UNIVERSITAT DE BARCELONA

FACULTAD DE FARMACIA

DEPARTAMENTO DE FISIOLOGÍA (FARMACIA)

Implicación de los metabolitos del ácido araquidónico producidos por las lipoxigenasas y citocromos P-450 sobre la proliferación de los fibroblastos 3T6

Diana Nieves Calatrava

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Implicación de los metabolitos del ácido araquidónico producidos por las lipoxigenasas y citocromos P-450 sobre la proliferación de los fibroblastos 3T6

Memoria presentada por Diana Nieves Calatrava para optar al título de doctor por la Universidad de Barcelona

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INFORMA:

Que la memoria titulada "Implicación de los metabolitos del ácido araquidónico producidos por las lipoxigenasas y citocromos P-450 sobre la proliferación de los fibroblastos 3T6" presentada por DIANA NIEVES CALATRAVA para optar al grado de Doctor por la Universidad de Barcelona, ha sido realizada bajo mi dirección en el Departamento de Fisiología (Farmacia), y considerándola finalizada, autorizo su presentación para ser juzgada por el tribunal correspondiente.

Y, para que así conste, firmo la presente en Barcelona, el día 30 de octubre de 2006.

Dr. Juan José Moreno Aznárez

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AA	Ácido araquidónico
1-ABT	1-Aminobenzotriazol
AFC	7-Amino-4-trifluorometil cumarina
BSA	Albúmina sérica bovina
COX	Ciclooxigenasa
CYP	Citocromo P450
CysLT	Cisteinil leucotrieno
DHETE	Ácido dihidroxieicosatetraenoico
DMEM	*Dulbecco's Modified Eagle's Medium*
DTT	Ditiotreitol
EDHF	Factor hiperpolarizante derivado del endotelio
EET	Ácido epoxieicosatrienoico
EGF	Factor de crecimiento epidermal
EFGR	Receptor de EGF
EH	Epóxido hidrolasa
mEH	Epóxido hidrolasa microsomal
sEH	Epóxido hidrolasa soluble
EPA	Ácido eicosapentaenoico
ERK	*Extracellular Signal-Regulated Kinase*
FABP	Proteína de unión a ácidos grasos
FBS	Suero fetal bovino
HETE	Ácido hidroxieicosatetraenoico
LOX	Lipoxigenasa
LT	Leucotrieno
MAPK	*Mitogen-Activated Protein Kinase*
NDGA	Ácido nordihidroguayarético
17-ODYA	Ácido 17-octadecinoico
P-Akt	Akt fosforilado
PBS	*Phosphate Buffered Saline*
PDGF	Factor de crecimiento derivado de plaquetas
PD098059	2-(2-Amino-3-metoxifenil)-4H-1-benzopiran-4-ona
PG	Prostaglandina
PGHS	Prostaglandina H sintasa
PKA	Proteína quinasa A
PKC	Proteína quinasa C
PMN	Leucocitos polimorfonucleados
PMSF	Fluorato de fenilmetilsulfonil

PPAR	*Peroxisome Proliferator-Activated Receptor*
PPOH	Ácido 6-(2-propargiloxifenil)hexanoico
PVDF	Difluorato de polivinilideno
ROS	Especies reactivas del oxígeno
SHR	Ratas espontáneamente hipertensas
SKF-525A	N,N-dietilaminoetil 2,2-difenilvalerato/proadifen
TdT	Deoxinucleotidil transferasa terminal
TNF	*Tumor Necrosis Factor*

1. INTRODUCCIÓN

1.1. Cascada del ácido araquidónico

La mayoría de las células de mamífero tienen cantidades importantes de ácido araquidónico (AA) esterificado en la posición sn-2 de los glicerofosfolípidos de las membranas. En ausencia de estímulos, la concentración celular de AA libre es muy baja. Sin embargo, lipasas específicas pueden liberar el AA que posteriormente será oxidado por los enzimas de la cascada del AA. Hace más de 30 años que se sabe que el AA puede ser metabolizado por las prostaglandina H sintasas (PGHSs), también llamadas **ciclooxigenasas** (COXs) y por las **lipoxigenasas** (LOXs). En 1981, Capdevila *et al.* observaron por primera vez que el AA también podía ser metabolizado por los **citocromos P-450** (CYPs).

Diversos estímulos tales como citocinas, factores de crecimiento o trauma mecánico son capaces de activar la liberación del AA, y posteriormente las PGHSs forman la prostaglandina (PG) H_2, un endoperóxido cíclico inestable (Funk, 2001). Existen dos isoformas de PGHSs, la PGHS-1/COX-1 y la PGHS-2/COX-2. Se considera que la COX-1 es la enzima encargada de la síntesis basal constitutiva de las PGs, mientras que la COX-2 es inducida en determinadas situaciones (Smith *et al.*, 2000). De forma específica en función del tipo celular, la PGH_2 puede ser metabolizada dando lugar a PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 (prostaciclina) o tromboxano A_2 (figura 1.1). Así, la tromboxano sintasa se ha encontrado en plaquetas y macrófagos, la prostaciclina sintasa en células endoteliales, la PGF sintasa en útero y la PGD sintasa en cerebro y mastocitos (Funk, 2001). Las PGs pueden ejercer efectos autocrinos o paracrinos, al unirse a receptores específicos que están acoplados a proteínas G y tienen siete dominios transmembrana. Así, se han descrito cuatro receptores para la PGE_2 (EP_1-EP_4), dos en el caso de la PGD_2 (DP_1 y DP_2), y un receptor al que se une la PGF_2 , PGI_2, y tromboxano A_2 (FP, IP, y TP, respectivamente) (Narumiya y FitzGerald, 2001). Las PGs también pueden unirse a receptores nucleares como los *peroxisome proliferator-activated receptors* (PPAR)-γ y regular los procesos inflamatorios (Zingarelli y Cook, 2005).

Además, el AA puede ser metabolizado por la 5-LOX formando el ácido 5-hidroxieicosatetraenoico (HETE) y el leucotrieno (LT) A_4. A partir del LTA_4 y mediante la LTA_4 hidrolasa se forma el LTB_4, un potente quimioatrayente de neutrófilos (Harvath, 1991) y estimulador de la adhesión de los leucocitos sobre las células endoteliales (Dahlen *et al.*, 1981). El LTA_4 también puede ser transformado a LTC_4 mediante la LTC_4 sintasa, y éste puede dar lugar a la formación de LTD_4 y LTE_4. El LTC_4, LTD_4 y LTE_4 se conocen con el nombre

genérico de cisteinil leucotrienos (CysLTs). Finalmente, la 12-LOX y la 15-LOX pueden producir HETEs y lipoxinos a partir del AA (Funk, 2001) (figura 1.1). Los LTs son sintetizados básicamente en células inflamatorias como leucocitos polimorfonucleados (PMN), macrófagos y mastocitos. Sus efectos biológicos dependen de su interacción con receptores específicos. Se han caracterizado cuatro receptores que también tienen siete dominios transmembrana y están acoplados a proteínas G. Así, hay dos receptores para el LTB_4, el BLT1 que es de alta afinidad y el BLT2 que es de baja afinidad (Tager y Luster, 2003); y dos receptores para los CysLTs, el CysLT1 y el CysLT2 (Kanaoka y Boyce, 2004). Además, se ha descrito que el LTB_4 puede interactuar con el receptor nuclear PPAR-α e inducir la expresión de genes implicados en la β-oxidación (Chinetti *et al.*, 2000).

Los eicosanoides juegan importantes papeles fisiológicos y además de su papel en la inflamación, están implicados en la fisiopatología de la diabetes, cáncer, arteriosclerosis e hipertensión (Capdevila y Falck, 2001). Debido a su importancia clínica, estos metabolitos y las enzimas responsables de su biosíntesis han sido estudiadas extensamente (Smith *et al.*, 2000; Cuendet y Pezzuto, 2000). Sin embargo, el papel biológico de los metabolitos derivados del AA vía CYPs todavía está bajo una intensa investigación.

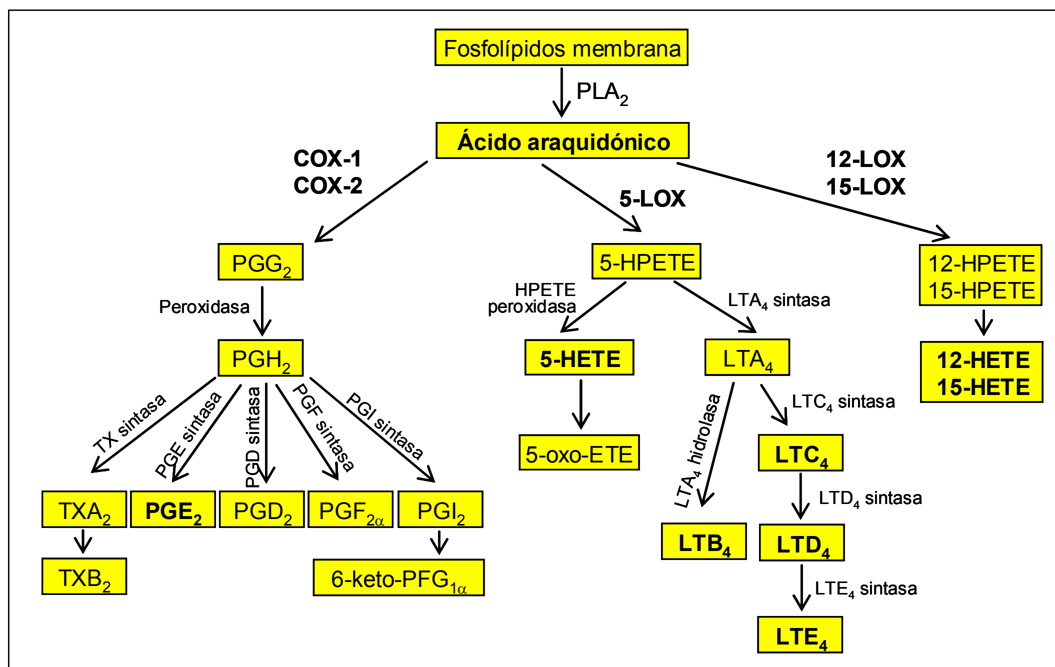

Figura 1.1: Principales metabolitos formados a partir del AA a través de las vías COX y LOX

1.2. Los citocromos P-450

Los CYPs son proteínas ubicuas que están ampliamente distribuidas en plantas, insectos y tejidos de animales, y se expresan en todos los tipos celulares de mamífero que se han estudiado. Hasta ahora, más de 400 enzimas CYP han sido identificados. Los CYPs de mamíferos forman al menos 17 familias, resultado de la expresión de los genes cuyas secuencias tienen más de un 40% de homología, tales como CYP**1**,CYP**2**,CYP**3**,CYP**4**...; 32 subfamilias, formadas por proteínas cuyos genes tienen secuencias de más de un 55% de homología, como CYP4**A**,CYP4**B**,CYP4**F**...; y más de 500 productos génicos específicos, tales como CYP4A**1**, CYP4A**2**, CYP4A**3**... (Capdevila y Falck, 2000).

Aunque muchos de estos CYPs se expresan constitutivamente de forma más o menos dependiente de especie, órgano/tejido, sexo y edad, la transcripción de muchos de ellos está regulada por factores como hormonas, citocinas, exposición a compuestos químicos, dieta, desnutrición, y varía en patologías como la diabetes e hipertensión (Capdevila y Falck, 2000). Así, en las células endoteliales la expresión de las epoxigenasas CYP2J es constitutiva, mientras que la de CYP2C aumenta al incrementar el calcio intracelular. En cambio, en riñón de rata la mayoría de isoformas constitutivas son miembros de la familia CYP2C (Capdevila y Falck, 2000). Se ha observado que la proteína CYP2C es bastante inestable, lo cual sugiere que los procesos post-transcripcionales pueden determinar los niveles de expresión de este enzima, siendo también importantes los estímulos fisiológicos en el control del nivel de los CYPs (Michaelis y Fleming, 2006). Algunos genes CYPs presentan en el promotor elementos de respuesta a la hipoxia, de esta manera los cambios en la concentración de oxígeno incrementarán o disminuirán su expresión (Marden *et al.*, 2003; Michaelis *et al.*, 2005). Dada la amplia existencia de proteínas CYPs, no es sorprendente que haya una considerable variación en la regulación de su expresión génica, la estabilidad de su mRNA y en las modificaciones post-transcripcionales de sus diferentes isoformas. En este sentido, los CYPs pueden ser fosforilados por la proteína quinasa A (PKA) modificando la actividad del enzima, su localización subcelular y su degradación en el proteasoma (Korsmeyer *et al.*, 1999; Oesch-Bartlomowicz *et al.*, 2001).

Los CYPs pertenecen a una superfamilia de hemoproteínas unidas a la membrana que sirven como oxidasas terminales de la cadena de transporte de electrones presente en el retículo endoplasmático (Hasler *et al.*, 1999). Los CYPs forman parte de un sistema multienzimático que incluye un FAD/FMN que

contiene una NADPH-citocromo P-450 reductasa y un citocromo b_5 (Fleming, 2001). Todos los CYPs contienen un grupo hemo prostético protoporfirina IX y participan en el metabolismo oxidativo de moléculas orgánicas lipofílicas tales como los xenobióticos, el colesterol, los esteroides, los ácidos grasos y las vitaminas. El grupo hemo tiolado está muy conservado y confiere unas propiedades espectrales, redox y catalíticas únicas a los CYPs. Así, la hemoproteína controla la inserción del oxígeno en el ácido graso, y determina el tipo de reacción, la regioselectividad y la enantioselectividad (Capdevila y Falck, 2000). Una vez liberado el AA, se une al sitio activo de la proteína CYP. A continuación la NADPH-P-450 reductasa transfiere un electrón del NADPH al hierro del CYP, catalizando la reducción del hierro, y captura el oxígeno molecular formando el complejo "oxi-P-450". En el complejo tiene lugar la reducción de un electrón, catalizado por la NADPH-P-450 reductasa. Seguidamente, se produce la escisión oxígeno-oxígeno, la protonación, la C-H inserción, y la liberación del producto, el substrato hidroxilado, y el agua (Capdevila y Falck, 2000). En resumen, durante la metabolización del AA a través del CYP se consume NADPH y oxígeno, y se produce agua y el substrato hidroxilado. En la figura 1.2 pueden observarse estas reacciones de modo esquemático.

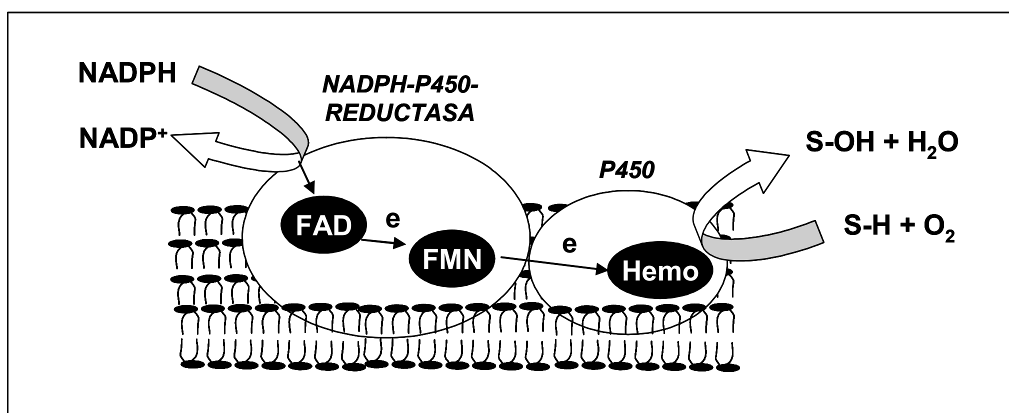

Figura 1.2: Reacciones producidas durante el ciclo catalítico del CYP. *S*, substrato

Mientras que los CYPs son monooxigenasas, las COXs y las LOXs son dioxigenasas que catalizan la reacción de forma parecida al ataque de los radicales libres sobre los ácidos grasos, son NADPH independiente y no activan un oxígeno, sino un carbono del substrato (Capdevila y Falck, 2000).

1.3. Metabolitos derivados del AA vía citocromos P-450

Los CYPs pueden metabolizar al AA por una o más de las siguientes reacciones: las oxidaciones *bis*-alílicas (reacciones tipo-lipoxigenasa), formando el 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE y 15-HETE; las hidroxilaciones en los carbonos C16-C20 (actividad ω/ω-1 hidroxilasa), dando lugar al 16-HETE, 17-HETE, 18-HETE, 19-HETE y 20-HETE; y finalmente la epoxidación olefín (actividad epoxigenasa) que produce los ácidos 5,6- epoxieicosatrienoico (EET), 8,9-EET, 11,12-EET y 14,15-EET Capdevila *et al.* (2000) (figura 1.3).

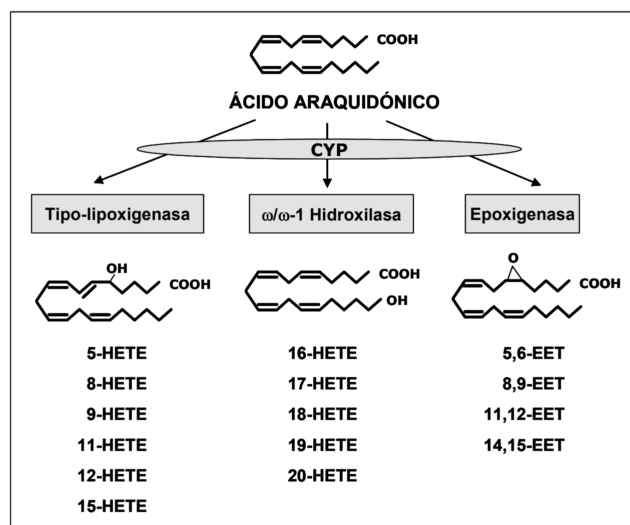

Figura 1.3: Principales metabolitos formados a partir del AA mediante las actividades de los CYPs (*figura adaptada de Capdevila et al., 2000*)

1.3.1. Oxidaciones *bis*-alílicas (tipo-lipoxigenasa)

A través de estas reacciones se forman los metabolitos 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE y 15-HETE (figura 1.3). El 5-HETE, 12-HETE y 15-HETE también puede ser formados por las LOXs correspondientes (Capdevila y Falck, 2000). Se ha demostrado mediante análisis estereoquímicos que en fracciones microsomales de hígado se cataliza la formación de 5-HETE, 8-HETE, 9-HETE, 11-HETE y 15-HETE como mezclas racémicas mientras que el 12-HETE se forma enantioselectivamente como isómero *R* (Capdevila *et al.*, 1986). Así, aunque se creía que el 12(*R*)-HETE era el principal enantiómero producido por los CYPs, y las 12-LOXs producían selectivamente el 12(*S*)-HETE, recientemente se han clonado y caracterizado dos LOXs capaces de producir 12(*R*)-HETE en piel humana (Boeglin *et al.*, 1998).

1.3.2. Hidroxilaciones en C16-C20 (ω/ω-1 hidroxilasa)

Los metabolitos formados a partir de la ω/ω-1 hidroxilación del AA son el 20-HETE, 19-HETE, 18-HETE, 17-HETE y 16-HETE (ω, ω-1, ω-2, ω-3 y ω-4 alcoholes, respectivamente) (figura 1.3). En términos de tasas de reacción y eficiencia catalítica los ácidos grasos saturados, y el ácido laúrico en particular son generalmente mejores substratos que el AA para las ω/ω-1 hidroxilasas (Capdevila y Falck, 2000).

En mamíferos, la mayoría de las reacciones ω/ω-1 hidroxilasa son catalizadas por las isoformas CYP4A. Se trata de una subfamilia génica muy conservada que codifica para proteínas que están especializadas en la oxidación de los ácidos grasos y prácticamente no tienen actividad sobre los xenobióticos. Al menos 13 isoformas CYP4A han sido clonadas y/o aisladas y purificadas en rata (CYP4A1, 4A2, 4A3 y 4A8), ratón (CYP4A10, 4A12 y 4A14), conejo (CYP4A4, 4A5, 4A6 y 4A7) y hombre (CYP4A9 y 4A11) (Capdevila y Falck, 2000). En la tabla 1.1 se muestran las actividades enzimáticas de las isoformas CYP4A en diversas especies.

Las isoformas CYP4F también tienen una gran actividad ω/ω-1 hidroxilasa (Kroetz y Zeldin, 2002). Los CYP 1A1, 1A2, 2E1, 4A2 y 4A3 también tienen actividad ω/ω-1 hidroxilasa y además pueden producir cantidades variables de EETs. Existen numerosos CYPs con actividad hidroxilasa que todavía han de caracterizarse (Capdevila *et al.*, 1992a).

Tabla 1.1: Metabolismo de los ácidos grasos y prostanoides a través de las diferentes isoformas CYP4A en varias especies *(tabla adaptada de Capdevila y Falck, 2001)*

ISOFORMA	ESPECIE	ACTIVIDAD ENZIMÁTICA
4A1	Rata	ω-oxidación de ácido laúrico y araquidónico
4A2	Rata	ω/ω-1 oxidación de ácido laúrico y araquidónico
4A3	Rata	ω/ω-1 oxidación de ácido laúrico y araquidónico
4A8	Rata	Desconocido
4A4	Conejo	ω–oxidación de ácido palmítico y araquidónico y PGA, PGE, PGD y $PF_{2\alpha}$
4A5	Conejo	ω/ω-1 oxidación de ácido laúrico y palmítico ω–oxidación de PGA_1 y ácido araquidónico
4A6	Conejo	ω–oxidación de ácido laúrico, palmítico y araquidónico
4A7	Conejo	ω–oxidación de ácido laúrico, palmítico, araquidónico y PGA_1
4A10	Ratón	ω–oxidación de ácido laúrico
4A12	Ratón	ω/ω-1 oxidación de ácido laúrico y araquidónico
4A14	Ratón	ω/ω-1 oxidación de ácido laúrico
4A11	Hombre	ω/ω-1 oxidación de ácido laúrico y araquidónico

1.3.3. Epoxidación olefín (epoxigenasa)

En mamíferos los CYPs son los únicos enzimas con actividad epoxigenasa y utilizan como substrato principalmente al AA (Capdevila *et al.*, 1985). Las epoxigenasas tienen una regioselectividad limitada pudiendo epoxidar asimétricamente sobre diferentes carbonos, así se forman el 5,6-EET, 8,9-EET, 11,12-EET o 14,15-EET (figura 1.3), y tienen una elevada enantioselectividad pudiendo formar por ejemplo el 14(*R*),15(*S*)EET o el 14(*S*),15(*R*)-EET. Los residuos aminoacídicos de los CYPs determinan las propiedades químicas y espaciales del lugar activo de la proteína, e influyen sobre la regio- y enantioselectividad de la epoxidación del AA. En la tabla 1.2 se muestra el grado de enantioselectividad de algunas isoformas de CYP2 (Capdevila y Falck, 2001).

Las CYP epoxigenasas producen los cuatro tipos de EETs, pero normalmente producen de forma predominante un o dos de ellos (Spector *et al.*, 2004). Así, en músculo cardíaco el CYP2J3 produce principalmente el 14,15-EET (41%) (Wu *et al.*, 1997), mientras que en riñón el CYP2J23 produce mayoritariamente el 11,12-EET (58%) (Karara *et al.*, 1993).

Tabla 1.2: Enantioselectividad de las isoformas CYP2 de rata en la síntesis del 8,9- y 11,12-EETs. Los valores representan el % de cada enantiómero
(tabla adaptada de Capdevila y Falck, 2001)

ISOFORMA	**8,9-EET**		**11,12-EET**	
CYP	***S,R***	***R,S***	***S,R***	***R,S***
2B1	86	14	86	14
2B2	90	10	84	16
2B12	39	61	70	30
2C11	46	54	21	79
2C23	6	94	11	89
2C24	61	39	25	75
2J3	40	60	38	62

Diversos autores mediante el uso de enzimas purificadas o recombinantes han demostrado que múltiples isoformas del CYP pueden formar EETs a partir del AA, como CYP1A, 2D, 2E, 2G, 2J, 2N y 4A (Kroetz y Zeldin, 2002). Aunque la mayoría de las epoxigenasas que metabolizan el AA son miembros de la familia génica CYP2. Algunas isoformas de las subfamilias CYP2B y 2C también se han identificado como epoxigenasas, tales como CYP2B1, 2B2, 2B4, 2B12, 2C11,

2C23 y 2C24 en rata; CYP2B4, 2C1 y 2C2 en conejo; CYP2C37, 2C38, 2C39 y 2C40 en ratón; y CYP2C8 y 2C9/2C10 en humanos (Capdevila *et al.*, 2002).

1.4. Metabolismo celular de los HETEs y EETs

Los HETEs pueden ser almacenados en los fosfolípidos de las membranas y modificar sus propiedades físico-químicas, como la permeabilidad o la actividad de las proteínas unidas a la membrana (Gordon y Spector, 1987). Estímulos hormonales como la angiotensina II pueden liberar los HETEs, aumentando la hidrólisis de los fosfolípidos mediada por receptor. Así, los HETEs unidos a los fosfolípidos representan una reserva en órganos como el riñón pudiendo ser liberados independientemente de la actividad de los CYPs (Carroll *et al.*, 1997).

Los HETEs pueden ser metabolizados por las COXs dando lugar a un análogo de la PGH_2. Así, a partir del 20-HETE se formaría el 20-OH PGH_2, el cual puede sufrir transformaciones adicionales mediante isomerasas formando los metabolitos 20-OH PGE_2, 20-OH PGI_2, 20-OH tromboxano A_2 y 20-OH $PGF_{2\alpha}$ (McGiff y Quilley, 1999). Estos metabolitos derivados de los HETEs también pueden ser fisiológicamente activos. Así, la endotelina-1 aumenta la síntesis de 20-HETE, éste es metabolizado por las COXs y forma análogos de las prostaglandinas que son vasoconstrictores (20-OH PGH_2), vasodilatadores y diuréticos (20-OH PGE_2 y 20-OH PGI_2), o vasoconstrictores y antidiuréticos (20-OH tromboxano A_2 y 20-OH $PGF_{2\alpha}$) (Oyekan y McGiff, 1998). Finalmente, las LOXs y los CYPs pueden hidroxilar los HETEs formando diHETEs. Así, en macrófagos peritoneales el 5-HETE puede ser metabolizado por la 12-LOX y 15-LOX produciendo el 5,12-diHETE y 5,15-diHETE, respectivamente (Hevko *et al.*, 2001). Y el 12-HETE puede ser ω-hidroxilado por los CYPs formando 12,20-diHETE en neutrófilos no estimulados (Marcus *et al.*, 1987).

En todos los tipos celulares donde se ha estudiado la captación de EETs radioactivos, se ha observado que hay una captación rápida al añadirlos en el medio de cultivo como ácidos grasos libres (Spector *et al.*, 2004). Pero no se sabe si los EETs atraviesan la membrana por difusión libre o a través de algún transportador de membrana. Estos EETs libres pueden ser incorporados a los fosfolípidos de membrana a través de un proceso acil coenzima A sintasa dependiente. Así, los EETs pueden esterificarse en la posición *sn-2* de varios fosfolípidos como la fosfatidilcolina, fosfatidiletanolamina o fosfatidilinositol (figura 1.4). Del mismo modo que los HETEs, los EETs incorporados a las membranas celulares pueden modificar su estructura así como sus propiedades

físico-químicas (Capdevila *et al.*, 2000), y ser liberados mediante reacciones hidrolíticas, independientemente de la actividad CYP epoxigenasa. De hecho, la mayoría de los EETs (85%) sintetizados por epoxigenasas que están presentes endógenamente en tejidos de mamíferos son incorporados a glicerofosfolípidos celulares y liberados bajo estimulación con varios agonistas. Karara *et al.* (1991) observaron que en el hígado aproximadamente el 92% de los EETs endógenos estaba esterificado en los fosfolípidos, un 4% en diglicéridos, un 4% en lípidos neutros y menos del 1% como ácidos grasos libres. Así, el 55% de los EETs se asociaron a fosfolípidos que contenían fosfatidilcolina. De este modo, los EETs son capaces de esterificarse *in vivo*, contrariamente a la mayoría de eicosanoides que son liberados fuera de la célula (Karara *et al.*, 1991).

Los EETs que están en el citosol sin esterificarse pueden unirse a las proteínas de unión a ácidos grasos (FABP) que actuarían como transportadores regulando su interacción con sus proteínas diana (Spector *et al.*, 2004) (Figura 1.4).

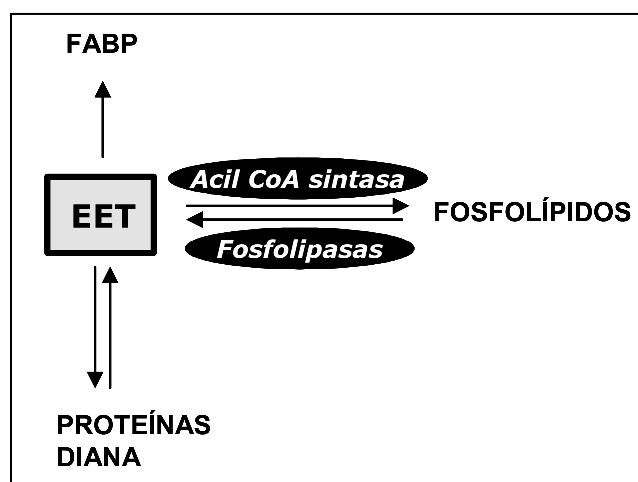

Figura 1.4: Vías de almacenaje y transporte de los EETs en la célula
(figura adaptada de Spector et al., 2004)

Por otro lado, las epóxido hidrolasas (EH) pueden metabolizar rápidamente los EETs formando los ácidos dihidroxieicosatetraenoicos (DHETEs) (figura 1.5). En los tejidos de mamíferos hay dos isoenzimas, la EH soluble (sEH) que se encuentra principalmente en el citosol, y la EH microsomal (mEH) que se localiza unida a las membranas intracelulares (Zeldin *et al.*, 1993 y 1995; Morisseau *et al.*, 2001). La sEH es la principal enzima responsable de la conversión de los EETs a DHETEs. Se han encontrado niveles altos de sEH en hígado, riñón, intestino y tejido vascular mientras que hay unos niveles bajos en testículos, pulmón, cerebro y bazo (Spector *et al.*, 2004). Se ha purificado la sEH

de hígado y riñón de diferentes especies incluida la humana. Parte de los EETs no esterificados son metabolizados formando DHETEs y la mayoría de éstos son liberados al medio extracelular (VanRollins *et al.*, 1993), mientras que una pequeña parte de los DHETEs también puede ser incorporada a los fosfolípidos celulares (VanRollins *et al.*, 1996). Además, los DHETEs son capaces de unirse a FABP citosólicas (Widstrom *et al.*, 2001), aunque tienen menos afinidad que los EETs.

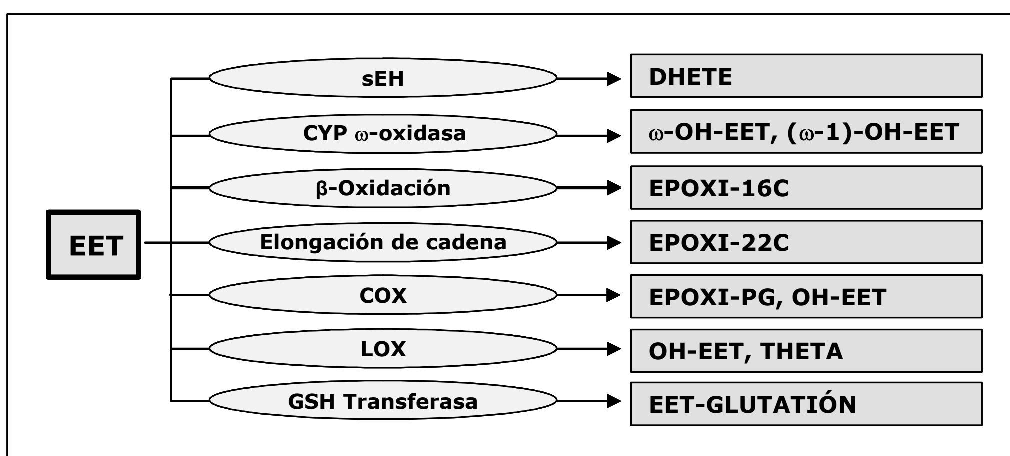

Figura 1.5: Principales vías de metabolización de los EETs
(figura adaptada de Spector et al., 2004)

Los metabolitos generados por la actividad CYP sobre el AA pueden ser reiterativamente oxidados. De este modo, los EETs compiten con el AA por el sitio activo del CYP y son rápidamente oxidados, incluso en presencia de un exceso de AA (figura 1.5), dando lugar a mezclas complejas de productos polioxidados. Todo esto complica la estimación de la actividad de los CYPs, la formación de metabolitos específicos para cada isoforma, y la contribución de una isoforma a la capacidad metabólica total de los CYPs (Capdevila *et al.*, 2000).

Los EETs también pueden ser metabolizados a través de otras reacciones como la β-oxidación, la elongación de cadena, la oxidación por las COXs, LOXs, o la conjugación con glutatión (figura 1.5) (Spector *et al.*, 2004).

1.5. Localización de las isoformas CYP y de los metabolitos derivados del AA-CYP

La ω/ω-1 hidroxilación se ha observado en fracciones microsomales procedentes de hígado, riñón, pulmón, intestino, epitelio olfatorio y pituitaria anterior (Capdevila y Falck, 2000). Pero ha sido en tejido renal donde estas reacciones se han caracterizado mejor y se han asignado numerosas funciones fisiológicas a los metabolitos producidos a partir de la metabolización del AA vía hidroxilación. Así, los CYP4A y 4F son las principales isoformas ω/ω-1 hidroxilasas que actúan sobre el AA. Tanto el CYP4A11 como el CYP4F2 se han identificado en los segmentos S2 y S3 de los túbulos proximales de riñón. Además, la isoforma CYP4F12 ha sido clonada a partir de riñón humano e intestino delgado y presenta actividad ω/ω-2 hidroxilasa para el AA (Kroetz y Zeldin, 2002).

El 20-HETE se ha encontrado en células endoteliales de arterias coronarias bovinas, en plasma de perro, astrocitos de rata y microsomas de riñón (Nithipatikom *et al.*, 2001). Además, es el eicosanoide producido en mayor cantidad en riñón, superando a la PGE_2 y a la PGI_2 (McGiff y Quilley, 1999).

Zeldin *et al.* (1996) identificaron la expresión de la subfamilia CYP2J, que produce EETs, en células epiteliales, células musculares de vasos y bronquios, y macrófagos endoteliales y alveolares, de pulmón de rata y hombre. En cambio, la isoforma 2J2 no se ha encontrado en tejidos como cerebro, riñón, ovario y testículos. En riñón de rata, las isoformas CYP2C23, 2C24 y 2C11 son las epoxigenasas más expresadas (Capdevila *et al.*, 1992b; Imaoka *et al.*, 1993; Capdevila *et al.*, 2000), mientras que en riñón humano la epoxigenasa más expresada es la 2C8 (Zeldin *et al.*, 1995). Las subfamilias CYP1A, CYP2B, CYP2C y CYP2E también están presentes en pulmón de conejo (Jacobs y Zeldin, 2001).

Se ha demostrado la formación de EETs a partir de CYPs purificados, fracciones microsomales y preparaciones celulares aisladas en diversos tejidos incluyendo hígado, riñón, pituitaria, cerebro, adrenales, endotelio, páncreas y ovarios (Capdevila *et al.*, 2002). Así, el producto mayoritario derivado de la actividad epoxigenasa en microsomas de cerebro es el 14,15-EET, mientras que en fracciones microsomales de hígado y riñón de rata el regioisómero predominante es el 11,12-EET (McGiff y Quilley, 1999; Capdevila *et al.*, 2000).

Se ha detectado la presencia de EETs a nivel de plasma, lo cual sugiere que pueden llegar a otros tejidos transportados con la sangre (Spector *et al.*, 2004). Así, la concentración de EETs en plasma de rata es de 10,2 ± 0,4 ng/mL

(Karara *et al.*, 1992) y alrededor del 90% están unidos a los fosfolípidos de las lipoproteínas. La mayor concentración se encuentra en las lipoproteínas de baja densidad, seguidas de las de alta densidad y de las de muy baja densidad. En el corazón, endotelio y plasma humano hay una cantidad similar de 8,9-EET, 11,12-EET y 14,15-EET, alrededor de 0,1-0,3 μM (Spiecker y Liao, 2005). Por otro lado, los DHETEs son metabolitos más estables que los EETs, lo que ha permitido su cuantificación en muestras de plasma y orina (Spiecker y Liao, 2005).

En un tipo celular específico varias isoformas de CYP pueden formar simultáneamente HETEs/EETs, y la contribución de cada una en la producción de estos metabolitos dependerá de su abundancia y de su eficiencia catalítica (Zeldin, 2001). Como se ha dicho anteriormente, el tipo de metabolitos derivados del AA a través de los CYPs es específico de tejido. Así, en el hígado de rata y conejo el producto mayoritario son los EETs, mientras que en el riñón los metabolitos derivan principalmente de la ω/ω-1 hidroxilación, y en la pituitaria de la actividad tipo-lipoxigenasa (tabla 1.3) (Capdevila *et al.*, 2000). En el riñón el CYP4A2 forma mezclas de 19-HETE y 20-HETE, y pequeñas cantidades de 11,12-EET, mientras que los queratinocitos presentan específicamente la epoxigenasa CYP2B12, que es altamente regioselectiva y forma en un 80% el 11,12-EET (Capdevila *et al.*, 2000).

Estos resultados demuestran que existe una biosíntesis endógena de los eicosanoides producidos por los CYPs y se sugiere un papel para este sistema enzimático en la fisiología celular.

Tabla 1.3: Especificidad de las actividades de los CYPs en varios tejidos
(tabla adaptada de Capdevila et al., 2000)

	HÍGADO	**RIÑÓN**	**PITUITARIA**
EPOXIGENASA	70	23	30
w/w-1 HIDROXILASA	20	77	16
LIKE-LIPOXIGENASA	9	-	45
OTROS	<1	<1	8

1.6. Receptores de los HETEs y EETs

Los metabolitos derivados del AA a través de la vía de los CYPs parecen ser importantes moduladores de las cascadas de señalización intracelular, pero no está claro cómo inician estos efectos. Es bien conocido que las PGs, los tromboxanos y los LTs median sus acciones por la interacción con receptores específicos de membrana. La clonación y caracterización de estos receptores ha permitido el desarrollo de agonistas y antagonistas específicos (Jacobs y Zeldin, 2001). En cambio, para los HETEs/EETs hay evidencias de lugares de unión en la membrana para el 14(*R*),15(*S*)-EET en células mononucleares de cobaya y monocitos U-937 sin que se hayan identificado receptores específicos (Wong *et al.*, 2000). De forma similar, usando análogos del 20-HETE en arterias renales se ha sugerido un efecto vasoactivo dependiente de receptor (Alonso-Galicia *et al.*, 1999). Hampson y Grimaldi (2002) demostraron que el 12(*S*)-HETE puede proteger a las neuronas de la excitotoxicidad mediante la activación de proteínas $G_{i/o}$ acopladas a receptor, limitando el flujo de calcio.

Las proteínas de membrana de unión al nucleótido guanina (proteínas G) $\alpha\beta$ están implicadas en la activación de los canales de potasio dependientes de calcio (Li y Campbell, 1997), y los EETs activan esta subunidad $G\alpha\beta$ de la proteína G heterotrimérica por ADP-ribosilación (Li *et al.*, 1999). Normalmente las proteínas G están asociadas a receptores con siete dominios transmembrana para iniciar cascadas de señalización, lo cual sugiere que los EETs se asociarían a estos receptores, dando lugar a un incremento del AMPc y a la activación de la PKA (Wong *et al.*, 1997 y 2000) (figura 1.6). En este sentido, se ha demostrado que el 11,12-EET aumenta la transcripción del gen activador del plasminógeno en células endoteliales a través de la activación de proteínas $G\alpha\beta$ y de la producción de AMPc (Node *et al.*, 2001).

Aunque ningún receptor ha sido clonado o caracterizado, es probable que existan receptores específicos de superficie de membrana para los HETEs/EETs, al igual que los hay para PGs y LTs. Si hubiese varias isoformas de receptores se podría explicar la variedad de respuestas bioquímicas que presentan estos metabolitos. Pero no podemos descartar la posibilidad de que los HETEs/EETs medien algunas de sus acciones sin la participación de receptores de membrana. Así, los HETEs/EETs son substancias lipofílicas y pueden incorporarse directamente en la membrana uniéndose a moléculas efectoras como ADP-ribosiltransferasas (Li *et al.*, 1999), Ras (Muthalif *et al.*, 2001) y PKA (Imig *et al.*, 1999).

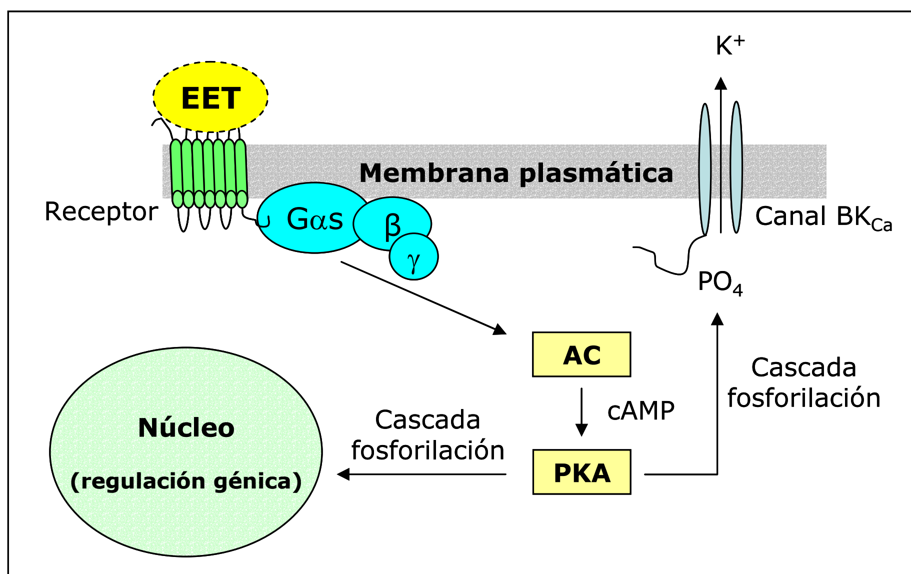

Figura 1.6: Mecanismo propuesto para la señalización de los EETs mediada por las proteínas G *(figura adaptada de Spector et al., 2004)*

También se han identificado como receptores intracelulares para los EETs o DHETEs a los PPARs. En este sentido, Cowart *et al.* (2002) demostraron que el 14,15-EET y el 14,15-DHETE pueden unirse al PPAR-α.

1.7. Funciones de los metabolitos derivados del AA por la vía de los CYPs

Se ha descrito el papel de los HETEs, EETs y DHETEs en el control de múltiples funciones fisiológicas tales como la regulación del tono vascular, la proliferación celular o la apoptosis. Además, la implicación de estos eicosanoides en patologías como la hipertensión hace que el estudio de estos metabolitos sea de gran interés, y que las enzimas que están implicadas en su formación y degradación puedan ser nuevas dianas terapéuticas.

1.7.1. Efectos de los HETEs y EETs sobre la inflamación

Numerosos estudios han demostrado que tanto los HETEs como los EETs pueden participar en procesos inflamatorios, jugando diversos papeles en función del tejido afectado y del origen del proceso inflamatorio.

1.7.1.1. HETEs e inflamación

El papel de los HETEs en la inflamación es controvertido. Por un lado, los HETEs pueden ser anti-inflamatorios. En este sentido, Bednar *et al.* (2000) observaron que el 16(*R*)-HETE producido por los PMN inhibía selectivamente la adhesión y agregación de estas células y la síntesis de LTB_4, durante los procesos de isquemia tras una apoplejía. En cambio, el 16(*S*)-HETE y el 20-HETE no presentaban estos efectos. Así, en los animales que recibían 16(*R*)-HETE se inhibía la función de los PMN y se reducía el daño causado por la isquemia como consecuencia de la supresión del aumento de la presión intracraneal. Contrariamente a estos efectos, Vafeas *et al.* (1998) demostraron que el 12(*R*)-HETE y 12(*R*)-HETrE tienen actividades pro-inflamatorias. Así, estímulos pro-inflamatorios como el LPS, inducían la síntesis de estos metabolitos en el epitelio de la córnea regulando el crecimiento de nuevos vasos que aparecen durante la inflamación crónica o en estadios de reparación durante un proceso agudo. Además, se ha observado la presencia de 12(*R*)-HETE en piel humana y aumenta su formación durante la psoriasis (Woollard, 1986). Por otro lado, el 12(*R*)-HETE también tiene propiedades quimiotácticas sobre los neutrófilos (Cunningham y Woollard, 1987).

1.7.1.2. EETs e inflamación

Actualmente se ha propuesto que los EETs tienen un papel eminentemente anti-inflamatorio. Así, los EETs disminuyen la expresión de las moléculas de adhesión inducidas por citocinas en las células endoteliales, previniendo la adhesión de los leucocitos a la pared vascular (Node *et al.*, 1999). Este efecto se debe a que los EETs producidos por las células endoteliales inhiben la IKK, y por lo tanto la activación de $NF\kappa\beta$, lo que evita que se expresen moléculas de adhesión impidiendo la adhesión de los leucocitos (figura 1.7). El 5,6-EET, 8,9-EET, 11,12-EET y 11,12-DHETE reducen la expresión de VCAM-1 en células estimuladas con *tumor necrosis factor* (TNF)-α. El 11,12-EET a concentraciones fisiológicas es el metabolito más activo en tejidos y plasma, mientras que el 14,15-EET no tiene efecto. Lo que sugiere que los diferentes regioisómeros pueden presentar diferentes actividades. Este efecto anti-inflamatorio de los EETs es independiente de su efecto sobre la hiperpolarización de la membrana (Node *et al.*, 1999). El 11,12-EET también inhibe la expresión de las moléculas de adhesión ICAM-1 y selectina-E inducidas por IL-1α y LPS

(Node *et al.*, 1999). Sin embargo, hay resultados contrarios a los mencionados anteriormente. Así, Pratt *et al.* (2002) observaron que los EETs no modifican la adhesión de los neutrófilos a la pared del vaso, y en cambio aumentan su agregación.

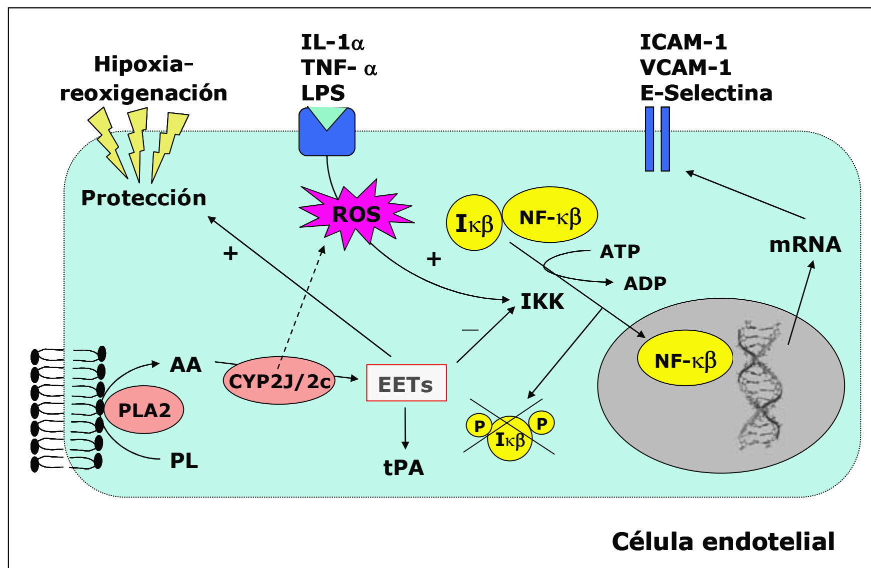

Figura 1.7: Efectos de los EETs sobre la actividad NF-*K*β y la expresión de los genes regulados por este factor de transcripción *(figura adaptada de Node et al., 1999)*

Un aspecto importante a destacar es que los efectos observados tras la aplicación exógena de EETs, puede ser diferente del que se obtiene como consecuencia de la activación endógena de los CYPs. Así, aunque la aplicación directa de EETs atenúa la actividad de NFκβ en las células endoteliales, la sobreexpresión de la proteína CYP2C9, que sintetiza EETs, tiene el efecto opuesto (Fleming, 2001). La razón de este efecto contradictorio es probablemente que el CYP2C además de EETs genera especies reactivas del oxígeno (ROS) que modulan la actividad NFκβ. Pero es importante señalar que no todos los CYPs producen la misma cantidad de ROS (Puntarulo y Cederbaum, 1998). Por otro lado, Yang *et al.* (2001) observaron que los EETs pueden proteger a las células endoteliales de aorta bovina o de corazón humano contra el daño inducido por la hipoxia-reoxigenación (figura 1.7), dado que los EETs presentan un efecto antioxidante al atenuar la producción de ROS y limitar la peroxidación lipídica inducida en estas condiciones experimentales.

Los EETs son capaces de inhibir la biosíntesis de tromboxanos en plaquetas (Yang *et al.*, 2001), y pueden inhibir la agregación plaquetaria

inducida por AA (Jacobs y Zeldin, 2001), lo que contribuiría a la explicación de su efecto anti-inflamatorio.

Además de estos efectos anti-inflamatorios los EETs pueden tener efectos fibrinolíticos (Kroetz y Zeldin, 2002). Así, en células vasculares endoteliales se ha demostrado que concentraciones fisiológicas de EETs o la sobreexpresión de CYP2J2 aumentan la expresión de la proteína t-PA, sin afectar a la expresión de su inhibidor PAI-1, produciéndose un incremento de la actividad fibrinolítica (figura 1.7).

El 11,12-EET y el 14,15-EET también pueden inhibir la migración de las células musculares lisas inducida por el factor de crecimiento derivado de plaquetas (PDGF-BB) o por el suero fetal (Spiecker y Liao, 2005). Los mecanismos mediante los que los EETs inhiben la migración celular no se conocen bien, aunque la actividad PKA y el AMPc intracelular estarían implicados.

En resumen, los EETs podrían jugar un papel importante como protectores vasculares ya que inhiben al factor de transcripción pro-inflamatorio NFκβ, atenúan la producción de ROS, protegen contra el daño inducido tras la isquemia, incrementan la fibrinolisis y reducen la migración de las células musculares vasculares.

1.7.2. Efectos de los HETEs y EETs sobre el tono vascular

1.7.2.1. HETEs y tono vascular

Los HETEs puede contraer las células musculares lisas de los vasos. Éstas células producen 20-HETE tras un aumento de la concentración del calcio intracelular y, una vez formado, aumenta el tono del músculo liso (Fleming, 2001) (figura 1.8). Este HETE inhibe los canales de K_{Ca}, lo que induce la despolarización y, finalmente produce un aumento de la concentración de calcio intracelular. En este proceso también puede darse la activación de la proteína quinasa C (PKC) y la inhibición de la Na^+-K^+-ATPasa (Nowicki *et al.*, 1997). A través de este mecanismo, el 20-HETE juega un papel importante en la regulación del tono vascular en órganos como el cerebro, riñón y corazón (Miyata y Roman, 2005). La formación de 20-HETE por las células musculares está estimulada por angiotensina II, endotelina, vasopresina y norepinefrina y está inhibida por el óxido nítrico (NO) (Miyata y Roman, 2005). Yu *et al.* (2003) observaron que el 5-HETE, 8-HETE, 12-HETE, 15-HETE, 16(*R*)-HETE y 19(*S*)-HETE tenían un efecto vasoconstrictor de menos del 20% del efecto del 20-HETE

en arteriolas interlobulares de riñón de rata. Por el contrario, Birks *et al.* (1997) observaron que el 20-HETE tiene efecto vasodilatador al actuar sobre la circulación pulmonar. También se ha descrito que el 16–HETE, 18–HETE y 19–HETE producen vasodilatación en los vasos que irrigan el riñón (Carroll *et al.*, 1996).

1.7.2.2. EETs y tono vascular

Se ha propuesto que los EETs son factores hiperpolarizantes derivados del endotelio (EDHF), ya que son substancias liberadas desde las células endoteliales y aumentan la hiperpolarización de las propias células endoteliales y de las células musculares lisas induciendo su relajación (Fleming, 2001). Esta respuesta tiene lugar a través de la activación de los canales K_{Ca} así como de la Na^+-K^+-ATPasa (figura 1.8). La activación de los canales de K_{Ca} podría darse a través de la ADP ribosilación de proteínas celulares como $G_{s\alpha}$, en lugar de actuar directamente sobre los canales. La mayoría de evidencias experimentales demuestran que el CYP2C es la epoxigenasa implicada en la respuesta del EDHF ya que la EDHF sintasa es una epoxigenasa homóloga al CYP2C8/9. En las células musculares lisas de traquea de conejo se ha demostrado el papel hiperpolarizante del 5,6-EET y el 11,12-EET (Jacobs y Zeldin, 2001). En condiciones basales los CYPs son inhibidos por el NO, y la vía EET/EDHF tienen un papel poco importante. En cambio, cuando se manifiesta una disfunción endotelial y disminuye la producción de NO, éste deja de inhibir a los CYPs y se puede formar una mayor cantidad de EETs que participarán en el mantenimiento de la vasodilatación (Fleming, 2001).

Sin embargo, otros autores han demostrado el papel vasoconstrictor del 5,6-EET en arterias intralobulares de pulmón de conejo, siendo esta contracción dependiente de la activación de Rho (Losapio *et al.*, 2005). Así, los EETs podrían tener un papel contrapuesto en la circulación sistémica y en la pulmonar, de forma similar a lo descrito previamente para el 20-HETE.

A los DHETEs también se les ha atribuido propiedades vasoactivas. En este sentido, se ha observado que pueden relajar la musculatura preglomerular (Imig *et al.*, 1996) y las arterias coronarias bovinas (Falck *et al.*, 2003), activan canales de K_{Ca} en miocitos de arterias coronarias de rata (Lu *et al.*, 2001), dilatan arteriolas coronarias caninas (Oltman *et al.*, 1998) y relajan las arterias coronarias porcinas con una intensidad parecida a la del 11,12-EET (Fang *et al.*, 1996).

El balance en la producción vascular de HETEs, EETs y DHETEs determinaría el control del tono vascular.

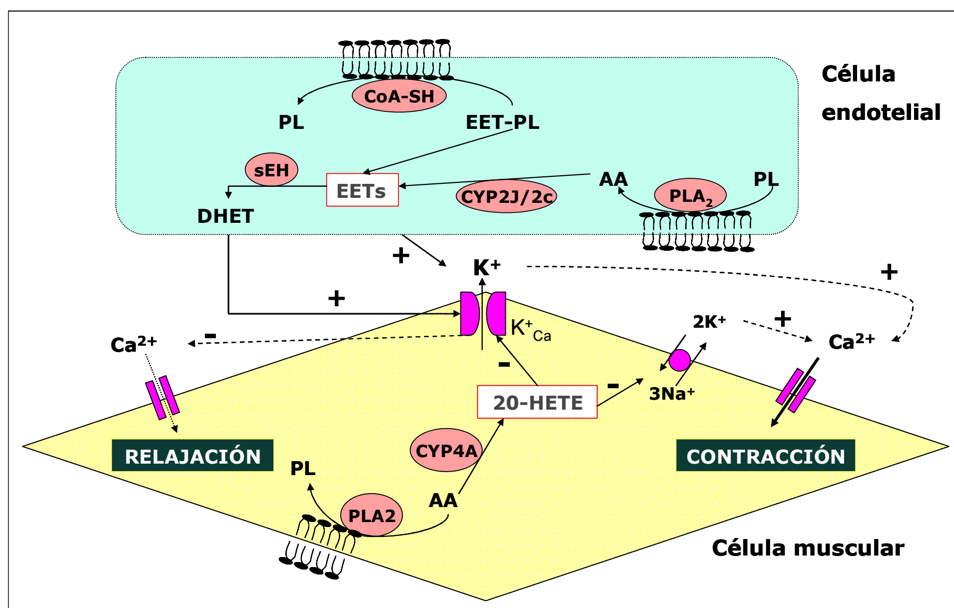

Figura 1.8: Papel de los EETs y 20-HETE sobre el mantenimiento del tono vascular *(figura adaptada de Fleming, 2001)*

1.7.3. Papel de los HETEs y EETs sobre la presión arterial

1.7.3.1. HETEs y presión arterial

Se ha demostrado que los productos derivados de la ω/ω-1 hidroxilación del ácido araquidónico están implicados en el aumento de la presión arterial (Capdevila *et al.*, 2002). Así, los niveles intrarenales de 20-HETE son muy elevados durante la hipertensión (Omata *et al.*, 1992). Además, la inhibición de la actividad ω-hidroxilasa en ratas hipertensas espontáneamente (SHR) reduce la producción de 20-HETE y la presión arterial. También se ha observado que el 20-HETE y el CYP4A2 que lo produce tienen un efecto hipertensivo en ratas Dahl (Iwai y Inagami, 1991). Recientemente se ha demostrado que la administración de oligonucleótidos antisentido contra el CYP4A1 reduce los niveles del CYP4 y la presión arterial en arterias mesentéricas (Wang *et al.*, 2001). Además, la hipertensión inducida con angiotensina II disminuye al tratarse con el inhibidor de la ω-hidroxilasa como el 1-ABT, sugiriendo la implicación del 20-HETE en esta patología (Muthalif *et al.*, 2000). Por otro lado, también se han encontrado niveles elevados de 12(*S*)-HETE en las plaquetas y orina de pacientes con hipertensión (González-Núñez *et al.*, 2001).

1.7.3.2. EETs y presión arterial

Yu *et al.* (2000a), observaron que la expresión del CYP2J y la síntesis de EETs aumentaba en el riñón de las ratas hipertensas SHR en comparación con las ratas normotensas Wistar-Kyoto. Las ratas SHR también mostraban un incremento de la expresión de sEH, de la hidrólisis de los EETs en el córtex renal, y de la consecuente excreción urinaria de DHETEs, sugiriendo que la actividad de la sEH está incrementada (Omata *et al.*, 1992). Así, estudios recientes han demostrado que la disrupción del gen de la sEH o la inhibición de la sEH reducen la presión arterial (Yu *et al.*, 2000b), sugiriendo que la sEH puede ser una potente diana farmacológica para el tratamiento de la hipertensión.

1.7.4. Implicación de los HETEs y EETs en la proliferación celular

Las actividades ω-hidroxilasa y epoxigenasa producen metabolitos, que junto con los ROS derivados de los CYPs, actúan como transductores de señales intracelulares que pueden afectar a la proliferación celular (Fleming, 2001).

1.7.4.1. HETEs y proliferación celular

Uddin *et al.* (1998) relacionaron la síntesis de HETEs y la activación de la vía de las *Mitogen Activated Protein Kinases* (MAPK). Así, observaron que la norepinefrina estimulaba fosfolipasas específicas, la liberación del AA desde los fosfolípidos de la membrana, la actividad ω/ω-1-hidroxilasa del CYP, la producción de 20-HETE y la estimulación de la proliferación de las células musculares lisas de aorta de rata. Además, estos autores observaron que el 20-HETE aumentaba la actividad de la MAPK *extracellular signal-regulated kinase* (ERK)1/2, mientras que el 19-HETE no tenía efecto sobre la proliferación de estas células. Uddin *et al.* (1998) propusieron que los HETEs podrían estimular la actividad Ras, permitiendo su unión a la membrana plasmática y la consecuente activación de la vía MAPK. Por otro lado, los HETEs podrían activar la vía MAPK a través del aumento del calcio intracelular.

También se ha demostrado el papel proliferativo del 5-HETE, 12-HETE y 15-HETE a través de la activación de las vías MAPK o PI3K/Akt en diversas líneas celulares (Rao *et al.*, 1994; Szekeres *et al.*, 2000; Ding *et al.*, 2003; Zhang *et al.*, 2005).

1.7.4.2. EETs y proliferación celular

Los primeros estudios que implicaron a los EETs en la proliferación celular fueron los de Chen *et al.* (1998, 1999) que observaron que en células epiteliales del túbulo proximal de riñón de cerdo todos los EETs estimulaban la incorporación de timidina tritiada, siendo el 14,15-EET el más potente, mientras que el 12(*R*)-HETE, 14,15-DHETE y 20-HETE no tenían efecto mitogénico. Estos autores observaron que el 14,15-EET aumentaba la fosforilación de PI3K y el receptor del factor de crecimiento epidermal (EGFR), y la unión PI3K-EGFR. Además, el 14,15-EET iniciaba una cascada de señalización donde se activaba la tirosín quinasa c-Src, se fosforilaba la proteína adaptadora SHC, ésta se asociaba con GRB2, y se reclutaba al factor de intercambio de nucleótidos guanina SOS, que daba lugar a la activación de las vías MAPK y PI3K (Chen *et al.*, 2000) (figura 1.9). Michaelis *et al.* (2003) también observaron que en células endoteliales humanas tras la sobreexpresión del CYP2C9 se activaba el EGFR y Akt, aumentaba la expresión de ciclina D1 y como consecuencia se inducía la proliferación celular y la angiogénesis.

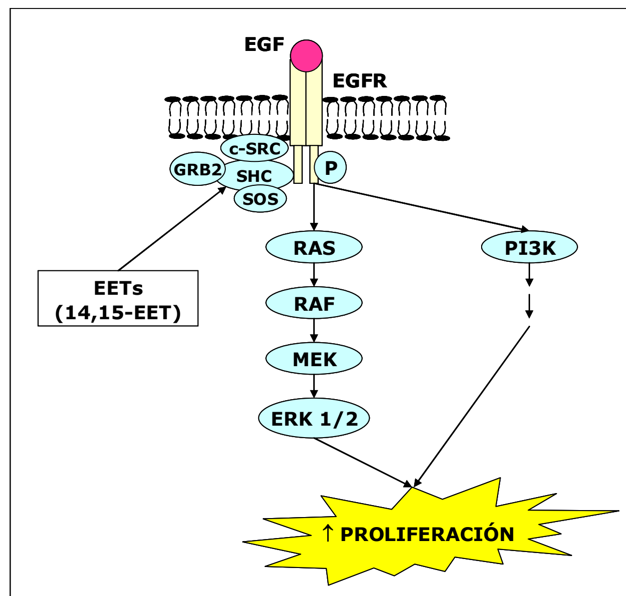

Figura 1.9: Efecto de los EETs sobre diversos mecanismos de transducción de señales implicados en el control de la proliferación celular.

Fleming *et al.* (2001) demostraron que al sobreexpresar el CYP2C8, se producía un aumento de la síntesis de 11,12-EET paralelo a un incremento de la fosforilación de ERK1/2 y p38, y consecuentemente se incrementaba la

proliferación de las células endoteliales, mientras que el 8,9-EET y el 14,15-EET no activaban dichos efectos. Por otro lado, Potente *et al.* (2002) observaron que la adición exógena del 11,12-EET o su producción a través de la sobreexpresión del CYP2C9 en células endoteliales, no tuvieron efecto sobre ERK1/2, provocó la activación de p38 y la inactivación de JNK. Este último efecto se relacionó con la expresión de la ciclina D1 y la proliferación endotelial. Estos mismos autores indicaron que el 11,12-EET induce proliferación celular de forma PI3K/Akt dependiente, provocando la fosforilación e inactivación de los factores de transcripción FOXO1 y FOXO3a, y consecuentemente disminuye la expresión del inhibidor del complejo CDK/ciclina $p27^{Kip1}$ (Potente *et al.*, 2003).

Recientemente, Pozzi *et al.* (2005) han demostrado que en células endoteliales murinas el 8,9-EET y el 11,12-EET estimulan la proliferación celular a través de la activación de p38, mientras que el efecto mitogénico del 5,6-EET y 14,15-EET está mediado por la activación de la PI3K. En cambio, en células endoteliales de aorta bovina se ha observado que la proliferación inducida por el 8,9-EET, 11,12-EET y 14,15-EET depende de las vías MAPK y PI3K (Wang *et al.*, 2005). Así, hasta el momento no está claro si las diferentes vías de señalización celular implicadas en el efecto de los EETs sobre la proliferación celular están conectadas entre ellas o simplemente se activan en paralelo.

Zhang y Harder (2002) observaron que los EETs liberados de astrocitos incrementaban la incorporación de timidina en las células endoteliales e inducían la formación de capilares. Estos autores mostraron que el 5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET, producidos en los astrocitos por el CYP2C11, y los EETs sintéticos promovían la mitogénesis en las células endoteliales y era un proceso sensible a la inhibición de las tirosín quinasas e independiente de PKC. Posteriormente se observó que al sobreexpresar el CYP2C9 y/o la aplicación de 11,12-EET o 14,15-EET exógenos aumentaban la angiogénesis en células endoteliales de pulmón humano (Medhora *et al.*, 2003). Además, este efecto se ha corroborado *in vivo*. Así, los EETs inducen angiogénesis en la membrana corioalantoica de pollo (Michaelis *et al.*, 2003), en ratas adultas tratadas con *Matrigel plugs* impregnados con EETs (Medhora *et al.*, 2003), y en un modelo de isquemia donde la sobreexpresión de diferentes isoenzimas CYP como CYP2C11 y CYP2J2 aumenta la densidad de capilares en el músculo (Wang *et al.*, 2005).

Davis *et al.* (2002) obtuvieron resultados opuestos a los estudios anteriores. Así, observaron que los EETs disminuían la proliferación inducida por PDGF en células musculares lisas vasculares. En cambio, recientemente estos mismos autores (2006) han observado que los EETs no alteran la proliferación

de estas células estimuladas con PDGF. Estos resultados coinciden con los de Sun *et al.* (2002) que no observaron ningún efecto de los EETs sobre la proliferación de las células musculares lisas. Así, demostraron que el tratamiento con 5,6-EET, 8,9-EET, 11,12-EET o 14,15-EET 1 μM durante 24 h, no tenía ningún efecto sobre la incorporación de timidina ni el crecimiento celular inducido con PDGF. En cambio, el 11,12-EET así como la sobreexpresión de CYP2J2 fueron capaces de inhibir la migración celular. Este efecto se correlacionó con la activación de la vía AMPc/PKA, y fue independiente de la vía COX.

Los cambios que inducen las actividades de los CYPs sobre el crecimiento celular no se pueden atribuir únicamente a la síntesis de EETs/HETEs, ya que el enzima también genera ROS. Algunos de ellos, como los aniones superóxido y el peróxido de hidrógeno, afectan a la expresión de las MAPK fosfatasas y por lo tanto la actividad MAPK y la proliferación celular (Potente *et al.*, 2002; Medhora *et al.*, 2003).

1.7.5. Efectos de los HETEs y EETs sobre la apoptosis

1.7.5.1. HETEs y apoptosis

El papel de los HETEs en el control de la apoptosis no está claro. Algunos autores han propuesto que el 15-HETE puede inhibir la proliferación celular e inducir apoptosis, mediante su interacción con el factor de transcripción PPAR-γ, en varias líneas celulares como macrófagos o las células de cáncer de colon (Shankaranarayanan y Nigam, 2003; Chen *et al.*, 2003). Sin embargo, otros autores han descrito que el 5-HETE y 12(*S*)-HETE tienen un efecto anti-apoptótico sobre células tumorales (Yang *et al.*, 2003; Szekeres *et al.*, 2002). Este efecto protector del 12(*S*)-HETE se propuso que es mediado por la activación de las vías MAPK y PI3K/Akt. Por otro lado, Romano *et al.* (2001) describieron el papel anti-apoptótico del 5(*S*)-HETE sobre células mesoteliales malignas de pleura humana donde se inhibió la vía 5-LOX.

1.7.5.2. EETs y apoptosis

Chen *et al.* (2001) describieron que el 14,15-EET inhibe la apoptosis inducida por peróxido de hidrógeno, etoposido, un exceso de AA o ausencia de suero, en células epiteliales de túbulo proximal de riñón. La vía de señalización PI3K/Akt parece implicada en este efecto anti-apoptótico, y no la vía MAPK.

También observaron que las células que sobreexpresan la 14,15-epoxigenasa están protegidas contra la inducción de la apoptosis. Así, estos autores propusieron que la actividad epoxigenasa promueve la supervivencia celular mediante la producción de 14,15-EET, previniendo la activación de la vía de la esfingomielinasa neutra que produce ceramida que es pro-apoptótica (figura 1.10).

Un efecto parecido lo han descrito Dhanasekaran *et al.* (2006) que observaron que el 8,9-, 11,12- y 14,15-EETs promueven la supervivencia de células endoteliales humanas a través de la vía PI3K. Recientemente, Jiang *et al.* (2005) demostraron que estos EETs protegen varias líneas celulares de carcinoma de la apoptosis inducida por TNF-α, mediante la activación de las vías MAPK y PI3K.

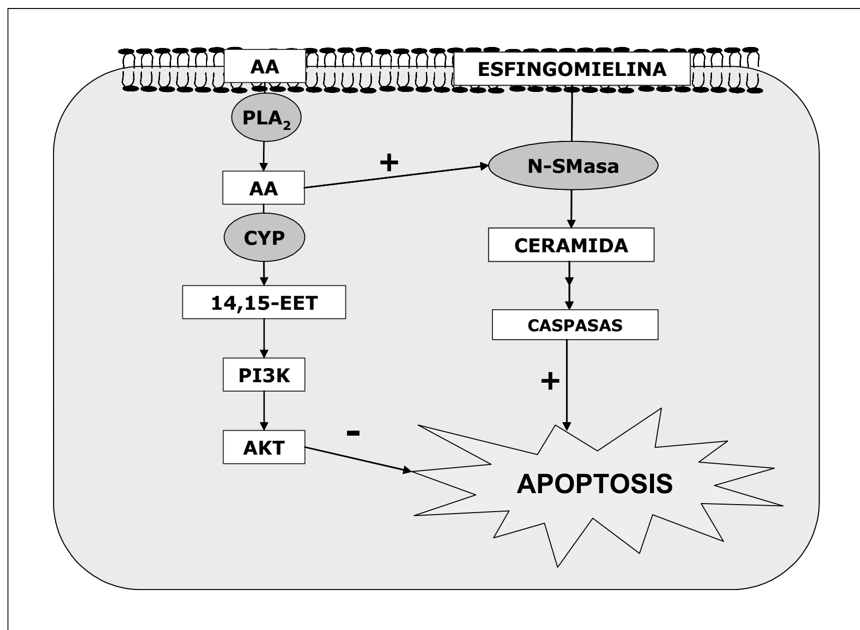

Figura 1.10: Papel del 14,15-EET en el desarrollo de la apoptosis
(figura adaptada de Chen et al., 2001)

2. OBJETIVOS

Estudios previos demostraron que la reducción de la liberación del AA, al inhibir la actividad PLA_2, reducía la proliferación de los fibroblastos 3T6 inducida por FBS (Sánchez y Moreno, 2001a). Al mismo tiempo, se demostró el papel de las PGs en el control del crecimiento de los fibroblastos 3T6 inducido por suero (Sánchez y Moreno, 2001b). Así, se observó que la inhibición de las COXs producía una reducción parcial de la proliferación celular (Sánchez y Moreno, 1999), mientras que los antagonistas receptoriales de la PGE_2 bloqueaban casi completamente el crecimiento de los fibroblastos 3T6 (Sánchez y Moreno, 2002; Sánchez y Moreno, 2006). Estos resultados sugirieron la implicación de los metabolitos del AA producidos a través de otras vías sobre el crecimiento de los fibroblastos 3T6. Considerando que el AA también puede ser metabolizado por las LOXs o por los CYPs produciendo LTs, HETEs y EETs, y que se ha sugerido la implicación de estos metabolitos en el control de la proliferación celular (Tong *et al.*, 2002a; Kroetz y Zeldin, 2002; Paruchuri y Sjölander, 2003), nos planteamos estudiar la implicación de estas vías sobre el control de la proliferación de los fibroblastos 3T6. De manera que el objetivo principal de la tesis ha sido evaluar el papel de los metabolitos derivados del AA vía LOXs, los LTs y HETEs, y de los metabolitos del AA producidos por la vía de los CYPs, los HETEs y EETs, sobre el control del crecimiento de los fibroblastos 3T6.

3. MATERIAL Y MÉTODOS

3.1 Materiales

Medio RPMI 1640, medio *Dulbecco's Modified Eagle's Medium* (DMEM), suero fetal bovino (FBS), penicilina, estreptomicina, tripsina-EDTA y Versene-EDTA fueron suministrados por BioWhittaker Europe (Verviers, Bélgica). LTB_4, LTB_5, LTD_4, factor de crecimiento derivado de plaquetas (PDGF), ácido nordihidroguayarético (NDGA), zileuton, ketoprofeno, SKF-525A (N,N-dietilaminoetil 2,2-difenilvalerato/proadifen), 17-ODYA (ácido 17-octadecinoico), ABT (1-aminobenzotriazol), PPOH (ácido 6-(2-propargiloxifenil)hexanoico), Bay u9773, PD098059 (2-(2-amino-3-metoxifenil)-4H-1-benzopiran-4-ona), wortmanina procedente de *penicillium funiculosum*, yoduro de propidio, Triton X-100, fluorato de fenilmetilsulfonil (PMSF), ácido dietilditiocarbámico, Igepal CA-630, aprotinina, leupeptina, ditiotreitol (DTT), ribonucleasa A de páncreas bovino, albúmina sérica bovina (BSA), naranja de acridina y bromuro de etidio fueron suministrados por Sigma Chemical Co. (St. Louis, MO, USA). Los ácidos 5,6-, 8,9-, 11,12- y 14,15-EET, los ácidos 5,6- y 11,12-DHETE, los ácidos 5(S)-, 12(S)-, 15(S)- y 20-HETE, la PGE_2 y PGE_3, y los antagonistas receptoriales de los leukotrienos U-75302, LY-171883, MK-571 y REV-5901 fueron suministrados por Cayman Chemical Co. (Ann Arbor, MI, USA). La baicaleína fue suministrada por Calbiochem (La Jolla, CA). La [Metil-^{3}H]timidina (20 Ci/mmol) fue proporcionada por American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). El resto de productos fueron de la calidad más alta comercializada.

3.2 Cultivos celulares

El trabajo se ha desarrollado con dos líneas celulares, los fibroblastos 3T6 que proceden de embriones murinos (ATCC CCL96) y se cultivaron con medio RPMI 1640; y los macrófagos RAW 264.7 murinos derivados de un tumor inducido por el virus de la leucemia de Abelson (ATCC TIB-71) que se mantuvieron con medio DMEM. Ambos medios contenían FBS 10%, penicilina (100 U/mL) y estreptomicina (100 µg/mL). Los cultivos se mantuvieron rutinariamente en frascos de 25 cm^2 (Costar), los fibroblastos se separaron de la superficie con tripsina-EDTA, y los macrófagos se despegaron raspando con Versene-EDTA. Para llevar a cabo los experimentos las células se sembraron en placas de 24 pocillos (tissue-culture cluster 24; Costar Cambridge, MA, USA) o en placas de petri de 60/100 mm (Costar). Los cultivos celulares se mantuvieron

en condiciones controladas, a una temperatura de 37ºC y atmósfera de aire con un contenido en CO_2 del 5% y saturada de humedad.

3.3 Ensayos de proliferación y viabilidad celular

Las células se sembraron en placas de 24 pocillos a una densidad de $20x10^3$ células/pocillo, con medio RPMI 1640 que contenía FBS 10% para los fibroblastos 3T6 o DMEM con FBS 10% para los macrófagos RAW 264.7. 24 h después, las células se incubaron con los tratamientos durante un periodo de 48 h. Finalmente, el sobrenadante de los pocillos se recogió para cuantificar el número de células despegadas. Por otro lado, las células pegadas se tripsinizaron en el caso de los fibroblastos 3T6 o se rasparon en el caso de los macrófagos RAW 264.7, y se contaron en un microscopio de fluorescencia usando una solución de tinción que contenía bromuro de etidio/naranja de acridina para determinar al mismo tiempo el número de células y la viabilidad celular (Parks *et al.*, 1979). El naranja de acridina entra en las células vivas, observándose en el microscopio con luz ultravioleta núcleos de color verde, y el bromuro de etidio penetra a través de las membranas celulares dañadas, tiñendo el núcleo de las células muertas de color naranja.

En éste y en el resto de ensayos todos los productos se disolvieron con DMSO y la concentración final nunca superó el 0,1%.

3.4 Análisis de la síntesis de DNA

La síntesis de DNA se cuantificó mediante el ensayo de incorporación de $[^3H]$timidina. Las células se sembraron en placas de 24 pocillos a una densidad de $20x10^3$ células/pocillo, con medio RPMI 1640 que contenía FBS 10% en el caso de los fibroblastos 3T6 o DMEM con FBS 10% para cultivar los macrófagos RAW 264.7. Después de 24 h, aplicamos los tratamientos durante 48 h, y las últimas 24 h añadimos la $[^3H]$timidina (0,25 µCi/pocillo). A continuación, el medio se aspiró y se descartó, y se lavaron los pocillos con una solución de BSA al 0,5% en *Phosphate Buffered Saline* (PBS), para eliminar el exceso de $[^3H]$timidina no incorporada a las células. Finalmente, las células se recogieron lisándolas con 300 µL de Tritón X-100 al 1% y se mezclaron con 3 mL de líquido de centelleo (*Cocktail Biogreeen 3,* Scharlau Chemie, Barcelona). La radioactividad presente en la fracción celular se midió con un contador de centelleo (*Packard Tri-Carb 1500*).

3.5 Análisis del ciclo celular por citometría de flujo

Las células se sembraron a una densidad de $60x10^3$ células/placa 100 mm con medio RPMI 1640 que contenía FBS 10% los fibroblastos 3T6 o DMEM con FBS 10% los macrófagos RAW 264.7. Pasadas 24 h, las células se sincronizaron incubándolas con medio sin suero durante 24 h, alcanzándose un porcentaje de células en fase G_0/G_1 superior al 80%. Entonces, las células se incubaron con los tratamientos el tiempo necesario. Seguidamente, las células se despegaron con tripsina en el caso de los fibroblastos 3T6 o con Versene-EDTA los macrófagos, se lavaron dos veces con PBS a 4ºC, se fijaron con etanol al 70% y se almacenaron a 4ºC durante al menos 2 h. Tras este tiempo, el DNA de bajo peso molecular se extrajo de las células con 500 µL de una solución que contenía Na_2HPO_4 0,2 M y ácido cítrico 0,1 M, y se incubaron las células durante 30 minutos a 37ºC en agitación. Luego, se tiñeron las células durante 1 h a temperatura ambiente con 1 mL de una solución que contenía 20 µg/mL de yoduro de propidio en PBS con Triton X-100 al 0,1% y 0,2 mg/mL de RNasa libre de DNasa. Finalmente, la cantidad de DNA que contenía cada célula se midió en un citómetro Epics XL (Coulter Corporation, Hialeah, Florida), y los resultados se analizaron (*Ploidy analysis*) en histogramas de fluorescencia simple por *Multicycle software* (*Phoenix Flow Systems*, San Diego, CA).

3.6 Cuantificación de 12(*S*)-HETE

Los fibroblastos 3T6 se sembraron en placas de 60 mm a una densidad de $60x10^3$ células/placa y se cultivaron durante 24 h con medio RPMI 1640 que contenía FBS 10%. A continuación, las células se incubaron 48 h con los tratamientos, se separó el sobrenadante de los cultivos y el 12(*S*)-HETE se purificó a través de unas columnas de extracción C_{18} de fase invertida. Las columnas previamente se habían acondicionado con 10 mL de etanol y 10 mL de agua desionizada. Las muestras se acidificaron con 50 µL de HCl 2M por cada 1 mL de muestra y se mantuvieron en hielo durante 15 minutos. Posteriormente, se centrifugaron las muestras y se pasaron por las columnas que retienen el 12(*S*)-HETE. Finalmente, lavamos las columnas con 10 mL de etanol al 15% y 10 mL de hexano, y se eluyó el 12(*S*)-HETE con 10 mL de acetato de etilo. La fase orgánica se evaporó bajo atmósfera de nitrógeno, el 12(*S*)-HETE se reconstituyó y se cuantificó por EIA, usando un kit de inmunoensayo (Assay Designs, Inc., Ann Arbor, MI, USA). El kit contenía un anticuerpo policlonal que

se unió de forma competitiva al 12(*S*)-HETE de la muestra o al 12(*S*)-HETE que estaba unido covalentemente a la fosfatasa alcalina. El anticuerpo fue 100% específico para el 12(*S*)-HETE, siendo la reactividad cruzada para el 12(*R*)-HETE inferior al 2,5%, y para otros eicosanoides inferior al 0,3%.

3.7 Cuantificación de LTB_4 / PGE_2

Las células se sembraron en placas de 24 pocillos a un densidad de $20x10^3$ células/pocillo, con medio RPMI 1640 que contenía FBS 10% los fibroblastos 3T6 o DMEM con FBS 10% en el caso de los macrófagos RAW 264.7. Seguidamente, se eliminó el suero del medio durante 24 h y las células se incubaron con los tratamientos. Finalmente, se tomó una alícuota de los sobrenadantes y se analizó la concentración de LTB_4 o PGE_2 por EIA utilizando un kit de immunoensayo (*Cayman Chemical Co*., Ann Arbor, MI, USA). El kit contenía un anticuerpo monoclonal que se unió de forma competitiva a la PGE_2/LTB_4 de las muestras o a la PGE_2/LTB_4 unida a acetilcolinesterasa. El anticuerpo fue 100% específico para la PGE_2/LTB_4.

3.8 Determinación de proteínas

La concentración de proteínas se determinó siguiendo el método descrito por Bradford (1976), usando como estándar la BSA. Se usó el reactivo BioRad que contiene *Comassie Brillant Blue G-250* que se combina rápidamente con las proteínas y forma un complejo estable durante aproximadamente 1 h de color azul. Así, mezclamos 50 µL de muestra o estándar con 50µL de ácido fórmico y 1,5 mL de reactivo BioRad diluido 5 veces, y se cuantificó la absorbancia mediante espectrofotometría a 595 nm.

3.9 Inmunodetección de Akt y P-Akt

Los fibroblastos 3T6 se sembraron en placas de 100 mm a una densidad de $25x10^4$ células con medio RPMI 1640 que contenía FBS 10% durante 24 h. A continuación, las células se cultivaron con medio sin suero durante 24 h y se trataron con los productos a ensayar durante 30 minutos. Las células se rasparon con tampón de lisis que contenía Tris-HCl 200 mM, NaCl 200 mM, Igepal CA-630 2%, NaF 400 µM, PMSF 20 µg/mL, aprotinina 20 µg/mL, leupeptina 20 µg/mL, DTT 200 µM y Na_3VO_4 400 µM, y se incuban 30 minutos a

4ºC. Seguidamente, se cuantificaron las proteínas de cada extracto celular y se cargaron 20 µg de proteína de cada muestra en un gel SDS-PAGE al 10%. Luego, las proteínas se transfirieron a una membrana de difluorato de polivinilideno (PVDF) de poro 0,2 µm (*Bio-Rad*, Hercules, CA) usando el sistema *MiniProtean II* (*Bio-Rad*). Las membranas se bloquearon con una solución de leche desnatada al 5% en PBS-Tween 20 al 0,1% durante 1h. Seguidamente, se incubaron durante la noche en presencia de los anticuerpos policlonales de conejo contra Akt o Akt fosforilado (P-Akt) (en la posición Ser 473) diluidos 1:1000. A continuación, se lavaron las membranas con PBS-Tween 20 al 0,1% y se incubaron durante 1h con el anticuerpo de cabra anti IgG asociado a peroxidasa de rábano (1:2000). Finalmente se desarrolló la reacción usando un kit de quimioluminiscencia. Los anticuerpos y el kit de quimioluminiscencia fueron suministrados por Cell Signalling (Beverly, MA).

3.10 Análisis de la unión de anexina V

Las células se sembraron en placas de 24 pocillos a un densidad de $20x10^3$ células/pocillo, con medio RPMI 1640 que contenía FBS 10% los fibroblastos 3T6 o DMEM con FBS 10% los macrófagos RAW 264.7. A continuación, las células se lavaron y se incubaron con los tratamientos durante 48 h. Al iniciarse la apoptosis las células exponen en la parte exterior de las membranas la fosfatidilserina, un fosfolípido que normalmente se encuentran en la cara interior. La anexina V es una molécula con gran capacidad de unión a la fosfatidilserina, por ello es usada como método de detección de apoptosis. Así, las células se tiñeron con anexina V marcada con fluoresceína y con yoduro de propidio (*Roche Diagnostics*, Mannheim, Alemania). De esta forma, las células marcadas con anexina V se encontraban en fase apoptótica temprana, las células con un marcaje doble de anexina V y yoduro de propidio estaban en apoptosis tardía, y las células marcadas solo con yoduro de propidio indicaban necrosis. Finalmente, las células se cuantificaron en un microscopio de fluorescencia invertido (*Nikon Eclipse TE200*) asociado a una cámara CCD.

3.11 Cuantificación de la actividad caspasa total

Los fibroblastos 3T6 se sembraron en placas de 24 pocillos a una densidad de $40x10^3$ células/pocillo con medio RPMI 1640 que contenía FBS 10%. A continuación, las células se lavaron y se cultivaron con medio que contenía

PDGF 100 ng/mL en presencia de EETs o DHETEs durante 48 h. Seguidamente, se eliminaron los tratamientos y las células se tiñeron con FITC-VAD-FMK, que se une a las caspasas activas (*Medical and Biological Laboratories*, Nagoya, Japan). Las muestras fueron observadas en un microscopio de fluorescencia invertido (*Nikon Eclipse TE200*) asociado a una cámara CCD, y se contaron las células caspasa positivas o negativas.

3.12 Cuantificación de las actividades caspasa-3/-8/-9/-12

Los fibroblastos 3T6 se sembraron en placas de 60 mm a un densidad de $40x10^4$ células, con medio RPMI 1640 que contenía FBS 10%. Tras 24 h, las células se lavaron y se incubaron con los tratamientos. Seguidamente, las células se rompieron con 50 µL del tampón de lisis adjunto en el kit (Medical and Biological Laboratories, Nagoya, Japan) durante 10 minutos en hielo. A continuación las actividades caspasa-3, -8, -9 y -12 fueron detectadas incubando las muestras con 5 µL de los substratos específicos fluorogénicos DEVD, IETD, LEHD o ATAD, respectivamente, conjugados con 7-amino-4-trifluorometil cumarina (AFC), junto con el tampón de reacción que contenía DTT 10 mM, durante 1,5 h a 37ºC. La actividad caspasa rompe el substrato y emite luz de fluorescencia amarillo-verde, que se cuantificó usando un fluorímetro (*Perkin Elmer LS 45*) con flitro de excitación a 400 nm y filtro de emisión a 505 nm. Los resultados fueron normalizados teniendo en cuenta la concentración de proteína de cada muestra.

3.13 Cuantificación de la fragmentación de DNA

La degradación del DNA cromosómico se evaluó mediante la técnica de TUNEL. Durante la apoptosis la cromatina es cortada por las endonucleasas, lo que genera fragmentos de DNA con extremos 3'-OH a los que puede unirse la fluoresceína-dUTP gracias a la acción de la deoxinucleotidil transferasa terminal (TdT). Las células se sembraron a una densidad de $20x10^4$ en placas de 60 mm, con medio RPMI 1640 que contenía FBS 10% en el caso de los fibroblastos 3T6 o DMEM con FBS 10% para los macrófagos RAW 264.7. 24 h después, las células se lavaron y se incubaron con los tratamientos durante 48 h. A continuación, se recogieron las células del sobrenadante y se tripsinizaron o se rasparon las células pegadas. Seguidamente, se lavaron con una solución de PBS que contenía BSA al 0,2% y se fijaron con paraformaldehído al 4% durante 30

minutos a 4ºC. Luego, se lavaron las células dos veces con PBS que contenía BSA al 0,2% y se permeabilizaron con 200 µL de etanol al 70% durante 30 minutos a -20ºC. Tras este tiempo, las células se lavaron 2 veces con PBS que contenía BSA al 0,2%, se añadió 30 µL de reactivo con TdT y se incubó durante 1 h a 37ºC. Finalmente, las células se lavaron dos veces con PBS que contenía BSA al 0,2% y se resuspendieron con 500 µL de esta misma solución. El DNA fragmentado se cuantificó por citometría de flujo (*Epics XL*, *Coulter Corporation*, Hialeah, Florida).

4. RESULTADOS

4.1. Publicación 1

Nieves D, Moreno JJ.

Hydroxyeicosatetraenoic Acids Released Through Cytochrome P-450 Pathway Regulate 3T6 Fibroblast Growth.

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Los eicosanoides participan en el control de múltiples funciones, como la regulación de la proliferación celular. Estudios previos en nuestro laboratorio demostraron que las PGs, metabolitos derivados del AA vía COX, están implicadas en la regulación de la proliferación de los fibroblastos 3T6 inducida con FBS 10%. Nuestros resultados, además, sugerían que otros metabolitos derivados del AA podían estar implicados en esta función. En el presente estudio se ha valorado el papel de las otras dos vías de metabolización del AA, a través de la acción de las LOXs y los CYPs. Mediante el uso de inhibidores de las LOXs y antagonistas receptoriales de los LTs demostramos que los metabolitos que derivan de la vía LOX, en especial la vía 5-LOX, no juegan un papel significativo en la regulación del crecimiento de los fibroblastos 3T6 inducido con suero. Por otro lado, valoramos el papel de los metabolitos derivados del AA vía CYP. Nuestros resultados demostraron que los fibroblastos 3T6 sintetizan 12(*S*)-HETE. Además, los inhibidores específicos de los CYPs como el SKF-525A, 17-ODYA, ABT y PPOH reducían los niveles de 12(*S*)-HETE, la proliferación de los fibroblastos 3T6 y la síntesis de DNA inducida por suero. La inhibición del crecimiento inducida por el SKF-525A fue revertida mediante la adición exógena de 5-, 12(*S*)-, 15(*S*)- y 20-HETEs. También observamos que el 5-, 12(*S*)- y 15(*S*)-HETEs eran capaces de estimular la proliferación celular y la incorporación de timidina tritiada, en ausencia de factores de crecimiento, y este efecto fue dependiente de la activación de la vía PI3K/Akt. En conclusión, estos resultados muestran que los HETEs están implicados en el control del crecimiento de los fibroblastos 3T6.

Hydroxyeicosatetraenoic acids released through the cytochrome P-450 pathway regulate 3T6 fibroblast growth

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Abstract Eicosanoids participate in the regulation of cellular proliferation. Thus, we observed that prostaglandin E_2 interaction with membrane receptors is involved in the control of 3T6 fibroblast growth induced by serum. However, our results suggested that another arachidonic acid pathway might be implicated in these events. Our results show that 3T6 fibroblasts synthesized hydroxyeicosatetraenoic acids (HETEs) such as 12-HETE through the cytochrome P-450 (CYP450) pathway. However, 3T6 fibroblasts did not produce leukotriene B_4 (LTB_4), and lipoxygenase inhibitors and LT antagonists failed to inhibit 3T6 fibroblast growth induced by FBS. In contrast, we observed that CYP450 inhibitors such as SKF-525A, 17-octadecynoic acid, 1-aminobenzotriazole, and 6-(2-propargyloxyphenyl)hexanoic acid reduced 12(*S*)-HETE levels, 3T6 fibroblast growth, and DNA synthesis induced by FBS. The impairment of DNA synthesis and 3T6 fibroblast growth induced by SKF-525A were reversed by exogenous addition of HETEs. Moreover, we report that 5-HETE, 12(*S*)-HETE, and 15(*S*)-HETE are mitogenic on 3T6 fibroblast in the absence of another growth factor, and this effect was dependent on the activation of the phosphatidylinositol-3-kinase pathway. In conclusion, our results show that HETEs, probably produced by CYP450, are involved in the control of 3T6 fibroblast growth.—Nieves, D., and J. J. Moreno. **Hydroxyeicosatetraenoic acids released through the cytochrome P-450 pathway regulate 3T6 fibroblast growth.** ***J. Lipid Res.*** **2006.** 47: **2681–2689.**

Supplementary key words cell proliferation • cell cycle • Akt kinase

Free arachidonic acid (AA) can be oxidized by three major metabolic pathways: the cyclooxygenases (COXs), which produce prostaglandins (PGs) and thromboxanes; the lipoxygenases (LOXs), which form leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs), and lipoxins; and the cytochrome P-450 monooxygenases (CYP450s) (1). The CYP450 proteins metabolized AA by one or more of the following reactions: bis-allylic oxidation (LOX-like reaction) to generate 5-, 8-, 9-, 11-, 12-, and 15-HETEs; ω/ω-1 hydroxylation gives 16-, 17-, 18-, 19-, and 20-HETEs; or olefin epoxidation, producing 5,6-, 8,9-, 11,12-, and 14,15-epoxyeicosatrienoic acids (EETs). Finally, the cytosolic epoxide hydrolases catalyze a rapid enzymatic hydration of the EETs to dihydroxyeicosatetraenoic acids (2).

Eicosanoids have numerous physiological effects (3), including cell proliferation and differentiation. Recent studies in our laboratory have suggested that the COX pathway is involved in serum-induced 3T6 fibroblast proliferation. On the one hand, we have demonstrated that COX-2 inhibition reduces 3T6 fibroblast proliferation in a concentration-dependent manner, producing an ~50% maximum effect (4). On the other hand, we have shown that EP_1 and EP_4 receptors of PGE_2 antagonists reduced 3T6 fibroblast growth in a concentration-dependent manner, but to almost complete inhibition (5). Furthermore, we have reported that phospholipase A_2 (PLA_2) inhibitors reduced serum-induced 3T6 fibroblast growth and [^{3}H]thymidine incorporation almost completely (6). These results suggested that other AA cascade pathways might be involved in these events (i.e., LOX or CYP450 pathway).

Little is known about LOX expression and LOX product biosynthesis in fibroblasts. Typically, LOX is expressed in platelets, leukocytes, neutrophils, monocytes, macrophages, endothelial cells, and smooth muscle cells (7–9). Kitzler and Eling (9) cloned, sequenced, and expressed 5-LOX cDNA from Syrian hamster embryo fibroblasts, although they did not detect 5-HETE. Furthermore, Newby and Mallet (10) found that human dermal fibroblasts produce 12-HETE and that without stimulation its concentration is higher than that of PGE_2. These authors also detected 15-HETE, whereas 5-HETE was not encountered. Other studies found that interleukin-1 increased the pro-

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Abbreviations: AA, arachidonic acid; ABT, 1-aminobenzotriazole; COX, cyclooxygenase; CYP450, cytochrome P-450; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LOX, lipoxygenase; LT, leukotriene; NDGA, nordihydroguaiaretic acid; 17-ODYA, 17-octadecynoic acid; PG, prostaglandin; PI3K, phosphatidylinositol-3-kinase; PLA2, phospholipase A2; PPOH, 6-(2-propargyloxyphenyl)-hexanoic acid.

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duction of 12- and 15-HETE in human dermal fibroblasts (11, 12). Additionally, it has been demonstrated that fibroblasts can respond to LOX products such as LTs or HETEs. For example, Hasegawa et al. (7) demonstrated that 12-HETE activates adenylyl cyclase via increasing intracellular $Ca^{2}+$ concentration in human normal fibroblast TIG-1 cells. Moreover, Chibana et al. (13) showed that human fetal lung fibroblast HFL-1 cells respond to LTC_4, and when they are stimulated with interleukin-13, cysteinyl LT receptor 1 mRNA and protein are upregulated.

With regard to CYP450s, several isoforms have been described in fibroblasts. Saeki et al. (14) demonstrated CYP450 1A1, 1B1, and 2E1 mRNA expression, whereas CYP450 1A2, 2A7, 2B6, and 3A4 mRNAs were not detected. Thus, CYP450s could release HETEs in fibroblast cultures. There are few studies about AA-CYP450 metabolites in fibroblasts, and the presence of EETs in fibroblasts has not been quantified. However, Fang et al. (15) demonstrated that human skin fibroblasts produce dihydroxyeicosatetraenoic acids when they are exposed to a relatively high concentration of EETs.

LOX and CYP450 pathway metabolites have been described as regulators of several functions, including cell proliferation. Thus, some studies show that LTD_4 and LTB_4 increase cell growth, inducing extracellular signal-regulated kinase 1/2 phosphorylation in intestinal epithelial cells and human pancreatic cancer cells, respectively (16, 17). It has also been demonstrated that 5-, 12-, 15-, and 20-HETEs stimulate cell proliferation through mitogen-activated protein kinase and Akt pathway activation in pancreatic cancer cells, human epidermoid carcinoma cells, and vascular smooth muscle cells (18–21). Finally, Chen et al. (22) found that all EETs stimulate [^{3}H]thymidine incorporation in renal epithelial cells, and their effects are mediated by the activation of the Src kinase and tyrosine kinase phosphorylation cascade.

Therefore, the aim of this study was to investigate the role of LOX and CYP450-dependent AA metabolism in the control of serum-induced 3T6 fibroblast proliferation.

MATERIALS AND METHODS

Materials

RPMI 1640 medium, FBS, penicillin, streptomycin, and trypsin-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). Nordihydroguaiaretic acid (NDGA), zileuton, 17-octadecynoic acid (17-ODYA), 1-aminobenzotriazole (ABT), 6-(2-propargyloxyphenyl)hexanoic acid (PPOH), wortmannin, *Penicillium funiculosum*, propidium iodide, Triton X-100, PMSF, Igepal CA-630, aprotinin, leupeptin, DTT, RNase A from bovine pancreas, acridine orange, and ethidium bromide were provided by Sigma Chemical Co. (St. Louis, MO). The LT antagonist receptors U-75302, LY-171883, and REV-5901 and the metabolites 5-, 12(*S*)-, 15(*S*), and 20-HETE and 11,12-EET were supplied by Cayman Chemical Co. (Ann Arbor, MI). Baicalein and SKF-525A hydrochloride (proadifen) were from Calbiochem (La Jolla, CA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All chemicals were of the highest quality available commercially.

Cell culture

Murine 3T6 fibroblasts (CCL96; American Type Culture Collection) were cultured in RPMI 1640 containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were harvested with trypsin-EDTA and seeded on 24-well plates (Tissue-Culture Cluster 24; Costar, Cambridge, MA) or in Tissue-Culture 60 mm dishes (Costar) for experimental purposes. Cell cultures were maintained in a temperature- and humidity-controlled incubator at 37°C with 95% air and 5% CO_2 for 24 h.

Cell growth assay

The effect of the treatments was assessed on 3T6 fibroblast plates at 20×10^3 cells/well on 24-well plates cultured for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of various compounds. Finally, cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to assess viability.

Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]thymidine incorporation assay, which involved culturing 3T6 fibroblasts on 24-well plates in RPMI 1640 with 10% FBS at a density of 20×10^3 cells/well. After 24 h of serum starvation, cells were incubated for 48 h with the treatments, and [^{3}H]thymidine (1 μCi/well) was added for the last 24 h. [^{3}H]thymidine-containing media were aspirated, and cells were overlaid with 1% Triton X-100 and then scraped off the dishes. Finally, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

Fluorescence-activated cell sorting analysis/flow cytometry cell cycle analysis

Fibroblasts were seeded in 60 mm dishes, and 24 h later they were serum starved. After 24 h without FBS, the percentage of cells in G_0/G_1 was ~80%. Cells were then cultured in 10% FBS RPMI containing the treatments. Thereafter, they were trypsinized, fixed with 70% ethanol, and stored at 4°C for at least 2 h. Next, low molecular weight DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corp., Hialeah, FL). DNA was analyzed (ploidy analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Assay of 12-HETE levels

Fibroblasts were seeded in 60 mm dishes at a density of 6×10^4 cells/dish and cultured for 24 h in RPMI 1640 supplemented with 10% FBS. Afterward, cells were incubated for 48 h in 10% FBS medium containing different compounds. Then, 12-HETE was extracted from culture supernatant medium through C_{18} reverse-phase extraction columns in ethyl acetate. Next, the organic phase was evaporated in a stream of nitrogen and 12(*S*)-HETE was measured using a 12(*S*)-HETE enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI) according to the manufacturer's protocol.

Measurement of PGE_2 and LTB_4

Cells were cultured at 25×10^3 cells/well on 24-well plates for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of several products. An aliquot of culture supernatant medium was acidified with 1 ml of 1% formic acid. PGE_2 and LTB_4 were extracted in ethyl acetate, and the

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organic phase was evaporated under a stream of nitrogen. Finally, PGE_2 and LTB_4 were determined using monoclonal enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

Western blot analysis of Akt and phosphorylated Akt

Fibroblast cultures were FBS-starved for 24 h and incubated for 30 min with HETEs. The total cellular fraction was obtained by scraping off the cells in lysis buffer containing 200 mM Tris-HCl, 200 mM NaCl, 2% Igepal CA-630, 400 μM NaF, 20 μg/ml PMSF, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 200 μM DTT, and 400 μM Na_3VO_4, followed by incubation for 30 min at 4°C. Immunoblot analyses were performed as follows: 20 μg of protein from cell lysates was separated on a 10% SDS-PAGE gel (23) and blotted for 1 h with a constant voltage of 100 V onto a polyvinylidene difluoride membrane (Immun-Blot membrane, 0.2 μm; Bio-Rad, Hercules, CA) using a MiniProtean II system (Bio-Rad). Membranes were blocked with 5% nonfat milk powder in PBS-0.1% Tween 20 for 1 h. Rabbit polyclonal antibodies against Akt or phospho-Akt (serine 473) were applied in a 1:1,000 dilution overnight. The blot was washed several times with PBS-0.1% Tween 20 and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody in a 1:2,000 dilution for 1 h. Finally, blots were developed using an enhanced chemiluminescence kit. Antibodies and the chemiluminescence kit were supplied by Cellular Signaling (Beverly, MA).

Statistics

Results are expressed as means ± SEM. Differences between control and treated cultures were tested using Student's *t*-test.

RESULTS

Effect of LOX inhibitors and LT receptor antagonists on the growth and DNA synthesis of 3T6 fibroblasts

First, we studied the role of the LOX pathway in the control of 3T6 fibroblast proliferation. We used several LOX inhibitors, such as zileuton, a specific 5-LOX inhibitor (24), the 5- and 12-LOX inhibitor baicalein (25), and the unspecific LOX inhibitor NDGA (26). As shown in **Fig. 1**, these treatments produced little growth inhibition and [^{3}H]thymidine incorporation inhibition compared with the COX inhibitor ketoprofen (27). We also studied the role of the LOX pathway on 3T6 fibroblast proliferation with LT receptor antagonists. We used U-75302, REV-5901, and LY-171883 as LTB_4, LTD_4, and CysLT receptor antagonists, respectively (28–30). All antagonists induced a low growth and [^{3}H]thymidine incorporation inhibition. On the other hand, we quantified the LTB_4 concentration in 3T6 fibroblast cultures stimulated with 10% FBS. **Table 1** shows that 3T6 fibroblasts produce a small quantity of this eicosanoid. Thus, our data indicate that the LOX pathway does not play a significant role in the control of 3T6 fibroblast growth.

Effect of CYP450 inhibitors on the growth, DNA synthesis, and cell cycle distribution of 3T6 fibroblasts

Once the LOX pathway was ruled out, we examined the role of CYP450 metabolites in the control of serum-induced 3T6 fibroblast growth. We used four structurally and mechanistically different CYP450 inhibitors. SKF-525A and 17-ODYA are nonselective CYP450 inhibitors that inhibit the ω-hydroxylation and epoxygenation of AA (31, 32). On the other hand, ABT is a specific ω-hydroxylation inhibitor (33), and PPOH is a potent and selective molecule that inhibits the conversion of AA into EETs (31). These compounds are reported as specific CYP450 enzyme inhibitors, but we checked to determine whether they have some effect on COX activity. Table 1 shows that some CYP450 inhibitors appreciably reduce PGE_2 production. Thus, ABT is the CYP450 inhibitor that has the greatest effect and reduces PGE_2 synthesis by ~50%. In parallel, we confirmed that CYP450 inhibitors significantly reduced 12(*S*)-HETE production induced by 10% FBS in 3T6 fibro-

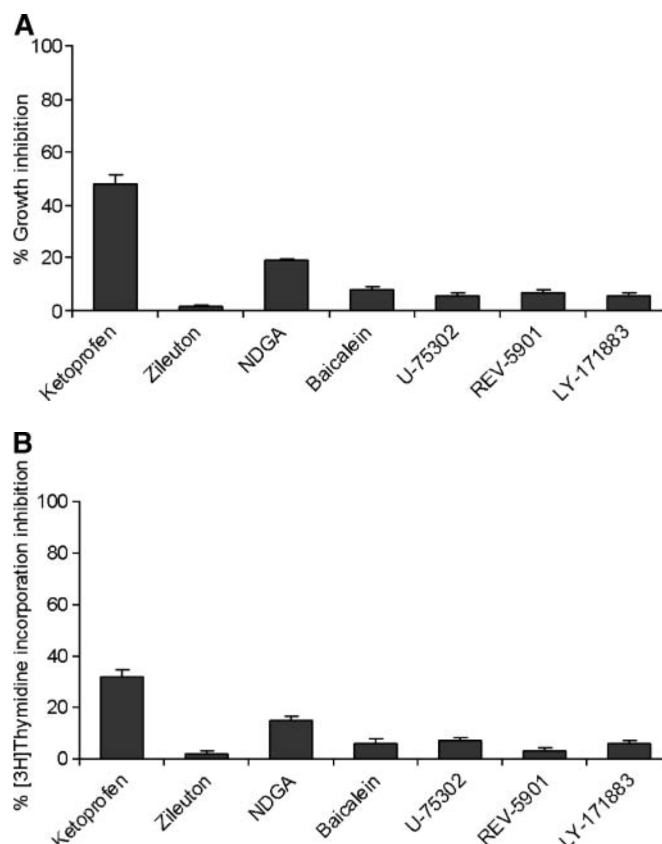

Fig. 1. Effect of lipoxygenase (LOX) inhibitors and leukotriene receptor antagonists on 10% FBS-induced 3T6 fibroblast proliferation and [^{3}H]thymidine incorporation. Fibroblasts (25×10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing ketoprofen (5 μM), zileuton (5 μM), nordihydroguaiaretic acid (NDGA; 5 μM), baicalein (30 μM), U-75302 (10 μM), REV-5901 (10 μM), or LY-171883 (100 μM). A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Cell number and [^{3}H]thymidine incorporation in control cells were 825,231 ± 79,254 cells and 176,344 ± 11,675 dpm, respectively. Results are means ± SEM from three experiments performed in triplicate.

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TABLE 1. Effects of CYP450 inhibitors on PGE_2, LTB_4, and 12(*S*)-HETE production in 3T6 fibroblast cultures

Treatment	Concentration		
	PGE_2	LTB_4	12(*S*)-HETE
		pg/ml	
FBS, 10%	608 ± 13.20	18 ± 1.5	1,154 ± 23.4
Ketoprofen	52 ± 2.2	24 ± 2.1	1,572 ± 21.3
SKF-525A	511 ± 12.1	ND	528 ± 11.6
17-ODYA	508 ± 6.1	ND	394 ± 13.7
ABT	288 ± 9.8	ND	756 ± 9.8
PPOH	458 ± 10.1	ND	826 ± 24.5

ABT, 1-aminobenzotriazole; CYP450, cytochrome P-450; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; ND, not determined; 17-ODYA, 17-octadecynoic acid; PG, prostaglandin; PPOH, 6-(2-propargyloxyphenyl)hexanoic acid. Cells were incubated for 48 h with 5 μM SKF-525A, 50 μM 17-ODYA, 2 mM ABT, or 50 μM PPOH. Eicosanoid concentrations were assayed as described in Materials and Methods. Data are expressed in percentages and are means ± SEM from three experiments performed in triplicate.

blast cultures. In this way, the effects of SKF-525A and 17-ODYA were found to be higher than those of ABT or PPOH.

As shown in **Fig. 2**, CYP450 inhibitors inhibit 3T6 fibroblast growth and [³H]thymidine incorporation in a concentration-dependent manner. Ethidium bromide/acridine orange staining and morphologic examination demonstrated that CYP450 inhibitors did not produce changes in cell structure and cell viability (**Table 2**). Thus, the effects on cell growth mentioned above do not appear to be a consequence of cytotoxicity.

Next, we chose the CYP450 inhibitor SKF-525A to examine cell cycle changes by flow cytometry. 3T6 fibroblast cultures were G_0/G_1 synchronized after 24 h serum starvation and incubated with 10% FBS medium containing SKF-525A (5 μM). Then, cells were collected and stained with propidium iodide and cell cycle distribution was analyzed. **Figure 3** provides data from a representative experiment and shows that SKF-525A produces a cell cycle delay. Thus, an increase in S population and a decrease in G_0/G_1 population were observed at 9 h in cultures incubated with 10% FBS, whereas these changes appeared at ~20 h in cells treated with SKF-525A (5 μM). This appreciable cell cycle delay could explain the inhibition of cell growth and [³H]thymidine incorporation observed in cultures containing SKF-525A.

Effect of AA-CYP450 metabolites on 3T6 fibroblast growth and DNA synthesis

To elucidate the role of AA-CYP450 metabolism in cell growth, we exogenously added some AA-CYP450 metabolites to cells stimulated with 10% FBS containing the CYP450 inhibitor SKF-525A (5 μM). As shown in **Fig. 4A**, SKF-525A produced a growth inhibition of ~50%, and the addition of hydroxylated AA-CYP450 metabolites such as 5-, 12-, 15-, and 20-HETE reversed this inhibitory effect. In contrast, epoxygenated AA-CYP450 metabolites such as 11,12-EET did not reverse this cell growth inhibition. With regard to DNA synthesis, SKF-525A also significantly inhibited [³H]thymidine uptake induced by 10% FBS, and

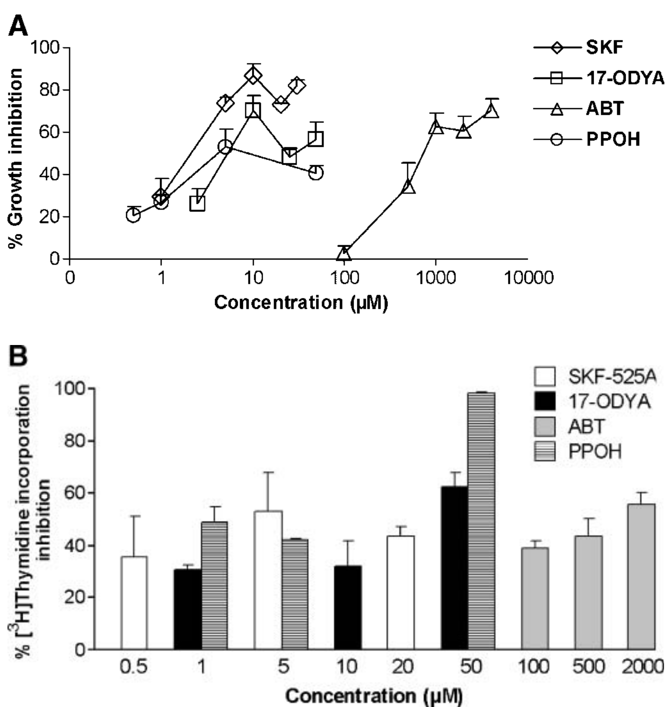

Fig. 2. Effect of cytochrome P-450 (CYP450) inhibitors on cell growth and [³H]thymidine incorporation. 3T6 fibroblasts (25 × 10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing SKF-525A, 17-octadecynoic acid (17-ODYA), 1-aminobenzotriazole (ABT), or 6-(2-propargyloxyphenyl)hexanoic acid (PPOH) at the indicated concentrations. A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [³H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Cell number and [³H]thymidine incorporation in control cells were 816,334 ± 69,474 cells and 155,251 ± 10,867 dpm, respectively. Results are means ± SEM from three experiments performed in triplicate.

5-, 12-, and 15-HETE reversed this effect, but 11,12-EET did not (Fig. 4B). Therefore, these results suggest that HETEs have comitogenic action together with growth factors present in FBS.

These data suggest than HETEs synthesized by CYP450 enzymes from AA are involved in the signal transduction pathways induced by FBS and consequently can be regarded as comitogenic factors. To determine the mitogenic effect of AA-CYP450 metabolites, we assayed the effect of HETEs on 3T6 fibroblast growth in the absence of growth factors (FBS). **Figure 5** shows that 5-, 12-, and 15-HETE significantly increase cell number. On the other hand, these HETEs also have a mitogenic effect on the [³H]thymidine incorporation assay.

Role of the Akt pathway in 3T6 fibroblast growth and DNA synthesis stimulated by HETEs

Once the comitogenic and mitogenic effects of HETEs on 3T6 fibroblasts were determined, we investigated through which cell signaling pathways these eicosanoids

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Treatment	Concentration	Viability
	μM	%
SKF-525A	0.5	98.15 ± 0.61
	5	98.24 ± 1.42
	20	96.11 ± 0.31
17-ODYA	1	98.72 ± 0.40
	10	95.89 ± 2.04
	50	95.56 ± 1.65
ABT	100	99.04 ± 0.22
	500	97.45 ± 0.27
	2,000	97.00 ± 0.95
PPOH	1	96.40 ± 1.12
	5	95.77 ± 0.75
	50	96.20 ± 0.69

TABLE 2. Effects of CYP450 inhibitors on 3T6 fibroblast viability

Cells were incubated for 48 h with SKF-525A, 17-ODYA, ABT, or PPOH at the concentrations indicated. Cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to assess viability. Data are means ± SEM from at least three experiments performed in triplicate.

act. For this purpose, we studied the classical phosphatidylinositol-3-kinase (PI3K)/Akt pathway that is activated by mitogens and growth factors and that plays an important role in the control of cell growth and differentiation.

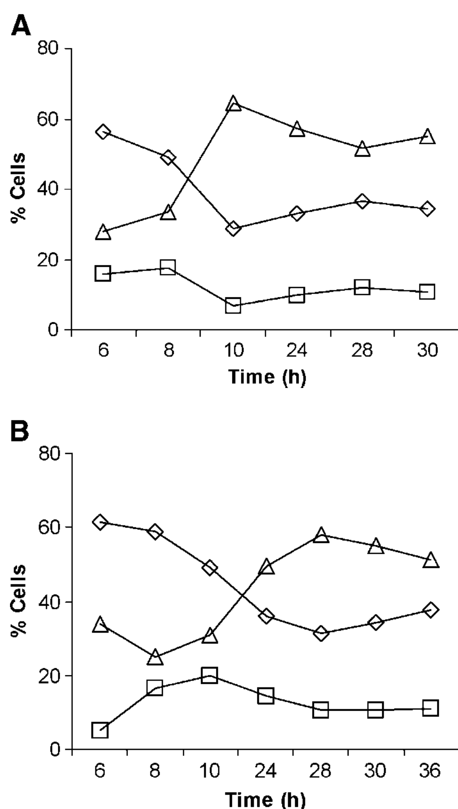

Fig. 3. Effect of the CYP450 inhibitor SKF-525A on cell cycle distribution in 3T6 fibroblast cultures. Cells were cultured with 10% FBS (A) or medium in the presence of 5 μM SKF-525A (B) for 6–36 h. Finally, cells were harvested and fixed with ethanol, and DNA was stained with propidium iodide. The DNA content was analyzed by fluorescence-activated cell sorting. Data are expressed as percentage of cells in the G_0/G_1 (diamonds), S (triangles), and G_2/M (squares) phases of the cell cycle. The results shown are representative of three independent experiments.

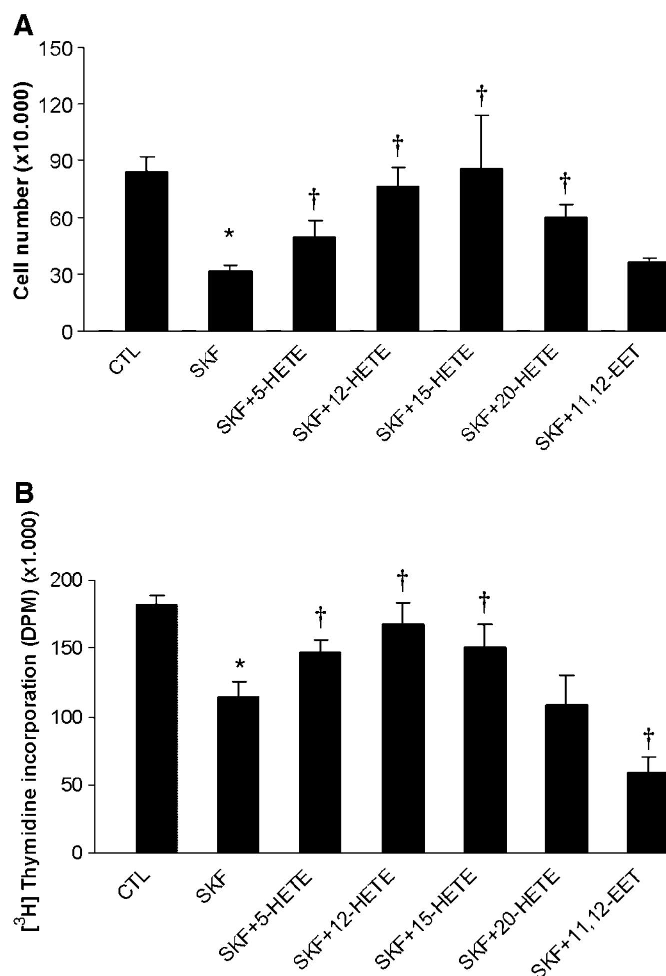

Fig. 4. Effect of exogenous hydroxyeicosatetraenoic acids (HETEs) on cell proliferation and [^{3}H]thymidine incorporation inhibited by SKF-525A. 3T6 fibroblasts (25 × 10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 h. Then, cells were incubated for 48 h with 10% FBS-RPMI containing 5 μM SKF-525A or the CYP450 inhibitor in the presence of 1 μM 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE, 20-HETE, or 11,12-epoxyeicosatrienoic acid (EET). A: After a 2 day treatment, cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Results are means ± SEM from three experiments performed in triplicate. * $P < 0.05$ compared with nontreated cells; † $P < 0.05$ compared with SKF-525A-treated cells. CTL, control.

Our results show that wortmannin, a PI3K inhibitor (34), inhibited the increasing effect of 5-, 12-, and 15-HETE on 3T6 fibroblast growth (Fig. 5A). In a similar way, wortmannin reduced the [^{3}H]thymidine incorporation induced by 5-, 12-, and 15-HETE (Fig. 5). Thus, these results suggest that the mitogenic effect of HETEs was via PI3K/Akt. To confirm the effect of HETEs on the PI3K/Akt pathway, we performed Western blot analysis of Akt and phosphorylated Akt levels. Our results show that 5-, 12-, and 15-HETE increased phosphorylated Akt levels (**Fig. 6**), which supports the role of the PI3K/Akt pathway on the effects of HETEs in 3T6 fibroblast proliferation.

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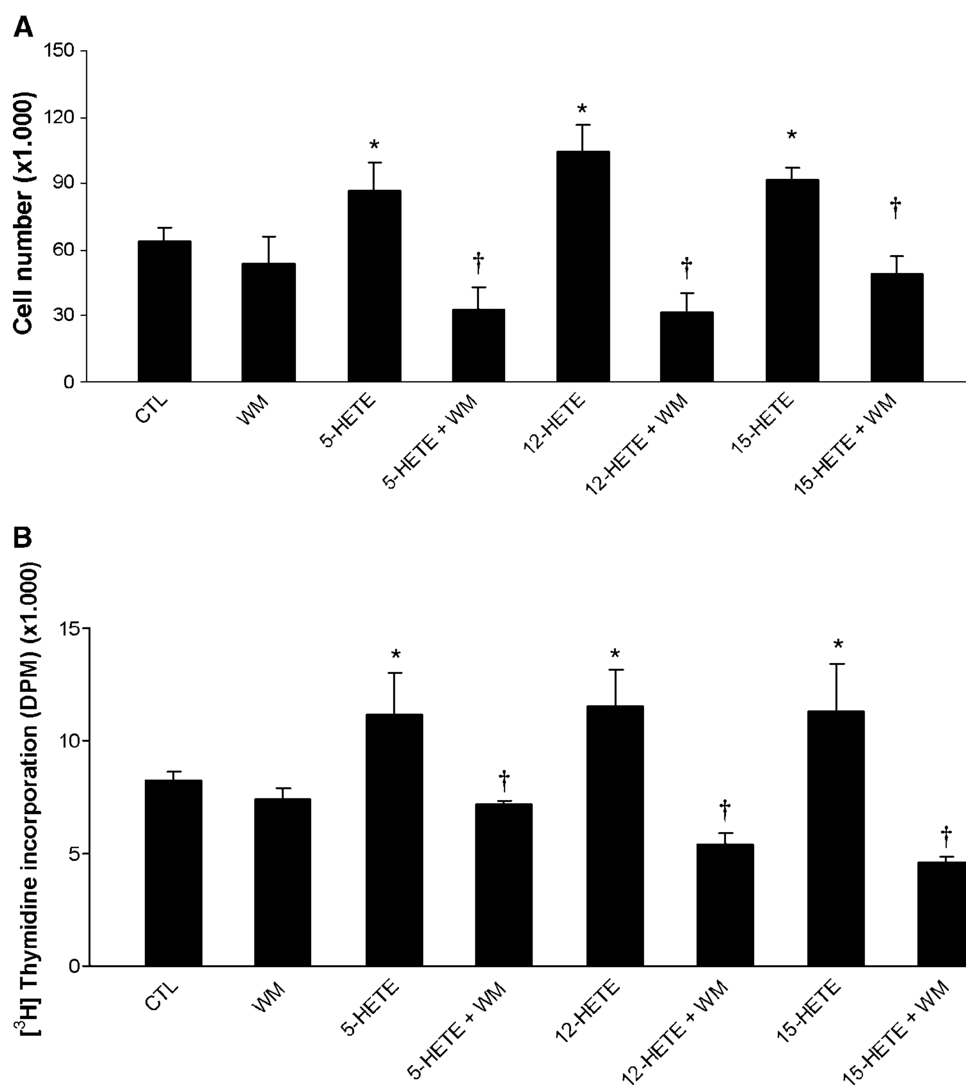

Fig. 5. Effect of exogenous HETEs on cell proliferation and [^{3}H]thymidine incorporation in 3T6 fibroblasts. Cells (25×10^3 cells/well) were plated and cultured in RPMI without FBS for 24 h. Then, cells were incubated for 2 days with RPMI without FBS containing 1 μM 5-HETE, 12(*S*)-HETE, or 15(*S*)-HETE in the absence or presence of 0.1 μM wortmannin (WM). A: Cells were trypsinized and counted using microscopy. B: For the last 24 h of treatment, [^{3}H]thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyzer. Results are means ± SEM from three experiments performed in triplicate. * $P < 0.05$ compared with nontreated cells. † $P < 0.05$ compared with cells treated with the corresponding HETE alone. CTL, control.

DISCUSSION

Calcium-independent cytosolic PLA_2 is involved in serum-induced 3T6 fibroblast proliferation (7), through AA release and the subsequent metabolism by the inducible form of COX-2, to synthesize PGs such as PGE_2 (5). The biological effects of PGE_2 have been attributed to its interaction with specific receptors. Thus, we provided evidence that PGE_2's interaction with EP_1 and EP_4 receptors is involved in the control of 3T6 fibroblast proliferation, which is associated with changes in D, E, and A cyclin levels (6). Recently, we also reported that the PGE_2-EP_3 receptor interaction may be involved in serum-induced 3T6 fibroblast growth as a result of their effects on cAMP levels and on the cell cycle machinery of the S phase (35). Thus, generation of and PGE_2 interaction with these receptors, in an autocrine or paracrine manner, may act as a necessary comitogenic signal.

When PG synthesis is abolished by COX inhibition, AA released after mitogenic stimuli by PLA_2 can be metabolized by LOXs and/or CYP450 enzymes. The mitogenic effect of other AA metabolites, which can be formed when the synthesis of PGs is blocked, may explain why COX inhibitors (36) have lower effects than PGE_2 antagonists (6) on 3T6 fibroblast growth. Considering these findings, the aim of this study was to elucidate the role of AA metabolism by LOX and CYP450 pathways in serum-induced 3T6 fibroblast growth.

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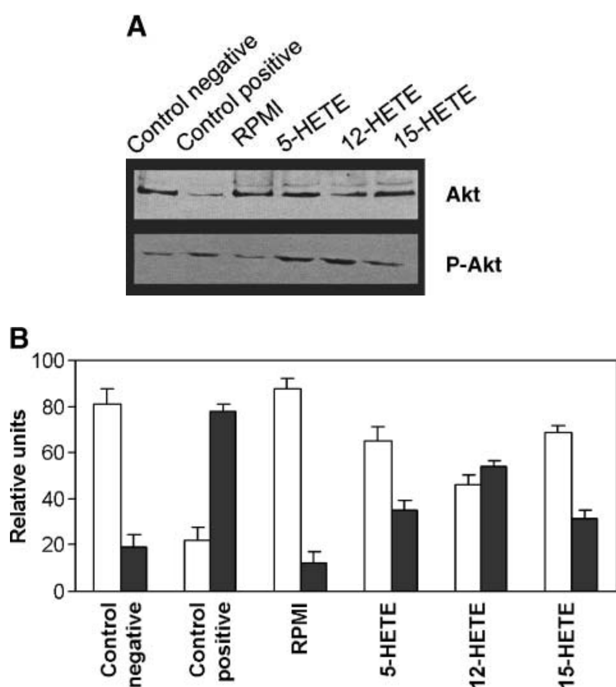

Fig. 6. A: Western blot of Akt and phosphorylated Akt (P-Akt) expression in cultured 3T6 fibroblasts. Cells were incubated with 1 μM 5-HETE, 12(*S*)-HETE, or 15(*S*)-HETE for 30 min, and Akt or phosphorylated Akt levels were determined using specific antibodies. Control negative was a LY-294002-treated Jurkat extract, and control positive was a calyculin A-treated Jurkat extract. Results are representative of three separate experiments. B: Akt (white bars) and phosphorylated Akt (black bars) values were normalized and are expressed as relative units. Results are means ± SEM from three experiments.

Our results show that serum induced PGE_2 and 12(*S*)-HETE synthesis by 3T6 fibroblasts but did not increase LTB_4 levels. Furthermore, we observed that COX inhibition by ketoprofen markedly enhanced 12(*S*)-HETE levels, suggesting that the inhibition of the COX pathway can upregulate the remaining pathways. Thus, we must consider that HETEs can be synthesized by LOXs or CYP450s. On the other hand, specific 5-LOX inhibitors (zileuton), 12-LOX inhibitors (baicalein), or LT antagonists (U-75302, REV-5901, and LY-171883) did not inhibit 3T6 fibroblast growth, whereas NDGA, a nonselective LOX inhibitor (25) and CYP450 inhibitor (37), had an appreciable effect on fibroblast growth. These preliminary results suggested that HETEs, probably produced through the CYP450 pathway, might be involved in the regulation of 3T6 fibroblast growth. To confirm this hypothesis, we measured the effect of CYP450 inhibitors such as SKF-525A, 17-ODYA, ABT, and PPOH on 12(*S*)-HETE synthesis and fibroblast proliferation. All inhibitors markedly reduced 12(*S*)-HETE levels, cell growth, and DNA synthesis. However, ABT and PPOH also reduced PGE_2 levels. Thus, their effects on cell proliferation could be a consequence of the impairment of both PG and HETE synthesis. In contrast, the effects of SKF-525A and 17-ODYA appear to be related to the inhibition of HETE synthesis.

To understand the effect of SKF-525A on 3T6 fibroblast growth, we investigated which point in the cell cycle is affected by the CYP450 inhibitor. Previously, Pidgeon et al. (38) demonstrated that the inhibition of 12(*S*)-HETE synthesis induced prostate cancer cell cycle arrest at G_0/G_1. In this study, we provide evidence that CYP450 inhibitors such as SKF-525A induce a cell cycle delay. Thus, serum induces entry into the cell cycle, and we observed the enhancement of S phase and the impairment of G_0/G_1 10 h later. However, these events were observed 24 h later, when SKF-525A was present. Therefore, it can be argued that the effect of SKF-525A on 3T6 fibroblast growth was the result of CYP450 inhibition and the subsequent impairment of HETE synthesis. The reversal of these SKF-525A effects by exogenous addition of HETEs confirms this hypothesis. Interestingly, 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE, and 20-HETE but not 11,12-EET reversed the effects of SKF-525A. These data suggest that HETEs are involved in the signal transduction pathways induced by FBS and that HETEs can be regarded as comitogenic factors. These observations agree with the comitogenic effect of 20-HETE in proximal tubular cells (39), 12-HETE in microvascular endothelial cells (40), 5-, 12-, and 15-HETE in mammary epithelial cells (41), and 12-HETE in corneal epithelial cells (42).

On the other hand, several authors reported that HETEs could be mitogenic agents. Thus, Palmberg et al. (43) reported that 15-HETE but not 5-HETE induced DNA synthesis in arterial smooth muscle cells. 12-HETE also regulates DNA synthesis in human lens epithelial cells (44), microvascular endothelial cells (40), and artery smooth muscle cells (45). Additionally, Zeng et al. (46) recently reported that 5-HETE induced DNA synthesis in human microvascular endothelial cells. Our results show that 5(*S*)-, 12(*S*)-, and 15(*S*)-HETE are mitogenic factors that induce 3T6 fibroblast growth and increase DNA synthesis.

Understanding the mechanism by which HETEs are involved in cell growth may be a critical issue in cell growth/cancer and lipid homeostasis. However, these molecular mechanisms have not been fully elucidated. No cellular receptors for HETEs have yet been identified, but some potential pathways by which they may stimulate cellular activities have been explored. This work provides the first evidence that 5-, 12-, and 15-HETE are mitogenic in fibroblasts through PI3K/Akt, a metabolic pathway involved in the control of cell growth (47) and cell survival (48). Thus, wortmannin, a PI3K inhibitor, blocked 3T6 fibroblast growth and DNA synthesis induced by the three HETEs. Moreover, we observed that 5-, 12-, and 15-HETE increased phosphorylated Akt levels. Recently, it was shown that 5-, 12-, and 15-HETE activate Akt toward stimulating growth in microvascular endothelial cells (46), prostate cancer cells (38), and dermal microvascular endothelial cells (49), respectively. Our findings clearly provide evidence for the efficacy of HETEs on stimulating 3T6 fibroblast growth.

In conclusion, we provide evidence indicating that 5-HETE, 12(*S*)-HETE, and 15(*S*)-HETE, probably synthesized from AA by CYP450, are involved in the control of 3T6 fibroblast proliferation induced by serum. Moreover,

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we observed that these HETEs are mitogenic on 3T6 fibroblasts and that this effect is dependent on activation of the PI3K pathway. Future studies are required to determine the receptors involved in these events and whether HETEs also regulate other pathways, such as mitogen-activated protein kinase, that are involved in fibroblast growth.

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4.2. Publicación 2

Nieves D, Moreno JJ

Role of 5-Lipoxygenase Pathway in the Regulation of RAW 264.7 Macrophage Proliferation.

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Posición en *Pharmacology and Pharmacy*: 38/193

El objetivo de este trabajo fue estudiar el papel de los metabolitos derivados del AA producidos por la 5-LOX en el control del crecimiento de los macrófagos RAW 264.7. Nuestros resultados mostraron que el zileuton, inhibidor de la 5-LOX, y el NDGA, inhibidor inespecífico de las LOXs, eran capaces de inhibir la proliferación de los macrófagos RAW 264.7 y la incorporación de timidina inducidas con FBS 10%, de forma concentración-dependiente. La inhibición del crecimiento inducida por el NDGA pudo ser debida a la inducción de la apoptosis, mientras que el zileuton no parecía inducir apoptosis. Además, ambos inhibidores alteraron el ciclo celular de los macrófagos. Por otro lado, los antagonistas receptoriales del LTD_4 (LY-171883 y MK-571), del LTB_4 (U-75302) y de los CysLTs (REV-5901 y Bay u9773) inhibieron la proliferación celular y la incorporación de timidina en los macrófagos RAW 264.7 de forma concentración-dependiente, y produjeron un retraso en el ciclo celular, sin inducir apoptosis. También observamos que al añadir LTB_4 o LTD_4 se revirtió el efecto inhibitorio sobre el crecimiento celular inducido por zileuton o los antagonistas receptoriales de los LTs. Finalmente mostramos que, en ausencia de factores de crecimiento, tanto el LTB_4 como el LTD_4 tienen efecto proliferativo, y presentamos evidencias preliminares de que en este efecto podrían estar implicadas las vías MAPK y PI3K. En resumen, nuestros resultados demuestran que la interacción del LTB_4 y LTD_4 con sus respectivos receptores están implicados en el control del crecimiento de los macrófagos RAW 264.7.

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Role of 5-lipoxygenase pathway in the regulation of RAW 264.7 macrophage proliferation

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Abbreviations:
AA, arachidonic acid
LOX, lipoxygenase
NDGA, nordihydroguaiaretic acid
LT, leukotriene
MAPK, mitogen-activated protein kinase
PI3K, phosphatydilinositol 3-kinase
PG, prostaglandin
COX, cyclooxygenase
HETE, hydroxyeicosatetraenoic acid
CysLT, cysteinyl leukotriene
CysLTR, cysteinyl leukotriene receptor
BLT, LTB_4 receptor
EGF, epidermal growth factor
DMEM, Dulbecco's modified Eagle's medium
FBS, foetal bovine serum
FACS, fluorescent-activated cell sorting

ABSTRACT

Arachidonic acid (AA) metabolites control cell proliferation, among other physiologic functions. RAW 264.7 macrophages can metabolise AA through the cyclooxygenase and lipoxygenase (LOX) pathways. We aimed to study the role of AA-metabolites derived from 5-LOX in the control of RAW 264.7 macrophage growth. Our results show that zileuton, a specific 5-LOX inhibitor, and nordihydroguaiaretic acid (NDGA), a non-specific LOX inhibitor, inhibit cell proliferation and $[^3H]$-thymidine incorporation in a concentration-dependent fashion. Growth inhibition induced by NDGA can be explained by an apoptotic process, while zileuton does not seem to induce apoptosis. Moreover, these treatments delay the cell cycle, as analysed by flow cytometry. On the other hand, the leukotriene (LT) B_4 receptor antagonist U-75302, the LTD_4 receptor antagonists LY-171883 and MK-571, and the cysteinyl-LT receptor antagonist REV-5901 also inhibit cell proliferation and $[^3H]$-thymidine incorporation in a concentration-dependent manner, and delay the RAW 264.7 cell cycle. However, these antagonists did not induce annexin V staining, caspase activation or DNA fragmentation. Furthermore, we demonstrated that exogenous addition of LTB_4 or LTD_4 revert the cell growth inhibition induced by zileuton or the leukotriene receptor antagonists mentioned above. Finally, we observed that LTB_4 and LTD_4, in the absence of growth factors, have pro-proliferative effects on macrophages, and we obtained preliminary evidences that this effect could be through mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. In conclusion, our results show that the interaction between LTB_4 and LTD_4 with its respective receptor is involved in the control of RAW 264.7 macrophage growth.

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1. Introduction

Arachidonic acid (AA) is usually esterified in the glycerophospholipids of membranes. Upon stimulation, it is released by selective lipases, and free intracellular AA can be oxidised by the enzymes of the AA cascade: the cyclooxygenases (COXs), which produce prostaglandins (PGs) and thromboxanes; the lipoxygenases (LOXs), which form leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins, and cytochrome P-450 monooxygenases. 5-, 12- and 15-LOX are considered the main LOXs catalyzing the biosynthesis of biologically active compounds. Thus, AA is metabolised by 5-LOX yielding 5-HETE and LTA_4, the precursor of LTB_4 and cysteinyl leukotrienes (CysLTs) such as LTC_4, LTD_4 and LTE_4. Finally, 12- and 15-LOX produce HETEs and lipoxins [1].

In macrophages, AA is mainly metabolised through COXs and LOXs enzymes, which have been described in this cell type [2]. 5-, 12- and 15-LOXs are expressed in murine macrophages [3–5]. Here, we focused on 5-LOX, the key enzyme of leukotriene biosynthesis, and its role in macrophage proliferation.

The biological effects of LTs depend on its interaction with specific receptors. Four leukotriene receptors that belong to the G protein-coupled seven transmembrane domain receptor family have been characterised: two receptors for LTB_4 (BLTs) and two receptors for $LTC_4/D_4/E_4$ (CysLTRs). BLT1 is a high-affinity receptor specific for LTB_4, whereas BLT2 is a low-affinity receptor that also binds other eicosanoids [6]. BLT2 is expressed ubiquitously, in contrast to BLT1, which is expressed predominantly in leukocytes. Pharmacological studies have determined that CysLTs activate at least two receptors, referred to as CysLTR1 and CysLTR2. In macrophages, the main receptor expressed is the former, which preferentially responds to LTD_4 [7].

Cell growth is controlled by mitogens like growth factors. The interaction of growth factors with their cell surface receptors leads to multiple signalling events including the activation of several phospholipases, which in turn can lead to the release of AA and the production of AA metabolites. Thus, epidermal growth factor (EGF) induces LOX metabolism in A431 cells, HeLa cells and fibroblasts [8], and LTB_4 synthesis in guinea pig gastric chief cells [9]. LOX metabolites are also involved in basic fibroblast growth factor-induced endothelial cell proliferation [10]. In addition, LTs and HETEs have recently been described as survival factors. Thus, there is evidence that 12-LOX regulates the serum-supported survival of W256 carcinosarcoma cells of monocytoid origin [11]. Moreover, Ghosh and Myers [12] observed that only 5-LOX inhibitors produce apoptosis in human prostate cancer cells, while 12-LOX, COX and cytochrome P-450 inhibitors do not induce programmed cell death. Thus, a number of studies have suggested that 5-LOX is involved in cell proliferation, although its effect on cell growth is cell-type specific. Thus, the 5-LOX pathway appears to facilitate the growth of several cell types such as pulmonary artery endothelial cells [13] and mesangial cells [14]. In contrast, it suppresses the growth of other cells, e.g. glioma cells [15] and murine Leidig cells [16].

Little is known about the role of the 5-LOX pathway in the proliferation of macrophages. Recently, Titos et al. [17] suggested that 5-LOX pathway metabolites participate in the regulation of Kupffer cell growth and survival in a model of liver fibrosis. The aim of this study was to explore the effects of 5-LOX pathway metabolites on serum stimulated RAW 264.7 macrophage proliferation. Our results suggest that LTB_4 and LTD_4 interaction with its specific receptors, are involved in RAW 264.7 cell cycle progression and serum-induced macrophage proliferation. Moreover, we show that the inhibition of macrophage proliferation by 5-LOX pathway inhibition was not consequence of a pro-apoptotic effect. Furthermore, we show that LTB_4 and LTD_4 have mitogenic effects in absence of growth factors, suggesting the involvement of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways in these events.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), penicillin, streptomycin and versene-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). Nordihydroguaiaretic acid (NDGA), zileuton, LTB_4, LTD_4, PD098059, wortmannin, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, acridine orange, ethidium bromide and Bay u9773 were provided by Sigma Chemical Co. (St. Louis, MO, USA). Leukotriene antagonist receptors U-75302, LY-171883, MK-571 and REV-5901 were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). All chemicals were of the highest quality available commercially.

2.2. Cell culture

Murine RAW 264.7 macrophages (ATCC TIB-71) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were seeded in 24-well plates (tissue-culture cluster 24; Costar Cambridge, MA, USA) or in tissue-culture 100 mm-dishes (Costar), and allowed to adhere at 37 °C in 5% CO_2/95% air for 24 h. Finally, cells were re-seeded by scraping off with versene-EDTA.

2.3. Cell growth assay

The effect of the treatments was assessed on macrophages plates at 25×10^3 cells/well in 24-well plates, cultured for 24 h in DMEM supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of various compounds. Finally, the medium was aliquoted to determine floating cells and attached cells were scraped off with versene-EDTA. Cells were counted under a microscope using ethidium bromide/acridine orange staining to assess viability.

2.4. Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]-thymidine incorporation assay, which involved culturing macrophages in

24-well plates in DMEM with 10% FBS at a density of 25×10^3 cells/well. After 24 h, serum starvation cells were incubated for 48 h with the treatments and [^{3}H]-thymidine (0.25 μCi/well) was added for the last 24 h. [^{3}H]-thymidine-containing media were aspirated, cells were overlaid with 1% Triton X-100 and then scraped off the dishes. Finally, the radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

2.5. *Fluorescent-activated cell sorting (FACS) analysis/flow cytometry cell cycle analysis*

Macrophages were seeded in 100 mm-dishes and 24 h later, they were serum starved. After 24 h without FBS, the percentage of cells in G_0/G_1 was about 80%. Cells were then cultured in 10% FBS–DMEM containing the treatments. Thereafter, they were scraped, fixed with 70% ethanol and stored at 4 °C for at least 2 h. Next, low-molecular weight DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 μg/ml propidium iodide solution in PBS containing 0.1% Triton X-100 and 0.2 mg/ml DNase-free RNase A. Cells were analysed on an Epics XL flow cytometer (Coulter Corporation, Hialeah, FL). DNA was analysed (Ploidy analysis) on single fluorescence histograms by Multicycle software (Phoenix Flow Systems, San Diego, CA).

2.6. *Measurement of LTB_4*

Cells were cultured in plates at 25×10^3 cells/well in 24-well plates for 24 h in DMEM supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in 10% FBS medium in the presence of several products. An aliquot of culture supernatant medium was analysed by an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's protocol.

2.7. *Measurement of annexin V binding*

RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h at 37 °C. Thereafter, treatments were removed and cells were stained with fluorescein-isothiocyanate-labelled annexin V and propidium iodide following the manufacturer instructions (Roche Diagnostics, Mannheim, Germany). Representative samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera.

2.8. *Caspase activity determination*

RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h at 37 °C. Thereafter, treatments were removed and cells were stained with FITC-VAD-FMK, which binds to activated caspases in apoptotic cells, following the manufacturer instructions (Medical and Biological Laboratories, Nagoya, Japan). Samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera, and caspase positive or negative cells were counted.

2.9. *Measurement of DNA fragmentation*

Degradation of chromosomal DNA was evaluated with TUNEL method. RAW 264.7 macrophages were cultivated in media containing 10% FBS with treatments for 48 h. Next, cells were fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labelled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analysed on a Epics XL flow cytometer (Coulter Corporation, Hialeah, FL).

2.10. *Data analysis*

Results are expressed as mean ± S.E.M. Differences between control and treated cultures were tested using Student's *t*-test.

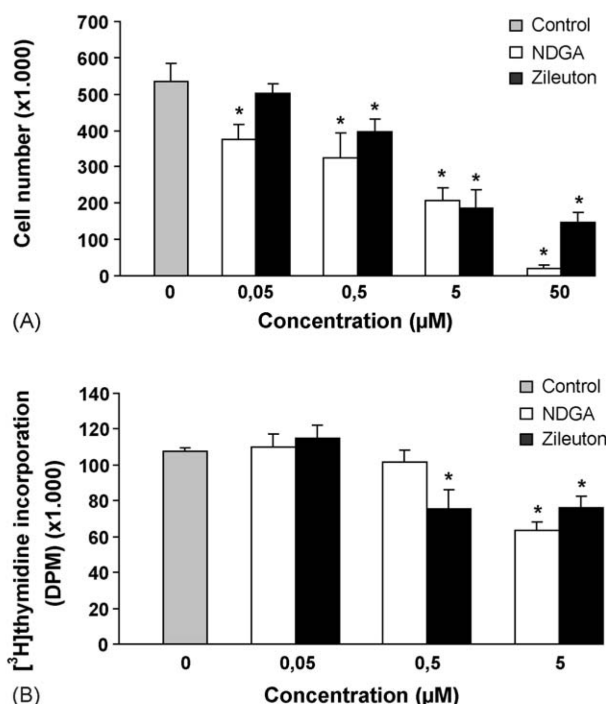

Fig. 1 – Effect of 5-LOX inhibitors on 10% FBS-induced macrophage proliferation and [^{3}H]-thymidine incorporation. Macrophages (25×10^3 cells/well) were plated and cultured in 10% FBS–DMEM. The next day, media were removed and free FBS–DMEM was added for 24 h. Cells were then incubated with 10% FBS–DMEM or with 10% FBS–DMEM containing NDGA or zileuton. After a 2-day treatment, cells were scraped off and counted (A), or the last 24 h [^{3}H]-thymidine (1 μCi/well) was added and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with non-treated cells. Baseline levels of cell number and [^{3}H]-thymidine incorporation in serum-free medium were 56.256 ± 1.725 cells and 6.182 ± 167 dpm, respectively.

3. Results

3.1. *Effect of LOX inhibitors on growth, DNA synthesis and cell cycle distribution of RAW 264.7 macrophages*

To determine the role of 5-LOX metabolites in macrophage growth induced by FBS, cells were exposed to the specific 5-LOX inhibitor zileuton [18] (0.05–50 μM). We compared the effect of zileuton with a non-selective LOX inhibitor as NDGA [19] (0.05–50 μM). As shown in Fig. 1A, both compounds significantly inhibited cell growth in a concentration-dependent manner. Ethidium bromide/acridine orange staining and morphologic examination revealed that the LOX inhibitors did not affect cell structure or cell viability (data not shown). Only 50 μM NDGA showed an appreciable reduced cell viability.

The effect of LOX inhibitors on RAW 264.7 growth was also examined by [^{3}H]-thymidine uptake. Serum strongly increases the rate of [^{3}H]-thymidine incorporation in RAW 264.7 macrophage cultures. Zileuton and NDGA induced a concentration-dependent inhibition of [^{3}H]-thymidine uptake by macrophages after 48 h of exposure (Fig. 1B).

Finally, we quantified cell cycle changes induced by LOX inhibitors by flow cytometry. RAW 264.7 macrophage cultures that had been G_0/G_1 synchronised by 24 h serum starvation were incubated with 10% FBS-medium containing zileuton (5 μM) or NDGA (5 μM). Cells were then collected and stained with propidium iodide and cell cycle distribution was analysed. Zileuton raises the percentage of G_0/G_1 population and reduces the percentage of cells in S phase at 24 and 36 h (Table 1). Thus, 5-LOX inhibition appears to induce an appreciable cell cycle delay. In contrast, NDGA at 24 h increases the percentage of cells in G_2/M phase and decreases the number of cells in G_0/G_1 phase, while at 36 h, the percentage of cells in each cell cycle phase is closer to control values.

In parallel, we have confirmed that zileuton and NDGA inhibit LTB_4 production induced by 10% FBS in RAW 264.7 cell culture macrophages. Thus, control culture medium presents 209.9 ± 30.5 pg LTB_4/ml, whereas macrophages incubated with zileuton (5 μM) and NDGA (5 μM) present 15.2 ± 1.1 and 10.7 ± 1.5 pg LTB_4/ml, respectively.

3.2. *Effect of leukotriene receptor antagonists on growth, DNA synthesis and cell cycle distribution of RAW 264.7 macrophages*

To provide further evidences of the role of 5-LOX pathway on RAW 264.7 macrophage proliferation, we used leukotriene receptor antagonists such as LY-171883 and MK-571 as LTD_4 receptor antagonists [20,21], U-75302 as LTB_4 receptor antagonist [22] and REV-5901 as CysLTs receptor antagonist [23]. Treatment with these specific antagonists and Bay u9773, a CysLTR1 and CysLTR2 antagonist [24], reduced cell number in

Table 1 – Effect of LOX inhibitors and leukotriene receptor antagonists on cell cycle distribution in RAW 264.7 cultures

	Percentage of cells		
	G_0/G_1	S	G_2/M
Time 24 h			
Control	59.1 ± 0.38	31.7 ± 1.90	9.2 ± 1.58
Zileuton	65.0 ± 0.12*	27.0 ± 0.20	8.0 ± 0.32
NDGA	47.9 ± 2.26*	31.3 ± 0.57	20.8 ± 2.64*
U-75302	63.5 ± 0.10*	29.5 ± 0.13	7.0 ± 0.21
LY-171883	67.2 ± 0.35*	25.5 ± 0.55*	7.4 ± 0.25
Time 36 h			
Control	56.2 ± 1.09	37.2 ± 1.36	6.6 ± 0.85
Zileuton	58.1 ± 0.60	30.5 ± 1.10*	11.4 ± 1.70
NDGA	57.4 ± 0.85	30.9 ± 1.55	11.8 ± 0.70*
U-75302	61.4 ± 0.50*	33.7 ± 1.40	4.9 ± 0.95
LY-171883	61.4 ± 0.85*	30.0 ± 0.20*	8.7 ± 0.65

Cells were cultured with DMEM–FBS 10% in presence of zileuton 5 μM, NDGA 5 μM, U-75302 10 μM or LY-171883 100 μM for 24 or 36 h. Finally, they were harvested and fixed with ethanol, and DNA was stained with propidium iodide. DNA content was analysed by FACS. Data are expressed as percentage of cells in the G_0/G_1, S and G_2/M phases of cell cycle. Data are the means ± S.E.M. of three experiments performed in duplicate. *$P < 0.05$ compared with non-treated cells.

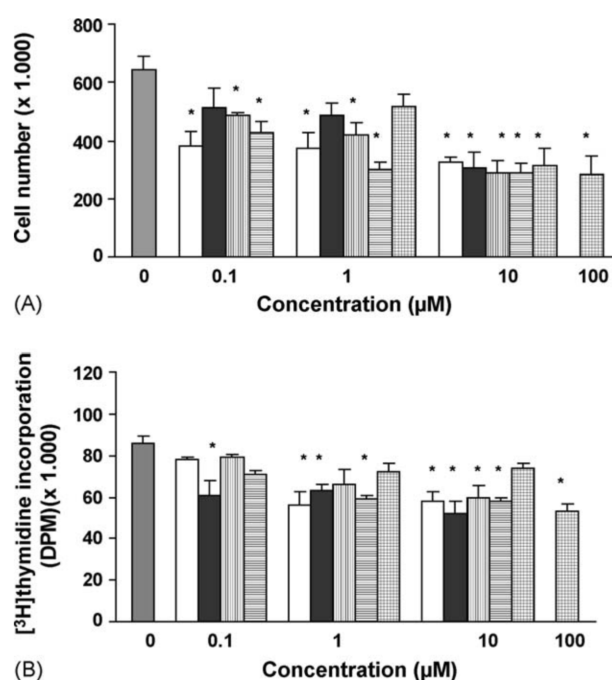

Fig. 2 – Effect of leukotriene receptor antagonists on 10% FBS-induced macrophage proliferation and [^{3}H]-thymidine incorporation. Cells (25×10^3 cells/well) were plated and cultured in 10% FBS–DMEM. The next day, media were removed and free FBS–DMEM was added for 24 h. Cells were then incubated with 10% FBS–DMEM or with 10% FBS–DMEM (grey bars) containing LY-171883 (square bars), MK-571 (black bars), U-75302 (white bars), REV-5901 (vertical line bars) and bay u9773 (horizontal line bars). After a 2-day treatment, cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added for the last 24 h and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with non-treated cultures.

a concentration-dependent manner. Thus, the treatments produced a maximum growth inhibition around of 50% for LY-171883 (100 μM), MK-571 (10 μM), U-75302 (10 μM), REV-5901 (10 μM) and Bay u9773 (1 μM), respectively (Fig. 2A). Similar data were obtained with the leukotriene receptor antagonists on [^{3}H]-thymidine incorporation by macrophages, although the effect was not as strong as in cell growth inhibition (Fig. 2B). Macrophages were also exposed to the receptorial antagonists to determine the effect of treatments on cell cycle progression. Table 1 shows that LTD_4 receptor antagonist (LY-171883, 100 μM) and LTB_4 receptor antagonist (U-75302, 10 μM) at 24 and 36 h markedly increased the number of cells in G_0/G_1 phase and impaired the percentage of cells in S phase. Moreover, to demonstrate the specific effect of zileuton and leukotriene receptor antagonists on cell proliferation, we added exogenously the corresponding treatments in presence of LTB_4 or LTD_4. Thus, our results show that zileuton effects on cell growth and DNA synthesis were reverted by LTB_4 and LTD_4 (Fig. 3A). In a similar way, the inhibitory effect of U-75302 and

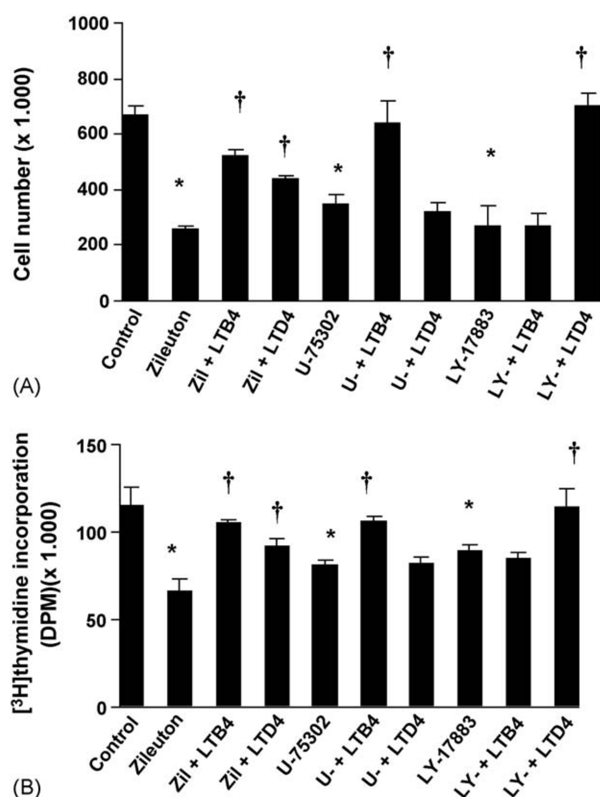

Fig. 3 – Effect of exogenous LTB_4/LTD_4 on cell proliferation and [^{3}H]-thymidine incorporation inhibited by zileuton or leukotriene receptor antagonists. Cells were treated for 48 h with zileuton (5 μM), U-75302 (1 μM) or LY-171883 (10 μM), or the inhibitor/antagonists in the presence of 0.01 μM LTB_4 or 0.1 μM LTD_4. Cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added to the cells for the last 24 h and radioactivity in cell lysates was determined in a scintillation analyser (B). Results are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with non-treated cultures. $^\dagger P < 0.05$ compared with cells treated only with the antagonist.

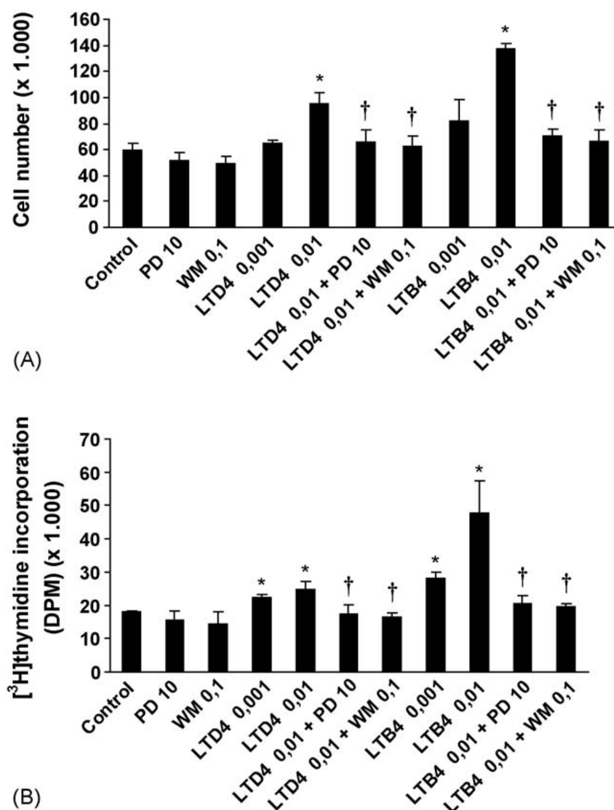

Fig. 4 – Mitogenic effects of LTD_4 and LTB_4 in absence of growth factors. Cells were incubated for 48 h with the leukotrienes at 0.001 and 0.01 μM in medium without fetal bovine serum, or leukotrienes at 0.01 μM and PD098059 (PD) 10 μM or wortmannin (WM) 0.1 μM. Cells were scraped off and counted (A) or [^{3}H]-thymidine (1 μCi/well) was added to the cells for the last 24 h and radioactivity in cell lysates were determined in a scintillation analyser (B). Data are means ± S.E.M. from three experiments performed in triplicate. $^*P < 0.05$ compared with the control group. $^\dagger P < 0.05$ compared with cells treated with LT at 0.01 μM.

LY-171883 on cell growth and [^{3}H]-thymidine uptake was also reverted by exogenous addition of LTB_4 or LTD_4, respectively (Fig. 3B).

The above results suggest that LTB_4 and CysLTs such as LTD_4 are involved in the signal transduction pathways induced by FBS, and that these eicosanoids can be regarded as co-mitogenic factors. To determine the mitogenic effect of 5-LOX pathway metabolites on RAW 264.7 macrophages, we performed the assays in absence of serum. LTB_4 and LTD_4 (0.001–0.01 μM) significantly increased [^{3}H]-thymidine uptake in our experimental conditions (Fig. 4B). LTB_4 was more mitogenic than LTD_4. Thus, LTB_4 (0.01 μM) doubled [^{3}H]-thymidine uptake. Since DNA synthesis can occur without cell division, the effects of LTs/CysLTs on RAW 264.7 growth were also determined. LTB_4 and LTD_4 also enhanced cell proliferation (Fig. 4A). Thus, LTB_4 (0.01 μM) approximately doubled cell numbers and LTD_4 (0.01 μM) also significantly increase cell growth although less than LTB_4. Next, we have checked the

Table 2 – Effect of LOX inhibitors and leukotriene receptor antagonists on apoptosis in RAW 264.7 macrophage cultures

Treatment	Apoptosis		
	Annexin V^+/PI^- (early apoptosis)	Annexin V^+/PI^+ (late apoptosis/necrosis)	Annexin V^-/PI^+ (necrosis)
Control	2.0 ± 0.26	3.6 ± 0.32	0.8 ± 0.14
Staurosporine (1 μM)	5.8 ± 0.84*	93.1 ± 0.99*	1.1 ± 0.37
NDGA (5 μM)	1.8 ± 0.58	7.5 ± 0.65*	1.3 ± 0.41
NDGA (50 μM)	12.1 ± 1.88*	81.1 ± 2.99*	6.8 ± 1.60*
Zileuton (5 μM)	3.5 ± 0.52	7.7 ± 1.37	2.3 ± 0.47
Zileuton (50 μM)	1.4 ± 0.17	6.8 ± 0.63*	5.4 ± 0.83*
REV-5901 (10 μM)	0.2 ± 0.14	0.7 ± 0.22	0.3 ± 0.11
MK-571 (10 μM)	2.0 ± 0.51	6.3 ± 1.63	2.0 ± 0.67
LY-171883 (100 μM)	1.7 ± 0.40	6.7 ± 0.62*	1.3 ± 0.22
U-75302 (10 μM)	1.0 ± 0.00	1.7 ± 0.11	0.1 ± 0.11

Cells were incubated for 48 h with DMEM–FBS 10% in the presence of NDGA, zileuton, REV-5901, MK-571, LY-171883 or U-75302. Apoptosis was measured by annexin-V and PI staining. Staurosporine was used as a positive control. Data are expressed in percentages and are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with non-treated cells.

metabolic pathways involved on this mitogenic effect. Thus, Fig. 4A and B shows that the MAPK kinase inhibitor PD98059 [25] inhibits cell proliferation and [^{3}H]-thymidine incorporation induced by LTB_4 and LTD_4. In the same way, the PI3K inhibitor wortmannin [26] also reduced cell growth and [^{3}H]-thymidine uptake induced by LTB_4 and LTD_4 on RAW 264.7 (Fig. 4A and B).

3.3. *Effect of LOX inhibitors and leukotriene receptor antagonists on RAW 264.7 macrophage apoptosis*

Previous studies have shown that 5-LOX inhibitors induce apoptosis. To test whether 5-LOX inhibitors or leukotriene receptorial antagonists decrease RAW 264.7 proliferation by induction of apoptosis, we measured annexin V and propidium iodide staining in these cells. The inside–outside translocation of phosphatidylserine from the plasma membrane is accentuated in the early stage of apoptosis. Annexin V staining was performed to determine this translocation. Zileuton and LTs/CysLT receptor antagonists did not markedly raise annexin V staining (Table 2), whereas NDGA (50 μM) significantly increased the percentage of annexin V-positive or annexin V- and propidium iodide-positive cells, suggesting that it induces apoptosis. These effects were correlated with the caspase activation and the DNA fragmentation. Thus, zileuton and LTs/CysLT receptor antagonist did not induce caspase activation, whereas cell treated with NDGA showed marked caspase activation (Fig. 5). Finally, we observed that zileuton and LTs/CysLT receptor antagonists did not induce an appreciable DNA fragmentation, whereas NDGA induced a marked DNA fragmentation (Fig. 5).

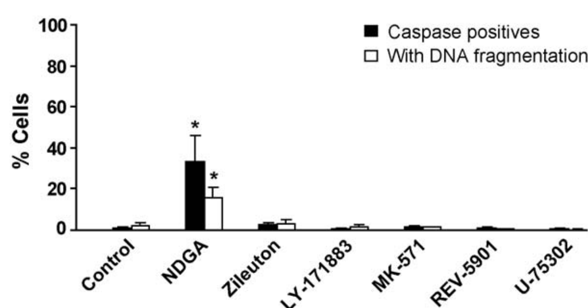

Fig. 5 – Effect of 5-LOX inhibitors and leukotriene receptor antagonists on caspase activation and DNA fragmentation in RAW 264.7 macrophage cultures. Macrophages were incubated for 48 h with DMEM–FBS 10% in the presence of NDGA 50 μM, zileuton 50 μM, LY-171883 100 μM, MK-571 10 μM, REV-5901 10 μM or U-75302 10 μM. Next, activated caspases were labelled and detected by fluorescence microscope. On the other hand, DNA ends derived from chromatin fragmentation were labelled and measured by flow cytometry. Results are means ± S.E.M. from three experiments performed in triplicate. *$P < 0.05$ compared with non-treated cultures.

4. Discussion

AA release and eicosanoid syntheses are early events of the mitogenic process induced by growth factors and subsequent cell proliferation. Thus, we observed that PGs and PGs interaction with its receptors are involved in the cell cycle progression and cell growth of 3T6 fibroblasts [27]. However, these cells failed to produce appreciable amounts of LTs. To gain more insight into the role of the 5-LOX pathway in the regulation of the mitogenic process, we examined the response of macrophages to 5-LOX inhibitors and leukotriene receptor antagonists, since these cells are able to produce LTB_4 and CysLTs [28].

There are four main structurally unrelated inhibitors of 5-LOX: redox inhibitors as NDGA, iron ligand inhibitors as BW 755C or BW A4C, AA analogs such as AA-861 or 5,8,11,14 eicosatetraynoic acid and N-hydroxyurea derivatives such as zileuton. NDGA is not selective to 5-LOX because inhibits 12-LOX [29] and 15-LOX [30] and other redox enzymes such as cyclooxygenases [31] and cytochromes P-450 [32] that are involved in AA metabolism. However, zileuton was not reported to affect these enzymes. Thus, zileuton appears a more specific 5-LOX inhibitor.

Our results shown that zileuton hinders cell growth and [^{3}H]-thymidine uptake, as well as LTB_4 synthesis in RAW 264.7 macrophage cultures. This treatment also appears to delay the RAW 264.7 macrophage cell cycle accumulating cells in G_0/G_1

phase. Thus, if a given response is inhibited by zileuton, the catalytic activity of 5-LOX pathway appears involved in RAW 264.7 macrophage proliferation. These results agree with Vargaftig and Singer [33], who suggested that zileuton reduces the fibroblast-like cell proliferation induced by interleukin-13.

Once secreted extracellularly, LTs act on specific receptors, and several studies have pointed to the presence of LTB_4 receptors [6] and CysLTRs [34] in macrophages. Thus, we aimed to elucidate the effect of LTs interaction with its receptors on the control of serum-induced RAW 264.7 macrophage progression through the cell cycle and subsequent proliferation.

REV-5901 inhibited RAW 264.7 macrophage proliferation and [^{3}H]-thymidine uptake without cytotoxicity at the concentrations and for the incubation times used. REV-5901 is a CysLTR antagonist but also a 5-LOX inhibitor. Thus, it can be argued that the effect of REV-5901 on RAW 264.7 growth was due to 5-LOX inhibition, as reported above for zileuton. To evaluate the role of CysLTRs in RAW 264.7 growth, we also used LY-171883 and MK-571, two selective antagonists of CysLTR1 that specifically blocked the binding of LTD_4 [20]. The effects of both compounds and the reversion of LY-171883 inhibition by exogenous LTD_4 addition clearly suggested that LTD_4 interaction with CysLTR1 is involved in FBS-induced RAW 264.7 proliferation. Moreover, we did not observed an additional macrophage growth inhibition when we used a dual CysLTR1 and CysLTR2 antagonist such as Bay u9773. These data suggest the main role of CysLTR1 versus CysLTR2 in the control of RAW 264.7 growth. On the other hand, a selective BLT1 receptor antagonist, U-75302 [22], inhibited FBS-induced RAW 264.7 proliferation, which was abolished by exogenous LTB_4. Thus, these results also implicate LTB_4 BLT1 interaction in RAW 264.7 growth. These findings agree with Vargaftig and Singer [33] who demonstrated that MK-571 reduces fibroblast-like cell proliferation, and Ciccarelli et al. [35] that reported the role of CysLTR1 in astrocyte growth. On the other hand, the LTD_4 receptor antagonist LY-171883 and the CysLTR antagonist REV-5901 have also been proposed as antipancreatic cancer agents because they inhibit thymidine incorporation and induce apoptosis in MiaPaCa-2 and AsPC-1 cells [36]. In contrast to our results, Przylipiak et al. [15] reported that LTD_4 inhibits thymidine incorporation in the human mammary carcinoma cell line MCF-7, which is reverted by LY-171883. On the other hand, Porreca et al. [37] showed that MK-571 inhibits the stimulatory effect of LTD_4 on thymidine incorporation in vascular smooth muscle cells in a dose-dependent manner. Here, inhibition of the 5-LOX pathway seriously hindered RAW 264.7 macrophage growth, whereas exogenous addition of 5-LOX pathway metabolites significantly reverted these effects, supporting the co-mitogenic effect of LTs/CysLTs.

Although LTB_4–BLT1 and LTD_4–CysLT1 interaction may be involved in FBS-induced RAW 264.7 proliferation, we cannot rule out the involvement of other receptors in the mechanisms of action of LTs/CysLTs in macrophage growth such as peroxisome proliferator-activated receptor-α.

Several authors reported the impairment of cell growth as consequence of apoptosis by 5-LOX inhibitors [12,17]. However, our results show that 5-LOX inhibitors such as zileuton and REV-5901 or LT/CysLTs antagonist such as U-45302, LY-171883 or MK-571 did not produce the detachment of macrophages, were not cytotoxic and did not induce a marked phosphatidylserine externalization, caspase activation or DNA fragmentation. Only NDGA induced annexin V staining and caspase activation and the subsequent DNA fragmentation. Recently, Romano et al. [38] observed that NDGA induces growth arrest of human malignant pleural mesothelial cells by an apoptotic process. Thus, although NDGA has been widely used as a 5-LOX inhibitor, the precise association between the inhibition of 5-LOX by NDGA and the events induced by NDGA described in this paper is not clear. We must consider that NDGA also inhibits 12-LOX and 15-LOX [29,30]. Furthermore, NDGA induces membrane alterations [39], increases intracellular calcium [40] and disrupts the filamentous actin cytoskeleton [41], events that may be related to cell detachment, the increase of cells in G_2/M phase and especially apoptosis. Thus, the inhibition of RAW 264.7 growth by NDGA may be consequence, at least in part, of the pro-apoptotic action, whereas the blockage of 5-LOX pathway by zileuton or LT/CysLT antagonists decrease RAW 264.7 macrophage proliferation without inducing apoptosis.

Panettieri et al. [42] noted that LTD_4 enhances EGF-induced human airway smooth muscle proliferation, but LTD_4 alone does not exert the same effect. In contrast, Paruchuri and Sjolander [43] demonstrated that LTD_4 increases the cell number and DNA synthesis of intestinal epithelial cells. In this way, Tong et al. [44] showed that LTB_4 stimulates thymidine incorporation in a concentration- and time-dependent manner and stimulate ERK1/2 phosphorylation in pancreatic cancer cells. In our study when LTD_4 or LTB_4 was added exogenously in absence of FBS, [^{3}H]-thymidine uptake and cell proliferation increased in RAW 264.7 cultures. It is noteworthy that the concentrations of exogenous LTs that evoke significant effects on RAW 264.7 proliferation are in a range similar to the amount released from macrophage cultures in our experimental conditions. Moreover, we have contributed suggesting that the pro-proliferative effect of LTB_4 and LTD_4 may be mediated by MAPK and PI3K cascades activation. Results in agreement with Klein et al. [45] who showed that LTB_4 can stimulate ERK and Akt phosphorylation in human neutrophils, and with Paruchuri et al. [46,47] who suggested that the mitogenic effect of LTD_4 is mediated by the activation of the MAPK ERK1/2 and Akt in intestinal epithelial cells.

To our knowledge, the effects of the 5-LOX-pathway on RAW 264.7 macrophage growth have not been described so far. In the present study, we provide several lines of evidence that support our hypothesis that the interaction of 5-LOX metabolites of AA (LTB_4 and LTD_4) with their plasma membrane receptors can influence RAW 264.7 macrophage cell cycle and proliferation.

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4.3. Publicación 3

Nieves D, Moreno JJ

Effect of Arachidonic and Eicosapentaenoic Acid Metabolism on RAW 264.7 Macrophage Proliferation.

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Las PGs y LTs derivados del AA son potentes mediadores de la inflamación y la proliferación celular. La ingesta de ácido eicosapentaenoico (EPA) parece beneficiosa para ambos procesos. Sin embargo, los mecanismos que explican esto efectos no están claros. Por esta razón, en este estudio hemos investigado el efecto del EPA sobre la incorporación del AA en los fosfolípidos de las membranas, la liberación de AA y su metabolismo, y consecuentemente, la síntesis de PGs. Nuestros resultados han demostrado que el [^{3}H]AA y el [^{14}C]EPA se incorporan de forma similar en las membranas de los macrófagos RAW 264.7, y que el patrón de redistribución entre los fosfolípidos es similar. El FBS 10% estimuló la liberación de [^{3}H]AA y [^{14}C]EPA de la misma forma, metabolizando unas 3 veces más de AA que de EPA. En este sentido, observamos que el AA podía ser metabolizado por la COX-1, por la COX-2 y por la 5-LOX, mientras que el EPA fue metabolizado por la COX-2 y la 5-LOX. Además, tanto el AA como el EPA fueron capaces de inducir la expresión de la COX-2. Al incubar [^{3}H]AA con EPA exógeno, observamos que el EPA no modificó la liberación del [^{3}H]AA inducida por el suero, pero la presencia de EPA disminuyó el metabolismo del [^{3}H]AA y por lo tanto, la síntesis de PGE_2. Por otro lado, estudiamos el efecto de los metabolitos del AA y el EPA sobre la proliferación de los macrófagos RAW 264.7. Así, nuestros resultados demostraron que la PGE_3 derivada del EPA estimula la proliferación celular de forma similar a la PGE_2, mientras que el LTB_5 producido a partir del EPA fue ligeramente menos efectivo que el LTB_4. Esto sugiere que los efectos del EPA sobre la proliferación celular pueden atribuirse en parte a la disminución de la liberación de eicosanoides, y no a la producción de metabolitos inhibidores de la proliferación.

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JOURNAL OF CELLULAR PHYSIOLOGY 208:428–434 (2006)

Effect of Arachidonic and Eicosapentaenoic Acid Metabolism on RAW 264.7 Macrophage Proliferation

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Prostaglandins (PGs) and leukotrienes (LTs) derived from arachidonic acid (AA) are potent mediators of inflammation and cell proliferation. Dietary intake of eicosapentaenoic acid (EPA) appears beneficial to both inflammatory processes and cell proliferation. However, there is no clear mechanism explaining these effects. In this study, we investigated the effect of EPA on the AA incorporation in phospholipid membranes, on AA release and metabolism, and consequently, on PG synthesis. Our results showed not only that [^{3}H]AA and [^{14}C]EPA were similar incorporated into RAW 264.7 macrophage membranes, but also that the redistribution pattern between phospholipids was alike. [^{3}H]AA or [^{14}C]EPA release was induced by fetal bovine serum (FBS) in a similar fashion with AA metabolizing 3-fold more than EPA. In this way, we observed that AA could be metabolized by cyclooxygenase (COX)-1, COX-2 and 5-lipoxygenase (5-LOX) whereas EPA was metabolized by COX-2 and 5-LOX pathways. Moreover, both fatty acids were able to induce COX-2 expression. When we incubated [^{3}H]AA labeled cells with exogenous EPA, we observed that EPA did not modify FBS-induced [^{3}H]AA release but that the presence of EPA decreased [^{3}H]AA metabolism and therefore PGE_2 synthesis. Moreover, we studied the effect of AA and EPA metabolites on macrophage proliferation. Our results showed that PGE_3 stimulated cell growth with a potency similar to that of PGE_2, whereas LTB_5 was less effective than LTB_4. These data suggest that the effects of EPA on cell growth might be attributable, at least in part, to the marked decrease of eicosanoid release. J. Cell. Physiol. 208: 428–434, 2006. © 2006 Wiley-Liss, Inc.

Arachidonic acid (AA), a common omega-6 (ϖ-6) polyunsaturated fatty acid (PUFA), is found esterified at the sn-2 position of membrane phospholipids. AA mobilization occurs through the activation of phospholipases, mainly phospholipases A_2 (PLA_2). When AA is released, it is oxidized by cyclooxygenases (COXs) to produce prostaglandin (PG)H_2, the common precursor of all prostanoids. Two COX isoforms have been described. COX-1 is constitutively expressed in nearly all cells whereas COX-2 is induced in many cells by a wide range of pro-inflammatory or mitogenic agents (Meade et al., 1993; Herschman, 1994). On the other hand, AA is also metabolized by lipoxygenases (LOXs) producing hydroxyeicosatetraenoic acids, leukotrienes (LTs) and lipoxins (Yamamoto et al., 1988). This large family of bioactive lipids modulates diverse physiological and pathological responses like cell growth and wound healing (Sanchez and Moreno, 2001; Sanchez and Moreno, 2002a; Moreno, 2003a).

The mitogenic effects derived from AA release and COX-2 induction are linked primarily to the excessive synthesis of PGE_2. Interestingly, AA and PGE_2 have been shown to amplify PGE_2 production by inducing COX-2 expression (Bagga et al., 2003; Yang et al., 2004). Thus, AA- and PGs-dependent amplification of COX-2 are hypothesized to be an important part of sustained proliferative conditions, and may explain the overexpression of COX-2 in growth cells and tumors (Moreno, 2005). In a similar way, AA metabolites produced by LOX pathway also exert profound effects on cell growth and cancer (Honn et al., 1994; Chen et al., 1994; Tang et al., 1996).

The availability of free AA and its subsequent metabolism are rate-limiting steps in the synthesis of PGs/LTs. Thus, the modulation of AA availability/metabolism could serve as a means of altering PGs/LTs synthesis and prevent/inhibit the effects associated with 2-series PGs/4-series LTs.

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are ϖ-3 PUFA found mainly in fish oils. EPA and DHA compete with AA at the level of incorporation into cell membrane phospholipids (Lands et al., 1992). In contrast to DHA, however, EPA can also function as a substrate for COX-2 and result in the synthesis of 3-series PG (Needleman et al., 1979). In a similar manner, 5-LOX is capable of metabolizing EPA to LTB_5 (Laegreid et al., 1988; Kobayashi et al., 1995).

Dietary supplements of fish oils rich in ϖ-3 PUFA are used as preventive measures against cancer (Connor, 2000). In addition to reducing the concentrations of 2-series PG, fish oil ingestion also results in increased 3-series PG in vivo (Fischer et al., 1988). Although similar in structure and stability, the 2-series of PG is considered to be more mitogenic and pro-inflammatory compared with the 3-series. However, only a few contradictory studies directly comparing the effects of 2-series versus 3-series PG or 4-series versus 5-series LT on cell proliferation have been performed. Thus, Mckenzie et al. (1994) reported that PGE_2 and PGE_3 stimulated murine mammary epithelial growth whereas Bagga et al. (2003) showed recently that PGE_3, unlike PGE_2, is not mitogenic in 3T3 fibroblast. On the other hand, Bortuzzo et al. (1996) reported that LTB_4 stimulated HT-29 proliferation while LTB_5 did not. Eicosanoids do not behave as mitogens or co-mitogens in all cell types, and in fact diminish the proliferation of lymphocytes. Thus, Shapiro et al. (1993) reported that PGE_3 and LTB_5 are as potent as PGE_2 and

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LTB_4 in inhibiting lymphocyte proliferation when added in vitro.

Taking into account the above-mentioned findings, we sought to investigate the effect of fetal bovine serum (FBS) growth factors on AA and EPA release, the effects of AA/EPA on COX-2 expression, and the subsequent eicosanoid synthesis. Finally, we studied the effects of PGE_2/PGE_3 and LTB_4/LTB_5 on cell growth using RAW 264.7 macrophage cultures.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle medium (DMEM), FBS, penicillin G, streptomycin and trypsin/EDTA were from Bio Whittaker Europe (Verviers, Belgium). [5,6,8,9,11,12,14,15 ^{3}H]AA (60–100 Ci/mmol), 5,8,11,14,17 [1-^{14}C]EPA (40–60 mCi/mmol) and [methyl-^{3}H]thymidine (20 Ci/mmol) were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). Aprotinin, AA, EPA, leupeptin, diethyldithiocarbamic acid, phenylmethylsulfonyl fluoride (PMSF), sodium fluoride, sodium orthovanadate (Na_3VO_4), Igepal CA-630, dithiothreitol (DTT), ethidium bromide, acridine orange, LTB_4, LTB_5, ketoprofen and zileuton were acquired from Sigma Chemical (St. Louis, MO). PGE_2, PGE_3, valeryl salicylate and NS-398 were from Cayman Chemical Co. (Ann Arbor, MI). Bromoenol lactone (BEL) and arachidonyl trifluoromethylketone ($AACOCF_3$) were acquired from Alexis Corp. (Lausen, Switzerland). Rabbit polyclonal antibody against COX-2, was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Culture of RAW 264.7 macrophages

Murine RAW 264.7 macrophages (TIB-71) from American Type Culture Collection (Manassas, VA) were grown in DMEM containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml), in a 95% air-5% CO_2-humidified atmosphere at 37°C. Cells were scraped off and passed to tissue culture 100 or 60 mm dishes (Costar, Cambridge, MA) for experimental purpose. Ethidium bromide/acridine orange staining was used to assess cell viability.

[^{3}H]AA/[^{14}C]EPA labeling and release from phospholipids

This experiment examined the distribution of [^{3}H]AA in phospholipids after labeling. Macrophages were labeled with [^{3}H]AA or [^{14}C]EPA. After cell stimulation, the supernatant medium was removed and cell lipids were then extracted with ethyl acetate (5 ml) acidified with 1% formic acid. The organic phase was evaporated under a nitrogen stream and resuspended in chloroform. An aliquot was used to measure the total amount of label in the lipids, a portion of the remainder separated by thin layer chromatography in a solvent system (chloroform:methanol:glacial acetic acid:water, 100:30:35:3, v:v:v:v) to determine the percentage of total [^{3}H]AA or [^{14}C]EPA in each phospholipid, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylcholine (PC). The percentage of total radioactivity in each lipid was determined by liquid scintillation spectroscopy. The amount of [^{3}H]AA/[^{14}C]EPA released from each phospholipid was then calculated as the difference between control and stimulated dishes. The percentage of release was calculated as the amount released from each phospholipid divided by the amount present in the unstimulated control.

Incorporation and release of [^{3}H]AA or [^{14}C]EPA

After cell replication and FBS starvation (6 h), the medium was removed and replaced by 0.5 ml DMEM containing 0.1% fatty acid-free BSA and 0.01 μCi [^{3}H]AA or 1 μCi [^{14}C]EPA (1.6–16 nM labeled PUFA approximately) for 24 h. [^{3}H]AA incorporation was performed in preconfluent cultures. Cells were then washed three times with medium containing 0.5% BSA-containing medium to remove unincorporated radioactivity. After a study period, the medium was removed to determine the amount of radioactivity released and the cells overlaid with 1% Triton X-100, and then scraped off the dishes. The amount of [^{3}H]AA/[^{14}C]EPA released into the medium was expressed as a percentage of cell-incorporated [^{3}H]AA/[^{14}C]EPA, which was determined in solubilized cells. The background released from non-stimulated cells ($7 \pm 0.5\%$ of ^{3}H incorporated and $6.5 \pm 1.1\%$ of ^{14}C incorporated) was subtracted from all data.

Measurement of the relative amounts of [^{3}H]AA or [^{14}C]EPA metabolized

To determine the amount of [^{3}H]AA or [^{14}C]EPA metabolized, RAW 264.7 cultures in presence of 10% FBS were incubated with [^{3}H]AA or [^{14}C]EPA for 1 h. Subsequently, 0.5 ml of cellular medium was extracted with ethyl acetate (5 ml) acidified with 1% formic acid. The organic phase was evaporated under a nitrogen stream, resuspended in 100 μl chloroform, and purified by ascending chromatography on silica thin-layer plates using a solvent mixture of diethyl ether:hexane:acetic acid (60:40:1, v:v:v) which resolves AA or EPA from different oxidized metabolites. Thin-layer chromatography plates were exposed to rhodamine B (0.2%) and activated at 100°C. [^{3}H]AA/[^{14}C]EPA and [^{3}H]AA/[^{14}C]EPA metabolites fluoresce under UV light (370 nm). Finally, the radioactive spots were scraped and quantified by scintillation counting.

Protein determination

Total protein was measured by the Bradford (1976) method, using the Bio-Rad protein assay (Hercules, CA) with BSA as standard.

Western Blot analysis

Cells were washed twice with ice-cold PBS, scraped off into PBS containing 2 mM sodium EDTA and pelleted. These pellets were sonicated in PBS containing 4 mM sodium EDTA, 500 μg/ml aprotinin, 500 μg/ml leupeptin, 500 μg/ml PMSF, and 400 μg/ml diethyldithiocarbamic acid and resuspended in lysis buffer containing 200 mM Tris-HCl, 200 mM NaCl, 2% Igepal CA-630, 400 μM NaF, 200 μM DTT.

Immunoblot analysis for COX-2 was performed as follows: 20 μg of protein from cell total lysates was separated by a 7.5% SDS–PAGE (Laemmli, 1970) and blotted for 1 h at a constant voltage of 100 V onto a nitro-cellulose membrane (Trans-Blot, 0.4 μm pore size, Bio-Rad) using a MiniProtean II system (Bio-Rad). A prestained SDS–PAGE protein standard (Bio-Rad) was used as a molecular-weight marker to verify transfer efficiency. Membranes were blocked with 5% non-fat milk powder in PBS-0.1% Tween-20 for 1 h. A rabbit polyclonal antiserum directed against COX-2 was applied at a dilution of 1:2,000 for 1 h. The specificity of antibody used was previously established (Martinez et al., 1997). The blots were washed several times in PBS-0.1% Tween-20 and incubated with a goat anti-rabbit antibody in a 1:2,000 dilution for 1 h. Antibody binding was visualized by an enhanced chemical luminescence technique using Supersignal West Dura Extended Duration Substrate Pierce (Rockford, IL) and Kodak Bio-Max light-2 film (Rochester, NY).

Cell growth assay

The influence of the treatments were assessed on macrophage plates at 25×10^3 cells/well in 24-well plates, and cultured for 24 h in DMEM supplemented with 10% FBS. After 24 h serum starved cells were incubated for 48 h in 10% FBS medium in the presence of different compounds. Finally, cells were scraped off with Versene-EDTA and counted under a microscope using ethidium bromide/acridine orange staining in order to count cells and to assess viability.

Analysis of DNA synthesis

DNA synthesis was measured using a [^{3}H]thymidine incorporation assay. This involved culturing macrophages in 24-well plates in DMEM with 10% FBS at a density of 25×10^3 cells/well. After 24 h serum starved cells were incubated for 48 h with the treatments and [^{3}H]thymidine (1 μCi/well) was

added the last 24 h. [^{3}H]thymidine-containing media were aspirated, cells were overlaid with 1% Triton X-100, and then scraped off the dishes. Finally, radioactivity present in the cell fraction was measured by scintillation counting using a Packard Tri-Carb 1500 counter.

Measurement of PGE_2

The influence of the treatments was assessed on macrophage plates at 25×10^3 cells/well in 24-well plates, and cultured for 24 h in DMEM supplemented with 10% FBS. After 24 h serum starved cells were incubated for 48 h in 10% FBS medium in the presence of different compounds. An aliquot of culture supernatant medium was analyzed by an enzyme immunoassay kit (Cayman Chemical Co.) following the manufacturer's protocol.

Statistical analysis

Results are expressed as mean ± standard error of the mean. Differences between non-treated and treated cells were tested by using Student's *t*-test followed by the least significant difference test when appropriate.

RESULTS

FBS-induced release and metabolization of AA and EPA

Our results showed that [^{3}H]AA and [^{14}C]EPA were similar incorporated into macrophage phospholipids, 38.2% and 35.3%, respectively. Furthermore, we found that [^{3}H]AA or [^{14}C]EPA was redistributed between phospholipids after a 24 h labeling period, and the pattern of this redistribution was similar after exogenous adding of AA or EPA (Table 1). Thus, AA or EPA was incorporated mainly into PE and PC phospholipids. When labeled cultures were challenged with FBS, the total [^{3}H]AA or [^{14}C]EPA released from the cells closely paralleled each other (Fig. 1). Furthermore, [^{3}H]AA and [^{14}C]EPA were mainly released from PC and PE phospholipids (Table 1). Moreover, we observed that FBS-stimulated [^{3}H]AA and [^{14}C]EPA release were sensitive to both $AACOCF_3$ and BEL treatments (Fig. 1), molecules that inhibit high-molecular phospholipases A_2 (Ackermann et al., 1995) and calcium-independent phospholipase A_2 (Riendeau et al., 1994), respectively.

We also determined the percentage of AA/EPA metabolized after fatty acids were mobilized from membranes. Our results showed that AA was metabolized 3-fold more than EPA (Fig. 2). Furthermore, we observed that valeryl salicylate, a COX-1 inhibitor (Johnson et al., 1995), NS-398, a specific COX-2 inhibitor (Barnett et al., 1994) and zileuton a 5-LOX inhibitor (Carter et al., 1991) reduced AA metabolism whereas EPA metabolism was only significantly inhibited by NS-398 and zileuton (Fig. 2). Our data suggested that AA might be metabolized by COX-1, COX-2 and 5-LOX whereas EPA is mainly metabolized by COX-2 and 5-LOX pathways.

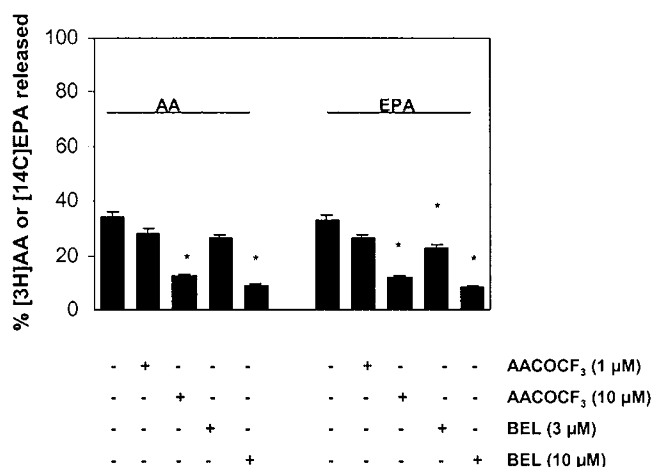

Fig. 1. [^{3}H]AA or [^{14}C]EPA released from RAW 264.7 macrophages by FBS. Cells were labeled with [^{3}H]AA or [^{14}C]EPA. Cells were incubated with FBS (10%) and treated with ($AACOCF_3$ 1–10 µM; BEL 3–10 µM) for 4 h. Finally, the percentage of [^{3}H]AA or [^{14}C]EPA released was calculated. Each bar represents the mean ± SEM of three determinations made in duplicate. *$P < 0.05$ respect to non-treated cells.

AA and EPA as well as PGE_2 and PGE_3 induced COX-2 overexpression

AA release is involved in the overexpression of COX-2 in inflammatory conditions (Sanchez and Moreno, 1999) and could also be involved in COX-2 overexpression in tumors (for review, see Moreno, 2005). Our results demonstrated that AA and EPA could be implicated in the control of COX-2 expression in RAW 264.7 macrophages. Thus, exogenous addition of AA or EPA to RAW 264.7 cultures induced an increase in cellular COX-2 levels (Fig. 3). However, EPA was less effective compared with AA. PGE_2-dependent amplification of COX-2 is also hypothesized to be an important part of proliferative and inflammatory processes (Murakami et al., 1997; Pang and Hoult, 1997), and may be involved in the overexpression of COX-2 (Fosslien, 2000). Our results have also shown that both PGE_2 and PGE_3 were able to induce the enhancement of COX-2 levels in RAW 264.7 cultures (Fig. 3).

EPA affects AA incorporation to membrane phospholipids as well as FBS-induced AA metabolism and PGE_2 synthesis

PG synthesis in macrophages depends on both the endogenous AA released from membrane phospholipids stores as well as the synthesis and activity of the COX-2

TABLE 1. Distribution of [^{3}H]AA or [^{14}C]EPA incorporated to cells and pattern of release in response to FBS stimulation

	PC	PE	PI	PS
Percentage of radioactivity incorporated (%)				
[^{3}H]AA	35.2 ± 2.3	46.3 ± 2.6	9.5 ± 1.1	4.8 ± 0.5
[^{14}C]EPA	36.7 ± 1.2	44.7 ± 1.8	8.9 ± 0.9	3.5 ± 0.3
Percentage of radioactivity released (%)				
[^{3}H]AA	42.4 ± 2.3	40.2 ± 2.1	28.3 ± 1.6	18.7 ± 1.1
[^{14}C]EPA	40.1 ± 1.7	39.1 ± 1.5	27.2 ± 1.5	19.2 ± 1.3

Cells were pulse-labeled with 0.01 µCi of [^{3}H]AA (1.6 nM) or 1 µCi of [^{14}C]EPA (16 nM) for 24 h, followed by incubation in unlabeled growth medium for 3 h. The percentage distribution of [^{3}H]AA or [^{14}C]EPA present in each phospholipid was determined after extraction of cell lipid and separation of lipids by thin-layer chromatography. The distribution of label in each phospholipid (PC, PE, PI, PS) as a percentage of total cell lipid is shown. The medium was then removed and replaced with DMEM or DMEM containing 10% FBS for 3 h. The percentage of [^{3}H]AA or [^{14}C]EPA released from each phospholipid and from all phospholipids (total) was also determined. (PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine). Data are the mean ± SEM of three experiments performed in duplicate.

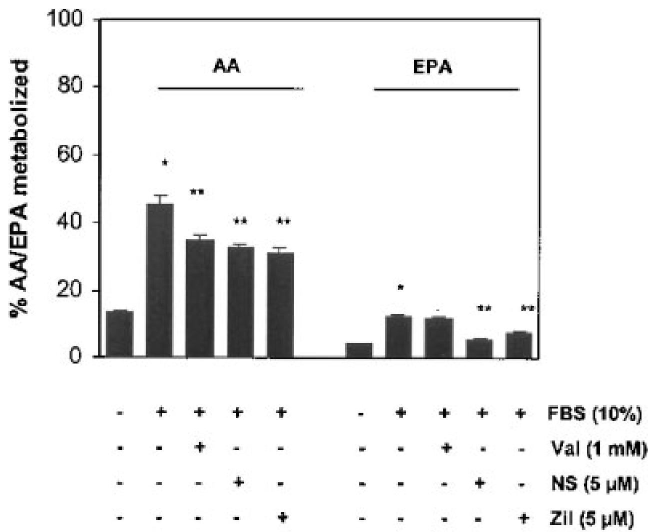

Fig. 2. Percentage of [^{3}H]AA or [^{14}C]EPA metabolized in RAW 264.7 macrophage cultures stimulated with FBS. RAW 264.7 macrophage were cultured with FBS 10% and incubated with [^{3}H]AA or [^{14}C]EPA for 1 h in presence of valeryl salicylate (Val), NS-398 (NS) or zileuton (Zil). The medium was then removed and [^{3}H]AA/[^{14}C]EPA metabolism was measured by thin-layer chromatography and liquid scintillation spectroscopy. Measurements were performed in duplicate and expressed as the mean ± SEM of three experiments. *$P < 0.05$ respect to cell cultured in absence of FBS. **$P < 0.05$ respect to non-treated cultures.

TABLE 2. Incorporation of [^{3}H]AA to cellular phospholipids in presence of EPA

	Percentage of [^{3}H]AA incorporated
EPA 0	38.5 ± 1.1
EPA 2.5	36.1 ± 0.8
EPA 5	30.2 ± 0.6*
EPA 10	26.7 ± 0.5*
EPA 15	23.4 ± 0.3*

Cells were pulse-labeled with 0.01 μCi of [^{3}H]AA (1.6 nM) in presence of EPA (0–15 μM) for 24 h. Finally, the percentage of [^{3}H]AA incorporated to phospholipids of RAW 264.7 macrophages was measured. Data are the mean ± SEM of three experiments performed in duplicate.
*$P < 0.05$ respect to experiments in absence of EPA.

protein (Reddy and Herschman, 1994). To determine whether membrane composition of fatty acids can affect substrate availability and COX-2-dependent PG synthesis, we assayed [^{3}H]AA labeling in presence of EPA. Table 2 shows that EPA induced a concentration-dependent decrease of [^{3}H]AA incorporation into macrophage phospholipids. On the other hand, we incubated [^{3}H]AA-labeled macrophages with EPA, and observed that EPA was not able to modify FBS-induced [^{3}H]AA release (Fig. 4A), although the presence of EPA decreased FBS-induced [^{3}H]AA metabolism (Fig. 4B). Thus, EPA induced a concentration-dependent inhibition of [^{3}H]AA incorporation into phospholipids and decreased AA metabolize induced by FBS. Consequently, FBS-induced PGE_2 synthesis was also significantly impaired, as shown Figure 4C. EPA at the concentrations and time periods assayed did not modify morphological appearance of the cells or cell viability (data not shown). Thus, these above-mentioned effects of EPA did not result from cytotoxicity.

ϖ-6 and ϖ-3 PUFA metabolites are involved in the control of RAW 264.7 macrophage proliferation

Our results have shown that FBS induces cell growth and [^{3}H]AA/[^{14}C]EPA release and metabolism in RAW 264.7 cultures. We also reported that macrophage growth was inhibited by COX inhibitors such as ketoprofen (Carabaza et al., 1996) and 5-LOX inhibitors such as zileuton (Fig. 5). Furthermore, we observed that PGE_2 and PGE_3 were able to revert cell growth inhibition induced by ketoprofen. In this way, we also observed that LTB_4 and LTB_5 reverted the effect of zileuton on RAW 264.7 growth (Fig. 5A), although LTB_4 was more effective than LTB_5. Similar results were obtained when we studied the effect of PGE_2/PGE_3 or LTB_4/LTB_5 on the inhibition of [^{3}H]thymidine incorporation induced by ketoprofen or zileuton in RAW 264.7 cultures. Thus, ketoprofen and zileuton inhibited the [^{3}H]thymidine uptake induced by FBS whereas ϖ-3 and ϖ-6 eicosanoids were able to revert these effects (Fig. 5B). The above data suggest than ϖ-6 and ϖ-3 PUFA metabolites are involved in the signal transduction pathways induced by FBS and that PGE_2/PGE_3 or LTB_4/LTB_5 can be regarded as co-mitogenic factors. To determine the mitogenic effect of AA and EPA metabolites on RAW 264.7 macrophages, we performed cell growth assays in the absence of growth factors. Our results have shown that ϖ-6, as well as ϖ-3 PGs and LTs, increase [^{3}H]thymidine uptake (Fig. 6B). Since DNA synthesis can occur without cell division, the effects of these eicosanoids on RAW 264.7 growth were also determined. PGs and LTs also enhanced cell proliferation (Fig. 6A). Thus, PGE_2 and PGE_3 (1–10 nM) increased cell numbers by 100% while LTB_4

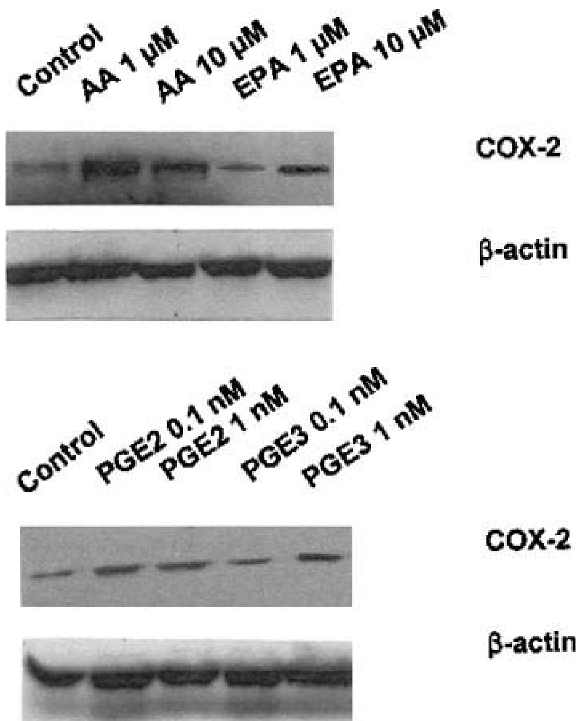

Fig. 3. COX-2 levels in RAW 264.7 macrophage cultures incubated with PUFA or PGs. Cells were incubated for 3 h with AA (1–10 μM), EPA (1–10 μM), PGE_2 (0.1–1 nM), or PGE_3 (0.1–1 nM) and scraped off to determine COX-2 levels. These results are representative of three blots.

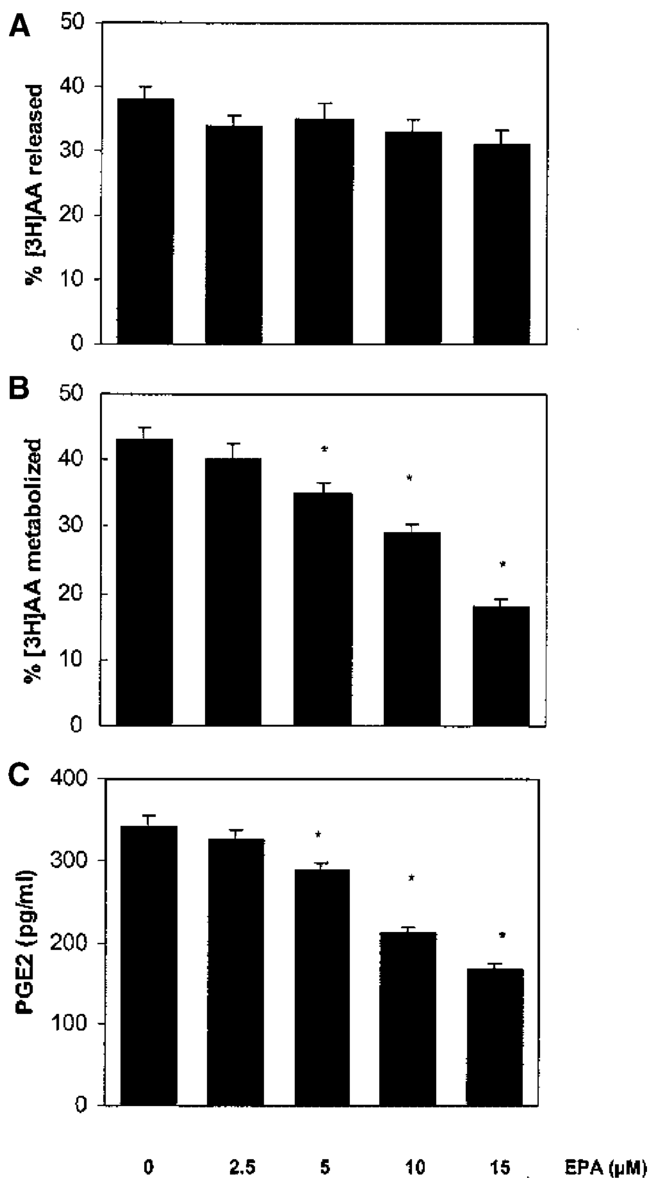

Fig. 4. Effect of EPA on [^{3}H]AA release, [^{3}H]AA metabolism and PGE_2 synthesis. **A**: RAW 264.7 macrophages labeled with [^{3}H]AA were incubated with FBS 10% in presence of EPA and the percentage of [^{3}H]AA release was determined. **B**: RAW 264.7 cells were cultured in FBS 10% and incubated with [^{3}H]AA in presence or absence of EPA for 1 h. Finally, the percentage of [^{3}H]AA metabolized was measured. **C**: RAW 264.7 cultures were stimulated with FBS 10% for 1 h in presence or absence of EPA and PGE_2 levels were assayed. Each bar represents the mean ± SEM of three determinations performed in duplicate. *$P < 0.05$ respect to cell cultures non-treated with EPA.

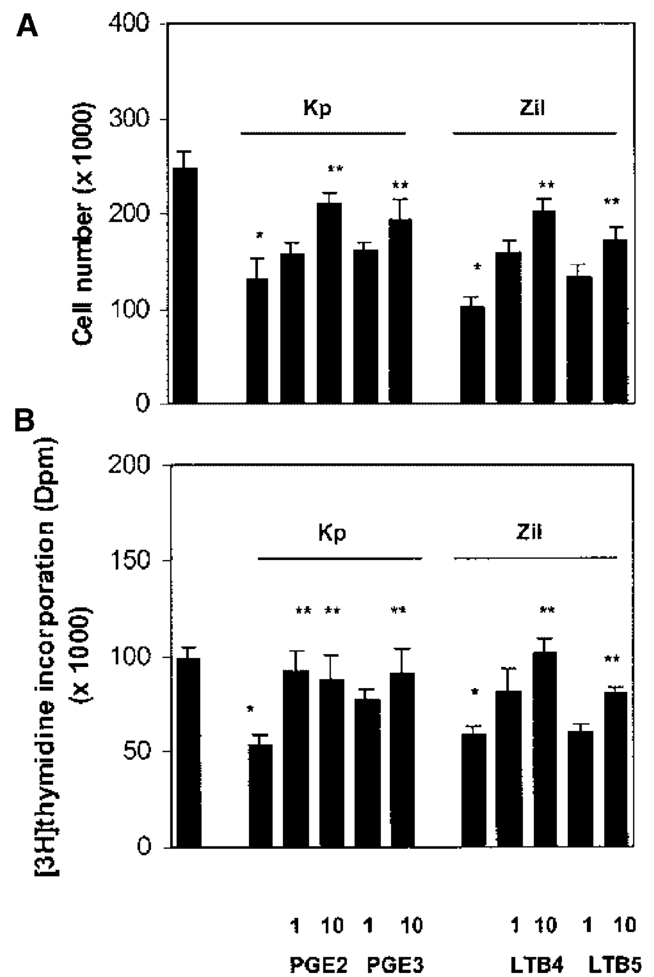

Fig. 5. Effect of COX and LOX inhibitors on cell growth and DNA synthesis in RAW 264.7 cultures. Cell growth (**A**) and DNA synthesis (**B**) induced by FBS (10%) were measured in presence of ketoprofen (Kp, 5 μM) or zileuton (Zil, 5 μM) with or without exogenous addition of PGE_2 (1–10 nM)/PGE_3 (1–10 nM) or LTB_4 (1–10 nM)/LTB_5 (1–10 nM), respectively. Results are mean ± SEM of three experiments performed in duplicate. *$P < 0.05$ respect to RAW 264.7 cultured with FBS 10%, **$P < 0.05$ respect to cell cultures incubated with Kp or Zil.

(10 or 100 nM) increased cell number by 50% and 75%, respectively. However, we observed that LTB_5 only induced cell growth at 100 nM. In this way, PGE_2, PGE_3, and LTB_4 induced [^{3}H]thymidine incorporation in absence of another growth factor. LTB_5 had a minor effect but LTB_5 100 nM was able to induce DNA synthesis in RAW 264.7 cultures (Fig. 6B).

DISCUSSION

Although dietary intake of fat and its association with the development of cancer remains surrounded by controversies, defining what exactly contributes to the risk of cancer is still an open question. However, nutritional, biochemical, and other experimental studies performed over the last 25 years have provided convincing evidence that dietary ϖ-3 PUFA play an important preventive role in tumorigenesis and on the growth of established tumors (Welsch, 1992; Klurfeld, 1995). Thus, epidemiological studies have shown an inverse relationship between blood levels of ϖ-3 fatty acids derived from fish oils and the risk for prostate cancer (Terry et al., 2001), colorectal cancer (Caygill and Hill, 1995), lung cancer (Takegaki et al., 2001), and melanoma (Veirod et al., 1997). Understanding the mechanism by which PUFA are involved in cell growth is a critical issue in cancer and lipid homeostasis. However, molecular mechanisms for the antiproliferative effects of EPA have not been fully elucidated. Dommels et al. (2003) suggested that the number of double bonds in the fatty acid carbon atom chains is more important for the anti-proliferative and cytotoxic effects of ϖ-3 fatty acids. Thus, these authors proposed that non-enzymatic lipid peroxidation products as well as COX-derived peroxidation products were probably responsible for the growth-inhibitory effects of

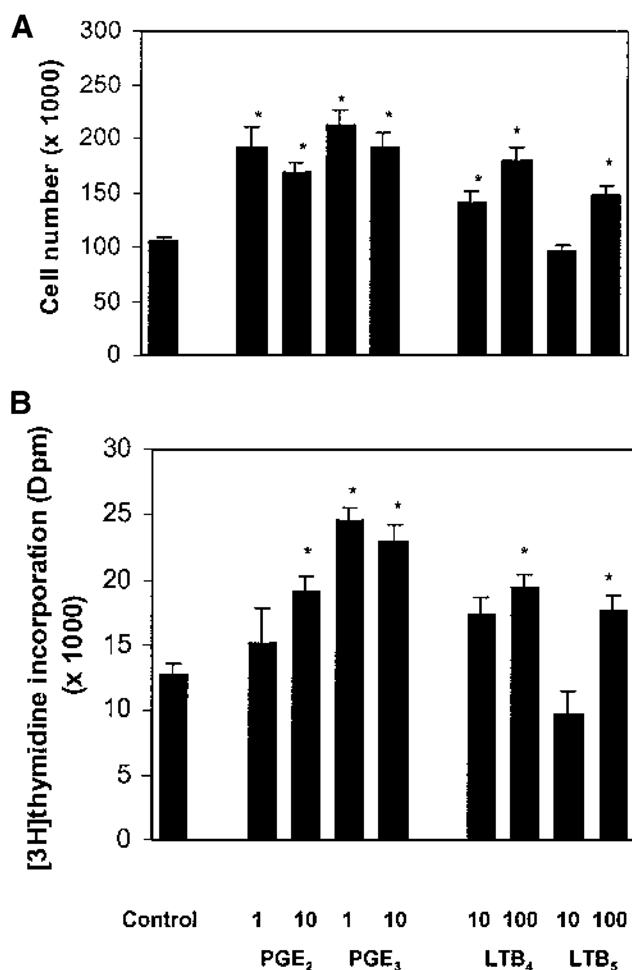

Fig. 6. Effect of exogenous ϖ-3 and ϖ-6 PGs and LTs on cell proliferation. The effect of exogenous PGE_2/PGE_3 (1–10 nM) or LTB_4/LTB_5 (10–100 nM) on cell proliferation (**A**) and [^{3}H]thymidine incorporation (**B**) were performed. Cells were incubated with eicosanoids for 48 h. [^{3}H]thymidine was added to the cells for the last 24 h. Finally, cells were scrapped off, counted and radioactivity in cell lysates was determined using a scintillation analyzer. Data are the mean ± SEM of three experiments performed in duplicate. $^*P < 0.05$ respect cell cultures non-incubated with eicosanoids.

PUFA-rich fatty acids such as EPA. However, these effects were only appreciable in presence of growth factors with fatty acid concentration (20–150 μM) higher than PUFA concentrations used in our study.

On the other hand, numerous studies have suggested that EPA effect on cell growth is associated with its ability to inhibit the synthesis of 2-series PGs. In addition to reducing the concentrations of 2-series PG, EPA ingestion also results in a 10- to 50-fold increase in 3-series PG (Fischer and Weber, 1984). The 2-series of PG is considered to be more mitogenic and proinflammatory compared with the 3-series of PG (Bagga et al., 2003). Thus, Bortuzzo et al. (1996) also reported that 5-series of LTs derived from EPA did not stimulate cell proliferation whereas LTB_4 induced HT-29 proliferation. Recently, Yang et al. (2004) reported that PGE_3 has anti-proliferative effects on human lung cancer A549 cells. Thus, the effect of EPA on cell growth could be a consequence of the impairment of pro-proliferative eicosanoid synthesis and the release of non pro-proliferative or anti-proliferative eicosanoids. However, our results have shown that both AA (PGE_2 and LTB_4) and EPA metabolites (PGE_3 and LTB_5) are involved in the FBS-induced cell signaling. The present work provides the first evidence that AA and EPA metabolites synthesized by COX or 5-LOX pathways are mitogenic in RAW 264.7 macrophage, cells capable of producing PGs and LTs (Moreno, 2003b). Thus, our results showed that PGE_3 stimulated macrophage proliferation with potency similar to that of PGE_2 whereas LTB_5 was less effective than LTB_4. This minor effect of LTB_5 could be due to minor affinity for LTB_4 receptors (Seya et al., 1988; Andazola et al., 1992). These results indicate that the inhibitory effect of EPA on cell growth (Bartsch et al., 1999) could not be attributable, at least exclusively, to the effects of PGE_3/LTB_5. Here, we proposed other mechanism to explain the effect of ϖ-3 PUFA on cell proliferation.

Our results show that FBS is capable of releasing AA as well as EPA from membrane phospholipids. Previously, we observed that AA release induced by a BEL sensitive PLA_2 was involved in the control of cell growth in 3T6 fibroblast (Sanchez and Moreno, 2001) and Caco-2 (Sanchez and Moreno, 2002b) cultures. An important observation made in this study was that a calcium-independent PLA_2 is also involved in AA and EPA release induced by FBS in RAW 264.7 macrophages. EPA can also appreciably interfere with AA release since both PUFA are released by a calcium independent PLA_2 to the same extent. Furthermore, FBS induced AA and EPA metabolism. Interestingly, COX-1, COX-2, and 5-LOX inhibition affected [^{3}H]AA metabolism whereas only COX-2 and 5-LOX inhibition modified [^{14}C]EPA metabolism. This could be because AA is the preferred substrate for COX-2 and 5-LOX as well as the exclusive substrate for COX-1. On the other hand, EPA and PGE_3, versus AA or PGE_2, induced minor COX-2 overexpression, an event that could also contribute to the impairment of EPA metabolism, and consequently, prostaglandin synthesis. All of these observations can explain why EPA was metabolized threefold less than AA. Furthermore, we observed that exogenous EPA addition decreases AA incorporation into macrophage phospholipids. Thus, considering the above, EPA incorporation to membrane phospholipids may be involved in the impairment of AA levels in membrane phospholipids, as we observed previously in animals fed fish oil, which would thereby affect ϖ-3/ϖ-6 ratios (Mitjavila et al., 1996). These events could explain the impairment of AA release and eicosanoid synthesis in resident peritoneal macrophages from rats fed fish oil (Moreno et al., 2001). Taken together, our data suggest that replacement of ϖ-6 PUFA (AA) with ϖ-3 PUFA (EPA) in membrane phospholipids can result in a decreased eicosanoid synthesis as cellular response to mitogenic stimuli. Considering that COX inhibition or 5-LOX inhibition induced the impairment of RAW 264.7 macrophage growth, our results suggest that the effects of EPA on cell growth might be attributable, at least in part, to the marked decrease of eicosanoid release as a consequence of the impairment of AA in phospholipid membranes, a PUFA that is more easily metabolized than EPA.

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4.4. Anexo 1

Role of Epoxyeicosatrienoic Acids on 3T6 Fibroblast Growth and Apoptosis.

(En preparación)

Se ha sugerido que la liberación del AA y su metabolización por las COXs están implicadas en la proliferación de los fibroblastos 3T6 inducida por suero, y que en este proceso pueden participar otras vías de metabolización del AA. Además, estudios previos demostraron que la reducción de la proliferación de los fibroblastos 3T6 con inhibidores de los CYPs puede ser revertida al añadir exógenamente HETEs, pero no el 11,12-EET. Por ello, nos planteamos estudiar el papel de los EETs/DHETEs sobre la proliferación de los fibroblastos 3T6 estimulados con PDGF. Nuestros resultados muestran que el 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE y 11,12-DHETE (0,1-1 μM) inhiben la proliferación y la síntesis de DNA en los fibroblastos 3T6 inducida con PDGF. Los metabolitos que inhibieron más la proliferación son el 11,12-EET, 14,15-EET y 11,12-DHETE. Además, este efecto sobre la proliferación se correlacionó con la inducción de la apoptosis. Así, observamos que los EETs y DHETEs (0,1-1 μM) en presencia de PDGF inducen la externalización de la fosfatidilserina (medida por unión a la anexina-V), la actividad caspasa y la fragmentación de DNA (cuantificada mediante la técnica TUNEL). El 11,12-EET, 14,15-EET y 11,12-DHETE fueron de nuevo los metabolitos que produjeron una mayor inducción de la apoptosis. También observamos que la activación de la caspasa-12 y la caspasa-3 están implicadas en estos procesos. Por lo tanto, los EETs y DHETEs tienen efectos anti-proliferativos y pro-apoptóticos sobre los fibroblastos 3T6 estimulados con PDGF.

Role of Epoxyeicosatrienoic Acids on 3T6 Fibroblast Growth and Apoptosis.

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Recent studies have suggested that arachidonic acid (AA) released and cyclooxygenase pathway are involved in serum-induced 3T6 fibroblast cycle progression and proliferation, but these results suggested that other AA cascade pathway might be also involved in these events. AA can be metabolized by the epoxygenase activity of the cytochrome P450 monooxygenases (CYP) producing epoxyeicosatrienoic acids (EETs). Finally, the cytosolic epoxide hydrolase catalyze the hydration of the EETs to dihydroxyeicosatetraenoic acids (DHETEs). We have studied the role of the EETs/DHETEs on 3T6 fibroblasts growth stimulated with PDGF. Our results show that PDGF stimulates 3T6 fibroblast proliferation and [^{3}H]thymidine incorporation, while addition of 5,6-EET, 8,9-EET, 11,12-EET or 14,15-EET (0,1-1 μM) inhibit these processes. Furthermore, 5,6-DHETE and 11,12-DHETE (0,1-1 μM) also inhibit cell proliferation and DNA synthesis. The metabolites that present more appreciable effect on proliferation are 11,12-EET, 14,15-EET and 11,12-DHETE. Interestingly, this growth inhibition were correlated with an induction of apoptosis. Thus, we observed that EETs or DHETEs (0,1-1 μM) in the presence of PDGF induce phophatidylserine externalization (measured by annexin V-binding), caspase activity and DNA fragmentation (quantified using a TUNEL assay). 11,12-EET, 14,15-EET and 11,12-DHETE are again the epoxygenase AA metabolites that induce more appreciable apoptosis. Our results show that caspase-12 and caspase-3 are involved in these events. Therefore, EETs and DHETEs have anti-proliferative and pro-apoptotic effects on PDGF-stimulated 3T6 fibroblasts.

INTRODUCTION

Free arachidonic acid (AA) can be metabolized by three pathways: the cyclooxygenases (COXs), which produces prostaglandins (PGs) and thromboxanes; the lipoxygenases (LOXs), which forms leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins; and the cytochrome P450 monooxygenases (CYPs) (Imig, 2000). The CYP proteins metabolized AA by one or more of the following reactions: *bis*-allylic oxidation (lipoxygenase-like reaction) to generate 5-, 8-, 9-, 11-, 12- and 15-HETEs; ω/ω-1 hydroxylation affords 16-, 17-, 18-, 19- and 20-HETEs; or olefin epoxidation producing 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs). Finally, the cytosolic epoxide hydrolases catalyze a rapid enzymatic hydration of the EETs to dihydroxyeicosatetraenoic acids (DHETEs) (Natarajan and Reddy, 2003).

EET synthesis has been identified in several tissues as human kidney cortex (Karara et al., 1990), rat liver (Karara et al., 1991) and bovine coronary endothelial cells (Rosolowsky and Campbell, 1996). These cells can release CYP-AA metabolites that influence surrounding cells, modulating their effects. EETs have been also detected in rat plasma lipoproteins (Karara et al., 1992) and then, they may be supplied to different tissues by the circulation. The presence of EETs in fibroblasts has not been quantified, but Fang et al. (2000) demonstrated that human skin fibroblasts produces DHETEs when they are exposed to a relatively high concentration of EETs. Furthermore, different cell types assessed as astrocytes (Shrivachar et al., 1995) or porcine aortic smooth muscle cells (Fang et al., 1995) when they were exposed to EETs in the media were able to take up it rapidly.

The mechanisms by which EETs initiate their actions are not clear. Some authors suggested the presence of a plasma membrane EET receptor on human U937 monocytes (Wong et al., 1997) and guinea pig mononuclear cells (Wong et al., 2000), but there is not yet a receptor purified and cloned. On the other hand, EETs can bind to cytosolic fatty acid protein binding (Widstrom et al., 2001), then they could be transported to specific targets and finally modulate gene expression.

CYP-derived EETs present numerous physiological effects (Spector et al., 2004) including a role in the regulation of cell proliferation and differentiation, among others. However, current studies about this role of EETs on cell growth are controversial. Thus, Chen et al. (1998) showed that all EETs stimulate $[^3H]$thymidine incorporation on the renal epithelial cell line LLCPKcl4, being

14,15-EET the most potent, and their effect are mediated by activation of Src kinase and tyrosine kinase phosphorilation cascade, whereas 14,15 DHETE was without effect (Chen et al. 1998). Recently Wang et al. (2005) and Pozzi et al. (2005) have demonstrated that 8,9-EET, 11,12-EET and 14,15-EET have angiogenic effect increasing proliferation and thymidine incorporation in endothelial cells. Similar effects were reported by Jiang et al. (2005) using human carcinoma cell lines. Moreover, Pozzi et al. (2005) proposed that the proliferative action of 8,9- and 11,12-EET are mediated by p38 mitogen-activated protein kinase (MAPK), while activation of phosphatidylinositol 3-kinase (PI3K) is necessary for 5,6-EET and 14,15-EET effect. However, the derived metabolites DHETEs did not present this effect. Concordingly, Potente et al. (2002, 2003) suggested that 11,12-EET generated by CYP2C9 induce human endothelial cell proliferation through MAPK and PI3K/Akt pathways. Besides mitogenic effect, EETs have also been described as survival factors. Thus, 14,15-EET inhibited apoptosis induced by serum withdrawal, H_2O_2, etoposide or excess free AA on renal epithelial cells, and this anti-apoptotic effect was proposed by activation of PI3K pathway (Chen et al., 2001). Recently, Jiang et al. (2005) showed that EETs protect carcinoma cells from apoptosis induced by tumor necrosis factor-α, because they reduced annexin-V staining, increased the levels of antiapoptotic proteins Bcl-2 and Bcl-xL and finally decreased levels of the proapoptotic protein Bax. All together suggest that EETs have pro-proliferative and antiapoptotic effects. However, Davis et al. (2002) demonstrated that EETs reduce platelet-derived growth factor (PDGF)-induced [^{3}H]thymidine incorporation in vascular smooth muscle cells. Therefore, it is not clear the action of EETs on cell growth and survival and the present study aim to clarify their role in the control of PDGF-induced 3T6 fibroblast proliferation. Thus, we have found that EETs and DHETEs reduce 3T6 fibroblast growth and [^{3}H]thymidine incorporation induced by PDGF. Furthermore, this effect is associated with an induction of apoptosis assessed by annexin-V staining, caspase activation and DNA fragmentation.

MATERIALS AND METHODS

Materials

RPMI 1640 media, FBS, penicillin, streptomycin and trypsin-EDTA were purchased from BioWhittaker Europe (Verviers, Belgium). PDGF, propidium iodide (PI), Triton X-100, ribonuclease A from bovine pancreas, acridine orange and ethidium bromide were provided by Sigma Chemical Co. (St. Louis, MO, USA). The metabolites 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET, 5,6-DHETE and 11,12-DHETE were supplied by Cayman Chemical Co. (Ann Arbor, MI, USA). [Methyl-^{3}H]thymidine (20 Ci/mmol) was from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). All chemicals were of the highest quality available commercially.

Cell culture

Murine 3T6 fibroblasts (ATCC CCL96) were cultured in RPMI 1640 containing 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were harvested with trypsin/EDTA and seeded in 24-well plates (tissue-culture cluster 24; Costar Cambridge, MA, USA) or in tissue-culture 60 mm-dishes (Costar) for experimental purposes. Cell cultures were maintained in a temperature- and humidity-controlled incubator at 37º with 95% air-5% CO_2 for 24 h.

Cell growth assay

The effect of the treatments was assessed on 3T6 fibroblasts plates at 20 x 10^3 cells/well in 24-well plates, cultured for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h serum starvation cells were incubated for 48 h in PDGF 100 ng/mL medium in the presence of EETs or DHETEs. Finally, the medium was taken to determine floating cells and attached cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to assess viability (Parks et al., 1979).

Analysis of DNA synthesis

DNA synthesis was measured by a [^{3}H]thymidine incorporation assay, which involved culturing 3T6 fibroblasts in 24-well plates in RPMI 1640 with 10% FBS at a density of 20 x 10^3 cells/well. After 24 h serum starvation cells were incubated for 48 h with PDGF 100 ng/mL containing EETs or DHETEs, and [^{3}H]thymidine (0,25 µCi/well) was added the last 24 h. [^{3}H]thymidine-containing media were aspirated, cells were washed and finally cells were overlaid with 1%

Triton X-100 and then scraped off the dishes. Finally, radioactivity present in the cell fraction was measured by scintillation counting, using a Packard Tri-Carb 1500 counter.

Measurement of PGE_2 levels

Cells were cultured at 25 x 10^3 cells/well on 24-well plates for 24 h in RPMI 1640 supplemented with 10% FBS. After 24 h of serum starvation, cells were incubated for 48 h in medium with PDGF (100 ng/ml) in the presence of several products. An aliquot of culture supernatant medium was acidified with 1 ml of 1% formic acid. PGE_2 was extracted in ethyl acetate, and the organic phase was evaporated under a stream of nitrogen. Finally, PGE_2 was determined using monoclonal enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's protocol.

Measurement of apoptosis by annexin V binding

3T6 fibroblasts were cultivated in media containing PDGF 100 ng/mL medium with EETs or DHETEs for 48 h. Thereafter, treatments were removed and cells were stained with fluorescein-isothiocyanate-labelled annexin V and propidium iodide following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Representative samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera.

Measurement of total caspase activity

Cells were cultivated in media containing PDGF 100 ng/mL medium with EETs or DHETEs for 48 h at 37°C. Thereafter, treatments were removed and cells were stained with FITC-VAD-FMK, which binds to activated caspases in apoptotic cells, following the manufacturer's instructions (Medical and Biological Laboratories, Nagoya, Japan). Samples were viewed with an inverted fluorescence microscope system (Nikon Eclipse TE200) linked to a CCD camera, and caspase positive or negative cells were counted.

Measurement of caspase-3/-8/-9/-12 activity

Cells were cultivated in media containing PDGF 100 ng/mL medium with EETs or DHETEs. Thereafter, cells were lysed and incubated with the suitable substrate. Caspase-3 activity was detected by using the specific caspase-3 fluorogenic substrate, DEVD peptide conjugated to 7-amino-4-trifluoromethyl coumarin (AFC). Caspase-8 activity was detected by using the specific caspase-8

fluorogenic substrate, IETD-AFC. Caspase-9 activity was detected by using the specific caspase-9 fluorogenic substrate, LEHD-AFC. Finally, caspase-12 activity was detected by using the specific caspase-12 fluorogenic substrate, ATAD-AFC. The substrates are cleaved by active caspases and emits yellow-green fluorescence, which is quantified using a luminescence spectrometer (Perkin Elmer LS 45), following the manufacturer instructions (Medical and Biological Laboratories, Nagoya, Japan). Results were normalised with protein concentration that was determined following Bradford's method (1976).

Measurement of DNA fragmentation

Degradation of chromosomal DNA was evaluated with TUNEL method. 3T6 fibroblasts were cultivated in media containing PDGF 100 ng/mL medium with EETs or DHETEs for 48 h. Next, cells present in the medium and attached cells trypsinized were collected, fixed with 4% paraformaldehyde and permeabilized with 70% ethanol. Thereafter, 3'-OH DNA ends generated by DNA fragmentation were labelled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analysed on a Epics XL flow cytometer (Coulter Corporation, Hialeah, Florida).

Statistics

Results are expressed as mean ± SEM. Differences between control and treated cultures were tested using Student's t-test.

RESULTS

Effect of EETs and DHETEs on growth and DNA synthesis of 3T6 fibroblasts

Firstly, we have studied the effect of EETs on 3T6 fibroblast proliferation. Figure 1 shows that cultures stimulated with 100 ng PDGF/mL markedly increase cell number (around 100%), and exogenous addition of EETs (0,1-1 μM) significantly reduces cell growth of 3T6 fibroblasts. These effects are concentration-dependent and 11,12-EET and 14,15-EET 1 μM were able to block 3T6 cell growth (Fig. 1A). In this way, EETs-derived metabolites 5,6-DHETE and 11,12-DHETE also reduce cell proliferation. This impairment of cell proliferation induced by EETs and DHETEs were correlated with the inhibition of [^{3}H]thymidine incorporation induced by PDGF (Fig. 1B). Interestingly, these effects were observed when we assessed EETs concentrations around to physiological relevant concentrations (0,1-1 μM) (Node et al., 1999). Dettached cells and viability were measured on cells incubated in presence of EETs/DHETEs. We only observed a significant but limited impairment of cell dettached/cell viability in presence of 11,12- and 14,15-EET at 1 μM (Table 1). Thus, these effects of EETs on 3T6 fibroblast viability can not explain the blockage of cell growth induced by four EETs and 5,6- and 11,12-DHETE.

Effect of EETs on PGs synthesis

Previously, we reported that PGs are involved in the control of 3T6 fibroblast growth (Martínez et al. 1997) and that PGH synthase pathway inhibition decreases 3T6 fibroblats proliferation (Sanchez and Moreno, 1999). Considering that Fang et al (1998) reported that 14,15 EET competitively inhibits PGH synthase activity and consequently PGs synthesis by vascular smooth muscle cells, we proposed to determine the effect of EETs on PGE_2 synthesis by 3T6 fibroblast. Our results show that 11,12 EET reduced PGE_2 formation by 15%, an effect that do not appear be responsible of the blockage of 3T6 fibroblast growth induced by this EET (Fig. 2).

Effect of EETs and DHETEs on apoptosis of 3T6 fibroblasts

Once we have observed that EETs are able to inhibit 3T6 fibroblast proliferation, we have studied if this action is through induction of an apoptotic process. Apoptosis is associated with characteristic morphological and biochemical changes. Thus, phosphatidylserine (PS), a phospholipid normally

confined to the cytoplasmic face of the plasma membrane, translocates to the outlayer. PS exposed to the external surface of cells is recognised by annexin-V, a phospholipid binding protein. Table 2 shows that all EETs and 5,6- and 11,12-DHETE induced an appreciable increase of double annexin-V^+-PI^+ staining cells, suggesting late apoptosis. Treatments that present a greater annexin-V^+-PI^+ staining are 11,12-EET, 14,15-EET and 11,12-DHETE. The time-course of annexin V^+ or annexin $V^{+/}PI^+$ staining show that 11,12-EET and 11,12-DHETE induced a gradual increase of the percentage of cells annexin V^+/PI^+ rise a significant effect after 24 h EET/DHETE addition (Fig. 3).

On the other hand, we have measured the activity of caspases because they are critical mediators of programmed cell death. Our results show that EETs/DHETEs were able to increase total caspase activity (Figure 4). There are three main pathways of caspase activation: the mitochondria-mediated pathway, the endoplasmic reticulum (ER)-mediated pathway and the cell surface death receptor-mediated pathway (Wang et al., 2005). We have studied which pathways activate EETs/DHETEs to induce apoptosis. Firstly, we have quantified activity of the caspase-3. This is an effector caspase and all three pathways converge at the level of this activation. Fig 5A shows that 11,12- and 14,15-EET increase caspase-3 activity with a maximum effect at 2-5 h after EET addition.

While TNF-α increases caspase-8, cells treated with 11,12- or 14,15-EET did not present marked caspase-8 activity (Figure 5B). Since the caspase-9 dependent mitochondrial pathway of apoptosis may activate caspase-3, we examined the effect of 11,12- and 14,15-EET on this caspase. Our results did not show any appreciable effect (Fig. 5C). The activity of caspase-12 was measured as the above caspases by fluorimetric assay, and a notable increase in this activity was observed when 3T6 fibroblast were cultured in presence of 11,12- or 14.15-EET (Fig. 5D). Our results also showed that caspase-12 activation was previous to the enhancement of caspase-3.

To determine the final effect of these caspase activation, we have quantified fragmentation of nuclear chromatin, as a late event in apoptosis. Figure 6 reported that the presence of EETs and 5,6- and 11,12-DHETE induced the enhancement of the percentage of cells with DNA fragmentation. Thus, only 3% of 3T6 fibroblasts growing with PDGF present DNA fragmentation whereas EETs/DHETEs exposure increase the percentage of cells with DNA fragmentation up to 30%.

Therefore, our results show that the inhibitory effect of EETs and DHETEs on cell growth is because these metabolites are inducing an apoptotic process.

DISCUSION

The last years has been made very progress in finding the functional effects of AA metabolites derived from cytochrome P-450 pathway such as EETs. But it is not yet clear the effects and the biochemical mechanisms through which EETs modulate cell growth. Most of studies suggested that EETs are pro-proliferative on renal epithelial cells and endothelial cells (Chen et al., 1998; Wang et al., 2005), which are cell types able to synthesised EETs. In contrast, Davis et al. (2002) described that EETs present the opposite effect reducing [^{3}H]thymidine incorporation induced by PDGF in smooth muscle cells. However, recently these authors (Davis et al., 2006) showed that EETs didn't have effect on smooth muscle cell proliferation. Concordingly, Sun et al. (2002) reported that EETs did not modify endothelial growth induced by PDGF. Therefore, this effect could be different depending of the cell type and stimuli used and there are contradictory results with the same cell and stimuli.

Cells surrounding vascular wall as smooth muscle cells or fibroblasts may receive EETs produced by endothelial cells and act as cell growth modulator, but there are not studies about this role of EETs on fibroblasts. Previously, we observed that another AA metabolites of cytochrome P-450 pathway such as HETEs stimulated 3T6 fibroblast growth (Nieves and Moreno, 2006). Considering all together, our aim is clarify the role of EETs on 3T6 fibroblast proliferation. Our results show that exogenous addition of 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET markedly inhibit 3T6 fibroblast growth and [^{3}H]thymidine incorporation induced by PDGF. In the present study, we also provide evidence that the same effect was observed when EETs-derived metabolites 5,6-DHETE and 11,12-DHETE were added. In contrast to these results, several authors suggested that EETs have biological effect while the respective DHETEs do not present this effect (Chen et al., 1998; Pozzi et al., 2005).

Once determined the impairment of 3T6 fibroblast growth induced by EETs/DHETEs, we proposed to assay if the anti-proliferative action is by means of induction of apoptosis considering that EETs were able to induce cell detachment and the decrease of fibroblast viability. Previously has been suggested that EETs act as survival factors on renal epithelial cells (Chen et al., 2001) and different human carcinoma cell lines (Jiang et al., 2005). However, our results demonstrate that EETs as well as 5,6-DHETE and 11,12-DHETE are pro-apoptotic because they increase phophatidylserine externalization as well as the enhancement of caspase activity and nuclear chromatin fragmentation in 3T6

fibroblast cultures in presence of PDGF. Effects that could explain the decrease of fibroblast proliferation induced by EETs/DHETEs.

Understanding the mechanisms by which EETs/DHETEs induced apoptosis is a critical issue to know the molecular mechanism for the anti-proliferative effects of these AA metabolites. In this way, caspases have emerged as the main players in the execution of the cell death program, with apoptotic caspases being divided into initiator caspases such as caspase-2, -8, -9, -12 and executioner caspases such as caspase-3, -6, -7 (Earnshaw et al., 1999). Caspases are critical mediators of several pro-apoptotic signalling pathways. These include the mitochondrial pathway in which pro-caspase-9 is activated (Reed and Paternostro, 1999), the death receptor pathway, leading to pro-caspase-8 activation (Sartorius et al., 2001) and the endoplasmic reticulum stress-mediated pathway, resulting in pro-caspase-12 processing (Nakagawa et al., 2000). These pathways converge upon the activation of effector caspases like caspase-3, resulting in the cleavage of several substrates, including poly (ADP-ribose) polymerase and DNA fragmentation, with consequently cellular disassembly. Our results show that 11,12-EET and 14,15-EET increased caspase-3 activity in 3T6 fibroblast and that this effect was consequence of a previous caspase-12 activation. However, 11,12-EET and 14,15-EET were not able to induce cascapse-9 or caspase-8 activity in our experimental conditions. All together suggest that endoplasmic reticulum stress-mediated caspase-12 could be involved in the pro-apoptotic action of 11,12-EET and 14,15-EET.

Previous studies demonstrated that elevation of cytosolic Ca^{2+} concentration (i[Ca^{2+}]) is an important component of the signalling pathways leasing to apoptosis (Caron-leslie and Cidlowski, 1991). In this way, Martínez and Moreno (2005) recently reported that EETs were able to induce a marked i[Ca^{2+}] influx in 3T6 fibroblast and that this event was sensible to receptor-operated Ca^{2+} channel blockers, but not to voltage-dependent Ca^{2+} channel blockers. Nakagawa and Yuan (2000) proposed that increase in i[Ca^{2+}] leads to movement of calpain to the endoplasmic reticulum surface, where it activates caspase-12. These findings led us to hypothesize that EETs may induce apoptosis to some extent via the activation of calpain and caspase-12, triggered by the elevation of i[Ca^{2+}] and that these events could be involved in the anti-proliferative action of these eicosanoids in 3T6 fibroblast cultures.

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FIGURES

Figure 1. Effect of EETs and DHETEs on PDGF-induced 3T6 fibroblast proliferation and [^{3}H]-thymidine incorporation. Fibroblasts (25 x 10^3 cells/well) were plated and cultured in 10% FBS-RPMI. The next day, media were removed and free FBS-RPMI was added for 24 hours. Then, cells were incubated for 48 h with 100 ng PDGF/mL containing 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE or 11,12-DHETE 0,1μM (black bars) or 1 μM (white bars). A, After two-day treatment, cells were trypsinized and counted in a microscopy. B, The last 24 h of treatment [^{3}H]-thymidine (1 μCi/well) was added to the cells. Radioactivity in cell lysates was determined in a scintillation analyser. Results are means ± SEM from three experiments performed in triplicate. *, p < 0,05 compared with PDGF-treated cells.

Figure 2. Effect of 11,12-EET on PGE_2 synthesis in 3T6 fibroblasts. Cells were incubated for 48 h in medium with PDGF (100 ng/ml) in the presence of 11,12-EET 1 μM, or with ketoprofen 5 μM as negative control. PGE_2 was extracted from culture supernatant medium and measured using monoclonal enzyme immunoassay kit. *, p < 0,05 compared with PDGF-treated cells.

Figure 3. Effect of 11,12-EET and 11,12-DHETE on phosphatidylserine externalization in 3T6 fibroblast cultures. Cells were incubated for 3, 6, 12, 24 or 48 hours with 100 ng PDGF/mL containing 11,12-EET or 11,12-DHETE 1 μM. Cells were stained with fluorescein-isothiocyanate-labelled annexin V and propidium iodide and counted with an inverted fluorescence microscope. Data are expressed in percentages and are means ± SEM from three experiments performed in triplicate. *, p < 0,05 compared with PDGF-treated cells.

Figure 4. Effect of EETs and DHETEs on total caspase activity of PDGF-induced 3T6 fibroblasts. Fibroblasts (80x10^3 cells/ well) were plated and cultured in 10% FBS-RPMI for 48 h. Then, cells were washed and incubated two days with 100 ng PDGF/mL containing 5,6-, 8,9-, 11,12-, 14,15-EETs, 5,6- or 11,12-DHETE 1 μM. Then, cells were stained and counted with an inverted fluorescence microscope. Results are means ± SEM from three experiments performed in triplicate. *, p < 0,05 compared with PDGF-treated cells.

Figure 5. Effect of 11,12-EET and 14,15-EET on caspase-3 (A), -8 (B), -9 (C) or -12 (D) activity of PDGF-induced 3T6 fibroblasts. Fibroblasts (80 x 10^3 cells/ well) were plated and cultured in 10% FBS-RPMI for 48 h. Then, cells were washed and incubated with 100 ng PDGF/mL containing 11,12-EET or 14,15-EET at 1 µM at times indicated. Next, cells were lysed, incubated with the suitable substrate for each caspase and fluorescence was quantified using a fluorometer. Results are means ± SEM from three experiments performed in triplicate.

Figure 6. Effect of EETs and DHETEs on DNA fragmentation of PDGF-induced 3T6 fibroblasts. Fibroblasts (25 x 10^4 cells/ 60 mm-dishes) were plated and cultured in 10% FBS-RPMI. The next day, cells were washed and incubated for 48 h with 100 ng PDGF/mL containing 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE or 11,12-DHETE 1 µM. Next, cells were detached, labelled following TUNEL assay and finally analysed by flow cytometry. Results are means ± SEM from three experiments performed in triplicate. *, $p < 0,05$ compared with PDGF-treated cells.

Figure 1A

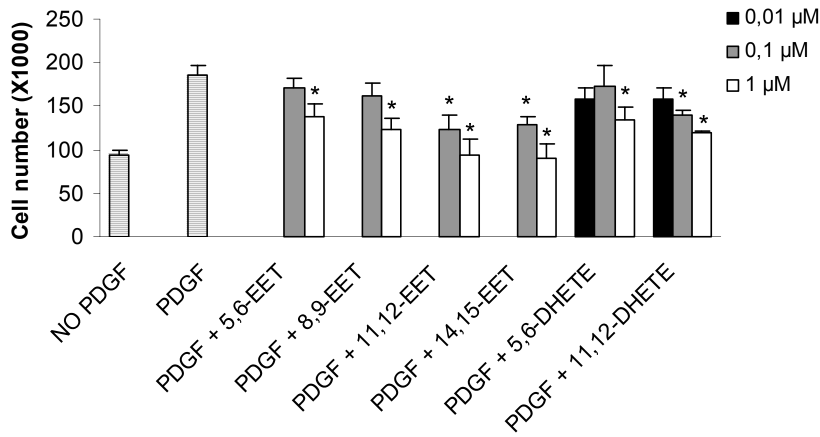


Figure 1B

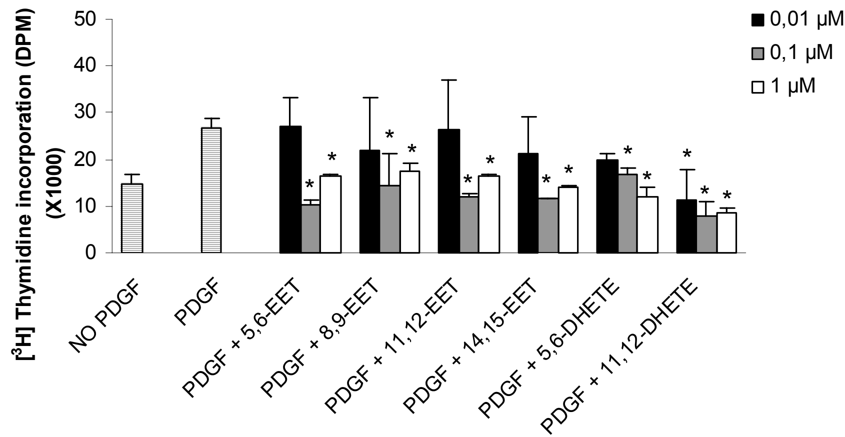


Figure 2

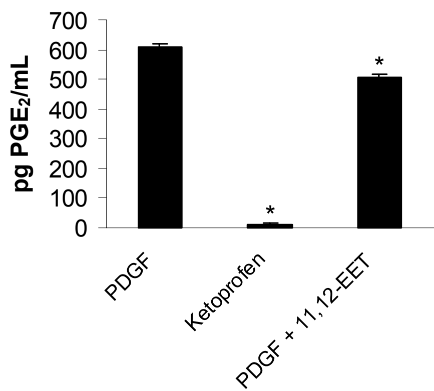
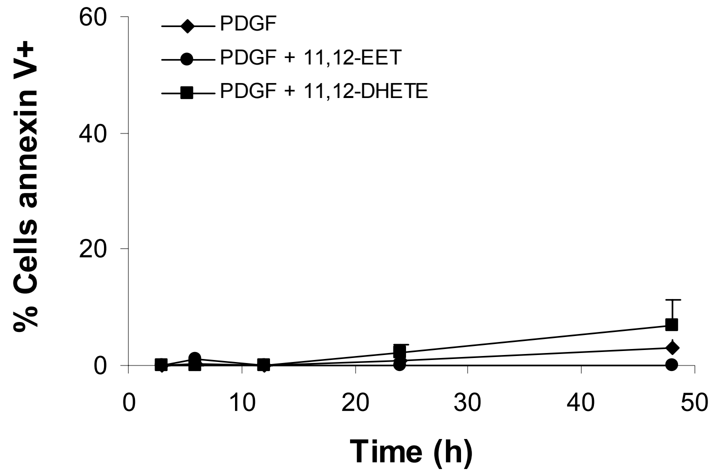
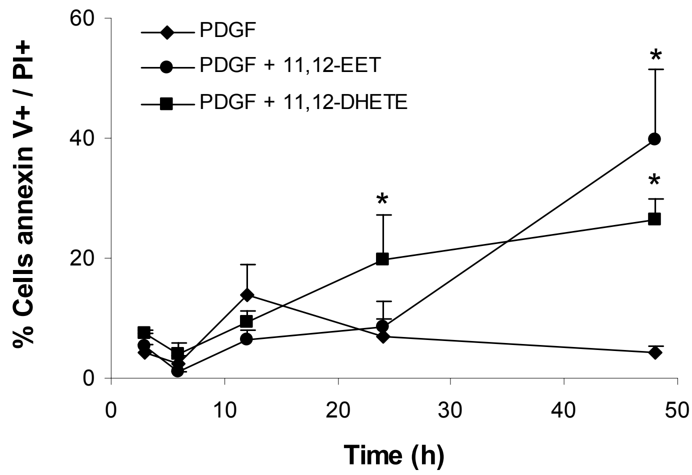


Figure 3





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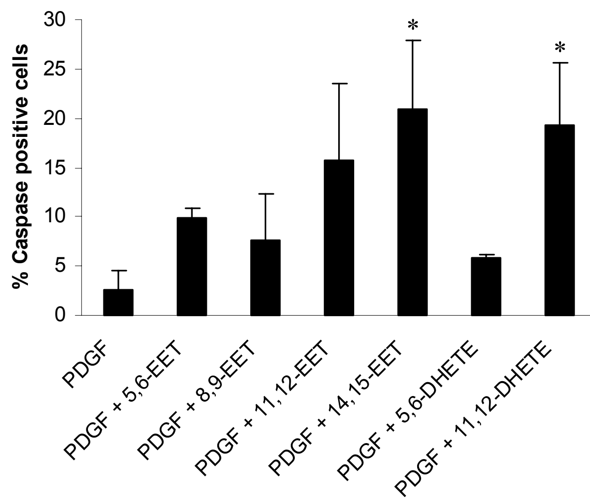

Figure 5A

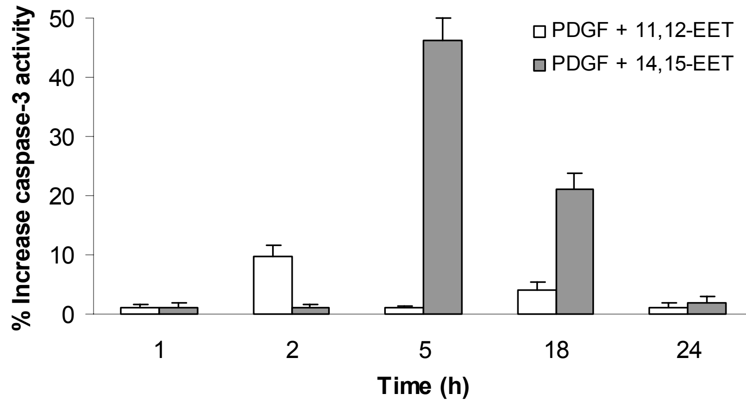

Figure 5B

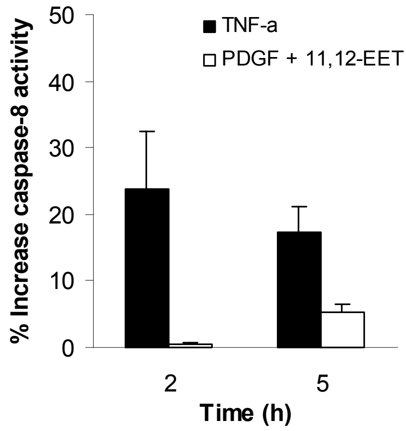

Figure 5C

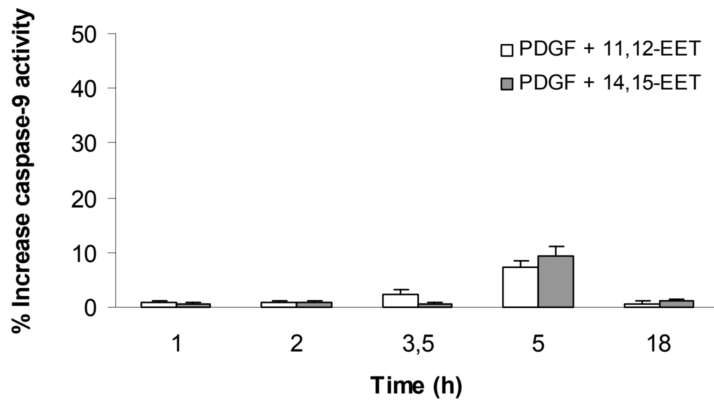

Figure 5D

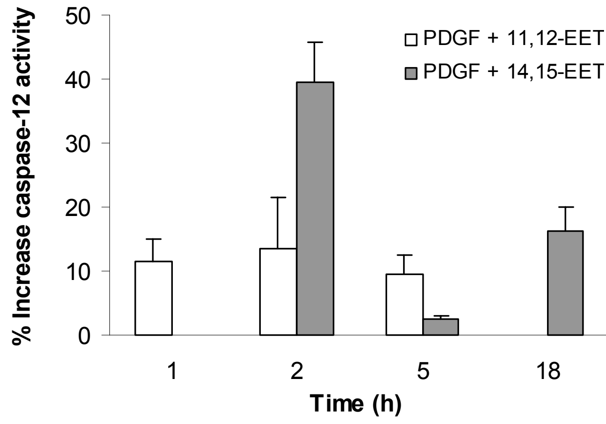

Figure 6

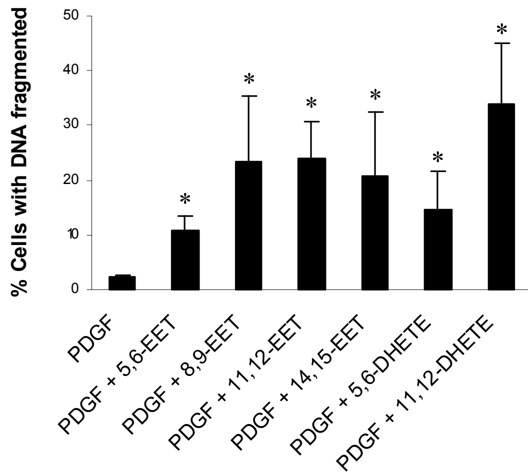

Table 1. Effect of EETs or DHETEs on 3T6 fibroblast viability.

	0,1 µM	1 µM
NO PDGF	94,0 ± 0,9	
PDGF	92,3 ± 1,6	
PDGF + 5,6-EET	93,2 ± 2,1	92,2 ± 3,0
PDGF + 8,9-EET	92,6 ± 1,5	94,5 ± 1,8
PDGF + 11,12-EET	90,4 ± 2,6	76,6 ± 5,7 *
PDGF + 14,15-EET	92,8 ± 3,4	85,6 ± 3,9 *
PDGF + 5,6-DHETE	93,0 ± 1,0	94,3 ± 1,7
PDGF + 11,12-DHETE	95,0 ± 1,5	94,7 ± 1,8

Cells were incubated for 48 h with 100 ng PDGF/mL containing 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE or 11,12-DHETE 1 µM. Cells were washed, trypsinized, and counted with a microscope using ethidium bromide/acridine orange staining to assess viability. Data are means ± SEM from at least three experiments performed in triplicate. *, $p < 0,05$ compared with PDGF-treated cells.

Table 2. Effect of EETs and DHETEs on phosphatidylserine externalization in 3T6 fibroblast cultures.

Treatment	Apoptosis		
	Annexin V+/PI- Early apoptotic cells	Annexin V+/PI+ Late apoptotic/necrotic cells	Annexin V-/PI+ Necrotic cells
NO PDGF	0,8 ± 0,65	3,8 ± 1,62	0,0 ± 0,00
PDGF	3,0 ± 1,27	4,3 ± 0,92	1,6 ± 0,76
PDGF + 5,6-EET	0,0 ± 0,00	9,8 ± 2,09 *	5,2 ± 3,46
PDGF + 8,9-EET	5,7 ± 5,67	9,0 ± 7,55	0,0 ± 0,00
PDGF + 11,12-EET	0,0 ± 0,00	39,8 ± 11,54 *	3,0 ± 2,45
PDGF + 14,15-EET	2,8 ± 1,96	42,0 ± 11,62 *	4,0 ± 2,58
PDGF + 5,6-DHETE	1,0 ± 1,00	8,3 ± 4,31	1,6 ± 1,41
PDGF + 11,12-DHETE	7,0 ± 4,36	26,3 ± 3,53 *	8,7 ± 2,19 *

Cells were incubated for 48 hours with 100 ng PDGF/mL containing 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE or 11,12-DHETE 1 µM. Cells were stained with fluorescein-isothiocyanate-labelled annexin V and propidium iodide and counted with an inverted fluorescence microscope. Data are expressed in percentages and are means ± SEM from three experiments performed in triplicate. *, $p < 0,05$ compared with PDGF-treated cells.

5. DISCUSIÓN GENERAL

Resultados preliminares obtenidos en nuestro laboratorio demostraron que la metabolización del AA por la COX-2, y la consecuente formación de PGs como la PGE_2, están implicadas en el control de la proliferación de los fibroblastos 3T6 inducida por suero (Sánchez y Moreno, 2001b). Así, la PGE_2 al interactuar con los receptores EP_1 y EP_4 modula las concentraciones de calcio y AMPc que regulan los niveles de las ciclinas D, E y A. De manera que, los antagonistas receptoriales del EP_1 y EP_4 bloquean el crecimiento de los fibroblastos 3T6 al acumular las células en G_0/G_1 o en la fase S, respectivamente (Sánchez y Moreno, 2002). Recientemente, también se ha demostrado que la interacción de la PGE_2 con el receptor EP_3 está implicada en el crecimiento de los fibroblastos 3T6 a través de sus efectos sobre los niveles de AMPc y la maquinaria de la fase S del ciclo celular (Sánchez y Moreno, 2006). Un efecto similar se observó al inhibir la liberación del AA bloqueando las PLA_2 implicadas en la movilización del AA inducida por FBS (Sánchez y Moreno, 2001a y 2001b). Por otro lado, la inhibición de las COXs produjo una reducción parcial de la proliferación celular muy inferior a la causada por los antagonistas de la PGE_2 o los inhibidores de la PLA_2 (Sánchez y Moreno, 2002). Estos resultados sugieren la implicación de otras vías de metabolización del AA diferentes a las COXs en el control de la proliferación de los fibroblastos 3T6. De manera que al inhibir las COXs, el AA liberado podría metabolizarse por una vía alternativa. Considerando todo ello, nos planteamos estudiar la implicación de los metabolitos producidos a partir del AA por las LOXs o los CYPs sobre la proliferación de los fibroblastos 3T6.

Nuestros resultados han demostrado que factores de crecimiento como el suero inducían la síntesis de PGE_2 y 12(*S*)-HETE en cultivos de fibroblastos 3T6, mientras que los niveles de LTB_4 se encontraban en el límite de detección (Nieves y Moreno, 2006a). Por otro lado, los inhibidores específicos de la 5-LOX (zileuton) (Carter *et al.*, 1991) y de la 12/15-LOXs (baicaleína) (Sekiya y Okuda, 1982), y los antagonistas de los receptores de los LTs (U-75302, REV-5901, LY-171883) (Fleisch *et al.*, 1985; Musser *et al.*, 1987; Falcone y Aharony, 1990) no inhibieron de forma significativa el crecimiento de los fibroblastos 3T6 ni la incorporación de timidina. Sin embargo, en los macrófagos RAW 264.7, una línea celular donde se ha demostrado la expresión de las 5-, 12- y 15-LOXs (Christmas *et al.*, 1999; Kawajiri *et al.*, 2002; Hong *et al.*, 2004) y donde el AA se metaboliza principalmente por las COXs y LOXs (Bonney y Humes, 1984), estos mismos tratamientos fueron capaces de inhibir la proliferación celular y la incorporación de timidina (Nieves y Moreno, 2006b). Además, el zileuton produjo

un retraso en el ciclo celular aumentando el número de células en la fase G_0/G_1. Estos resultados sugieren la implicación de la 5-LOX y los receptores de los LTs en el control de la proliferación de los macrófagos RAW 264.7. En este sentido, Vargaftig y Singer (2003) sugirieron que el zileuton y el MK-571 reducen la proliferación de las células *fibroblast-like*, y Ciccarelli *et al.* (2004) describieron el papel del CysLTR1 en el crecimiento de los astrocitos. Porreca *et al.* (1996) demostraron que el MK-571 inhibía el efecto estimulatorio del LTD_4 sobre la incorporación de timidina en células de músculo liso vasculares. Por otro lado, el LY-171883 y el REV-5901 han sido propuestos como agentes contra el cáncer de páncreas ya que inhiben la incorporación de timidina e inducen apoptosis en las células MiaPaCa-2 y AsPC-1 (Tong *et al.*, 2000b y 2002a). En cambio, Przylipiak *et al.* (1998) demostraron que el LTD_4 inhibe la incorporación de timidina en la línea celular de carcinoma de mama humano MCF-7, y es revertido con el LY-171883.

Además, hemos comprobado que la inhibición del crecimiento por parte de los inhibidores de la 5-LOX y los antagonistas receptoriales de los LTs no es debida a la inducción de citotoxicidad o apoptosis en los macrófagos RAW 264.7, tal como han sugerido otros autores (Ghosh y Myers, 1998). Todo esto sugiere que los LTs pueden, a través de una acción autocrina/paracrina, tener un efecto co-mitogénico junto con otros factores de crecimiento. Por otro lado, nuestros resultados demuestran que el efecto mitogénico de la adición exógena de LTB_4 o LTD_4, en ausencia de suero, induce un incremento del número de células y de la incorporación de timidina de los macrófagos RAW 264.7. Estos resultados concuerdan con los de Paruchuri y Sjölander (2003) donde el LTD_4 aumentaba el número de células y la incorporación de timidina en células epiteliales intestinales. En este sentido, Tong *et al.* (2002a) demostraron que el LTB_4 estimulaba la incorporación de timidina de forma concentración- y tiempo-dependiente y aumentaba la fosforilación de ERK1/2 en células de cáncer de páncreas. Nuestros resultados también sugieren que el efecto proliferativo del LTB_4 y LTD_4 está mediado por la activación de las vías MAPK y PI3K/Akt. Estos resultados coinciden con los de Klein *et al.* (2001) que demostraron que el LTB_4 estimulaba la fosforilación de ERK y Akt en neutrófilos humanos, y con Paruchuri *et al.* (2002, 2005) quienes sugirieron que el efecto mitogénico del LTD_4 está mediado por la activación de ERK1/2 y Akt en células epiteliales intestinales.

Los macrófagos RAW 264.7 son un buen modelo celular para estudiar el papel de los metabolitos derivados del AA vía COX y LOX sobre el crecimiento

celular ya que ambas vías se encuentran implicadas, en contraposición a los fibroblastos 3T6 donde solo parece estar implicada la vía COX.

El EPA compite con el AA al ser metabolizado por la COX-2, formando las PGs de la serie-3, y por la 5-LOX, dando lugar al LTB_5 (Fischer y Weber, 1984). Diversos autores han sugerido que el efecto del EPA sobre el crecimiento celular sería consecuencia de la inhibición de la síntesis de las PGs de la serie-2 (Denkins *et al.*, 2005). Las PGs de la serie-2 se consideran más mitogénicas y pro-inflamatorias que las PGs de la serie-3 (Bagga *et al.*, 2003). En este sentido, Bortuzzo *et al.* (1996) demostraron que los LTs de la serie-5 derivados del EPA no estimulaban la proliferación celular, mientras que el LTB_4 inducía la proliferación de las células HT-29. Recientemente, Yang *et al.* (2004) demostraron que la PGE_3 tiene efectos anti-proliferativos en las células A549 de cáncer de pulmón humano. Así, se ha propuesto que el efecto del EPA sobre el crecimiento celular podría ser debido a una disminución de la síntesis de eicosanoides proliferativos y la liberación de eicosanoides no proliferativos o anti-proliferativos. Sin embargo, nuestros resultados demuestran que la PGE_3 estimulaba la proliferación de los macrófagos con una potencia similar a la de la PGE_2, mientras que el LTB_5 tuvo menos efecto que el LTB_4 (Nieves y Moreno, 2006c). Esto podría ser debido a la menor afinidad del LTB_5 por los receptores del LTB_4 (Seya *et al.*, 1988; Andazola *et al.*, 1992). De manera que estos resultados indican que el efecto inhibitorio del EPA sobre el crecimiento celular descrito por otros autores (Bartsch *et al.*, 1999), no puede ser atribuido a la producción de eicosanoides sin actividad pro-proliferativa.

Dado que la vía de las LOXs no parecía implicada en el control de la proliferación de los fibroblastos 3T6, centramos nuestro interés en estudiar la implicación de los metabolitos del AA producidos por los CYPs. Tal como hemos comentado en la introducción, los CYPs pueden tener actividad ω/ω-1 hidroxilasa y tipo-lipoxigenasa formando los HETEs, o epoxigenasa produciendo los EETs. Nuestros resultados demostraron que el suero induce la síntesis de 12(*S*)-HETE en los fibroblastos 3T6, y que la inhibición de las COXs con ketoprofeno incrementa el nivel de 12(*S*)-HETE, sugiriendo que la inhibición de la vía COX puede estimular las vías de metabolización del AA que dan lugar a este metabolito. Además, el uso de inhibidores de los CYPs como el SKF-525A, 17-ODYA, ABT y PPOH (Zou *et al.*, 1994; Su *et al.*, 1998; Jacobs y Zeldin, 2001) inhibieron la síntesis de 12(*S*)-HETE, el crecimiento celular y la síntesis de DNA. Sin embargo, el ABT y el PPOH también redujeron los niveles de PGE_2, de manera que su efecto sobre la proliferación celular podía ser debido a la

reducción de HETEs y/o PGs. En cambio, el SKF-525A y el 17-ODYA solo inhibieron significativamente la síntesis de 12(*S*)-HETE. Para entender el efecto del SKF-525A sobre el crecimiento de los fibroblastos 3T6, investigamos su efecto sobre el ciclo celular. Previamente, Pidgeon *et al.* (2002) demostraron que la inhibición de la síntesis de 12(*S*)-HETE inducía una parada del ciclo celular en G_0/G_1 en células de cáncer de próstata. En nuestro estudio el SKF-525A produjo un retraso en el ciclo celular, que podía explicar el efecto inhibitorio de estos tratamientos sobre el crecimiento y la incorporación de timidina. Además, el 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE y 20-HETE revertieron la inhibición del crecimiento celular inducida por el SKF-525A, mientras que el 11,12-EET no lo hizo. Así, todos estos resultados sugieren que los HETEs producidos por los CYPs están implicados en las vías de transducción de señales inducidas por el FBS en los fibroblastos 3T6 y pueden actuar como factores co-mitogénicos. Estas observaciones coinciden con el efecto co-mitogénico del 20-HETE en las células del túbulo proximal (Lin *et al.*, 1995), del 12-HETE en células endoteliales microvasculares (Tang *et al.*, 1995), del 5-HETE, 12-HETE y 15-HETE en células epiteliales de mamífero (Bandyopadhyay *et al.*, 1988) y del 12-HETE en células epiteliales de córnea (Ottino *et al.*, 2003).

Por otro lado, diversos autores han sugerido que los HETEs pueden ser agentes mitogénicos. Así, Palmberg *et al.* (1991) observaron que el 15-HETE pero no el 5-HETE inducía la síntesis de DNA en células de músculo liso. El 12-HETE también regula la síntesis de DNA en células epiteliales humanas (Arora *et al.*, 1996), células endoteliales (Tang *et al.*, 1995) y células de músculo liso (Preston *et al.*, 2006). En este sentido, Zeng *et al.* (2002) demostraron recientemente que el 5-HETE inducía la síntesis de DNA en células endoteliales. Nuestros resultados muestran que el 5-HETE, 12(*S*)-HETE y 15(*S*)-HETE inducen el crecimiento de los fibroblastos 3T6 y la síntesis de DNA, mientras que la adición de wortmanina, un inhibidor de la PI3K, inhibió este efecto. Además, confirmamos que los efectos mitogénicos de estos metabolitos se producían a través de la activación de la vía PI3K/Akt, ya que el 5-HETE, 12(*S*)-HETE y 15(*S*)-HETE aumentaron los niveles de P-Akt. Recientemente, también ha sido demostrado que el 5-HETE, 12(*S*)-HETE y 15(*S*)-HETE aumentan el crecimiento celular a través de la activación de Akt en células endoteliales (Zeng *et al.*, 2002, 2005) y células de cáncer de próstata (Pidgeon *et al.*, 2002). No está claro cómo los HETEs inician las cascadas de señalización en estas células que finalmente inducirán la proliferación celular. Debido a que las PGs y los LTs median sus efectos a través de la interacción con receptores específicos de

membrana, se ha sugerido que los metabolitos derivados del AA por los CYPs también podrían iniciar sus acciones de esta manera. Existen evidencias de que los HETEs pueden unirse a receptores de membrana (Alonso-Galicia *et al.*, 1999; Hampson y Grimaldi, 2002), aunque todavía no se han identificado receptores específicos. También se ha sugerido que los HETEs pueden unirse a los receptores nucleares PPARs. Así, se ha descrito que el 15(*S*)-HETE puede unirse a PPAR-γ y modular la proliferación de las células PC3 de cáncer de próstata (Shappell *et al.*, 2001). En este sentido, Muga *et al.* (2000) demostraron que el 8(*S*)-HETE puede unirse al PPAR-α y participar en el control de la diferenciación de los queratinocitos.

Una vez descrito el papel de los HETEs en el control de la proliferación de los fibroblastos 3T6, centramos nuestro estudio en los metabolitos derivados de la actividad epoxigenasa de los CYPs. Hasta el momento, el efecto de los EETs en el control de la proliferación celular no está claro. La mayoría de estudios sugieren que los EETs tiene un papel proliferativo en células renales epiteliales y células endoteliales (Chen *et al.*, 1998; Wang *et al.*, 2005), que son tipos celulares capaces de sintetizar estos metabolitos. En cambio, otros autores muestran que los EETs presentan el efecto opuesto reduciendo la síntesis de DNA inducida por el PDGF en células musculares lisas (Davis *et al.*, 2002). Sin embargo, recientemente estos autores han demostrado que los EETs no tienen efecto sobre la proliferación de las células musculares lisas (Davis *et al.*, 2006), resultados que concuerdan con los de Sun *et al.* (2002) quienes demostraron que los EETs no modifican el crecimiento de las células musculares lisas vasculares inducido con PDGF. Como vemos, el efecto de los EETs sobre el crecimiento celular podría ser diferente en función del tipo celular y el estímulo usado.

Los EETs producidos por las células endoteliales podrían ser captados por las células que rodean los vasos sanguíneos como las células musculares lisas o los fibroblastos, y actuar como moduladores del crecimiento celular, aunque no hay estudios en fibroblastos. Así, nuestro objetivo ha sido esclarecer el papel de los EETs sobre la proliferación de los fibroblastos 3T6. Nuestros resultados demuestran que la adición exógena de 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE o 11,12-DHETE inhibe la proliferación de los fibroblastos 3T6 y la incorporación de timidina inducida por PDGF. Contrariamente a estos resultados, varios autores han sugerido que los EETs tienen efecto sobre la proliferación de células epiteliales y endoteliales mientras que los respectivos DHETEs no presentan este efecto (Chen *et al.*, 1998; Pozzi *et al.*, 2005).

Estudios previos sugieren que los EETs actúan como factores de supervivencia en células epiteliales renales (Chen *et al.*, 2001) y diversas líneas celulares de carcinomas humanos (Jiang *et al.*, 2005). Sin embargo, hemos observado que los EETs inducen que los fibroblastos 3T6 se despeguen y reducen su viabilidad celular. Además, nuestros resultados sugieren que el 5,6-EET, 8,9-EET, 11,12-EET, 14,15-EET, 5,6-DHETE y 11,12-DHETE son pro-apoptóticos ya que aumentan la externalización de la fosfatidilserina, la actividad caspasa total y la fragmentación de la cromatina nuclear. Estos efectos podrían explicar la reducción de la proliferación de los fibroblastos 3T6 inducida por los EETs/DHETEs.

Entender los procesos mediante los que los EETs/DHETEs inducen la apoptosis es un punto básico para conocer los mecanismos moleculares del efecto anti-proliferativo de estos metabolitos. En este sentido, las caspasas juegan un papel importante en la ejecución de la muerte celular programada, dividiéndose en dos grupos, las caspasas iniciadoras como la caspasa-2, -8, -9 y -12, y las caspasas ejecutoras como la caspasa-3, -6 y -7 (Earnshaw *et al.*, 1999). Las caspasas tienen un papel central en las vías de señalización apoptóticas. Éstas incluyen la vía mitocondrial en la que participa la caspasa-9 (Reed y Paternostro, 1999), la vía iniciada por los receptores de muerte celular en la que está implicada la caspasa-8 (Sartorius *et al.*, 2001), y la vía mediada por estrés en el retículo endoplasmático, en la que se activa la caspasa-12 (Nakagawa *et al.*, 2000). Estas vías convergen hacia la activación de las caspasas efectoras como la caspasa-3, dando lugar al procesamiento de varios substratos, incluyendo la poli (ADP-ribosa) polimerasa y la fragmentación de DNA. Nuestros resultados muestran que EETs como el 11,12-EET y 14,15-EET aumentan la actividad caspasa-3 en los fibroblastos 3T6, y este efecto fue consecuencia de una activación previa de la caspasa-12. Sin embargo, estos metabolitos no fueron capaces de inducir apreciablemente la actividad caspasa-9 ni caspasa-8 en nuestras condiciones experimentales. Por lo tanto, nuestros resultados sugieren que la vía mediada por estrés en el retículo endoplasmático podría ser la implicada en la acción pro-apoptótica del 11,12-EET y 14,15-EET.

Estudios previos demostraron que el aumento de la concentración citosólica de calcio (i[Ca^{2+}]) es un componente importante en la inducción de la apoptosis (Caron-Leslie y Cidlowski, 1991). Nakagawa y Yuan (2000) propusieron que el incremento de i[Ca^{2+}] permite el desplazamiento de las calpaínas hacia la superficie del retículo endoplasmático, donde activan a la caspasa-12. En este sentido, Martínez y Moreno (2005) demostraron

recientemente que los EETs pueden inducir el flujo de i[Ca^{2+}] en los fibroblastos 3T6, y este proceso fue sensible a bloqueadores de los canales de Ca^{2+} vía receptor, pero no los bloqueadores de los canales de Ca^{2+} dependientes de voltaje. Estos resultados nos permiten hipotetizar que los EETs inducen apoptosis mediante la activación de las calpaínas y caspasa-12, provocados por el incremento del i[Ca^{2+}] inducido por los EETs, y estos acontecimientos estarían implicados en el efecto anti-proliferativo de estos eicosanoides sobre los fibroblastos 3T6. En este sentido, para confirmar esta hipótesis faltaría demostrar que los EETs son capaces de activar las calpaínas.

En conclusión, los metabolitos derivados del AA vía LOXs no están implicados en la regulación de la proliferación de los fibroblastos 3T6, mientras que los HETEs derivados del AA mediante los CYPs tienen efecto proliferativo sobre estas células, y parte de este efecto podría deberse a la activación de la vía PI3K/Akt. Los EETs, producidos por la actividad epoxigenasa de los CYPs, y los DHETEs inhiben el crecimiento celular e inducen apoptosis en nuestras condiciones experimentales. Por lo tanto, el papel de los metabolitos derivados del AA por la vía de los CYPs sobre la proliferación de los fibroblastos 3T6 vendría determinado por el balance en la producción de HETEs y EETs.

6. CONCLUSIONES

- Los metabolitos derivados del AA vía LOXs como el LTB_4 y los CysLTs no parecen estar implicados en el control de la proliferación de los fibroblastos 3T6.

- Los metabolitos del AA producidos por la vía 5-LOX participan en la regulación de la proliferación de los macrófagos RAW 264.7. El LTB_4 y LTD_4 tienen efecto co-mitogénico sobre los macrófagos RAW 264.7 cultivados en presencia de suero.

- En ausencia de factores de crecimiento, el LTB_4 y LTD_4 presentan efecto proliferativo, y en este proceso participan las vías MAPK y PI3K/Akt.

- La PGE_3 y el LTB_5 derivados del EPA estimulan la proliferación de los macrófagos RAW 264.7 de forma similar a la PGE_2 y el LTB_4 formados a partir del AA.

- El 5-HETE, 12(*S*)-HETE, 15(*S*)-HETE y 20-HETE, metabolitos derivados del AA mediante la actividad hidroxilasa de los CYPs, parecen tener un papel co-mitogénico sobre los fibroblastos 3T6 estimulados con suero.

- En ausencia de factores de crecimiento, el 5-HETE, 12(*S*)-HETE y 15(*S*)-HETE inducen la proliferación de los fibroblastos 3T6, y este efecto es dependiente de la activación de la vía PI3K/Akt.

- El 5,6-EET, 8,9-EET, 11,12-EET y 14,15-EET, metabolitos derivados de la actividad epoxigenasa de los CYPs, y el 5,6-DHETE y 11,12-DHETE, provenientes de la hidratación de los EETs, tienen efecto anti-proliferativo y pro-apoptótico sobre fibroblastos 3T6 estimulados con PDGF.

- El efecto pro-apoptótico inducido por el 11,12-EET y el 14,15-EET parece mediado a través de la activación de la caspasa-12 y la caspasa-3.

- En resumen, la proliferación de los fibroblastos 3T6 estaría regulada por los metabolitos del AA producidos por la vía de las COXs y por la vía de los CYPs. En este último caso, están implicados tanto los metabolitos derivados de la actividad hidroxilasa, los HETEs, como de la actividad epoxigenasa, los EETs, con efectos contrapuestos.

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