar, que a diferència de la resta de TZDs, la troglitazona (veure Figura 6) també presenta altres efectes que per exemple no presenta la rosiglitazona, com la inhibició de l'ACS, impedint així, l'activació dels àcids grassos (Fulgencio i col., 1996).

Finalment, ratolins transgènics deficients en PPARγ al múscul esquelètic presenten resistència a la insulina. Malgrat això, aquests ratolins responen igual al tractament amb TZDs. Aquests resultats demostren com l'expressió de PPARγ al múscul juga un paper fonamental en el manteniment de la homeòstasi metabòlica a l'organisme, malgrat la seva reduïda expressió. A més, també suggereixen l'existència d'alguna molècula capaç d'intercomunicar el múscul amb el teixit adipós i el fetge, ja que aquests ratolins desenvolupen resistència a la insulina en aquests teixits. El fet que la resposta al tractament amb TZDs sigui la mateixa, reforça el paper clau que juga el teixit adipós com a principal diana d'aquests fàrmacs (Hevener i col., 2003; Norris i col., 2003), però no exclou que puguin tenir algun efecte directe sobre el múscul.

ABREVIATURES

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AAS	Àcid acetilsalicílic
AB	*Aortic Banding* (Constricció de l'aorta abdominal)
AC	Adenilat ciclasa
ACC	Acetil-CoA carboxilasa
ACO	Acil-CoA oxidasa
ACS	Acil-CoA sintetasa
AF	*Activation function*
AGL	Àcids grassos lliures
AINE	Antiinflamatoris no esteroideus
Akt	Proteïna cinasa B (PKB)
AMPK	*AMP-activated protein kinase*
AP-1	*Activator protein-1*
ASK-1	*Apoptosis signal-regulating kinase 1*
CBP/p300	*CREBP-binding protein*
COUP-TF	*Chicken ovalbumin upstream promoter transcriptional factor*
COX	Ciclooxigenasa
CPT-I	Carnitina-palmitoiltransferasa-I
DAG	Diacilglicerol
DBD	*DNA-binding domain*
DM2	Diabetis mellitus de tipus 2
DNA	Àcid desoxirribonucleic
DR-1	*Direct repeat 1*
eNOS	Sintasa d'òxid nítric endotelial
ERK	*Extracellulary responsive kinases*
ERRα	*Estrogen-related receptor α*
ET-1	Endotelina-1
FAT/CD36	Translocasa d'àcids grassos
FATP	Proteïna transportadora d'àcids grassos
FE	Fenilefrina
FKBP	*FK-binding protein*
GDP	Guanosin difosfat
GPCR	*G protein coupled receptors* (Receptors acoblats a proteïna G)
GRK	Cinases dels GPCR
GSK-3β	Glicògen sintasa cinasa-3β

GTP	Guanosin trifosfat
HETE	Àcid hidroxieicosatetraènic
HMG-CoA	3-hidroxi-metilglutaril coenzima A
HODE	Àcid hidroxi-octadecadienoic
Hsp	*Heat shock protein*
HTB	Àcid 3-hidroxi-4-trifluorometilbenzoic
IECA	Inhibidors de l'enzim conversor de l'angiotensina
IKK	*IκB kinase complex*
IP_3	Inositol 1,4,5-trifosfat
IRS	*Insulin receptor substrates*
JNK	*c-Jun N-terminal kinases*
LBD	*Ligand-binding domain*
LCAD	Acil-CoA deshidrogenasa de cadena llarga
LDL	*Low density lipoprotein* (Lipoproteïna de baixa densitat)
LPL	Lipoproteïna lipasa
LPS	Lipopolisacàrid
LT	Leucotriè
MAPK	*Mitogen-Activated Protein Kinases*
MCAD	Acil-CoA deshidrogenasa de cadena intermitja
MCD	Malonil-CoA decarboxilasa
MCP-1	*Monocyte chemoattractant protein 1*
mRNA	Àcid ribonucleic missatger
mTOR	*Mammalian target of rapamycin*
NCoR	*Nuclear receptor co-repressor*
NF-κB	Factor nuclear κB
NFAT	*Nuclear factor of activated T-cells*
NRF	*Nuclear respiratory factor*
PDH	Piruvat deshidrogenasa
PDK-1	Cinasa dependent de PtdIns
PDK-4	Piruvat deshidrogenasa cinasa-4
PEPCK	Fosfoenolpiruvat-cinasa
PG	Prostaglandines
PGC-1	*PPARγ coactivator-1*
PI3K	Fosfatidininositol 3-cinasa
PIP_2	Fosfatidininositol bifosfat
PKA	Proteïna cinasa A
PKC	Proteïna cinasa C

PLB	Fosfolambà
PLC	Fosfolipasa C
PPAR	*Peroxisome proliferator-activated receptor* (Receptors activats per proliferadors peroxisòmics)
PPRE	*Peroxisome proliferator response element* (Element de resposta a proliferadors peroxisòmics
PtdIns	Fosfatidininositol
RIP-140	*Receptor-interacting protein-140*
ROS	*Reactive oxygen species* (Espècies reactives a l'oxigen)
RTK	Receptors tirosina cinasa
RXR	*Retinoic X receptor* (Receptor del retinoid X o de l'àcid 9-*cis*-retinoic)
RyR	Receptor de la Rianodina
SERCA	Ca^{2+}-ATPasa del reticle sarcoplasmàtic
SMRT	*Silencing mediator of retinoid and thyroid receptors*
SRBP	*Sterol regulatory element binding protein*
SRC-1	*Steroid receptor coactivator-1*
STAT	*Signal transducer and activator of transcription*
TNFα	*Tumor necrosis factor-α*
TNFR	Receptor de TNFα
TZD	Tiazolidinediones
UCP	*Uncoupling protein* (Proteïna desacobladora)
Wy-15643	Àcid 4-cloro-6-(2,3-xilidin)-2-pirimidiniltio acètic

OBJECTIUS

Les alteracions en el metabolisme dels àcids grassos estan implicades tant en l'aparició de la hipertròfia cardíaca com de la resistència a la insulina, encara que els mecanismes responsables són poc coneguts.

Per aquesta raó, l'objectiu general d'aquesta tesi doctoral ha estat aprofundir en el coneixement dels mecanismes responsables de l'aparició d'aquestes dues patologies, així com els efectes de diferents fàrmacs sobre la hipertròfia cardíaca i la resistència a la insulina. Per a la consecució d'aquest objectiu general s'han realitzat una sèrie d'estudis amb els següents objectius específics:

I. Determinar els efectes que produeix l'activació de NF-κB durant la hipertròfia cardíaca sobre l'expressió dels gens implicats en el metabolisme dels àcids grassos i l'efecte produit pels inhibidors d'aquest factor de transcripció.

II. Avaluar si l'activador de PPARβ/δ, L-165041, és capaç de revertir la hipertròfia cardíaca en cardiomiòcits neonatals, i en cas que així sigui, determinar el mecanisme responsable.

III. Examinar nous mecanismes potencials a través dels quals l'activació de NF-κB afecta l'acció dels PPARs en un model de rates amb constricció de la aorta abdominal que desenvolupen hipertròfia cardíaca i determinar l'efecte del tractament amb estatines en aquest model.

IV. Estudiar l'efecte de les estatines sobre l'activitat de PPARβ/δ en cèl·lules H9c2 estimulades amb LPS i la possible participació del cofactor PGC-1 en aquest procés.

V. Determinar si el tractament amb inhibidors de NF-κB afecta a la via Akt-GSK3β implicada en l'aparició de la hipertròfia cardíaca.

VI. Examinar els efectes de l'HTB sobre la hipertròfia induïda per fenilefrina en cardiomiòcits neonatals i del triflusal en un model de rates amb constricció de la aorta abdominal.

VII. Estudiar com el tractament amb troglitazona afecta a l'expressió de l'Akt i a l'activitat d'AMPK al múscul esquelètic i la seva implicació en els efectes d'aquest fàrmac sobre el metabolisme dels àcids grassos i la glucosa.

RESULTATS I DISCUSSIÓ

I

Nuclear Factor-κB Activation Leads to Down-regulation of Fatty Acid Oxidation During Cardiac Hypertrophy

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Nuclear Factor-κB Activation Leads to Down-regulation of Fatty Acid Oxidation During Cardiac Hypertrophy

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Summary

Little is known about the mechanisms responsible for the fall in fatty acid oxidation during the development of cardiac hypertrophy. We focused on the effects of Nuclear Factor (NF)-κB activation during cardiac hypertrophy on the activity of Peroxisome Proliferator-Activated Receptor (PPAR)β/δ, which is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism. Phenylephrine-induced cardiac hypertrophy in neonatal rat cardiomyocytes caused a reduction in the expression of pyruvatedehydrogenase kinase 4 (PDK4), a target gene of PPARβ/δ involved in fatty acid utilization, and a fall in palmitate oxidation that was reversed by NF-κB inhibitors. LPS-stimulation of NF-κB in embryonic rat-heart derived H9c2 myotubes, which only express PPARβ/δ, caused both a reduction in PDK4 expression and in the DNA binding activity of PPARβ/δ to its response element, effects that were reversed by NF-κB inhibitors. Coimmunoprecipitation studies demonstrated that LPS strongly stimulated the physical interaction between the p65 subunit of NF-κB and PPARβ/δ, providing an explanation for the reduced activity of PPARβ/δ. Finally, we assessed whether this mechanism was present *in vivo* in pressure overload-induced cardiac hypertrophy. In hypertrophied hearts of banded rats the reduction in the expression of PDK4 was accompanied by activation of NF-κB and enhanced interaction between p65 and PPARβ/δ. These results indicate that NF-κB activation during cardiac hypertrophy downregulates PPARβ/δ activity, leading to a fall in fatty acid oxidation, through a mechanism that involves enhanced protein-protein interaction between the p65 subunit of NF-κB and PPARβ/δ.

Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction, and cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia, and sudden death(1;2). Cardiac hypertrophy is associated with an increase in glucose utilization and a decrease in fatty acid oxidation, which is characteristic of fetal heart(3;4). It is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are a consequence or the reason for cardiac hypertrophy. However, several factors support a role for cardiac metabolism in the development of cardiac hypertrophy. Thus, defects in mitochondrial fatty acid oxidation enzymes cause childhood hypertrophic cardiomyopathy(5), and perturbation of fatty acid oxidation in animal models causes cardiac hypertrophy(6;7), demonstrating that substrate utilization is important in the pathogenesis of hypertrophy. Nonetheless, little is known about the molecular mechanisms linking cardiac hypertrophy and the fall in the expression of genes involved in cardiac fatty acid metabolism.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation(8). The PPAR subfamily consists of three subtypes, PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ/δ (NR1C2) and PPARγ (NR1C3)(9). PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle(10). PPARβ/δ is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts. In order to be transcriptionally active, PPARs need to heterodimerize with the 9-*cis* retinoic acid receptor (RXR) (NR2B). PPAR-RXR heterodimers binds to DNA specific sequences

called peroxisome proliferator-response elements (PPREs), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). These sequences have been characterized within the promoter regions of PPAR target genes. However, the regulation of gene transcription by PPARs extends beyond their ability to transactivate specific target genes. PPARs are also able of regulating gene expression independently of binding to DNA through a mechanism termed receptor-dependent *trans*repression(11). One of these mechanisms involves a physical interaction of PPARα with nuclear factor (NF)-κB, that may lead to suppression activity of the former(12). Interestingly, NF-κB signaling pathway plays a pivotal role in the hypertrophic growth of the myocardium and its inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes(13-16).

Given that Gilde and co-workers(17), using neonatal rat cardiomyocytes as well as the embryonic rat heart-derived H9c2 cells, clearly demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism, we focused on the effects of NF-κB activation during cardiac hypertrophy on PPARβ/δ activity. Further, in order to avoid the interference of the other PPAR subtypes we took advantage of the use of the embryonic rat heart-derived H9c2 myotubes, which only express PPARβ/δ. Our findings indicate that NF-κB activation downregulates PPARβ/δ activity, leading to a fall in fatty acid oxidation through a mechanism involving enhanced protein-protein interaction between the p65 subunit of NF-κB and PPARβ/δ.

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MATERIALS AND METHODS

Cell Culture-Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously(18). The media was changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 hours before treatments. In this study phenylephrine (PE) was used to stimulate neonatal rat cardiomyocytes in the absence or in the presence of 10 μmol/L parthenolide for the last 6 hours. Animal handling and disposal were performed in accordance with law 5/1995, 21st July, of the Generalitat de Catalunya.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA analysis H9c2 cells were stimulated with LPS (10 ng/mL) for 24 hours in the presence or the absence of 10 μmol/L parthenolide for the last 6 hours.

Incorporation of [^{3}H]leucine-To examine the effect of PE on protein synthesis, the incorporation of [^{3}H]leucine was measured essentially by the method of Thaik et al.(19). Cultured neonatal rat ventricular myocytes were treated with PE in the presence or in the absence of pathenolide and coincubated with [^{3}H]leucine (1 μCi/mL) for 24 hours. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4ºC for 30 minutes to precipitate the protein. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted with scintillitation counter.

Fatty Acid Oxidation-Experiments to determine the oxidation capacity of 9,10-[^{3}H]palmitate (Amersham) oxidation was performed as previously described(20).

Pressure overload-induced cardiac hypertrophy-Male Sprague-Dawley rats (225 to 250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1ºC). They were fed standard diet (Panlab, Barcelona, Spain) for five days before the studies began. The animals were randomly distributed into two groups: sham-operated rats and pressure overloaded rats. Pressure overload was induced by constriction of the abdominal aorta at the suprarenal level with 7-0 nylon strings by ligation with a blunted 25-gauge needle, which was then pulled out. For the age-matched sham operation, the identical procedure was performed except that the suture was not tied around the aorta. Hearts were harvested 15 days after the surgical operation. The heart weight/body weight (HW/BW) ratio was calculated and the heart samples were frozen in liquid nitrogen and then stored at -80ºC. Animal handling and disposal were performed in accordance with the law 5/1995, 21st July, from the Generalitat de Catalunya.

RNA preparation and analysis-Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described(21). The sequences of the sense and antisense primers used for amplification were: ANF, 5'-TCCTCTTCCTGGCCTTTTGGC-3' and 5'-AGACGGGTTGCTTCCCCAGTC-3'; monocyte chemoattractant protein 1 (MCP-1), 5'-GGGCCTGTTGTTCACAGTTGC-3' and 5'-GGGACACCTGCTGCTGGTGAT-3'; pyruvatedehydrogenase kinase 4 (PDK4), 5'-GAACACCCCTTCCGTCCAGCT-3' and 5'-TGTGCCATCGTAGGGACCACA-3'; and APRT (adenosyl phosphoribosyl transferase), 5'-

GCCTCTTGGCCAGTCACCTGA-3' and 5'-CCAGGCTCACACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, MCP-1: 157 bp, PDK4: 168 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

Immunoblotting-Cell lysates and nuclear extracts from H9c2 cells were obtained as previously described(21). Proteins (50 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against p65 (Santa Cruz Biotechnology, Inc), PPARβ/δ (gift of Dr. Walter Wahli) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel. Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

Electrophoretic mobility shift assay (EMSA)-H9c2 cells were pretreated with inhibitors of NF-κB, pyrrolidine dithiocarbamate (PDTC) (5 mM), parthenolide (10 μM) or the PPARb/d activator L-165041 (10 μM) 24 hours before LPS (10 ng/ml) stimulation for 1 hour. Isolation of nuclear extracts and EMSA were performed as previously described(21).

Coimmunoprecipitation-Cell nuclear extracts were brought to a final volume of 0.5 mL with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, BSA 2% and 1 mg/ml nonfat milk for 6 h at 4ºC and incubated with 4 μg of anti-p65. Immunocomplex was captured by incubating the samples with protein A-agarose suspension overnight at 4ºC on a rocking platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After

microcentrifugation, the pellet was washed with 60 μl of SDS-PAGE sample buffer and boiled for 5 min. at 100ºC. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against PPARβ/δ.

Statistical Analyses-Results were obtained from at least four independent experiments and presented as mean±S.D. Significant differences were established by Student's *t*-test or ANOVA, according to the number of groups compared. When significant variations were found, the Tukey-Kramer multiple comparisons test was performed (GraphPad Software V2.03) (GraphPad Softwware Inc., San Diego, CA). Differences were considered significant at $P<0.05$.

RESULTS

Down-regulation of Fatty Acid Oxidation in Hypertrophied Neonatal Rat Cardiomyocytes is Reversed by NF-κB Inhibitors-Cardiac hypertrophy is characterized by increase protein content (e.g. [^{3}H]leucine uptake) and induction of fetal-type genes (e.g. ANF). PE-induced cardiac hypertrophy enhanced [^{3}H]leucine incorporation (1.6-fold, $P<0.001$) and mRNA levels of ANF in neonatal rat cardiomyocytes (approximately 2-fold induction, $P<0.01$) (Figure 1A and B). Next, we evaluated whether induction of cardiac hypertrophy led to a reduction in the expression of cardiac genes involved in lipid metabolism. We examined the expression of the PPARβ/δ target gene(22) PDK4, which suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization(23). Induction of cardiac hypertrophy by PE led to a reduction in the transcript levels of PDK4 (30%, $P<0.05$). Since it has been previously demonstrated that PE-induced cardiac hypertrophy involves NF-κB activation(14), we confirmed NF-κB activation by measuring in neonatal rat cardiomyocytes the mRNA levels of MCP-1, a gene under the transcriptional control of NF-κB(24). PE stimulation enhanced 2-fold ($P<0.01$) the expression of this gene (Figure 1C). These findings indirectly suggested a link between activation of NF-κB and the reduction in the expression of genes involved in cardiac fatty acid metabolism during the development of cardiac hypertrophy. To clearly demonstrate this relationship we assessed the effects of NF-κB inhibitors on the expression of these genes and in palmitate oxidation. The changes in PDK-4 and MCP-1 mRNA levels in PE-induced cardiac hypertrophy were prevented when cardiomyocytes were incubated with parthenolide, that specifically inhibits activation of NF-κB by preventing IκB degradation(27) (Figure 2). Thus, the 33% reduction ($P<0.05$) observed in the transcript levels of PDK4 after PE stimulation was prevented by parthenolide treatment and even a 5-fold induction was observed compared to control cells (Figure 2A). Similarly, the 1.8-fold induction ($P<0.05$) assessed by PE

was prevented when cells were incubated with parthenolide (Figure 2B). Further, we determined whether these changes affected the palmitate oxidation rate in neonatal rat cardiomyocytes either in the absence or in the presence of NF-κB inhibitors such as parthenolide and SN-50, a cell-permeable peptide highly selective against NF-κB(25) that inhibits NF-κB by blocking its translocation to the nucleus(26). PE-induced cardiac hypertrophy led to a 44% reduction ($P<0.001$) in palmitate oxidation compared to untreated cardiomyocytes (Figure 2C). In contrast, when PE-treated cardiomyocytes were coincubated with either parthenolide or SN-50 statistically significant increases were observed in palmitate oxidation compared with cardiomyocytes treated only with PE.

Reduced DNA Binding Activity of PPARβ/δ in LPS-stimulated H9c2 myotubes. Restoration by NF-κB Inhibitors- Data obtained in neonatal rat cardiomyocytes clearly establish a link between NF-κB activation during cardiac hypertrophy and the fall in fatty acid oxidation. However, since PPARα and PPARβ/δ are expressed in neonatal and adult heart(17) it is difficult to discard the potential involvement of the former in the changes reported. Thus, to clearly establish the role of PPARβ/δ in the changes observed we used H9c2 myotubes, which only express the PPARβ/δ subtype(17) (Figure 3A), whereas PPARα is undetectable. In order to activate NF-κB, H9c2 myotubes were stimulated for 1 hour with LPS, which has been reported to activate NF-κB in cardiomyocytes(28). LPS-induced activation of NF-κB in H9c2 myotubes was confirmed by EMSA and by assessing the expression of MCP-1. EMSA showed that the NF-κB probe formed three complexes with cardiac nuclear proteins (complexes I to III, Figure 3B). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures. NF-κB binding activity, mainly of specific complex II, increased in cells stimulated with LPS for 1 hour. Characterization of NF-κB

was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of NF-κB. Addition of this antibody to incubation mixtures resulted in complete supershift of complex II, demonstrating that this complex contained p65. As expected, a robust induction (7-fold, $P<0.001$) was observed in the mRNA levels of the NF-κB target gene MCP-1 after LPS stimulation (Figure 3B). This increase was significantly reduced in the presence of the NF-κB inhibitor, parthenolide. Furthermore, stimulation of H9c2 myotubes with LPS also caused a 40% reduction in the mRNA levels of PDK4 that was completely prevented in the presence of parthenolide (Figure 3C). Therefore, two different stimulus leading to NF-κB activation caused a reduction in the expression of PDK4 in cardiac cells, strengthening the correlation between both processes.

We next sought to determine the molecular mechanism by which NF-κB activation leads to reduced expression of PPARβ/δ–target genes, such as PDK4. EMSA were performed to examine the interaction of PPARs with its *cis*-regulatory element using a ^{32}P-labeled PPRE probe and cardiac nuclear extracts from H9c2 myotubes stimulated with LPS for 1 hour. The PPRE probe formed two main complexes with cardiac nuclear proteins (complexes I and II, Figure 3E). Competition studies performed with a molar excess of unlabeled probe revealed that both complexes represented specific PPRE-protein interactions. Supershift studies performed using an antibody against PPARδ/β demonstrated that complex I contained this PPAR subtype. In nuclear extracts from LPS-stimulated H9c2 myotubes a significant reduction was observed in the binding activity of complex I compared to nuclear extracts from control cells (Figure 3F). In contrast, this binding activity was restored when LPS-stimulated H9c2 myotubes were coincubated with two inhibitors of NF-κB, PDTC and parthenolide. Nuclear extracts from LPS-stimulated H9c2 cells incubated with the selective PPARβ/δ activator L-165041 were used as a positive control to demonstrate that enhanced complex I binding activity was due to increased PPARβ/δ activity.

These results clearly indicate that NF-κB activity regulates the binding activity of PPARβ/δ to its *cis*-regulatory element.

NF-κB activation enhances the Interaction of p65 with PPARβ/δ-The reduction in the DNA binding activity of PPARβ/δ after LPS treatment may result from different molecular mechanisms. First, these changes may be caused by a reduction in the expression of PPARβ/δ. However, this possibility is unlikely since no significant changes were observed in the protein expression of either PPARβ/δ or the p65 subunit of NF-κB after LPS stimulation (Figure 4A). In addition, the p65 subunit of NF-κB may interact physically with PPARs. This association has been described for PPARα and prevents this nuclear receptor from binding to its response element and thereby inhibits its ability to induce gene transcription(12). It is not known yet if a similar mechanism may affect PPARβ/δ in cardiac cells. In order to evaluate this possibility we performed coimmunoprecipitation studies with isolated nuclear extracts using antibodies against the p65 subunit of NF-κB and PPARβ/δ. Data shown in Figure 4B demonstrate that LPS stimulation strongly enhanced the physical interaction between p65 and PPARβ/δ, suggesting that increased association between these two proteins is the mechanism through which PPARβ/δ activity is reduced after LPS stimulation.

Enhanced p65-PPARβ/δ interaction in pressure overload-induced cardiac hypertrophy-Finally, we wanted to evaluate whether the mechanism proposed was also observed *in vivo* in pressure overload-induced cardiac hypertrophy. Cardiac hypertrophy was assessed by measuring the HW/BW ratio. This parameter significantly increased (1.35-fold, $P<0.001$) after aortic constriction compared with sham-operated rats (Figure 5A). Further, pressure overload also led to approximately 2-fold induction in the mRNA levels of ANF compared with sham-operated rats

(data not shown). In the heart of banded rats PDK4 mRNA levels were downregulated by 35% ($P<0.01$) compared with sham-operated rats (Figure 5B), which is in agreement with the reduction in fatty acid oxidation during the development of cardiac hypertrophy(3;4). We also performed EMSA to demonstrate NF-κB activation in pressure overload-induced cardiac hypertrophy. These studies showed that the NF-κB probe formed four main specific complexes with cardiac nuclear proteins (complexes I to IV, Figure 5C), based on competition experiments performed by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures. NF-κB binding activity increased in banded rats, especially complex III, compared with sham-operated rats. Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of this transcription factor (Figure 5D). No changes were observed in the DNA binding of cardiac nuclear proteins to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (Figure 5E). Finally, we evaluated whether the impairment in the expression of PPARβ/δ target gene PDK4 observed during cardiac hypertrophy may result from enhanced p65-PPARβ/δ interaction. Nuclear extracts isolated from hearts were immunoprecipitated using anti-p65 antibody coupled to protein A-agarose beads. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with anti-PPARβ/δ antibody. Data shown in Figure 5F demonstrate that pressure overload-induced cardiac hypertrophy enhanced the physical interaction of p65 with PPARβ/δ, suggesting that increased association between these proteins is a mechanism contributing to the reported reduction in the expression of PPARβ/δ-target genes involved in fatty acid metabolism.

DISCUSSION

Fuel generation in the adult myocardium relies on the oxidation of long chain fatty acids by the mitochondria for production of energy. Development of cardiac hypertrophy is associated with a suppression of fatty acid oxidation and metabolic reversion of the heart toward increased glucose utilization, which is characteristic of fetal heart(3;4). It is unclear at present, however, which consequences might result from impaired oxidation of fatty acids in the heart, but several studies have demonstrated that substrate utilization is important in the pathogenesis of cardiac hypertrophy(5-7). In the present study we demonstrate that the NF-κB signaling pathway, which is one of the most important signal transduction pathways involved in the hypertrophic growth of the myocardium, may also be involved in the downregulation of fatty acid oxidation. Therefore, inhibition of NF-κB activation during cardiac hypertrophy may also ameliorate cardiac fatty acid oxidation, achieving a better improvement in the prevention or inhibition of this pathological process. Further, these data may also contribute to explain how, given the complexity of the hypertrophic response, inhibition of NF-κB may be sufficient to prevent cardiac hypertrophy(29).

Barger et al.(30) demonstrated that during hypertrophic growth cardiac PPARα gene expression fell and its activity was altered at the post-transcriptional level via the extracellular signal-regulated kinase mitogen-activated protein kinase pathway. However, nothing was known until now about the effects of cardiac hypertrophy on PPARβ/δ activity. Gilde et al.(17) recently suggested that PPARβ/δ plays an important function in the heart. In fact, these authors shown that both PPARα and PPARβ/δ were expressed in comparable levels in heart, whereas PPARγ was barely detectable. Further, PPARβ/δ was fatty acid inducible and activated the expression of PPARα target genes involved in fatty acid utilization in cardiac myocytes. The authors of this study suggested that PPARα and PPARβ/δ shared similar functions in cardiac cells regarding

cardiac fatty acid metabolism. In agreement with this idea, Muoio *et al.*(31) shown that fatty acid oxidation in skeletal muscle of PPARα$^{-/-}$ mice was not impaired, probably because of PPARβ/δ compensated for the lack of PPARα in these mice.

In the present study stimulation of rat neonatal cardiomyocytes with PE, which leads to NF-κB activation(14), caused cardiac hypertrophy that was accompanied by a fall in the expression of PDK4, a PPARβ/δ target gene involved in fatty acid metabolism(22). Further, the fall in fatty acid oxidation observed in PE-stimulated rat neonatal cardiomyocytes was restored by NF-κB inhibitors. These data pointed to the involvement of NF-κB in the downregulation of fatty acid oxidation during the development of cardiac hypertrophy. However, given that both PPARα and PPARβ/δ are expressed in rat neonatal cardiomyocytes(17), this could lead to a potential interference caused by PPARα in the changes observed. This possibility was avoided by using H9c2 myotubes, which only express the PPARβ/δ subtype(17). Stimulation of H9c2 myotubes with LPS, which has been reported to activate NF-κB in cardiomyocytes(28), also lead to the reduction in the expression of PDK4, supporting a link between NF-κB activation and the reduced expression of PPARβ/δ target genes involved in fatty acid oxidation. All these findings suggest that the impairment of PPARβ/δ activity plays an important role in the fall of myocardial fatty acid oxidation during cardiac hypertrophy. In agreement with this idea, a recent study demonstrated that cardiomyocyte-restricted PPARβ/δ deletion reduced myocardial fatty acid oxidation and the mRNA expression of genes involved in this process, such as PDK4, and led to cardiomyopathy(32).

The mechanism by which activation of NF-κB results in reduced expression of PPARβ/δ target genes seems to involve reduced interaction of this PPAR subtype with its *cis*-regulatory element, since exposure of cardiac cells to LPS caused a dramatic reduction in the binding of PPARβ/δ

protein to the PPRE probe. This reduction was partially reversed by coincubation of the cells with NF-κB inhibitors, confirming the involvement of this transcription factor in the changes observed. Therefore, the reduced binding activity of PPARβ/δ seemed to be related to the activation of NF-κB in cardiac cells. However, it remained to establish through which mechanism NF-κB activation avoided the interaction of PPARβ/δ with its response element. NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits. However, after activation this heterodimer translocates to the nucleus and regulates the expression of genes involved in inflammatory and immune processes. Our results indicate that once the p65 subunit of NF-κB reaches the nucleus interacts with PPARβ/δ. This association prevents PPARβ/δ from binding to its response element and thereby inhibits its ability to induce gene transcription, leading to a reduction in the expression of PDK4. These findings are in concordance with the results reported by Westergaard et al.(33), who showed that PPARβ/δ physically interacts with p65 in psoriatic lesions. Further, they showed a p65-dependent repression of PPARβ/δ, but not of PPARα or PPARγ.

In summary, in the present study we show that the NF-κB signaling pathway, which plays a pivotal role in the hypertrophic growth of the myocardium, downregulates PPARβ/δ activity *in vitro* and *in vivo* through a mechanism that involves enhanced protein-protein interaction of the p65 subunit of NF-κB with this PPAR subtype. These data link NF-κB activation during the development of cardiac hypertrophy with the fall in fatty acid oxidation.

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Figure 1. Downregulation of fatty acid oxidation in PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μmol/L PE for 24 hours. A, [^{3}H]leucine incorporation was determined by coincubating cardiac myocytes with 1.0 μCi/mL [^{3}H]leucine for 24 hours. Data are expressed as mean ± SD (n=6) of the treated-to-control ratio. Analysis of the mRNA levels of ANF (B) and PDK4 (C) and MCP-1 (D) in PE-stimulated cardiomyocytes. A representative autoradiogram normalized to the APRT mRNA levels is shown. Data are expressed as mean ± S.D. of 4-5 different experiments. Data are expressed as mean ± S.D. of 6 different experiments. ***P<0.001, **P<0.01, * P<0.05 vs control cells.

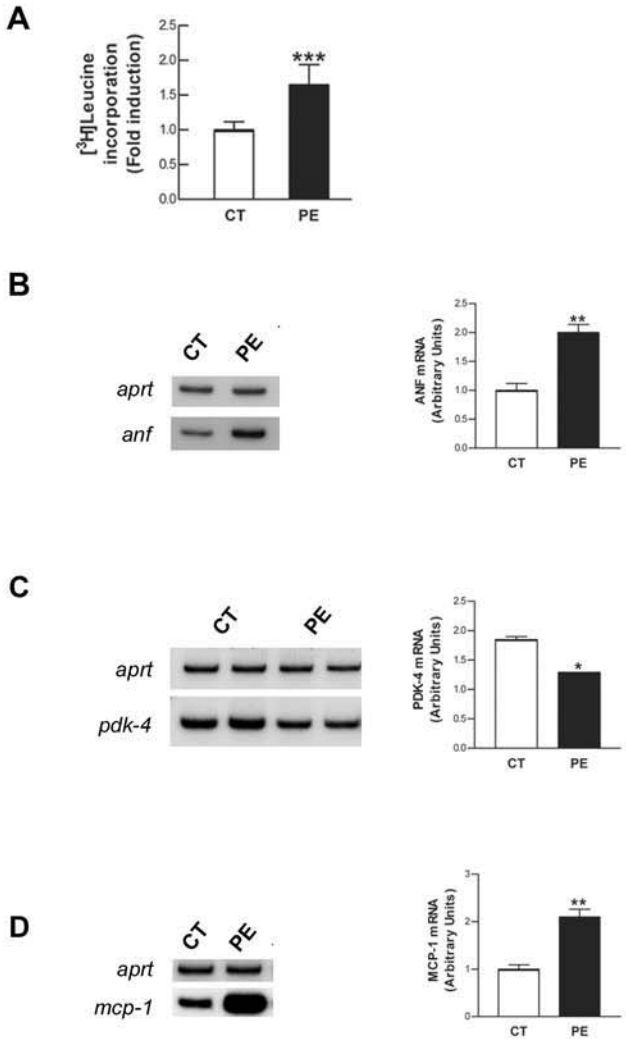
Figure 2. Downregulation of fatty acid oxidation in PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes is reversed by NF-κB inhibitors. Cardiac myocytes were stimulated with 100 μmol/L PE for 24 hours. A, Analysis of the mRNA levels of PDK4 (A) and MCP-1 (B) in PE-stimulated cardiomyocytes in the presence or in the absence of 10μM parthenolide. A representative autoradiogram normalized to the APRT mRNA levels is shown. Data are expressed as mean ± S.D. of 4-5 different experiments. C, Effect of PE-stimulated cardiac hypertrophy on fatty acid oxidation. The oxidation of radiolabeled palmitate was measured as the production of ^3H_2O in the incubation medium for 60 minutes in neonatal rat cardiomyocytes in the absence or in the presence of 10 μM parthenolide or 10 μM SN-50. Results are expressed as percentage compared with the control, which was arbitrarily set at 100% and corresponds with 0.14±0.008 nmol palmitate/min per mg protein. Data are expressed as mean ± S.D. of 6 different experiments. ***P<0.001, **P<0.01, * P<0.05 vs control cells. $^{\#\#}$P<0.01, $^{\#}$P<0.05 vs PE-stimulated cells.

Figure 3. Downregulation of PDK4 in LPS-stimulated H9c2 myotubes. A, Nuclear extracts (NE) from rat heart and H9c2 myotubes were assayed for western-blot analysis with PPARβ/δ and PPARα antibodies. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and NE from H9c2 myotubes stimulated with LPS for 1 hour. Supershift analysis was performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. The supershifted immune complex (IC) is denoted by the arrow. Autoradiograph data are representative of three separate experiments. Analysis of the mRNA levels of MCP-1 (C) and PDK4 (D) in LPS-stimulated H9c2 myotubes. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 5 different experiments. * $P<0.05$ and *** $P<0.001$ vs control. $^{\#}P<0.05$ vs LPS-stimulated myotubes. E, Autoradiograph of EMSA performed with a ^{32}P-labeled PPRE nucleotide and NE. Two specific complexes (I to II), based on competition with a molar excess of unlabeled probe, are shown. The supershift immune complex (IC) obtained by incubating NE with an antibody directed against PPARβ/δ is also shown. F, Autoradiograph of EMSA performed with a ^{32}P-labeled PPRE nucleotide and nuclear extracts from cells stimulated with LPS for 1 hour either in the presence or in the absence of PDTC or parthenolide. All the autoradiograph data are representative of three separate experiments.

Figure 4. Enhanced protein-protein interaction between the p65 subunit of NF-κB and PPARβ/δ in LPS-stimulated H9c2 myotubes. A, LPS stimulation affects neither the protein levels of p65 nor PPARβ/δ. Nuclear protein extracts from H9c2 myotubes stimulated with LPS for 1 hour were assayed for western-blot analysis with PPARβ/δ or p65 antibodies. B, Nuclear extracts from H9c2 myotubes stimulated with LPS for 1 hour were subjected to immunoprecipitation using anti-p65 antibody coupled to protein-A agarose beads. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with an anti-PPARβ/δ antibody. The blot data are representative of three separate experiments.

Figure 5. Enhanced protein-protein interaction between the p65 subunit of NF-κB and PPARβ/δ in pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. At 15 days after operation, hearts were excised and weighed. A, Analysis of the HW/BW ratio in sham-operated and in banded rats (aortic banding, AB). Data are expressed as mean ± S.D. of 4 different experiments. *** $P<0.001$ vs sham-operated rats. B, Analysis of the PDK4 cardiac mRNA levels in sham-operated and in banded rats (aortic banding, AB). A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 4 different experiments. ** $P<0.01$ vs sham-operated rats. C, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and cardiac nuclear extracts (NE) from sham-operated and banded rats (aortic banding, AB). Four specific complexes (I to IV), based on competition with a molar excess of unlabeled probe, are observed. D, Supershift analysis performed by incubating cardiac NE with an antibody directed against the p65 subunit of NF-κB. The supershifted immune complex (IC) is denoted. E, Autoradiograph of EMSA performed with a ^{32}P-labeled Oct-1 nucleotide. F, enhanced association

of the p65 subunit of NF-κB with PPARβ/δ in pressure overload-induced cardiac hypertrophy. NE (equalized by protein concentrations) from sham-operated and banded rats (aortic banding, AB) were subjected to immunoprecipitation using anti-p65 antibody coupled to protein A-agarose beads. Immunoprecipitates were subjected to SDS-PAGE, and immunoblotted with anti-PPARβ/δ antibody. Arrowheads represent the PPAR subtypes or IgG signal. All autoradiograph and blot data are representative of three separate experiments.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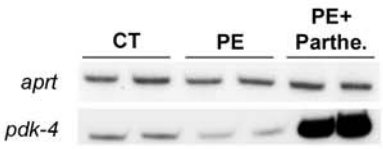
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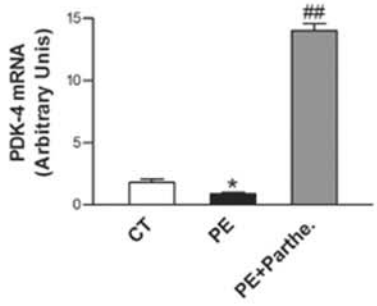
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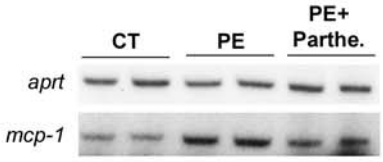
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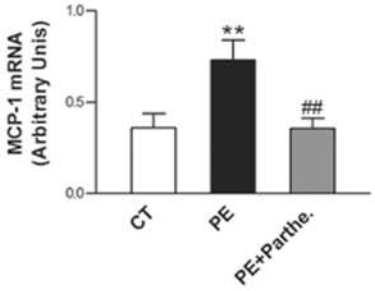
aprt
mcp-1


MCP-1 mRNA
(Arbitrary Unis)
1.0
0.5
0.0
**
##
CT
PE
PE+Parthe.

C

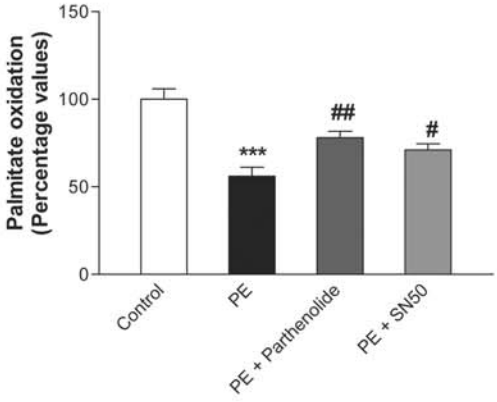

Palmitate oxidation
(Percentage values)
150
100
50
0
***
##
#
Control
PE
PE + Parthenolide
PE + SN50

Figure 2

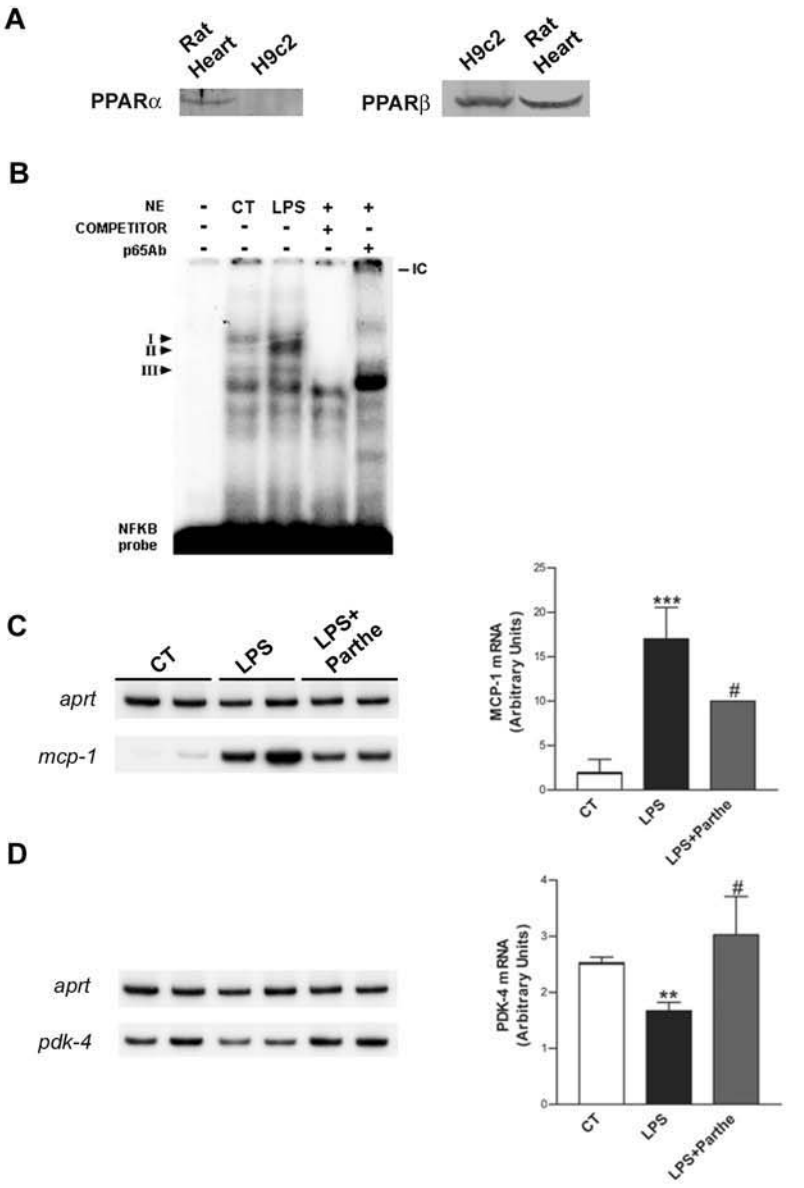

A
Rat Heart
H9c2
PPARα
H9c2
Rat Heart
PPARβ
B
NE - CT LPS + +
COMPETITOR - - - + -
p65Ab - - - - +
IC
I
II
III
NFKB probe
C
CT
LPS
LPS+ Parthe
aprt
mcp-1
MCP-1 mRNA (Arbitrary Units)
***
#
CT
LPS
LPS+Parthe
D
aprt
pdk-4
PDK-4 mRNA (Arbitrary Units)
**
#
CT
LPS
LPS+Parthe
E
NE - + + +
COMPETITOR - - + -
PPARβAb - - - +
IC
I
II
PPRE probe
F
NE -
CT
LPS
LPS+PDTC
LPS+Parth.
LPS+L165041
I
II
PPRE probe

Figure 3

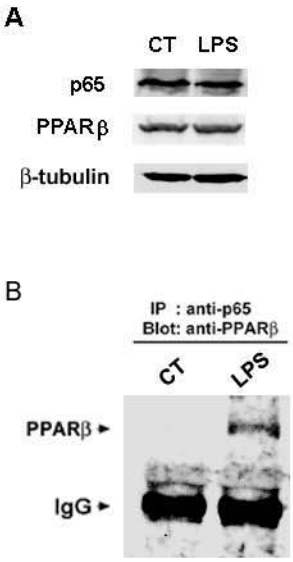

A
CT
LPS
p65
PPARβ
β-tubulin
B
IP : anti-p65
Blot: anti-PPARβ
CT
LPS
PPARβ ►
IgG ►

Figure 4

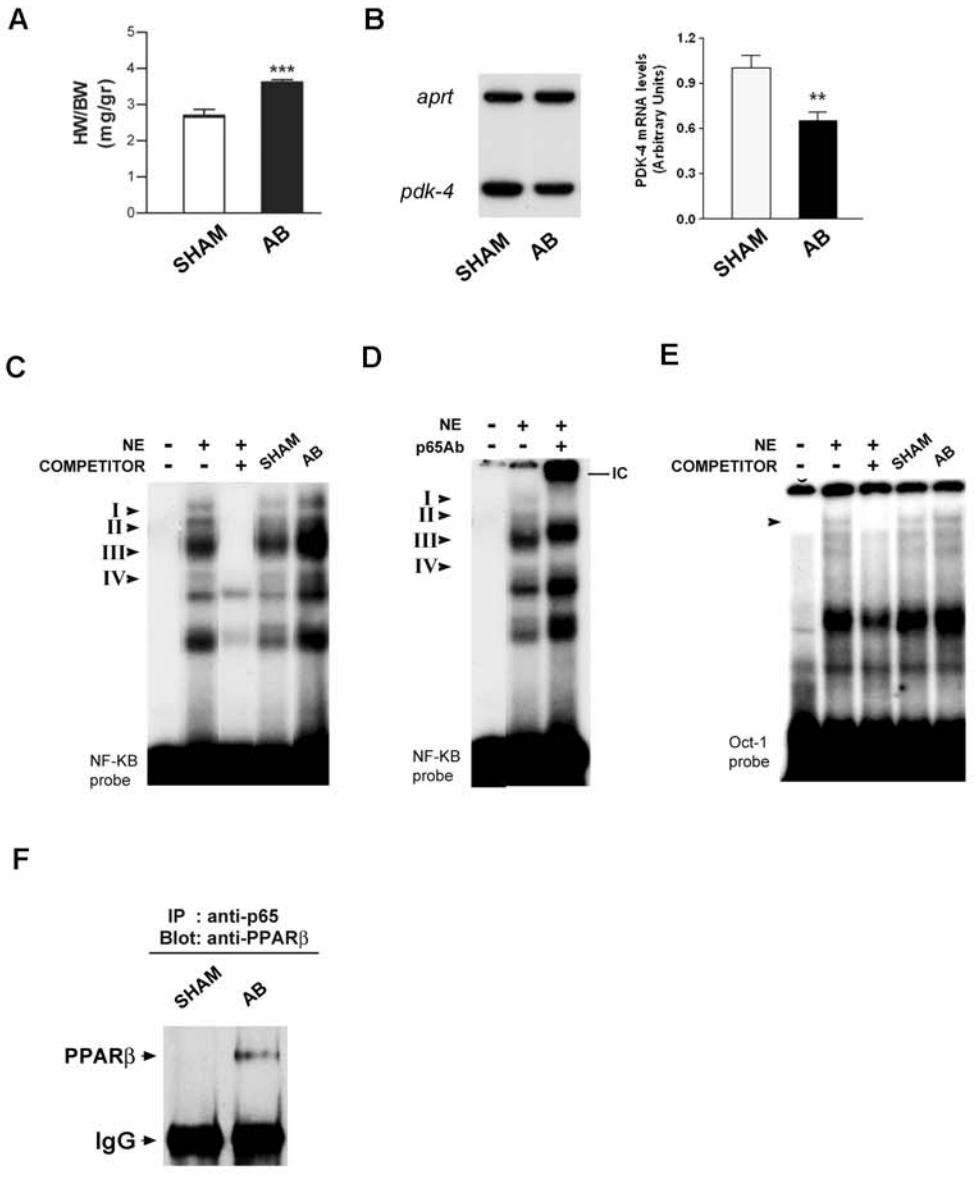

A
HW/BW (mg/gr)
5
4
3
2
1
0
***
SHAM
AB
B
aprt
pdk-4
SHAM
AB
PDK-4 mRNA levels (Arbitrary Units)
1.2
0.9
0.6
0.3
0.0
**
SHAM
AB
C
NE - + +
COMPETITOR - - +
SHAM
AB
I
II
III
IV
NF-KB probe
D
NE - + +
p65Ab - - +
IC
I
II
III
IV
NF-KB probe
E
NE - + +
COMPETITOR - - +
SHAM
AB
Oct-1 probe
F
IP : anti-p65
Blot: anti-PPARβ
SHAM
AB
PPARβ
IgG

Figure 5

II

Peroxisome proliferator-activated receptor β/δ activation inhibits hypertrophy in neonatal rat cardiomyocytes.

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Peroxisome proliferator-activated receptor β/δ activation inhibits hypertrophy in neonatal rat cardiomyocytes

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Abstract

Objective: Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism. However, the role of PPARβ/δ activators in cardiac hypertrophy is not yet known.

Methods and Results: In cultured neonatal rat cardiomyocytes, the selective PPARβ/δ activator L-165041 (10 μmol/L) inhibited phenylephrine (PE)-induced protein synthesis ([^{3}H]leucine uptake), induction of the fetal-type gene atrial natriuretic factor (ANF) and cardiac myocyte size. Induction of cardiac hypertrophy by PE stimulation also led to a reduction in the transcript levels of both muscle-type carnitine palmitoyltransferase (50%, $P<0.05$) and pyruvatedehydrogenase kinase 4 (30%, $P<0.05$), and these changes were reversed in the presence of the PPARβ/δ agonist L-165041. Stimulation of neonatal rat cardiomyocytes with PE and embryonic rat heart-derived H9c2 cells with lipopolysaccharide (LPS) enhanced the expression of the nuclear factor (NF)-κB-target gene monocyte chemoattractant protein 1 (MCP-1). The induction of MCP-1 was reduced in the presence of L-165041, suggesting that this compound prevented NF-κB activation. Electrophoretic mobility shift assay (EMSA) revealed that L-165041 significantly decreased LPS-stimulated NF-κB binding activity in H9c2 myotubes. Finally, coimmunoprecipitation studies showed that L-165041 strongly enhanced the physical interaction between PPARβ/δ and the p65 subunit of NF-κB, suggesting that increased association between these two proteins is the mechanism responsible for antagonizing NF-κB activation by PPARβ/δ activators.

Conclusion: These results suggest that PPARβ/δ activation inhibits PE-induced cardiac hypertrophy and LPS-induced NF-κB activation.

Keywords: Cardiac hypertrophy; NF-κB; L-165041; p65

***This article is referred to in the Editorial by N.N. Petrashevskaya and A. Schwarz (pages 770–771) in this issue*.**

Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction and hypertrophic cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia and sudden death [1,2]. Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role, since it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes [3–6]. In addition, cardiac hypertrophy is associated with an increase in glucose utilization and a decrease in fatty acid oxidation, which is

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characteristic of fetal heart [7,8]. Defects in mitochondrial fatty acid oxidation enzymes cause childhood hypertrophic cardiomyopathy [9], and perturbation of fatty acid oxidation in animal models causes cardiac hypertrophy [10,11], demonstrating that substrate utilization is important in the pathogenesis of hypertrophy. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation [12]. The PPAR subfamily consists of three subtypes, PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ (also known as PPARδ) (NR1C2) and PPARγ (NR1C3) [13]. PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle [14]. PPARβ/δ is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts. In order to be transcriptionally active, PPARs need to heterodimerize with the 9-*cis* retinoic acid receptor (RXR) (NR2B). PPAR-RXR heterodimers binds to DNA specific sequences called peroxisome proliferator-response elements (PPREs), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). These sequences have been characterized within the promoter regions of PPAR target genes. However, the regulation of gene transcription by PPARs extends beyond their ability to transactivate specific target genes. PPARs are also able of regulating gene expression independently of binding to DNA through a mechanism termed receptor-dependent *trans*repression [15]. One of these mechanisms involves a physical interaction of PPARα with NF-κB, leading to suppression activity of the latter [16].

It has been demonstrated that, of the three PPAR subtypes, activation of both PPARα [17,18] and PPARγ [19,20] results in inhibition of cardiac hypertrophy. However, the role of PPARβ/δ in the development of this process is unknown. The recent availability of specific synthetic ligands for PPARβ/δ, such as L-165041, now makes possible to study the role of this nuclear receptor in cardiac cells. Thus, recently, Gilde et al. [21], using neonatal rat cardiomyocytes as well as the embryonic rat heart-derived H9c2 cells, clearly demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism, suggesting that PPARβ/δ, similarly to PPARα and γ, may play an important role in cardiac disease.

In this study, we examined the role of PPARβ/δ activation in phenylephrine (PE)-induced hypertrophy in neonatal rat cardiac myocytes and in lipopolysaccharide (LPS)-estimulated H9c2 myotubes. We found that activation of PPARβ/δ inhibits PE-induced hypertrophy and LPS-induced NF-κB activation.

1. Methods

1.1. Materials

L-165041 was synthesized according to Berger et al. [22]. [γ-^{32}P]dATP (3000 Ci/mmol) and [^{3}H]leucine (50 Ci/mmol) were purchased from Amersham Pharmacia Biotech KK. Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories and Alexa flouro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were from Molecular Probes. All other chemicals were purchased from Sigma.

1.2. Cell culture

Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague–Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously [23]. The media was changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 h before treatments. In this study, PE was used to stimulate neonatal rat cardiomyocytes. Animal handling and disposal were performed in accordance with NIH guidelines.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA analysis H9c2 cells were treated with 10 μmol/L L-165041 and LPS (10 ng/mL) for 24 h.

1.3. Incorporation of [^{3}H]leucine

To examine the effect of PE on protein synthesis, the incorporation of [^{3}H]leucine was measured essentially by the method of Thaik et al. [24]. Cultured neonatal rat ventricular myocytes were treated with PE in the presence or in the absence of L-165041 and coincubated with [^{3}H]leucine (1 μCi/mL) for 24 h. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4 °C for 30 min to precipitate the proteins. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted with scintillation counter.

1.4. Immunocytochemistry

Neonatal rat ventricular myocytes were fixed in ice-cold 100% methanol for 10 min. Anti-α-actinin antibody and anti-ANF polyclonal antiserum were added at dilutions 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 h at room temperature. Secondary antibodies,

Alexa flouro 488 goat anti-rabbit and Alexa flouro 568 goat anti-mouse, were used at a dilution of 1:300 in PBS containing 5% rat serum and incubated for 30 min at room temperature. Immunofluorescence was visualized using a confocal laser fluorescence microscope Olympus Fluoview FV500. Photographic images were taken from five random fields.

1.5. RNA preparation and analysis

Relative levels of specific mRNAs were assessed by the reverse transcription–polymerase chain reaction (RT–PCR) as previously described [25]. The sequences of the sense and antisense primers used for amplification were: ANF, 5′-TCCTCTTCCTGGCCTTTTGGC-3′ and 5′-

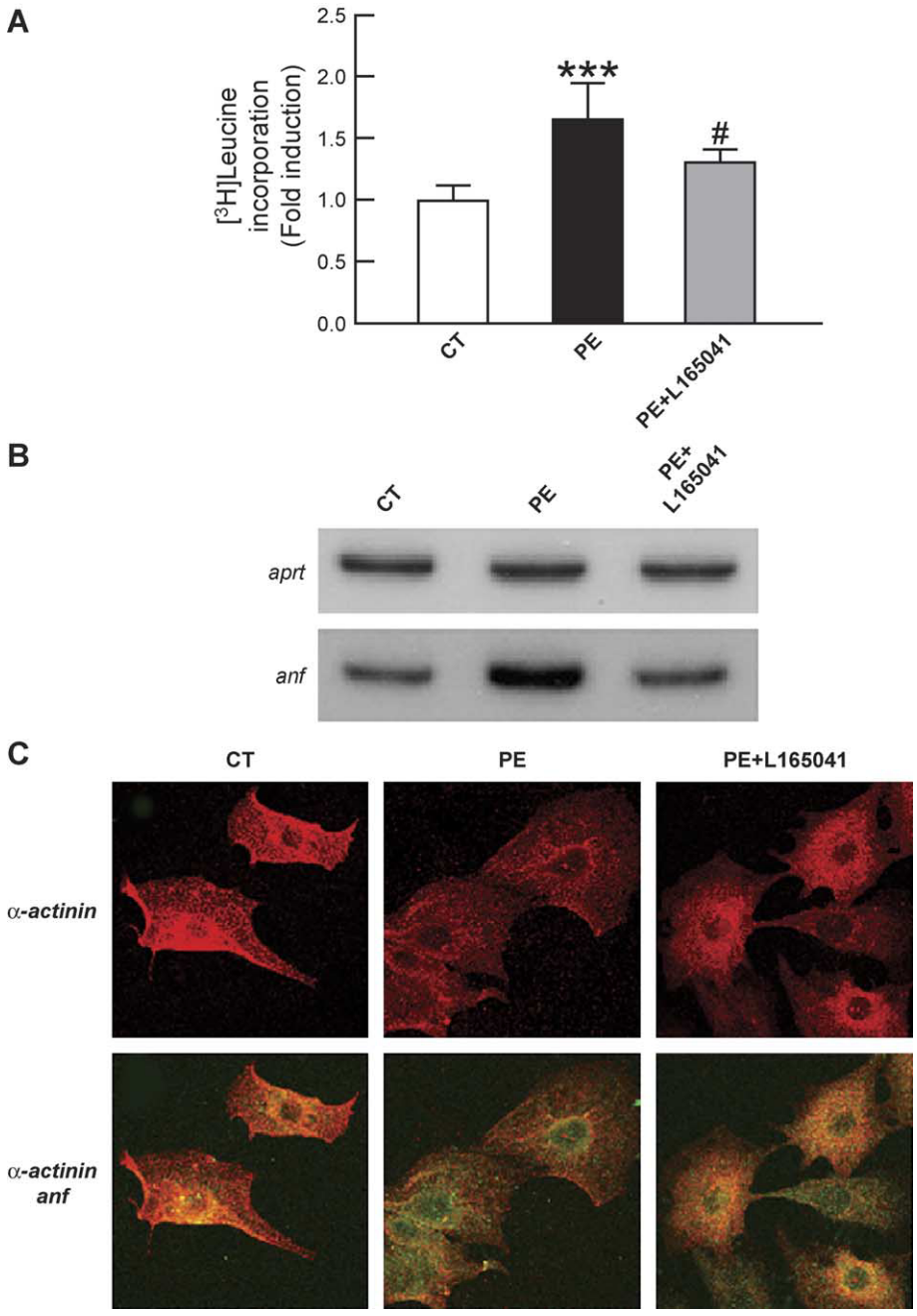

Fig. 1. The PPARβ/δ activator L-165041 inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μmol/L PE in the presence or absence of 10 μmol/L L-165041 that was added 30 min before experiments. (A) [^{3}H]leucine incorporation was determined by coincubating cardiac myocytes with 1.0 μCi/mL [^{3}H]leucine for 24 h. Data are expressed as mean±S.D. (n=6) of the treated-to-control ratio. (B) Analysis of the mRNA levels of ANF in PE-stimulated cardiomyocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram is shown. (C) Effects of PE with and without L-165041 on cardiac myocyte ANF protein expression and cardiac myocyte size. Double immunofluorescent microscopy was performed using specific antibodies to α-actinin (upper panel, red color) and ANF (lower panel, green color). Experiments were performed three times with similar results. ***P<0.001 vs. control. $^{\#}P$<0.05 vs. PE-stimulated cardiac myocytes.

AGACGGGTTGCTTCCCCAGTC-3′; gp91, 5′-CACCTGCAGCCTGCCTGAATT-3′ and 5′-ATGGTGTGAATGGCGGTGTGA-3′; inducible nitric oxide synthase (iNOS), 5′-GCATGGACCAGTATAAGGCAAGCA-3′ and 5′-GCTTCTGGTCGATGTCATGAGCAA-3′; malonyl-CoA decarboxylase (MCD), 5′-TACGGTGAGAAGCACCGAGGC-3′ and 5′-GGGGCCTGTCTCCTCCAGGTA-3′; monocyte chemoattractant protein 1 (MCP-1), 5′-GGGCCTGTTGTTCACAGTTGC-3′ and 5′-GGGACACCTGCTGCTGGTGAT-3′; muscle-type carnitine palmitoyltransferase (M-CPT-I), 5′-TTCACTGTGACCCCAGACGGG-3′ and5′-AATGGACCAGCCCCATGGAGA;pyruvatedehydrogenase kinase 4 (PDK-4), 5′-GAACACCCCTTCCGTCCAGCT-3′ and 5′-TGTGCCATCGTAGGGACCACA-3′; PPARγ coactivator-1 (PGC-1), 5′-AGAAAGGGCCCGAGCAATCTG-3′ and 5′-AGATGTGCCCCTGCCAGTCAC-3′; p22, 5′-CCCCGGGGAAAGAGGAAAAAG-3′ and 5′-GGATGGCTGCCAGCAGGTAGA-3′; and APRT (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-3′. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, gp91: 200 bp, iNOS: 198 bp, MCD: 231 bp, MCP-1: 157 bp, M-CPT-I: 222 bp, PDK-4: 168 bp, PGC-1: 234 bp, p22: 215 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

1.6. Immunoblotting

Cell lysates and nuclear extracts from H9c2 cells were obtained as previously described [25]. Proteins (50 μg) were separated by SDS–PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride mem-

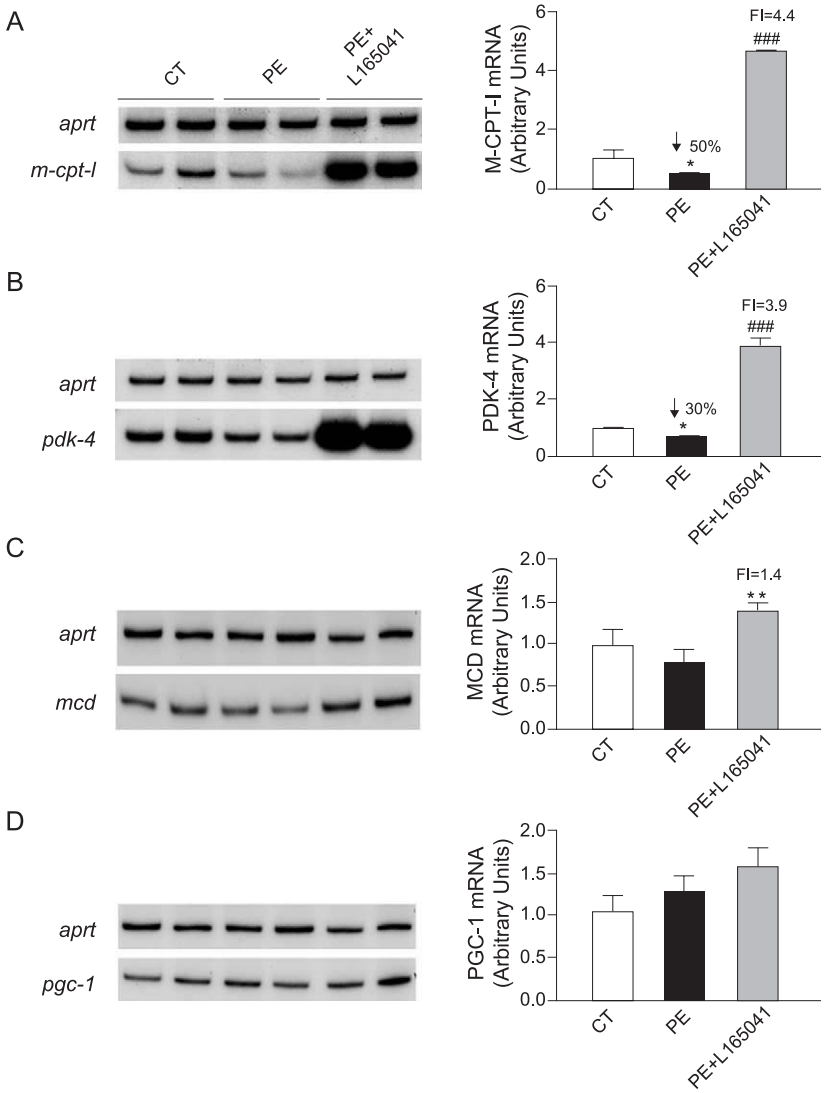

Fig. 2. L-165041 prevents downregulation of the expression of several genes involved in fatty acid lipid metabolism in PE-stimulated neonatal rat cardiomyocytes. Analysis of the mRNA levels of M-CPT-I (A), PDK-4 (B), MCD (C) and PGC-1 (C) in PE-stimulated cardiac myocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean±S.D. of five different experiments. *$P<0.05$ and **$P<0.01$ vs. control. $^{###}P<0.001$ vs. PE-stimulated cardiac myocytes.

branes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against IκBα, IκBβ, p65 and PPARβ/δ (Santa Cruz Biotechnology) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

1.7. Electrophoretic mobility shift assay (EMSA)

H9c2 cells were pretreated with 10 μmol/L L-165041 for 24 h before stimulation with LPS (10 ng/ml) for 1 h. Isolation of nuclear extracts and EMSA were performed as previously described [25].

1.8. Coimmunoprecipitation

Cell nuclear extracts were brought to a final volume of 0.5 mL with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, BSA 2% and 1 mg/ml nonfat milk for 6 h at 4 °C and incubated with 4 μg of anti-p65. Immunocomplex were captured by incubating the samples with protein A-agarose suspension overnight at 4 °C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of SDS–PAGE sample buffer and boiled for 5 min at 100 °C. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS–PAGE and immunoblotted with an antibody against PPARβ/δ.

1.9. Statistical analyses

Results were obtained from at least four independent experiments and presented as mean±S.D. Comparisons between groups were performed with one-way ANOVA using the computer program GraphPad Instat (GraphPad Softwware, San Diego, CA). When significant variations were found, the Tukey–Kramer multiple comparisons test was performed. Differences were considered significant at $P<0.05$.

2. Results

2.1. PPARβ/δ activation by L-165041 inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes

Cardiac hypertrophy leads to significant increases in protein content (e.g., [^{3}H]leucine uptake), induction of fetal-type genes (e.g., ANF) and cardiac myocyte size [26]. Therefore, we first examined the effects on these parameters of the specific PPARβ/δ agonist L-165041, which was previously shown to be selective for this PPAR-subtype at 10 μmol/L [27]. The cells were pretreated with either vehicle or L-165041 for 30 min and subsequently stimulated with 100 μmol/L PE for 24 h. As shown in Fig. 1A, [^{3}H]leucine incorporation was significantly increased by PE (1.6-fold, $P<0.001$) and this was inhibited by L-165041 (−20%, $P<0.05$). PE-induced cardiomyocyte hypertrophy also led to approximately twofold induction in the mRNA levels of the fetal cardiac gene ANF (Fig. 1B). In contrast, in the presence of L-165041 PE-induced ANF expression was abolished. Immunostaining of cardiac myocytes for the sarcomere-associated protein α-actinin and ANF clearly shown an increase in cardiac myocyte size and ANF protein expression following PE stimulation (Fig. 1C). These changes were blocked by the presence of the PPARβ/δ agonist L-165041.

2.2. Treatment with the PPARβ/δ agonist L-165041 prevents the reduction in the expression of genes involved in lipid metabolism caused by PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes

An important molecular adaptation in cardiac hypertrophy is the increase in glucose utilization and decrease in fatty acid oxidation associated to a downregulation of the expression of the mRNA levels of genes involved in fatty acid metabolism [8]. Interestingly, PPARβ/δ activates the expression of several PPAR target genes involved in fatty acid utilization in cardiac myocytes [21], including M-CPT-I, which determines the flux of mitochondrial β-oxidation [28], and PDK-4, which suppresses glucose

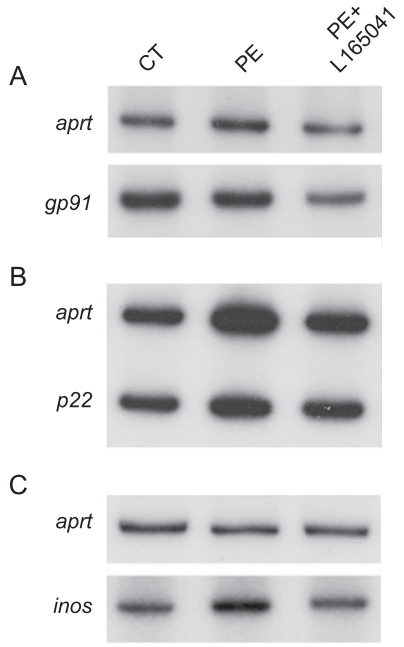

Fig. 3. Effects of L-165041 of the expression of NADPH oxidase subunits and iNOS. Analysis of the mRNA levels of gp91 (A), p22 (B) and iNOS (C) in PE-stimulated cardiac myocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram is shown. Experiments were performed three times with similar results.

oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization [29]. Taking into account these data, we next assessed the effects of PE-induced cardiomyocyte hypertrophy on the mRNA levels of these genes in the presence or in the absence of L-165041. Induction of cardiomyocyte hypertrophy by PE led to a reduction in the transcript levels of both M-CPT-I (50%, $P<0.05$) and PDK-4 (30%, $P<0.05$). In contrast, in the presence of the PPARβ/δ agonist, PE did not reduce the levels of these genes and even a robust induction (about fourfold, $P<0.001$) was observed compared to control values (Fig. 2A and B). PE treatment did not affect the mRNA levels of MCD and PGC-1, two genes involved in lipid metabolism, whereas coincubation with L-165041 caused a significant increase in MCD expression (Fig. 2C and D).

2.3. Treatment with L-165041 inhibits the upregulation of MCP-1 caused by PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes

PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes is mediated through NF-κB activation via the generation of reactive oxygen species (ROS) [4]. NADPH oxidase is one of the systems generating ROS whose expression is increased in cardiac hypertrophy [30]. We therefore evaluated whether PPARβ/δ activation by L-165041 affected the expression of the NADPH oxidase subunits gp91 and p22 (Fig. 3A and B). No changes were observed in the mRNA levels of these genes in neonatal rat cardiomyocytes, making unlikely that reduced NADPH oxidase expression may account for the antihypertrophic effect of L-165041. Similarly, L-165041 treatment did not

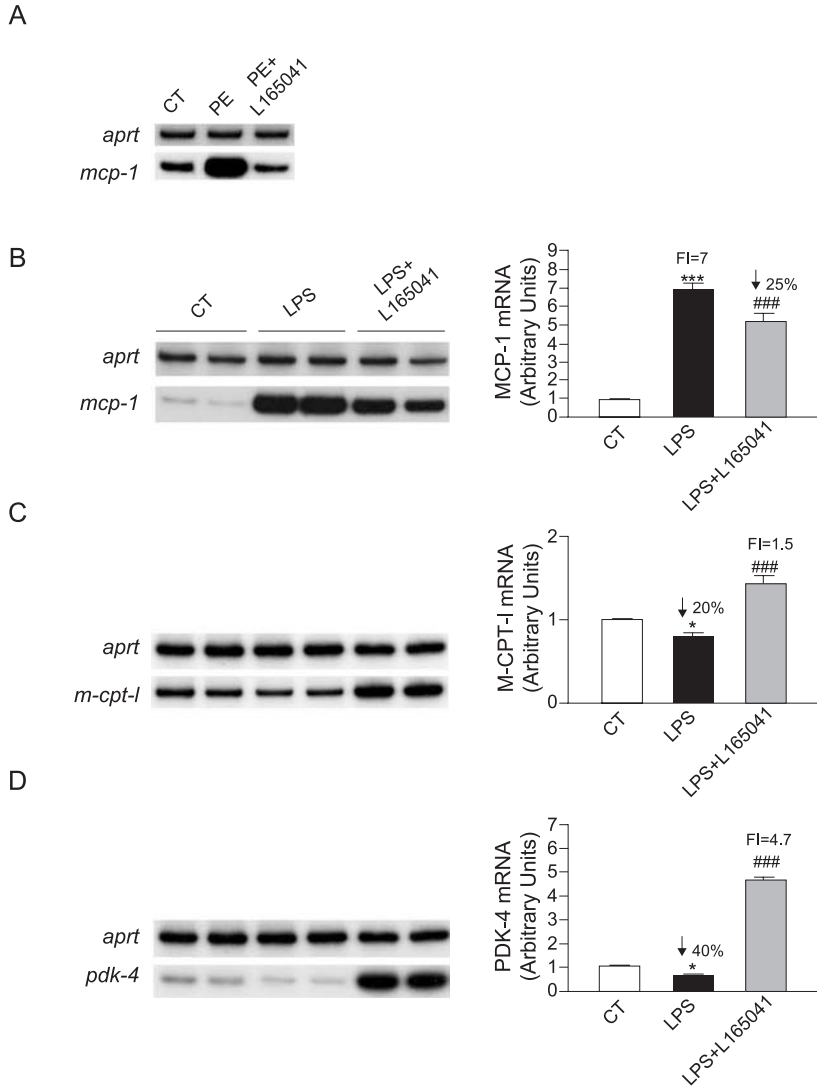

Fig. 4. L-165041 inhibits the upregulation of MCP-1 caused by PE and LPS stimulation in myocytes. Analysis of the mRNA levels of MCP-1 in PE-stimulated in neonatal rat cardiomyocytes (A) in the presence or absence of 10 μmol/L L-165041. Analysis of the mRNA levels of MCP-1 (B), M-CPT-I (C) and PDK-4 (D) in LPS-stimulated H9c2 cells in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean±S.D. of six different experiments. *$P<0.05$ and ***$P<0.001$ vs. control. $^{###}P<0.001$ vs. either PE- or LPS-stimulated cells.

affect the expression of iNOS, which has been previously involved in maladaptive consequences of cardiac hypertrophy [31] (Fig. 3C). Next, we determined the effects of L-165041 on the expression of MCP-1, a gene under the transcriptional control of NF-κB [32], in neonatal rat cardiomyocytes. Stimulation of cardiac myocytes with PE enhanced twofold the expression of this gene and this was abolished by L-165041 (Fig. 4A). This data suggests that prevention of NF-κB activation may be involved in the antihypertrophic effect attained by PPARβ/δ activation. Because of limited amount of mRNA and proteins obtained from neonatal rat cardiomyocytes, we continue our studies in the embryonic rat-heart derived H9c2 cells to confirm the involvement of NF-κB in the changes observed after L-165041 treatment. Gilde et al. [21] recently reported that H9c2 cells abundantly express the PPARβ/δ subtype, whereas PPARα and γ were undetectable. This fact converts H9c2 cells in a proper tool to investigate the role of PPARβ/δ activation without the potential interference of the other PPAR subtypes. In order to activate NF-κB, H9c2 cells were stimulated for 24 h with LPS, which has been reported to activate NF-κB in cardiomyocytes [33]. As expected, a robust induction (sevenfold, $P<0.001$) was observed in the mRNA levels of the NF-κB target gene MCP-1 (Fig. 4B), that was significantly reduced (−25%, $P<0.001$) in the presence of L-165041. The stimulation of H9c2 cells with LPS also caused a similar pattern of changes in the expression of genes involved in fatty acid metabolism to those observed in PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes. Thus, a 20% and a 40% reduction were observed in the mRNA levels of M-CPT-I and PDK-4, respectively, and these changes were prevented in the presence of L-165041 (Fig. 4C and D).

2.4. Treatment with the PPARβ/δ Activator L-165041 reduces LPS-induced NF-κB activation

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes [3–6] and MCP-1 transcription

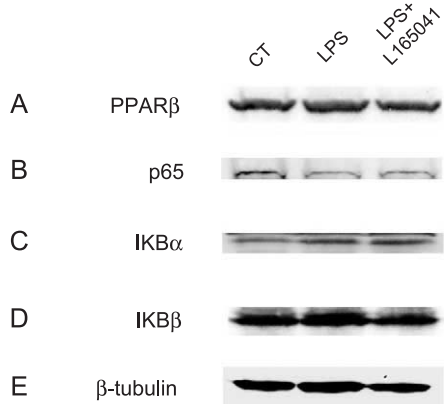

Fig. 6. Treatment with L-165041 does not affect the protein levels of IκBα. Protein extracts from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041 were assayed for Western blot analysis with PPARβ/δ (A), p65 (B), IκBα (C) IκBβ (D) and β-tubulin (E) antibodies.

is regulated by this transcription factor, we performed EMSAs to investigate whether the PPARβ/δ activator L-165041 inhibited LPS-induced NF-κB activation in H9c2 cells. EMSA studies shown that the NF-κB probe formed three complexes with cardiac nuclear proteins (complexes I to III, Fig. 5). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures (Fig. 5A). NF-κB binding activity, mainly of specific complex II, increased in cells stimulated with LPS for 1 h (Fig. 5B). In contrast, in the presence of L-165041 the LPS-induced increase in NF-κB binding activity was abolished. Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of NF-κB. Addition of this antibody to incubation mixtures resulted in complete supershift of complex II, thus showing that this complex contained p65. No changes were observed in the DNA binding of nuclear proteins to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (data not

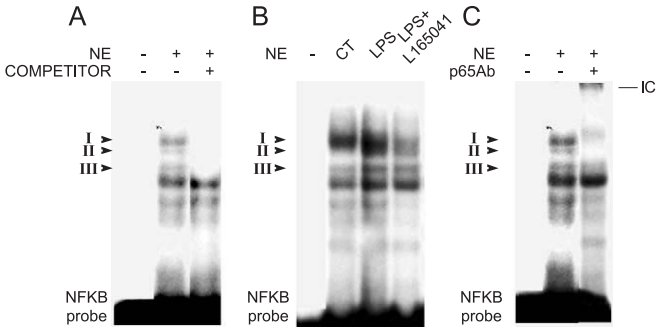

Fig. 5. Treatment with the PPARβ/δ activator L-165041 reduces LPS-induced NF-κB activation in H9c2 myotubes. (A) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and crude nuclear protein extract (NE) shows three specific complexes (I to III), based on competition with a molar excess of unlabeled probe. (B) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and NE from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041. (C) Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-kB. Supershifted immune complex (IC) is denoted.

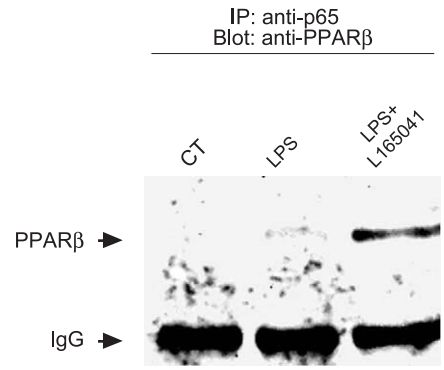

Fig. 7. L-165041 enhances PPARβ/δ association with the p65 subunit of NF-κB. Nuclear extracts from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041 were subjected to immunoprecipitation using anti-p65 antibody coupled to protein-A agarose beads. Immunoprecipitates were subjected to SDS–PAGE and immunoblotted with an anti-PPARβ/δ antibody. The blot data are representative of three separate experiments.

shown). Overall, these data demonstrate that PPARβ/δ activation by L-165041 inhibits LPS-induced NF-κB activation.

2.5. Treatment with the PPARβ/δ activator enhances its interaction with the p65 subunit of NF-kB

Finally, we sought to determine the molecular mechanism by which the PPARβ/δ activator L-165041 inhibits LPS-induced NF-κB activation. Since it has been proposed that PPARβ/δ and PPARα share similar biological roles [21], we studied whether PPARβ/δ inhibited NF-κB signaling through mechanisms similar to those reported for PPARα. Activation of PPARα may result in inhibition of NF-κB signaling through different mechanisms. First, PPARα activators have been reported to induce the expression of IκBα, which forms a cytoplasmic inactive complex with the p65–p50 heterodimeric complex [34,35]. We did not observe significant changes in the protein expression of PPARβ/δ, the p65 subunit of NF-κB, IκBα or IκBβ after L-165041 treatment (Fig. 6), suggesting that PPARβ/δ activation did not act through this mechanism. In addition, PPARα activators may act through DNA-binding independent mechanisms that may involve a physical interaction with NF-κB. This association prevents NF-κB from binding to its response element and thereby inhibits its ability to induce gene transcription [16]. In order to evaluate whether PPARβ/δ activation acts through a similar mechanism, we performed coimmunoprecipitation studies with isolated nuclear extracts using antibodies against the p65 subunit of NF-κB and PPARβ/δ. Data shown in Fig. 7 demonstrate that addition of the PPARβ/δ agonist L-165041 strongly enhanced the physical interaction between p65 and PPARβ/δ, suggesting that increased association between these two proteins is the mechanism through which PPARβ/δ activation prevents NF-κB activation.

3. Discussion

In the present study, we demonstrate that activation of PPARβ/δ by the specific ligand L-165041 inhibits PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes. Treatment with L-165041 also inhibited PE-induced expression of the NF-κB-target gene MCP-1, suggesting that the antihypetrophic effect of this compound involves downregulation of NF-κB signaling pathway. Further, it is shown that L-165041 may inhibit LPS-induced NF-κB activation through enhanced physical interaction of PPARβ/δ with the p65 subunit of NF-κB.

Several studies have reported that both PPARα and PPARγ activators inhibit cardiac hypertrophy [36–39]. In contrast, the biologic role of PPARβ/δ activation in cardiac hypertrophy was unknown. The availability of selective PPARβ/δ ligands, such as L-165041, opened the possibility of studying the role of this PPAR subtype in cardiac cells. Thus, previous data of a recent study [21] pointed to an important function of PPARβ/δ in the heart. The authors demonstrated that both PPARα and PPARβ/δ were expressed in comparable levels in heart, whereas PPARγ was barely detectable. Further, PPARβ/δ was fatty acid inducible and activated the expression of PPARα target genes involved in fatty acid utilization in cardiac myocytes. The authors of this study suggested that PPARα and PPARβ/δ shared similar functions in cardiac cells regarding cardiac fatty acid metabolism. In agreement with this idea, Muoio et al. [40] shown that fatty acid oxidation in skeletal muscle of PPARα$^{-/-}$ mice was not impaired, probably because of PPARβ/δ compensated for the lack of PPARα in these mice. In the present study, we define a new role for PPARβ/δ activation, inhibition of cardiomyocyte hypertrophy. Therefore, given the abundant expression of both PPARα and PPARβ/δ in heart and the fact that PPARα activation also inhibits cardiac hypertrophy [41,42], these PPAR subtypes may also share similar roles in the development of cardiac hypertrophy.

It is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are the consequence or the reason for cardiac hypertrophy. However, several factors support a role for cardiac metabolism in the development of cardiac hypertrophy. Thus, an increase in the activities of several glycolytic enzymes has been reported prior to cardiac hypertrophy [7]. Moreover, the fact that PPARα gene influences human left ventricular growth in response to exercise and hypertension, indicates that maladaptative cardiac substrate utilization can play a causative role in the pathogenesis of left ventricular hypertrophy [43]. In the present work, stimulation of rat neonatal cardiomyocytes with PE, which leads to NF-κB activation [4], caused cardiomyocyte hypertrophy that was accompanied by a fall in the expression of genes involved in fatty acid metabolism, such as M-CPT-I and PDK-4. This effect was abolished by the addition of the PPARβ/δ activator

L-165041, which strongly induced the expression of these genes. Further studies are necessary to clearly establish whether pharmacological modulation of cardiac fatty acid metabolism with either PPARα or PPARβ/δ activators is enough to alleviate or inhibit cardiac hypertrophy. However, it is worth noting that treatment of H9c2 cells with LPS for 24 h caused a similar pattern of changes in the expression of M-CPT-I and PDK-4 to those observed in PE-induced cardiomyocyte hypertrophy. Since both PE-induced cardiomyocyte hypertrophy and LPS lead to NF-κB activation, these data point to the involvement of this transcription factor in the downregulation of genes involved in fatty acid metabolism. Activation of PPARβ/δ would inhibit NF-κB signaling pathway avoiding both cardiomyocyte hypertrophy and downregulation of genes involved in fatty acid metabolism. Furthermore, and although, it is not the objective of this study, apoptosis is considered an important factor in the progression from cardiac hypertrophy to heart failure. Activation of NF-κB is involved in direct regulation of both anti- and proapoptotic effects [44] and the latter maybe stimulated by LPS.

Interestingly, L-165041 reduced the induction of the NF-κB target gene MCP-1 in cardiac cells stimulated by either PE or LPS, suggesting that PPARβ/δ may antagonize NF-κB activation. Enhanced myocardial MCP-1 has been described in the hypertrophied and failing heart [45] and may lead to the infiltration and activation of inflammatory cells, such as monocytes/macrophages and lymphocytes. In addition, it has been reported that activation of MCP-1 expression contributes to left ventricular remodeling and failure after myocardial infarction [46]. Therefore, PPARβ/δ activation may become a therapeutic option to reduce the expression of MCP-1 in heart. It is important to note that the inhibitory effect of L-165041 on LPS-induced MCP-1 expression was of lower intensity that the observed for PE. This probably reflects the higher induction achieved by LPS stimulation (sevenfold induction) compared to PE (twofold induction) and/or differences in the two cell system used. The use of H9c2 myotubes, which only express PPARβ/δ, offers the advantage of avoiding the interference of other PPAR subtypes and, therefore, permits to adscribe the changes observed to this transcription factor. However, LPS-treatment of H9c2 myotubes was performed to achieve NF-κB activation, but not cardiac hypertrophy, and consequently the findings observed in these cells should be limited to the activation of NF-κB by LPS.

PPARα activators may inhibit NF-κB signaling through different mechanisms [16,47,48]. One of these mechanisms involves physical interaction of PPARα and the p65 subunit of NF-κB [16]. Here, we demonstrate that PPARβ/δ activation by L-165041 enhances the protein-protein association between PPARβ/δ and p65, indicating that this mechanism may interfere NF-κB transactivation capacity. Therefore, PPARα and PPARβ/δ may also share similar mechanisms of action inhibiting NF-κB signaling. Further studies are necessary to investigate whether PPARβ/δ activation may inhibit the NF-κB signaling pathway through additional mechanisms or affects the activity of other transcription factors involved in cardiac hypertrophy, such as nuclear factor of activated T lymphocyte (NFAT).

In summary, in the present study, we show that PPARβ/δ activation inhibits PE-induced cardiomyocyte hypertrophy in neonatal rat ventricular cardiomyocytes. PPARβ/δ activation also inhibits LPS-induced NF-κB activation through a mechanism that may involve enhanced protein–protein interaction between this PPAR subtype and the p65 subunit of NF-κB. These data indicate that inhibition of the NF-κB signaling pathway may be the underlying mechanism responsible for the inhibition of cardiomyocyte growth.

Acknowledgments

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III

Atorvastatin improves peroxisome proliferator-activated receptor signaling in cardiac hypertrophy by preventing nuclear factor-κB activation

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Atorvastatin improves peroxisome proliferator-activated receptor signaling in cardiac hypertrophy by preventing nuclear factor-κB activation

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Abstract

Nuclear factor (NF)-κB signaling pathway plays a pivotal role in cardiac hypertrophy. Although it has been reported that statins inhibit cardiac hypertrophy by reducing generation of reactive oxygen species, it is not yet known whether statins prevent NF-κB activation and whether this effect can be related to the reduction in the peroxisome proliferator-activated receptor (PPAR) pathway. In this study, we examined the role of atorvastatin on NF-κB activity and PPAR signaling in pressure overload-induced cardiac hypertrophy. Our findings indicate that atorvastatin inhibits cardiac hypertrophy and prevents the fall in the protein levels of PPARα and PPARβ/δ. Further, atorvastatin treatment avoided NF-κB activation during cardiac hypertrophy, reducing the protein–protein association between these PPAR subtypes and the p65 subunit of NF-κB. These findings indicate that negative cross-talk between NF-κB and PPARs may interfere with the transactivation capacity of the latter, leading to a fall in the expression of genes involved in fatty acid metabolism, and that these changes are prevented by statin treatment.

Keywords: Hypertrophy; Myocyte; Energy metabolism; Statin

1. Introduction

Cardiac hypertrophy represents an initial physiological adaptive response to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction, and cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia, and sudden death [1,2]. Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role. Thus, it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes [3–6]. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, the p65 and p50 subunits. In unstimulated cells, NF-κB is found in the cytoplasm and is bound to IκB, which prevents it from entering the nucleus. When these cells are stimulated, specific kinases phosphorylate IκB causing its rapid degradation by proteasomes. The release of IκB from NF-κB results in the passage of this heterodimer to the nucleus, where it binds to specific sequences in the promoter regions of target genes. Through this mechanism NF-κB plays a critical role in mediating immune and inflammatory responses.

On the other hand, development of cardiac hypertrophy is associated with an increase in glucose utilization and a decrease in fatty acid oxidation, which is characteristic of fetal heart [7]. The expression of cardiac genes involved in glucose and fatty acid metabolism is mainly regulated by peroxisome proliferator-activated receptors (PPARs). The PPAR subfamily consists of three subtypes, PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ/δ (NR1C2) and PPARγ (NR1C3). PPARα is expressed primarily in

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tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle [8]. PPARβ/δ is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts. Interestingly, a negative cross-talk has been described between PPARα and NF-κB [9,10].

Recent reports have suggested that PPARs may play an important role in cardiac disease. Thus, it has been reported that the shift in the substrate utilization from fatty acids to glucose observed during the cardiac hypertrophic growth is associated with deactivation of PPARα [11]. These results suggest that reduced activity of this transcription factor may account for the down-regulation of enzymes involved in fatty acid oxidation. However, the role of PPARβ/δ in the development of this process is largely unknown. Recently, Gilde et al. [12], using neonatal rat cardiomyocytes as well as the embryonic rat heart-derived H9c2 cells, clearly demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism, suggesting that similarly to PPARα, PPARβ/δ may play an important role in cardiac disease.

Current treatments for cardiac hypertrophy are limited to vasodilators or afterload reducers, with few therapies directed at the myocardial process. The 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors, or statins, are widely prescribed cholesterol-lowering agents that decrease the incidence of myocardial infarction and ischemic stroke. In addition to inhibiting cholesterol synthesis, statins also inhibit the synthesis of important isoprenoid intermediates that are important lipid attachments required for the subcellular localization and function of a variety of proteins. Further, it has been recently reported that statins prevent cardiac hypertrophy by inhibiting generation of reactive oxygen species [13]. However, it is not yet known whether statins prevent NF-κB activation and whether activation of this transcription factor affects the expression of target genes involved in fatty acid metabolism. In this study, we examined new potential mechanisms by which NF-κB activation affects the expression of PPAR signaling in pressure overload-induced cardiac hypertrophy. Further, we evaluated the effects of atorvastatin treatment on these mechanisms.

2. Methods

2.1. Materials

Atorvastatin was provided by Pfizer Laboratories. [γ-^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech KK. All other chemicals were purchased from Sigma.

2.2. RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described [14]. The sequences of the sense and antisense primers used for amplification were: atrial natriuretic factor (ANF), 5′-TCCTCTTCCTGGCCTTTTGGC-3′ and 5′-AGACGGGTTGCTTCCCCAGTC-3′; α-actinin, 5′-GGCTGTGTTCCCATCCATCGT-3′ and 5′-CCCGGTTAGCTTTGGGGTTCA-3′; pyruvatedehydrogenase kinase 4 (PDK-4), 5′-GAACACCCCTTCCGTCCAGCT-3′ and 5′-TGTGCCATCGTAGGGACCACA-3′; and APRT (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-3′. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, α-actinin: 266 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

2.3. Pressure overload-induced cardiac hypertrophy

Male Sprague–Dawley rats (225 to 250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21±1 °C). They were fed standard diet (Panlab, Barcelona, Spain) for 5 days before the studies began. The animals were randomly distributed into three groups as follows: (1) sham-operated rats, (2) pressure overloaded rats and (3) pressure overloaded rats treated with atorvastatin. Five days before the surgical procedure, rats were fed either a control diet or a diet containing 0.05% (wt/wt) atorvastatin (which resulted in approximately 15 mg/kg/day) and continued for 15 days post-surgery. The diets were prepared as previously described [15]. Throughout the study, the weight and daily food intake were measured. Pressure overload was induced by constriction of the abdominal aorta at the suprarenal level with 7–0 nylon strings by ligation with a blunted 25-gauge needle, which was then pulled out. For the age-matched sham operation, the identical procedure was performed except that the suture was not tied around the aorta. Hearts were harvested 15 days after the surgical operation. The heart weight/body weight (HW/BW) ratio was calculated and the heart samples were frozen in liquid nitrogen and then stored at −80 °C. Animal handling and disposal were performed in accordance with the law 5/1995, 21st July, from the Generalitat de Catalunya.

2.4. Immunoblotting

Cell lysates and nuclear extracts from hearts were obtained as previously described [14]. Proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and

transferred to Immobilon polyvinylidene diflouride membranes (Millipore, Bedford, MA). Western blot analysis was performed using rabbit polyclonal antibodies against IκBα, IκBβ and p65 (Santa Cruz Biotechnology, Inc.) and mouse monoclonal antibody against β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies). Bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging).

2.5. Electrophoretic mobility shift assay (EMSA)

Isolation of nuclear extracts was performed as previously described [14]. EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the NF-κB nucleotide (5′AGTTGAGGGGACTTTCCCAGGC-3′) and Oct-1 (5′-TGTCGAATGCAAATCACTAGAA-3′). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5× kinase buffer, 1 μl of T4 polynucleotide kinase (10 U/μl), and 2.5 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) incubated at 37 °C for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris–HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) according to the manufacturer's instructions. Ten micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer (10 mM Tris–HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA and 50 μg/ml poly(dI–dC)), in a final volume of 15 μl. Labeled probe (approximately 60.000 cpm) was added and the reaction was incubated for 15 min at 4 °C. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4 °C. Protein–DNA complexes were resolved by electrophoresis at 4 °C on a 5% acrylamide gel and subjected to autoradiography.

2.6. Coimmunoprecipitation

Cell nuclear extracts were brought to a final volume of 0.5 ml with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, BSA 2% and 1 mg/ml nonfat milk for 6 h at 4 °C and incubated with 4 μg of anti-p65. Immunocomplex was captured by incubating the samples with protein A–agarose suspension overnight at 4 °C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of SDS-PAGE sample buffer and boiled for 5 min at 100 °C. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against PPARβ/δ.

2.7. Statistical analyses

Results were obtained from at least four independent experiments. Band intensities were analyzed by densitometry. Significant differences were established by one-way ANOVA, using the computer program GraphPad Instat V2.03 (GraphPad Softwware Inc., San Diego, CA). When significant variations were found, the Tukey–Kramer multiple comparisons test was performed. Differences were considered significant at $P<0.05$.

3. Results

3.1. Atorvastatin treatment prevents pressure overload-induced cardiac hypertrophy

Cardiac hypertrophy is characterized by increased HW/BW ratio, induction of fetal-type genes (e.g., ANF) and sarcomeric disorganization. Therefore, we first examined the effects of atorvastatin on these parameters in the pressure-overload model of cardiac hypertrophy in order to clearly demonstrate the antihypertrophic effects of this drug. The HW/BW ratio significantly increased (1.35-fold, $P<0.001$) after aortic constriction compared with sham-operated rats (Fig. 1A). Treatment with atorvastatin completely abolished the increase in the HW/BW ratio ($P<0.01$ vs. banded rats). Further, pressure overload also led to approximately twofold induction in the mRNA levels of ANF compared with sham-operated rats (Fig. 1B). However, in the heart of atorvastatin-treated rats ANF expression nearly disappeared. The fall in the expression levels of ANF after atorvastatin treatment below the control values (sham-operated rats) is in agreement with previous studies [16]. Similarly, mRNA levels of the sarcomere-associated protein α-actinin increased twofold compared with sham-operated rats (Fig. 1C) and this induction was not observed in the hearts of atorvastatin-treated rats.

3.2. Atorvastatin treatment prevents NF-κB activation in pressure overload-induced cardiac hypertrophy

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes [17–20] and it has been reported that statins inhibit the activation of this redox transcription factor in vascular cells [21], we performed EMSA studies to investigate whether atorvastatin inhibited NF-κB activation in pressure overload-induced cardiac hypertrophy. These studies showed that the NF-κB probe formed four main specific complexes with cardiac nuclear proteins (complexes I to IV, Fig. 2A), based on competition experiments performed by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures. NF-κB binding

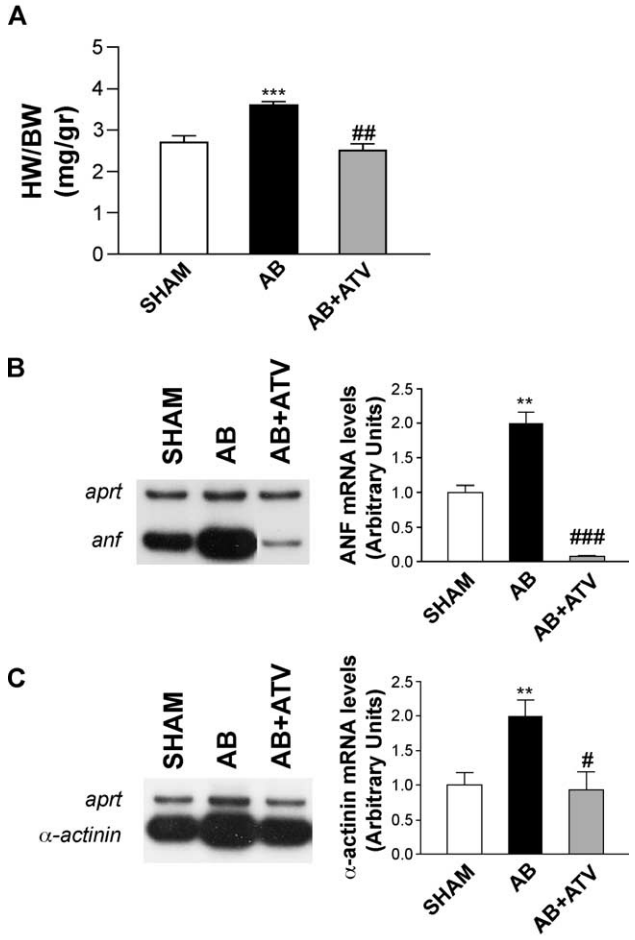

Fig. 1. Atorvastatin inhibits pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. Treatment with atorvastatin was initiated 5 days before operation and continued for 15 days as food admixture at a concentration of 0.05% (wt/wt). At 15 days after operation, hearts were excised and weighed. (A) Analysis of the HW/BW ratio in sham-operated rats, in banded rats (aortic banding, AB) and in banded rats treated with atorvastatin (AB+ATV). Analysis of the mRNA levels of ANF (B) and α-actinin (C) in pressure overload-induced cardiac hypertrophy. A representative autoradiogram of the four animals studied per treatment group is shown. Data are expressed as mean±S.D. of four different experiments. $^{**}P<0.01$, $^{***}P<0.001$ vs. sham-operated rats. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. banded rats.

activity increased in banded rats, especially complex III (2.5-fold increase, $P<0.01$ vs. sham-operated rats) (Fig. 2B), and this effect was abolished by atorvastatin treatment ($P<0.01$ vs. banded rats). Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of this transcription factor. Addition of this antibody to incubation mixtures resulted in complete supershift of complex III, thus showing that this complex contained p65 (Fig. 2C). No changes were observed in the DNA binding of cardiac nuclear proteins from the different groups of rats to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (Fig. 2D). Overall, these data demonstrate that atorvastatin inhibits NF-κB activation in pressure overload-induced cardiac hypertrophy and that this mechanism may contribute to the antihypertrophic effect of this compound.

3.3. Atorvastatin prevents down-regulation of both PPARα and PPARβ/δ in pressure overload-induced cardiac hypertrophy

It has been reported that induction of cardiac hypertrophy leads to down-regulation of the expression of PPARα mRNA levels and several of its target genes involved in fatty acid metabolism [11]. However, nothing is known about the effects of cardiac hypertrophy on PPARβ/δ expression. Therefore, we next evaluated the consequences of pressure overload-induced cardiac hypertrophy and atorvastatin treatment on the protein expression of these transcription factors. As shown in Fig. 3, a fall in the expression of PPARα protein (60% reduction, $P<0.01$) was observed in the heart of banded rats compared with sham-operated rats. Interestingly, atorvastatin treatment prevented the reduction of PPARα ($P<0.01$ vs. banded rats). Likewise, cardiac hypertrophy also resulted in a fall in the expression of PPARβ/δ protein (50% reduction, $P<0.05$ vs. sham-operated rats) and atorvastatin treatment prevented the fall in the expression of this transcription factor ($P<0.05$ vs. banded rats). These data indicate that the expression pattern of both PPARα and PPARβ/δ is similarly regulated during the development of cardiac hypertrophy. Finally, the expression of the NF-κB subunit p65 was not significantly affected by either aortic banding or atorvastatin treatment.

In order to assess whether atorvastatin treatment also prevented the down-regulation of PPAR-target genes involved in fatty acid metabolism, we determined the mRNA levels of PDK-4, a target gene for both PPARα and PPARβ/δ [22] that suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization [23]. As shown in Fig. 4A, PDK-4 mRNA levels were down-regulated by 35% ($P<0.01$) in the heart of banded rats, compared with sham-operated rats. In contrast, in the hearts of atorvastatin-treated rats no changes were observed, suggesting that prevention of NF-κB activation avoided changes in fatty acid metabolism.

Further, the protein levels of the PPAR-target gene medium chain medium chain acyl-CoA dehydrogenase (MCAD) [24] were also reduced by 40% ($P<0.05$ vs. sham-operated rats) (Fig. 4B). Again, atorvastatin treatment avoided the fall in the expression of MCAD protein ($P<0.05$ vs. banded rats).

3.4. Atorvastatin treatment inhibits p65-PPAR interaction in pressure overload-induced cardiac hypertrophy

Reduced activity of PPARα during cardiac hypertrophy has been related to phosphorylation of this protein [11]. In this work, we sought to determine whether other mechanisms might contribute to the impairment of the PPAR

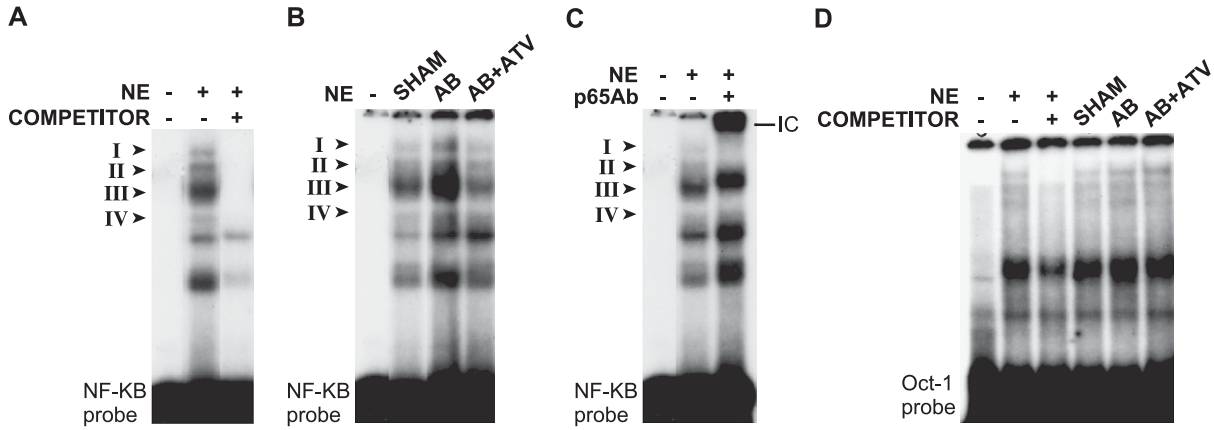

Fig. 2. Treatment with atorvastatin prevents NF-κB activation in pressure overload-induced cardiac hypertrophy. (A) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear protein extract (NE) shows four specific complexes (I to IV), based on competition with a molar excess of unlabeled probe. (B) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and cardiac NE from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV). (C) Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. Supershifted immune complex (IC) is denoted. (D) Autoradiograph of EMSA performed with a ^{32}P-labeled Oct-1 nucleotide. All autoradiograph data are representative of four separate experiments.

pathway. Since it has been reported that a physical interaction exists between PPARα and the p65 subunit of NF-κB [9,10] and that NF-κB inhibits the expression of PPARα-target genes [25], we next evaluated whether cardiac hypertrophy resulted in enhanced p65-PPAR interaction. Nuclear extracts isolated from hearts were immunoprecipitated using anti-p65 antibody coupled to protein A–

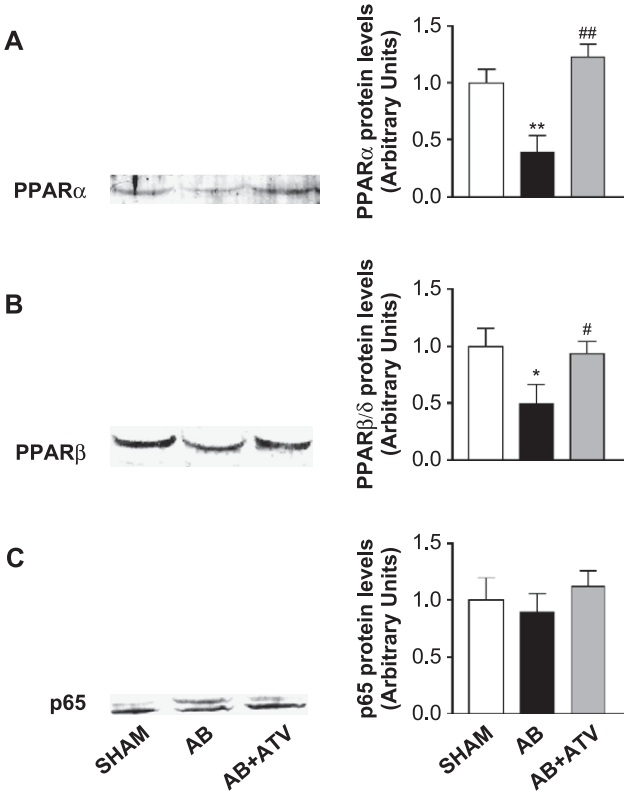

Fig. 3. Treatment with atorvastatin prevents the reduction in protein levels of PPAR in pressure overload-induced cardiac hypertrophy. Cardiac protein extracts from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV) were assayed for Western blot analysis with PPARα (A), PPARβ/δ (B) and p65 (C) antibodies. A representative blot normalized to the β-tubulin levels is shown. Data are expressed as mean±S.D. of four different experiments. *P<0.05 and **P<0.01 vs. sham-operated rats. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs. banded rats.

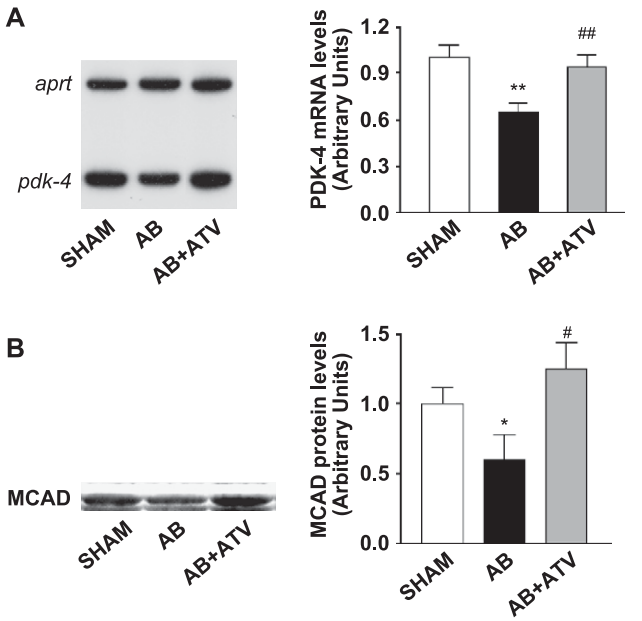

Fig. 4. Atorvastatin restores the expression of PDK-4 and MCAD in pressure overload-induced cardiac hypertrophy. (A) Analysis of the cardiac mRNA levels in sham-operated rats, in banded rats (aortic banding, AB) and in banded rats treated with atorvastatin (AB+ATV). A representative autoradiogram normalized to the APRT mRNA levels is shown. Data are expressed as mean±S.D. of four different experiments. (B) Treatment with atorvastatin prevents the reduction in protein levels of MCAD in pressure overload-induced cardiac hypertrophy. Cardiac protein extracts from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV) were assayed for Western blot analysis with MCAD antibody. A representative blot normalized to the β-tubulin levels is shown. Data are expressed as mean±S.D. of four different experiments. *P<0.05 and **P<0.01 vs. sham-operated rats. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 vs. banded rats.

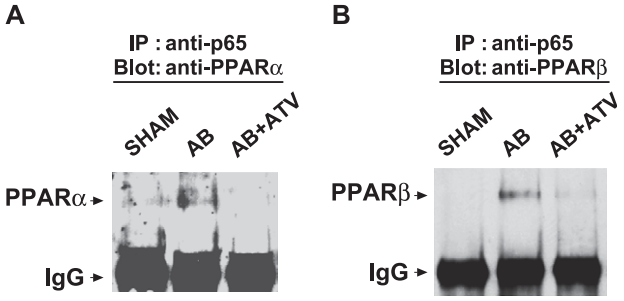

Fig. 5. Atorvastatin reduces PPAR association with the p65 subunit of NF-κB in pressure overload-induced cardiac hypertrophy. Nuclear extracts (equalized by protein concentrations) from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV) were subjected to immunoprecipitation using anti-p65 antibody coupled to protein A–agarose beads. Immunoprecipitates were subjected to SDS-PAGE, and immunoblotted with both anti-PPARα (A) and anti-PPARβ/δ (B) antibodies. Arrowheads represent the PPAR subtypes or IgG signal. The blot data are representative of four separate experiments.

agarose beads. Immunoprecipitates were then subjected to SDS-PAGE and immunoblotted with both anti-PPARα and anti-PPARβ/δ antibodies. Data shown in Fig. 5 demonstrate that pressure overload-induced cardiac hypertrophy enhanced the physical interaction of p65 with both PPARα and PPARβ/δ, suggesting that increased association between these proteins is a mechanism contributing to the reported reduction in the expression of PPAR-target genes involved in fatty acid metabolism. Interestingly, in the hearts of atorvastatin-treated rats no enhanced physical interaction between p65 and PPAR was observed, indicating that prevention of NF-κB activation avoids this interaction.

4. Discussion

Whereas a plethora of signaling cascades have been implicated in the development of cardiac hypertrophy [26], relatively little is known about the intrinsic mechanisms with the potential to inhibit or even reverse hypertrophy. In the present study, we demonstrate that atorvastatin, similarly to other statins [13], inhibits pressure overload-induced cardiac hypertrophy. Further, our findings indicate for the first time that during the development of cardiac hypertrophy two processes may reduce the expression of PPAR-target genes. First, reduced protein expression of both PPARα and PPARβ/δ in hypertrophied hearts. Second, NF-κB activation during cardiac hypertrophy resulting in increased protein–protein association between PPARs and the p65 subunit of NF-κB. In addition, we report that atorvastatin treatment prevents both processes during cardiac hypertrophy, ameliorating the expression of PPAR-target genes involved in fatty acid metabolism.

It is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are the consequence or the reason for the shift of cardiac metabolism from fatty acids to glucose observed in cardiac hypertrophy. However, several factors support a role for cardiac metabolism in the development of cardiac hypertrophy. Defects in mitochondrial fatty acid oxidation enzymes cause childhood hypertrophic cardiomyopathy [27], and perturbation of fatty acid oxidation in animal models causes cardiac hypertrophy [28,29], demonstrating that substrate utilization is important in the pathogenesis of hypertrophy. Moreover, the fact that PPARα gene influences human left ventricular growth in response to exercise and hypertension indicates that maladaptative cardiac substrate utilization can play a causative role in the pathogenesis of left ventricular hypertrophy [30]. In this work, we show that induction of cardiac hypertrophy, which leads to NF-κB activation [31], was accompanied by a fall in the expression of genes involved in fatty acid metabolism, such as MCAD and PDK-4. Changes in the expression of PPAR-target genes may result from deactivation of PPARα, as previously reported [11]. In addition, we have demonstrated that the levels of PPARβ/δ, which is expressed in comparable amounts in heart to PPARα [12], are reduced in cardiac hypertrophy. It has been suggested that PPARα and PPARβ/δ share similar functions in cardiac fatty acid metabolism and that the second probably compensates for the lack of PPARα in skeletal muscle of PPARα$^{-/-}$ mice, avoiding the impairment of fatty acid oxidation in these mice [32]. Therefore, the fall in the expression of both PPAR subtypes during the development of cardiac hypertrophy seems necessary to down-regulate the expression of genes involved in fatty acid metabolism. It is important to note that similarly to the data here presented, we have shown in a previous study that atorvastatin prevented the down-regulation of hepatic PPARα in sucrose-fed rats [33], suggesting that this statin may avoid metabolic derangements caused by different stimuli.

Interestingly, the changes caused by cardiac hypertrophy on the expression of genes involved in fatty acid metabolism were not observed when enhanced NF-κB activity was prevented by atorvastatin treatment. These data pointed to the involvement of NF-κB in these changes. In agreement with this hypothesis, a recent study demonstrated that inhibition of NF-κB increased the expression of the well-known PPARα-target gene apoA-I [25], confirming the negative cross-talk between NF-κB and PPARα. Therefore, we evaluated whether, in addition to the reported reduction in the expression of PPARs during cardiac hypertrophy (Ref. [11] and this study), the physical interaction between PPARs and the p65 subunit of NF-κB [9,10] may contribute to the changes in the expression of genes involved in cardiac fatty acid metabolism. The results here shown demonstrate that cardiac hypertrophy enhances the protein–protein association between p65 and both PPARα and PPARβ/δ, indicating that this mechanism may interfere with PPAR transactivation capacity, contributing to a fall in the expression of genes involved in fatty acid metabolism. On the contrary, prevention of NF-κB activation by

atorvastatin treatment avoided the PPAR–p65 interaction and restored the expression of these genes.

In summary, in the present study we show for the first time that the reduction in fatty acid oxidation observed during cardiac hypertrophy may result from the down-regulation of PPARα and β/δ and the enhanced protein–protein interaction between p65 and PPARs, and that these changes are prevented by statin treatment.

Acknowledgments

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IV

Lipopolysaccharide-stimulated Nuclear Factor(NF)-κB Activation in Heart-derivated H9c2 Cells Reduces the Interaction between Peroxisome Proliferator-Activated Receptor β/δ and PPARγ Coactivator 1. Restoration by Atorvastatin Treatment

Anna Planavila, Rosa M. Sánchez, Juan C. Laguna, Manuel Vázquez-Carrera

En avaluació

Lipopolysaccharide-stimulated Nuclear Factor(NF)-κB Activation in Heart-derived H9c2 Cells Reduces the Interaction between Peroxisome Proliferator-Activated Receptor β/δ and PPARγ Coactivator 1. Restoration by Atorvastatin Treatment

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Running title: NF-κB activation reduces PPARβ/δ-PGC-1 interaction

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Abstract

Although abnormalities in cardiac fatty acid metabolism are involved in the development of several cardiac pathologies, the mechanisms underlying these changes are not well understood. Given the prominent role played by peroxisome proliferator-activated receptor β/δ (PPARβ/δ) in cardiac fatty acid metabolism, the aim of this study was to examine the effects of nuclear factor (NF)-κB activation on the activity of this nuclear receptor. Embryonic rat heart-derived H9c2 cells stimulated with lipopolysaccharide (LPS) showed a reduction (38%, $P<0.05$) in the mRNA levels of the PPARβ/δ-target gene pyruvatedehydrogenase kinase 4 (PDK4) that was prevented in the presence of the NF-κB inhibitors parthenolide (10 μM) and atorvastatin (10 μM). Electrophoretic mobility shift assay revealed that both parthenolide and atorvastatin significantly decreased LPS-stimulated NF-κB binding activity in H9c2 cardiac cells. LPS-stimulation of H9c2 cardiac cells also led to a 30% reduction ($P<0.05$) in the mRNA levels of PPPARγ Coactivator 1 (PGC-1) that was consistent with the reduction in the protein levels of this coactivator. In the presence of either atorvastatin or parthenolide the reduction in PGC-1 expression was prevented. Co-immunoprecipitation studies showed that LPS-stimulation led to a reduction in the physical interaction between PGC-1 and PPARβ/δ and that this reduction was prevented in the presence of atorvastatin. Finally, electrophoretic mobility shift assay revealed that parthenolide and atorvastatin prevented LPS-mediated reduction in PPARβ/δ binding activity in H9c2 cardiac cells. These results suggest that LPS-mediated NF-κB activation inhibits the expression of genes involved in fatty acid metabolism by a mechanism involving reduced expression of PGC-1, which in turn affects the PPARβ/δ transactivation of target genes involved in cardiac fatty acid oxidation.

Key words: PGC-1, PPARβ/δ, NF-κB, atorvastatin.

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation [1]. The PPAR subfamily consists of three subtypes, PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ/δ (NR1C2) and PPARγ (NR1C3) [2]. PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle [3]. PPARβ/δ is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whith limited amounts in other tissues such as skeletal muscle and heart. To be transcriptionally active, PPARs need to heterodimerize with the 9-*cis* retinoic acid receptor (RXR) (NR2B). PPAR-RXR heterodimers bind to DNA specific sequences called peroxisome proliferator-response elements (PPREs), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). These sequences have been characterized within the promoter regions of PPAR target genes. However, the regulation of gene transcription by PPARs extends beyond their ability to transactivate specific target genes. PPARs also regulate gene expression, independently of their binding to DNA, through a mechanism termed receptor-dependent *trans*repression [4]. One of these mechanisms involves a physical interaction of PPARα with nuclear factor (NF)-κB, that may lead to suppression activity of the former [5].

It is now clear that coactivators have a crucial role in the regulation of gene expression by PPARs[6]. Coactivators stimulate gene expression, acetylate histones, and mediate interactions between transcription factors [6]. The peroxisomal proliferator-activator receptor-γ coactivator 1 (PGC-1) was initially identified as a protein that interacted with PPARγ [7]. Several isoforms of PGC-1 have been cloned including PGC-1α, PGC-1β and the PGC-related coactivator [8-10]. PGC-1α is highly expressed in tissues with high metabolic rates, such as heart, muscle and brown adipose tissue [6]. A recent study showed that PGC-1α acts as a transcriptional coactivator for PPARβ/δ and activates the expression of genes involved in fatty acid metabolism together with PPARβ/δ [11]. Further, it has been demonstrated that PPARβ/δ physically interacts with PGC-1α [12] and that activators of this PPAR subtype stimulate PGC-1α mRNA levels both *in vitro* and *in vivo* [13].

Abnormalities in cardiac fatty acid metabolism are involved in the development of several cardiac pathologies. Thus, defects in mitochondrial fatty acid oxidation enzymes cause childhood hypertrophic cardiomyopathy [14], and perturbation of fatty acid oxidation in animal models causes cardiac hypertrophy [15,16]. Gilde and co-workers [17], using neonatal rat cardiomyocytes as well as embryonic rat heart-derived H9c2 cells, demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism. In agreement with the role of PPARβ/δ in cardiac fatty acid metabolism, a recent study reported that cardiomyocyte-restricted PPARβ/δ deletion perturbed myocardial fatty acid oxidation and led to cardiomyopathy [18]. Given the crucial role played by PPARβ/δ in fatty acid metabolism, knowledge of the mechanisms governing PPARβ/δ-mediated regulation of cardiac fatty acid metabolism may be useful for preventing or treating cardiac diseases. Little is known about the molecular mechanisms leading to the fall in the expression of genes involved in cardiac fatty acid metabolism, but NF-κB may be involved since this transcription factor inhibits PPAR activity [5].

The 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors, or statins, are widely prescribed cholesterol-lowering agents that decrease the incidence of myocardial infarction and ischemic stroke. In addition to inhibiting cholesterol synthesis, statins also inhibit the synthesis of important isoprenoid intermediates that are important lipid attachments required for the subcellular location and function of a variety of proteins. As a result, statins may affect several signaling pathways, including NF-κB activation [19]. It is still not known whether statins prevent NF-κB activation in cardiac cells and whether this effect can be related to changes in fatty acid metabolism. In this study, we examined the effects of NF-κB inhibitors on PPARβ/δ activity in LPS-stimulated H9c2 cardiac cells.

2. Materials and Methods

2.1. Materials

Atorvastatin was provided by Pfizer Laboratories. [γ-^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech KK. All other chemicals were purchased from Sigma.

2.2. Cell Culture

The embryonic rat-heart-derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5,000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. H9c2 cells were treated with LPS (10 ng/mL) for 24 hours.

2.3. RNA preparation and analysis

Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR), as previously described [20]. The sequences of the sense and antisense primers used for amplification were: monocyte chemoattractant protein 1 (*Mcp-1*), 5'-GGGCCTGTTGTTCACAGTTGC-3' and 5'-GGGACACCTGCTGCTGGTGAT-3'; *Pgc-1*, 5'-AGAAAGGGCCCGAGCAATCTG-3' and 5'-AGATGTGCCCCTGCCAGTCAC-3'; pyruvatedehydrogenase kinase 4 (*Pdk4*), 5'-GAACACCCCTTCCGTCCAGCT-3' and 5'-TGTGCCATCGTAGGGACCACA-3' and *Aprt* (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGGCCAGTCACCTGA-3' and 5'-CCAGGCTCACACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (*Mcp-1*: 157 bp, *Pdk4*: 168 bp, *Pgc-1*: 234 bp and *Aprt*: 329 bp). The results for the expression of specific mRNAs are always given in relation to the expression of the control gene (*Aprt*).

2.4. Immunoblotting

Cell lysates and nuclear extracts from H9c2 cells were obtained as previously described [20]. Proteins (50 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against PGC-1, p65 and PPARβ/δ (Santa Cruz Biotechnology, Inc) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL

chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

2.5. Electrophoretic mobility shift assay (EMSA)

H9c2 cells were pre-treated with either 5μmol/L atorvastatin or 10 μmol/L parthenolide for 24 hours before stimulation with LPS (10 ng/ml) for 1 hour. Isolation of nuclear extracts and EMSA were performed as previously described [20].

2.6. Coimmunoprecipitation

Cell nuclear extracts were brought to a final volume of 0.5 mL with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, 2% BSA and 1 mg/ml nonfat milk for 6 h at 4ºC and incubated with 4 μg of anti-PGC-1. Immunocomplexes were captured by incubating the samples with protein A-agarose suspension overnight at 4ºC on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of SDS-PAGE sample buffer and boiled for 5 min. at 100ºC. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS-PAGE and immunoblotted with an antibody against PPARβ/δ.

2.7. Statistical Analyses

Results were obtained from at least four independent experiments and presented as mean±S.D. Groups were compared with one-way ANOVA using the computer program GraphPad Instat (GraphPad Software Inc., San Diego, CA). When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Differences were considered significant at $P<0.05$.

3. Results

3.1. NF-κB inhibitors Prevent the Down-regulation of PDK4 and the Upregulation of MCP-1 Caused by LPS in H9c2 cells

Gilde *et al.* [17] recently reported that H9c2 cardiac cells abundantly express the PPARβ/δ subtype, whereas PPARα and γ are undetectable. This converts H9c2 cells into a proper tool to investigate the function of PPARβ/δ in cardiac fatty acid metabolism without the potential interference of the other PPAR subtypes. To study the effects of NF-κB activation on PPARβ/δ activity we stimulated cells for 24 h with LPS, which has been reported to activate NF-κB in cardiomyocytes [21]. We examined the expression of the PPARβ/δ target gene PDK4 [13], which suppresses glucose oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization [22]. LPS stimulation caused a 38% reduction ($P<0.05$) in PDK4 mRNA levels. However, co-incubation of LPS-stimulated cells with the NF-κB inhibitors parthenolide, which specifically inhibits activation of NF-κB by preventing IκB degradation [23], or atorvastatin, prevented this reduction (Figure 1A). These data suggest that NF-κB may be involved in the reduced expression of PDK4 after LPS stimulation. Then, we tested whether atorvastatin reduced NF-κB activation in LPS-stimulated H9c2 cardiac cells by measuring the mRNA levels of the NF-κB target gene MCP-1 [24]. As expected, LPS stimulation led to a strong induction of the mRNA levels of MCP-1 (8.5-fold, $P<0.001$) (Figure 1B), whereas coincubation of LPS-stimulated cells with either parthenolide or atorvastatin significantly reduced LPS-mediated induction of MCP-1. Thus, in the presence of parthenolide LPS stimulation led to a 5–fold induction ($P<0.05$ vs LPS-stimulated cells), whereas atorvastatin achieved a 5.5-fold induction ($P<0.05$ vs LPS-stimulated cells). These findings suggest that atorvastatin, like parthenolide, inhibits NF-κB activation.

3.2. NF-κB Inhibitors Prevent LPS-Induced NF-κB Activation

The results shown above suggest that atorvastatin restores the expression of PDK4 by its capacity to prevent NF-κB activation. To demonstrate this fully, we performed EMSA to investigate whether atorvastatin and parthenolide prevented NF-κB activation in LPS-stimulated H9c2 cardiac cells. These studies showed that the NF-κB probe formed three

complexes with cardiac nuclear proteins (complexes I to III, Figure 2). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures (Figure 2A). NF-κB was characterized by incubating nuclear extracts with an antibody directed against the p65 subunit of NF-κB. Addition of this antibody to incubation mixtures resulted in a supershift of complexes I and II, thus showing that these complexes contained p65. NF-κB binding activity, mainly of specific complexes I and II, increased in cells stimulated with LPS for 1 hour (Figure 2B). In contrast, in the presence of parthenolide or atorvastatin the LPS-induced increase in NF-κB binding activity was abolished.

3.3. NF-κB inhibitors Prevent PGC-1 downregulation in H9c2 LPS-stimulated cells

Next, we sought to determine the molecular mechanism by which LPS stimulation led to a fall in the expression of PDK4 mRNA. As stated above, PDK4 expression is under the control of PPARβ/δ and the activity of this transcription factor is influenced by the coactivator PGC-1 [13]. The analysis of the mRNA levels of PGC-1 (Figure 3A) showed that LPS stimulation led to a 30% reduction ($P<0.05$), whereas when cells were stimulated by LPS in the presence of either parthenolide or atorvastatin this reduction was prevented. We then analyzed PGC-1 protein expression. As shown in Figure 3B, LPS stimulation affected neither the p65 subunit of NF-κB nor PPARβ/δ expression. However, the protein expression of PGC-1 was reduced by LPS stimulation, but the fall in its expression was prevented when LPS-stimulated H9c2 cardiac cells were incubated in the presence of either parthenolide or atorvastatin.

3.4. LPS Stimulation Reduces the Interaction of PGC-1 with PPARβ/δ.

Wang *et al.* [12] clearly demonstrated that PGC-1 is a powerful coactivator of PPARβ/δ and that both proteins physically interact in skeletal muscle cells. Therefore, we evaluated whether the fall in the expression of PGC-1 caused by LPS stimulation affected their interaction with PPARβ/δ by performing co-immunoprecipitation studies with antibodies against PGC-1 and PPARβ/δ. Data shown in Figure 4 demonstrate that LPS stimulation led to a reduction in the physical interaction between PGC-1 and PPARβ/δ, whereas this reduced interaction was prevented by atorvastatin treatment. These data indicate that reduced PGC-1-PPARβ/δ association may contribute to the reduction in PPARβ/δ-mediated expression of PDK4.

3.5. NF-κB Inhibitors Prevent the LPS-stimulated Fall in PPARβ/δ binding activity.

Finally, we assessed whether the reduced protein-protein interaction between PPARβ/δ and PGC-1 affected the binding activity of the former in LPS-stimulated cells in the presence or in the absence of parthernolide or atorvastatin. As shown in Figure 5, the PPRE probe used formed two main complexes with H9c2 nuclear extracts and the incubation with an antibody against PPARβ/δ led to a supershift of complex I, suggesting that this complex contained PPARβ/δ. Interestingly, the profile of bands obtained with the PPRE probe was the opposite of that reported for the NF-κB probe. Thus, LPS stimulation reduced the binding activity of PPARβ/δ, whereas this binding activity was restored in LPS-stimulated cells treated with either parthenolide or atorvastatin.

4. Discussion

In the present study we show that stimulation of H9c2 cardiac cells with LPS, which leads to NF-κB activation [25], caused a fall in the expression of PDK4, a PPARβ/δ–target gene involved in fatty acid metabolism [13]. By using H9c2 cardiac cells, which only express the PPARβ/δ subtype [17], we excluded the potential interference caused by PPARα in the changes observed. Further, the fall in the expression of PDK4 in LPS-stimulated H9c2 cells was prevented by the NF-κB inhibitors atorvastatin and parthenolide, pointing to the involvement of NF-κB in the down-regulation of this gene. All these findings suggest that the impairment of PPARβ/δ activity has an important effect on the fall of PDK4 expression in cardiac cells. A recent study corroborated this view demonstrating that cardiomyocyte-restricted PPARβ/δ deletion reduced myocardial fatty acid oxidation and the mRNA expression of genes involved in this process, such as PDK4, and led to cardiomyopathy [18].

The mechanism by which LPS-induced activation of NF-κB results in a fall in the expression of PDK4 seems to involve reduced expression of the PGC-1 coactivator, although other mechanisms may also contribute. It has been reported that PGC-1α acts as a transcriptional coactivator for PPARβ/δ and activates the expression of genes involved in fatty acid metabolism together with PPARβ/δ [11]. In addition, PPARβ/δ physically interacts with PGC-1α, leading to enhanced PPARβ/δ activity [12]. Therefore, the reduced expression of PGC-1 in LPS-stimulated H9c2 cells may affect PPARβ/δ activity, affording an explanation for the reduction in the expression of the PPARβ/δ–target gene PDK4. In fact, in this study we demonstrate that the fall in the expression of PGC-1 in LPS-stimulated cells results in a reduction in the physical interaction between PGC-1 and PPARβ/δ. Furthermore, we found that exposure of H9c2 cardiac cells to LPS caused a dramatic reduction in the binding of the PPARβ/δ protein to the PPRE probe. This reduction was reversed in the presence of the NF-κB inhibitors atorvastatin and parthenolide, confirming the involvement of this redox transcription factor in the changes observed. Therefore, the reduced binding activity of PPARβ/δ seemed to be related to the activation of NF-κB by LPS.

Growing evidence suggests that PPARβ/δ plays an important role in the control of fatty acid metabolism in healthy heart and in cardiac hypertrophy [17,18,26]. In fact, it is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are the consequence of cardiac hypertrophy or the reason for it. However, several factors

support the view that cardiac metabolism affects the development of cardiac hypertrophy. Thus, an increase in the activities of several glycolytic enzymes has been reported prior to cardiac hypertrophy [27]. Moreover, the fact that the PPARα gene influences human left ventricular growth in response to exercise and hypertension, indicates that maladaptative cardiac substrate utilization can play a causative role in the pathogenesis of left ventricular hypertrophy [28]. Knowledge of the mechanisms controlling fatty acid metabolism in cardiac cells may be crucial for preventing or treating cardiac hypertrophy. The findings of the present study indicate that NF-κB activation, which plays a pivotal role in cardiac hypertrophy since inhibition of this transcription factor blocks or attenuates the hypertrophic response of cultured cardiac myocytes [25,29-31], may affect cardiac lipid metabolism by reducing PGC-1 expression. Interestingly, the fact that the expression of PGC-1 drops in cardiac hypertrophy [32] is in agreement with this possibility. Furthermore, since atorvastatin prevents the fall in PGC-1, this mechanisms may contribute to the reported antihypertrophic effect of statins [33,34].

In summary, in the present study we show that LPS-mediated activation of NF-κB results in a fall in the expression of PGC-1. This results in a reduction in the physical interaction between this coactivator and PPARβ/δ, which in turn may lead to a reduced binding of PPARβ/δ to its *cis*-regulatory element, causing a fall in the expression of genes such as PDK4 involved in cardiac fatty acid metabolism. Interestingly, all these changes were prevented in the presence of the NF-κB inhibitors atorvastatin and parthenolide, suggesting that activation of this transcription factor was responsible for the changes observed. These findings suggest that PGC-1 down-regulation in cardiac cells after NF-κB activation may affect the expression of PPARβ/δ–target genes involved in cardiac fatty acid oxidation.

Acknowledgments

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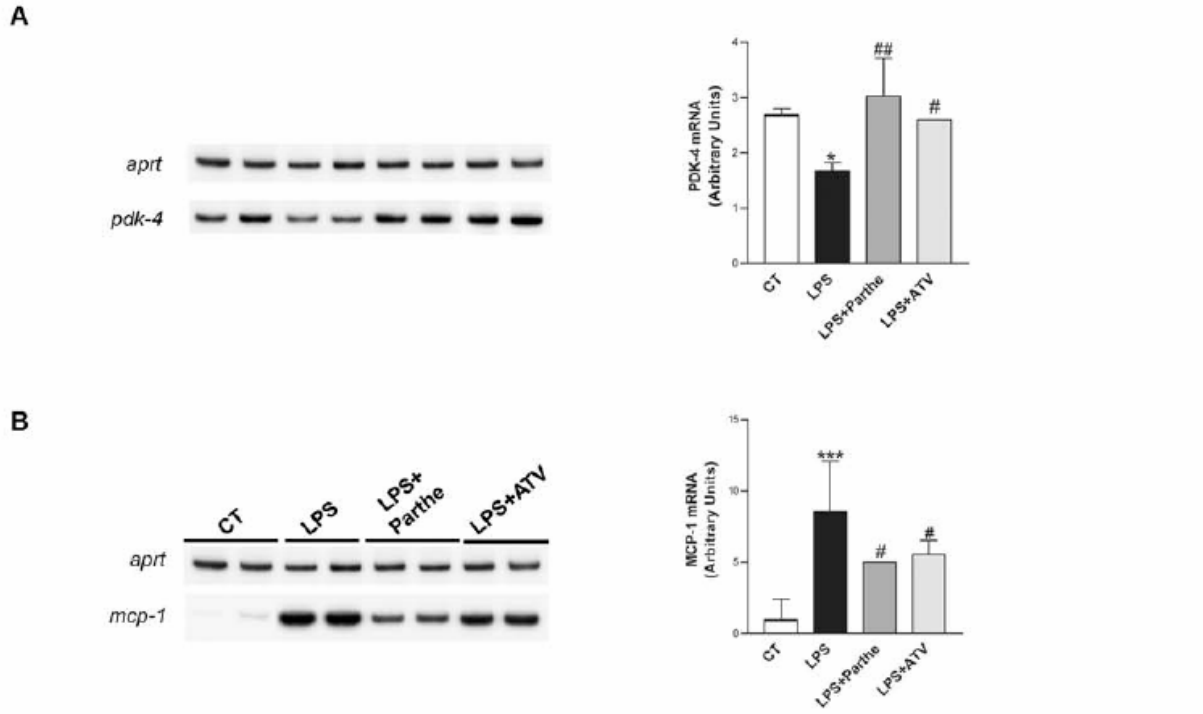

Figure 1. Down-regulation of *Pdk4* in LPS-stimulated H9c2 cardiac cells. Analysis of the mRNA levels of *Pdk4* (A) and *Mcp-1* (B) in LPS-stimulated H9c2 cardiac cells in the presence or in the absence of either 10μM parthenolide or 10μM atorvastatin. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 5 different experiments. * P<0.05 and *** P<0.001 vs control. #P<0.05 and ##P<0.01 vs LPS-stimulated H9c2 cardiac cells.

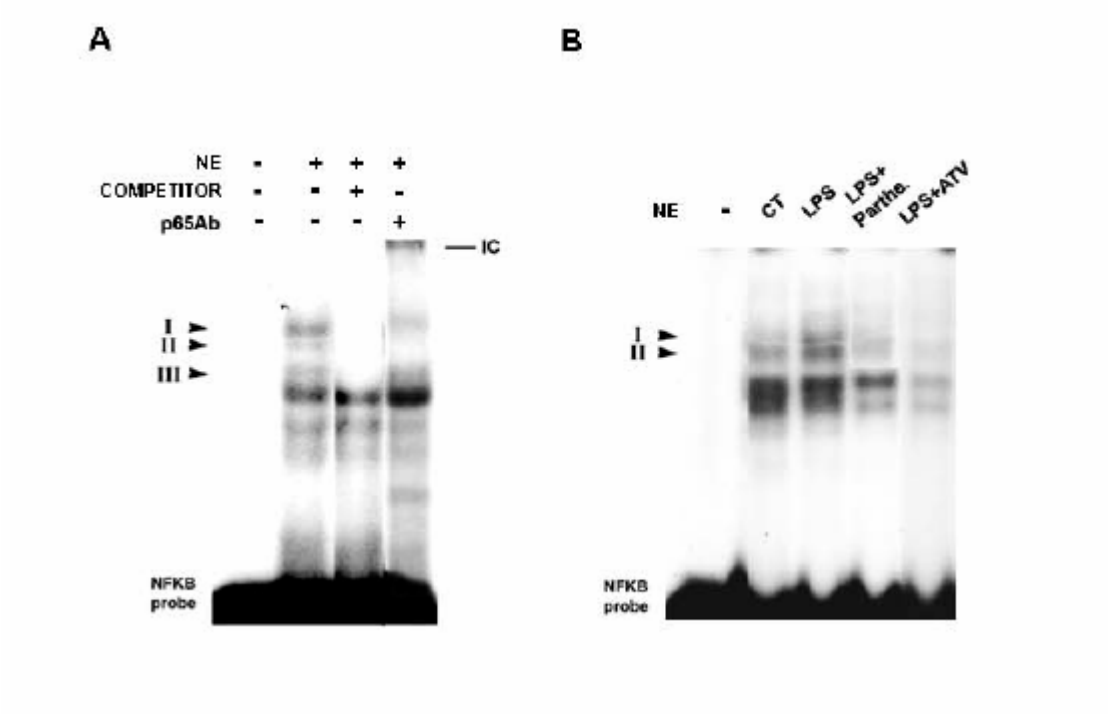

Figure 2. NF-κB inhibitors prevent LPS-induced NF-κB activation in H9c2 cardiac cells. A, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear extracts (NE). Three specific complexes (I to III), based on competition with a molar excess of unlabeled probe, are shown. The supershift immune complex (IC) obtained by incubating NE with an antibody directed against the p65 subunit of NF-κB is also shown. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and NE from H9c2 cardiac cells stimulated with LPS for 1 hour in the presence or in the absence of either 10μM parthenolide or 10μM atorvastatin. Autoradiograph data are representative of three separate experiments.

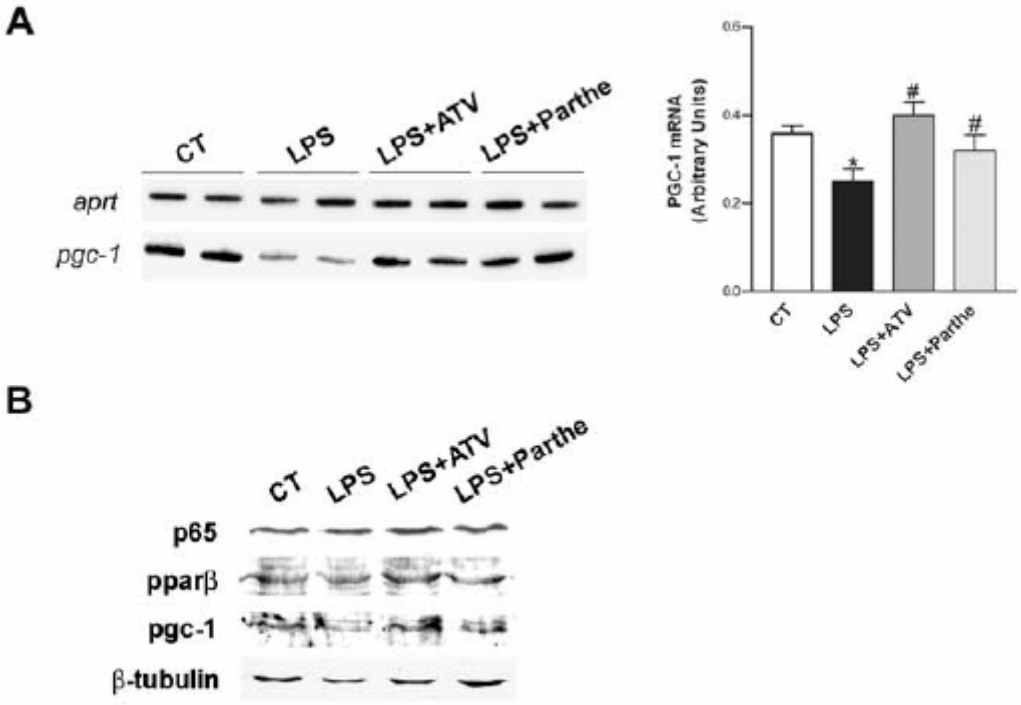

Figure 3. Downregulation of *Pgc-1* in LPS-stimulated H9c2 cardiac cells. A, Analysis of the mRNA levels of *Pgc-1* in LPS-stimulated H9c2 cardiac cells in the presence or in the absence of either 10μM parthenolide or 10μM atorvastatin. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 5 different experiments. * $P<0.05$ vs control. $^{\#}P<0.05$ vs LPS-stimulated H9c2 cardiac cells. B, Protein extracts from H9c2 cardiac cells stimulated with LPS for 1 hour in the presence or the absence of either 10μM parthenolide or 10μM atorvastatin were assayed for Western-blot analysis with p65 (A), PPARβ/δ (B), Pgc-1 (C) and β-tubulin (D) antibodies. The blot data represent three separate experiments.

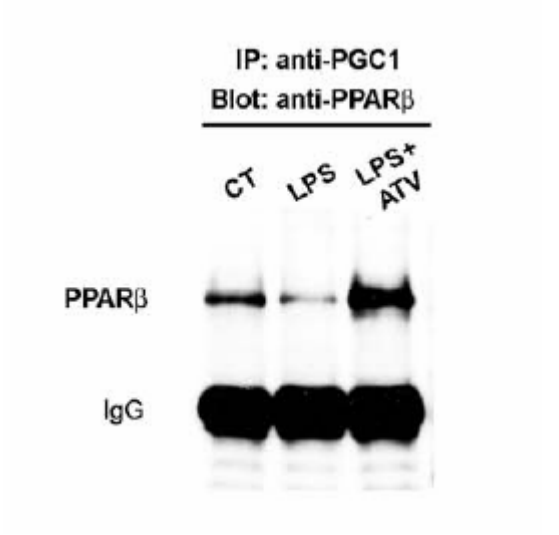

Figure 4. Atorvastatin prevents the reduction in the protein-protein interaction between Pgc-1 and PPARβ/δ. Nuclear extracts from H9c2 cardiac cells stimulated with LPS for 1 hour in the presence or the absence of 10 μM atorvastatin were subjected to immunoprecipitation using anti-Pgc-1 antibody coupled to protein-A agarose beads. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted with an anti-PPARβ/δ antibody. The blot data are representative of three separate experiments.

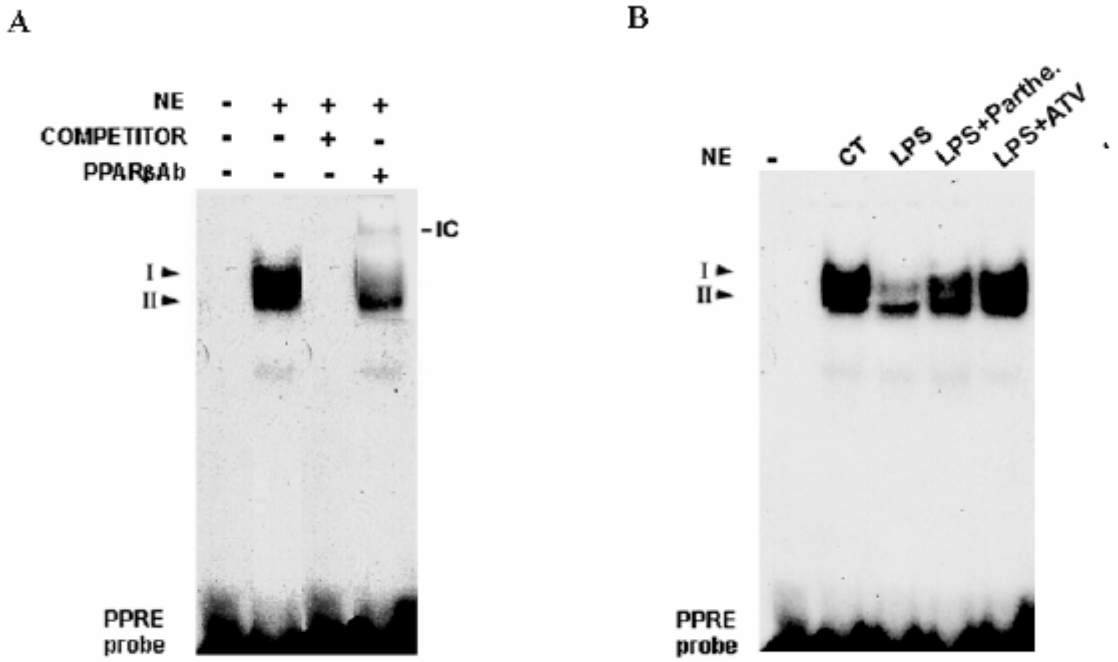

Figure 5. NF-κB Inhibitors Prevent the LPS-mediated Reduction in PPARβ/δ binding activity. A, Autoradiograph of EMSA performed with a ^{32}P-labeled PPRE nucleotide and NE. Two specific complexes (I to II), based on competition with a molar excess of unlabeled probe, are shown. The supershift immune complex (IC) obtained by incubating NE with an antibody directed against PPARβ/δ is also shown. B, Autoradiograph of EMSA performed with a ^{32}P-labeled PPRE nucleotide and nuclear extracts from H9c2 cardiac cells stimulated with LPS for 1 hour either in the presence or in the absence of either parthenolide or atorvastatin. All the autoradiograph data are representative of three separate experiments.

V

Atorvastatin Suppresses the Stimulation of the Akt/Glycogen Synthase Kinase-3β Pathway by Cardiac Hypertrophic Stimuli

A Novel Potential Antihypertrophic Mechanism of Statins

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En avaluació

Atorvastatin Suppresses the Stimulation of the Akt/Glycogen Synthase Kinase-3β Pathway by Cardiac Hypertrophic Stimuli

A Novel Potential Antihypertrophic Mechanism of Statins

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Abstract

Objective: Although it has been reported that statins inhibit cardiac hypertrophy by reducing the generation of reactive oxygen species, it is not yet known whether other mechanisms contribute to the antihypertrophic effect of these drugs.

Methods and Results: In this study, we examined the effect of the statin atorvastatin on the Akt/GSK-3β pathway. Our findings indicate that atorvastatin inhibits cardiac hypertrophy and prevents NF-κB activation in pressure-overload cardiac hypertrophy. Furthermore, the increase in the phosphorylation of Akt and GSK-3β caused by pressure-overload cardiac hypertrophy was suppressed by atorvastatin treatment. To test whether the effect of atorvastatin on the phosphorylation of Akt and GSK-3β was direct we performed *in vitro* studies using embryonic rat heart-derived H9c2 cells and neonatal rat cardiomyocytes. LPS stimulation of H9c2 myotubes enhanced NF-κB activity and Akt/GSK-3β phosphorylation, whereas preincubation with atorvastatin prevented these changes. Two inhibitors of NF-κB, SN-50 and TPCK, caused a similar effect, suggesting that atorvastatin suppressed the activity of this transcription factor. Finally, TNFα-stimulated neonatal rat cardiomyocytes showed increased phosphorylation of Akt and GSK-3β, whereas preincubation with atorvastatin before stimulation with TNF-α abolished these changes.

Conclusion: These findings point to a new potential antihypertrophic effect of statins and place NF-κB upstream of Akt/GSK-3β activation in the sequence of signaling events leading to cardiac hypertrophy.

Key words: Cardiac hypertrophy, NF-κB, Atorvastatin, Akt, GSK-3β, TNFα.

Introduction

Many pathological stimuli (such as arterial hypertension, valvular heart disease, myocardial infarction, and cardiomyopathy) induce the heart to undergo adaptive hypertrophic growth that temporarily augments cardiac function. Although the initial hypertrophic response may be beneficial, sustained hypertrophy often undergoes a transition to heart failure, which is a leading cause of mortality and morbidity worldwide, and is characterized by progressive deterioration in cardiac function. The signaling mechanisms leading to cardiac hypertrophy have been extensively investigated over the past decade [1-4]. However, the nature of the cross talk between these pathways remains unclear. Equally important is the dissection of the pathways counteracting these prohypertrophic signaling mechanisms, which thereby alleviates the hypertrophic responses.

Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role, since it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes [5-8]. Given the complexity of the hypertrophic response it remains unknown how the inhibition of this single transcription factor is sufficient for preventing the development of the hypertrophic response. Inhibition of NF-κB may interfere other pathways, thus explaining the key role of this transcription factor in cardiac hypertrophy. The Akt/Glycogen synthase kinase-3β (GSK-3β) pathway is one of the most remarkable signaling pathways converging with NF-κB in cardiac hypertrophy. Activation of the hypertrophic program by stimulation of β-adrenergic receptors, Gq-coupled receptors and Fas receptors inactivates endogenous GSK-3β via Ser9 phosphorylation, predominantly through the phosphatidylinositol 3-kinase/Akt (protein kinase B) pathway [9-14]. Although it is well established that activation of Akt stimulates NF-κB activity (given that IκB kinase is a substrate of Akt), whether NF-κB inhibition affects the Akt/GSK-3β pathway during cardiac hypertrophy remains unknown.

The 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) reductase inhibitors, or statins, are widely prescribed cholesterol-lowering agents that decrease the incidence of myocardial infarction and ischemic stroke. In addition to inhibiting cholesterol synthesis, statins also inhibit NF-κB activation [15]. Moreover, it has been recently reported that statins prevent cardiac

hypertrophy by inhibiting the generation of reactive oxygen species [16]. However, it is not yet known whether other mechanisms contribute to the antihypertrophic effects of statins. In this study, we report that atorvastatin prevents the activation of the Akt/GSK-3β pathway, by a mechanism that involves NF-κB inhibition. These findings reveal a new potential antihypertrophic effect of statins and place NF-κB upstream of Akt/GSK-3β activation in the sequence of signaling events leading to cardiac hypertrophy.

Methods

Materials

Atorvastatin was provided by Pfizer Laboratories. [γ-^{32}P]dATP (3000 Ci/mmol) was purchased from Amersham Pharmacia Biotech KK. All other chemicals were from Sigma.

Pressure overload-induced cardiac hypertrophy

Male Sprague-Dawley rats (225 to 250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1ºC). They were fed standard diet (Panlab, Barcelona, Spain) for five days before the studies began. The animals were randomly distributed into three groups as follows: (1) sham-operated rats, (2) pressure overloaded rats and (3) pressure overloaded rats treated with atorvastatin. Five days before the surgical procedure rats were fed either a control diet or a diet containing 0.05% (wt/wt) atorvastatin (which resulted in approximately 15 mg/kg/day) and continued for the 15-days post-surgery. The diets were prepared as described elsewhere [17]. Throughout the study, weight and daily food intake were measured. Pressure overload was induced by constriction of the abdominal aorta at the suprarenal level with 7-0 nylon strings by ligation with a blunted 25-gauge needle, which was then pulled out. For the age-matched sham operation, the identical procedure was performed except that the suture was not tied around the aorta. Hearts were harvested 15 days after the surgical operation. The heart weight/body weight (HW/BW) ratio was calculated and the heart samples were frozen in liquid nitrogen and then stored at -80ºC. Animal handling and disposal were performed in accordance with the law 5/1995, 21st July, from the Generalitat de Catalunya.

Cell Culture

Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described elsewhere [18]. The media were changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 hours before treatments. In this study, TNFα (10 ng/ml for 15 minutes) was used to stimulate neonatal rat cardiomyocytes. Atorvastatin (5 μmol/L) was added to the medium 24 h before the addition of TNFα, whereas SN-50 (10 μmol/L) was added 1 h prior

to the addition. Animal handling and disposal were performed in accordance with law 5/1995, 21st July, of the Generalitat de Catalunya.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced by differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA analysis H9c2 cells were stimulated with LPS (10 ng/mL) for 24 hours in the presence or the absence of either atorvastatin (5 μmol/L) or parthenolide (10 μmol/L), which was added for the last 6 hours. For EMSA studies, H9c2 cells were treated for 24 h with atorvastatin (5 μmol/L) and parthenolide (10 μmol/L) before stimulation with LPS (10 ng/ml) for 1h. For Western analysis, atorvastatin (5 μmol/L) was added 24 h before LPS (10 ng/ml for 1h) stimulation, whereas SN-50 (10 μmol/L) and TPCK (10 μmol/L) were added 1 h before stimulation with LPS.

RNA preparation and analysis

Total RNA was isolated using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as described elsewhere [19]. The sequences of the sense and antisense primers used for amplification were: atrial natriuretic factor (ANF), 5'-TCCTCTTCCTGGCCTTTTGGC-3' and 5'-AGACGGGTTGCTTCCCCAGTC-3'; α-actinin, 5'-GGCTGTGTTCCCATCCATCGT-3' and 5'-CCCGGTTAGCTTTGGGGTTCA-3'; monocyte chemoattractant protein 1 (MCP-1), 5'-GGGCCTGTTGTTCACAGTTGC-3' and 5'-GGGACACCTGCTGCTGGTGAT-3' and APRT (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGGCCAGTCACCTGA-3' and 5'-CCAGGCTCACACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, α-actinin: 266 bp, MCP-1: 157 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

Immunoblotting

Cell lysates and nuclear extracts from hearts and cells were obtained as described elsewhere [19]. Proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). Samples were analyzed for the phosphorylation status of Akt on Ser^{473} and GSK-3β on Ser^{9} (Cell Signaling Technology, Inc, Beverly, MA) by Western blot analysis using phosphorylation site-specific antibodies. In addition, Western blot analysis of total Akt was performed using an antibody that recognizes both the phosphorylated and unphosphorylated proteins (Santa Cruz Biotechnology, Santa Cruz, CA) and IκBα was analyzed using a rabbit polyclonal antibody against this protein (Santa Cruz Biotechnology, Inc). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). The size of detected proteins was estimated using protein molecular-mass standards (Life Technologies). Bands were quantified by video-densitometric scanning (Vilbert Lourmat Imaging).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were isolated as described elsewhere [19]. EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the nuclear factor-κB (NF-κB) nucleotide (5'AGTTGAGGGGACTTTCCCAGGC-3') and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3'). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5x kinase buffer, 1 μl of T4 polynucleotide kinase (10 U./μl), and 2.5 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) incubated at 37ºC for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP, the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) following the manufacturer instructions. Ten micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer (10 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA and 50 μg/ml poly(dI-dC)), in a final volume of 15 μl. Labeled probe (approximately 60.000 cpm) was added and the reaction was incubated for 15 min. at 4ºC. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4ºC. Protein-DNA complexes were resolved by electrophoresis at 4ºC on a 5% acrylamide gel and subjected to autoradiography.

Statistical Analyses

Results were obtained from at least four independent experiments. Bands intensities were analyzed by densitometry. Significant differences were established by Student's *t*-test or one-way ANOVA, according to the number of groups compared, using the computer program GraphPad Instat V2.03 (GraphPad Softwware Inc., San Diego, CA). When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Differences were considered significant at $P<0.05$.

Results

Atorvastatin treatment prevents pressure overload-induced cardiac hypertrophy

Cardiac hypertrophy is characterized by increased HW/BW ratio, induction of fetal-type genes (e.g. ANF) and sarcomeric disorganization. Therefore, we first examined the effects of atorvastatin on these parameters in the pressure-overload model of cardiac hypertrophy in order to clearly demonstrate the antihypertrophic effects of this drug. The HW/BW ratio significantly increased (1.35-fold, $P<0.001$) after aortic constriction compared with sham-operated rats (Figure 1A). Treatment with atorvastatin completely abolished the increase in the HW/BW ratio ($P<0.01$ vs banded rats). Further, pressure overload also roughly doubled the mRNA levels of ANF compared with sham-operated rats (Figure 1B). However, in the heart of atorvastatin-treated rats the expression levels of ANF after atorvastatin treatment dropped below control values (sham-operated rats), as reported elsewhere [20]. Similarly, the mRNA levels of the sarcomere-associated protein α-actinin were 2-fold higher than those of sham-operated rats (Figure 1C). This induction was not observed in the heart of atorvastatin-treated rats.

Atorvastatin Treatment Prevents NF-κB Activation and Akt/GSK-3β Phosphorylation in Pressure Overload-Induced Cardiac Hypertrophy

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes [5-8] and it has been reported that statins inhibit the activation of this redox transcription factor in vascular cells [5], we performed EMSA studies to test whether atorvastatin inhibits NF-κB activation in pressure overload-induced cardiac hypertrophy. These studies showed that the NF-κB probe formed four main specific complexes with cardiac nuclear proteins (complexes I to IV, Figure 2A), based on competition experiments performed by adding excess unlabeled NF-κB oligonucleotide to incubation mixtures. NF-κB binding activity increased in banded rats, especially complex III (2.5-fold increase, $P<0.01$ *vs* sham-operated rats) (Figure 2B), which was abolished by atorvastatin treatment ($P<0.01$ *vs* banded rats). NF-κB was characterized by incubating nuclear extracts with an antibody directed against the p65 subunit of this transcription factor. Addition of this antibody to incubation mixtures resulted in complete supershift of complex III, showing that this complex contained p65 (Figure 2C). No changes were observed in the DNA binding of cardiac nuclear proteins from each group of rats to an Oct-1 probe, indicating that the increase in the NF-κB probe was specific (Figure

2D). Overall, these data demonstrate that atorvastatin inhibits NF-κB activation in pressure overload-induced cardiac hypertrophy and that this mechanism may contribute to the antihypertrophic effect of this compound.

We next examined the effects of cardiac hypertrophy and atorvastatin treatment on the Akt/GSK-3β pathway. In banded rats, Akt and GSK-3β phosphorylation were higher than in sham-operated rats (Figure 3). In contrast, atorvastatin-treated rats showed no increase in the phosphorylation of these proteins. These findings do not demonstrate the direct effect of atorvastatin on the Akt/GSK-3β pathway, since this may be indirectly abolished as a result of the inhibition of the hypertrophic growth of the heart. To test whether atorvastatin directly inhibits the phosphorylation of Akt and GSK-3β, we performed *in vitro* studies using embryonic rat heart-derived H9c2 cells. LPS is a compound commonly used to activate NF-κB in cardiomyocytes [21]. As expected, H9c2 cells stimulated with LPS for 24 hours showed robust induction (7-fold, $P<0.001$) of the mRNA levels of the NF-κB target gene, MCP-1 (Figure 4 A). This induction was significantly reduced when cells treated with LPS were preincubated with atorvastatin (-35%, $P<0.05$ compared with cells stimulated with LPS) or the NF-κB inhibitor parthenolide (40-%, $P<0.05$ compared with cells stimulated with LPS), which specifically inhibits the activation of NF-κB by preventing IκB degradation [22]. To clearly demonstrate the effects of atorvastatin on NF-κB activity in H9c2 cells after LPS treatment, we performed EMSA assays. NF-κB probe formed three complexes with cardiac nuclear proteins (complexes I to III, Figure 4B). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding excess unlabeled NF-κB oligonucleotide to incubation mixtures. LPS stimulation for 1 hour increased binding activity, especially that of specific complex II, which was prevented when cells were preincubated with atorvastatin or parthenolide. NF-κB was characterized by incubating nuclear extracts with an antibody directed against the p65 subunit of NF-κB. Addition of this antibody to incubation mixtures resulted in complete supershift of complex II, demonstrating that this complex contained p65. Finally, we analyzed the potential direct effects of atorvastatin and NF-κB inhibitors on Akt and GSK-3β phosphorylation. LPS stimulation for 1 hour increased Akt and GSK-3β phosphorylation, whereas when these cells were preincubated with atorvastatin, Akt phosphorylation was reduced to values similar to those present in control cells (Figure 4C). To test whether the effect caused by atorvastatin was due to the inhibition of NF-κB, we used SN-50, a cell-permeable peptide that inhibits NF-κB by blocking its translocation to the nucleus [23], and TPCK, another commonly used pharmacological

inhibitor of NF-κB that acts by inhibiting IκB degradation [24-26]. Treatment with SN-50, which is highly selective for NF-κB and has no effect on the activities of any other signaling molecule [27], reduced Akt phosphorylation by LPS (Figure 4D), but not GSK-3β phosphorylation. In constrast, treatment with TPCK did not affect the phosphorylation of Akt, but reduced GSK-3β phosphorylation caused by LPS. Differences between these two NF-κB inhibitors, SN-50 and TPCK, may arise from their distinct mechanisms of action. In any case, both inhibitors blocked Akt/GSK-3β at different steps, either Akt or GSK-3β.

Atorvastatin Prevents TNF-α-induced Akt/GSK-3β Phosphorylation in Neonatal Rat Cardiomyocytes

To clearly demonstrate the effects of atorvastatin on the Akt/GSK-3β pathway during cardiac hypertrophy, we used neonatal rat cardiomyocytes. These cells undergo cardiac hypertrophy when exposed to stimuli such as TNFα [5,28]. This cytokine is a commonly used activator of NF-κB, supporting the link between NF-κB activation and the development of cardiac hypertrophy. TNFα treatment causes rapid degradation of IκB proteins, thus allowing the nuclear translocation of NF-κB, which enhances the DNA binding activity of NF-κB [29,30]. As expected, TNFα treatment for 15 minutes stimulated IκBα degradation (Figure 5). Preincubation with either atorvastatin or SN-50 did not modify the effects of TNFα on IκBα protein levels (Figure 5). Stimulation with TNFα enhanced Akt and GSK-3β phosphorylation. In neonatal rat cardiomyocytes preincubated with atorvastatin for 24 hours before stimulation with TNF-α a reduction in Akt and GSK-3β phosphorylation was observed. Preincubation with SN-50 for 1 hour prior LPS stimulation reduced Akt phosphorylation. In contrast, the effect of this NF-κB inhibitor on the phosphorylation of GSK-3β was less intense. These findings demonstrate that atorvastatin attenuates the Akt/GSK-3β pathway in rat neonatal cardiomyocytes exposed to hypertrophic stimuli such as TNF-α.

Discussion

Whereas a plethora of signaling cascades have been implicated in the development of cardiac hypertrophy [3], relatively little is known about the intrinsic mechanisms with the potential to inhibit or even reverse hypertrophy. In the present study we demonstrate that atorvastatin, similarly to other statins [16], inhibits pressure overload-induced cardiac hypertrophy. Further our findings indicate for the first time that treatment with this statin may inhibit cardiac hypertrophy by a new mechanism that involves inhibition of the Akt/GSK-3β pathway. This Akt/GSK-3β pathway plays an important role in the development of cardiac hypertrophy. Thus, cardiac-specific overexpression of active mutants of Akt induces hypertrophy in transgenic mice [31,32] and GSK-3β functions as a negative regulator of cardiac hypertrophy [9-13]. The inhibition of the Akt/GSK-3β pathway caused by atorvastatin seems to involve the NF-κB transcription factor, since pharmacological inhibitors of NF-κB also inhibit the Akt/GSK-3β pathway. Our findings also place NF-κB upstream of Akt/GSK-3β, which is in agreement with a previous report in neurons [33]. In addition, the finding that the NF-κB and the Akt/GSK-3β pathway converge during cardiac hypertrophy may explain how the inhibition of NF-κB prevents a complex process, such as cardiac hypertrophy, which involves several pathways.

TNFα is a cytokine produced in the myocardium under pressure or volume overload but not present in normal rats [34-36]. Under short-term experimental conditions, TNFα induces negative inotropic effects [36,37], but when chronically overexpressed in the myocardium, TNFα leads to hypertrophy, cardiac enlargement, and death [38,39]. Interestingly, our findings demonstrate that atorvastatin suppresses the phosphorylation of Akt and GSK-3β in TNFα-stimulated neonatal rat cardiomyocytes, suggesting that treatment with this statin reduces the contribution of the Akt/GSk-3β pathway to the development of cardiac hypertrophy. Furthermore, cardiac hypertrophy after transplantation is associated with persistent expression of TNFα [40], whereas the contribution of hypertension is minimal. Thus, the use of statins may block the effects of TNFα on Akt/GSK-3β phosphorylation and attenuate the development of cardiac allograft hypertrophy.

The mechanism responsible for the abolishment of the Akt/GSK-3β pathway by atorvastatin seems to involve inhibition of the NF-κB pathway, since inhibitors of this transcription factor

exerted similar effects. However, in this study the pharmacological inhibitors of NF-κB used differed. Atorvastatin was the most potent inhibitor of this pathway, since in both H9c2 myotubes stimulated with LPS and neonatal rat cardiomyocytes stimulated with TNFα, statin treatment blocked the phosphorylation of Akt and GSK-3β. In contrast, some differences were observed between SN-50 and TPCK. The former mainly reduced Akt phosphorylation in H9c2 myotubes and neonatal rat cardiomyocytes, whereas the latter reduced the phosphorylation of GSK3-β in H9c2 myotubes. These differences may be due to their distinct mechanisms of action.

In summary, atorvastatin inhibits the activation of the Akt/GSK-3β pathway by hypertrophic stimuli through a mechanism that may involve the suppression of NF-κB activity and that may contribute to the antihypertrophic effects of statins.

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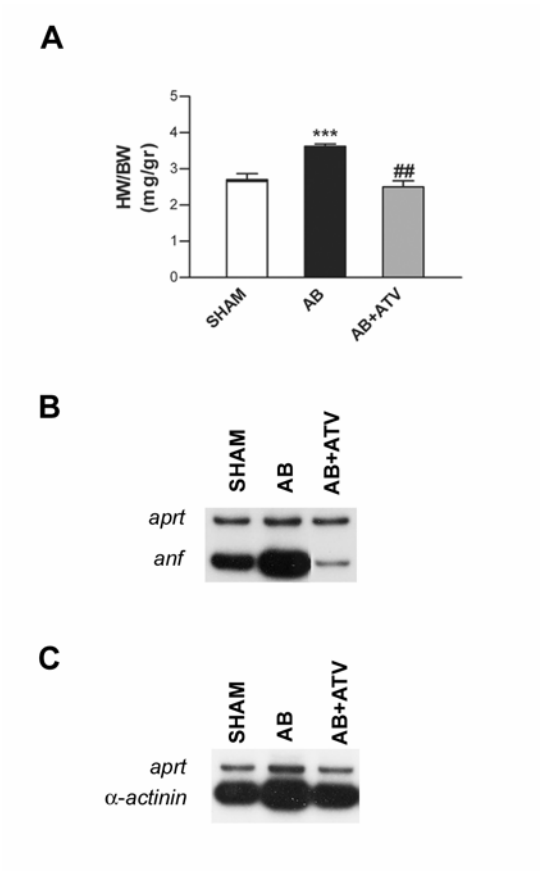

FIG. 1. Atorvastatin inhibits pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. Treatment with atorvastatin was initiated 5 days before operation and continued for 15 days as food admixture at a concentration of 0.05% (wt/wt). At 15 days after operation, hearts were excised and weighed. A, Analysis of the HW/BW ratio in sham-operated rats, in banded rats (aortic banding, AB) and in banded rats treated with atorvastatin (AB+ATV). Analysis of the mRNA levels of ANF (B) and α-actinin (C) in pressure overload-induced cardiac hypertrophy. A representative autoradiogram of the four animals studied per treatment group is shown. Data are expressed as mean ± S.D. of 4 different experiments. **P <0.01, *** P<0.001 vs sham-operated rats. # P<0.05, ## P<0.01 vs banded rats.

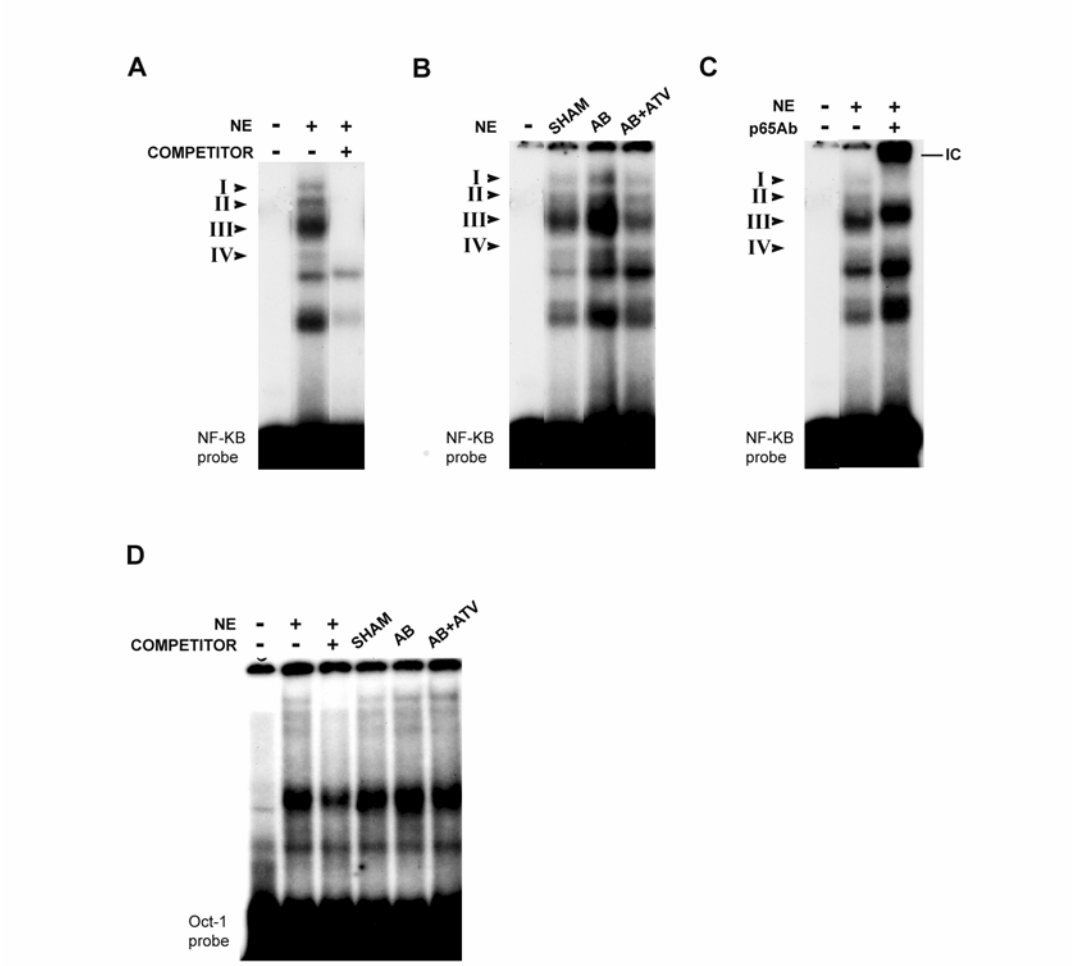

FIG. 2. Treatment with atorvastatin prevents NF-κB activation in pressure overload-induced cardiac hypertrophy. A, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear protein extract (NE) shows four specific complexes (I to IV), based on competition with a molar excess of unlabeled probe. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and cardiac NE from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV). C, Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. Supershifted immune complex (IC) is denoted. D, Autoradiograph of EMSA performed with a ^{32}P-labeled Oct-1 nucleotide. All autoradiograph data are representative of four separate experiments.

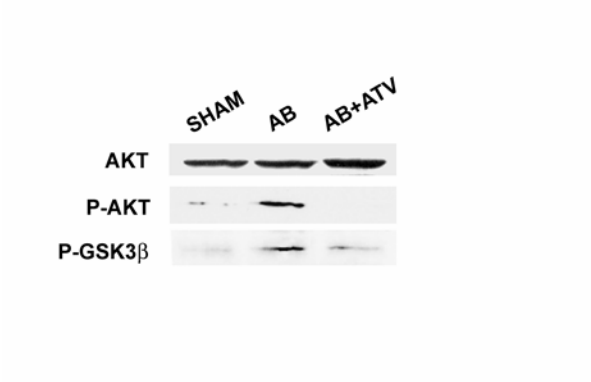

FIG. 3. Atorvastatin inhibits the Akt/GSK-3β pathway in pressure overload-induced cardiac hypertrophy. Protein extracts from hearts from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with atorvastatin (AB+ATV) were subjected to immunoblot analysis as described under "Material and Methods". Representative immunoblots using anti-Akt antibody, anti-phospho-Akt (ser-473) antibody and anti-phospho-GSK-3β (ser-9) antibody are shown. Blot data are representative of three separate experiments.

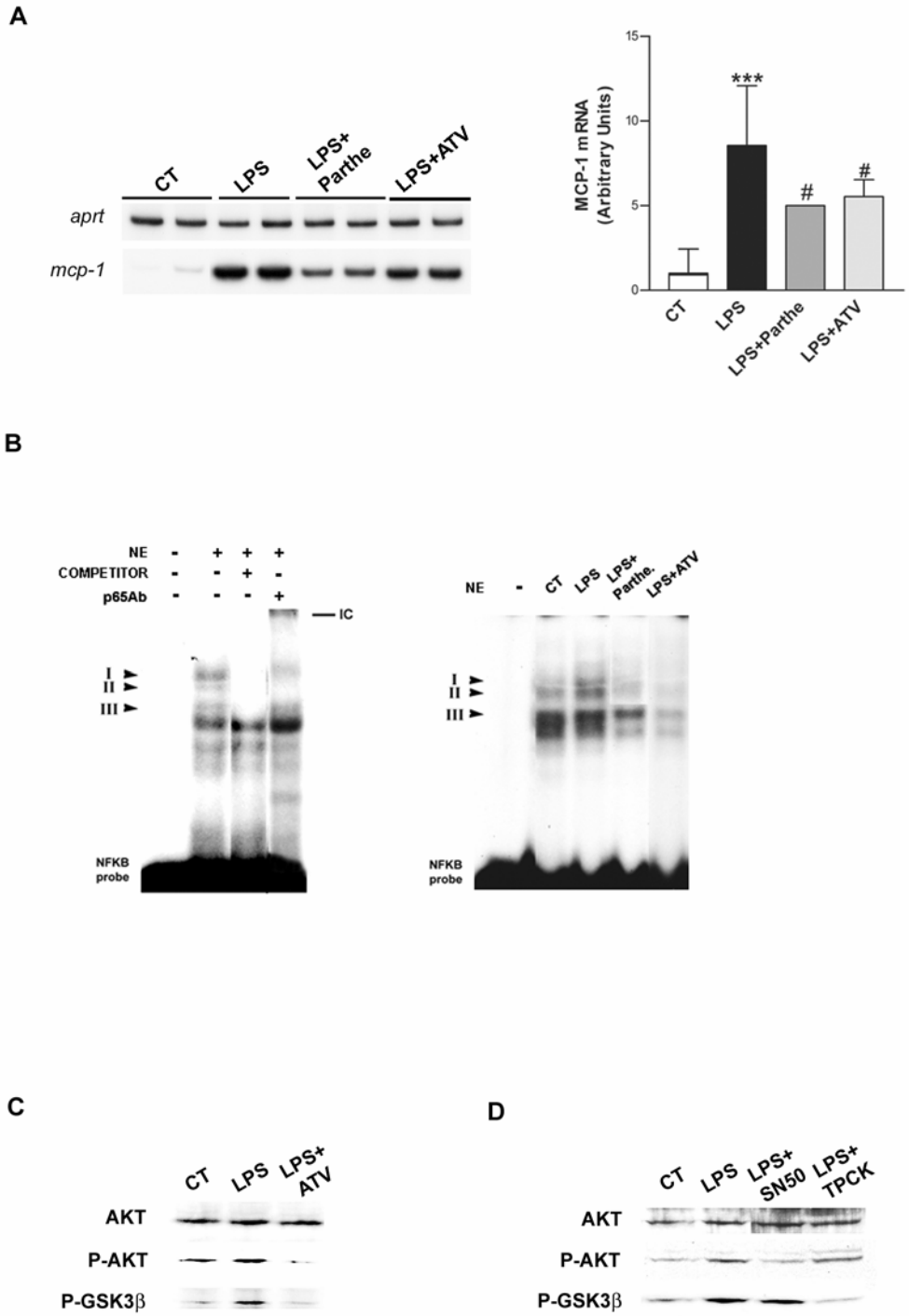
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FIG. 4. Atorvastatin prevents NF-κB activation and Akt/GSK-3β phoshorylation in LPS-stimulated H9c2 myotubes. A, Analysis of the mRNA levels of MCP-1 in LPS-stimulated H9c2 myotubes in the presence or in the absence of either 5 μmol/L atorvastatin or 10 μmol/L parthenolide. A representative autoradiogram and the quantification normalized to the APRT mRNA levels is shown. Data are expressed as mean ± S.D. of 5 different experiments. *** $P<0.001$ vs control. $^{\#}P<0.05$ vs LPS-stimulated myotubes. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear extracts (NE) from H9c2 myotubes stimulated with LPS for 1 hour. Supershift analysis was performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. The supershifted immune complex (IC) is denoted by the arrow. Autoradiograph data are representative of three separate experiments. C, Effects of atorvastatin (C) and NF-κB inhibitors on the Akt/GSK-3β pathway from LPS-stimulated H9c2 myotubes. Cells were non-stimulated or stimulated with LPS (10 ng/ml for 1 h). When indicated atorvastatin (5 μmol/L) was added 24 h prior LPS (10 ng/ml for 1h) stimulation, whereas SN-50 (10 μmol/L) and TPCK (10 μmol/L) were added 1 h prior stimulation with LPS. Whole cell lysates were prepared and subjected to immunoblot analysis as described under "Material and Methods". Representative immunoblots using anti-Akt antibody, anti-phospho-Akt (ser-473) antibody and anti-phospho-GSK-3β (ser-9) antibody are shown. Blot data are representative of three separate experiments.

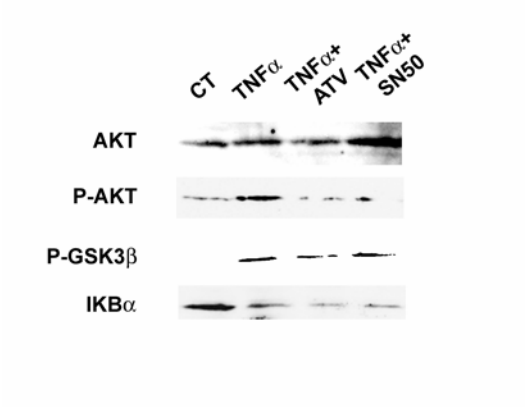

FIG. 5. Atorvastatin prevents Akt/GSK-3β phoshorylation in TNFα-stimulated neonatal rat cardiomyocytes. Cells were non-stimulated or stimulated with TNFα (10 ng/ml for 1 h). When indicated atorvastatin (5 μmol/L) was added 24 h before TNFα stimulation, whereas SN-50 (10 μmol/L) was added 1 h before stimulation. Whole cell lysates were prepared and subjected to immunoblot analysis as described under "Material and Methods". Representative immunoblots using anti-Akt antibody, anti-phospho-Akt (ser-473) antibody, anti-phospho-GSK-3β (ser-9) antibody and IκBα antibody are shown. Blot data are representative of three separate experiments.

VI

Inhibition of Cardiac Hypertrophy by Triflusal (4-Trifluoromethyl Derivative of Salicylate) and Its Active Metabolite

Anna Planavila, Ricardo Rodríguez-Calvo, Manuel Merlos, Juan C. Laguna, Manuel Vázquez-Carrera

En avaluació

Inhibition of Cardiac Hypertrophy by Triflusal (4-Trifluoromethyl Derivative of Salicylate) and Its Active Metabolite

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Running title: Triflusal and its active metabolite inhibit cardiac hypertrophy

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Abstract

Aims Nuclear Factor (NF)-κB signaling pathway is an important intracellular mediator of cardiac hypertrophy. The aim of the present study was to determine whether triflusal (2-acetoxy-4-tri-fluoromethylbenzoic acid), a salicylate derivative used as antiplatelet agent, and its active metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB) inhibit cardiac hypertrophy *in vitro* and *in vivo* by blocking the NF-κB signaling pathway.

Methods and results In cultured neonatal rat cardiomyocytes HTB (300 μmol/L, a concentration reached in clinical use) inhibited phenylephrine(PE)-induced protein synthesis ([^{3}H]leucine uptake), induction of the fetal-type gene atrial natriuretic factor (ANF) and sarcomeric disorganization. Assessment of the effects of triflusal in pressure overload-induced cardiac hypertrophy by aortic banding resulted in a significant reduction in the ratio heart weight/body weight and in a reduction of the mRNA levels of the cardiac hypertrophy markers ANF and α-actinin compared with untreated banded rats. Electrophoretic mobility shift assay revealed an increase in the NF-κB binding activity in cardiac nuclear extracts of banded rats that was prevented by triflusal treatment. Interestingly, banded rats treated with oral triflusal showed enhanced protein levels of IκBα, which forms a cytoplasmic inactive complex with the p65-p50 heterodimeric complex, compared with untreated rats. Finally, treatment of embryonic rat heart-derived H9c2 cells with HTB increased levels of both mRNA and protein levels of IκBα, suggesting that the changes caused by triflusal on IκBα expression occur at the transcriptional level.

Conclusion These results indicate that triflusal, a drug with a well-characterized pharmacological and safety profile currently used as antiplatelet, inhibits cardiomyocyte growth by interfering NF-κB signaling pathway through enhanced IκBα expression.

Key words: Cardiac hypertrophy, triflusal, NF-κB.

Introduction

Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction, and cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia, and sudden death [1,2]. Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role, since it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes [3-6]. The transcription factor NF-κB can be activated by a wide array of exogenous and endogenous stimuli and plays a critical role in mediating immune and inflammatory responses. In resting cells, NF-κB is present in the cytoplasm as an inactive heterodimer, consisting of the p50 and p65 subunits complexed with an inhibitor protein subunit, IκB. After stimulation, a serine kinase cascade is activated leading to the phosphorylation of IκB. This event converts IκB in a substrate for ubiquitination and subsequent degradation, releasing the NF-κB heterodimer, which then translocates to the nucleus and regulates the expression of genes involved in inflammatory and immune processes.

Non-steroid anti-inflammatory drugs (NSAID), such as salicylates, are potent inhibitors of inflammatory processes, which act by blocking prostaglandin synthesis via inhibition of cyclooxygenase activity [7,8], and more recently their activity has been also associated to their ability to inhibit the activation of the transcription factor NF-κB [9-12]. Triflusal (2-acetoxy-4-tri-fluoromethylbenzoic acid) is a NSAID structurally related to the salicylate group of compounds, with a characterized pharmacological profile (Figure 1). Triflusal has an antiplatelet effect and has been largely used for the prevention and/or treatment of vascular thromboembolisms [13]. Recent studies have shown that triflusal and its deacetylated metabolite 2-hydroxy-4-trifluoromethylbenzoic acid (HTB) block the inflammatory related transcription factor NF-κB more effectively than aspirin [14-17]. Unlike aspirin, the effects of triflusal are found at concentrations reached in its therapeutic use as antiplatelet agent [13,14]. The key role played by NF-κB activation in the development of cardiac hypertrophy [3-6] may suggest a potential role for triflusal in the inhibition of cardiac hypertrophy.

In this study, we examined the effects of HTB on phenylephrine(PE)-induced hypertrophy in neonatal rat cardiac myocytes and of triflusal in pressure overload-induced cardiac

hypertrophy in rats. We found that these drugs inhibit cardiac hypertrophy through a mechanism that may involve downregulation of NF-κB signaling pathway by increasing the expression of IκBα.

Methods

Materials

Triflusal and HTB were from Uriach Laboratories. [γ-^{32}P]dATP (3000 Ci/mmol) and [^{3}H]leucine (50 Ci/mmol) were purchased from Amersham Pharmacia Biotech KK. Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories and Alexa flouro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were from Molecular Probes. All other chemicals were purchased from Sigma.

Cell Culture

Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously [18]. The media was changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 hours before treatments. In this study phenylephrine (PE) was used to stimulate neonatal rat cardiomyocytes. Animal handling and disposal were performed in accordance with law 5/1995, 21st July, of the Generalitat de Catalunya.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA and protein analysis H9c2 cells in DMEM medium were treated with 500 μmol/L HTB for 24 hours.

Incorporation of [^{3}H]leucine

To examine the effect of PE on protein synthesis, the incorporation of [^{3}H]leucine was measured essentially by the method of Thaik et al. [19]. Cultured neonatal rat ventricular myocytes were treated with PE in the presence or in the absence of drugs and coincubated with [^{3}H]leucine (1 μCi/mL) for 24 hours. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4ºC for 30 minutes to precipitate the protein. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted with scintillation counter.

Immunocytochemistry

Neonatal rat ventricular myocytes were fixed in ice-cold 100% methanol for 10 min. Anti-α–actinin antibody and anti-ANF polyclonal antiserum were added at dilutions 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 hour at room temperature. Secondary antibodies, Alexa flouro 488 goat anti-rabbit and Alexa flouro 568 goat anti-mouse, were used at a dilution of 1:300 in PBS containing 5% rat serum and incubated for 30 min at room temperature. Immunofluorescence was visualized using a confocal laser fluorescence microscope Olympus Fluoview FV500. Photographic images were taken from five random fields.

RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx, Houston). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR) as previously described [20]. The sequences of the sense and antisense primers used for amplification were: ANF, 5'-TCCTCTTCCTGGCCTTTTGGC-3' and 5'-AGACGGGTTGCTTCCCCAGTC-3'; α-actinin, 5'-GGCTGTGTTCCCATCCATCGT-3' and 5'-CCCGGTTAGCTTTGGGGTTCA-3'; IκBα, 5'-TGAAGGGAGACCTGGCCTTCC-3' and 5'-GTGGCCGTTGTAGTTGGTGGTGGC-3' and APRT (adenosyl phosphoribosyl transferase), 5'-GCCTCTTGGCCAGTCACCTGA-3' and 5'-CCAGGCTCACACACTCCACCA-3'. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, α-actinin: 266 bp, IκBα: 263 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

Pressure overload-induced cardiac hypertrophy

Male Sprague-Dawley rats (225 to 250 g) were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature (21 ± 1ºC). They were fed standard diet (Panlab, Barcelona, Spain) for five days before the studies began. The animals were randomly distributed into three groups as follows: (1) sham-operated rats, (2) pressure overloaded rats and (3) pressure overloaded rats with triflusal. Five days before the surgical procedure, rats were fed either a control diet or a diet containing 0.05% (wt/wt) triflusal (which resulted in approximately 15 mg/kg/day). The diets were prepared as previously described [21]. Throughout the study, the

weight and daily food intake were measured. Pressure overload was induced by constriction of the abdominal aorta at the suprarenal level with 7-0 nylon strings by ligation with a blunted 25-gauge needle, which was then pulled out. For the age-matched sham operation, the identical procedure was performed except that the suture was not tied around the aorta. Hearts were harvested 15 days after the surgical operation. The heart weight/body weight (HW/BW) ratio was calculated and the heart samples were frozen in liquid nitrogen and then stored at -80ºC. Animal handling and disposal were performed in accordance with the law 5/1995, 21st July, from the Generalitat de Catalunya.

Immunoblotting

Cell lysates and nuclear extracts from hearts were obtained as previously described [20]. Proteins (30 μg) were separated by SDS-PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride membranes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against IκBα, IκBβ and p65 (Santa Cruz Biotechnology, Inc) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek Ltd., Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies). Further, IκBα was identified by using a blocking peptide (Santa Cruz Biotechnologies) against this protein.

Electrophoretic mobility shift assay (EMSA)

Isolation of nuclear extracts was performed as previously described. EMSA was performed using double-stranded oligonucleotides (Promega, Madison, WI) for the consensus binding site of the nuclear factor-κB (NF-κB) nucleotide (5'AGTTGAGGGGACTTTCCCAGGC-3') and Oct-1 (5'-TGTCGAATGCAAATCACTAGAA-3'). Oligonucleotides were labeled in the following reaction: 2 μl of oligonucleotide (1.75 pmol/μl), 2 μl of 5x kinase buffer, 1 μl of T4 polynucleotide kinase (10 U./μl), and 2.5 μl of [γ-^{32}P] ATP (3000 Ci/mmol at 10 mCi/ml) incubated at 37ºC for 1 h. The reaction was stopped by adding 90 μl of TE buffer (10 mM Tris-HCl pH 7.4 and 1 mM EDTA). To separate the labeled probe from the unbound ATP the reaction mixture was eluted in a Nick column (Pharmacia, Sant Cugat, Spain) according to the manufacturer's instructions. Ten micrograms of crude nuclear proteins were incubated for 10 min on ice in binding buffer (10 mM Tris-HCl pH 8.0, 25 mM KCl, 0.5 mM DTT, 0.1 mM EDTA pH 8.0, 5% glycerol, 5 mg/ml BSA, 100 μg/ml tRNA and 50 μg/ml poly(dI-dC)), in a

final volume of 15 μl. Labeled probe (approximately 60.000 cpm) was added and the reaction was incubated for 15 min. at 4ºC. Where indicated, specific competitor oligonucleotide was added before the labeled probe and incubated for 10 min on ice. p65 antibody was added 15 min before incubation with the labeled probe at 4ºC. Protein-DNA complexes were resolved by electrophoresis at 4ºC on a 5% acrylamide gel and subjected to autoradiography.

Statistical Analyses

Results were obtained from at least four independent experiments and presented as mean±S.D. Significant differences were established by Student's *t*-test or one-way ANOVA, according to the number of groups compared, using the computer program GraphPad Instat V2.03 (GraphPad Softwware Inc., San Diego, CA). Differences were considered significant at $P<0.05$.

Results

HTB, but not Aspirin, Inhibits PE-induced Cardiac Hypertrophy in Neonatal Rat Cardiomyocytes

Cardiac hypertrophy is characterized by increase protein content (e.g. [^{3}H]leucine uptake), induction of fetal-type genes (e.g. ANF) and sarcomeric disorganization. Therefore, we first examined the effects of HTB on these parameters in a primary culture of neonatal rat cadiomyocytes. Cells were pretreated with either vehicle or drugs for 30 minutes and subsequently stimulated with 100 μmol/L PE for 24 hours. As shown in Figure 2A, [^{3}H]leucine incorporation was significantly increased by PE (1.5-fold, $P<0.001$) and this was inhibited by HTB at 300 μmol/L (-74%, $P<0.01$ compared with PE-induced cells), whereas this drug had no effect at 100 μmol/L. In contrast, 300 μmol/L aspirin (Acetyl Salicylic Acid, ASA) did not affect [^{3}H]leucine incorporation. PE-induced cardiac hypertrophy also led to approximately 2-fold induction in the mRNA levels of the sarcomere-associated protein α-actinin (Figure 2B). In contrast, in the presence of 300 μmol/L HTB PE-induced α-actinin expression was abolished. Immunostaining of cardiac myocytes for α-actinin and the fetal cardiac protein ANF clearly shown an increase in cardiac myocyte size and ANF protein expression following PE stimulation (Figure 2C). These changes were blocked in the presence of HTB in the culture medium.

Triflusal Treatment Inhibits Pressure Overload-Induced Cardiac Hypertrophy

In order to evaluate whether our *in vitro* findings had physiological relevance we evaluated the effects of triflusal using the pressure overload model of cardiac hypertrophy. HW/BW ratio significantly increased (1.35-fold, $P<0.001$) after aortic constriction compared with sham-operated rats (Figure 3A). Treatment with triflusal attenuated the increase in the HW/BW ratio (1.12-fold, $P<0.01$ vs banded rats). Further, pressure overload enhanced mRNA levels of the cardiac hypertrophy markers ANF and α-actinin, compared with sham-operated rats (Figure 3B and C), and these changes were abolished by triflusal treatment.

Triflusal Treatment Inhibits NF-κB Activation in Pressure Overload-Induced Cardiac Hypertrophy

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes [3-6] and it has been reported that triflusal inhibits the activation of this redox transcription factor [14-17],

we performed EMSA studies to investigate whether triflusal inhibited NF-κB activation in pressure overload-induced cardiac hypertrophy. These studies shown that the NF-κB probe formed four specific complexes with cardiac nuclear proteins (complexes I to IV, Figure 4A), based on competition experiments performed by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures. NF-κB binding activity increased in banded rats, especially of complex III, compared with sham-operated rats (Figure 4B) and this effect was abolished by triflusal treatment. Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of this transcription factor. Addition of this antibody to incubation mixtures resulted in complete supershift of complex III, thus showing that this complex contained p65 (Figure 4C). No changes were observed in the DNA binding of cardiac nuclear proteins from the different groups of rats to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (Figure 4D). Overall, these data demonstrate that triflusal inhibits NF-κB activation in pressure overload-induced cardiac hypertrophy and that this mechanism may contribute to the antihypertrophic effect of this compound.

Triflusal treatment enhances IκBα levels in heart and cardiomyocytes in culture

Finally, we sought to determine the molecular mechanism by which triflusal inhibits NF-κB activation. Inhibition of NF-κB signaling may occur through different mechanisms. One of these mechanisms may involve enhanced expression of IκBα, which forms a cytoplasmic inactive complex with the p65-p50 heterodimeric complex. When we determined the protein levels of the p65 subunit of NF-κB, IκBβ and IκBα, we observed that triflusal did no affect the expression of these proteins, except of IκBα. Triflusal significantly increased the protein levels of this inhibitor of NF-κB, suggesting that this was the mechanism responsible for the inhibition of this transcription factor (Figure 5). In order to assess whether HTB treatment resulted in increased expression of IκBα *in vitro*, we used the embryonic rat heart-derived H9c2 cells. Treatment of H9c2 cells with 500 μmol/L HTB for 24 hours resulted in enhanced levels of both mRNA (similar results were obtained treating cells with 300 μmol/L HTB for 24 hours, data not shown) and protein levels of IκBα (Figure 6), suggesting that the changes caused by triflusal on IκBα expression occur at the transcriptional level.

Discussion

Whereas a plethora of signaling cascades have been implicated in the development of cardiac hypertrophy [22], relatively little is known about the intrinsic mechanisms with the potential to inhibit or even reverse hypertrophy. In the present study we demonstrate that triflusal, which is currently used as an antiaplatelet agent, and its main metabolite, HTB, inhibit PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes and in the pressure overload animal model of cardiac hypertrophy. Further, our findings indicate that the antihypetrophic effect of these salicylate-derived compounds involves downregulation of NF-κB signaling pathway through enhanced expression of the NF-κB inhibitor IκBα.

Recent studies by several groups have implicated the activation of NF-κB as a causal event in the cardiac hypertrophy response [3-6]. Further, increased NF-κB activation was also observed in the myocardium of patients with congestive heart failure [23,24]. This association between NF-κB and myocyte hypertrophy is interesting taking into account the hypothesis that inflammatory cytokines are involved in cardiomiopathic disease states and that NF-κB itself is regulated by several of these cytokines. Here we demonstrate that triflusal and its main metabolite inhibit NF-κB activation in cardiac cells and that this mechanism may be responsible for their antihypertrophic effect. Our findings are consistent with several studies reporting that prevention of NF-κB activation leads to inhibition of cardiac hypertrophy. Thus, both peroxisome proliferator-activated receptor γ (PPARγ) activators [25] and antioxidants [4,26] can abolish the hypertrophic response of cardiomyocytes through inhibiting NF-κB activation. All these studies support the contention that NF-κB inhibition represents a potential therapeutic approach for preventing or reversing cardiac hypertrophy. Therefore, the discovery that triflusal, a drug with a well-characterized pharmacological and safety profile currently used in therapy, inhibits cardiac hypertrophy may lead to the potential use of this agent in the treatment of this pathology. Triflusal is currently used in patients with vascular occlusive diseases and it is associated with an incidence of gastrointestinal bleeding lower than aspirin [27,28]. The concentrations found to inhibit cardiac hypertrophy in this work (300 μmol/L) are easily reached after the approved dosification of triflusal (600-900 mg/day), where HTB plasma levels of near 1 mM are found [13].

Inhibition of the activity of NK-κB can be performed at different stages. For instance, antioxidants can inhibit generation of reactive oxygen species, which are a stimulus for NF-

κB activation. Interestingly, NF-κB is regulated by subcellular localization. It is retained in the cytosol by being bound to inhibitors of κB, IκB. In the present study we demonstrate that triflusal and HTB increase the expression of IκBα. Therefore, increased expression of this inhibitor of κB results in persistent binding to NF-κB, blocking its translocation to the nucleus and thus its activity. In addition, although the primary function of IκBα is to retain the NF-κB proteins in the cytoplasm, it has been reported that this inhibitor is also involved in the removal of NF-κB proteins from the nucleus [29,30]. Thus, IκB has both cytoplasmic and nuclear roles in regulating the NF-κB pathway. Regarding the mechanism responsible for the enhanced expression of IκBα in cardiac cells our findings indicate that HTB increases both IκBα protein and mRNA levels, suggesting the involvement of a transcriptional increase in the expression of this gene as the responsible for the changes observed. Further studies are necessary to investigate whether triflusal may increase IκBα expression through additional mechanisms.

Previous studies have demonstrated that triflusal and HTB inhibit NF-κB activation in endothelial cells, in brain cells and *in vivo* [14-17] more effectively than aspirin. In our studies we tested the effect of aspirin at the same concentration that HTB. In contrast to the metabolite of triflusal, aspirin treatment did not affect [^{3}H]leucine incorporation. Thus, the introduction of the trifluoromethyl group in the 4-position of salicylates confers new properties to the molecule of triflusal.

In summary, in the present study we show that triflusal and HTB inhibit cardiac hypertrophy *in vitro* and *in vivo* through a mechanism that may involve inhibition of the NF-κB signaling pathway, an important intracellular mediator of this process. Therefore, these findings suggest that triflusal may become a therapeutic option to reduce cardiac hypertrophy.

Acknowledgements

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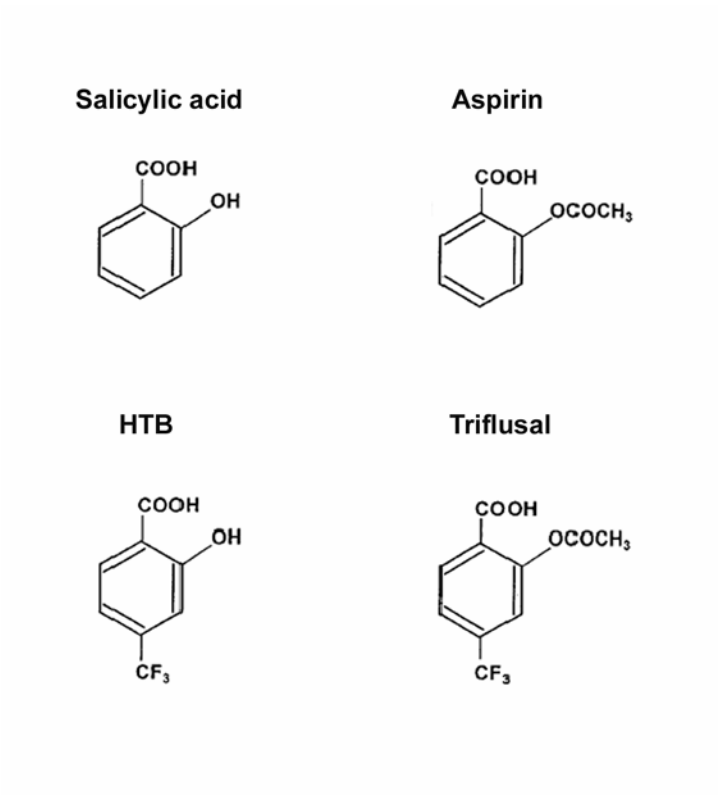
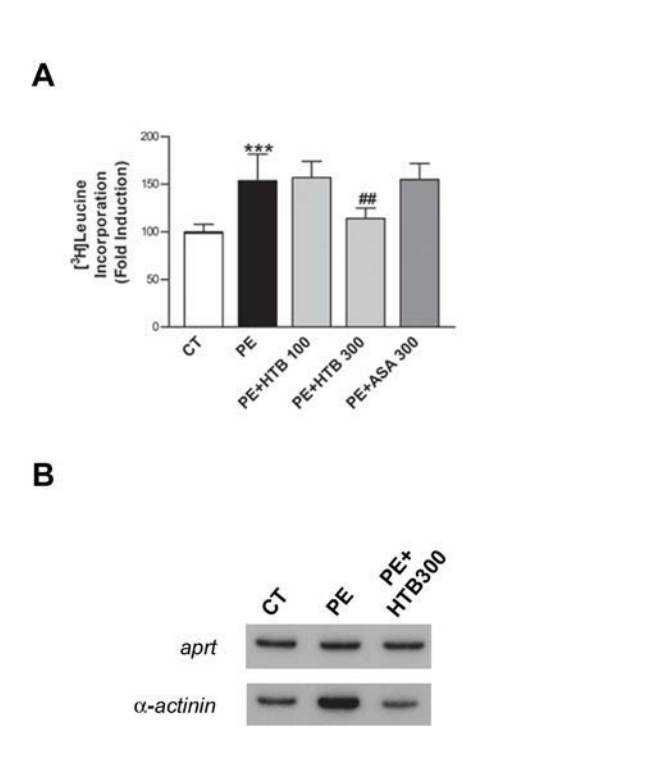


Figure 1. Chemical structures of triflusal, HTB, aspirin and salicylic acid.



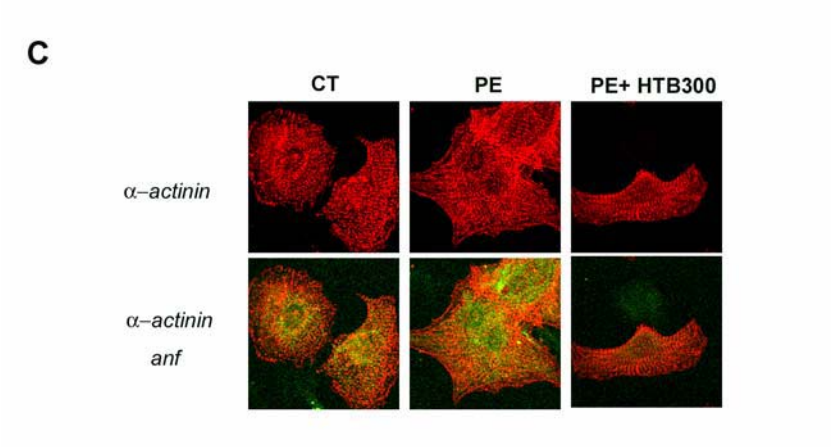


Figure 2. The main metabolite of triflusal, HTB, inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μmol/L PE in the presence or absence of HTB (100 or 300 μmol/L) or aspirin (acetylsalicylic acid, ASA) (300 μmol/L) that were added 30 minutes before experiments. A, [^{3}H]leucine incorporation was determined by coincubating cardiac myocytes with 1.0 μCi/mL [^{3}H]leucine for 24 hours. Data are expressed as mean ± SD (n=6) of the treated-to-control ratio. B, Analysis of the mRNA levels of α-actinin in PE-stimulated cardiomyocytes in the presence or absence of 300 μmol/L HTB. C, Effects of PE with and without 300 μmol/L HTB on cardiac myocyte ANF protein expression and sarcomeric organization. Double immunofluorescent microscopy was performed using specific antibodies to α-actinin (upper panel, red color) and ANF (lower panel, green color). Experiments were performed three times with similar results.*** $P<0.001$ vs control. ## $P<0.01$ vs PE-stimulated cardiac myocytes. All blot data are representative of at least four separate experiments.

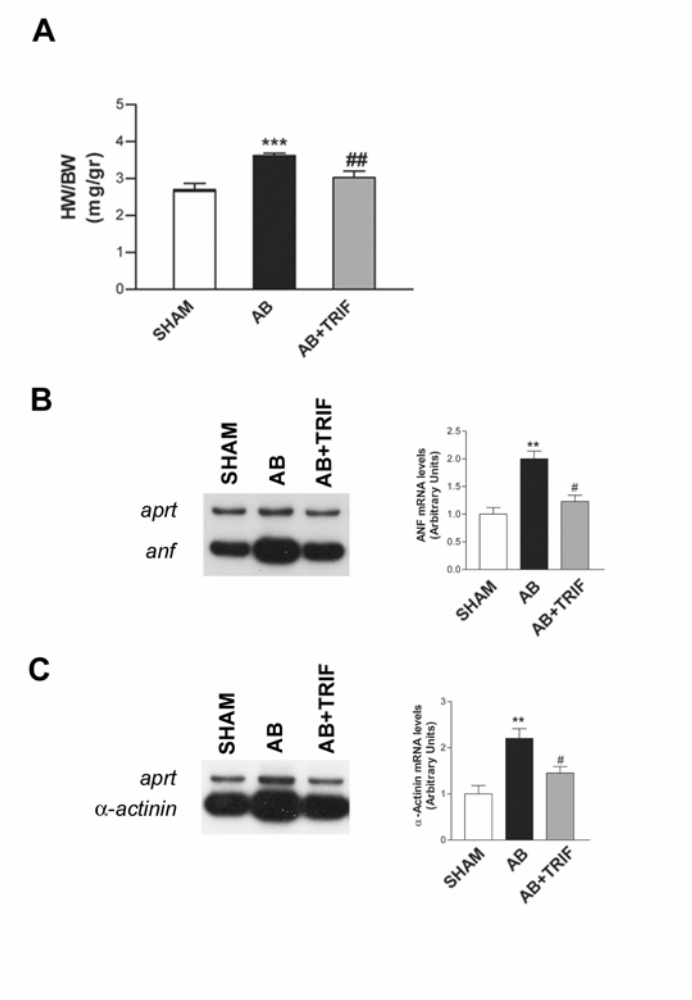


Figure 3. Triflusal inhibits pressure overload-induced cardiac hypertrophy. Pressure overload was produced by constriction of the abdominal aorta. Treatment with triflusal was initiated 5 days before operation and continued for 15 days as food admixture at a concentration of 0.05% (wt/wt). At 15 days after operation, hearts were excised and weighed. A, Analysis of the HW/BW ratio in sham-operated rats, in banded rats (aortic banding, AB) and in banded rats treated with triflusal (AB+TRIF). Analysis of the mRNA levels of ANF (B) and α-actinin (C) in pressure overload-induced cardiac hypertrophy. Data are expressed as mean ± S.D. of 6 different experiments. *** $P<0.001$ vs sham-operated rats. ## $P<0.01$ vs banded rats.

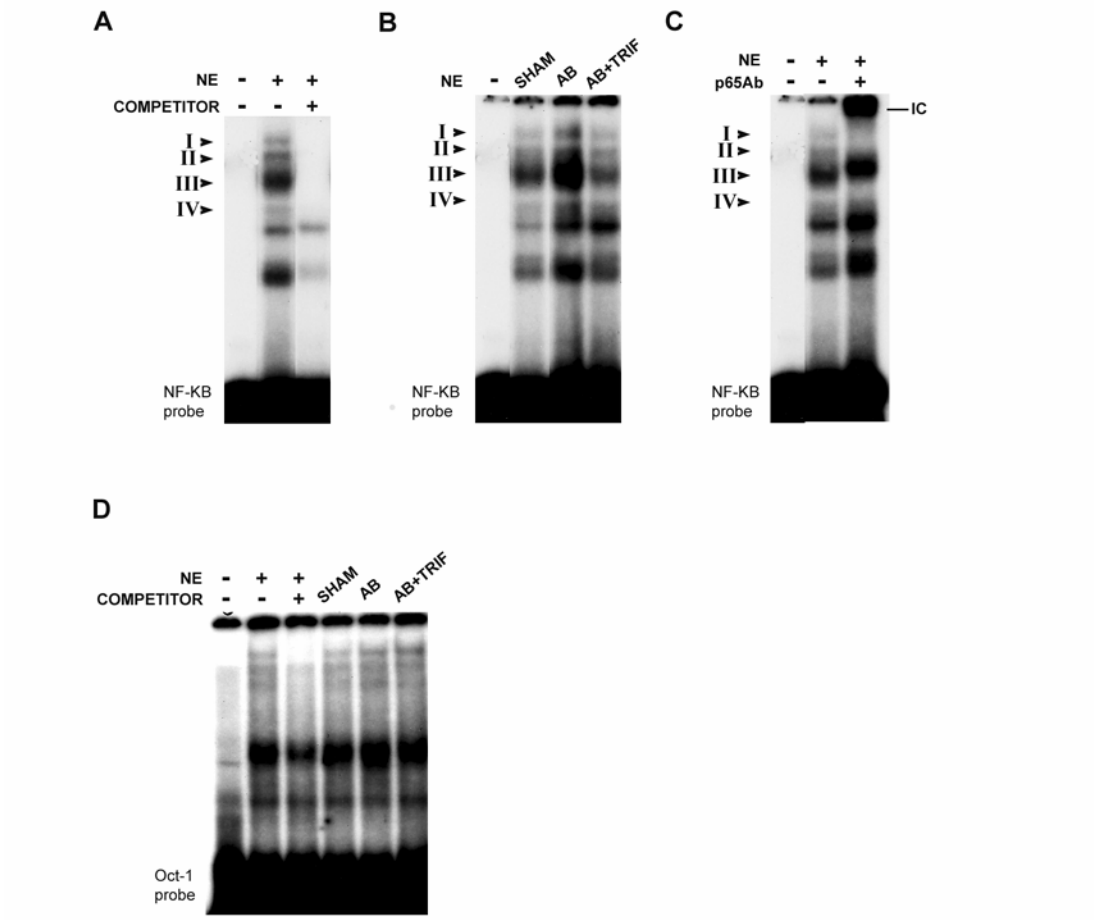


Figure 4. Treatment with triflusal prevents NF-κB activation in pressure overload-induced cardiac hypertrophy. A, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and nuclear protein extract (NE) shows four specific complexes (I to IV), based on competition with a molar excess of unlabeled probe. B, Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and cardiac NE from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with triflusal (AB+TRIF). C, Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-κB. Supershifted immune complex (IC) is denoted. D, Autoradiograph of EMSA performed with a ^{32}P-labeled Oct-1 nucleotide. All blot data are representative of at least three separate experiments.

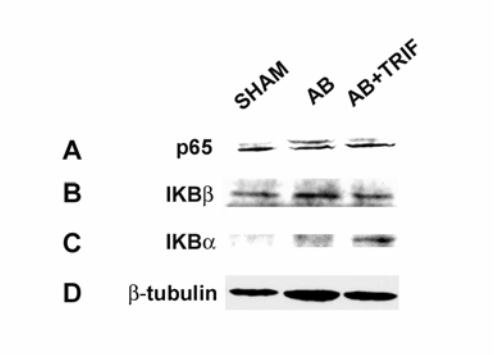


Figure 5. Triflusal treatment increases the protein levels of IκBα. Cardiac protein extracts from sham-operated rats, banded rats (aortic banding, AB) and banded rats treated with triflusal (AB+TRIF) were assayed for western-blot analysis with p65 (A), IκBβ (B), IκBα (C) and β-tubulin (D) antibodies. All blot data are representative of at least three separate experiments.

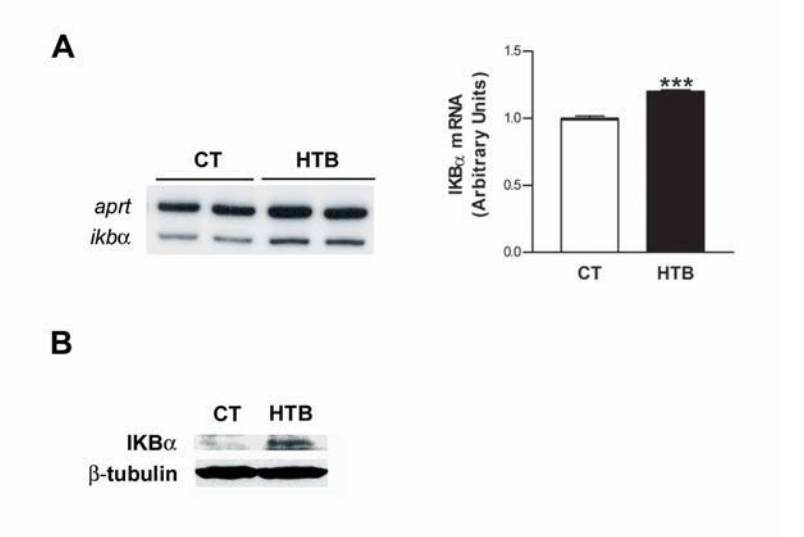


Figure 6. The active metabolite of triflusal, HTB, induces the expression of IκBα in the embryonic rat heart-derived H9c2 cells. Analysis of the mRNA levels (A) and protein levels (B) of IκBα in H9c2 myocytes in the presence or absence of 500 μmol/L HTB for 24 hours. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of 6 different experiments. *** P<0.001 vs control (CT).

VII

Increased Akt protein expression is associated with decreased ceramide content in skeletal muscle of troglitazone-treated mice

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Increased Akt protein expression is associated with decreased ceramide content in skeletal muscle of troglitazone-treated mice

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Abstract

Although it is generally believed that thiazolidinediones ameliorate insulin resistance by lowering circulating free fatty acids, direct effects of these drugs in skeletal muscle may also contribute to their antidiabetic action. We report that troglitazone administration to mice for 1 day increased the protein expression of Akt (two-fold induction, $P < 0.001$) in skeletal muscle without significant changes in the levels of free fatty acids in plasma. Increased Akt protein expression was associated with reduced phospho-AMP-activated protein kinase abundance and with a fall in the phosphorylation of acetyl-CoA carboxylase, which in turn resulted in an increase in the content of muscular malonyl-CoA (2.4-fold, $P < 0.05$) and lactate (1.4-fold, $P < 0.05$). Troglitazone treatment did not affect the mRNA levels of either Akt1 or Akt2, suggesting that a transcriptional mechanism was not involved, but caused a dramatic reduction in the content of muscular ceramides (76%, $P < 0.001$), lipid-derived second messengers known to increase Akt degradation. Our data indicate that troglitazone treatment inhibited de novo ceramide synthesis, since the content of its precursor, palmitoyl-CoA, was reduced (55%, $P = 0.05$). These results were confirmed in C2C12 myotubes, where troglitazone treatment increased Akt protein expression and prevented the reduction of this protein and the increase in ceramide levels caused by palmitate. These findings implicate ceramide as an important intermediate in the regulation of Akt after troglitazone treatment.

Keywords: Akt; PPAR; Troglitazone; AMPK; Malonyl-CoA; Ceramide

1. Introduction

Insulin resistance is a common metabolic abnormality associated with obesity, hypertension and type 2 diabetes mellitus [1]. Skeletal muscle accounts for the majority of insulin-stimulated glucose utilization and is, therefore, the major site of insulin resistance. During the development of insulin resistance in skeletal muscle an impairment of glucose utilization and insulin sensitivity has been related to the presence of increased availability of certain lipid-derived second messengers, such as ceramides, which can attenuate several insulin signaling pathways [2] leading to insulin resistance.

Thiazolidinediones (ciglitazone, pioglitazone, rosiglitazone and troglitazone) are oral antidiabetic agents that improve insulin sensitivity and glucose homeostasis in type 2 diabetic patients as well as in various animal models of diabetes and obesity [3–5]. These drugs act as ligands of the peroxisome proliferator-activated receptor-γ (PPARγ), which belongs to the nuclear hormone receptor superfamily of transcription factors [6,7]. PPARγ is highly expressed in adipose tissue and plays a pivotal role in fat cell differentiation [6,7]. Upon activation by thiazolidinediones PPARγ regulates gene expression by binding as a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) to DNA response elements called peroxisome proliferator-response elements (PPRE), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (direct repeat-1). PPARγ response elements have been identified in the regulatory regions of several genes involved in fatty acid and carbohydrate metabolism [8]. Through these transcriptional changes thiazolidinediones promote accumulation of free

Abbreviations: ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; CTE, cytosolic acyl-CoA thioesterase; M-CPT-I, muscle-type carnitine palmitoyl-transferase; PGC-1, PPARγ coactivator 1; PPAR, peroxisome proliferator-activated receptor

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fatty acids in adipocytes. Thus, this close association of PPARγ-induced lipid lowering with the enhancement of insulin action and the fact that PPARγ is highly expressed in adipose tissues, compared with muscle or liver, have lead to the hypothesis that thiazolidinediones improve muscle insulin action by sequestering lipids in adipocytes, a mechanism that ultimately reduces lipid accumulation in skeletal muscle. Accordingly, the enhanced insulin action in muscle, and perhaps in liver, after thiazolidinedione treatment is generally considered secondary to a systemic lipid-lowering effect via a principal action in adipose tissue [6,9].

However, some evidences also suggest that thiazolidinediones may directly enhance muscle insulin action, independent of the lowering of free fatty acids. Thus, in mice with lipodystrophy in which adipose tissue is essentially absent, troglitazone still improves insulin sensitivity [10]. In agreement with this fact, recent studies in isolated skeletal muscle have shown that thiazolidinediones have direct effects on muscle lipid metabolism independent of PPARγ-mediated gene expression [11]. Therefore, changes in the levels of intracellular lipids and lipid-derived second messengers (such as ceramides) after thiazolidinedione treatment may account for some of the direct antidiabetic actions of these drugs in skeletal muscle. Insulin stimulation of Akt (also referred as protein kinase B) in skeletal muscle is one of the insulin signaling pathways that may be attenuated by ceramides leading to insulin resistance [12]. Akt is a serine/threonine protein kinase that is stimulated by a variety of growth factors including insulin via a multistep pathway involving a phosphatidylinositol 3-kinase-dependent mechanism [13]. Once Akt is phosphorylated and activated, it can promote glucose uptake and subsequent metabolism via translocation of glucose transporter (GLUT) 4 to the plasma membrane [14,15]. In addition, the phosphorylation and inhibition of glycogen synthase kinase 3 by Akt activates glycogen synthase and thereby promotes glycogen synthesis [16]. On the other hand, recent evidences indicate that Akt may be related to fatty acid metabolism through its cross-talk with AMP-activated protein kinase (AMPK), since it has been reported that Akt activation can lead to decreased AMPK activity [17]. AMPK plays an important role in fatty acid metabolism in skeletal muscle since its activation stimulates a rapid phosphorylation and inactivation of acetyl-CoA carboxylase (ACC), leading to a fall in malonyl-CoA concentration, an inhibitor of carnitine palmitoyltransferase I (CPT-I), the enzyme that controls the transfer of long-chain fatty acyl-CoA into mitochondria. Therefore, AMPK activation results in an increase in fatty acid oxidation [18,19]. This cross-talk between Akt and AMPK may be one of the underlying mechanisms responsible for the inhibition of lipid oxidation and the release of lactate (anaerobic glycolysis) in isolated skeletal muscle after troglitazone treatment [11,20].

The aim of the present study was to investigate whether troglitazone treatment affected the expression of Akt and the activity of AMPK in skeletal muscle and their involvement in the effects of this drug on fatty acid and glucose metabolism. The results shown here demonstrate that troglitazone treatment increases Akt protein expression in skeletal muscle, which is associated with a reduction in the activity of AMPK to phosphorylate ACC and an increase in the levels of malonyl-CoA, a known inhibitor of fatty acid oxidation. In addition, we observed that troglitazone treatment reduced the content of ceramides in skeletal muscle, which are involved in the degradation of Akt protein. Further, troglitazone treatment increased Akt protein expression in C2C12 myotubes and prevented the reduction in the expression of this protein and the increase in ceramide levels caused by palmitate. These findings implicate ceramide as an important intermediate in the regulation of Akt afforded by troglitazone treatment.

2. Materials and methods

2.1. Materials

Troglitazone was kindly provided by Glaxo Wellcome.

2.2. Animals and treatment

Twelve male Swiss mice from Harlan (Barcelona) were used. They were maintained under standard conditions of illumination (12-h light/dark cycle) and temperature ($21 \pm 1\ ^{\circ}C$) and fed a standard diet. The mice were randomly distributed into two groups. Each group was administered, respectively, either 0.5% carboximethyl cellulose (control group) or 100 mg/kg/day of troglitazone (dissolved in 0.5% carboximethyl cellulose). Each compound was administered per os once a day for 1 day (1 ml/100 g of body weight). Food and water were given ad libitum. Twenty-four hours after administration, mice were killed under pentobarbitone anesthesia to collect blood samples and to isolate soleus skeletal muscle.

2.3. Plasma determinations

Plasma triglycerides (Sigma), non-esterified fatty acids (Wako) and glucose (Sigma) concentrations were determined by colorimetric tests.

2.4. Cell culture

Mouse C2C12 myoblasts (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin. When cells reached confluence, the medium was switched to the differentiation medium containing DMEM and 2% horse serum, which was changed every other day. After 4 additional days, the differentiated C2C12 cells had fused into myotubes. Lipid-containing media were prepared by conjugation of

palmitate with fatty acid-free bovine serum albumin, by a method modified from that described by Chavez et al. [12]. Briefly, palmitate was dissolved in ethanol and diluted 1:100 in DMEM containing 2% (w/v) fatty-acid-free bovine serum albumin. Myotubes were incubated for 16 h in serum free-DMEM containing 2% bovine serum albumin in either the presence or absence of 0.5 mM palmitate and 100 μM troglitazone. After the incubation, RNA was extracted from myotubes as described below.

2.5. RNA preparation and analysis

Total RNA was isolated by using the Ultraspec reagent (Biotecx). The total RNA isolated by this method is undegraded and free of protein and DNA contamination. Relative levels of specific mRNAs were assessed by the reverse transcription-polymerase chain reaction (RT-PCR). Complementary DNA was synthesized from RNA samples by mixing 0.5 μg of total RNA, 125 ng of random hexamers as primers in the presence of 50 mM Tris–HCl buffer (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 200 U Moloney murine leukemia virus reverse transcriptase (Invitrogen), 20 U RNAsin (Invitrogen) and 0.5 mM of each dNTP (Sigma) in a total volume of 20 μl. Samples were incubated at 37 °C for 60 min. A 5 μl aliquot of the RT reaction was then used for subsequent PCR amplification with specific primers.

Each 25-μl PCR reaction contained 5 μl of the RT reaction, 1.2 mM $MgCl_2$, 200 μM dNTPs, 1.25 μCi [^{32}P]-dATP (3000 Ci/mmol, Amersham Biosciences), 1 unit of Taq polymerase (Invitrogen), 0.5 μg of each primer and 20 mM Tris–HCl, pH 8.5. To avoid unspecific annealing, cDNA and Taq polymerase were separated from primers and dNTPs by using a layer of paraffin (reaction components contact only when paraffin fuses, at 60 °C). The sequences of the sense and antisense primers used for amplification were: PPARα, 5′-GGCTCGGAGGGCTCTGTCATC-3′ and 5′-ACATGCACTGGCAGCAGTGGA-3′; muscle-type carnitine palmitoyltransferase I (M-CPT-I), 5′-TTCACTGTGACCCCAGACGGG-3′ and 5′-AATGGACCAGCCCCATGGAGA; PPARγc coactivator-1 (PGC-1), 5′-AGAAAGGGCCCGAGCAATCTG-3′ and 5′-AGATGTGCCCCTGCCAGTCAC-3′; Akt1, 5′-GCAAGGGCACCTTTGGGAAAG-3′ and 5′-ACACGCGCTCTCGAGACAGGT-3′; Akt2, 5′-GAGAAGGCCACTGGCCGCTAT-3′ and 5′-CATAGGCGGTCATGGGTCTGG-3′; cytosolic acyl-CoA thioesterase (CTE), 5′-CAGCCACCCCGAGGTAAAAGG-3′ and 5′-CCTTGAGGCCATCCTTGGTCA-3′; and APRT (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-3′. PCR was performed in an MJ Research Thermocycler equipped with a peltier system and temperature probe. After an initial denaturation for 1 min at 94 °C, PCR was performed for 23 (PGC-1 and Akt1), 25 (PPARα, M-CPT-I, Akt2) and 27 (CTE) cycles. Each cycle consisted of denaturation at 92 °C for 1 min, primer annealing at 60 °C, and primer extension at 72 °C for 1 min and 50 s. A final 5-min extension step at 72 °C was performed. A volume of 5 μl of each PCR sample was separated on a 1-mm-thick 5% polyacrylamide gel. The gels were dried and subjected to autoradiography using Kodak X-ray films to show the amplified DNA products. Amplification of each gene yielded a single band of the expected size (PPARα: 645 bp, M-CPT-I: 222 bp, PGC-1: 234 bp, Akt1: 264 bp, Akt2: 167 bp, CTE: 224 bp and APRT: 339 bp). Preliminary experiments were carried out with various amounts of cDNA to determine non-saturating conditions of PCR amplification for all the genes studied. Therefore, under these conditions, relative quantification of mRNA was assessed by the RT-PCR method used in this study [21]. The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

2.6. Immunoblotting

Soleus muscles and C2C12 myotubes were homogenized in cold lysis buffer (5 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5.4 μg/ml aprotinin). The homogenate was centrifuged at 10,000 × *g* for 30 min at 4 °C. Protein concentration was measured by the Bradford method. Proteins (50 μg) were separated by SDS-PAGE on 10% separation gels (7% for ACC-P determination) and transferred to Immobilon polyvinylidene diflouride membranes (Millipore). Samples were analyzed for the phophorylation status of Akt on Ser^{473} (Cell Signaling Technology), AMPK on Thr^{172}, ACC on Ser^{79} by Western blot analysis using phosphorylation site-specific antibodies. In addition, total Akt, using an antibody that recognize both the phosphorylated and unphosphorylated proteins (Santa Cruz Biotechnology), heat shock protein (Hsp)27 and Hsp90 (Stressgen Biotechnologies), and poly(ADP-ribose) polymerase (PARP) (Cell Signaling) were measured by Western blot analysis. Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries). Equal loading of proteins was assessed by red phenol staining. Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

2.7. Skeletal muscle malonyl-CoA, palmitoyl-CoA and lactate measurement

Approximately 50 mg of frozen skeletal muscle powder were homogenized in 1 ml of ice-cold 3.6% perchloric acid. The denaturated protein was removed by centrifugation and an aliquot of the supernatant was used directly for analysis of skeletal muscle malonyl-CoA levels by HPLC as previously described [22]. A second aliquot was used for the extraction of lipids, including long-chain acyl-CoAs, with saturated $(NH_4)_2SO_4$ (25 μg/ml of extract) and $CHCl_3$-MeOH (1:2 vol/vol) followed by further $CHCl_3$-MeOH (1:2)

extraction. The resulting supernatants were evaporated and then reconstituted in 1 ml 50 mM KH_2PO_4 (pH 5.3). Extracts were loaded onto a C18 reverse-phase column and long-chain acyl-CoAs were separated following the method described by Ellis et al. [23]. Palmitoyl-CoA detection was performed by comparing sample peak with a standard. Finally, 20 mg of frozen skeletal muscle powder were homogenized in 1 ml of ice-cold 0.5 M perchloric acid and 1 mM EDTA, neutralized to pH 7.0 with 2 M $KHCO_3$ and analyzed for lactate content using a commercial kit (Sigma).

2.8. Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) was determined by means of the PeroxiDetect kit (Sigma).

2.9. Determination of ceramide levels

The content of ceramides in skeletal muscle and C2C12 myotubes was determined by the diacylglycerol kinase method. Briefly, lipids were extracted from approximately 50 mg of skeletal muscle with 600 μl of chloroform, methanol, 1 N HCl (100:100:1). After agitation and centrifugation, the lower phase containing the chloroform-extracted lipids was transferred to a new microfuge tube. Chloroform was evaporated under a N2 stream. Dried lipids were resuspended in 300 μl of 0.1 N KOH in methanol and incubated for 1 h at 37 °C to eliminate diacylglycerol. Then, 300 μl of PBS was added, and lipid extraction was repeated as indicated above. Lipids were resuspended in 100 μl of reaction buffer (150 ng/100 μl cardiolipin, 280 μM diethylenetriaminepentaacetic acid, 51 mM octyl β-D-glucopyranoside, 50 mM NaCl, 51 mM imidazol, 1 mM EDTA, 12.5 mM $MgCl_2$, 2 mM dithiothreitol, 0.7% glycerol, 70 μM β-mercaptoethanol, 500 μM ATP, 5 μCi/100 μl [γ-^{32}P]ATP), and 35 ng of diacylglycerol kinase was added to each sample. Reactions were incubated at 30 °C for 30 min and stopped by the addition of 170 μl of stop buffer (135 mM NaCl, 1.5 mM CaCl2, 0.5 mM glucose, 10 mM Hepes, pH 7.2) and 30 μl of 100 mM EDTA. Lipids were extracted again with 1 ml chloroform, methanol, 1 N HCl (100:100:1), resuspended in 40 μl of chloroform, spotted onto silica gel TLC plates (Whatman Inc.), and resolved using chloroform:methanol:acetic acid (65:15:5) as a solvent. Plates were measured in a PhosphoImager (BioRad). Quantification of ceramide mass was obtained by comparison with a standard curve ranging from 0 to 1000 pmol of ceramide-1-phosphate (Sigma), which was processed in parallel to the samples.

2.10. Statistical analyses

Results are expressed as mean ± S.D. of six mice. Significant differences were established by Student's *t*-test or one-way ANOVA, according to the number of groups compared. When significant variations were found, the Tukey-Kramer multiple comparisons test was performed. Statistical analyses were performed using the computer program GraphPad Instat (GraphPad Software V2.03) (GraphPad Softwware Inc.).

3. Results

3.1. Troglitazone increases Akt protein expression in soleus muscle of normoglycemic mice

First we determined the effects of troglitazone administration for 1 day (100 mg/kg/day) on several plasma energy substrates. Plasma triglyceride, free fatty acids and glucose levels were not significantly affected by thiazolidinedione treatment for 24 h (data not shown), in agreement with previous studies [24]. We next analyzed the effect of troglitazone on Akt expression in the soleus muscle using both anti-phospho-Akt (Ser-473) and anti-Akt antibodies in the basal state. Thiazolidinedione treatment did not affect the expression of phospho-Akt, which is consistent with the lack of insulin infusion in the animals used in this study (Fig. 1A). In contrast, Akt protein expression was two-fold higher ($P < 0.001$) in skeletal muscle of troglitazone-treated mice than in untreated animals (Fig. 1B).

3.2. Increased Akt protein expression after troglitazone treatment does not reduce the mRNA levels of genes involved in fatty acid oxidation

Since chronic Akt activation has been related to down-regulation of important genes involved in fatty acid oxidation, such as PPARα and PPARγ coactivator-1(PGC-1) [25], we next studied whether the increased protein Akt expression was responsible for the inhibition of fatty acid oxidation reported after troglitazone treatment [11]. The mRNA levels of PPARα, the transcription factor that controls the expression of several genes involved in fatty acid oxidation, were up-regulated (2.7-fold induction, $P < 0.01$) in skeletal muscle of thiazolidinedione-treated mice compared with untreated animals (Fig. 2A). In contrast, no changes were observed in the transcript levels of

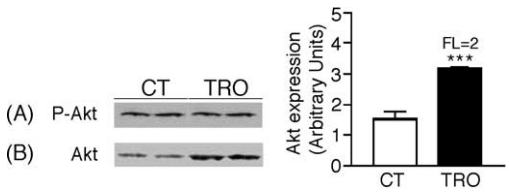

Fig. 1. Increased expression of Akt protein in soleus muscle of troglitazone-treated mice. Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Representative immunoblots using anti-phospho-Akt (ser-473) antibody (A) and anti-Akt antibody (B) are shown. Data are expressed as mean ± S.D. of six mice. $^{***}P < 0.001$ compared with control animals.

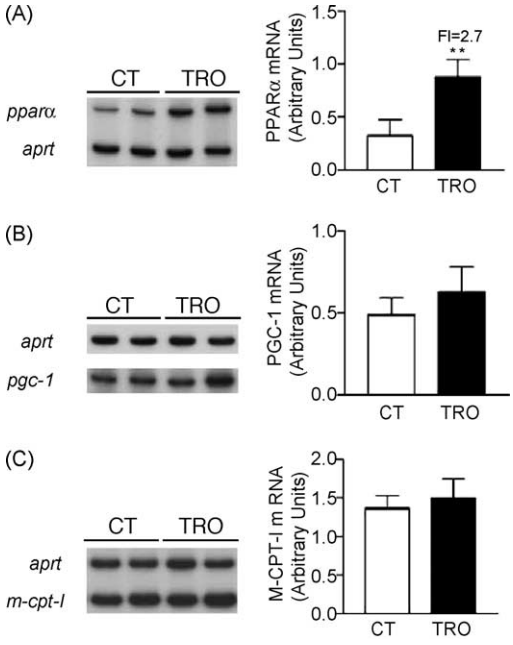

Fig. 2. Troglitazone treatment does not reduce the mRNA levels of genes involved in fatty acid metabolism in soleus muscle. Analysis of the mRNA levels of PPARα (A), PGC-1 (B) and M-CPT-I (C) in soleus muscle of untreated (control) and troglitazone-treated mice. An amount of 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six mice. $^{**}P < 0.01$ compared with control animals.

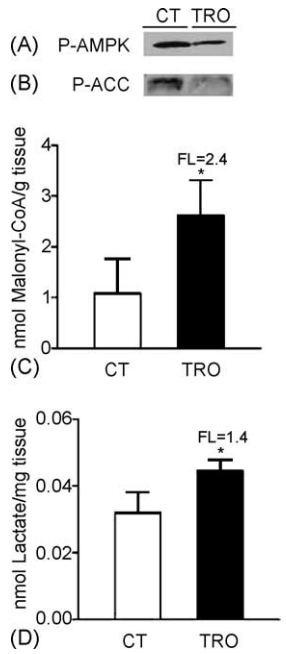

Fig. 3. Effects of troglitazone treatment on AMPK and ACC phopshorylation, malonyl and lactate content in skeletal muscle. Analysis of the phosphorylation of AMPK (A) and ACC (B). Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Equal protein loading was assessed by phenol red staining of the lower portion of the gel (data not shown). Analysis of the intracellular content of malonyl-CoA (C) and lactate (D) in soleus muscle of control and troglitazone-treated mice. Data are expressed as mean ± S.D. of six mice. $^{*}P < 0.05$ compared with control animals.

PGC-1, a coactivator for many factors in the nuclear hormone receptor family that has been implicated in mitochondrial biogenesis, respiration and thermogenesis [26] (Fig. 2B). Similarly, the mRNA expression of muscle-type CPT-I (M-CPT-I), that catalyses the entry of long-chain fatty acids into the mitochondrial matrix for fatty acid oxidation, was not altered by drug treatment (Fig. 2C). Therefore, these data suggest that increased Akt protein expression in skeletal muscle after troglitazone treatment does not result in a down-regulation of genes involved in fatty acid oxidation.

3.3. Increased Akt protein expression is associated with reduced AMPK activity in skeletal muscle of troglitazone-treated mice

Since Akt activation can lead to decreased AMPK activity [17,27] we next evaluated whether the changes in Akt protein expression affected AMPK activity. As shown in Fig. 3A, troglitazone treatment reduced the abundance of phospho-AMPK in skeletal muscle compared with untreated animals. AMPK plays a key role in controlling the switch between carbohydrate and fatty acid utilization in skeletal muscle through phopshorylation of acetyl-CoA carboxylase (ACC), given that this enzyme catalyses the formation of malonyl-CoA, an inhibitor of mitochondrial fatty acid oxidation through feedback inhibition of M-CPT-I. Thus, with reduced phospho-AMPK abundance, one would expect to find a reduction in the phosphorylation of ACC and an increase in the malonyl-CoA levels. Both changes were observed in skeletal muscle of troglitazone-treated animals (Fig. 3B and C). A dramatic fall in the phosphorylation of ACC was observed, whereas the concentration of malonyl-CoA was 2.4-fold ($P < 0.05$) higher in skeletal muscle of troglitazone-treated mice. Furthermore, the decrease in AMPK activity has been related to an increase in lactate production [27] and, according to this fact, a higher content of lactate (1.4-fold, $P < 0.05$) was present in skeletal muscle of troglitazone-treated mice, indicating that drug treatment increased glycolysis (Fig. 3D).

3.4. The increase in Akt protein expression achieved by troglitazone does not involve changes in its mRNA nor Hsp protein levels

Most of the effects caused by activation of the PPARγ transcription factor by troglitazone are mediated through changes in the mRNA expression of its target genes. In order to assess what was the mechanism involved in the observed increase in Akt protein expression after troglitazone treatment, we determined the mRNA levels of Akt. Troglitazone treatment did affect neither the transcript levels of Akt1 nor Akt2 (Fig. 4A and B). These results

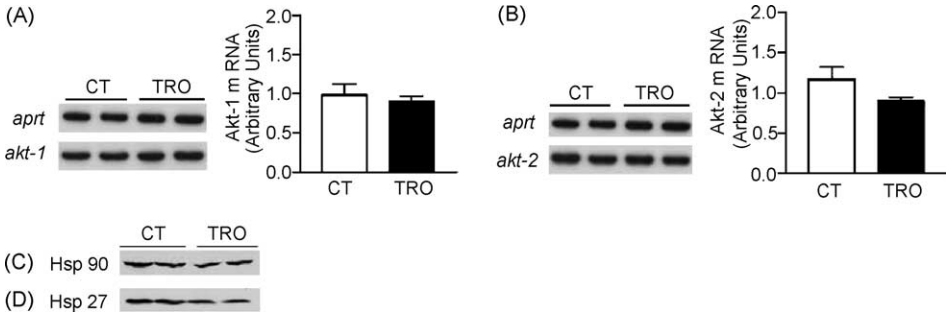

Fig. 4. Troglitazone treatment does not affect the mRNA levels of Akt1 or Akt2 nor Hsp protein expression in skeletal muscle. Analysis of the mRNA levels of Akt1 (A) and Akt2 (B) in soleus muscle of untreated (Control) and troglitazone-treated mice. An amount of 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six mice. Effects of troglitazone treatment on Hsp90 and Hsp27 protein and the generation of H_2O_2 in skeletal muscle. Extracts from soleus skeletal muscle from untreated (control) and troglitazone-treated mice were subjected to immunoblot analysis as described in Section 2. Representative immunoblots using anti-Hsp90 (C) and anti-Hsp27 (D) are shown. Data are expressed as mean ± S.D. of six mice.

make unlikely that the changes observed in Akt expression after troglitazone treatment may occur as a result of a transcriptional mechanism and suggest that other mechanisms, such as the control of protein degradation, may be involved. Regarding the degradation of Akt protein it is important to note that Akt binds to heat shock protein 90 (Hsp90), a family of protein chaperones that protect proteins against degradation [28]. Akt forms complexes with Hsp90 and 27, and complex formation with the former facilitates kinase activation by preventing both dephosphorylation and Akt degradation [29]. Therefore, troglitazone may act promoting changes in Hsp expression that would lead to a reduction in Akt degradation. However, when we determined the expression of Hsp90 and Hsp27 no changes were observed in the expression of these proteins (Fig. 4C and D). These data indicate that the effects of troglitazone on Akt expression do not involve a reduction in its degradation as a result of enhanced expression of Hsp proteins.

3.5. The increase in Akt protein expression caused by troglitazone is associated with a reduction in the levels ofpalmitoyl-CoA and ceramides

According to recent studies Akt protein levels are also regulated by ceramide and hydrogen peroxide (H_2O_2) levels, which induce a caspase-3-independent degradation of Akt [30]. Thus, to gain further insight into the mechanism by which troglitazone increases Akt protein expression we determined the levels of H_2O_2 and ceramides in skeletal muscle of troglitazone-treated and untreated mice. Changes in the content of H_2O_2 may activate a wide array of proteases, leading to the degradation of Akt and other proteins, such as PARP [30], a well-established substrate of caspase-3 in vivo [31]. No changes were observed in the content of H_2O_2 in skeletal muscle of troglitazone-treated compared with untreated mice (data not shown), indicating that the cell redox state was unchanged. In agreement with the lack of changes in the levels of H_2O_2 we found that the content of PARP, which shows a similar pattern of degradation that Akt in the presence of increased levels of H_2O_2 [30], was unaffected (data not shown). Ceramides are the other factor governing the degradation of Akt [30]. Palmitoyl-CoA is a precursor of sphingolipid synthesis, since the initial step of de novo ceramide synthesis is the formation of 3-ketodihydrosphingosine from palmitoyl-CoA and L-serine. When we analyzed the content of ceramides a dramatic reduction (76%, $P < 0.001$) in the skeletal muscle of troglitazone-treated mice compared to untreated mice was observed (Fig. 5A). Moreover, the levels of the precursor of de novo ceramide synthesis, palmitoyl-CoA showed a 55% ($P = 0.05$) reduction in skeletal muscle after troglitazone treatment (Fig. 5B). These results indicate that the increase in the content of Akt protein observed in troglitazone-treated mice may result from the inhibition of de novo ceramide synthesis. The reduction in the content of palmitoyl-CoA may be due to the increase in the mRNA levels of cytosolic acyl-CoA thioesterase (CTE) (3.2-fold induction, $P < 0.05$) (Fig. 5C), which hydrolyses fatty acyl-CoAs to free fatty acids and CoA.

In order to confirm the effects of troglitazone in vivo we performed an additional study in C2C12 myotubes. Treatment with troglitazone for 24 h led to a four-fold increase ($P < 0.01$) in the protein levels of Akt (Fig. 6A), confirming the results obtained in vivo. Further, we studied whether in palmitate-induced insulin resistance troglitazone treatment prevented the reduction in Akt protein expression and the increase in the cellular ceramide content. In myotubes treated with 0.5 mM palmitate for 16 h a 40% reduction ($P < 0.05$) in the protein levels of Akt was observed. In contrast, co-incubation with troglitazone prevented this reduction and even led to a three-fold increase ($P < 0.05$) compared to palmitate-treated cells (Fig. 6B). Finally, the analysis of the ceramide content showed that palmitate caused a 2.5-fold increase ($P < 0.001$) compared to control cells and that in the presence of troglitazone this increase was completely abolished (Fig. 6C).

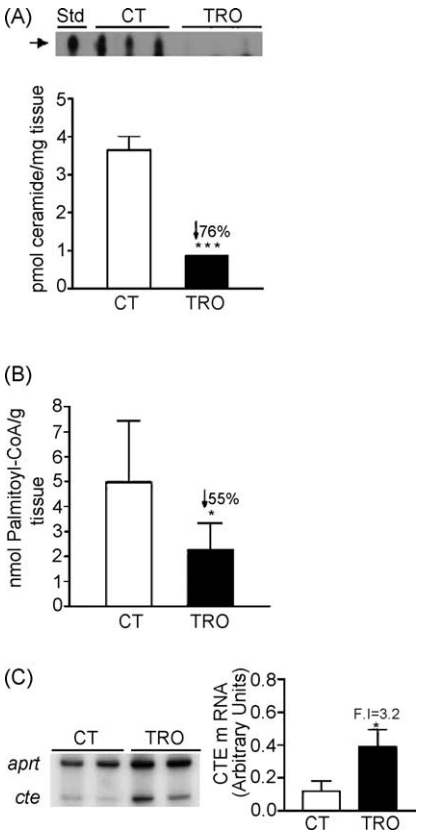

Fig. 5. Troglitazone treatment reduces ceramide levels in skeletal muscle. Measurement of ceramide levels in skeletal muscle of untreated (control) and troglitazone-treated mice. Lipid extracts from skeletal muscle were prepared and assayed for ceramides as detailed in Section 2. (A) Phosphorimage of phosphorylated ceramides from samples and standards, separated by TLC, and its quantification (mean ± S.D.) is shown. (B) Quantification of palmitoyl-CoA levels in skeletal muscle. (C) Analysis of the mRNA levels of CTE in soleus muscle of untreated (control) and troglitazone-treated mice. An amount of 0.5 μg of total RNA was analyzed by RT-PCR. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean ± S.D. of six mice.

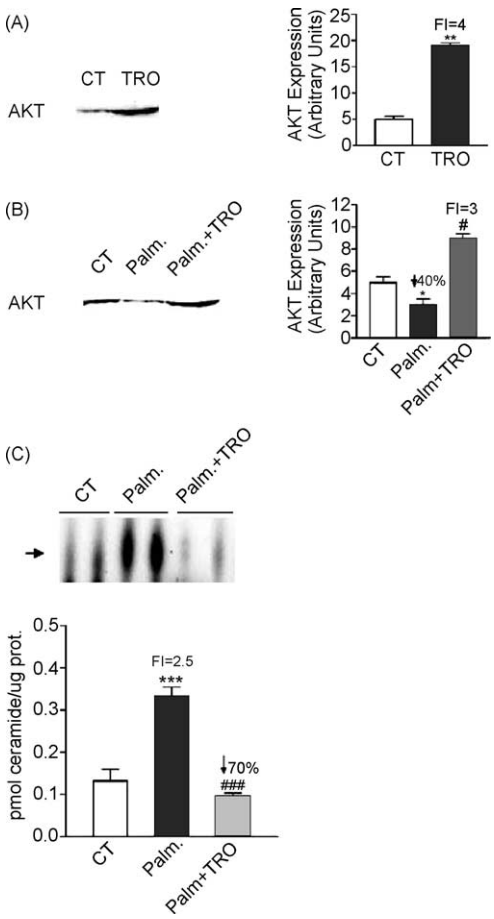

Fig. 6. Troglitazone treatment increases Akt expression and reduces ceramide levels in C2C12 myotubes. (A) Representative immunoblot analysis using anti-Akt antibody of total protein extracts of C2C12 myotubes incubated for 24 h in the presence or in the absence of troglitazone. C2C12 myotubes incubated (16 h) with 0.5 mM palmitate in the presence or in the absence of troglitazone were assayed for (B) immunoblot analysis using anti-Akt antibody or (C) measurement of ceramide levels. Lipid extracts from cells muscle were prepared and assayed for ceramides as detailed in Section 2. Data are expressed as mean ± S.D. of four different experiments. $^{***}P < 0.001$ and $^{*}P < 0.05$ vs. control. ### and # vs. palmitate-treated cells.

4. Discussion

Thiazolidinediones are a recent new class of oral agents for the treatment of type 2 diabetes that improve glycemic control by increasing insulin sensitivity in target tissues, such as skeletal muscle and adipose tissue [9]. Although it is clear that thiazolidinediones work mostly by increasing insulin sensitivity in the periphery, the mechanism(s) by which this occurs remain obscure [32,33]. These drugs are known to ameliorate existing insulin resistance associated with chronic lipid accumulation. It is generally believed that lowering of circulating lipids reduces lipid availability to tissues, resulting in improved insulin sensitivity [6]. In the present study we report that troglitazone treatment increases the expression of Akt protein in skeletal muscle. Akt has been proposed to be an intermediate in the signaling pathway by which insulin, controls glucose uptake [34]. Thus, overexpression of constitutively active forms of Akt induced glucose uptake, GLUT4 translocation, and glycogen synthesis [35,36]. In addition, it has been demonstrated that mice deficient in Akt2 were insulin resistant in muscle and liver [37]. All these data indicate that Akt is a central mediator of the insulin effects. Exposing skeletal muscle cells to free fatty acids, specially saturated free fatty acids, inhibits insulin stimulation of Akt [12]. Interestingly, troglitazone treatment increases the phosphorylation of Akt in skeletal muscle [38], effect that may account for part of the mechanisms leading to improved insulin sensitivity of glucose uptake and glucose storage in skeletal muscle. However, this effect has been related to the reduction in plasma free fatty acids achieved by troglitazone treatment [38]. In contrast, in this study we report that the increase in Akt protein expression occurs without significant changes in the levels of free fatty acids in plasma in the basal state.

Although this study was performed in the soleus, a slow-twitch oxidative muscle, and it may differ in other muscle types, the increase in the expression of Akt in skeletal muscle after troglitazone treatment may have beneficial consequences in the prevention and treatment of type 2 diabetes mellitus. Thus, it has been previously reported that prior treatment with the thiazolidinedione pioglitazone preserved insulin sensitivity in normal rats during acute fatty acid elevation, mainly through protecting liver insulin sensitivity, an effect that was independent of the plasma lipid-lowering of this drug [39]. Our experiments suggest that one additional factor involved in the protection afforded by these drugs against insulin resistance induced by lipid oversupply may be the increase in Akt protein in skeletal muscle. Animals treated with thiazolidinediones would be provided with a high level of Akt in muscle, leading to a preservation of insulin signaling and glucose uptake in lipid-induced insulin resistance. Although the data here presented need to be confirmed in humans, a previous report suggests that a similar effect could be attained by troglitazone in humans. Thus, in agreement with our results, Meyer et al. [38] recently reported that troglitazone treatment showed a tendency to increase Akt protein expression, although differences did not reach statistical significance, in vastus lateralis skeletal muscle of normoglycemic subjects at risk for the development of type 2 diabetes mellitus.

It has been reported that in isolated rat soleus muscle strips troglitazone caused lactate release as well as inhibition of mitochondrial palmitate oxidation by a PPARγ-independent mechanism [11]. Several facts raise the possibility that increased Akt protein expression in skeletal muscle after troglitazone treatment may lead to changes in intracellular lipid metabolism, which are not related to its lipid-lowering action. First, although chronic Akt overexpression has been related with the reduction in the expression of PPARα and PGC-1 [25] we did not find a fall in their expression. These data suggest down-regulation of PPARα and PGC-1 is not involved in the effects of troglitazone on palmitate oxidation and glycolisis. Second, the reported cross-talk between Akt and AMPK [17] may result in changes in intracellular lipid metabolism after troglitazone treatment. In our study we observed that up-regulation of the expression of Akt was accompanied by a reduction in phospho-AMPK content, which is a reflex of a decrease in its activity, leading to a fall in the phosphorylation of ACC and an increase in the malonyl-CoA levels, an inhibitor of mitochondrial fatty acid oxidation through feedback inhibition of M-CPT-I. In agreement with previous studies [27], the decrease in AMPK activity has been associated with an increase in the content of lactate, suggesting that troglitazone may increase the rate of glycolisis, although additional experiments are needed to confirm this point. Therefore, the increase in Akt protein expression after troglitazone treatment may lead to a reduction in AMPK activity and subsequent changes in malonyl-CoA content that may reduce mitochondrial fatty acid oxidation.

Regarding the mechanism of action responsible for the induction in the expression of Akt protein after troglitazone treatment, neither Akt1 nor Akt2 mRNA level was affected by troglitazone, suggesting that transcriptional changes were not involved. Since Akt protein expression is regulated by proteolytic degradation, we next explored whether up-regulation of Akt protein after troglitazone treatment was the result of changes in factors regulating this process. Degradation of Akt is enhanced by generation of H_2O_2 and ceramide [30]. Activation of proteases involved in the H_2O_2-induced degradation of Akt also result in PARP proteolysis. The lack of changes in the intracellular content of H_2O_2 and PARP protein indicates that troglitazone does not affect these factors involved in Akt degradation. In contrast, when we analyzed the content of ceramide, we observed a dramatic fall in the levels of these lipid-derived second messengers after troglitazone treatment. Ceramides are a family of sphingolipids that differ in the fatty acyl moeity and are known to induce insulin resistance and apoptosis in cultured cells [40,41]. Interestingly, a recent study shown that ceramide content is increased in skeletal muscle from obese insulin-resistant humans [42], suggesting that reduction in the content of ceramides attained by troglitazone may contribute to part of its effects. As an intermediate in the sphingomyelin pathway, ceramide can be generated by hydrolysis of sphingomyelin or can be produced by de novo synthesis [43]. Palmitate, once activated to palmitoyl-CoA by acyl-CoA synthase (ACS), is the precursor of de novo ceramide synthesis. The results presented here indicate that troglitazone inhibits de novo ceramide synthesis through reducing the levels of palmitoyl-CoA. Since in our study troglitazone treatment did not lower free fatty acids in plasma it is unlikely that plasma lipid availability may be involved in palmitoyl-CoA changes. In contrast, the data presented here suggest that the reduction in the intracellular content of palmitoyl-CoA may be the result of the increase in the expression of CTE, which hydrolyses fatty acyl-CoAs to free fatty acids and CoA. In addition, we cannot exclude that the well-known inhibitory effect of troglitazone on ACS may also contribute to reduce the content of intracellular palmitoyl-CoA [44].

In summary, in the present study we show that troglitazone treatment increases Akt protein expression in skeletal muscle. The mechanism responsible for the increase in Akt protein expression seems to involve a reduction in de novo ceramide synthesis, since these lipid-derived second messengers accelerate Akt protein degradation. Our results suggest that prior troglitazone treatment may lead to a preservation of insulin signaling and glucose uptake in lipid-induced insulin resistance.

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RESUM GLOBAL

HIPERTRÒFIA CARDÍACA

La hipertròfia cardíaca és una adaptació fisiològica del cor que en un primer moment pot resultar beneficiosa, ja que actua com a mecanisme compensador normalitzant l'estrès sobre la paret, però si perdura en el temps pot donar lloc a complicacions més severes, arribant fins i tot a provocar insuficiència cardíaca o mort súbita (Levy i col., 1990).

Existeixen diferents processos implicats en l'aparició i el desenvolupament d'aquesta alteració, molts dels quals encara no es coneixen bé; malgrat això, dos d'ells apareixen habitualment associats a la hipertròfia cardíaca. El primer d'aquests processos consisteix en alteracions del metabolisme energètic dels àcids grassos i la glucosa, on es produeix una reversió cap a l'estat fetal amb una major utilització de glucosa per part del cor (Van Bilsen i col., 1998). El segon d'aquests mecanismes és l'activació de NF-κB, fonamental perquè es desenvolupi la hipertròfia cardíaca, ja que si s'inhibeix aquest factor, la hipertròfia no es desenvolupa (Purcell i col., 2001).

Aquests dos processos són regulats pels PPARs (Desvergne i Wahli, 1999; Kersten i col., 2000). Així, diversos estudis han demostrat, com els PPARs regulen el metabolisme energètic a través de mecanismes com la trans-activació de gens (Braissant i col., 1996; Akiyama i col., 2001), o la trans-repressió de factors com NF-κB (Deleviere i col., 1999b; Zhou i col., 1999). Com a resultat d'aquests efectes, s'ha confirmat que els activadors de PPARα i PPARγ són capaços de revertir la hipertròfia cardíaca (Liang i col., 2003; Irukayama-Tomobe i col., 2004; Yamamoto i col., 2001; Asakawa i col., 2002). D'altra banda, Barger i col. (2000) van demostrar que durant el creixement hipertròfic, l'expressió de PPARα es reduïa i la seva activitat s'alterava a nivell post-transcripcional a través de la via de les MAPK. Pel contrari, el paper de PPARβ/δ en aquest procés és poc conegut, tot i que s'expressa en quantitats similars a PPARα en cor i sembla tenir funcions similars a PPARα i fins i tot pot compensar la seva absència el metabolisme dels àcids grassos (Gilde i col., 2003).

I. Efectes de l'activació de NF-κB sobre el metabolisme dels àcids grassos

Segons tot el que s'acaba de comentar, en el primer dels objectius d'aquesta tesi doctoral vam evaluar els efectes provocats per l'activació de NF-κB durant la hipertròfia cardíaca i el paper jugat per PPARβ/δ en el desenvolupament d'aquest procés. Els resultats obtinguts demostren que la inducció d'hipertròfia cardíaca amb fenilefrina (FE), que provoca l'activació de NF-κB, anava acompanyada d'una reducció de l'expressió de la PDK-4, gen diana de PPARβ/δ, implicada en el metabolisme dels àcids grassos. A més, la inducció de la hipertròfia cardíaca també va provocar una reducció en l'oxidació dels àcids grassos que es va revertir amb inhibidors de NF-κB. Aquestes dades semblen implicar a NF-κB en el control de l'oxidació dels àcids grassos que es produeix durant el desenvolupament de la hipertròfia cardíaca. Malgrat això, aquests canvis podrien ser causats per la interferència de NF-κB amb PPARα, ja que tant PPARα com PPARβ/δ s'expressen en cardiomiòcits neonatals. Per tal de descartar aquesta possibilitat, vam utilitzar miotubs H9c2 que només expressen el subtipus PPARβ/δ (Gilde i col., 2003). L'estimulació d'aquestes cèl·lules amb LPS, un estímul capaç d'activar NF-κB en cardiomiòcits (Takano i col., 2000), també va provocar una disminució en l'expressió de la PDK-4, fet que reforça la hipòtesi d'un lligam entre l'activació de NF-κB i la reducció en l'expressió dels gens diana de PPARβ/δ. Aquests resultats suggereixen que la reducció en l'activitat de PPARβ/δ juga un paper molt important en la disminució de l'oxidació dels àcids grassos que es produeix durant la hipertròfia cardíaca.

D'altra banda, l'exposició de les cèl·lules cardíaques H9c2 a LPS va provocar un forta reducció de la unió de la proteïna PPARβ/δ a la sonda PPRE. Sembla que l'activació de NF-κB seria la responsable d'aquest efecte i, en conseqüència, de la disminució observada en l'expressió dels gens diana de PPARβ/δ, donat que els inhibidors de NF-κB ho reverteixen. Aquests resultats suggereixen que l'activació de NF-κB està relacionada amb aquesta reducció d'activitat del factor de transcripció PPARβ/δ. Per tal de determinar-ne el motiu vam realitzar estudis de co-immunoprecipitació que van demostrar la interacció entre la subunitat p65 de NF-κB i PPARβ/δ. Aquesta associació evitaria la unió de PPARβ/δ al seu element de resposta, impedint així la inducció de la transcripció dels seus gens diana, entre els quals es troba la PDK-4. Aquests resultats concorden amb els obtinguts per Westergaard i col. (2003), que van demostrar la interacció física entre PPARβ/δ (però no de PPARα ni PPARγ) i p65 en les lesions soriàtiques.

Finalment, per tal de determinar si el mecanisme proposat era reproduible *in vivo*, vam utilitzar un model d'hipertròfia cardíaca *in vivo* mitjançant la constricció de l'aorta abdominal que produeix una sobrecàrrega de pressió. En aquest cas, també es va produir l'activació de NF-κB i la reducció dels gens diana de PPARβ/δ. Els estudis de co-immunoprecipitació, també van demostrar la interacció física entre PPARβ/δ i NF-κB durant el desenvolupament d'aquesta patologia.

En resum, en aquest estudi s'ha demostrat que l'activació de NF-κB reprimeix l'activitat de PPARβ/δ, tant *in vitro* com *in vivo*, a través de la interacció entre ambdues proteïnes. A més, l'activació de NF-κB sembla ser responsable de la reducció en la capacitat oxidativa dels àcids grassos durant el desenvolupament de la hipertròfia cardíaca.

Donada la importància tant de PPARβ/δ com de NF-κB durant el desenvolupament de la hipertròfia cardíaca, fàrmacs capaços d'activar i inhibir, respectivament, aquests dos factors de transcripció podrien ser útils per al tractament d'aquesta patologia. Aquest va ser l'objectiu dels següents estudis presentats en aquesta tesi.

II. Efecte d'un activador de PPARβ/δ sobre la hipertròfia cardíaca

Com ja s'ha comentat, estudis recents han demostrat que l'activació tant de PPARα com PPARγ reverteix la hipertròfia cardíaca (Liang i col., 2003; Irukayama-Tomobe i col., 2004; Yamamoto i col., 2001; Asakawa i col., 2002), mentre que es desconeix quin és el paper de PPARβ/δ durant el desenvolupament d'aquest fenomen. Gilde i col (2003) van demostrar que PPARβ/δ i PPARα s'expressen en quantitats similars al cor, mentre que PPARγ s'expressa en quantitats molt baixes, quasi indetectables. Tanmateix aquests autors van demostrar que PPARβ/δ s'indueix en presència d'àcids grassos i que és capaç d'activar l'expressió dels gens diana de PPARα implicats en l'oxidació dels àcids grassos en cardiomiòcits. Aquests autors també suggereixen que ambdos subtipus tindrien funcions similars en la regulació del metabolisme lipídic cardíac. Tenint en compte tot això, i gràcies a la recent disponibilitat d'agonistes selectius per aquest subtipus de PPAR com L-165041, el segon objectiu d'aquesta tesi doctoral va ser determinar l'efecte de l'activador selectiu de PPARβ/δ, L-165041**,** sobre la hipertròfia cardíaca.

Els resultats presentats indiquen que el tractament de cadiomiòcits neonatals amb L-165041 va inhibir la hipertròfia induïda amb FE i, a més, també va revertir la disminució dels gens implicats en l'oxidació dels àcids grassos com la M-CPT-I i la PDK-4. Tal i com vam fer en el primer estudi, per tal de determinar l'especificitat de PPARβ/δ, vam reproduir els experiments en H9c2, estimulant les cèl·lules amb LPS. En aquest cas vam observar una reducció en l'expressió de la M-CPT-I i la PDK-4, que es revertia en presència de L-165041. El tractament amb aquest activador també va reduir la inducció del gen diana de NF-κB, MCP-1, provocada tant pel tractament amb FE com amb LPS, suggerint que PPARβ/δ podria antagonitzar l'activació de NF-κB. Shioi i col (1997) van descriure increments de MCP-1 en el cor hipertrofiat i en insuficiència cardíaca, els quals es consideren els responsables de la infiltració i activació de les cèl·lules inflamatòries, com monòcits/macròfags i limfòcits. A més també s'ha descrit que l'activació de l'expressió de MCP-1 contribueix al remodelat ventricular després d'un infart (Hayashidani i col., 2003). Segons això, la reducció de l'expressió de MCP-1 observada en presència de l'activador PPARβ/δ, podria ser un bon punt de partida per estudiar el tractament d'aquestes patologies.

Existeixen diversos mecanismes a través dels quals PPARβ/δ podria reprimir l'activitat de NF-κB. Un d'aquests mecanismes és l'increment en l'expressió de les proteïnes repressores de NF-κB, com les IκB (α i β) (Evans i col., 2002), però els nivells d'expressió d'aquestes proteïnes no es van modificar amb el tractament amb L-165041. Un altre mecanisme potencial podria ser la inhibició de l'activitat NF-κB mitjançant la interacció directa entre PPARβ/δ i NF-κB. Els estudis de co-immunoprecipitació van demostrar que en presència de L-165041 es produïa una interacció física entre PPARβ/δ i la subunitat p65 de NF-κB, indicant que aquest mecanisme podria ser el responsable d'interferir en la capacitat de trans-activació de NF-κB.

En resum, l'activació de PPARβ/δ inhibeix la hipertròfia induïda per FE en cardiomiòcits neonatals. L'activació de PPARβ/δ també inhibeix l'activació de NF-κB induïda per LPS en miotubs H9c2 a través d'un mecanisme que implicava la interacció proteïna-proteïna entre aquest subtipus de PPAR i la subunitat p65 de NF-κB. Sembla doncs, que l'activació de PPARβ/δ inhibeix la via de senyalització de NF-κB revertint, tant la hipertròfia cardíaca com la repressió dels gens implicats en el metabolisme dels àcids grassos.

Així doncs, tenint en compte els resultats del primer i segon objectiu sembla que la interacció entre la subunitat p65 de NF-κB i PPARβ/δ pot resultar o bé en una repressió de PPARβ/δ en absència de lligand per aquest receptor o bé en una reducció de l'activitat NF-κB en presència de lligand PPARβ/δ. Cal dir que en aquest últim cas l'associació entre PPARβ/δ i p65 és molt més elevada que l'associació que es va aconseguir per l'estimulació de NF-κB amb LPS en el primer estudi.

III. Efecte de les estatines sobre la hipertròfia cardíaca

Donat l'important paper de NF-κB en el desenvolupament de la hipertròfia cardíaca, els fàrmacs capaços d'inhibir-lo també podrien revertir aquest procés. Ortego i col. (1999) van demostrar que les estatines inhibeixen l'activació de NF-κB i estudis més recents han demostrat que el tractament amb estatines evita el desenvolupament de la hipertròfia cardíaca (Takemoto i col., 2001). El tercer objectiu d'aquesta tesi doctoral va ser estudiar l'efecte de l'atorvastatina sobre la hipertròfia cardíaca en un model de rates amb sobrecàrrega de pressió per constricció de l'aorta abdominal.

En aquest model animal, la inducció de la hipertròfia cardíaca va provocar l'activació de NF-κB i la reducció de l'expressió de gens implicats en el metabolisme dels àcids grassos com la MCAD i la PDK-4. Prèviament ja s'havia descrit que aquests canvis podrien ser causats per la desactivació de PPARα (Barger i col., 2000). De fet, els nostres resultats demostren una reducció de PPARα en aquest model d'hipertròfia. Però a més, en aquest estudi vam demostrar com els nivells de PPARβ/δ també es trobaven reduïts durant la hipertròfia cardíaca. Aquesta reducció en l'expressió dels dos subtipus de PPAR sembla ser necessària per tal de reprimir l'expressió dels gens implicats en l'oxidació dels àcids grassos. Aquests canvis no els vam observar quan la inducció en l'activitat de NF-κB s'inhibia pel tractament amb atorvastatina, fet que suggereix la implicació de NF-κB en aquest procés. Un estudi recent va demostrar que la inhibició de NF-κB incrementava l'expressió de l'apoA-I, gen diana de PPARα (Morishima i col., 2003), confirmant així un interacció negativa entre tots dos. Els nostres resultats també van demostrar com la hipertròfia cardíaca incrementava l'associació entre la subunitat p65 i els dos subtipus de PPAR, indicant que aquest mecanisme podria interferir en la capacitat de transactivació dels PPARs, contribuint així al descens en l'expressió dels gens implicats en el metabolisme dels àcids grassos. Pel contrari,

el tractament amb atorvastatina va evitar la interacció entre PPAR-p65 i va restablir l'expressió d'aquests gens.

A partir dels resultats d'aquest estudi podem concloure que la reducció en l'oxidació dels àcids grassos observada durant la hipertròfia cardíaca, està associada a la reducció dels nivells de proteïna tant de PPARα com de PPARβ/δ i de l'augment de la interacció entre p65 i els PPARs, i que aquests canvis es revertien amb el tractament amb atorvastatina.

La reducció de l'oxidació dels àcids grassos en cardiomiòcits que es produeix durant processos com la hipertròfia cardíaca, a banda d'afectar als PPARs també pot afectar als seus cofactors com la PGC-1, que són necessaris per la funció d'aquests factors de transcripció. La sobreexpressió d'aquest coactivador en cardiomiòcits activa l'expressió dels gens implicats en el transport i l'oxidació dels àcids grassos (Lehman i col., 2000). Estudis recents han demostrat la interacció física entre PPARβ i PGC-1α (Wang i col., 2003) i també que els activadors d'aquest subtipus de PPAR estimulen els nivells de RNAm de la PGC-1α tant *in vivo* com *in vitro* (Tanaka i col., 2003). En el quart objectiu d'aquesta tesi doctoral vam evaluar l'efecte de l'atorvastatina sobre el cofactor PGC-1.

En aquest estudi, el tractament amb atorvastatina i partenolide de cèl·lules H9c2, prèviament estimulades amb LPS, també va recuperar l'expressió de la PDK-4 (com ja succeïa en el treball anterior però en aquest cas *in vitro*), implicant a NF-κB en aquest procés. Estudis previs ja havien descrit que PGC-1α actua com a coactivador de PPARβ/δ, activant la transcripció dels gens implicats en el metabolisme dels àcids grassos (Dressel i col., 2003). A més, altres autors havien demostrat l'existència d'una interacció física entre tots dos factors que incrementava l'activitat de PPARβ/δ (Wang i col., 2003). En el nostre cas vam observar una reducció en l'expressió d'aquest coactivador en les cèl·lules estimulades amb LPS que podria ser la responsable de la reducció en l'activitat de PPARβ/δ i del seu gen diana PDK-4. A més en aquest estudi també vam demostrar com el descens d'expressió de PGC-1 en les cèl·lules estimulades amb LPS provocava una reducció en la interació física entre PGC-1 i PPARβ/δ. Finalment, el tractament amb LPS també va provocar una reducció en la unió de PPARβ/δ a la sonda PPRE que es va veure revertida en presència dels inhibidors de NF-κB, atorvastatina i partenolide, confirmant la implicació d'aquest factor redox en els canvis observats.

Els resultats d'aquest estudi indiquen que l'activació de NF-κB podria afectar al metabolisme lipídic cardiac a través de la reducció en l'expressió del coactivador de PPARβ/δ, PGC-1. Aquests resultats juntament amb els descrits per Lehman i col. (2002), que van demostrar que en hipertròfia cardíaca l'expressió de PGC-1 estava reduïda, semblen indicar que la reducció de PGC-1 també pot estar implicada en processos que produeixen una reducció de l'oxidació dels àcids grassos com la hipertròfia cardíaca. A més, en aquest treball també vam observar com l'atorvastatina revertia l'expressió de la PGC-1 i la seva unió a PPARβ/δ, suggerint un altre possible mecanisme d'acció antihipertròfic d'aquest fàrmac.

En resum, en aquest quart treball vam descriure com l'activació de NF-κB, estimulat per LPS, provocava una reducció en l'expressió de la PGC-1, fet que reduia la interacció física entre aquest coactivador i PPARβ/δ. A la vegada, aquest efecte pot disminuir la unió de PPARβ/δ al seu element de resposta, reduint-se així l'expressió dels seus gens diana com la PDK-4 implicats en el metabolisme lipídic cadíac. Finalment, vam desmostrar com tots aquests canvis es prevenien en presència d'inhibidors de NF-κB com l'atorvastatina i el partenolide, implicant a NF-κB en els canvis observats.

Finalment, en el nostre darrer treball amb estatines, vam voler determinar com aquests fàrmacs afectaven a la via Akt/GSK3β. Aquesta via de senyalització, juga un paper molt important en el desenvolupament de la hipertròfia cardíaca, ja que la sobreexpressió de l'Akt en ratolins provoca hipertròfia (Cook i col., 2002; Shioi i col., 2002) i la GSK3β funciona com un regulador negatiu d'aquest procés (Badorff i col., 2002). En aquest treball vam obsevar com l'atorvastatina inhibia aquesta via en el model de rates amb constricció de l'aorta abdominal. Malgrat això, aquest efecte podia ser indirecte, degut simplement a la inhibició del procés de la hipertròfia. Per aquesta raó vam tractar els cardiomiòcits amb un estímul prohipertròfic com el TNFα.

El TNFα és una citocina que produeix el cor quan es donen increments de pressió o de volum, però no està present en rates normals (Torre-Amione i col., 1995; Torre-Amione i col., 1996; Yokoyama i col., 1993). En condicions experimentals a curt termini, TNFα indueix efectes inotròpics negatius (Yokoyama i col., 1993; Finkel i col., 1992), però quan es sobreexpressa crònicament al miocardi, provoca hipertròfia i mort (Bryant i col., 1998;

Kubota i col., 1997). En aquest treball vam demostrar que l'atorvastatina evitava la fosforilació de l'Akt i la GSK-3β en cardiomiòcits neonatals tractats amb aquesta citocina, suggerint que el tractament amb estatines reduia la contribució d'aquesta via al desenvolupament de la hipertròfia cardíaca. A més, després d'un transplantament la hipertròfia que es desenvolupa s'associa amb una expressió permanent de TNFα (Stetson i col., 2001) i per tant, el tractament amb estatines podria bloquejar aquests efectes de TNFα sobre la via Akt/GSK3β i prevenir el desenvolupament d'aquest procés.

El mecanisme a través del qual l'atorvastatina reduiria la via Akt-GSK3β amb el tractament amb atorvastatina sembla implicar novament el factor NF-κB, ja que els seus inhibidors exerceixen efectes similars. Sembla ser, però, que l'atorvastatina és l'inhibidor més potent d'aquesta via ja que tant en cèl·lules H9c2 estimulades amb LPS com en cardiomiòcits neonatals estimulats amb TNFα, el tractament amb estatines va bloquejar la fosforilació tant d'Akt com de GSK3β. En contrast amb això, s'observen algunes diferències entre els inhibidors de NF-κB, SN-50 i TPCK. El primer d'ells només redueix la fosforilació d'Akt en cèl·lules H9c2, mentre que el segon només redueix la fosforilació de GSK3β, probablement per diferències en el mecanisme d'inhibició de NF-κB. Aquests resultats també situen a NF-κB per sobre de l'Akt en la via de senyalització, ja que els inhibidors farmacològics de NF-κB també inhibeixen la via Akt/GSK3β, dades que concorden amb el que prèviament ja s'havia descrit en neurones (Meng i col., 2002). A més, aquest fet que situa NF-κB per sobre de la via Akt/GSK3β durant la hipertròfia cardíaca, podria explicar perquè únicament inhibint aquest factor es reverteix un procés tan complex que implica diverses vies.

En resum, en aquest treball vam demostrar que l'atorvastatina inhibia l'activació de la via Akt/GSK3β a través d'un mecanisme que implicava una reducció de l'activitat de NF-κB, contribuint així als efectes antihipertròfics de les estatines.

IV. Efecte del triflusal sobre la hipertròfia cadíaca

El darrer dels fàrmacs que ens vam proposar estudiar va ser el triflusal, que com ja s'ha comentat és capaç també d'inhibir el factor de transcripció NF-κB. La utilització de

fàrmacs ja comercialitzats amb un bon perfil de seguretat i eficàcia pot ser una possibilitat molt eficaç per al tractament de la hipertròfia cardíaca. El triflusal s'utilitza habitualment per al tractament de malalties vasculars i s'associa amb una menor incidència de problemes gastrointestinals en comparació amb l'aspirina. En aquest estudi vam demostrar com el triflusal i el seu metabòlit actiu HTB, inhibien la hipertròfia cardíaca tant en cardiomiòcits neonatals com en el model de rates amb constricció de la aorta abdominal. Aquesta inhibició es produia a una concentració (300 μmol/L), fàcilment assolible amb la dosssificació aprovada per aquest fàrmac (600-900 mg/dia), on els nivells d'HTB en plasma són de l'ordre de 1 mM (McNeely i Goa, 1998). Els resultats obtinguts semblen indicar que aquesta inhibició de la hipertròfia cardíaca és deguda a que aquest fàrmac inhibeix l'activació de NF-κB en les cèl·lules cardíaques.

La inhibició de la hipertròfia cardíaca sembla deguda a un increment en l'expressió de la proteïna IκBα que es troba incrementada tant pel triflusal com pel seu metabòlit actiu HTB. Aquesta proteïna és la responsable de retenir NF-κB al citoplasma i també sembla que estaria implicada en el retorn de NF-κB des del nucli (Tam i col., 2000; Yamamoto i Gaynor, 2004). Sembla que la inducció d' IκBα es produeix tant a nivell de mRNA com de proteïna, i que per tant els canvis observats en l'expressió d'aquesta proteïna es produirien a nivell transcripcional. Seran necessaris més estudis per tal de determinar els mecanismes que provoquen l'increment d' IκBα pel triflusal.

Estudis previs havien demostrat que aquest fàrmac inhibia l'activació de NF-κB en cèl·lules endotelials, en cèl·lules de cervell i *in vivo* més eficientment que l'aspirina (Bayon i col., 1999; Hernández i col., 2001; Fernández de Arriba i col., 1999; Acarin i col., 2000). En el nostre estudi també vam utilitzar l'aspirina a la mateixa concentració que l'HTB. En contrast amb aquest, l'aspirina no modificava la incorporació de [^{3}H]leucina. Així doncs, la incorporació del grup trifluorometil a la posició 4 dels salicilats aporta noves propietats a la molècula del triflusal.

En resum, en aquest estudi vam demostrar que tant el triflusal com els seu metabòlit actiu, HTB, inhibien la hipertròfia cardíaca tant *in vivo* com *in vitro* a través d'un mecanisme que implicava la inhibició de la via de senyalització de NF-κB, important mediador

intracel·lular d'aquest procés. Segons això, sembla que el triflusal podria esdevenir una opció terapèutica pel tractament d'aquesta patología.

RESISTÈNCIA A LA INSULINA

Les tiazolidinediones són un nou tipus d'agents antidiabètics que s'utilitzen per al tractament de la diabetis mellitus de tipus 2. Aquests fàrmacs milloren la sensibilitat a la insulina en els teixits perifèrics però encara no es coneix del tot a través de quin mecanisme. Un dels mecanismes responsables sembla ser la reducció dels àcids grassos lliures circulants, en canvi es desconeix si les TZDs milloren directament la resistència a la insulina en el múscul esquelètic, independentment de la reducció dels àcids grassos lliures. L'objectiu d'aquest estudi va ser determinar si la troglitazona afectava directament la via de senyalització de la insulina, en concret la via de l'Akt o proteïna cinasa B, al múscul esquelètic. Aquesta proteïna és un intermediari en la via de senyalització de la insulina que controla la captació de glucosa per part de la cèl·lula (Hajduch i col., 2001). Diversos estudis havien demostrat com la sobreexpressió constitutiva de la forma activa d'aquesta proteïna induia la captació de glucosa, la translocació de GLUT4 i la síntesi de glicògen (Ueki i col., 1998; Kohn i col., 1996). A més, ratolins deficients en Akt2 eren resistents a la insulina al múscul i al fetge (Cho i col., 2001). Totes aquestes dades indiquen que l'Akt és un intermediari important en la via de senyalització de la insulina. També l'exposició de cèl·lules musculars esquelètiques a àcids grassos lliures, inhibeix l'activació de l'Akt per part de la insulina (Chavez i col., 2003). D'altra banda el tractament amb troglitazona incrementa la fosforilació de l'Akt al múscul esquelètic, efecte que s'ha relacionat amb la reducció dels àcids grassos lliures en plasma provocats pel tractament (Meyer i col., 2002). A més, els resultats d'aquesta d'aquesta tesi doctoral demostren un increment d'expressió d'aquesta proteïna *in vivo,* sense que es donin diferències significatives en els nivells d'àcids grassos lliures en plasma, i també en cèl·lules C_2C_{12}.

Aquests resultats suggereixen que un altre factor addicional que contribueix a la millora de la sensibilitat a la insulina provocada per la troglitazona podria ser l'increment de l'expressió de la proteïna Akt al múscul esquelètic. Els animals tractats amb tiazolidinediones presentarien uns nivells més elevats d'Akt al múscul, que mantindrien la via de senyalització de la insulina activa i també la captació de glucosa, en situacions de resistència a la insulina. Aquests efectes però, haurien de ser confirmats en humans. En aquest sentit, Meyer i col. (2002) van descriure com el tractament amb troglitazona en individus normoglicèmics amb risc de desenvolupar diabetis de tipus 2, presentaven una tendència a incrementar l'expressió de la proteïna Akt, encara que les diferències no van arribar a ser significatives.

Ja s'havia descrit com el tractament amb troglitazona en múscul de rata aïllat, provocava l'alliberació de lactat i la inhibició de l'oxidació de palmitat, per un mecanisme independent de PPARγ (Brunmair i col., 2001). D'altra banda s'havia descrit una relació negativa entre Akt i AMPK (Kovacic col., 2003), fet que podria alterar el metabolisme dels àcids grassos. En el nostre estudi vam observar que l'increment d'Akt s'associava amb una disminució del contingut d'AMPK fosforilada, reflexant una reducció de la seva activitat. Aquest fet també va provocar un descens en la fosforilació de l'ACC i un increment dels nivells de malonil-CoA, conegut inhibidor de la M-CPT-I. D'acord amb altres estudis (Itani i col., 2003), la reducció d'AMPK incrementa el contingut en lactat, suggerint que la troglitazona podria incrementar la glucòlisi. Segons tot això, els nostres resultats semblen indicar que l'increment de l'expressió de la proteïna Akt provocat per la troglitazona reduiria l'activitat de l'AMPK provocant canvis en els nivells de malonil-CoA, fet que podria reduir l'oxidació mitocondrial dels àcids grassos.

A continuació vam intentar determinar el mecanisme d'acció responsable de la inducció de la proteïna Akt. No vam observar canvis en els nivells de mRNA d'Akt1 ni d'Akt2, fet que suggeria que no es produien canvis a nivell transcripcional. Com que l'Akt també es regula a nivell proteolític, ens vam plantejar si els factors que la regulen a aquest nivell estaven implicats en els canvis observats. La degradació d'Akt s'incrementa tant per increments d'H_2O_2 com de ceramides (Martin i col., 2002). Els nostres resultats indiquen com els nivells d'H_2O_2 no es van veure modificats pel tractament, mentre que els de ceramides es van veure fortament reduïts després del tractament amb troglitazona. El contingut de palmitoil-CoA, precursor de la síntesi *de novo* de ceramides, també es trobava disminuït en el múscul dels animals tractats, indicant que la troglitazona inhibia aquesta via perquè reduïa els nivells intracel·lulars del seu precursor. Com que els nivells d'àcids grassos circulants en el nostre estudi no es troben modificats després del tractament amb troglitazona, aquests no podien ser els responsables de la reducció dels nivells de palmitoil-CoA. Nosaltres suggerim que l'increment que observem dels nivells de mRNA de la CTE, que hidrolitza els acil-CoAs a àcids grassos lliures sigui el responsable de la reducció de palmitoil-CoA. Malgrat això, no podem excloure que l'efecte inhibidor de la troglitazona sobre l'ACS (Fulgencio i col., 1996), contribueixi a reduir el contingut intracel·lular de palmitoil-CoA.

CONCLUSIONS

CONCLUSIONS

I. L'activació del factor NF-κB durant la hipertròfia cardíaca redueix l'activitat de PPARβ/δ tant *in vivo* com *in vitro*, a través d'un mecanisme que implica la interacció entre la subunitat p65 de NF-κB i PPARβ/δ.

II. L'activació de PPARβ/δ amb un agonista selectiu inhibeix la hipertròfia cardíaca induïda per fenilefrina en cardiomiòcits neonatals. Aquest tractament també inhibeix l'activació de NF-κB induïda per LPS a través d'un mecanisme que implica la interacció física entre aquest subtipus de PPAR i la subunitat p65 de NF-κB.

III. La reducció de l'oxidació dels àcids grassos observada durant la hipertròfia cardíaca, sembla ser el resultat de la reducció tant dels nivells de PPARα com de PPARβ/δ i de la interacció dels dos subtipus de PPAR i p65. Aquests canvis es reverteixen després del tractament amb atorvastatina.

IV. L'activació de NF-κB induïda per LPS redueix l'expressió de la PGC-1. Aquest fet disminueix la interacció d'aquest coactivador i PPARβ/δ provocant un descens en l'expressió dels gens implicats en l'oxidació dels àcids grassos. Aquests canvis es reverteixen en presencia d'inhibidors de NF-κB com l'atorvastatina i el partenolide.

V. L'atorvastatina inhibeix l'activació de la via Akt/GSK3β per estímuls hipertròfics, a través d'un mecanisme que implica la supressió de l'activitat NF-κB.

VI. El triflusal, i el seu metabòlit actiu HTB, eviten la hipertròfia cardíaca tant *in vitro* com *in vivo* a través de la inhibició de NF-κB.

VII. El tractament amb troglitazona incrementa l'expressió de la proteïna Akt en múscul esquelètic, a través de la reducció dels nivells intracel·lulars de ceramides.

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