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ESTUDIO DE LA RESPUESTA POSTPRANDIAL DE CÉLULAS VASCULARES Y SU IMPLICACIÓN EN LA ATEROSCLEROSIS

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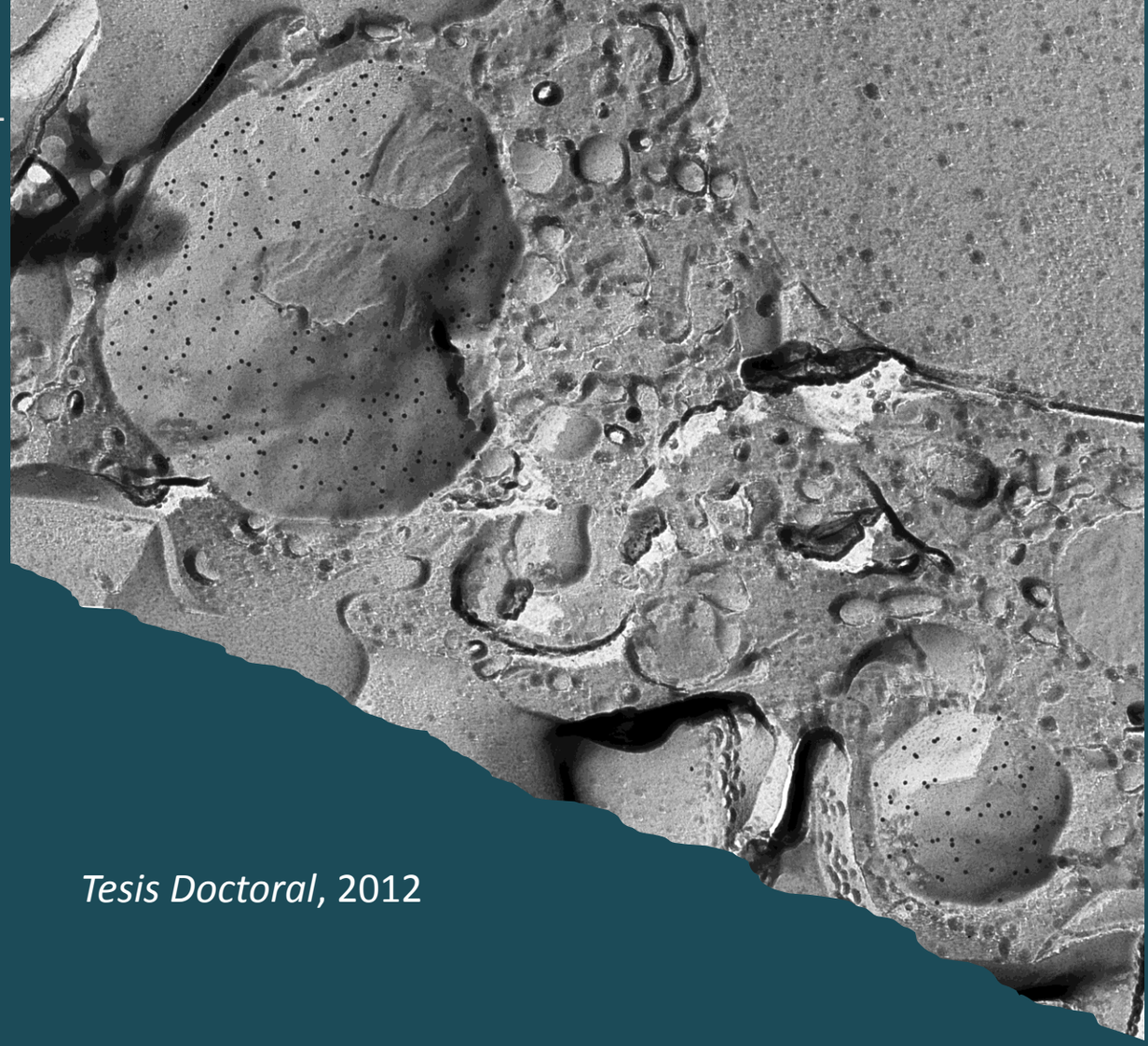

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Tesis Doctoral

"ESTUDIO DE LA RESPUESTA POSTPRANDIAL DE CÉLULAS VASCULARES Y SU IMPLICACIÓN EN LA ATEROSCLEROSIS"

Lourdes María Varela Pérez, 2012

Tesis presentada para optar al Grado de Doctor por la
Universidad de Sevilla:

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CERTIFICAN: que la Tesis Doctoral titulada "ESTUDIO DE LA RESPUESTA POSTPRANDIAL DE CÉLULAS VASCULARES Y SU IMPLICACIÓN EN LA ATEROSCLEROSIS", realizada por Lourdes María Varela Pérez para optar al grado de Doctor, ha sido llevada a cabo bajo nuestra dirección en el laboratorio de Nutrición Celular y Molecular del Instituto de la Grasa, perteneciente al Consejo Superior de Investigaciones Científicas.

Sevilla, Junio de 2012

Fdo: Dra. Rocío Abia González Fdo: Dr. Francisco J García Muriana Fdo: Dr. Sergio López Martín

A mis padres y a Daniel,

«La vida está hecha de momentos, trocitos de mica en un estrecho y largo camino de granito. Sería maravilloso que nos llegaran intactos, pero eso no puede ocurrir. Tenemos que aprender a vivir, vivir realmente… amar el viaje, no el destino»

Anna Quindlen (novelista y periodista)

ABREVIATURAS

ACV	Accidente cerebrovascular
ADMA	Dimetilarginina asimétrica
ADRP	*Adipose differentiation-related protein*/perilipina 2
AGE	Productos finales avanzados de la glicosilación
Apo	Apolipoproteína
ApoB48R	Receptor de apoB48
ASO	Oligonucleótidos antisentido
ATGL	Lipasa de triglicéridos de tejido adiposo
bFGF	Factor de crecimiento de fibroblastos básico
BMP	Proteína morfogenética ósea
CETP	Proteínas de transferencia de ésteres de colesterol
CM	Quilomicrones
CREB	*cAMP response element-binding*
DHA	Ácido docosahexaenoico
ECV	Enfermedades cardiovasculares
EGF	Factor de crecimiento de la epidermis
EPA	Ácido eicosapentaenoico
ETS-1	Factor de transcripción de la familia *E-twenty six*
FABP4/aP2	Proteína 4 de unión a los ácidos grasos
FF2/ALX	Receptor de la lipoxina
fMLP	N-formil-metionil-leucil-fenilalanina
GEF	Factor de intercambio de nucleótidos de guanina
HbA1c	Hemoglobina glucosilada
HB-EGF	*Heparin-binding EGF-like growth factor*
hCASMCs	Células de músculo liso de arteria coronaria humana
HDL	Lipoproteínas de alta densidad
HO-1	Hemo oxigenasa-1
HSL	Lipasa sensible a hormonas
IDL	Lipoproteínas de densidad intermedia
IGF	Factor de crecimiento como la insulina

IMC	Índice de masa corporal
LCAT	Lecitina colesterol acil-transferasa
LDL	Lipoproteínas de baja densidad
LDLR	Receptores de LDL
LPL	Lipoproteína lipasa
LPS	Lipopolisacáridos
LRP	Proteína relacionada con LDLR
MAPK	Quinasas activadas por proliferadores peroxisomales
MARCO	*Macrophage receptor with collagenous structure*
MMP	Metaloproteinasas de la matriz
MUFA	Ácidos grasos monoinsaturados
NO	Óxido nítrico
OXPAT	*Oxidative tissue-enriched PAT protein*
PAD	Presión arterial diastólica
PAI-1	Inhibidor tipo 1 del activador del plasminógeno
PAS	Presión arterial sistólica
PC	Perímetro de cintura
PDGF	Factor de crecimiento derivado de plaquetas
PGC-1α	Coactivador-1 alfa de PPARγ
PKA	Proetína quinasa A
PKR	Proteína quinasa de ARN de doble cadena
PLIN	Perilipina 1
PLTP	Proteína de transferencia de fosfolípidos
PMA	Forbol-12-miristato-13-acetato
PP	Presión de pulso
PPAR	Receptores activados por proliferadores peroxisomales
PPRE	Elementos de respuesta a los PPAR
PUFA	Ácidos grasos poliinsaturados
S-1-P	Esfingosina-1-fosfato
Sf	Índice de flotación de Svedberg
SFA	Ácidos grasos saturados
SMC	Células de músculo liso
SCARA-5	Receptor residual A-5

SR	Receptores residuales
SR-A	Receptores residuales de la clase A
SRCL	Receptores residuales con lectina del tipo C
S-X-PR	Receptores de esfingosina-X-fosfato acoplados a proteínas G
TGFβ1	Factor β1 de crecimiento transformante
TIMP	Inhibidores tisulares específicos de MMP
TIP47	*Tail-interacting protein of 47 kDa*/perilipina 3
TNFα	Factor de necrosis tumoral alfa
t-PA	Activador del plasminógeno tipo tisular
TLR	*Toll-like receptors*
TRL	Lipoproteínas ricas en triglicéridos
uPAR	Receptor activador del plasminógeno tipo-uroquinasa
UPR	Respuesta a la desnaturalización de proteínas
VLDL	Lipoproteínas de muy baja densidad
VLDLR	Receptor de VLDL
8-OHdG	8-hidroxi-2-deoxiguanosina

ÍNDICE

INTRODUCCIÓN

1. DIETA, NUTRICIÓN, Y ENFERMEDAD CARDIOVASCULAR

1.1. Enfermedades cardiovasculares

Las enfermedades cardiovasculares (ECV) son la primera causa de muerte en todo el mundo. Son aquellas que afectan al corazón y/o a los vasos sanguíneos. Entre ellas se incluyen las cardiopatías coronarias (ataque cardíaco/infarto agudo de miocardio), las enfermedades cerebrovasculares (accidente cerebrovascular o ACV/apoplejía/ictus), las vasculopatías periféricas, las cardiopatías reumáticas, las cardiopatías congénitas, y la insuficiencia cardíaca (**Figura 1**).

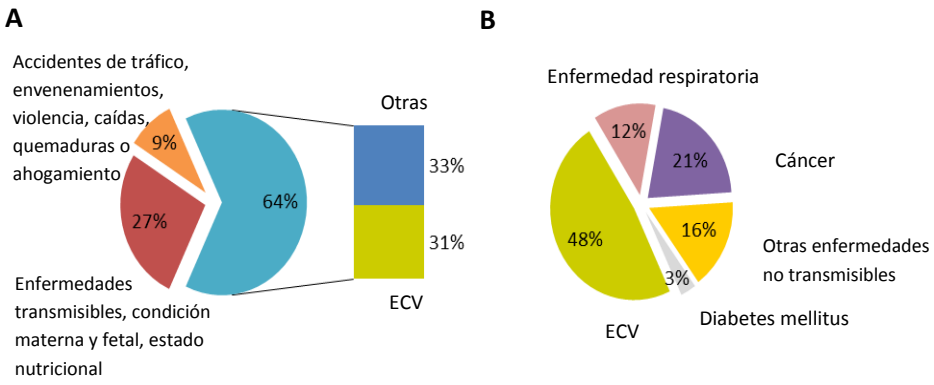

Figura 1. Tasa de mortalidad en todo el mundo por causa de fallecimiento (A) y por enfermedades no transmisibles (B), para ambos sexos.

Las cardiopatías isquémicas y las enfermedades cerebrovasculares son las principales causas de muerte en hombres y en mujeres, respectivamente. Según las estimaciones de la Organización Mundial de la Salud (OMS), más de 17 millones de personas mueren cada año en el mundo de infarto agudo de miocardio o de ACV (OMS, 2011). Se calcula que en 2030 esta cifra supere los 23 millones de personas. En España, los últimos datos aportados por el Instituto Nacional de Estadística (INE), ponen de manifiesto que las ECV han sido responsables del 31,2% de los fallecimientos en 2009. También se estima

que el 80% de las muertes prematuras por ECV podrían evitarse, mediante cambios en los hábitos de vida, especialmente a través de una alimentación y actividad física adecuada, y un continuo progreso en las técnicas de diagnóstico y los tratamientos.

1.2. Factores de riesgo cardiovascular

Un factor de riesgo es aquel hábito adquirido o signo biológico que se asocia con una mayor frecuencia de padecer una determinada enfermedad. Entre los factores de riego para las ECV están los no modificables o intrínsecos de cada persona, como la edad, el sexo, y los antecedentes familiares; y los modificables o evitables, como la dieta inadecuada, el sedentarismo, el consumo de tabaco, el sobrepeso/obesidad, la diabetes tipo 2, la hipertensión arterial, y las dislipemias (FEC, 2009). Un aspecto importante a considerar es que la modificación favorable de dichos factores de riesgo se asocia a una reducción en el número de eventos cardiovasculares, tanto si se inicia antes de la aparición de las primeras manifestaciones clínicas (prevención primaria) como después (prevención secundaria). Las prevalencias estandarizadas de los principales factores de riesgo cardiovascular modificables en la población española de 35 a 74 años son las siguientes (Grau et al., 2011):

- Hipertensión arterial y dislipemias > 40%
- Obesidad y consumo de tabaco: 27%
- Diabetes: 13%

La hipertensión arterial (presión arterial sistólica (PAS) $\geq$140 mm Hg y diastólica (PAD) $\geq$90 mm Hg) está relacionada con el 46,4% de los fallecimientos por ACV y con el 42% por cardiopatías isquémicas (Gómez-Marcos et al., 2009). Este incremento de riesgo de episodios cardiovasculares suele estar asociado a la aparición de lesiones en diferentes órganos, como la lesión renal (microalbuminuria), la hipertrofia ventricular izquierda o el incremento del grosor de la capa íntima-media de la carótida como marcadores subrogados de la aterosclerosis (García-Ortiz et al., 2009). En la

población general, la relación entre la morbimortalidad cardiovascular con la PAS y la PAD es continua (aumenta de forma lineal a partir de valores de 115 mm Hg de PAS y de 75 mm Hg de PAD), tanto para ACV, como para cardiopatías isquémicas (Lewington et al., 2002; Guo et al., 2011). Sin embargo, en estudios observacionales, especialmente en ancianos, se ha verificado una relación directa de dicha morbimortalidad cardiovascular con la PAS, pero inversa con la PAD, manteniendo una relación directa y significativa con la presión de pulso (PP) o presión diferencial (Pinto, 2007; Frese et al., 2011). El aumento de la PP indica un aumento de la rigidez de las grandes arterias y, en consecuencia, de lesión en un órgano diana (Laurent et al., 2006).

En la práctica clínica actual, los parámetros que habitualmente se manejan para valorar el riesgo cardiovascular asociado a las dislipemias son el colesterol total, el colesterol LDL (lipoproteínas de baja densidad), el colesterol HDL (lipoproteínas de alta densidad), y en menor medida los triglicéridos en plasma y en ayunas. Mientras que el colesterol total (≥5,2 mmol/L) y el colesterol LDL (≥3,4 mmol/L) elevados son factores de riesgo cardiovascular, los estudios epidemiológicos han establecido que bajas concentraciones (<1,0 mmol/L en hombres y <1,2 mmol/L en mujeres) de colesterol HDL también aumentan el riesgo cardiovascular (Cooney et al., 2009; Elis et al., 2011). En distintos estudios, como en el Framingham (pionero en 1948), se ha establecido que el colesterol HDL protege frente a las cardiopatías isquémicas. Estos estudios han sugerido que una concentración baja de colesterol HDL puede ser incluso un factor de riesgo más determinante que una concentración elevada de colesterol LDL (Barter, 2011). Adicionalmente, las concentraciones elevadas (≥1,7 mmol/L) de triglicéridos (en forma de VLDL, lipoproteínas de muy baja densidad) también aumentan el riesgo relativo de cardiopatías isquémicas (Bayturan et al., 2010). A la vista de las evidencias científicas actuales, las recomendaciones recogidas en las guías internacionales de prevención cardiovascular incluyen, cada vez más frecuentemente, la consideración de objetivos terapéuticos para el colesterol HDL y los triglicéridos (NCEP, 2004; ESC, 2011).

En las sociedades industrializadas, la prevalencia de la obesidad (índice de masa corporal, IMC ≥30 kg/m^2) sigue aumentando con carácter epidémico, del mismo modo que la diabetes tipo 2, y al contrario de lo que sucede con otros factores de riesgo cardiovascular, como la hipertensión arterial o el colesterol LDL elevado (Villar et al., 2007). El tejido adiposo, particularmente la grasa intra-abdominal (visceral), es un órgano endocrino metabólicamente activo que puede inducir hiperinsulinemia y resistencia a la insulina, dislipemia, hipertensión arterial, y un aumento en la secreción de ácidos grasos libres, mecanismos a través de los cuales aumenta el riesgo cardiovascular (Guía Europea de Prevención Cardiovascular en la Práctica Clínica, 2011). La reducción del peso está recomendada en las personas obesas y debe considerarse en los casos de sobrepeso (IMC ≥25 y <30 kg/m^2). No obstante, la distribución corporal del tejido adiposo puede ser más determinante que el peso corporal total para predecir las ECV; por ello, la OMS estima conveniente utilizar el índice perímetro de la cintura (PC <102 cm en hombres y <88 cm en mujeres) como un indicador adicional de riesgo cardiovascular en cada categoría de IMC (OMS, 2005).

La diabetes es una enfermedad metabólica que se caracteriza por hiperglucemia. Está causada por defectos en la secreción de insulina, en la acción de la insulina o en ambos mecanismos. Los criterios de diagnóstico incluyen la hiperglucemia (≥11,1 mmol/L, y en presencia de síntomas como la poliuria, polidipsia o pérdida de peso inexplicada) al azar; la hiperglucemia (≥7 mmol/L) en ayunas; la hiperglucemia (≥11,1 mmol/L) a las 2 h tras una sobrecarga oral de 75 g de glucosa; y una hemoglobina glucosilada (HbA1c) ≥6,5% (ADA, 2012). En la diabetes tipo 1, el páncreas no produce insulina; ocurre con mayor frecuencia en los niños y adultos jóvenes (Peters et al., 2011). En la diabetes tipo 2, se produce una disminución de la sensibilidad periférica a la insulina, en adultos, obesos, sedentarios, y con historia familiar de diabetes (Uusitupa et al., 2011). Las mujeres embarazadas también pueden desarrollar la denominada diabetes gestacional (3-10% de los casos). La aceleración del proceso aterosclerótico en el diabético es un hecho constatado y probado que ha llevado a establecer, desde el punto de vista del riesgo

cardiovascular, la equivalencia entre ser diabético y la prevención secundaria de un evento coronario (Leosdottir et al., 2011). Aproximadamente el 65% de los diabéticos fallecen a consecuencia de ECV, en parte debido a la propia diabetes (el riesgo cardiovascular se multiplica por dos en hombres y por cuatro en mujeres), pero también debido a su frecuente asociación con otros factores de riesgo cardiovascular, como la hipertensión arterial, las dislipemias, y la obesidad (Raj, 2012).

1.3. La dieta influye en la salud

Los alimentos, en toda su variedad cultural, determinan en gran medida el crecimiento, el desarrollo y la salud de las personas que los consumen. Las proteínas, los hidratos de carbono, y las grasas representan los macronutrientes que proporcionan la energía, los elementos constructivos y de mantenimiento, y los reguladores de los procesos metabólicos necesarios para adquirir y mantener un estado completo de bienestar físico, mental, y social (OMS, 2006).

Los estudios realizados sobre aspectos evolutivos de la dieta indican que los cambios ocurridos al menos en los últimos 100 años, especialmente en la ingesta del tipo y cantidad de grasas, han aumentado considerablemente la incidencia de ECV (Zatoński et al., 2011). En la actualidad, las sociedades industrializadas se caracterizan, desde el punto de vista nutricional, por un desequilibrio energético, es decir, se consume más de lo que se necesita; y esta sobrealimentación, en lo que se refiere a las grasas, se caracteriza por una mayor ingesta de grasas saturadas (Márquez-Sandoval et al., 2008).

1.3.1. Los ácidos grasos en la dieta

Las grasas alimentarias incluyen todos los lípidos de los tejidos vegetales y animales que se ingieren como alimentos. Las grasas (sólidas a temperatura

ambiente) o aceites (líquidos a temperatura ambiente) están constituidas preferentemente por los triglicéridos [ésteres de 3 moléculas de ácidos grasos con una molécula de glicerol, (**Figura 2**)]. En función del tipo de ácidos grasos que predomine en dichas grasas, y en particular por el grado de insaturación (número de dobles enlaces) de dichos ácidos grasos, las grasas alimentarias también se distinguen en grasas saturadas, monoinsaturadas, y poliinsaturadas.

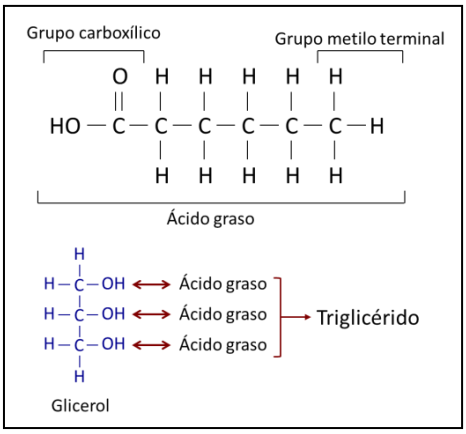

Figura 2. Estructura de los triglicéridos.

Los ácidos grasos son cadenas hidrocarbonadas generalmente lineales, con número par de átomos de carbono (suele oscilar entre 4 y 36), con un grupo carboxilo en uno de los extremos. Cada átomo de carbono se une al siguiente y al precedente mediante un enlace covalente sencillo o doble (**Figura 3**). La fórmula básica de una molécula de ácido graso con todos sus enlaces sencillos (completamente saturada) es CH_3-$(CH_2)_n$-COOH (ácidos grasos saturados, SFA). También existen ácidos grasos insaturados, con dobles enlaces en su cadena alquílica: un doble enlace (ácidos grasos monoinsaturados, MUFA) o varios dobles enlaces (ácidos grasos poliinsaturados, PUFA). En la naturaleza, la configuración que predomina en los dobles enlaces de los ácidos grasos es la *cis* (~95%), donde los dos átomos de hidrógeno, cada uno perteneciente a los átomos de carbono unidos por el doble enlace, se localizan en el mismo eje de

simetría (Steinhart et al., 2003). Esta configuración confiere una alta flexibilidad y se asocia, en parte, con las funciones biológicas de estos ácidos grasos *cis* (Law et al., 2005). Sin embargo, la isomería *trans* (átomos de hidrógeno en diferente plano de simetría) es termodinámicamente más estable que la *cis*; por lo cual, mediante procedimientos físicos (temperatura, presión) y/o químicos (pH, catalizadores metálicos), como los que se producen durante la hidrogenación parcial de las grasas vegetales (Gebauer et al., 2011), los dobles enlaces *cis* pueden cambiar su geometría a la configuración *trans* (Ferreri & Chatgilialoglu, 2005). Los ácidos grasos *trans* también se generan en el rumen de los animales poligástricos (Iwata et al., 2011).

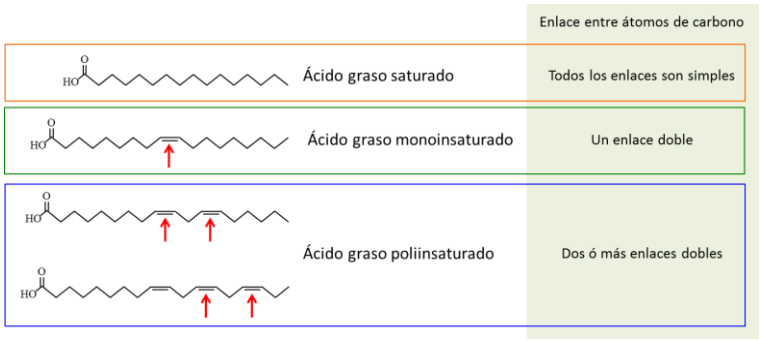

Figura 3. Tipos de ácidos grasos (de acuerdo al número de dobles enlaces entre átomos de carbono).

La mayoría de los ácidos grasos tienen un nombre común, pero además, todos ellos se pueden nombrar mediante la nomenclatura sistemática y la abreviada. La nomenclatura sistemática sigue las reglas adoptadas por la IUPAC, donde se indica la longitud de la cadena y el número, posición y configuración de los dobles enlaces contando el número de átomos de carbono a partir del grupo carboxilo (Δ). En la nomenclatura abreviada, el ácido graso se simboliza por dos números separados por dos puntos, indicando el primero la longitud de la cadena y el segundo el número de dobles enlaces. A continuación, se indica la posición en la que se encuentra el doble enlace, ya sea contando el número de átomos de carbono a partir del grupo carboxilo (Δ)

o a partir del grupo metilo terminal (n ó ω). Finalmente, se indica la configuración *cis* (c) o *trans* (t) del doble enlace (**Tabla 1**). La nomenclatura que se usa en nutrición y en medicina, con frecuencia obvia la letra Δ. Como ejemplos: el ácido palmítico es el más común de los SFA, su nombre sistemático es ácido hexadecanoico (abreviatura: C16:0; 16 átomos de carbono, ningún doble enlace); el ácido oleico es el principal MUFA, su nombre sistemático es ácido *cis*-9-octadecenoico (C18:1c9, C18:1ω-9; 18 átomos de carbono, con un doble enlace *cis* en el carbono 9 ó ω-9); entre los PUFA, el ácido linoleico con nombre sistemático de ácido *cis*,*cis*-9,12-octadecadienoico (C18:2c9c12, C18:2ω-6; 18 átomos de carbono, con dos dobles enlaces *cis* en los carbonos 9 y 12 ó ω-6 y ω-9, respectivamente) y el ácido alfa-linolénico (C18:3c9c12c15, C18:3ω-3; 18 átomos de carbono, con tres dobles enlaces *cis* en los carbonos 9, 12, y 15 ó ω-3, ω-6, y ω-9, respectivamente). Por lo tanto, el ácido oleico pertenece a la familia de ácidos grasos ω-9, el ácido linoleico a la familia ω-6, y el ácido alfa-linolénico a la familia ω-3. Los ácidos grasos linoleico y alfa-linolenico se consideran ácidos grasos esenciales. Otros ácidos grasos de gran interés biológico son el ácido eicosapentaenoico (C20:5ω-3; EPA) y el ácido docosahexaenoico (C22:6ω-3; DHA).

En la **Figura 4**, se recoge la composición media de ácidos grasos de distintas fuentes de grasa utilizadas en la dieta.

Tabla 1. Nomenclatura de algunos ácidos grasos de los alimentos.

Nombre común	Nombre sistemático	Nombre abreviado
Láurico	Dodecanoico	12:0
Mirístico	Tetradecanoico	14:0
Palmítico	Hexadecanoico	16:0
Esteárico	Octadecanoico	18:0
Araquídico	Eicosanoico	20:0
Lignocérico	Tetracosanoico	24:0
Palmitoleico	Cis-9-hexadecenoico	16:1 7c
Oleico	Cis-9-octadecenoico	18:1 9c
Erúcico	Cis-13-docasaenoico	22:1 9c
Linoleico	Cis,cis-9,12-octadecadienoico	18:2 6c,9c
α-linolénico	Cis,cis,cis-9,12,15-octadecatrienoico	18:3 3c,6c,9c
γ-linolénico	Cis,cis,cis-6,9,12-octadecatrienoico	18:3 6c,9c,12c
dihomo-γ-linolénico	Cis,cis,cis-8,11,14-eicosatrienoico	20:3 6c,9c,12c
Araquidónico	All-cis-5,8,11,14-eicosatetraenoico	20:4 6c, 9c, 12c, 15c
EPA	All-cis-5,8,11,14,17-eicosapentaenoico	20:5 3c, 6c, 9c, 12c, 15c
DHA	All-cis-4,7,10,13,16,19-docosahexaenoico	22:6 3c, 6c, 9c, 12c, 15c, 18c

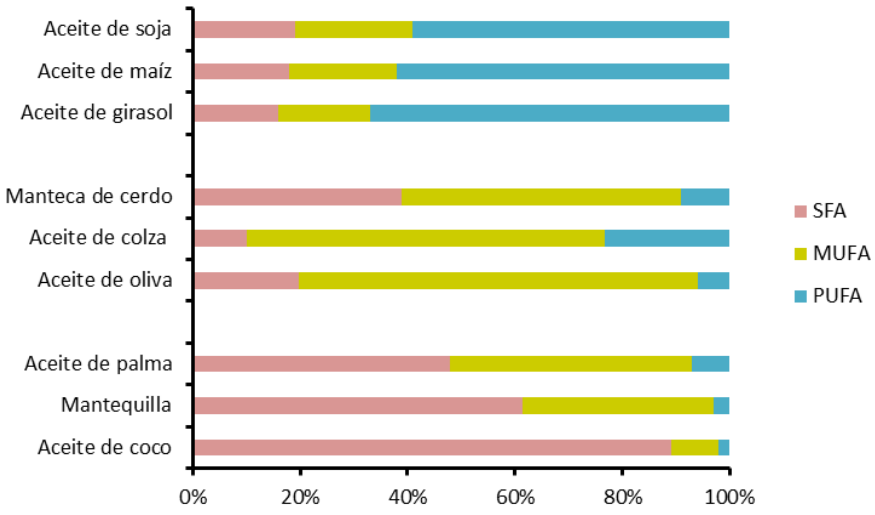

Figura 4. Porcentaje de los distintos tipos de ácidos grasos presentes en algunas grasas comestibles.

1.3.2. Ácidos grasos y factores de riesgo cardiovascular

Una dieta equilibrada es aquella que proporciona los alimentos en cantidad y diversidad adecuada para mantener un estado de salud (física, mental, y social). Depende de múltiples factores como el sexo, la altura, el peso, la edad, la actividad física, el clima, y el entorno. El primer objetivo de la dieta es el aporte de la energía requerida para llevar a cabo los procesos metabólicos y de trabajo físico diario; para ello existen las recomendaciones nutricionales de ingesta calórica diaria, que mayoritariamente coinciden con una distribución porcentual de calorías de los macronutrientes: 55-60% de los hidratos de carbono, 12-15% de las proteínas, y 30-35% de las grasas (EUFIC; www.eufic.org/index/es/). Respecto a la ingesta de grasas, estos valores no son sólo cuantitativos sino también cualitativos, de tal manera que se recomienda un consumo <10% de SFA, <7% de PUFA (~5% de la familia ω-6 y ~1% de la familia ω-3), <1% de ácidos grasos *trans*, y el resto (hasta el 30-35% del total de la energía) de MUFA.

Numerosos estudios epidemiológicos han establecido una relación causal entre los ácidos grasos de la dieta y el riesgo de ECV (Shay et al., 2011; Larsson et al., 2012). En 1970, las investigaciones de Ancel Keys (el Estudio de los Siete Países) aportaron la primera evidencia de los beneficios cardiovasculares de una dieta rica en ácido oleico (Keys, 1970). En países como Grecia, donde el aporte de calorías procedentes de las grasas superaba el 40% del total (con predominio del ácido oleico por la ingesta de aceite de oliva), el índice de mortalidad por ECV era más bajo que en países con menor ingesta de grasas pero con predominio de las saturadas. A raíz de este estudio surge el concepto de un estilo de vida, basado en una dieta rica en productos vegetales (aceite de oliva, verduras, legumbres, y fruta) y reducida en productos de origen animal (excepto en pescado azul), que se denominó dieta mediterránea (Moreiras-Varela, 1989). Como recurso didáctico, se diseñó una guía dietética en forma piramidal (pirámide de la dieta mediterránea) dividida en dos grandes zonas: la próxima a la base, que contiene los alimentos de consumo diario o semanal, y la próxima al vértice, que recoge los alimentos de consumo

ocasional (**Figura 5**). El 16 de noviembre de 2010, en Nairobi (Kenia), el Comité Intergubernamental de la UNESCO para la Salvaguardia del Patrimonio Cultural Inmaterial acordó inscribir la Dieta Mediterránea en la Lista representativa del Patrimonio Cultural Inmaterial de la Humanidad.

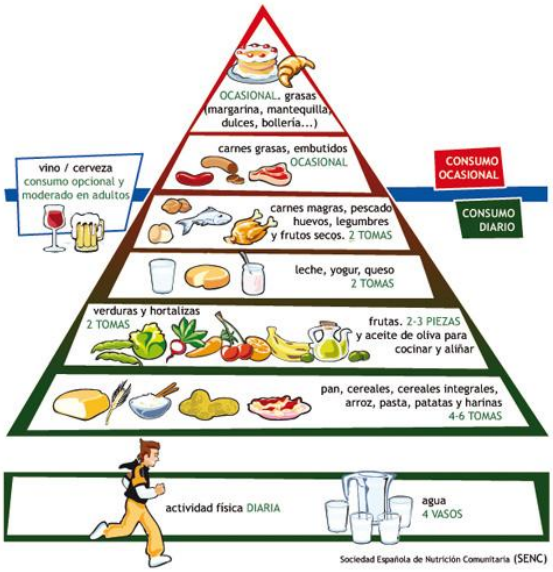

Figura 5. La pirámide nutricional de la dieta mediterránea muestra los principales alimentos y su frecuencia recomendada de consumo. También fomenta la actividad física regular.

A partir de del estudio de los Siete países, muchos otros han tratado de dilucidar los procesos metabólicos, relacionados con el desarrollo y la progresión de las ECV, que se afectan de manera diferencial por el tipo de ácidos grasos en la dieta. Los trabajos clásicos realizados en este campo se han basado en las modificaciones del perfil lipídico en plasma durante el metabolismo inter-digestivo (ayuno), fomentados por la aceptación de la hipótesis del colesterol total y sobretodo del colesterol asociado a las LDL (hipótesis de la hipercolesterolemia) (Yamada, 2011). Existe un consenso sobre el papel pro-aterogénico de los SFA (que aumentan la concentración de colesterol LDL) y el anti-aterogénico de los MUFA y PUFA (que reducen el

colesterol LDL), de forma particular en personas hipercolesterolémicas (Clifton et al., 2009). Así, la reducción plasmática de LDL tras la sustitución isocalórica de tan sólo el 5% de la energía procedente de los SFA por MUFA (ó PUFA) puede reducir hasta un 40% el riesgo de padecer ECV (Kris-Etherton, 1999). Entre los SFA, el ácido esteárico (C18:0) no induce los efectos hipercolesterolémicos del ácido palmítico (C16:0), ácido láurico (C14:0), y ácido mirístico (C12:0) (Tojo & Leis, 2005). Con respecto al colesterol HDL, se conoce que el consumo de MUFA eleva su concentración plasmática (López-Miranda et al., 2010) y que los PUFA de la familia ω-6 la disminuyen (Kralova Lesna et al., 2008); este efecto de los MUFA, como aceite de oliva, tiene lugar tanto en el contexto de la dieta mediterránea (Fitó et al., 2007) como fuera de éste (Covas et al., 2006).

La aterogenicidad de las LDL también se asocia con la susceptibilidad de ser modificadas mediante reacciones oxidativas (hipótesis de la modificación oxidativa de las LDL) (Steinberg, 2009). Las LDL ricas en PUFA son más proclives a la oxidación que LDL ricas en MUFA (Cicero et al., 2008). Se conoce que los componentes menores del aceite de oliva (virgen), destacando la presencia de α-tocoferol (por su acción como vitamina E), el β-caroteno como provitamina A, y los polifenoles, protegen a las LDL del estrés oxidativo (Raederstorff, 2009; Covas, 2008).

Algunos autores han descrito que los MUFA, cuando sustituyen a los SFA en la dieta, pueden disminuir la concentración plasmática de triglicéridos totales en personas sanas (Belarbi et al., 2011). Este efecto igualmente se observa incluso con dosis encapsuladas de aceite de oliva (4 g/día) en personas con concentraciones ligeramente altas de colesterol en plasma (Violante et al., 2009). Una dieta enriquecida con ácido oleico (aceite de oliva), a diferencia de una dieta enriquecida con ácido α-linolénico (nueces), contribuye además a reducir el contenido de colesterol, triglicéridos, y la proporción triglicéridos/apoB en las VLDL de personas con un alto riesgo cardiovascular (Perona et al., 2010). Se ha demostrado que los PUFA de cadena larga de la familia ω-3, EPA y DHA, también tienen un marcado efecto

hipotrigliceridémico, tanto en personas sanas como en hiperlipémicas (Kobayashi et al., 2007; Skulas-Ray et al., 2011).

Además de los efectos sobre los lípidos plasmáticos, en el Estudio de los Siete Países se observó una menor prevalencia de la hipertensión arterial en las poblaciones que consumían aceite de oliva (ácido oleico como principal ácido graso en la dieta) (Keys, 1980). Estudios posteriores han confirmado el efecto hipotensor de los MUFA en personas con una ingesta similar de sodio, potasio, y calcio, y sin diferencias en la excreción urinaria de sodio (Lahoz et al., 1999). Dicho efecto no parece estar regulado por las prostaglandinas, la liberación de renina, o las catecolaminas plasmáticas, sino, al menos en parte, por cambios en la composición lipídica de las membranas celulares, alterando la fluidez y el transporte iónico transmembrana (Barceló et al., 2009). Es probable que un aumento de la biodisponibilidad de óxido nítrico (NO) sea uno de los mecanismos implicados en la capacidad hipotensora del aceite de oliva, puesto que su ingesta se relaciona con una disminución de la concentración plasmática de dimetilarginina asimétrica (ADMA; inhibidor endógeno de la NO sintasa) y de la proporción L-arginina/ADMA (Thomazella et al., 2011). También se ha descrito una correlación positiva entre la presión arterial y la insulina (Schlaich et al., 2012). La insulina puede aumentar la retención de sodio y la actividad simpática, modificar la respuesta vascular y la actividad de ciertos factores de crecimiento (Vicent et al., 2003; Eringa et al., 2012). De hecho, dietas ricas en MUFA (aceite de oliva), cuando se comparan con dietas pobres en grasa, disminuyen la glucemia en ayunas en personas asintomáticas con diferentes factores de riesgo cardiovascular (Estruch et al., 2006). Es interesante destacar que dietas ricas en MUFA (>12% del total de la ingesta calórica total a partir del ácido oleico), respecto a dietas pobres en MUFA ($\leq$12%), inducen a medio y largo plazo una disminución de la concentración en plasma en ayunas de glucosa, insulina, HbA1c, y de la resistencia periférica a la insulina en personas con diabetes tipo 2, con una reducción del 11% en el riesgo de padecer ECV (Schwingshacki et al., 2011; Schwingshackl & Strasser, 2012). Los ácidos grasos EPA y DHA disminuyen los valores de presión arterial (2-5 mm Hg) en normotensos e hipertensos mediante diferentes mecanismos

de acción (Poudyal et al., 2011; Mozaffarian & Wu, 2011), pero no parecen evidentes sus efectos en el control glucémico, al menos a dosis supranutricionales (Akinkuolie et al., 2011).

En su conjunto, estas observaciones determinan la importancia (superioridad) nutricional y funcional (valor biológico) de los MUFA (ácido oleico del aceite de oliva) sobre los PUFA y SFA en la prevención primaria y secundaria de las ECV.

2. ATEROSCLEROSIS

La patología de base de las ECV es la aterosclerosis, una enfermedad que se caracteriza por la acumulación de lípidos, células inflamatorias, y tejido fibroso en la pared de las arterias, que constituye la placa de ateroma (Woollard & Geissmann, 2010). Este proceso se inicia durante la infancia o la adolescencia y su progresión está relacionada con los factores de riesgo cardiovascular (Insull, 2009).

La placa de ateroma se localiza comúnmente en sitios de bifurcación del árbol circulatorio, o bien en aquellos lugares en los que se producen cambios súbitos de velocidad y/o de dirección del flujo sanguíneo (Kalva & Mueller, 2008), y supone modificaciones en la estructura normal de la pared arterial, que consta de tres capas celulares bien diferenciadas: la íntima, formada por una monocapa de células endoteliales asociadas entre sí para formar el lumen que contacta con la sangre, una membrana basal subendotelial, y una lámina elástica interna; la media, constituida por múltiples capas de células de músculo liso (SMC) entremezcladas con fibras elásticas; y la adventicia, formada por pequeños capilares y tejido conjuntivo. Los procesos proliferativos que se originan en las capas íntima y media, en respuesta a los acúmulos fibroadiposos, causan la invasión de la luz de las arterias que, junto a procesos trombóticos, pueden comprometer la funcionalidad circulatoria y generar situaciones de índole isquémicas (Badimon et al., 2011).

El desarrollo de la aterosclerosis puede clasificarse en dos grandes etapas: etapa temprana (silente y reversible) y etapa avanzada (puede ser no reversible y con complicaciones clínicas leves o severas dependiendo del grado de la lesión) (**Figura 6**).

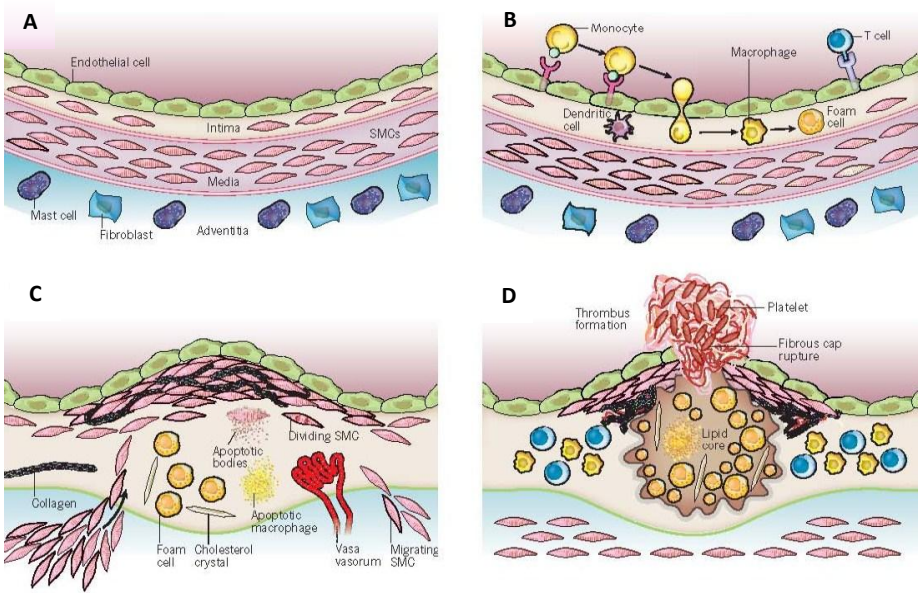

Figura 6. Diagrama que muestra el desarrollo de la lesión aterosclerótica. (A) Capas de pared vascular; (B) reclutamiento leucocitario y formación de células espumosas; (C) formación de la placa fibrosa; y (D) ruptura de la placa que puede generar un trombo.

2.1. Etapa temprana

La disfunción endotelial (descompensación en las funciones biológicas del endotelio) es uno de los principales fenómenos patológicos que se producen en la fase inicial de la aterosclerosis (Tousoulis et al., 2010); se caracteriza por un incremento de la permeabilidad del endotelio a las lipoproteínas plasmáticas (y de su modificación oxidativa), de la adhesión de leucocitos (reclutamiento leucocitario y migración directa de leucocitos), de producción de factores pro-trombóticos, anti-fibrinolíticos, y estimulantes del crecimiento

celular, y por un desequilibrio entre sustancias vasoactivas (dilatadoras y constrictoras) (Hirase & Node, 2012).

La estría grasa es la primera lesión detectable en estudios anatómicos de arterias, y se caracteriza por la acumulación de macrófagos derivados de monocitos con abundante contenido lipídico en su interior, también denominadas como células espumosas. Los linfocitos T que se infiltran en el espacio subendotelial desempeñan un papel importante en esta fase de la aterosclerosis. Los linfocitos T colaboradores o *helper* del linaje Th1 se activan por las citoquinas IL-12 e IFNγ secretadas por las células dendríticas; una vez activados, los linfocitos Th1 producen IL-2, IFNγ, TNFα (factor de necrosis tumoral alfa), y linfotoxina (TNFβ ó LTα) (Vinson et al., 2011). La principal función de los linfocitos Th1 es la activación de los monocitos por la vía clásica; estos monocitos se diferencian a macrófagos M1 con fenotipo pro-inflamatorio, que son los precursores de las células espumosas. Otro linaje de linfocitos es el de los linfocitos Th2, que se inducen de forma muy potente por IL-4 a partir de células TCD4 indiferenciadas (Murray & Wynn, 2011). Los linfocitos Th2 producen IL-4, IL-5, IL-9, IL-10, e IL-13, y su principal función es la activación y expansión de los linfocitos B y la producción de inmunoglobulinas (IgM, IgA, e IgE), aunque también inducen la activación de monocitos por la vía alternativa; estos monocitos se diferencian a macrófagos M2 con fenotipo anti-inflamatorio (Chawla et al., 2011). La estría grasa progresa cuando comienzan a predominar los macrófagos M1 (Bie et al., 2011).

2.2. Etapa avanzada

La lesión aterosclerótica, hasta su forma avanzada y fibrosa, evoluciona fundamentalmente conforme a tres procesos celulares (Miller et al., 2010): 1) la adhesión de monocitos circulantes e ingreso continuo en la íntima vascular, donde se diferencian a macrófagos, y la proliferación de las SMC y linfocitos; 2) la acumulación excesiva de lípidos, procedentes de LDL y lipoproteínas ricas en triglicéridos (TRL), en el espacio intra- y extracelular de macrófagos y SMC, con

el consiguiente aumento del número de células espumosas, y 3) la formación de una extensa matriz de tejido conectivo por la migración y transformación fenotípica de las SMC, que incrementan la síntesis de colágeno, fibras elásticas, y proteoglucanos.

La fase evolutiva de la estría grasa a la lesión fibroadiposa implica el aumento de las proporciones relativas de células espumosas, linfocitos T, y SMC. Por lo general, esta lesión está constituida por capas alternativas de células espumosas y SMC entre cantidades variables de tejido conjuntivo. En su interior puede formarse un núcleo lipídico, constituido por colesterol en forma cristalina y esterificado. El material lipídico extracelular procede de la necrosis y/o apoptosis de las células espumosas, lo que provoca una reacción inflamatoria-fibroproliferativa que es precursora de la placa fibrosa (Zheng et al., 2011).

La placa fibrosa tiene una morfología muy característica, pues su superficie está recubierta por una cápsula fibrosa de densidad variable, constituida por SMC con morfología aplanada y rodeadas de una matriz de tejido conjuntivo cuya composición básica es colágeno, fibras elásticas, y proteoglucanos. Esta matriz está a su vez envuelta por tejido conjuntivo laxo que contiene cantidades variables de macrófagos y linfocitos T. El núcleo de la cápsula fibrosa contiene células espumosas, células necróticas, desechos celulares, lípidos y, con más frecuencia a medida que avanza la lesión, cristales de bioapatita (fosfato cálcico) (Bennet, 2011). Todo este proceso suele acompañarse de una disminución continua del grosor de la media subyacente, debido probablemente a la migración de SMC desde la media hasta la íntima (Perrotta, 2011).

Las lesiones ateroscleróticas de mayor riesgo son aquellas en las cuales la capa fibrosa es menos densa, principalmente en los márgenes de la misma, con un menor número de SMC y un mayor número de macrófagos y linfocitos T, y más frágil a las alteraciones del flujo sanguíneo (Fagerber et al., 2010). Este estrés de la pared vascular puede resultar en fractura, fisura o ulceración de la

lesión, y la formación de trombo intravascular o hemorragia en el interior de la placa fibroadiposa, con la eventual oclusión aguda del vaso afectado (Derksen et al., 2011). No obstante, es posible revertir las lesiones ateroscleróticas, no sólo en las fases iniciales, sino también, aunque con mayor lentitud, en ciertas formas de lesiones más avanzadas (Insull, 2009).

2.3. Retención y modificación de lipoproteínas

Las lipoproteínas son las partículas encargadas del transporte de los lípidos en la sangre. Los ácidos grasos, en forma de triglicéridos re-sintetizados en el intestino o en el hígado, y el colesterol procedente de la dieta o de la síntesis hepática, se solubilizan en estas partículas; que están formadas por una fracción apolar (triglicéridos y ésteres de colesterol) en el interior y una envoltura polar (fosfolípidos, colesterol libre, y apolipoproteínas) (**Figura 7**).

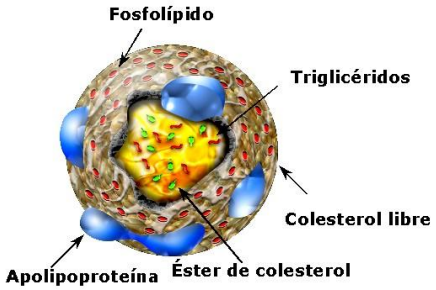

Figura 7. Representación de la estructura y composición de una lipoproteína.

Existen diferentes tipos de lipoproteínas, en función de su composición relativa de lípidos y apolipoproteínas (apo): quilomicrones (CM), VLDL, lipoproteínas de densidad intermedia (IDL), LDL, y HDL (**Tabla 2**).

Tabla 2. Características físico-químicas de las principales lipoproteínas plasmáticas. EC, ésteres de colesterol; FL, fosfolípidos; CL, colesterol libre.

	Densidad (g/mL)	Diámetro (nm)	Triglicéridos (%)	EC (%)	FL (%)	CL (%)	Apolipoproteína principal
CM	<0.95	80-100	90-95	2-4	2-6	1	apoB48
VLDL	0.95-1.006	30-80	50-65	8-14	12-16	4-7	apoB100
IDL	1.006-1.019	25-30	25-40	20-35	16-24	7-11	apoB100
LDL	1.019-1.063	20-25	4-6	34-35	22-26	6-15	apoB100
HDL	1.063-1.210	8-13	7	10-20	25	5	apoA-I

Los CM se forman en el intestino, tras la ingesta de alimentos grasos. Los triglicéridos se digieren y se absorben en forma de ácidos grasos y 2-monoglicéridos por los enterocitos. Estas mismas células, utilizando esos sustratos, re-sintetizan nuevos triglicéridos y los ensamblan con una molécula de apoB48 (además de apoA-I, apoA-II, y apoA-IV), fosfolípidos, y la proteína microsomal de transporte de triglicéridos. Mediante exocitosis, los CM se secretan a los capilares linfáticos del plexo mesentérico, de donde pasan a los vasos linfáticos, y finalmente al torrente circulatorio. En los capilares de los tejidos, los triglicéridos de los CM se hidrolizan parcialmente por la enzima lipoproteína lipasa (LPL). Los CM remanentes y no remanentes pueden interaccionar con las células sanguíneas y con cualquier tipo celular accesible, incluyendo las células de la pared vascular; también intercambian lípidos y proteínas con otras lipoproteínas circulantes de origen endógeno a través de las proteínas de transferencia de ésteres de colesterol (CETP) y de fosfolípidos (PLTP), consecuencia de lo cual disminuyen su contenido en apoA-I y apoA-IV, y aumentan el de ésteres de colesterol, apoC-I, apoC-II, apoC-III, y apoE (Xiao & Lewis, 2011).

El suministro de ácidos grasos a los tejidos extra-hepáticos durante el ayuno se realiza mediante la producción en el hígado y la liberación al torrente circulatorio de las VLDL. El 65% de los lípidos en las VLDL son triglicéridos y el

componente proteico está constituido mayoritariamente por una molécula de apoB100, aunque también transportan apoC-I, apoC-II, apoC-III, y apoE. Al igual que los CM, los triglicéridos de las VLDL también se hidrolizan parcialmente por la LPL, y se transforman en IDL. Los CM, VLDL, y sus remanentes son las TRL. Los ácidos grasos liberados se incorporan a las células donde pueden ser utilizados para obtener energía tras su β-oxidación (músculo), o bien ser almacenados como triglicéridos (tejido adiposo) (Mattijssen & Kersten, 2012). Las IDL, tras sucesivas hidrólisis de sus triglicéridos remanentes por la LPL y el enriquecimiento relativo en ésteres de colesterol, también asociado a la acción de CETP y PLTP, se transforman en LDL. Las LDL satisfacen las necesidades de colesterol de las células. Habitualmente son reconocidas (mediante la apoB100 y la apoE) por receptores específicos (receptores de LDL, LDLR) en el hígado donde son metabolizadas (Orsó et al., 2011).

Las HDL también se sintetizan *de novo* en el hígado e intestino delgado como partículas en forma de disco ricas en apolipoproteínas (apoA-I, apoC-I, apoC-II, y apoE) y casi desprovistas de colesterol. Estas HDL nacientes se unen mediante la apoA-I con la proteína ABCA1 de las células para la transferencia del colesterol y se transforman en HDL pre-beta (HDL discoidal); la esterificación del colesterol a través de la lecitina colesterol acil-transferasa (LCAT) genera partículas maduras y esféricas (HDL_2 y HDL_3). Esta función de las HDL, de transportar el colesterol desde las células de los tejidos periféricos hasta el hígado, se conoce como transporte reverso del colesterol (Díaz Díaz et al., 2011).

Entre las distintas teorías relacionadas con la patogenia y el desarrollo de la aterosclerosis, la más aceptada es la propuesta por los investigadores Kevin Williams e Ira Tabas en el año 1995, conocida como “teoría de la respuesta a la retención de las lipoproteínas” (Williams & Tabas, 1995). Esta teoría postula que el proceso aterogénico principal es la acumulación y retención subendotelial de las LDL por los proteoglucanos de la matriz extracelular, lo cual desencadena una respuesta inflamatoria en la pared vascular que

favorece la formación de la placa de ateroma. Ya en el apartado 1.2 de esta Tesis, se indica que la dislipemia aterogénica (aumento de la concentración plasmática de las LDL y disminución de las HDL) representa uno de los principales factores de riesgo cardiovascular (Cooney et al., 2009; Elis et al., 2011). Sin embargo, existen suficientes evidencias que sugieren un papel de los triglicéridos de los CM y las VLDL en la aterosclerosis tan relevante como el colesterol de las LDL (Chapman et al., 2011). De hecho, el perfil metabólico de la hipertrigliceridemia, hipercolesterolemia (LDL densas y de pequeño tamaño), y disminución de HDL en plasma se considera el mayor determinante en el aumento del riesgo cardiovascular en pacientes con síndrome metabólico, resistencia a la insulina, y diabetes tipo 2. En este contexto, el aumento de la adiposidad (Grundy, 2006) y de la ingesta de hidratos de carbono (Parks & Hellerstein, 2000) incrementa de forma significativa la retención en la pared vascular de las LDL, VLDL, y la incidencia de las ECV.

La presencia de una molécula de apoB100 en las LDL y VLDL, y de una molécula de apoB48 en los CM, es fundamental para la retención intravascular de dichas lipoproteínas, así como los proteoglucanos en la pared vascular. La apoB100 es una de las mayores proteínas en el ser humano, con 4536 aminoácidos; mientras que la apoB48 es una forma truncada de la apoB100 y consta del 48% de sus aminoácidos desde el extremo N-terminal (Liu et al., 2010). Los proteoglucanos (la decorina y los biglucanos son los más comunes) se secretan principalmente por las SMC y están formados por una proteína unida covalentemente a una o varias cadenas de glucosaminoglucanos, entre los que se encuentran el heparán, chondroitán, dermatán, y keratán sulfato (Walters & Wrenn, 2011). Tanto apoB100 como apoB48 disponen de dominios cargados positivamente que interaccionan mediante fuerzas electrostáticas con los dominios cargados negativamente de estos glucosaminoglucanos (**Figura 8**).

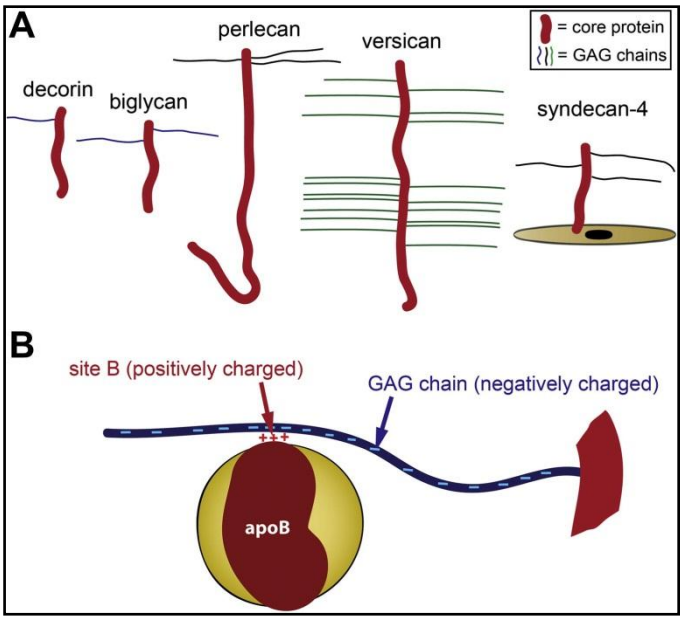

Figura 8. Principales proteoglucanos en la pared vascular (A) e interacción de los dominios cargados positivamente que aporta la apoB con los dominios cargados negativamente de los proteoglucanos (B).

Entre los factores que influyen en la elongación y sulfatación de las cadenas de los glucosaminoglucanos y su afinidad por apoB100 y apoB48 están los factores de crecimiento, los componentes de la matriz extracelular, las propias lipoproteínas (normalmente oxidadas), y los ácidos grasos (Fogelstrand & Borén, 2012). Apenas existen diferencias entre la afinidad de apoB100 y apoB48 por los proteoglucanos.

Otras apolipoproteínas, como la apoE y el amiloide A sérico, también tienen dominios de unión a proteoglucanos. Por ejemplo, la presencia de apoB48 y apoE en los CM puede aumentar su retención y contribuir significativamente a la deposición de triglicéridos en la pared vascular (Tannock et al., 2002). Las HDL contienen apoE y amiloide A sérico; las HDL oxidadas se detectan en lesiones ateroscleróticas humanas (Feng & Li, 2009) y co-localizan con los biglucanos (King et al., 2010). Por lo tanto, aunque las HDL

circulantes ejercen efectos anti-aterogénicos, si son retenidas en el espacio subendotelial, pueden llegar a ser pro-aterogénicas. La apoC-III, que se encuentra en CM, VLDL, HDL, y LDL, incrementa la retención de estas lipoproteínas; por ejemplo, altas concentraciones de apoC-III en las LDL aumenta su susceptibilidad a la oxidación (y a otras modificaciones, como la acetilación), la inflamación vascular, y el riesgo de ECV (Hiukka et al., 2009).

Las lipoproteínas, en su forma nativa y cuando son retenidas en la pared vascular, tienen una capacidad limitada para inducir respuestas pro-aterogénicas. La retención *per se* estimula la internalización de las lipoproteínas por parte sobretodo de los macrófagos, mediante cambios estructurales que también facilitan la oxidación y la agregación/fusión de estas lipoproteínas. Así, los procesos metabólicos de las células circundantes (células endoteliales, SMC, y macrófagos) conducen a la secreción al medio extracelular de productos, como el radical anión superóxido y lipoperóxidos, que provocan la oxidación parcial de las lipoproteínas (Weber & Noels, 2011). La oxidación de las lipoproteínas mínimamente oxidadas progresa por la acción de mieloperoxidasas y lipoxigenasas, que se secretan por SMC y macrófagos activados, hasta la oxidación total. Las lipoproteínas oxidadas (las LDL oxidadas han sido las más estudiadas) son reconocidas por receptores especializados en los macrófagos (también en las SMC); esta captación de LDL oxidadas transforma a los macrófagos (y SMC) en células espumosas, que cuando se saturan de lípidos se rompen, incrementando de manera notable la inflamación y el tamaño del núcleo lipídico-necrótico de la placa de ateroma (Mitra et al., 2011). Además, este ambiente estimula la secreción por los macrófagos de esfingomielinasa, fosfolipasas, y LPL que potencian la retención de más lipoproteínas (Gustafsson et al., 2007). Es interesante destacar que los CM no necesitan ser oxidados para ejercer efectos pro-aterogénicos y que, a diferencia de las LDL, la retención intravascular de los CM es irreversible (Graham et al., 2011).

2.4. Receptores de lipoproteínas implicados en la acumulación lipídica

Las lipoproteínas que han sido retenidas en la pared vascular se captan por los macrófagos y SMC preferentemente a través de los receptores residuales (SR, *scavenger receptors*), cuya expresión se fomenta durante la diferenciación de los monocitos infiltrados a macrófagos (Badimon et al., 2011) y de las SMC con fenotipo sintético al proliferativo (Yu et al., 2010), y tras la captación de lipoproteínas modificadas, aunque otras células también expresan estos receptores. Se pueden distinguir diferentes tipos o clases de receptores residuales: A, B, C, D, E, F, G, y H (**Figura 9**).

Los receptores residuales de la clase A (SR-A) se caracterizan por tener un dominio similar al colágeno, que es esencial para la unión al ligando, y una estructura homotrimérica (Moore & Freeman, 2006). Entre los SR-A se encuentran SR-AI, SR-AII, SR-AIII, MARCO (*macrophage receptor with collagenous structure*), SCARA-5 (*scavenger receptor* A-5), y SRCL (SR con lectina del tipo C). Se ha demostrado que SR-AI y SR-AII son los que mayoritariamente se expresan en la superficie de los macrófagos, aunque también se han detectado en células endoteliales y SMC en lesiones ateroscleróticas, y son responsables del 80% de la captación de LDL oxidadas y acetiladas. SR-AI y SR-AII también reconocen células apoptóticas, el péptido amiloide β, fosfolípidos aniónicos (cardiolipina, fosfatidilserina, fosfatidilinositol, ácido fosfatídico), y productos finales avanzados de la glucosilación; e incluso participan en las respuestas inmune innata y adaptativa mediante el reconocimiento de patógenos y moléculas asociadas a patógenos (Plüddemann et al., 2007; Kzhyshkowska et al., 2012).

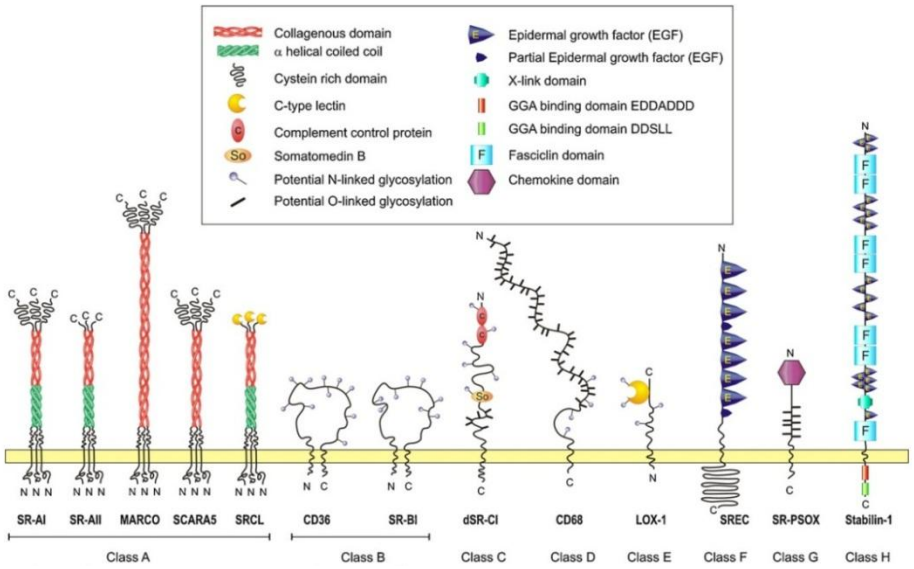

Figura 9. Diagrama esquemático de la familia de receptores residuales implicados en la aterosclerosis.

Los principales receptores de la clase B son CD36 y SR-BI (SCARB1). CD36, a diferencia de SR-AI y SR-AII, no reconoce LDL acetiladas ni LDL completamente oxidadas, sino LDL con una oxidación moderada (Riazy et al., 2011). CD36 se expresa en monocitos, macrófagos, adipocitos, endotelio microvascular, plaquetas, y precursores de eritrocitos. Los ligandos de CD36 son comunes a los de SR-A, pero CD36 puede además reconocer lipoproteínas nativas (LDL, HDL, y VLDL), así como tromboespondina-1, colágeno, ligandos derivados de patógenos, y ácidos grasos. SR-BI tiene una homología del 30% con CD36 en la secuencia de aminoácidos y comparte afinidad por los mismos ligandos, aunque SR-BI facilita la captación y transferencia del colesterol de las HDL, por lo que desempeña un papel importante en el transporte reverso del colesterol (Demetz et al., 2012). SR-BI se expresa en hígado, macrófagos, y tejidos esteroidogénicos con una demanda continua de colesterol. Por lo tanto, dependiendo del contexto, SR-BI puede tener implicaciones pro-aterogénicas o anti-aterogénicas.

Otros receptores residuales son CD68 y LOX-1 (Stephen et al., 2010), que pertenecen a la clase D y E, respectivamente, de receptores residuales. CD68 se expresa en los endosomas y lisosomas de macrófagos; participa activamente en el procesamiento endolisosomal de las LDL oxidadas. LOX-1 es una proteína transmembrana del tipo II, como la lectina, se expresa en macrófagos, SMC, y células endoteliales; reconoce a ligandos típicos de los receptores residuales y LDL oxidadas. Receptores residuales de la clase F son SREC-I y SREC-II, que se expresan en células endoteliales; aunque ambos receptores reconocen LDL modificadas, solo SREC-I las internaliza (Plüddemann et al., 2007). SR-PSOX es un receptor residual de la clase G; es una proteína casi idéntica a CXCL16 y se expresa en macrófagos, SMC, y células endoteliales; reconoce fosfatidilserina y LDL oxidadas (Liu et al., 2011). Stabilin-1 pertenece a la clase H; se expresa en células endoteliales sinusoidales de hígado, bazo y ganglios linfáticos; media la endocitocis de LDL. Además, este receptor se ha detectado en monocitos de pacientes con hipercolesterolemia familiar (Mosig et al., 2009) y en lesiones ateroscleróticas humanas (Brochériou et al., 2011). La acumulación de lípidos en las células de la pared vascular también puede llevarse a cabo mediante macropinocitosis (Anzinger et al., 2010).

Además de los receptores residuales, las células vasculares pueden captar lipoproteínas nativas y mínimamente modificadas a través de los receptores LRP (proteína relacionada con LDLR), VLDLR (receptor de VLDL), y de apoB48 (apoB48R). Todos ellos son ampliamente expresados en lesiones ateroscleróticas, a excepción de LDLR (Bermúdez, 2007). LRP y VLDLR son mayoritariamente expresados en células endoteliales y SMC, mientras que los receptores residuales y apoB48R actúan preferentemente en macrófagos.

VLDLR es una proteína transmembrana que reconoce las TRL y comparte un alto grado de homología con los receptores de la familia LDLR. Consta de cinco dominios que abarcan 846 aminoácidos; un extremo N-terminal, que comprende la región de unión al ligando con regiones ricas en cisteínas, muy característica de LDLR; un dominio homólogo al precursor del factor de crecimiento epidérmico; una región inmediatamente anterior a la membrana

plasmática enriquecida con residuos de serinas y treoninas; un segmento transmembrana; y un dominio citoplasmático donde se incluye la secuencia FDNPVY (Phe-Asp-Asn-Pro-Val-Tyr), necesaria para la invaginación de la membrana e internalización del complejo receptor-lipoproteína al citoplasma (Wang et al., 2011). VLDLR se expresa en musculatura, corazón, y tejido adiposo, lo que sugiere que este receptor desempeña un papel importante en el catabolismo de las TRL en esos tejidos (Tao & Hajri, 2011).

LRP (LRP-1) es un receptor multifuncional endocítico de 600 kDa que presenta en su estructura los cinco dominios básicos altamente conservados de la familia LDLR. Entre los distintos ligandos para este receptor se encuentran las TRL enriquecidas en apoE; de hecho, fue identificado como un receptor específico de apoE y de la macroglobulina alfa-2 activada (Behl et al., 2009). También favorece la captación de complejos formados por TRL unidas a LPL (Morita et al., 2003) y de LDL agregadas (Llorente-Cortés et al., 2007). Este receptor es capaz de reconocer adicionalmente más de 40 ligandos, como proteinasas, factores de coagulación sanguínea, factores de crecimiento, proteínas de matriz extracelular, chaperonas, y proteínas víricas y bacterianas. Recientemente, se ha descrito un papel relevante de LRP-6 en la aterosclerosis (Keramati et al., 2011).

Uno de los receptores menos conocido en la captación de lípidos procedentes de las lipoproteínas es el receptor apoB48R (**Figura 10**). Los CM son las únicas lipoproteínas que contienen apoB48 y habitualmente son captados por el hígado a través de apoE; sin embargo, estudios en animales de experimentación indican que fracciones significativas de CM pueden ser internalizadas por células del sistema reticuloendotelial (Ross & Zilversmit, 1977; Nagata & Zilversmit, 1987), de forma independiente de apoE (Hussain et al., 1989). A raíz de estos hallazgos se llevó a cabo el aislamiento (de monocitos humanos THP-1) y clonación/caracterización de apoB48R (Brown et al., 2000), el único receptor hasta ahora descrito para apoB48. El gen apoB48R se localiza en el cromosoma 16p11 (GenBank accession no. AF141332). Este receptor es una proteína transmembrana que consta de 1088 residuos de aminoácidos. El

cDNA de apoB48R (3744 bp) codifica una proteína sin homología con otros receptores de lipoproteínas. A diferencia de otros receptores, que disponen de dominios funcionales ricos en cisteína, apoB48R tiene solo 8 residuos de cisteína distribuidos a lo largo de la secuencia de aminoácidos; el receptor mantiene su plena funcionalidad tanto en condiciones reductoras como no reductoras, indicando que los residuos de cisteína no son esenciales en la capacidad de apoB48R para reconocer su ligando. Es una proteína altamente polar, con 242 aminoácidos con carga negativa y 122 con carga positiva, y contiene dos dominios hidrofóbicos, uno de ellos de 23 residuos de aminoácidos y relacionado con la porción transmembrana del receptor (aunque con una hidrofobicidad atípicamente baja). Otra diferencia respecto a la familia LDLR es que apoB48R carece de dominio de internalización basado en residuos de tirosina, por el contrario contiene dominios de di-leucina (ExxxLL) implicados en la endocitosis en células del sistema inmune (Pandey, 2009). Se ha descrito que la expresión de apoB48R en macrófagos se regula mediante rutas dependientes de RhoA, una GTPasa de la superfamilia Ras relacionada con la α-actina del citoesqueleto en la formación de fibras de estrés (Kawakami et al., 2005), y los factores de transcripción PPARα y PPARγ (receptores activados por proliferadores peroxisomales) (Haraguchi et al., 2003). ApoB48R tiene una alta afinidad por los CM, aunque también une a VLDL carentes de apoE (tripsinizadas), pero no a VLDL y LDL nativas. Se expresa preferentemente en monocitos (THP-1), macrófagos (derivados de monocitos THP-1), y células endoteliales; también en cerebro, corazón, riñón, hígado, pulmón, páncreas, y placenta. Mediante inmunohistoquímica se ha demostrado que apoB48R está presente en células espumosas de lesiones ateroscleróticas humanas (Brown et al., 2000). Por lo tanto, apoB48R y sus rutas intracelulares representan un modelo único para explicar, al menos en parte, el riesgo cardiovascular asociado al metabolismo postprandial de las grasas.

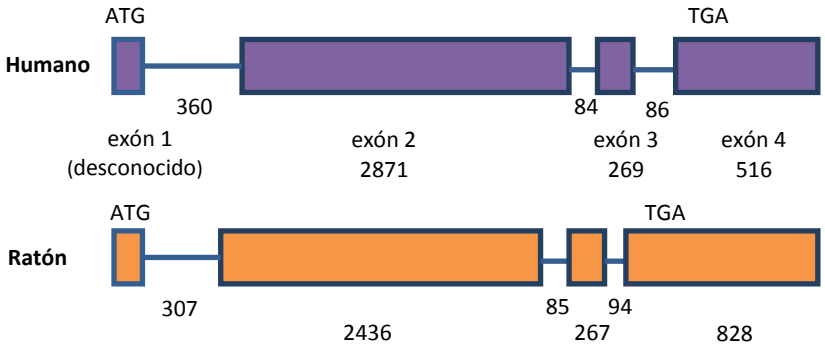

Figura 10. Organización genómica del receptor apoB48R humano y de ratón.

2.5. Proteínas estructurales implicadas en la acumulación lipídica

La acumulación de lípidos en el interior de las células de la pared vascular tiene lugar en unos orgánulos especializados, llamados gotas lipídicas (Gong et al., 2011). Las gotas lipídicas citoplasmáticas son originadas en el retículo endoplásmico (Robenek et al., 2008) y su importancia celular como reserva energética se aprecia en los casos dónde su función se ve alterada y afectada, la consecuencia de ello es la aparición de patologías como la aterosclerosis, la diabetes o la obesidad (Paul et al., 2008). Además, intervienen en la regulación de diversas funciones celulares, como son la β-oxidación de ácidos grasos, la biogénesis de membranas, la síntesis de hormonas esteroides, los mecanismos de señalización intracelular, y el almacenamiento y degradación de proteínas (Fujimoto et al., 2008).

Las gotas lipídicas tienen una estructura similar a la de las lipoproteínas circulantes, con un núcleo central constituido por lípidos neutros (triglicéridos, ésteres de colesterol, y ácidos grasos), recubierto por una monocapa de fosfolípidos y proteínas específicas (Robenek et al., 2011). Gracias a la proteómica, se han podido identificar diversas proteínas asociadas a las gotas lipídicas, incluyendo enzimas, transportadores de lípidos, y caveolinas (Yang et al., 2012), aunque las más abundantes pertenecen a la familia de proteínas PAT, la cual incluye 5 miembros en los mamíferos (Ducharme et al., 2008). Los

3 primeros miembros identificados dieron nombre a la familia: PLIN (perilipina 1), ADRP (*adipose differentiation-related protein* o perilipina 2), y TIP47 (*tail-interacting protein of 47 kDa* o perilipina 3). Posteriormente, se incluyeron dos proteínas adicionales en la familia: S3-12 (perilipina 4) y OXPAT (*oxidative tissue-enriched PAT protein*, MLDP, LSDP5 o perilipina 5) (Bickel et al., 2009). (**Figura 11**). PLIN y ADRP se localizan de forma constitutiva en la superficie de las gotas lipídicas (proteínas no intercambiables), mientras que el resto de las proteínas PAT se localizan en el citosol y, tras la estimulación con diferentes sustancias, se translocan hacia la superficie de las gotas lipídicas (proteínas intercambiables) (Wolins et al., 2006).

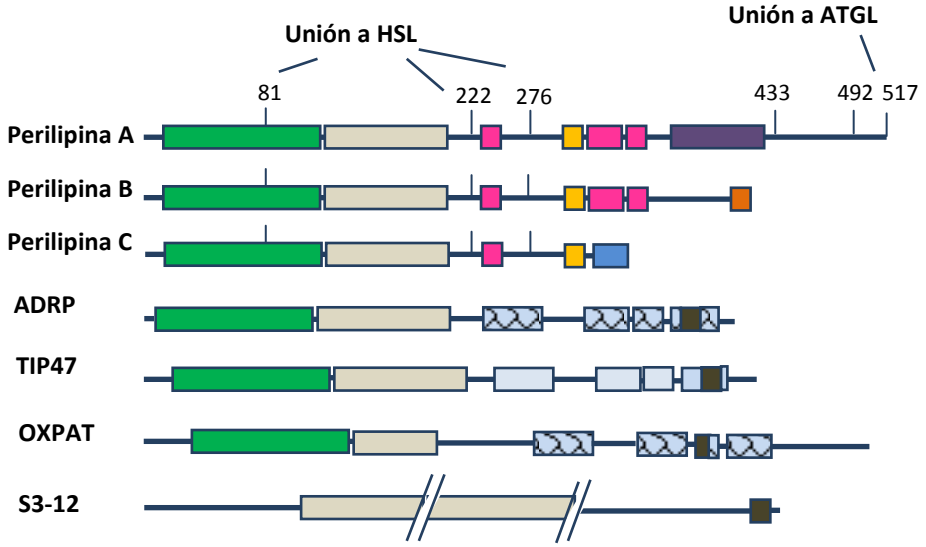

Figura 11. Homología de la secuencia de aminoácidos de las proteínas de la familia PAT.

PLIN es la proteína mejor caracterizada de la familia de proteínas PAT. Se expresa en adipocitos y células esteroidogénicas (Londos et al., 2005). Sin embargo, también se expresa en macrófagos en lesiones ateroscleróticas y en hepatocitos de personas con esteatosis hepática (Hofnagel et al., 2007; Straub et al., 2008). La expresión de PLIN está regulada por PPARγ (Arimura et al., 2004) y EERα (Akter et al., 2008). Existen 3 isoformas de esta proteína: A, B y C; siendo la isoforma A la predominante y esencial en la regulación de la lipolisis

(Brasaemle et al., 2009). Es el único miembro de la familia cuya función depende del estado de fosforilación, estando el mecanismo regulado por PKA (proteína quinasa A) dependiente de AMPc (Granneman et al., 2007). En condiciones basales, PLIN está asociada a la proteína CGI-58, de forma que inhibe la lipolisis al impedir el acceso de las lipasas, HSL (lipasa sensible a hormonas) y ATGL (lipasa de triglicéridos de tejido adiposo), a los lípidos almacenados en el núcleo de las gotas lipídicas. Tras la estimulación β-adrenérgica, aumentan los niveles de AMPc y se activa PKA, la cual fosforila a PLIN y HSL. La fosforilación de PLIN conlleva la pérdida de afinidad por CGI-58, la cual se disocia y dispersa en el citoplasma, donde se une a ATGL para promover la hidrólisis de triglicéridos (Yamaguchi, 2010).

ADRP fue identificada en estudios relacionados con los procesos de diferenciación de adipocitos (Jiang et al., 1992); con posterioridad, se demostró que también se expresa en fibroblastos, células endoteliales y epiteliales, y constituye un marcador de acumulación lipídica (Dahl et al., 2011). La expresión de ADRP está regulada por los PPAR (Targett-Adams et al., 2005). En la familia de las proteínas PAT, ADRP es la proteína más involucrada en la formación de células espumosas y en la patogénesis de la aterosclerosis (Paul et al., 2008). El mecanismo mediante el cual ADRP induce acumulación lipídica no se conoce con exactitud, aunque se plantea que, al igual que otras proteínas PAT, pueda regular la acción de las lipasas. Un aumento de la expresión de ADRP se ha relacionado con una disminución de la unión de ATGL a las gotas lipídicas, de modo que imposibilita la hidrólisis de los triglicéridos y facilita su acumulación (Listenberger et al., 2007). En macrófagos, ADRP estimula la expresión de genes relacionados con la inflamación, como TNFα, MCP-1, e IL-6 (Chen et al., 2010); siendo otro mecanismo que relaciona a ADRP con el desarrollo de la aterosclerosis.

TIP47 fue descrito como una proteína citosólica que facilitaba el reciclado del receptor de manosa-6-fosfato a través de endosomas tardíos hacia la red trans del Golgi, formando un complejo con dicho receptor y la proteína Rab9 (Bulankina et al., 2009). A diferencia de PLIN y ADRP, TIP47 no está regulada

por los PPAR (Bickel et al., 2009). Se ha demostrado que TIP47 interviene en la acumulación celular de triglicéridos (Gu et al., 2010), y que actúa como una proteína transportadora de ácidos grasos, atribuyéndole un papel en la formación de células espumosas (Buers et al., 2009). Por su parte, S3-12 se expresa en el tejido adiposo blanco y en menor medida en el músculo esquelético y cardíaco (Li et al., 2010); OXPAT también se localiza en el músculo esquelético y cardíaco, donde inhibe la lipolisis y favorece la β-oxidación de los ácidos grasos y la acumulación intracelular de triglicéridos (Wolins et al., 2006).

2.6. Receptores activados por proliferadores peroxisomales

La transcripción de los genes requiere de múltiples moléculas, entre las que se encuentran los receptores nucleares; de hecho, estos receptores también se conocen como factores de transcripción. Son proteínas que comparten estructura y dominios funcionales altamente conservados (**Figura 12**): un dominio amino-terminal de unión a ADN (la propiedad que les diferencia del resto de receptores), para anclar la proteína a secuencias específicas (región promotora); un dominio en su extremo carboxilo de unión a ligando; y un dominio de transactivación/transrrepresión, donde se unen co-activadores/co-rrepresores que facilitan/inhiben la transcripción (Poulsen et al., 2012).

Estos receptores se han clasificado habitualmente en dos grupos: a) los receptores nucleares con ligandos conocidos, entre los cuales se incluyen los receptores para hormonas esteroideas, tiroideas, y vitamina D; y b) los receptores nucleares huérfanos, para muchos de los cuales todavía no se han identificado los ligandos endógenos que controlan su actividad transcripcional. Entre los receptores nucleares huérfanos, el subgrupo de los receptores heterodiméricos es el más relevante desde el punto de vista de la regulación del metabolismo lipídico. Durante su activación por ligandos específicos, estos receptores nucleares forman un complejo heterodimérico que resulta de la

interacción de dos proteínas independientes: el receptor del ácido 9 cis-retinoico, denominado RXR, que es el elemento constante del complejo; y otra proteína homóloga que determina la respuesta a los ligandos específicos. Una vez formado el complejo heterodimérico, éste interactúa con los elementos de respuesta ubicados en la región promotora de los genes diana, determinando la activación de la expresión de los mismos y, en último término, la síntesis de las proteínas codificadas por dichos genes.

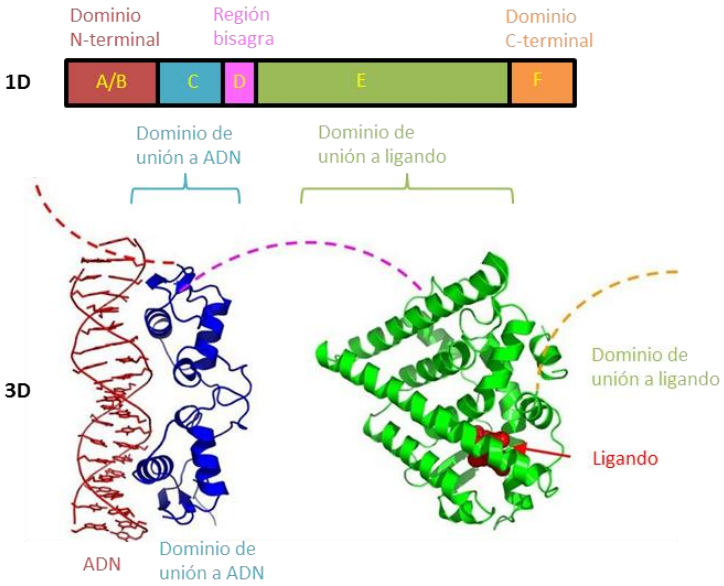

Figura 12. Esquema de la secuencia de aminoácidos de un receptor nuclear (1D) y estructura 3D de las regiones de unión a ADN y de unión a ligando.

Entre las proteínas homólogas que se unen a RXR está la subfamilia de receptores PPAR; los ácidos grasos y moléculas derivadas de ellos son ligandos naturales de los PPAR (Plutzky, 2011). La unión del ligando a los PPAR permite la heterodimerización con RXR y su unión a los elementos de respuesta PPRE (**Figura 13**) Se han descrito tres isoformas de PPAR: PPARα, PPARγ, y PPARβ/δ (Tyagi et al., 2011).

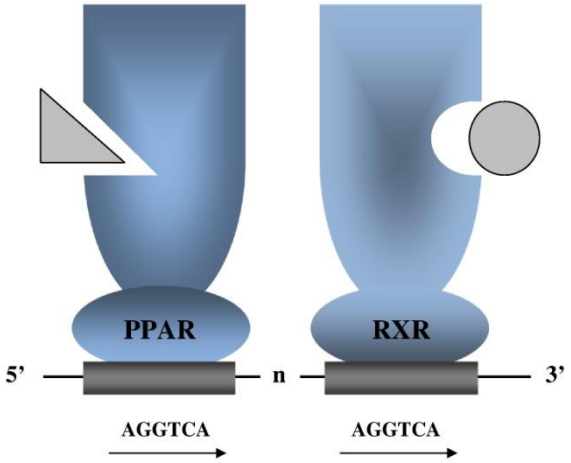

Figura 13. Mecanismos de la unión específica al ADN de los receptores nucleares.

PPARα se expresa en hígado, tejido adiposo, corazón, músculo esquelético, corteza renal, en células del sistema inmune, SMC, y en menor cantidad en pulmón, cerebro, intestino, páncreas, y placenta (Azhar, 2010). PPARα está involucrado en procesos relacionados con el metabolismo lipídico y de las lipoproteínas, principalmente en la captación y oxidación de los ácidos grasos (Alsaleh et al., 2011). Otros efectos derivados de la activación de PPARα se relacionan con una disminución de la resistencia a la insulina y de genes implicados en la inflamación, estrés oxidativo, crecimiento, y migración celular (Yessoufou & Wahli, 2010). Aunque existe controversia, la mayoría de los estudios sobre PPARα tienden a atribuirle un efecto protector contra la aterosclerosis; de hecho, PPARα fue el primero susceptible de aplicación clínica mediante el desarrollo de los fibratos como principios activos para el tratamiento de las dislipemias (Wilding, 2012).

PPARγ se divide a su vez en cuatro isoformas (PPARγ1, PPARγ2, PPARγ3, y PPARγ4). PPARγ1 se expresa prácticamente en todos los tejidos, incluyendo el corazón, músculo esquelético, colon, riñón, páncreas, y bazo; PPARγ2 en tejido adiposo; PPARγ3 en monocitos/macrófagos, intestino, y tejido adiposo blanco; y PPARγ4 en células endoteliales (Tyagi et al., 2011). Este receptor promueve la

adipogénesis, controlando el programa de diferenciación del adipocito; y participa en el metabolismo lipídico, regulando la expresión de genes relacionados con la captación y almacenamiento de ácidos grasos en el tejido adiposo (Semple et al., 2005). Al igual que PPARα, PPARγ desempeña un papel esencial en la homeostasis de la glucosa y en la sensibilización a la insulina (Abbas et al., 2012). La mayoría de los estudios señalan un papel protector de PPARγ contra la aterosclerosis, debido en parte a la regulación negativa de la actividad transcripcional en macrófagos; así, la activación de PPARγ inhibe la cascada de señalización de NF-κB, atenuando la expresión de genes pro-inflamatorios, además de reducir la expresión de MCP-1 y TNFα (Seno et al., 2011). También se ha descrito que la activación de PPARγ influye en la diferenciación de monocitos a macrófagos hacia el fenotipo anti-inflamatorio M2 (Bouhlel et al., 2007). Actualmente, PPARγ representa una diana terapéutica, con las tiazolidinedionas (glitazonas) como principios activos para el tratamiento de las alteraciones del metabolismo lipídico y de la glucosa asociadas a la obesidad, diabetes tipo 2, y el síndrome metabólico (Derosa & Maffioli, 2011). Los agonistas de PPARγ aumentan significativamente el colesterol HDL por efecto de una mayor expresión del transportador ABCA1 (Komatsu & Node, 2010). En términos de beneficio contra la aterosclerosis, el uso de rosiglitazona reduce significativamente el tamaño de las placas de ateroma de la aorta ascendente en animales de experimentación deficientes en LDLR y sometidos a una dieta rica en grasas saturadas (Collins et al., 2009). Esta observación se ha confirmado por la disminución del grosor de la íntima de la arteria carotidea tras el tratamiento con pioglitazona en personas con diabetes tipo 2 (Mizoguchi et al., 2011).

PPARβ/δ está ampliamente expresado en la mayoría de los tejidos, incluyendo hígado, tejido adiposo, músculo esquelético, pulmón, cerebro, y piel. Aunque los efectos biológicos de PPARβ/δ son todavía poco conocidos, los estudios recientes resaltan su papel favorable en el metabolismo lipídico, la sensibilidad a la insulina, la reducción de mediadores inflamatorios y moléculas de adhesión (Salvadó et al., 2012).

Diversos estudios han demostrado que la naturaleza de los ácidos grasos mayoritarios en la dieta puede modular la activación de los receptores PPAR (Bays et al., 2008). Por ejemplo, el ácido oleico es eficaz para invertir el efecto inhibidor en la producción de insulina de la citoquina inflamatoria TNFα, mediante la activación de PPARγ (Vassiliou et al., 2009). La afinidad de los tres isotipos de PPAR por los distintos ácidos grasos va a depender de la longitud y del grado de insaturación de la cadena hidrocarbonada. En general, los ácidos grasos tienen mayor afinidad por PPARα, seguido por PPARγ y PPARβ/δ (Robinson & Grieve, 2009). Dentro de los PUFA, los ácidos linoleico, α-linolénico, araquidónico, eicosapentaenoico, y docosahexaenoico han sido identificados como los activadores más potentes de PPARα; mientras que los SFA de cadena larga, como el palmítico o el esteárico; y los SFA de cadena corta, son activadores débiles. Los MUFA como el oleico, y los ácidos grasos *trans* muestran afinidades comparables para los tres isotipos de PPAR (Ringseis & Eder, 2010). Además, el ácido linoleico conjugado también es capaz de unirse y activar a PPARα, PPARγ, y PPARβ/δ (Ringseis & Eder, 2009). Aunque durante mucho tiempo los ácidos grasos de la dieta han sido considerados únicamente como fuente de energía, recientemente se les reconoce también como reguladores de la inflamación, donde los receptores PPAR juegan un papel muy importante en la transducción de las señales derivadas de la ingesta alimentaria de los lípidos (Varga et al., 2011).

3. METABOLISMO LIPÍDICO POSTPRANDIAL

El periodo postprandial es un fenómeno fisiológico, agudo y transitorio, que ocurre tras la ingesta de los alimentos y durante el cual se produce la absorción y utilización metabólica de los nutrientes (y no nutrientes). El metabolismo lipídico postprandial se refiere a los procesos metabólicos del periodo postprandial tras la ingesta de los alimentos grasos.

3.1. Grasas de la dieta y lipemia postprandial

La lipemia postprandial es el aumento de la concentración en sangre de triglicéridos, fundamentalmente en forma de CM, en el periodo postprandial (Lambert & Parks, 2012). Este aumento comienza a los pocos minutos de la ingesta y es gradual hasta un máximo de concentración que suele coincidir a las 2-4 horas. Seguidamente se produce una disminución paulatina de la lipemia debido a una menor producción intestinal de CM, a la lipolisis en los capilares sanguíneos, y a la captación de CM, VLDL, y sus remanentes por el hígado y los tejidos periféricos. Tras esta disminución o aclaramiento, y coincidiendo con la secreción hepática de las VLDL, puede aparecer un segundo pico aunque mucho menor de triglicéridos, de tal manera que hasta pasadas 6-8 horas no se alcanzan los valores basales. Según los hábitos de alimentación, es posible pasar gran parte de nuestra vida en este estado metabólico postprandial, ya que cuando realizamos una segunda comida aún pueden estar transportándose y asimilándose las grasas de la comida anterior (Lairon et al., 2007). Únicamente a primeras horas de la mañana nos encontramos en un estado de ayuno casi total (**Figura 14**).

Una mayor ingesta de grasas puede aumentar la magnitud de la respuesta lipémica postprandial. Algunos estudios también sugieren que el tipo de ácidos grasos en la dieta puede influir en la velocidad de formación de las TRL postprandiales (Karupaiah et al., 2011). Sin embargo, el proceso de digestión de las grasas (y su posterior entrada en plasma en forma de CM) está condicionado por muy diversos factores como la palatabilidad del alimento y la velocidad del vaciado gástrico. Más aceptada es la relación entre el efecto de los ácidos grasos de la dieta y la amplitud y duración de la lipemia postprandial (Ortega et al., 2012). Los PUFA favorecen una reducción rápida de los valores de lipemia postprandial, probablemente al favorecer el aclaramiento de las TRL postprandiales por la LPL y de sus remanentes vía hepática (Shearer et al., 2012). Entre los PUFA, los omega-3 estimulan más que los omega-6 la lipolisis de los CM (Davidson, 2006). Hay pocas referencias sobre el efecto de los MUFA, aunque las existentes (utilizando aceite de oliva como fuente de ácido

oleico) indican que producen una disminución más rápida de la lipemia postprandial en comparación con los SFA (Pacheco et al., 2006; López et al., 2008). Por lo tanto, y en términos generales, la amplitud y la duración de la respuesta lipémica postprandial se afecta por el tipo de ácidos grasos en la dieta, según el siguiente orden: SFA > MUFA = PUFA.

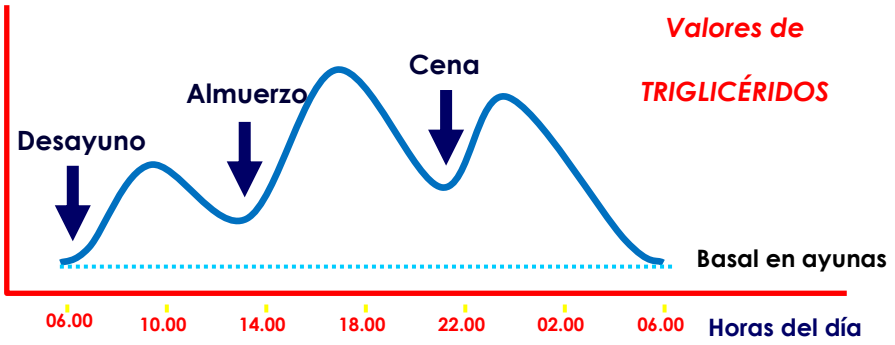

Figura 14. Hiperlipemia postprandial a lo largo del día.

3.2. Relación entre lipemia postprandial y aterosclerosis

La lipemia postprandial implica la existencia de variaciones diurnas de triglicéridos considerablemente mayores a las de LDL y HDL, que pasan desapercibidas en muestras de sangre en ayunas. Se ha estimado que la variabilidad de los triglicéridos a lo largo del día puede superar el 20%, frente a valores del 6% para el colesterol total (Sundvall et al., 2011). Como ya se ha indicado, las grasas de la dieta pueden modular ciertos factores de riesgo cardiovascular, que podrían calificarse como "clásicos" (Getz et al., 2007). Sin embargo, todavía son pocos los estudios que abordan el metabolismo lipídico postprandial y su relación con la aterosclerosis. El riesgo cardiovascular aumenta cuanto mayor sea el pico de máxima concentración de triglicéridos y el tiempo de aclaramiento (Notø et al., 2008; Bravo et al., 2010), y cuanto menor sea el tamaño de las TRL postprandiales (Zhang et al., 2008; Weinstein et al., 2010).

Los estudios llevados a cabo por Zilversmit en 1979 fueron los primeros en relacionar la lipemia postprandial con las ECV (Zilversmit, 1979). De hecho, algunos autores consideran a la lipemia postprandial como un nuevo factor de riesgo cardiovascular independiente al colesterol (Nakatani et al., 2011); incluso se ha asociado la presencia en plasma de TRL postprandiales con casos de muerte súbita cardíaca (Nakajima et al., 2008). Algunos estudios prospectivos han comprobado que la lipemia postprandial exacerbada aumenta el riesgo de infarto agudo de miocardio y de enfermedad coronaria, tanto en hombres como en mujeres (Nordestgaard et al., 2007; Langsted et al., 2011; Varbo et al., 2011). Por lo tanto, la respuesta lipémica postprandial puede ser un parámetro complementario y muy informativo para el diagnóstico del riesgo cardiovascular, pues su valor predictivo puede ser más importante que la concentración de triglicéridos en ayunas (Stalenhoef & de Graaf, 2008; Boullart et al., 2012). La aterogenicidad de las TRL postprandiales depende de su interacción, al menos, con las células endoteliales, los macrófagos, y las SMC.

3.2.1. Disfunción endotelial

En su teoría de las lesiones ateroscleróticas inducidas por las TRL postprandiales, Zilversmit fue uno de los primeros investigadores en proponer que la interacción de estas lipoproteínas con la superficie del endotelio, junto con la liberación de ácidos grasos tras la hidrólisis de sus triglicéridos por la acción de la LPL, podrían ser la causa de un deterioro de la función endotelial (Zilversmit, 1995). Estudios posteriores han confirmado, mediante la medida del flujo por pletismografía (Shimabukuro et al., 2007) y de la vasodilatación por hiperemia (Marin et al., 2011), que la ingesta de una comida rica en grasas produce disfunción endotelial. Aunque no se conocen con exactitud los mecanismos implicados, hay evidencias de la formación de hidroperóxidos y producción del radical anión superóxido en las células endoteliales, que reaccionan con NO y contribuyen a la formación de peroxinitritos; el resultado es la neutralización de la actividad biológica de NO y la disminución de su

biodisponibilidad (Kar & Kavdia, 2011). Otros estudios han descrito la disminución de la actividad plasmática de glutatión peroxidasa (enzima antioxidante) y el aumento de la excreción urinaria de 8-epi-prostaglandina F-2α (marcador de estrés oxidativo) (Tsai et al., 2004), así como el aumento de la producción endotelial de IL-6, TNFα, MCP-1, VCAM-1, ICAM-1, ICAM-2, y ICAM-3 (marcadores de inflamación vascular) mediante la activación de los factores de transcripción CREB (*cAMP response element-binding*) y NF-κB (Norata et al., 2006, 2007) durante la lipemia postprandial. Esta inflamación vascular es concomitante con la activación de neutrófilos y monocitos circulantes (Klop et al., 2012) e implica un aumento de su adhesión al endotelio activado (Alipour et al., 2008a; Bentley et al., 2011). Las TRL postprandiales también pueden ser citotóxicas para las células endoteliales y provocar alteraciones en la permeabilidad y homeostasis del endotelio (Yang et al., 2011). Sin embargo, los MUFA y PUFA de la familia omega-3 son menos perjudiciales e incluso pueden revertir los efectos adversos de los SFA sobre la reactividad vascular postprandial (Perez-Martinez et al., 2010; Newens et al., 2011).

La transmigración de los monocitos al espacio subendotelial permite la diferenciación a macrófagos. Las TRL postprandiales y los productos de su hidrólisis interaccionan *in situ* con las células del entorno vascular, incluidos los macrófagos y SMC; incluso las células endoteliales (Rose & Babensee, 2007) y los macrófagos en respuesta a los ácidos grasos (Chung et al., 2012) pueden tener efectos paracrinos sobre las SMC.

3.2.2. Migración y proliferación de células de músculo liso

Los procesos de migración y proliferación de las SMC son fundamentales en el desarrollo y estabilidad de la placa de ateroma (Doran et al., 2008). Las SMC migran desde la media hasta la íntima (Aramaki et al., 2008), y es allí donde proliferan (Kawakami et al., 2003) y pueden transformarse en células espumosas (Kawakami & Yoshida, 2005; Allahverdian et al., 2012). Durante la

hiperplasia de la íntima, las SMC con el fenotipo proliferativo (sintético) expresan menos proteínas contráctiles, como α-actina, cadenas pesadas de miosina (SM-1 y SM-2), calponina, transgelina (SM-22α), y liselina (*smoothelin*); además, son altamente respondedoras a factores de crecimiento y citoquinas liberadas por las células endoteliales, macrófagos, y linfocitos T (Mitra et al., 2005). Entre los mediadores que regulan la desdiferenciación (reversible) de las SMC del fenotipo contráctil al proliferativo están PDGF (factor de crecimiento derivado de plaquetas), bFGF (factor de crecimiento de fibroblastos básico), EGF (factor de crecimiento de la epidermis), IGF (factor de crecimiento como la insulina), angiotensina-II, endotelina-1, serotonina, α-trombina, Factor Xa, tromboxanos, y ácidos lisofosfatídicos insaturados (Beamish et al., 2010). Entre los factores preventivos de la transición de las SMC al fenotipo proliferativo se encuentran la heparina, TGFβ1 (factor β1 de crecimiento transformante), angiotensina-II, y IGF-1; sus mecanismos de acción se relacionan con la activación de la proteína quinasa de ARN de doble cadena (PKR) y el bloqueo de la transición G_1-S (Beamish et al., 2010).

Al proliferar, las SMC producen más proteoglucanos y por lo tanto se favorece la retención de lipoproteínas portadoras de apoB; este efecto se estimula por los SFA y PUFA de la familia ω-6 (Rodríguez-Lee et al., 2006). Resulta interesante destacar que las TRL postprandiales promueven la transición G_1-S (Bermúdez et al., 2008) e inducen proliferación en SMC mediante la activación de rutas en las que participan ERK1 y ERK2 (Pacheco et al., 2002) y el eje esfingomielina-ceramida (Pacheco et al., 2003). El tipo de ácido graso en las TRL postprandiales influye en esta respuesta proliferativa de las SMC, pues TRL ricas en SFA, cuando se comparan con TRL ricas en MUFA o PUFA de la familia ω-3, producen un aumento de la expresión de genes proliferativos, incluyendo ciclina D1, PCNA, y pRB, y otros genes reguladores de la proliferación (MAP3K1, DYRK1A, y PLK-3) (Bermúdez et al., 2008); los SFA en las TRL postprandiales, a diferencia de los MUFA, inducen en SMC la sobreexpresión de genes pro-inflamatorios, como NF-κB, E2F-1, y SP-1. Estos resultados concuerdan con estudios recientes en los que el medio de macrófagos incubados con ácido palmítico estimula la migración y

proliferación de SMC (Chung et al., 2012); además de COX-2, IL-6, y TNFα, en los efectos paracrinos de estos macrófagos intervienen BMP-2 (proteína morfogenética ósea 2) y BMP-4. Otros estudios indican que los ácidos grasos pueden regular la migración (y proliferación) de SMC a través mecanismos asociados al sistema fibrinolítico (t-PA, el activador del plasminógeno tipo tisular estimula la actividad de MMP (metaloproteinasas de la matriz); este proceso está regulado por PAI-1, inhibidor tipo 1 del activador del plasminógeno) (Delbosc et al., 2008). Los MUFA y SFA en las grasas de la dieta influyen en las concentraciones postprandiales de t-PA y PAI-1 (Pacheco et al., 2006) y por lo tanto podrían influir en la migración de las SMC.

Las MMP son endopeptidasas con capacidad de escindir prácticamente todos los componentes proteicos de la matriz extracelular, como el colágeno, elastina, gelatina, y caseína (Back et al., 2010). Se pueden distinguir distintos grupos de MMP, en función de su estructura y los componentes de la matriz extracelular que preferentemente son capaces de degradar: colagenasas (MMP1, MMP8, y MMP13), que degradan colágenos tipo I, II, y III; gelatinasas (MMP2 y MMP9), que degradan gelatina, fibronectina, colágenos de la membrana basal o formadores de red (tipo IV), y en menor medida colágenos fibrilares (tipo I y V); estromalisinas (MMP3, MMP10, y MMP11), que degradan glucoproteínas y proteoglucanos; matrilisinas (MMP7 y MMP26), que degradan colágeno tipo IV y X, laminina, gelatina, elastina, fibronectina, y proteoglucanos; y MMP unidas a membrana (MMP14, MMP15, MMP16, MMP17, MMP24, y MMP25), que degradan colágeno tipo I-III, laminina, elastina, gelatina, fibronectina, vitronectina, y agrecano. Todas las MMP comparten las siguientes características: presentan una homología estructural; un dominio catalítico que contiene átomos de zinc; son secretadas en forma de zimógenos inactivos, y activadas por la lisis de una secuencia pro-peptídica y por reacciones autolíticas posteriores; y son inhibidas por los inhibidores tisulares específicos de MMP (TIMP), que proporcionan un equilibrio endógeno muy importante para prevenir la degradación excesiva de la matriz extracelular (Raffetto & Khalil, 2008).

Entre las estrategias esenciales para minimizar el desarrollo de la hiperplasia de la íntima está favorecer un fenotipo contráctil estable de las SMC e impedir su migración. Los mecanismos que regulan la migración de las SMC, y en particular aquellos relacionados con la lipemia postprandial, son muy poco conocidos. Tan sólo se ha descrito un estudio en los últimos cinco años en el que las TRL remanentes inducen la migración de SMC a través del receptor LOX-1 (Aramaki et al., 2008). La expresión de LOX-1 también se induce por TNFα, HB-EGF (*heparin-binding EGF-like growth factor*), y LDL oxidadas; y recientemente, entre sus ligandos, se han descrito la proteína C reactiva (Shih et al., 2009) y la angiotensina-II (Luo et al., 2011). Sin embargo, además de este receptor residual y sobretodo apoB48R, se considera que la familia de GTPasas Rho (pequeñas proteínas acopladas a nucleótidos de guanina, con actividad guanosina trifosfatasa) puede desempeñar un papel clave en la migración de las SMC (Surma et al., 2011).

En mamíferos, la familia de GTPasas Rho incluye RhoA, RhoB, y RhoC (proteínas Rho), Rac1, Rac2, y Rac3 (proteínas Rac), Cdc42, TC10, TCL, Wrch1, Chp/Wrch2, RhoD y RhoG; también RhoH, Rnd1, Rnd2, y Rnd3, aunque carecen de la actividad GTPasa y su estado constitutivo es el activado (Riou et al., 2010). Estas proteínas desempeñan su función conforme a un ciclo entre la conformación activa unida a GTP y la conformación inactiva unida a GDP. Las proteínas de GTPasas Rho más estudiadas han sido RhoA (Lacolley et al., 2012), Cdc42 (Lengfeld et al., 2012), y Rac1 (Cheng et al., 2012). Tienen la capacidad de inducir el reordenamiento dinámico de la α-actina del citoesqueleto asociada a la membrana plasmática, y de regular la contractibilidad de la actomiosina y la formación de microtúbulos. Rac1 (**Figura 15**) puede ser activada por productos originados durante las modificaciones oxidativas de las LDL (Kaneyuki et al., 2007) y tiene la particularidad de integrar rutas de señalización asociadas al sistema fibrinolítico y estrés oxidativo (Touré et al., 2012); así, los antioxidantes pueden inhibir la migración de SMC mediante la inhibición de la activación de Rac1 (Kumerz et al., 2011). Sin embargo, se desconoce si en concreto las TRL postprandiales, y los tipos de ácidos grasos que transportan, regulan la expresión y funcionalidad de Rac1 en SMC.

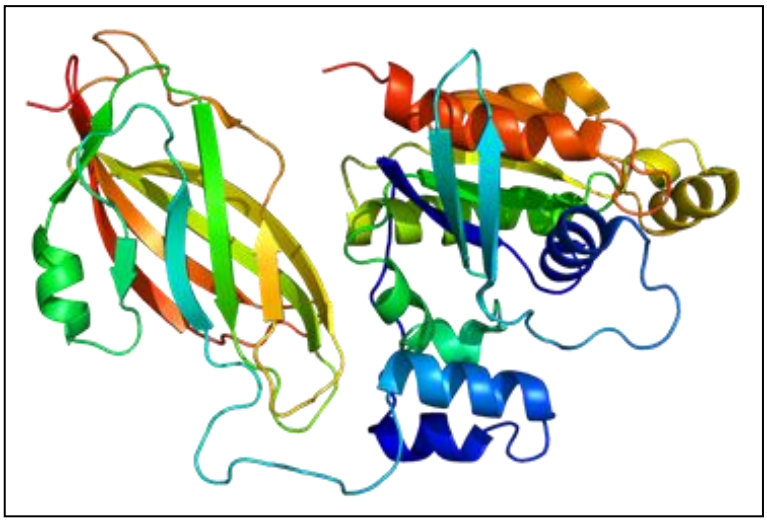

Figura 15. Estructura 3D de Rac1.

OBJETIVOS

HIPÓTESIS

La hipótesis de esta tesis supone que durante el metabolismo postprandial de las grasas de la dieta se generan lipoproteínas ricas en triglicéridos de origen intestinal que pueden interaccionar con leucocitos circulantes, macrófagos, y células de la musculatura lisa de la pared vascular, ocasionando cambios en la expresión de genes y de comportamiento relacionados con la aterosclerosis. También creemos que estos efectos pueden ser dependientes de la composición de los ácidos grasos de la dieta.

OBJETIVOS

1. Evaluar el efecto de la ingesta de una comida enriquecida con grasa sobre la expresión del gen apoB48R y la acumulación intracelular de lípidos en monocitos de personas sanas durante el periodo postprandial.

2. Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la expresión del gen apoB48R, la acumulación intracelular de lípidos, y los mecanismos moleculares implicados en monocitos y macrófagos humanos.

3. Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la expresión de ADRP y TIP47 en macrófagos y su transformación en células espumosas.

4. Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la migración, incluyendo las rutas intracelulares implicadas, en células de la musculatura lisa de arteria coronaria humana.

RESULTADOS

El trabajo de esta Tesis Doctoral se ha centrado en identificar algunos de los acontecimientos celulares y moleculares que participan en el desarrollo de las ECV asociadas al metabolismo de las grasas, de forma especial en las fases agudas de la alimentación. El abordaje general ha consistido en la utilización de un modelo humano postprandial, pues se aproxima perfectamente a nuestros hábitos de alimentación. Se han llevado a cabo estudios para determinar: (i) los efectos *in vivo* de la hipertrigliceridemia postprandial sobre la expresión de un gen de un receptor clave en el metabolismo de las lipoproteínas postprandiales ricas en triglicéridos y la acumulación intracelular de lípidos en monocitos circulantes de personas sanas; y (ii) los efectos *in vitro* de las lipoproteínas postprandiales ricas en triglicéridos con diferente composición en ácidos grasos sobre ese mismo gen en macrófagos humanos y sobre la capacidad migratoria de células humanas de la musculatura lisa.

Los estudios realizados se plantearon para responder a las siguientes preguntas:

1) ¿Pueden los monocitos circulantes acumular lípidos a través del receptor apoB48R durante el periodo postprandial en personas sanas, antes de que estos monocitos puedan infiltrarse en la pared arterial? (**Capítulo 3**).
2) ¿Pueden los ácidos grasos de la dieta modular diferencialmente la expresión del gen apoB48R y la acumulación intracelular de lípidos en monocitos y macrófagos humanos? ¿Qué mecanismos moleculares intervendrían en dicha regulación? (**Capítulo 4**).
3) ¿Pueden los ácidos grasos de la dieta modular diferencialmente la expresión y distribución intracelular de ADRP y TIP47 en macrófagos y su transformación en células espumosas? (**Capítulo 5**).
4) ¿Pueden los ácidos grasos de la dieta modular diferencialmente la capacidad de migrar de las células de la musculatura lisa de arteria coronaria humana? ¿Qué rutas intracelulares intervendrían en dicha regulación? (**Capítulo 6**).

Además de los trabajos de investigación planteados, se incluyen dos artículos de revisión, uno trata de la relación entre los distintos ácidos grasos de la dieta y los cambios en el metabolismo de los lípidos y la glucosa durante el periodo postprandial (**Capítulo 1**); el otro artículo trata de los efectos particularmente beneficiosos del aceite de oliva respecto a otras grasas comestibles sobre diversos factores de riesgo cardiovascular durante el periodo postprandial (**Capítulo 2**).

CAPÍTULO 1

"Dietary fatty acids linking postprandial metabolic response and chronic diseases"

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En este capítulo se presenta información y se discute de la relación entre los distintos ácidos grasos de la dieta y los cambios en el metabolismo de los lípidos y la glucosa durante el periodo postprandial.

RESUMEN: El consumo excesivo de grasas se ha relacionado con el aumento del riesgo de enfermedades crónicas, como las enfermedades cardiovasculares. Sin embargo, este riesgo es diferente según los tipos de ácidos grasos (saturados, monoinsaturados o poliinsaturados) que predominan en dichas grasas y además puede estar supeditado a los cambios metabólicos que se producen durante el periodo postprandial. En esta revisión, se analiza el estado del arte de la relación que existe entre el metabolismo postprandial de los ácidos grasos de la dieta y los desórdenes metabólicos asociados al aumento de la concentración en plasma de triglicéridos y de insulina. Las recomendaciones nutricionales vigentes establecen el consumo máximo del 10% del total de la energía a partir de los ácidos grasos saturados, 6-10% de los ácidos grasos poliinsaturados, y el resto, hasta un máximo del 35% del total de la energía, de los ácidos grasos monoinsaturados (>20%). La identidad y el reconocimiento de algunos ácidos grasos como esenciales (en el más amplio sentido del término) son aspectos claves para la comprensión de la utilidad metabólica de los ácidos grasos de la dieta en el marco de las recomendaciones nutricionales indicadas. En este sentido, las anormalidades

metabólicas en plasma de los lípidos, en particular de los triglicéridos, y de la insulina en el control glucémico postprandial son muy poco conocidas. Se destaca que la ingesta de los ácidos grasos monoinsaturados tiene ventajas, sobretodo respecto a los ácidos grasos saturados, en la respuesta hipertrigliceridémica e hiperinsulinémica postprandial. Ello determina un papel beneficioso, hasta ahora desconocido, de los ácidos grasos monoinsaturados en la prevención de las enfermedades crónicas asociadas con las hiperlipemias y la diabetes, y en las estrategias nutricionales para combatirlas.

Dietary fatty acids linking postprandial metabolic response and chronic diseases

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Abstract

Chronic diseases are by far the leading cause of mortality in the world. One of the current global recommendations to counteract disability and premature death resulting from chronic diseases is to decrease the consumption of energy-dense high-fat diets, particularly those rich in saturated fatty acids (SFA). The most effective replacement for SFAs in terms of risk factor outcomes for chronic disease are polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). The biochemical basis for healthy benefits of such a dietary pattern has been widely evaluated under fasting conditions. However, the increasing amount of data available from multiple studies suggest that the postprandial state, i.e., “the period that comprises and follows a meal”, plays an important, yet largely silent, role in the genesis of numerous pathological conditions. In this review, the potential of MUFA, PUFA, and SFA to postprandially affect selected metabolic abnormalities related to chronic diseases is discussed.

1. INTRODUCTION

The consumption of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in place of saturated fatty acids (SFA) is considered one of the major aggressive and worldwide nutrition policies for the prevention and control of chronic diseases. This recommended community-wide action arises because we fully recognise the essential role of dietary fatty acids in inducing substantial negative and positive effects on health throughout life. Most importantly, MUFA, PUFA, and SFA adjustment may not only influence present health, but may determine whether an individual will develop obesity, diabetes, hypertension, dyslipidemia, cardiovascular disease, stroke or some types of cancer much later in life (1).

First, the general dietary goal is to restrict SFA consumption to less than 10% of the total daily calories and less than 7% for high-risk people. Then, the consumption of the other fatty acids is adjusted: PUFA, which is also limited to less than 10% of the total daily calories (ranging from 6% up to 10%), and MUFA, which may supply daily calories that are at least equivalent to those provided by SFA and PUFA together (**Figure 1**). The daily total fat intake will range from 15% up to 35% of total daily calories, depending on age, gender, activity, adequate body weight, and the type of dietary fats (e.g., up to 40% of total daily calories is even allowed if virgin olive oil is the main source of MUFA in the diet) (2). The monitoring of metabolic parameters, routinely in the fasting state, is often used to ensure successful long-term outcomes. However, transient but repetitive changes in the postprandial lipid metabolism occur every time we eat a fatty meal. In the postprandial state, dietary fatty acids are largely incorporated into nascent triglyceride-rich lipoproteins, which are released from the small intestine into the blood and thereafter cleared by extracellular lipolytic (3) and non-lipolytic (4) platforms in the tissues. MUFA, PUFA, and SFA have dissimilar postprandial effects on the risk factors for chronic diseases, (2, 5-8) suggesting that short-term outcomes in response to dietary fatty acid adjustment could be useful to finely tune fat consumption, even for preventing diet-related chronic diseases. In this review, we summarise

the evidence in support of the influence of the metabolic control of dietary fatty acids on the risk and pathogenesis of chronic diseases in the context of the postprandial state.

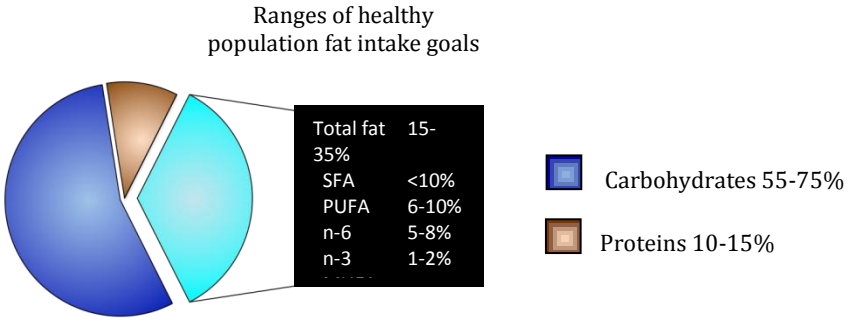

Figure 1. Ranges of population fat intake goals. SFA: saturated fatty acids, PUFA: polyunsaturated fatty acids, MUFA: monounsaturated fatty acids.

2. DIETARY FATTY ACID IDENTITIES

A fatty acid is a carboxylic acid that often has a long unbranched aliphatic chain. Fatty acids are divided into SFA and unsaturated fatty acids based on structural and chemical properties (**Figure 2**). SFA do not contain any double bonds or other functional groups along the chain, which is fully saturated with hydrogen atoms. Palmitic acid [16:0 (PA)] is composed of 16 carbon atoms and is the principal SFA in the diet. SFA is found chiefly in animal products, including meats and dairy foods, but is also found in some plant sources, including coconut, cottonseed, and palm kernel oils. MUFA are unsaturated fatty acids that contain one pair of carbon atoms linked by a *cis* double bond. The major dietary MUFA is oleic acid [18:1n-9 (OA)], which has 18 carbon atoms with the double bond occurring 9 carbon atoms away from the methyl end of the fatty acid molecule. OA is the primary component of olive oil, but also can be found in hazelnut, canola, and peanut oils. A carbon chain that contains two or more *cis* double bonds with the first double bond located between the third and fourth or sixth and seventh carbon atom from the

methyl end of the fatty acid molecule characterises the families of n-3 or n-6 PUFA. These families cannot be synthesised by the human body (double bonds can be introduced into all positions of the fatty acid chain except for the n-3 and n-6 positions) and must be obtained from the diet either as alpha-linolenic acid [18:3n-3 (αLN)] and linoleic acid [18:2n-6 (LA)], or their long-chain PUFA derivatives (9). Of these fatty acids, eicosapentaenoic acid [20:5n-3 (EPA)], docosahexaenoic acid [22:6n-3 (DHA)], dihomo-gamma linolenic acid [20:3n-6 (DGLA)], and arachidonic acid [20:4n-6 (AA)] are the most metabolically significant. While conversion of LA to DGLA and AA is typically very efficient, conversion of αLN to EPA and DHA is much less so (10). This fact has particular importance in people with compromised αLN availability or conversion enzyme activity. Therefore, not only αLN and LA but also EPA and DHA should be considered as essential fatty acids. LA and αLN can be found in vegetable oils, LA in safflower, sunflower, soybean, maize, and cottonseed oils, and αLN in flaxseed, blackcurrant, walnut, rapeseed, and soybean oils. EPA and DHA are abundant in cold-water fatty fish, including herring, sardines, mackerel, salmon, tuna, and shellfish. The competition between EPA and DHA with DGLA and AA and the opposing effects of their oxidative metabolites suggest the importance of an optimum ratio for the consumption of n-3 and n-6 PUFA (11). It is widely accepted that the ratio of n-3 to n-6 consumption in a typical Western diet is imbalanced and extends into the range of 1:10 to 1:25, which is far from the currently established ideal consumption ratio of between 1:1 and 1:4 (12).

Requirements in MUFA, n-3 and n-6 PUFA are satisfied by the diet. MUFA can be synthesised from acetyl-CoA within mammalian tissues. However, it is unclear whether the entire MUFA requirement can be met by de novo metabolic machinery. MUFA, and specifically OA, represent one of the core components of the Mediterranean diet (mainly due to the liberal use of virgin olive oil), which represents a prototypical dietary model associated with a long life expectancy and a low occurrence of chronic diseases (2).

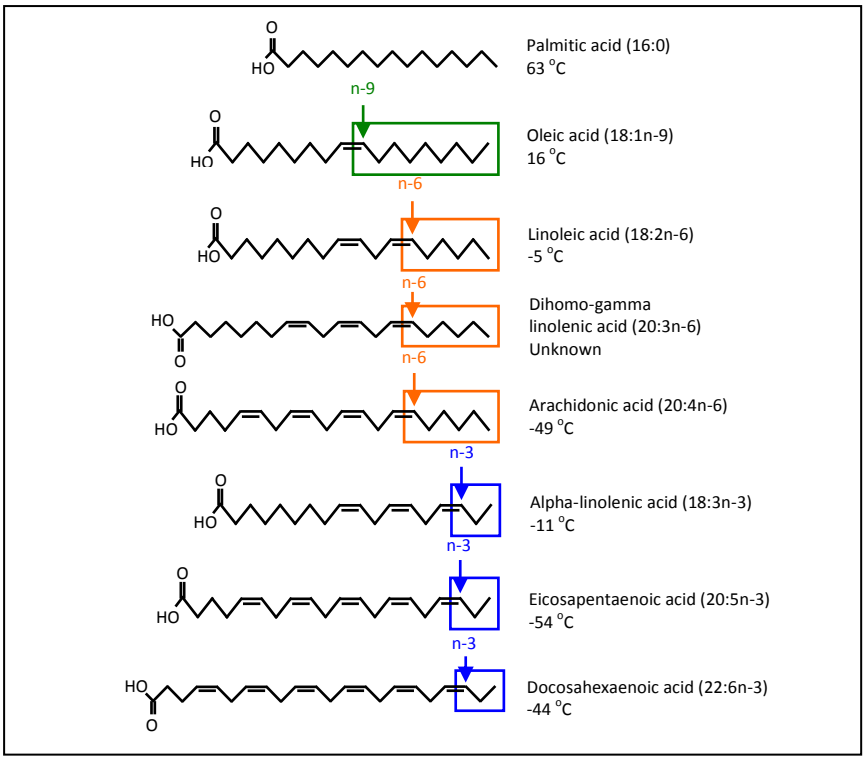

Figure 2. The structure and melting point of the most significant dietary fatty acids.

3. THE ROLE OF DIETARY FATTY ACIDS IN THE RISK FACTORS FOR CHRONIC DISEASES

There is increasing evidence to suggest that postprandial hypertriglyceridemia, which also severely affects insulin secretion and action (6), contributes to the pathogenesis of type 2 diabetes, atherosclerosis, coronary heart disease (CHD), and stroke (13-15). Worldwide, these chronic diseases led to approximately 30% of all deaths, and the proportion is predicted to substantially increase in the next few years. The most important behavioural risk factor for type 2 diabetes, CHD, and stroke is unhealthy diet, (16, 17) mainly due to a high-intake of SFA. However, changes in the quality of dietary fatty acids and their effects on postprandial TRL and insulin excursions

have received little attention as simple and cost-effective strategies for the prevention and management of these chronic diseases.

3.1. Blood lipid abnormalities

The small intestine can secrete two different triglyceride-rich lipoproteins: chylomicrons (CM) and very low-density lipoproteins (VLDL), (18, 19) both referred herein to as TRL (**Figure 3**). CM are formed after eating fatty foods, whereas VLDL are the major lipoproteins secreted during fasting. Studies involving lipid infusion in animals and lipid interaction in an *in vitro* model of human intestinal cells have shown that triglycerides and phospholipids induce the formation of CM and VLDL, respectively (20). The function of the postprandial TRL is to collect the absorbed dietary lipids and fat-soluble components, stabilising them for transport in the aqueous plasma environment. CM and VLDL also carry one molecule of apolipoprotein (apo)-B48 and apoB100 per particle, respectively. However, exaggerated postprandial hypertriglyceridemia, which is characterised by increased production and decreased clearance of postprandial TRL, is emerging as an important risk factor for chronic diseases (13-15). The fatty acid composition of dietary triglycerides influences the size and number of postprandial TRL (21), which may be better predictors of atherosclerosis than conventional triglyceride measurements (13). A recent proton NMR spectroscopic study of nonfractionated plasma revealed that compared to an SFA-based meal, the consumption of a MUFA-rich meal leads to the formation of fewer large TRL particles (21). There is no consensus on the effects of MUFA, PUFA, and SFA on the intensity of the postprandial triglyceride peak. Some studies indicate that SFA-rich meals elicit either a lower, higher or comparable postprandial peak value for triglycerides than meals rich in MUFA and PUFA (22-26). However, it is consistent that postprandial TRL enriched in MUFA and PUFA are more efficiently cleared compared to SFA-containing postprandial TRL. This efficiency probably depends on several factors, including the extent of triglyceride intraluminal lipolysis, the ability of triglyceride-depleted particles

to accept cholesterol from donors, and the rate of receptor-mediated uptake in hepatic and extrahepatic tissues (21, 27, 28).

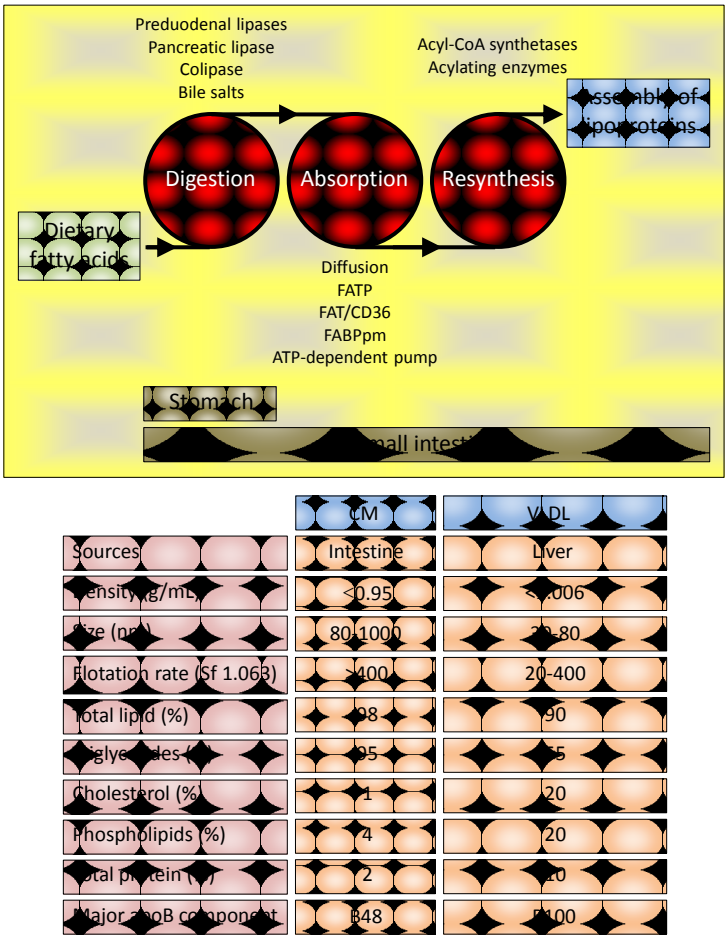

Figure 3. Digestion of triglycerides containing dietary fatty acids, absorption of fatty acids (and sn-2-monoglycerides), resynthesis to triglycerides for intestinal lipoprotein assembly, and physical properties and composition of intestinal lipoproteins: chylomicrons (CM) and very low-density lipoproteins (VLDL). FATP: fatty acid-transport proteins, FAT/CD36: fatty acid translocase/cluster of differentiation 36, FABPpm: plasma membrane-associated fatty acid-binding protein, Sf: Svedberg flotation, apoB: apolipoprotein-B. Values are approximate.

Dietary fatty acids might modulate the clearance of triglycerides in postprandial TRL using a lipoprotein lipase (LPL)–mediated lipolytic platform, which is tethered to the capillary endothelium via heparin sulphate proteoglycans (29, 30). A recent study indicates that dietary fatty acids can modulate the LPL inhibitor angiopoietin-like protein 4 (Angptl4) (31). In a transgenic mouse model of Angptl4 deletion, it was found that Angptl4 protects against the severe proinflammatory effects in mesenteric lymph nodes and resident macrophages of PA-rich meals and that this effect is specific for PA because OA and LA did not cause an inflammatory response. Interestingly, OA and LA were much more potent inducers of Angptl4 expression compared to PA, suggesting that dietary SFA may disturb the homeostatic negative feedback mechanism of Angptl4 on LPL. Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein-1 (GPIHBP1) has the ability to stabilise LPL and partially prevents LPL inhibition by Angptl4 (32). GPIHBP1, which is expressed on the luminal side of the endothelium, has been also suggested to bind postprandial TRL and to facilitate their processing (33). The tissue pattern of GPIHBP1 gene expression is similar to that of LPL. GPIHBP1 and Angptl4 genes are transcriptionally regulated by peroxisome proliferator-activated receptors (PPAR). Fatty acids can be intracellularly generated from postprandial TRL by enzymatic and non-enzymatic mechanisms (34), and they are the best-known bonafide PPAR activators (35). Therefore, it can be hypothesised that distinctive effects of MUFA, PUFA, and SFA on PPAR activity could play a role on GPIHBP1 and Angptl4-mediated TRL lipolysis in the postprandial state.

Delayed clearance of postprandial TRL has been reported to increase lipid exchange between postprandial TRL and cholesterol-rich lipoproteins mediated by cholesterol ester transfer protein (36). This reciprocal exchange leads to the formation of cholesterol-enriched postprandial TRL, whereas cholesterol-rich lipoproteins are enriched in triglycerides (37). Moreover, MUFA-rich meals, when compared to SFA and PUFA-rich meals, promote the largest area-under-the-curve for cholesterol in postprandial TRL (38), suggesting a high lipid exchange turnover with cholesterol-rich lipoproteins.

These observations further support the importance of dietary fatty acids in TRL and cholesterol-rich lipoprotein remodelling in the postprandial state.

Defective clearance of postprandial TRL likely influences the lipid accumulation into the arterial wall (39), which is considered an initial step in atherogenesis. Lipid deposition begins with the entry of TRL and low-density lipoproteins (LDL) and the initiation of a proinflammatory cascade that attracts monocytes into the subendothelial space. Postprandial TRL and oxidised or modified LDL can be taken up by infiltrated macrophages, eventually becoming foam cells. However, monocytes circulate together with TRL in the postprandial bloodstream and may start accumulating lipids even prior to their migration to tissues and differentiation to macrophages (4). *In vivo*, postprandial TRL are removed by circulating monocytes via the apoB48 receptor (apoB48R). Monocytes from healthy volunteers displayed an early time-dependent lipid accumulation in response to a high-fat meal that was paralleled by increased apoB48R mRNA levels and activation. These effects were coincident with an increase of plasma TRL carrying apoB48, with a decrease of plasma free fatty acid levels, and with no change of plasma fractions containing VLDL and cholesterol-rich lipoproteins. During the late postprandial phase, the acceleration in the rate of plasma triglyceride clearance was hypothesised to be a harbinger of apoB48R gene transcription, lipid accumulation, and activation decline in monocytes. Interestingly, when compared to postprandial TRL rich in SFA and PUFA, postprandial TRL rich in MUFA diminish the apoB48R transcriptional activity, lipid accumulation, and activation in human primary and THP-1 monocytes (unpublished data). These findings suggest that postprandial TRL are involved in the process of atherosclerosis prior to their entry into the subendothelial space and highlight the role of dietary fatty acids in modulating pro-atherogenic events in the postprandial state.

3.2. Blood insulin abnormalities

The damage to beta-cell function and the promotion of insulin resistance are known to contribute to the development of type 2 diabetes, which is a metabolic disorder that progresses over the course of months to years. Lifestyle modification is the cornerstone of both treatment and attempts to prevent type 2 diabetes. Compensatory hyperinsulinemia due to enhanced beta-cell function is considered to be an obligate accompanying feature in insulin resistance syndromes (40). Euglycemic clamps or frequently sampled intravenous glucose tolerance tests are the reference methods to determine beta-cell sensitivity to glucose and the sensitivity of body tissues to insulin (41). However, these tests are far from physiological because insulin secretion or activity is only measured in the steady-state. Empirical and model-based indices based on the oral glucose tolerance test (OGTT) provide a reasonable approximation of postprandial beta-cell function and whole-body insulin sensitivity (41, 42). An important caveat of the OGTT is that the events associated with the ingestion of a pure glucose solution are not wholly equivalent to the numerous metabolic events associated with eating a mixed high-fat meal when both carbohydrates and fatty acids are ingested.

It has been hypothesised that insulin resistance syndromes might be a postprandial phenomenon linked to the acute metabolism of dietary fatty acids (43). Exaggerated postprandial hypertriglyceridemia is indeed an inherent feature of diabetic dyslipidemia and is frequently found even in diabetic patients with normal fasting triglycerides (44). There is strong evidence that SFA selectively desensitises the response of peripheral tissues to insulin, whereas MUFA may counteract this effect (45). Such phenomena would be consistent with studies linking SFA-rich meals to dysfunctions in insulin secretion and the frequency of type 2 diabetes (6, 46). It is probable that MUFA, PUFA, and SFA could compete at the level of the beta-cell (**Figure 4**). The islet tissue, which expresses LPL, could access triglycerides from postprandial TRL as a source of free fatty acids, in which case, the type and concentration of the fatty acid in the immediate vicinity of the beta-cells is

likely to be dependent on the nature of the dietary fatty acids (6). The input of fatty acids into the beta-cell can be mediated by cell surface G protein-coupled receptors (i.e., GPR40) (47) and fatty acid-translocase FAT/CD36 (48). ApoE-dependent and independent recognition sites could also cooperate with LPL to selectively remove postprandial TRL and to immediately generate intracellular fatty acids via catabolic pathways (49-53). Free fatty acid deprivation in islet tissue has been reported to impede glucose-stimulated insulin secretion, a process rapidly reversed by replacement with exogenous free fatty acids (54). *In vitro*, OA elicits half of the insulinotropic potency of PA or stearic acid (18:0) (55). When mixed high-fat meals with different proportions of dietary fatty acids are administered to healthy subjects, they become less insulin resistant postprandially as the proportion of MUFA to SFA in dietary fatty acids increase (56). In addition, when compared to SFA-rich meals, the MUFA and PUFA-rich meals induce a lower early postprandial insulin response (56, 57). These findings are consistent with the notion that in comparison with SFA, MUFA might moderate the compensatory hyperactivity of beta-cells in the postprandial state (6), although whether this maintenance of glucose tolerance during feeding periods could prevent or delay the development of overt type 2 diabetes remains to be elucidated.

Fasting hypertriglyceridemia results from either overproduction of triglycerides by the liver, impaired lipolysis, or a combination of both. In hypertriglyceridemic patients, the overproduction of triglycerides is disproportionately greater than the increase in apoB100 production, resulting in the formation of large triglyceride-rich VLDL particles (58). Obesity and insulin resistance result in increased hepatic supply of fatty acids and overproduction of triglycerides (59). Insulin inhibits VLDL production in an effort to reduce the postprandial triglyceride response to a high-fat meal. A recent randomised and within-subject crossover study in volunteers who were newly diagnosed with type IIb or IV hyperlipoproteinemia revealed that postprandial beta-cell function and insulin sensitivity are improved with MUFA when compared to SFA (60), therefore extending the relationship between MUFA-rich meals and the benefits on postprandial glucose homeostasis

observed in subjects with normal fasting triglyceride levels (56) to a population of subjects with high fasting triglyceride levels (60). Furthermore, with regard to resistance to insulin-mediated glucose disposal, SFA was found to stimulate additional insulin secretion to maintain postprandial glucose homeostasis, suggesting a mechanism of lipid-induced deterioration of insulin sensitivity coupled with compensatory insulin secretion that is distinctively modulated by dietary fatty acids. Whether lipoprotein receptor-mediated signalling pathways in skeletal muscle and other insulin target tissues, such as adipose tissue and liver, are sensitive to the types of fatty acids in postprandial TRL, which largely depend on the nature of dietary fatty acids embodied in the meal, and whether they are connected to insulin signalling pathways, should be established.

4. CONCLUSIONS

Dietary fatty acids are nutrient signals that play a relevant role in modulating hypertriglyceridemia and compensatory hyperinsulinemia to decreased insulin sensitivity in the postprandial state, which are major risk factors for chronic diseases such as atherosclerosis and type 2 diabetes. Contrary to SFA, MUFA leads to improved acute lipid tolerance and insulin action. PUFA does not appear to confer additional benefit over MUFA. Therefore, this review is consistent with dietary policies that specifically promote the consumption of MUFA in place of SFA and that highlight the need to study the mechanisms and pathways that might account for transient but repetitive daily dietary fatty acid-induced postprandial metabolic abnormalities. Such phenomena would imply a greater propensity towards chronic diseases, as a metabolic abnormality becomes more exaggerated and prolonged in the postprandial state. This largely subclinical and silent condition further links the metabolism of SFA with atherogenic and diabetogenic disorders and suggests a new role for MUFA, particularly OA, as a critical player in preventing and controlling chronic diseases.

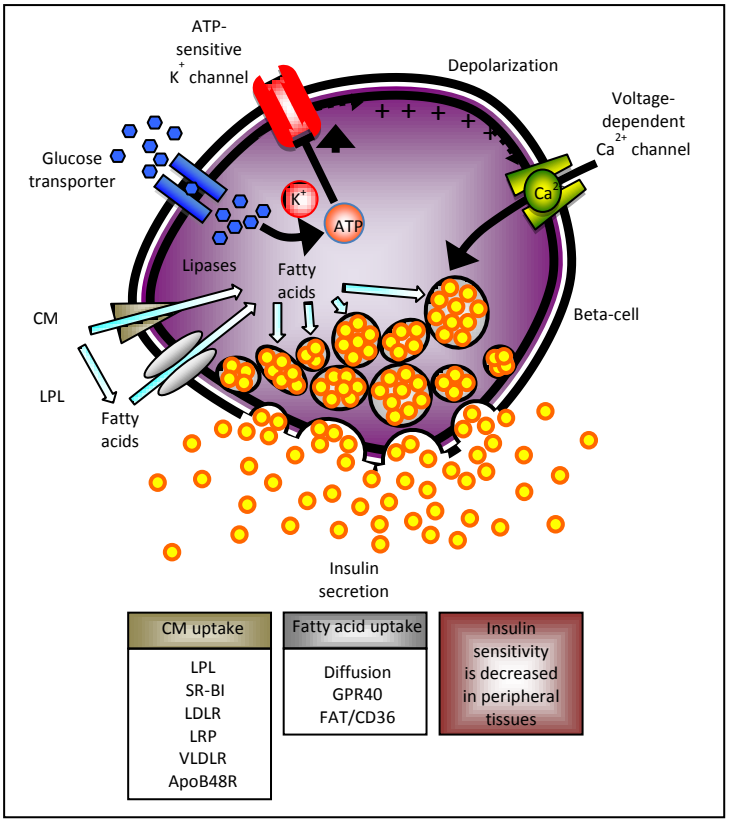

Figure 4. The potential impact of dietary fatty acids on beta-cell function in the postprandial state. In concert or not with LPL, postprandial TRL could be taken up in islets by different receptors and then hydrolyzed inside the beta-cell. Fatty acids from local LPL activity could enter the beta-cell via passive diffusion and different receptors. Elevated beta-cell fatty acid levels could modulate insulin secretion, which largely would depend on the type of fatty acid in the diet. The postprandial state is characterized by a decrease in insulin sensitivity. LPL: lipoprotein lipase, TRL: triglyceride-rich lipoproteins, CM: chylomicrons, SR-BI: scavenger receptor class B member 1, LDLR: low-density lipoprotein receptor, LRP: LDL receptor-related protein, VLDLR, very low-density lipoprotein receptor, apoB48R: apolipoprotein-B48 receptor, GPR40: G protein-coupled receptor 40, FAT/CD36: fatty acid translocase/cluster of differentiation 36.

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CAPÍTULO 2

"Olive oil is superior to other dietary fats for reducing cardiovascular risk in the fed state: a lipid and comprehensive perspective"

Lourdes M. Varela, Almudena Ortega, Sergio López, Beatriz Bermúdez, Yolanda M. Pacheco, José Villar, Rocío Abia and Francisco J.G. Muriana

Olive Oil and Health, Nova Science Publishers Inc, 351-370 (2009)
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En este capítulo se presenta información y se discute de los efectos particularmente beneficiosos del aceite de oliva respecto a otras grasas comestibles sobre diversos factores de riesgo cardiovascular durante el periodo postprandial.

RESUMEN: Evidencias epidemiológicas y estudios nutricionales en humanos han permitido establecer una relación directa entre el consumo de aceite de oliva y la prevención de enfermedades cardiovasculares. En esta revisión se analizan los estudios más recientes sobre los efectos saludables del aceite de oliva, detallando los aspectos relacionados con la digestión y absorción de sus componentes, la producción de lipoproteínas ricas en triglicéridos de composición similar a la del aceite de oliva, y el impacto sobre los principales factores de riesgo cardiovascular durante el periodo postprandial. Se destaca el papel del aceite de oliva, sobretodo respecto a la mantequilla, en la formación de un menor número y mayor tamaño de lipoproteínas postprandiales ricas en triglicéridos; lo cual influye en un rápido aclaramiento y menor intercambio con otras lipoproteínas plasmáticas. También se definen mecanismos de respuesta inmediata a la ingesta de aceite de oliva que reducen la hipercoagulabilidad, la hipertensión, y la inflamación, y que mejoran la función endotelial, el tono vascular, y la diabetes tipo 2. Se

concluye que el aceite de oliva es una grasa comestible natural (zumo de la aceituna) con propiedades nutricionales, más allá de las meramente energéticas, y saludables únicas. Todas estas consideraciones implican que el aceite de oliva es superior a cualquier otra grasa conocida en la prevención de las enfermedades cardiovasculares y el riesgo futuro de mortalidad de origen cardiovascular.

Olive oil is superior to other dietary fats for reducing cardiovascular risk in the fed state: a lipid and comprehensive perspective

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Abstract

Evidence from epidemiological studies suggests that a higher proportion of monounsaturated fatty acids, notably oleic acid, in the diet is linked with a reduction in the risk of coronary heart disease (CHD). To achieve this benefit, olive oil, a major source of oleic acid, should replace a similar amount of saturated fats with no increase in the total daily calorie intake. The biochemical basis for the ameliorative effect of oleic acid has been widely evaluated under fasting conditions. However, the increasing amount of data available from multiple studies suggest that the postprandial state, in other words “the period that comprises and follows a meal”, plays an important, yet largely silent, role in the genesis of numerous pathological conditions, including CHD. The purpose of this chapter is to describe our current understanding of the ability of olive oil to favorably influence postprandial

metabolic processes related to several cardiovascular risk factors and to draw a comparison with other dietary fats.

1. INTRODUCTION

Olive oil is a fruit oil obtained from the olive (Olea europaea; family Oleaceae along with lilacs, jasmine and ash trees), a traditional tree crop of the Mediterranean Basin, that can be consumed in its natural state, known as extra and/or virgin olive oil, or as a refined product. One of the main differences between olive oil and other oils is its high oleic acid content, which makes up 56–84% of total fatty acids complexed in the form of triglycerides.

Evidence from epidemiological studies suggests that a higher proportion of monounsaturated fatty acids (MUFAs), notably oleic acid, in the diet is linked to a reduction in the risk of coronary heart disease (CHD). To achieve this benefit, olive oil, a major source of oleic acid, should replace a similar amount of saturated fat with no increase in the total daily calorie intake. The biochemical basis for the ameliorative effects of oleic acid is thought to involve modification of plasma lipid and lipoprotein concentrations (1), inhibition of coagulation (2), recovery of endothelial function (3), regulation of blood pressure (4), attenuation of inflammation (5) and oxidative status (6), and improved glucose homeostasis (7) under fasting conditions.

More recently, however, a body of evidence has grown to support the hypothesis that postprandial metabolism of dietary fats plays a causal role in the pathogenesis and progression of CHD. Postprandial lipemia is characterized by increased levels of exogenous triglycerides present in triglyceride-rich lipoproteins (TRLs). Postprandial hypertriglyceridemia results from the competition between TRLs for lipoprotein lipase (LPL) and hepatic receptors. As a result of these processes, low- (LDL) and high-density lipoprotein (HDL) particles become abundant in triglycerides, small and dense LDLs are produced, and HDLs are metabolized in the liver and kidneys at a higher rate.

Postprandial hypertriglyceridemia is usually evaluated on the basis of the change in the area-under-the-curve (AUC) of triglyceridemia, assessed at consecutive hours after a standard meal (**Figure 1**). This procedure can be achieved for any other plasma biomarker. Relevant data can also be obtained from the analysis of absolute and relative lipid changes (compared to the fasting value) evaluated at a particular point in time, preferably when changes in triglyceride levels are most pronounced, usually 2–3 h after the meal. There are many factors that may impact inter-individual postprandial response to the meal, some of which are outlined in **Table 1**. However, the association between maximum fasting and postprandial triglyceride levels or postprandial changes in cardiovascular risk factors remains unclear. In this chapter, we show that olive oil could be beneficial, and therefore be considered as a nutritional determinant, for the regulation of several cardiovascular risk factors during the postprandial state compared to other dietary fats.

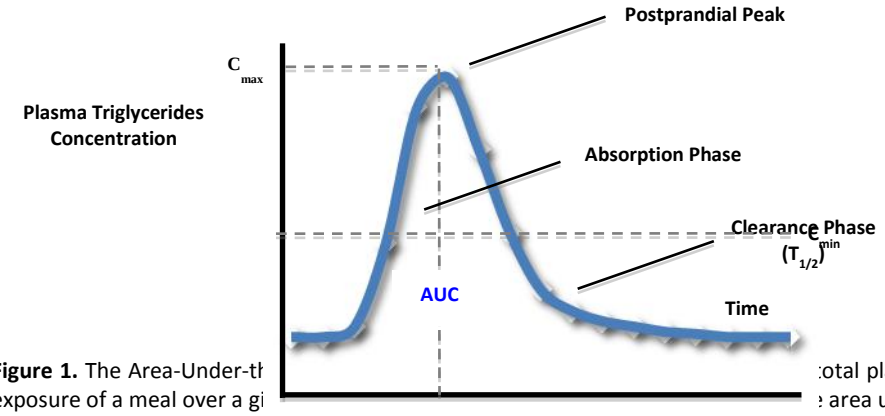

Figure 1. The Area-Under-tl :otal plasma exposure of a meal over a gi : area under the plasma triglycerides concentration-time curve and is estimated by taking multiple concentration measurements at various time points in order to predict AUC. The AUC for triglycerides is determined by the administered fat dose and the clearance of the plasma triglycerides. Cmax: maximum plasma concentration; Cmin: minimum plasma concentration; T_{max}: time to reach Cmax; $T_{1/2}$: half-life.

Table 1. Sources of Postprandial Variability

Subject intrinsic factors
• Genetic variability resulting in differences in absorption, metabolic and clearance

pathways
- Disease state
- Circadian rhythm
- Sex
- Age
- Body surface

Other factors
- Composition of the meal
- Mode of administration
- Background diet
- Subject diet
- Subject compliance

1.1. A brief description of the key facts regarding olive oil digestion and absorption

The digestion and absorption of olive oil is a dynamic, complex and very efficient process (**Figure 2**) that is only partially understood at the molecular level. The hydrophobicity of lipids is a limiting factor for their digestion because of the hydrophilic character of lipases. During the initial gastric process, partially emulsified triglycerides, notably triolein, are attacked by lingual and gastric lipases that are similar in terms of their structure and physicochemical characteristics (8). However, the activity of these lipases towards short-, medium- and long-chain fatty acids is not the same (9). The presence of long-chain free fatty acids (FFAs) per se does not inhibit lipolysis, since oleic acid in amounts close to, or even higher than, those generated during physiological gastric lipolysis does not alter the kinetics of triolein hydrolysis by gastric lipase (10). During gastric lipolysis, FFAs, notably oleic acid, have a higher affinity for the surface than the core of the lipid droplets. The accumulation of FFAs at the droplet surface leads to an inhibition of lipolysis by gastric lipase. The mechanism by which this happens involves the formation of clusters at the surface of the lipid droplets. Isolation of these clusters has shown that they are particles about 200 nm in diameter consisting mainly of FFAs (approximately 74%) along with phospholipids (PLs; 16%), monoglycerides (MGs; 5%), diglycerides (DGs; 4%), free cholesterol (1%), triglycerides (<1%), and gastric lipase.

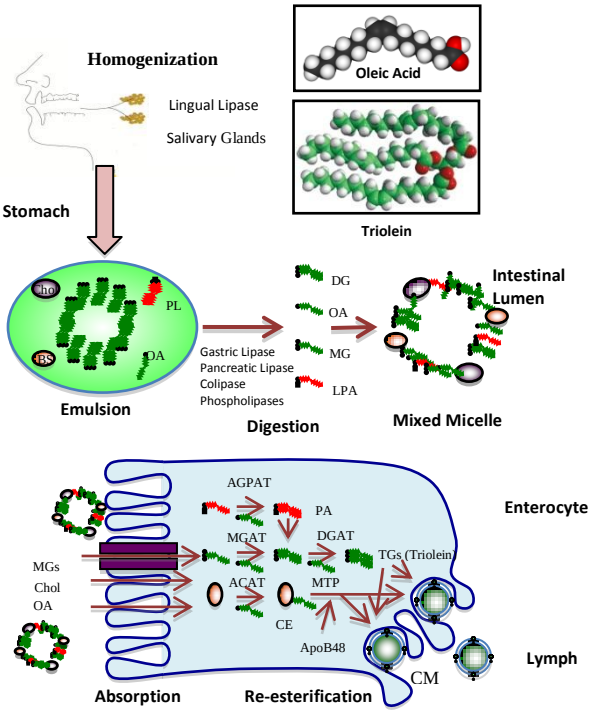

Figure 2. Dietary Lipid (Olive Oil) Digestion and Absorption. Dietary lipid digestion begins in the mouth, where lipids are subjected to partial digestion by lingual lipase. In the stomach, gastric lipase favors the formation of large fat globules with hydrophobic triglycerides (TGs) cores surrounded by polar molecules, including phospholipids (PLs), cholesterol (Chol), fatty acids and ionized proteins. The digestive processes are completed in the intestinal lumen, where large emulsions of fat globules are mixed with bile salts (BS) and pancreatic juice containing lipid digestive enzymes. Monoglycerides (MGs), diglycerides (DGs) and fatty acids that are released by lipid hydrolysis join BS, Chol, lysophosphatidic acid (LPA) and fat-soluble vitamins to form mixed micelles that provide a continuous source of digested dietary products for absorption at the brush-border membranes of the enterocytes. If olive oil is ingested, TGs, DGs, MGs and fatty acids are mainly triolein, sn-1,2 dioleoyl glycerol, sn-2 monooleoyl glycerol and oleic acid (OA), respectively. Fatty acids and MG enter the enterocytes by passive diffusion and are facilitated by transporters. They are then re-esterified sequentially inside the endoplasmic reticulum by MG acyltransferase (MGAT) and DG acyltransferase (DGAT) to form TGs (triolein). LPA is acylated by 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT) to form phosphatidic acid (PA), which is also converted into TGs. Dietary Chol is acylated by acyl-CoA:cholesterol acyltransferase (ACAT) to cholesterol esters (CEs). Facilitated by microsomal triglyceride transfer protein (MTP), TGs

joins CEs and apolipoprotein B48 (ApoB48) to form nascent chylomicrons (CMs) that enter circulation through the lymph.

The oleic acid-rich lipid emulsion enters the intestine as fine droplets less than 0.5 μm in diameter (11). These fat droplets, which are covered with bile salts, are not accessible to pancreatic lipase, although the co-lipase enzyme allows the pancreatic lipase molecule to bind to the lipid aqueous interface, thereby facilitating the stabilization of emulsified triglycerides. Pancreatic lipase does not remain irreversibly adsorbed to the oil–water interface, however, and exchanges rapidly between oil droplets (12). Pancreatic lipase cleaves the sn-1 and sn-3 positions of triglycerides to mainly produce sn-2 monooleoyl glycerol (MOG) and oleic acid molecules (9).

Absorption of these lipid molecules takes place in the epithelial cells of the small intestine, mainly in the proximal jejunum but also in parts that are more distal, in a process that involves both micellar and non-micellar mechanisms. Enterocytes may also take up FFAs via energy-dependent and carrier-mediated processes. This carrier probably plays an important role in the early stages of olive oil intake, whereas passive diffusion is likely to predominate in the late postprandial period. Up to 95% of total fatty acids at the sn-2 position in olive oil are oleic acid, which means that olive oil acts as a supplier of oleic acid-rich hydrocarbon skeletons for the cellular synthesis of triglycerides and PLs (13).

Triglyceride re-synthesis in the enterocyte begins with the activation of FFAs to acyl-CoA. In this form, fatty acids are sequentially transferred into sn-2 MG, notably sn-2 MOG, by MG and DG acyltransferases. This process contributes to 80% of the intestinal triglyceride re-synthesis in the fed state. On the other hand, acyl-coA can also be transferred to α-glycerophosphate (derived from glucose metabolism) by the phosphatidic acid pathway, which accounts for the remaining 20% (9). The regulation of triglyceride re-synthesis is complex and involves transcriptional and post-transcriptional steps that respond to specific hormones, metabolites (derived from individual meals and long-term diet exposures), fasting and re-feeding cycles, and exercise-

mediated energy expenditure (14). The major cellular compartments of the enterocyte where TRLs assembly takes place include the rough and smooth (SER) endoplasmic reticulum and the Golgi apparatus. Furthermore, newly synthesised apoB48 and oleic acid-rich triglycerides accumulate in the SER membrane and are transferred into the lumen in a microsomal triglyceride transfer protein (MTP)-dependent step. Secretion occurs at the basolateral membrane of the enterocytes and TRLs enter the lymphatic capillaries of intestinal microvilli, which drain into the lymphatic channels, thereby reaching systemic circulation through the thoracic duct.

1.2. The influence of olive oil on postprandial plasma lipids

The composition of dietary triglycerides influences the size and number of postprandial TRLs. Furthermore, the degree of unsaturation of fatty acids tends to increase the size of postprandial TRLs (15), and the configuration of the double bonds can also affect their size (16). Indeed, the size and number of TRL particles may be a better predictor of atherosclerosis than conventional triglyceride measurements (17). There is no consensus on the effects of olive oil on the size and number of postprandial TRLs. Results from the study by Jackson et al. (18), for example, showed that olive oil increases the number of postprandial TRLs compared with other oils, including saturated and n-6 and n-3 polyunsaturated-enriched oils. These findings were derived from indirect but consistent measurements where the structural protein apoB48 was used to determine the number and size of TRL particles after each of the test meals. A possible mechanism could involve modulation of the activity or expression of MTPs in the enterocytes, or an mRNA editing enzyme that involves the co-translational enrichment of apoB48 in lipids and further lipidation of the primordial particle along the secretory pathway. On the other hand, a recent 1H NMR spectroscopic study of nonfractionated plasma revealed that consumption of an olive oil-rich meal leads to the formation of a lower number of larger TRL particles than a butter-based meal (19).

The size and number of TRLs influence their fate and rate of clearance. Endothelial cells initially participate in the clearance of postprandial TRLs by way of heparan sulfate proteoglycan-dependent lipoprotein lipase (LPL) and endothelial lipase (EL). LPL (which mainly has glyceride hydrolase activity) and EL (which mainly has phospholipase activity) act together to remodel the monolayer of TRL particles, a step that is essential in order to access the TRL triglyceride core. The largest TRL particles have the highest affinity for LPL, and an inverse correlation has been demonstrated between the size of TRLs and their affinity for LPL (20). Hydrolysis of postprandial TRLs results in the formation of cholesterol ester-rich particles (remnant TRLs) after the removal of 70-90% of the triglycerides. These remnant particles interact with hepatic lipase (HL), which further hydrolyses triglycerides and PLs and prepares the remnant particles to be taken up in a liver receptor-mediated process.

Olive oil increases postprandial triglyceridemia compared with other dietary fats, including safflower oil and milk fat (21). Similar findings have been described by Mekki et al. (22), who found that a meal rich in butter produces a lower postprandial lipemic response than sunflower and olive oils. The time to reach the triglyceride postprandial peak is also higher with olive oil (Jackson et al., 2002). TRL-triglycerides from olive oil are cleared much more efficiently than other MUFA-containing oils during postprandial metabolism, which depends on the distribution of fatty acids into triglyceride and PL molecules (23). Oleic acid is the main fatty acid found in the PL fraction of postprandial TRLs after a meal rich in olive oil, whereas stearic and linoleic acids are found after a meal rich in high-oleic sunflower oil; both the latter exhibit higher clearance times. This metabolic process is also affected by the nature of the triglycerides themselves, with triolein (OOO) being removed more quickly than palmitoyl-dioleoyl glycerol (POO) and dioleoyl-linoleoyl glycerol (OOL) (13).

Olive oil elicits lower triglyceride-to-cholesterol ratios than both butter and high-palmitic sunflower oil in the postprandial TRLs of healthy men (24). The high-fat meals had no influence on fasting triglycerides and cholesterol, thus indicating that postprandial processing of TRLs contributed the most to

changes in the triglyceride-to-cholesterol ratio. The mechanisms responsible for the aforementioned remodeling of postprandial TRLs are not entirely understood but could be the result of different clearance rates for postprandial TRLs, depending on the type of dietary fat present in the meal, the extent of triglyceride intraluminal lipolysis and the ability of triglyceride-depleted particles to accept cholesterol from donors. Previous studies reported that dietary fatty acids might modulate the clearance of triglycerides in postprandial TRLs by LPL-mediated lipolysis (25). Furthermore, fatty acids have been shown to exert some regulatory cellular effects on LPL, although they also compete for LPL binding sites.

Delayed postprandial clearance of triglycerides has been reported to increase the exchange of lipids between postprandial TRLs and cholesterol-rich lipoproteins mediated by cholesterol ester transfer protein (CETP) (26). Such reciprocal exchange leads to the formation of cholesterol-enriched postprandial TRLs, whereas cholesterol-rich lipoproteins are enriched in triglycerides. Accordingly, the slopes for the triglyceride-to-cholesterol ratio and triglyceride clearance in postprandial TRLs have been found to be steep after olive oil-enriched meals (24). In addition, olive oil induced the largest net AUC for cholesterol in postprandial TRLs, thereby suggesting a high lipid exchange turnover with cholesterol-rich lipoproteins. However, oleic acid is a worse CETP activator than palmitic acid. As proposed in previous trials, the transferability of cholesterol from donors might be related to its affinity for CETP, which varies according to the fatty acid composition of the diet and the (surface) size of the acceptors (27). The ingestion of olive oil-containing meals has been shown to result in the formation of large postprandial TRLs, thereby facilitating lipid transfer with cholesterol-rich lipoproteins. These observations further support the importance of the magnitude and duration of the postprandial response to high-fat meals in postprandial TRL remodeling and the effect of olive oil on the production of less atherogenic lipid and on the lipoprotein profile during the postprandial state (**Figure 3**).

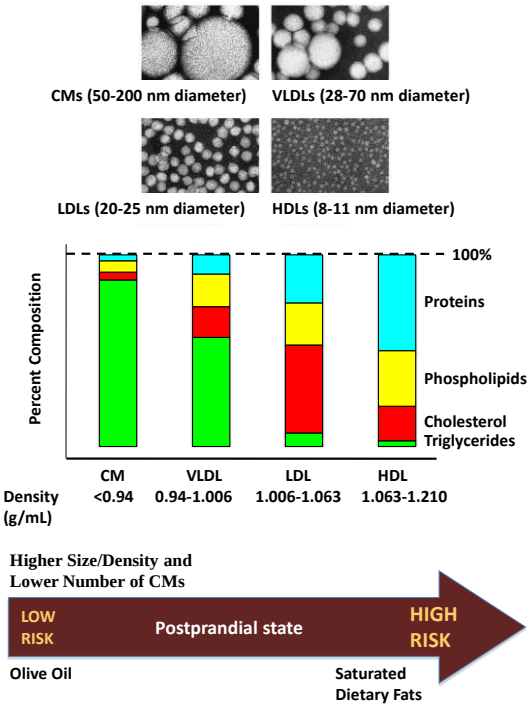

Figure 3. Size and Composition of Lipoproteins in the Fasting and Postprandial Plasma. The size/density of plasma lipoprotein particles is mainly related to the content of triglycerides (CMs > VLDLs > LDLs > HDLs). The ingestion of olive oil leads to the production of higher size/density and lower number of CMs than saturated dietary fats. This postprandial lipoprotein particle profile indicates that olive oil has a lower risk of causing cardiovascular disease.

1.3. The influence of olive oil on postprandial homeostasis

The extrinsic clotting cascade, which is triggered by tissue factor (TF) bound to, or shed from, blood cells and the disrupted endothelium, is thought to play a crucial role in the shift of the haemostatic balance. TF is a small integral transmembrane glycoprotein that acts as a cofactor in the proteolytic activity of factor VII/VIIa toward factor IX and factor X, with higher TF levels resulting in higher plasma fibrinogen levels. In contrast, plasminogen and its

activators, including tissue plasminogen activator (tPA), mediate the proteolytic degradation of fibrin. Indeed, the endothelial release of tPA is considered to be a primary endogenous defense mechanism against thrombosis. Plasminogen activator inhibitor-1 (PAI-1), a member of the serine protease inhibitor (serpin) superfamily, is the main physiological inhibitor of tPA in the fibrinolytic system, along with PAI-2, PAI-3, α_2-macroglobulin, and C1 esterase inhibitor. Thus, endogenous tPA is rapidly neutralized by PAI-1, which binds to the active site of t-PA to form a stable 1:1 stoichiometric (tPA/PAI-1) complex.

Although some studies have indicated that thrombotic complications might be mediated by high levels of circulating TF (28) and accelerated during postprandial lipemia (29), there is little available data regarding the postprandial effects of dietary fats on TF. In contrast, much more information exists regarding the effects of dietary fat composition on postprandial levels of activated factor VII (FVIIa) (30). Activation of FVII by TF represents a critical event in thrombogenesis. Indeed, the transient rise in FVIIa after a fat-rich meal is generally detectable 2–3 h postprandially and persists for at least 8 h, thus displaying dose-response characteristics similar to those reported for TF in healthy subjects. This increase in FVIIa is correlated with fasting triglyceride levels but not with postprandial triglyceride levels (31). Interestingly, oleic acid is one of the main dietary determinants of postprandial changes in FVIIa (32), which agrees with the ability of olive oil to decrease the postprandial response for TF in healthy subjects (33).

Circulating PAI-1 is derived from a variety of sources, including the vascular endothelium, adipose tissue and liver, and is involved in the onset of obesity, diabetes and CHD. It was reported that high-fat meals could induce a PAI-1 peak 2 h postprandially in healthy subjects (33). As was the case with TF, olive oil induced a lower PAI-1 net AUC than butter. It has also been reported that a temporary increase in postprandial PAI-1 antigen levels leads to an impaired fibrinolytic activity after a meal enriched in saturated fatty acids (SFAs), in this case palmitic acid (34), whereas improved fibrinolytic activity has been

associated with a decline in postprandial PAI-1 after high-MUFA (oleic acid) meals (35). These observations could have important clinical consequences as the morning peak in PAI-1 antigen corresponds with the circadian peak in the incidence of acute myocardial infarction.

The tPA antigen has been used to predict subsequent acute coronary syndromes in prospective clinical and multicenter studies involving angina pectoris and post-infarction patients. tPA is a 68 kDa serine protease of 530 amino acids encoded by a gene on chromosome 8 that is mainly generated in the endothelium. Acute release of tPA and PAI-1 antigens has a U-shaped characteristic that depends on their strong diurnal variations in net fibrinolytic activity. There is evidence that the ingestion of high-fat meals leads to a marked decline in postprandial tPA levels in healthy subjects (33). However, the postprandial response of tPA appears not to be directly dependent on the type of dietary fatty acid but rather to postprandial triglyceride levels elicited by dietary fats. Remarkably, fasting tPA antigen is linked to metabolic syndrome and is positively related to fasting triglyceride and insulin and to the waist-to-hip ratio in familial combined hyperlipidemia (36).

These data are consistent with the view that olive oil may have a beneficial regulatory influence on certain thrombogenic and fibrinolytic markers during the postprandial state in humans. A greater decrease in the postprandial levels of TF (anti-thrombotic effect) and PAI-1 (pro-fibrinolytic effect) was observed after the ingestion of olive oil when compared to other dietary fats, such as butter, thereby suggesting that the Mediterranean diet can reduce cardiovascular events in part due to the high oleic acid content in olive oil and its impact on the postprandial haemostatic system.

1.4. The influence of olive oil on postprandial endothelial function

The endothelium, once considered to be merely a selectively permeable barrier between the bloodstream and the outer vascular wall, is now recognized as being a biologically active dynamic organ that is fundamental for regulation of the vascular tone and structure and is involved in both physiological and pathological processes. Endothelial cells are able to synthesize and secrete a broad spectrum of anti-atherosclerotic substances, the best known of which is nitric oxide (NO), a gas that is generated upon metabolism of L-arginine by endothelial NO synthase (eNOS), which is constitutively expressed in endothelial cells (37). The loss of NO results in an increased vascular tone and abnormal endothelium adhesiveness, which in turn promote platelet aggregation and leukocyte trafficking at the vessel wall (38). Such a loss may result from reduced activity of eNOS and/or, more frequently, a decreased bioavailability of NO due to increased breakdown by reactive oxygen species (ROS). Along with NO deficiency, a dysfunctioning endothelium also produces other substances and mediators that are detrimental to the arterial wall, including endothelin-1, tromboxane A2, prostaglandin H2, and ROS. The presence of endothelial dysfunction, whether primary or after cardiovascular risk factors, has been implicated in the pathogenesis of atherosclerosis and thrombosis, both for the loss of its protective capability and the induction of proatherothrombotic mechanisms. Importantly, endothelial membrane composition, and hence eNOS activity, can be modified by dietary fats (39).

The vascular endothelium responds to different types of dietary fats in different ways, although both the negative and positive effects on endothelial function as a consequence of dietary fat intake tend to diminish after 3 h in healthy subjects (40). This could be due the fact that the healthy vascular endothelium may be able to develop unknown defensive mechanisms after short-term alterations. In particular, a comparison of meals enriched with olive oil, butter and walnuts suggested that olive oil induces a greater vasodilatory response and NO bioavailability than butter and walnuts (41). Patients with diabetes invariably show an impairment of endothelium-dependent vasodilation, partly due to the frequent association of this disease with other

cardiovascular risk factors, including hypertension, obesity, and dyslipidemia. Endothelial dysfunction occurs early in the course of type-2 diabetes and contributes to the development of macrovascular complications of the disease. The consumption of a single olive oil-rich meal does not impair endothelial function in subjects with type-2 diabetes (42), whereas consumption of a butter-rich meal exerts a noxious effect on endothelial function that starts at 2 h and is maintained up to 6 h postprandially. Notably, the differential effects of olive oil and butter on endothelial function were observed for similar changes in plasma glucose, insulin, and lipid concentrations, total plasma antioxidant capacity and reactive hyperemia. Consumption of olive oil may therefore preserve postprandial endothelial function in both healthy and sick patients (43).

1.5. The influence of olive oil on postprandial blood pressure

High blood pressure (BP) is one of the key cardiovascular disease risk factors, accounting as it does for nearly two-thirds of all strokes and half of all ischemic heart disease. It is estimated that 7.6 million premature deaths (about 13.5% of the global total) and 92 million deaths and disability-adjusted life years (DALYS; 6.0% of the global total) are attributable to high BP. This risk factor can be classified as either essential or secondary. The former refers to a type of hypertension not associated with a single identifiable cause but to a cluster of factors, including genetics, age, body weight, environment, and diet, whereas the latter refers to the fact that the high BP happens to be a symptom of other medical conditions such as renal disease, narrowing of certain arteries, and adrenal cortical disorders. A reduction in BP is associated with significant decreases in cardiovascular morbidity and mortality.

Previous studies have shown that in the postprandial state, and especially through postprandial hypertriglyceridemia, there are either no effects, or significant, mostly unfavorable, alterations in BP and arterial stiffness (44, 45).

The potential lowering effects of olive oil on postprandial wave reflections may therefore have major cardiovascular implications.

It has been demonstrated recently in an animal model that oleic acid is responsible for the acute hypotensive effects of olive oil (46), which, unlike soybean oil, induced marked and significant reductions of systolic BP after acute (2 h) administration. Interestingly, although the hypotensive effects of a single dose of olive oil were transient, reductions in BP were both marked and stable after 3 or 4 days of olive oil intake. It was hypothesized that oleic acid, as a main digestive product of olive oil, is easily transferred from the small intestine to blood vessels, where it can regulate cell signaling in vascular cells. Once incorporated into the cell membranes, oleic acid could augment their nonlamellar (HII) phase propensity, which appears to facilitate the insertion of bulky isoprenyl moieties into membrane microdomains, such as those found after post-translational modification of the Gγ subunits of trimeric G proteins. It is known that G protein heterotrimers prefer HII-prone regions whereas Gαi monomers prefer lamellar-prone structures, which are normally enriched in SFAs (47). Altered G protein levels and function have been reported in both hypertensive humans and experimental models of hypertension. These findings suggest that the activity of the adrenoreceptor signaling pathway can be regulated by olive oil, thus enhancing the production of vasodilatory stimuli and restricting vasoconstriction pathways (48).

Hemodynamics after combined consumption of olive oil and wine has been explored postprandially in healthy subjects (49) and the augmentation index (AIx) calculated to characterize wave reflections. Aortic AIx was defined as the change of aortic systolic pressure (which is generated by the return of reflected waves at the central aorta), expressed as a percentage of pulse pressure. The combination of olive oil and wine significantly reduced wave reflections (as expressed by AIx) in the postprandial state, thus indicating that they contain vasoactive components that play an important and favorable role in vessel function. It is noteworthy that all combinations of olive oil (refined and virgin) and wine (red and white) were effective in reducing AIx when

compared to the control group. Although this study did not reveal which specific component(s) of olive oil and wine might be more effective in reducing BP, it provided *in vivo* evidence concerning the combined effects of two essential components of the Mediterranean diet on arterial wave reflections in the postprandial state.

1.6. The influence of olive oil on postprandial systemic inflammation

Inflammation plays an instrumental role in all stages of atherosclerosis, with several inflammatory mediators, including adhesive and signaling mechanisms, being involved in early atherosclerotic lesion formation. These mediators participate in the early stages, including recruitment of monocyte–leukocytes from circulating blood by vascular endothelial cells, migration of leucocytes into the intima, and transition of monocytes into macrophages and eventually into lipid-laden foam cells. Inflammatory processes involving activation of cytokines, proteolytic enzymes, tissue factor, and growth factors continue to play a role in the subsequent development of atherosclerotic lesions into complicated plaques (50).

The initial stages of atherosclerosis are characterized by adhesion of circulating leukocytes to the endothelial cells and subsequent transendothelial migration. This process is mediated, in part, by members of the immunoglobulin superfamily, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin, which are expressed on the endothelial membrane in response to several inflammatory cytokines. Soluble forms of ICAM-1 (sICAM-1) and VCAM-1 (sVCAM-1) are found in plasma as a result of A disintegrin and metalloproteinase (ADAM)-mediated shedding from the surface of activated endothelial cells (51). The up-regulation of sICAM-1 and sVCAM-1 is synonymous with endothelial activation and vascular inflammation. It is interesting to note that both sICAM-1 and sVCAM-1 are positively associated with low HDL concentrations in subjects with fasting hypertriglyceridemia (52)

but not in subjects with normal triglyceride levels (53). These observations highlight the importance of triglyceride enrichment of HDL particles secondary to fasting hypertriglyceridemia, and the potential effect of postprandial hypertriglyceridemia on the values of sICAM-1 and sVCAM-1. Indeed, postprandial levels of sICAM-1 and sVCAM-1 have been found to increase with postprandial triglycerides in healthy subjects and hypertriglyceridemic patients, whereas olive oil induced a lower net AUC for sICAM-1 and sVCAM-1 than a high-palmitic sunflower oil enriched meal (54). These observations are fully consistent with the results of *in vitro* experiments which showed that MUFAs, but not SFAs, acutely inhibited cytokine-induced expression of ICAM-1 and VCAM-1 (55). The mechanism of this effect is uncertain, although it may be the result of fatty acid changes in the lipid moieties (PLs and mainly triglycerides) of nascent TRLs, which could be "transferred" to other lipoproteins as a result of postprandial TRL metabolism. This assumption is in accordance with studies indicating that SFAs adversely affect the postprandial anti-inflammatory (antiadhesive) properties of HDLs and that postprandial LDLs are more effective than postabsorptive LDLs in up-regulating ICAM-1 on the surface of the endothelium.

There is some evidence that postprandial hypertriglyceridemia is related to the pro-inflammatory state due to the high expression of activation markers in the circulating neutrophils and monocytes of healthy subjects (56). Ingestion of dietary SFAs, such as cream, may increase postprandial inflammatory activity and plasma IL-6 by increasing the generation of ROS, which are known to upregulate nuclear transcription factor-κB (NF-κB)-mediated inflammation (57). Interestingly, butter and walnuts, but not olive oil, elicited postprandial activation of NF-κB (58) and transcriptional activity of TNF-α (59) in peripheral blood mononuclear cells in healthy men, therefore it is conceivable that diets rich in olive oil may lead to a reduction in the risk of CHD in subjects with a transiently or permanently activated endothelium by improving their vascular inflammatory response postprandially.

1.7. The influence of olive oil on postprandial insulin secretion and sensitivity

A loss of β-cell function and insulin sensitivity is known to contribute to the development of diabetes, a metabolic disorder that develops over a course of months to years. It has been hypothesized that insulin resistance syndromes might be a postprandial phenomenon linked to acute dietary fat metabolism, as suggested from frequently sampled intravenous glucose tolerance test data (60). Exaggerated postprandial hypertriglyceridemia is indeed an inherent feature of diabetic dyslipidemia and is frequently found even in diabetic patients with normal fasting triglycerides (42). There is also strong evidence that SFAs selectively desensitize the response of peripheral tissue to insulin, whereas unsaturated fatty acids (including oleic acid) may counteract this effect (61). Such phenomena would be in accordance with studies linking the nature of dietary fats to dysfunctions in insulin secretion and the frequency of type 2 diabetes. According to empirical indices of □-cell function and insulin sensitivity, it is possible to postprandially discriminate the influence of dietary fats on glucose homeostasis (62). Thus, in healthy subjects with similar fasting insulin sensitivity, as assessed by HOMA-IR, rQUICKI, and basal-Belfiore indices for glycemia and blood FFAs, meals enriched with olive oil and butter elicit superimposable postprandial glucose responses. However, the higher content of oleic acid in olive oil is involved in the decrease of postprandial insulinemic peak and AUC for insulin and FFAs. Indeed, even a high-fat meal increased postprandial β-cell function when assessed on the basis of the insulinogenic index (IGI), IGI/HOMA-IR, AUCI/AUCG and HOMA-B, and decreased postprandial insulin sensitivity as assessed by ISGTTTM, OGISGTTTM and the postprandial Belfiore indices for glycemia and blood FFAs. Furthermore, subjects became less insulin resistant postprandially as the proportion of oleic acid to palmitic acid increased in dietary fats.

Dietary fats, particularly those containing SFAs, are known to promote insulin secretion and resistance. Indeed, β-cells are particularly sensitive to the degree of unsaturation of the fatty acids (63). It is likely that oleic acid and

palmitic acid could compete at the level of the β-cell, in line with a previous model to explain the ability of fatty acids to trigger insulin secretion by glucose-responsive triglyceride/FFA cycling (64). The islet tissue, which expresses LPL, could access postprandial triglycerides as a source of FFAs, in which case the FFA type and concentration in the immediate vicinity of the β-cells is likely to be dependent on the nature of dietary fats. This system could be linked to the local promotion of both intracellular triglyceride lipolysis and fatty acid esterification. FFA deprivation in islet tissue has indeed been reported to impede glucose-stimulated insulin secretion, a process rapidly reversed by replacement with exogenous FFAs (65). Oleic acid was found to elicit half the insulinotropic potency of palmitic or stearic acids. Thus, it has been hypothesized that, in comparison with saturated fats, olive oil might moderate postprandial hyperactivity of β-cells, although whether this maintenance of glucose tolerance during feeding periods could prevent or delay the development of overt type 2 diabetes is still to be determined.

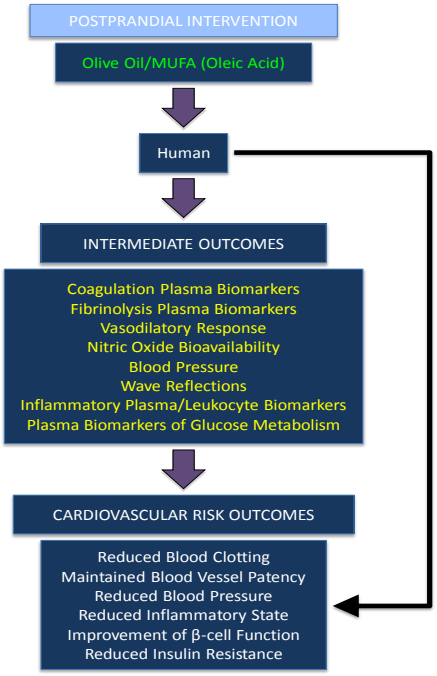

Figure 4. Analytic Framework of Evidence that Olive Oil Arrest Cardiovascular Risk Factors During Postprandial State. This framework highlights how olive oil postprandially impacts outcome measures/parameters associated with cardiovascular disease.

2. CONCLUSION

These data highlight the need to study the mechanisms and pathways that might account for transient but repetitive (daily) dietary fat-induced postprandial changes to cardiovascular disease-related biomarkers and conditions, and could signal a shift in our understanding of the principles underlying atherosclerosis. This new view links postprandial metabolism of dietary fats with cardiovascular disorders and the risk of future cardiovascular morbidity and mortality. However, it is still unclear whether specific

modulation of these responses to the ingestion of high-fat meals can alter cardiovascular outcomes. Nevertheless, we conclude that, mainly due to its high oleic acid (MUFA) content, olive oil is useful when designing optimal dietary fat intake to postprandially counteract several cardiovascular risk factors (**Figure 4**).

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CAPÍTULO 3

"A high-fat meal promotes lipid-load and apolipoprotein B-48 receptor transcriptional activity in circulating monocytes"

Lourdes M. Varela, Almudena Ortega, Beatriz Bermúdez, Sergio López, Yolanda M. Pacheco, José Villar, Rocío Abia and Francisco J.G. Muriana

The American Journal of Clinical Nutrition 93, 918-925 (2011)

En este trabajo se incluyen los estudios llevados a cabo para cumplir con el **Objetivo 1** de esta Tesis Doctoral: Evaluar el efecto de la ingesta de una comida enriquecida con grasa sobre la expresión del gen apoB48R y la acumulación intracelular de lípidos en monocitos de personas sanas durante el periodo postprandial.

RESUMEN: Tras la ingesta de una comida rica en grasas, es decir, durante el periodo postprandial, se produce un aumento de la producción de lipoproteínas ricas en triglicéridos (quilomicrones, CM) en el intestino. En el proceso de ensamblaje de estos CM, se incluye una molécula de apolipoproteína B48 (apoB48) que sirve para el reconocimiento y la captación de CM por células y tejidos. El receptor que reconoce apoB48, y por lo tanto las lipoproteínas que lo transportan, se denomina receptor de apoB48 (apoB48R). Este receptor ha sido descrito en macrófagos humanos y su función fisiológica es muy poco conocida. En este artículo se describe por primera vez la existencia de apoB48R y la correlación de su expresión génica con la acumulación de lípidos en monocitos circulantes durante el periodo postprandial en personas sanas. Se comprueba la hipótesis provocativa de que los monocitos, antes de infiltrarse en el espacio subendotelial de la pared vascular de las arterias y de transformarse en macrófagos y luego en células espumosas, pueden captar CM a través de apoB48R y acumular lípidos de

forma independiente a la concentración plasmática de ácidos grasos libres y de otros receptores como VLDLR, LRP1/LRP-1, y SCARBI/SR-BI. También se detectó la activación temprana de estos monocitos postprandiales, mediante la expresión relativa del gen que codifica el marcador ITGAM/CD11b. Este estudio demuestra un nuevo y relevante aspecto de la aterogenicidad de las lipoproteínas postprandiales ricas en triglicéridos e identifica al receptor apoB48R como una diana terapéutica en la prevención de enfermedades cardiovasculares, especialmente en personas con hipertrigliceridemia postprandial exacerbada.

A high-fat meal promotes lipid-load and apolipoprotein B-48 receptor transcriptional activity in circulating monocytes[1-4]

Lourdes M. Varela, Almudena Ortega, Beatriz Bermúdez, Sergio López, Yolanda M. Pacheco, José Villar, Rocío Abia and Francisco J.G. Muriana

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Abstract

Background: The postprandial metabolism of dietary fats results in the production of apolipoprotein B48 (apoB48)-containing triglyceride-rich lipoproteins (TRLs), which cause rapid receptor-mediated macrophage lipid engorgement via the apoB48 cell surface receptor (apoB48R). Monocytes circulate together with apoB48-containing TRLs in the postprandial bloodstream and may start accumulating lipids even prior to their migration to tissues and differentiation to macrophages.

Objectives: We sought to determine whether circulating monocytes are equipped with apoB48R and whether, in the postprandial state, circulating monocytes accumulate lipids and modulate apoB48R transcriptional activity after intake of a high-fat meal.

Design: In a crossover design, we studied the effect of a high-fat meal on fasting and postprandial concentrations of triglycerides, free fatty acids, cholesterol, and insulin in 12 healthy men. TRLs and monocytes were freshly isolated at fasting, hourly until the postprandial peak, and at the late postprandial phase. TRLs were subjected to triglycerides, apoB-48, and apoB100 analyses; and lipid accumulation and apoB48R mRNA expression levels were measured in monocytes.

Results: Monocytes showed a time-dependent lipid accumulation in response to the high-fat meal, which was paralleled by an increased in apoB48R mRNA expression levels. These effects were coincident only with an increase in apoB48-containing TRLs in the postprandial phase and were also observed *ex vivo* in freshly isolated monocytes incubated with apoB48-containing TRLs.

Conclusion: In a setting of abundant plasma apoB48-containing TRLs, these findings highlight the role of dietary fat in inducing lipid accumulation and apoB48R gene transcription in circulating monocytes.

1. INTRODUCTION

Increasing evidence suggests that postprandial hypertriglyceridemia contributes to the pathogenesis of atherosclerosis, including coronary heart disease and subsequent sudden death (1, 2). In the postprandial state, the lipid moieties in circulation are mainly in the form of triglyceride-rich lipoproteins (TRLs). These lipoproteins carry one molecule of apolipoprotein (apo) B-48 (apoB48) that lacks the C-terminal domain of the apoB100 (apoB100) that binds to the LDL receptor (3). However, apoB48 protein has a proteoglycan-binding site in the amino terminal region that confers as equal atherogenicity to apoB48- and apoB100-containing lipoproteins (4). TRLs and their remnants are the only lipoproteins that cause rapid receptor-mediated macrophage lipid engorgement via the cell surface apoB48 receptor (apoB48R) (5). The apoB48R is an apoE-independent receptor that binds to apoB48 or to a like domain of apoB100 in TRLs and may function as a nutritional receptor to provide dietary fatty acids and lipid-soluble vitamins to cells, and this pathway is not regulated by sterol (6).

According to the current paradigm, the atherosclerotic process is primarily initiated in the vascular wall itself, where macrophages derived from circulating monocytes transform to foam cells by accumulating lipids. Furthermore, apoB48-containing TRLs have the ability to enter into the arterial intima, and they are even trapped preferentially over LDL (7). However, monocytes circulate together with apoB48-containing TRLs in the postprandial bloodstream (8) and may start accumulating lipids even before their migration to tissues and differentiation to macrophages. The purpose of our study was to establish, in healthy volunteers, whether circulating monocytes are equipped with apoB48R and whether in the postprandial state, circulating monocytes engulf lipids as a result of abrupt plasma increase of apoB48-containing TRLs after consumption of a high-fat meal.

2. SUBJECTS AND METHODS

2.1. Subjects

Studies were performed in 12 healthy men whose mean (±SD) age was 24 ± 3 years with a mean body mass index (in kg/m^2) of 22.1 ± 1.2. All subjects had a normal blood count and serological measures, and they took no medication known to affect lipoprotein metabolism. This study was conducted according to good clinical practice guidelines. Before the study, all protocols were approved by the local institutional review board (Ethics Committee of the Hospitales Virgen del Rocio, Seville), and written consent was obtained from each participant. The study was conducted according to the principles expressed in the Helsinki Declaration by the World Medical Association. Recruitment for this study began in March 2008.

2.2. Methods

2.2.1. Plasma measurements

Plasma was separated from blood collected in EDTA-containing Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by centrifugation (2000× g, 20 min, 4 ^{o}C), and triglycerides and cholesterol were quantified by using commercially available reagents on a Hitachi Modular Analytics D-2400 analyzer (Roche Diagnostic, Basel, Switzerland). Plasma free fatty acid (FFA) concentrations were measured by using an ACS-ACOD assay (Wako Chemicals GmbH, Neuss, Germany) on a COBAS Mira-Plus analyzer. Plasma insulin was measured by using a specific enzyme-linked immunosorbent assay (Dako, Cambridge, United Kingdom) on a Hitachi Modular Analytics E-170 analyzer.

Postprandial TRLs [Svedberg flotation unit (S_f) > 400, density > 0.93 g/mL] were isolated from plasma layered with sodium chloride solution (density = 1.006 g/mL) by ultracentrifugation (95,000× g, 42 min, 4 °C) (9).

Ultracentrifugation was performed using an SW 41 Ti rotor in a Beckman L8-70M preparative ultracentrifuge (Beckman Instruments, Barcelona, Spain). Triglycerides in postprandial TRLs were measured by using an enzyme-based colorimetric kit supplied by Thermo Trace (Madrid, Spain); apoB100 and apoB48 in postprandial TRLs were separated by sodium dodecyl sulfate polyacrylamide slab gel electrophoresis and scanned with laser densitometry (10).

2.2.2. Monocyte isolation and determinations

Monocytes were immediately isolated from blood collected into citrate-containing Vacutainer CPT tubes (Becton Dickinson) by magnetic cell sorting with CD14 MicroBeads according to the manufacturer's instructions (MACS/VarioMACS, Myltenyi Biotec, Madrid, Spain). Hematoxylin and Oil Red O were used for nuclei and intracellular neutral lipid staining, and the stained cells were examined by using an Olympus model IX81 inverted phase microscope fitted with a DP71 Olympus digital camera. Cell R software (Olympus, Barcelona, Spain) was used for image acquisition and further image analysis. Monocytes were tested for purity by CD14 fluorescein isothiocyanate (FYTC) labeling and fluorescence-activated cell sorter (FACS) analysis. Briefly, 5×10^5 cells were stained with FITC-conjugated CD14 (Becton Dickinson), and 5×10^5 cells were stained with FITC-conjugated mouse IgG_{2a} as a negative isotype control. Cells were incubated in phosphate-buffered saline, 0.1% sodium azide, and 20 µL of the conjugated antibody at room temperature for 15 min, washed, and resuspended in 300 µL phosphate-buffered saline. A FACScanto II flow cytometer and FACSDiva software (Becton Dickinson) were used for the analysis.

In *ex vivo* experiments, monocytes isolated from blood at fasting (referred to as primary monocytes) were exposed to RPMI medium containing postprandial TRL (100 µg triglycerides/mL, ~0.1 mmol/L) at indicated times. Cellular lipids were extracted by using a hexane:isopropyl alcohol (3:2)

mixture. Triglycerides were determined by enzymatic assay using a kit from Thermo Trace.

2.2.3. RNA preparation

Total RNA was also extracted from monocytes by using Trizol Reagent (Roche), as instructed by the manufacturer. RNA quality was assessed on the basis of the A_{260}/A_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). RNA (1 μg) was subjected to reverse transcription at 42 °C for 1 h. The reaction mixture contained 1 × reverse transcription buffer, 1 mmol dNTP mix/L, 20 units RNase inhibitor, and 200 units revertAid M-MuLV reverse transcriptase (Fermentas, Madrid, Spain). Of the resulting cDNA, 20 ng was used as a template for real-time polymerase chain reaction (PCR) amplifications. The expression levels of mRNA for specific genes were measured by real-time PCR in a MX3000P system (Stratagene, Madrid, Spain). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Master mix (Stratagene) containing the primer pairs for apoB48R, VLDL receptor (VLDLR), LDL receptor-related protein 1 (LRP1/LRP-1), scavenger receptor class B member 1 (SCARBI/SR-BI), cluster differentiation molecule 11b (ITGAM/CD11b), ribosomal protein large P0 (RPLP0), or hypoxanthine phosphoribosyltransferase as housekeeping genes (**Table 1**). All amplification reactions were performed in triplicate, and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change in mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (RPLP0 and hypoxanthine phosphoribosyltransferase) gene content and expressed as the fold change over the controls.

3. DESIGN

The study was designed as a single-blind, randomized and within-subject crossover in which the volunteers attended the Research (Internal Medicine) Unit at the Hospitales Virgen del Rocío. Fasting blood samples (t = 0) were taken at 08:00 after the subjects fasted overnight (12 h). Immediately afterwards, the subjects ingested a high-fat meal within 15 min, which consisted of dietary fat (butter; 50 g/m^2 body surface area), a portion of plain pasta (30 g/m^2 body surface area), one slice of brown bread, and one skimmed yogurt (11). The average total energy provided by the high-fat meal was ~800 kcal (~10 kcal/kg) with a profile of 72% fat, 22% carbohydrate, and 6% protein. The subjects also consumed the same test meal containing no fat as a control meal. There was an interval of ~2 weeks between meals. Blood samples were collected 1, 2, 3, 4, 6, and 8 hours after ingestion of the meals.

4. STATISTICAL ANALYSIS

Individual data from each subject were plotted and evaluated qualitatively, and the general pattern was described. A repeated-measures analysis of variance model was used to assess the effect of the meals on plasma triglycerides, FFAs, insulin responses, and monocyte gene expression. Area under the curve (AUC) and incremental AUC (iAUC) (area above baseline) were calculated by using the trapezoidal rule. A Bonferroni correction was used for the post hoc detection of significant pairwise differences. The data were analyzed by using STATVIEW (5.0) for WINDOWS (SAS Institute, Cary, NC). The designated level of significance was $P < 0.05$.

5. RESULTS

Plasma concentrations of postprandial triglycerides induced by the high-fat meal are shown in **Figure 1A**. As expected, no plasma triglycerides appeared

when no fat was included in the meal. However, a marked increase ($P < 0.05$) in plasma triglycerides was observed when fat was consumed, which peaked at 3-4 h. The high-fat meal induced higher ($P < 0.05$) AUC and iAUC values (0-8 h) for plasma triglycerides than did the no-fat meal (**Figure 1B**). Likewise, the high-fat meal induced significantly ($P < 0.05$) higher mean plasma FFA and insulin concentrations (**Figure 1C**) and AUC and iAUC values (0-8 h) for FFAs and insulin (**Figure 1D**).

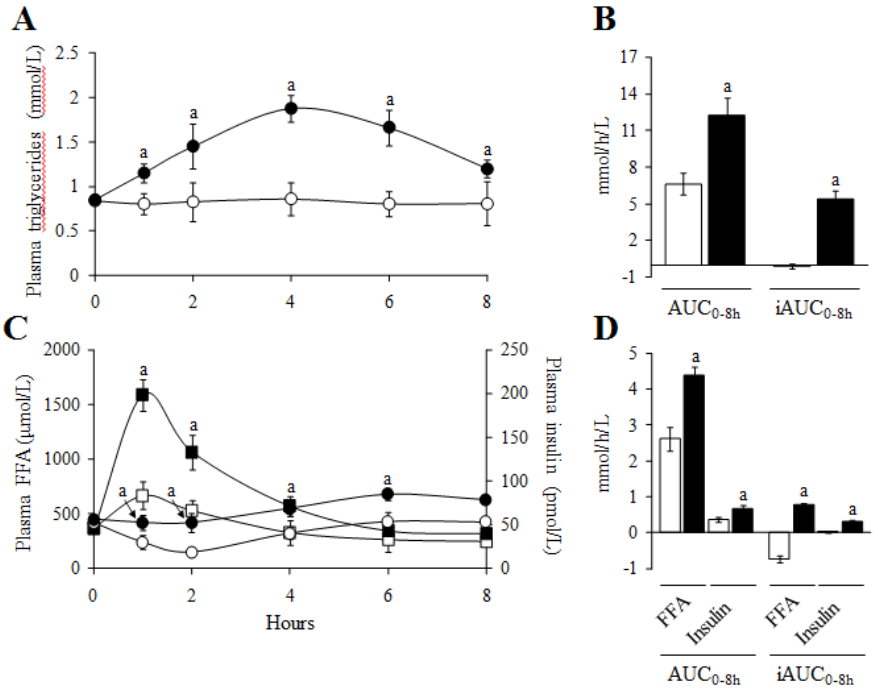

Figure 1. Mean (±SD) postprandial plasma concentrations of triglycerides, free fatty acids (FFA), and insulin, and the area under the curve (AUC) and the incremental AUN (iAUC) between 0 and 8 h for plasma triglycerides, FFAs ($x10^{-3}$), and insulin ($x10^{-3}$) after ingestion of the no-fat meal (open symbols and bars) or the high-fat meal (closed symbols and bars). A and B: Tiglycerides. C and D: FFAs (circles) and insulin (squares). [a]Significantly different from the no-fat meal, $P < 0.05$ (repeated-measures ANOVA with Bonferroni correction. $n = 12$.

The TRL fraction (S_f > 400) was isolated by centrifugation from fasting and postprandial plasma. In the fasting state, the mean plasma triglycerides concentrations was 0.85 ± 0.22 mmol/L, and the mean TRL triglyceride concentration was 0.27 ± 0.08 mmol/L (32% of plasma triglycerides). At the time point of the maximal increase in triglycerides after the high-fat meal, the mean plasma triglycerides concentration was 1.86 ± 0.15 mmol/L, and the mean TRL triglyceride concentration was 1.36 ± 0.16 mmol/L. Therefore, nearly 75% of the plasma triglycerides were contained in the TRL fraction 3-4 h after the high-fat meal; the remaining 25% of plasma triglycerides was in the S_f 20-400 lipoproteins. When the early postprandial phase (0-4 h) was analyzed for triglyceride, cholesterol, and FFA responses in plasma and for triglycerides in TRL fractions and S_f 20-400 lipoproteins, only an increase in the iAUC value (0-4 h) for triglycerides in plasma and in TRLs was observed (**Figure 2A**). Furthermore, apoB48 was found to be predominant (5:1) over apoB100 in the postprandial TRL fraction (referred to as apoB48-containing TRLs) (**Figure 2B**).

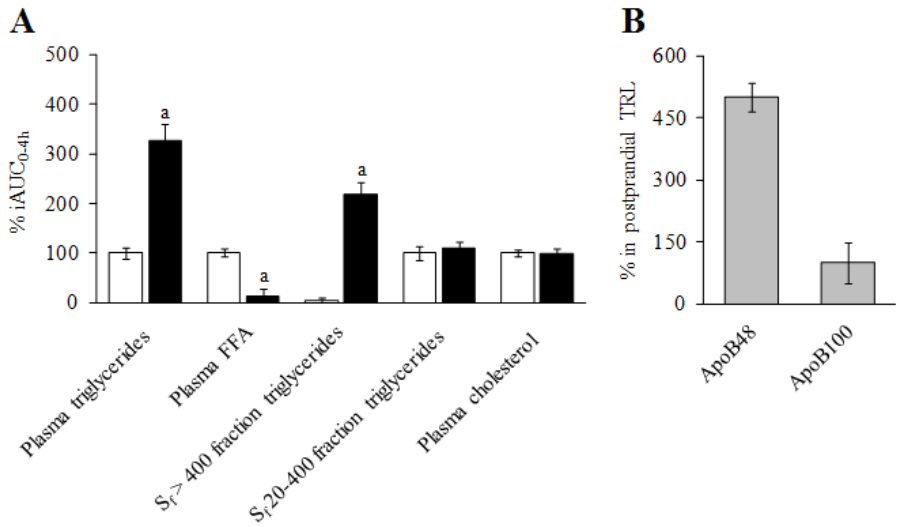

Figure 2. A. Mean (±SD) percentage incremental areas under the curve (iAUC) in the early postprandial phase (0-4 h) for plasma triglycerides, free fatty acids (FFA), cholesterol, and triglycerides with a Svedberg flotation unit (Sf) > 400 (postprandial triglyceride-rich lipoproteins, TRLs) or 20-400 after the ingestion of the no-fat meal (open bars) or the high-fat meal (open bars). B: Mean (±SD) percentage of apolipoprotein B-48 (ApoB48) relative to apolipoprotein B-

100 (ApoB100) in postprandial TRLs. [a]Significantly different from the no-fat meal, $P < 0.05$ (repeated-measures ANOVA with Bonferroni correction. $n = 12$.

For circulating monocyte isolation, peripheral blood was obtained at fasting, hourly until the postprandial peak, and at the late postprandial phase (6 and 8 h). The monocyte fractions contained mostly (91.4 ± 2.6%) a population of $CD14^+$ cells (**Figure 3A**). Cytoplasmic lipid droplets were virtually not present in monocytes at fasting and throughout the postprandial period after the meal with no fat (**Figure 3B**, upper panels). However, an accumulation of lipid droplets was observed in the cytoplasm of monocytes at the postprandial peak after the high-fat meal (**Figure 3B**, bottom panels). A logarithmic function was fitted to the triglyceride content in monocytes during the time-course experiments from 0 to 4 h (**Figure 3C**). Monocyte triglycerides decreased (marginally significant; $P < 0.08$) at the late postprandial phase. Furthermore, a strong correlation ($P < 0.05$) was observed between iAUC values (0-1, 0-2, 0-3, and 0-4 h) for the triglyceride content in monocytes and iAUC values (0-1, 0-2, 0-3, and 0-4 h) for triglycerides in plasma and in TRL (Sf > 400) (**Figure 3D**). This time-dependent cumulative effect of lipids in postprandial monocytes did not correlate with corresponding iAUC values for FFAs and cholesterol in plasma and for triglycerides in S_f 20-400 lipoproteins (**Figure 3E**).

The relative content of apoB48R mRNA in monocytes was examined by using real time-PCR. The predicted 206-bp amplified DNA fragment from apoB48R had a melting temperature of 86.8 ^oC, with Ct values ranging from 19 to 24 for different DNA concentrations (**Figure 4A**). RPLP0 and hypoxanthine phosphoribosyltransferase genes were used as endogenous controls to ensure that nearly equal amounts of cDNA template were used from monocytes. Each cellular sample was tested twice; for all samples, repeat PCR analysis yielded consistent results. ApoB48R protein expression was not analyzed because there is no commercially antibody available. Expression levels of apoB48R mRNA in monocytes did not change postprandially when no fat was included in the meal (**Figure 4B**). However, we observed an increase of apoB48R mRNA expression in monocytes from fasting to the postprandial peak after the high-

fat meal. A logarithmic function was fitted to the apoB48R mRNA concentration in monocytes during the time-course experiments from 0 to 4 h (**Figure 4C**), which strongly correlated with monocyte lipid accumulation (**Figure 4D**) after the high-fat meal. Monocyte apoB48R mRNA expression decreased at the late postprandial phase (6 and 8 h), when plasma concentrations of triglycerides (and thereby apoB48-containing TRLs) also decreased (**Figure 4E**). The steepness of the trend line for the decrease in plasma triglycerides was greater than that for monocyte apoB48R mRNA expression. Analysis of the expression of genes encoding other lipoprotein receptors, including VLDLR, LRP1/LRP-1, and SCARBI/SR-BI, were assessed (**Figure 4F**). VLDLR mRNA was undetectable, whereas LRP1/LRP-1 and SCARBI/SR-BI mRNA expression levels were down-regulated ($P < 0.05$) in monocytes in response to the high-fat meal. In further *ex vivo* experiments, apoB48-containing TRLs induced apoB48R transcriptional activity and the accumulation of lipids in primary monocytes (**Figure 4G**).

Because the increased expression of the β integrin heterodimer ITGAM/CD11b is a typical feature of leukocyte activation (8), we assessed the effect of the high-fat meal on the expression of ITGAM/CD11b in monocytes by real time-PCR. The relative content of ITGAM/CD11b mRNA in monocytes increased significantly ($P < 0.05$) after the high-fat meal, but did not change significantly in monocytes after the meal with no fat when compared with monocytes at fasting (**Figure 5**). In addition, monocyte ITGAM/CD11b gene expression decreased in the late postprandial phase (8 h).

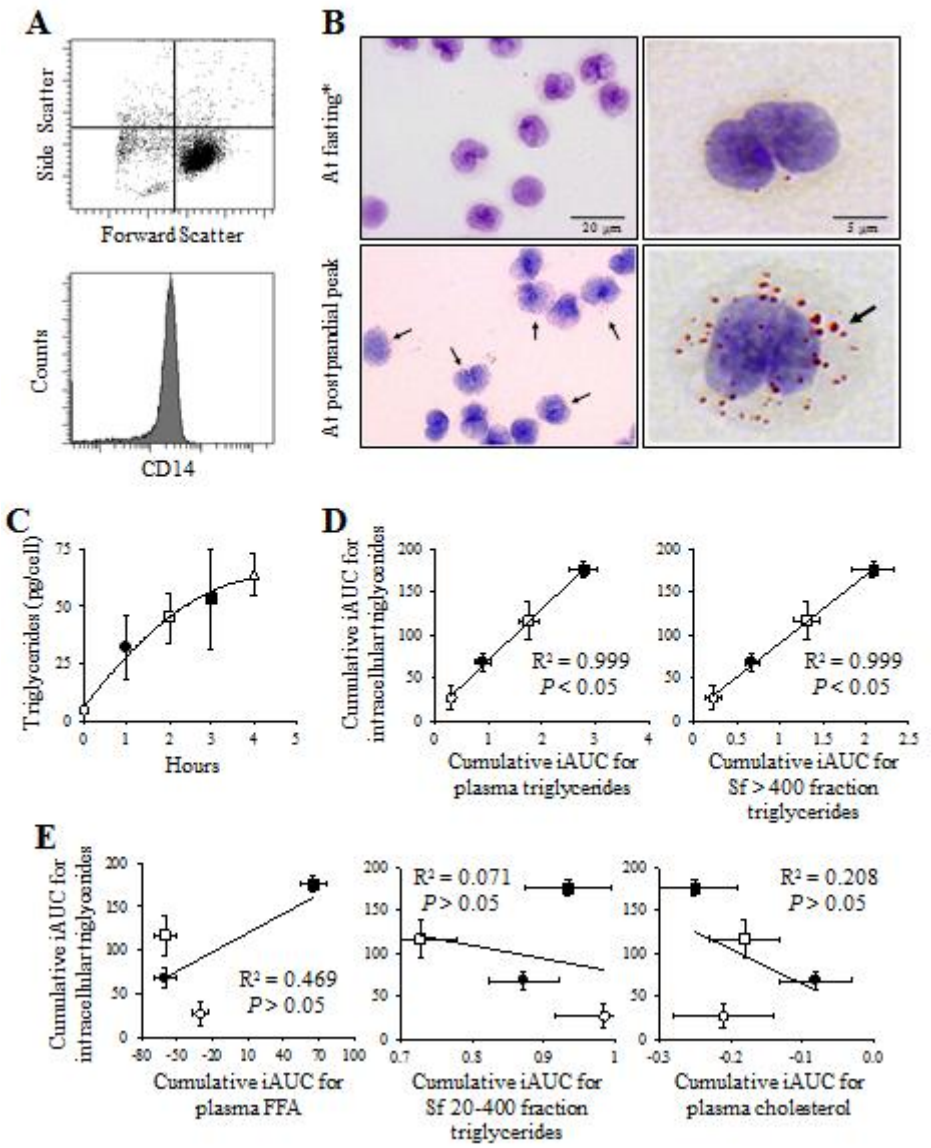

Figure 3. Mean (±SD) lipid accumulation in freshly isolated monocytes after ingestion of the high-fat meal. A: Representative assessment of the purity of monocytes with anti-CD14 (a side scatter/forward scatter representative histogram of monocytes is shown at the top of the panel). B: Representative monocytes stained with hematoxylin and oil red O (asterisk refers to similar staining for monocytes throughout the postprandial period after the no-fat meal; arrows indicate lipid droplets; panels on the right represent magnified views of the panels on the left). C: Time-course increase (0–4 h) and decrease (4–8 h) in triglycerides in monocytes. D and E:

Plots and correlations of incremental areas under the curve (iAUC) at 0–1 h (□), 0–2 h (■), 0–3 h (Δ), and 0–4 h (▲) for triglycerides in monocytes with the corresponding iAUC values for triglycerides in plasma and in triglyceride-rich lipoproteins [Svedberg flotation unit (Sf) . 400] and or free fatty acids (FFA) in plasma, triglycerides in Sf 20–400 lipoproteins, and cholesterol in plasma. *n* = 12. Data were analyzed by simple linear regression, and the square of the Pearson product-moment correlation coefficient (R^2) was determined (D and E).

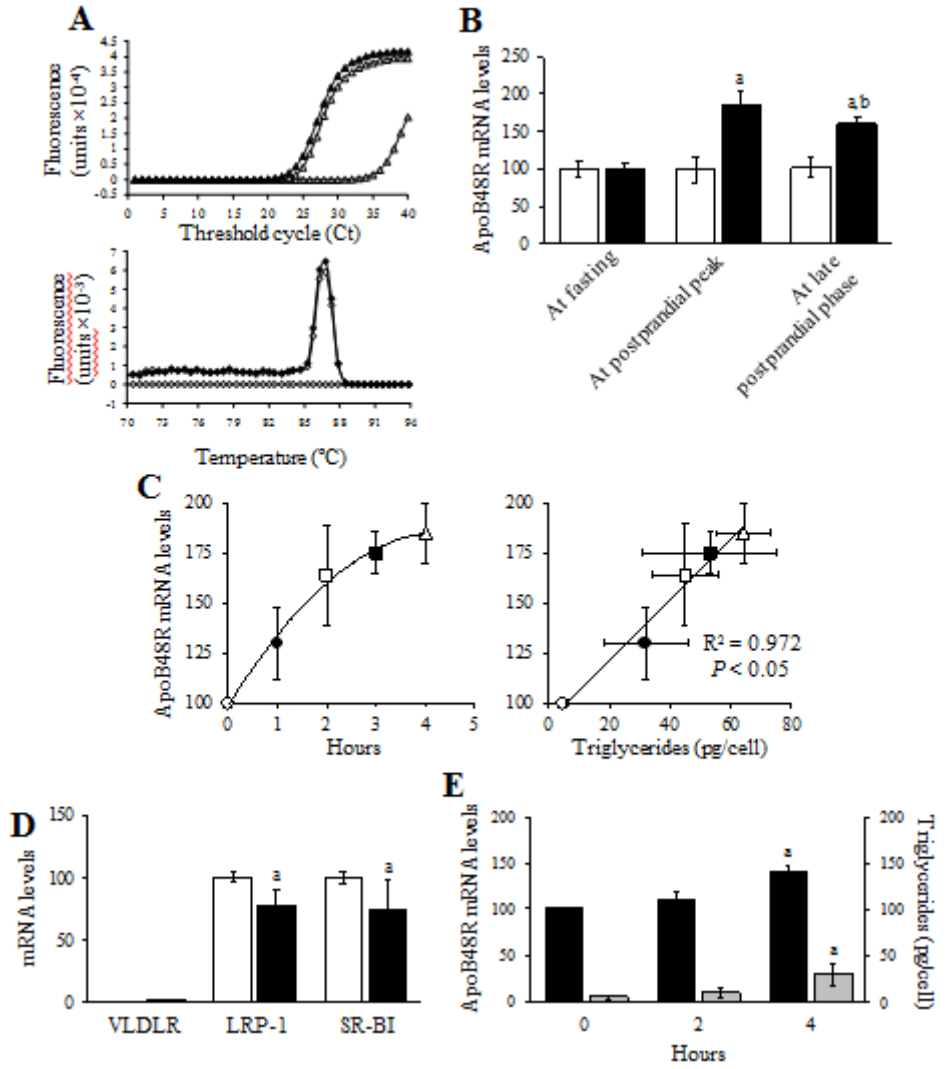

Figure 4. Mean apolipoprotein B-48 receptor (ApoB48R) gene expression in freshly isolated monocytes after ingestion of the high-fat meal or after their incubation with apoB48-containing

lipoproteins. A: Amplification (triangles) and dissociation (diamonds) curve plots of apoB48R polymerase chain reaction products from monocytes, including no template controls (gray symbols). B: Transcript levels of apoB48R in monocytes after the no-fat meal (open bars) or the high-fat meal (closed bars). C: Time-course increase (0–4 h) and decrease (4–8 h) in apoB48R mRNA in monocytes. D: Plot and correlation of increased apoB48R mRNA expression levels with accumulated triglycerides in monocytes at 0–4 h. E: Plot and correlation of decreased apoB48R mRNA expression levels with plasma triglycerides at 6 and 8 h (100% at 4 h). F: Transcript levels of VLDL receptor (VLDLR), LDL receptor–related protein 1 (LRP1/LRP-1), and scavenger receptor class B member 1 (SCARBI/SR-BI) in monocytes after the no-fat meal (open bars) or the high-fat meal (closed bars). G: Transcript levels of apoB48R (closed bars) and triglycerides (gray bars) in monocytes incubated at different times with apoB48-containing lipoproteins obtained 3–4 h after the high-fat meal. [a,b]Significantly different from monocytes at fasting (a) or at the postprandial peak (b) (B) or from monocytes after the no-fat meal (F) or from monocytes at lower incubation times (G) ($P < 0.05$, repeated-measures ANOVA with Bonferroni correction). Error bars represent SDs; $n = 12$. Data were analyzed by simple linear regression, and the square of the Pearson product-moment correlation coefficient (R2) was determined (D and E).

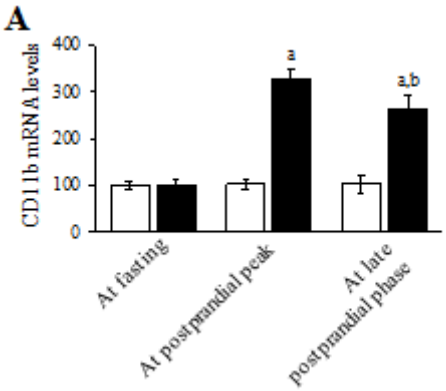

Figure 5. Mean (±SD) cluster differentiation molecule 11b (ITGAM/CD11b) gene expression in freshly isolated monocytes after ingestion of the no-fat meal (open bars) or the high-fat meal (closed bars). [a,b]Significantly different from monocytes at fasting (a) or at the postprandial peak (b), $P < 0.05$ (repeated-measures ANOVA with Bonferroni correction). $n = 12$.

Table 1. Polymerase chain reaction primers for gene expression analysis

Target	Gen Bank accession number	Direction	Sequence (5´→3´)	Predicted size
apoB48R	NM_182804	Forward Reverse	GCCAGTCACCTCTTCCTCTG AGGATGCACAGACTGGCTCT	206 bp
VLDLR	NM_003383	Forward Reverse	CCAATTCCAGTGCACAAATG TGAACCATCTTCGCAGTCAG	196 bp
LRP1/LRP-1	NM_002332	Forward Reverse	CAGCTTAACGGGAGCAATGT GTCACCCCAGTCTGTCCAGT	239 bp
SCARBI/SR-BI	NM_005505	Forward Reverse	CTGTGGGTGAGATCATGTGG GCCAGAAGTCAACCTTGCTC	216 bp
ITGAM/CD11b	NM_001145808	Forward Reverse	AGAACAACATGCCCAGAACC GCGGTCCCATATGACAGTCT	245 bp
RPLP0	NM_001002	Forward Reverse	TCGACAATGGCAGCATCTAC ATCCGTCTCCACAGACAAGG	223 bp
HPRT	NM_000194	Forward Reverse	ACCCCACGAAGTGTTGGATA AAGCAGATGGCCACAGAACT	248 bp

HPRT, hypoxanthine phosphoribosyltransferase; VLDLR, VLDL receptor; LRP1/LRP-1, LDL receptor-related protein; SCARBI/SR-BI, scavenger receptor class B member 1; ITGAM/CD11b, cluster differentiation molecule 11b; apoB48R, apolipoprotein B-48 receptor, RLP0, ribosomal protein large P0; bp, base pairs.

6. DISCUSSION

We undertook a comparison of circulating monocytes isolated from healthy volunteers at fasting and throughout the postprandial period after a high-fat meal and a meal with no fat to examine the accumulation of lipids and the apoB48R transcriptional activity. Monocytes displayed a time-course increase in lipid droplet accumulation during early postprandial hypertriglyceridaemia that was paralleled by increased apoB48R mRNA expression. These effects were coincident with an increase in plasma TRLs carrying apoB48, a decrease in plasma FFA concentration, and no change in plasma fractions containing VLDL and cholesterol-rich lipoproteins. Both apoB48R transcriptional activity and cytoplasmic lipid accumulation were

promoted in primary monocytes after incubation with apoB48-containing TRLs. These observations suggest depot characteristics and apoB48R mRNA up-regulation in human monocytes in response to plasma apoB48-containing TRLs.

A recent study reported that lipolysis products, such as FFAs generated extracellularly from postprandial VLDL, induced the formation of lipid-filled bodies within cultured THP-1 monocytes (12). These authors also speculated that elevated VLDL lipolysis could be responsible for freshly isolated postprandial monocytes containing similar intracellular lipid structures. However, in our study, we did not observe a temporal relation between the intracellular lipid staining in postprandial monocytes and plasma FFA concentrations. The iAUC for plasma FFAs was indeed negative in the early postprandial phase (0-4 h) after the high-fat meal, which was the period of intracellular lipid accumulation. The control meal with no fat induced the lowest plasma FFA and insulin concentrations, but did not elicit any lipid accumulation in postprandial monocytes. This agrees with an early decrease in plasma FFAs by suppression of peripheral lipolysis rather than by monocyte-mediated FFA handling and disposal after the high-fat meal and the meal with no fat. These findings, the time-course accumulation of lipids in monocytes that paralleled the appearance of apoB48-containing TRLs in plasma after the high-fat meal, and *ex vivo* accumulation of lipid droplets in monocytes by apoB48-containing TRLs suggest that dietary fat, particularly the apoB48-containing lipoproteins, was primarily responsible for the accumulation of lipids in postprandial monocytes.

We were able to detect transcript apoB48R in monocytes from healthy volunteers at fasting, and, more importantly, high expression levels of apoB48R mRNA were found in postprandial monocytes after the high-fat meal but not after the meal with no fat. However, the transcription of genes encoding other lipoprotein receptors was not detectable (VLDLR) or even down-regulated (LRP1/LRP-1 and SCARBI/SR-BI) after the high-fat meal. A strong high-fat-meal-dependent correlation was observed between apoB48R

mRNA expression levels and cytoplasmic lipid accumulation in monocytes in the early postprandial phase. Our observations also suggest that acceleration in the rate of plasma triglyceride clearance could be a harbinger of a decrease in apoB48R gene transcription in monocytes in the late postprandial phase. In addition, we found *ex vivo* evidence of apoB48-containing TRL-mediated apoB48R gene expression in primary monocytes. These data are consistent with the notion that under a metabolic environment enriched in apoB48-containing TRLs, circulating monocytes rapidly initiate an adaptive program to up-regulate the expression of apoB48R, which renders them more prone to collect apoB48-containing TRLs and to accumulate lipids in the cytoplasm. VLDLR, LRP1/LRP-1, and SCARBI/SR-BI are probably not involved in the postprandial accumulation of lipids in monocytes, but we do not exclude the possibility for the participation of other untested receptors. Furthermore, whether apoB48R is dominantly involved in monocyte lipid accumulation remains to be unsolved, because the loss-of-function and gain-of-function of apoB48R in monocytes have not been explored in this study. Similarly to our findings, a lipid overload and an increased uptake of apoB100-containing cholesterol-rich lipoproteins (native LDL and ox-LDL), which is accompanied by an up-regulation of genes encoding receptors for the precise acquisition of such lipoproteins, has been shown to be common in circulating monocytes from patients with homozygous familial hypercholesterolemia (13).

The repercussions of intracellular lipid accumulation, which have been well documented in macrophages (14), are not clear and merit further study in circulating monocytes. In contrast with lipid-storing cells, monocytes have virtually no lipid bodies under resting conditions in healthy subjects (12). Note that lipid accumulation is characteristically evoked in circulating cells in association with diverse inflammatory responses, including neutrophils and eosinophils (15). In line with this concept, the *ex vivo* uptake of postprandial TRLs or lipid emulsions in the hypertriglyceridemic range by monocytes was recently shown to induce monocyte activation, even better than the synthetic peptide f-Met-Leu-Phe (8). In our study, we observed *in vivo* monocyte lipid accumulation and activation by increased ITGAM/CD11b gene expression at

the postprandial peak after the high-fat meal. Of interest was the finding that both apoB48R and ITGAM/CD11b mRNA expression levels decreased similarly in the late postprandial phase. Therefore, we hypothesize a causal relation between monocyte lipid accumulation and monocyte activation in which apoB48R could play a major role during the postprandial period of high-fat meal challenges. Our data suggest that apoB48-containing TRLs may be involved in the process of atherosclerosis before their entry into the subendothelial space.

In conclusion, our data highlight the role of dietary fat in inducing lipid accumulation in circulating monocytes in the postprandial period. Moreover, we showed for the first time that apoB48R gene transcription is regulated in human circulating monocytes in a setting of abundant plasma apoB48-containing TRLs. Whether apoB48-containing TRLs directly drive apoB48R gene expression by a feed-forward loop to postprandially modulate lipid homeostasis in human monocytes remains to be elucidated; *ex vivo* and *in vitro* studies to examine this hypothesis are currently ongoing in our laboratory. The modulation of apoB48R in monocytes may represent a possible novel approach for interventions targeting lipid-laden foam cells, which might be particularly relevant to the clinical outcomes of subjects who have pathologically exacerbated and delayed postprandial responses to the ingestion of dietary fats.

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CAPITULO 4

"***Triglyceride-rich lipoprotein regulates APOB48 receptor gene expression in human THP-1 monocytes and macrophages"***

Beatriz Bermúdez, Sergio López, **Lourdes M. Varela**, Almudena Ortega, Yolanda M. Pacheco, Wenceslao Moreda, Rafael Moreno-Luna, Rocío Abia and Francisco J.G. Muriana

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En este trabajo se incluyen los estudios llevados a cabo para cumplir con el **Objetivo 2** de esta Tesis Doctoral: Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la expresión del gen apoB48R, la acumulación intracelular de lípidos, y los mecanismos moleculares implicados en monocitos y macrófagos humanos.

RESUMEN: Conocida la expresión del gen apoB48R en monocitos humanos, se estudia *in vitro* los mecanismos moleculares de su regulación en células THP-1 como modelo experimental de monocitos humanos. Se descubre que las propias lipoproteínas postprandiales ricas en triglicéridos y no las VLDL, aisladas de personas sanas, modulan como un sistema de retroalimentación la expresión del gen apoB48R, tanto en monocitos como en macrófagos derivados (mediante inducción química) de dichos monocitos. Hasta ahora, los macrófagos y no los monocitos eran considerados como uno de los principales ejecutores de la respuesta inmune innata en el espacio subendotelial de la pared vascular de las arterias, responsables de la captación de LDL oxidadas, y progenitores de las células espumosas. Con este estudio se comprueba que las lipoproteínas postprandiales ricas en triglicéridos también pueden participar en estos procesos pro-ateroscleróticos. Mediante el empleo de agonistas y antagonistas químicos y de oligonucleótidos de siRNA, se demuestra que estas

lipoproteínas ejercen efectos promotores en monocitos y represores en macrófagos sobre la expresión del gen apoB48R, que estos efectos correlacionan con la acumulación intracelular de lípidos, que dependen de la disponibilidad intracelular de ácidos grasos, y que tienen lugar mediante la acción coordinada de los factores de transcripción PPARα, PPARγ, y RXRα. Mediante este estudio se identifica a las lipoproteínas postprandiales ricas en triglicéridos como partículas aterogénicas con capacidad para generar células espumosas.

ANEXO (Tablas y Figuras al final de este capítulo):

Adicional y como complemento a este estudio, se realizaron experiencias para determinar el efecto de las TRL postprandiales de diferente composición en ácidos grasos en la expresión del gen apoB48R en monocitos y macrófagos THP-1. Además de la mantequilla, se probaron el aceite de oliva refinado, una mezcla de aceites vegetales suplementada con aceite de pescado azul, y el aceite de girasol alto-palmítico. Los resultados demuestran que, a medida que aumenta la proporcionalidad entre el ácido oleico (MUFA) y el ácido palmítico (SFA) en las TRL postprandiales, sus efectos sobre la expresión del gen apoB48R se atenúan en monocitos y se potencian en macrófagos. También se estudió la expresión del gen apoB48R en monocitos circulantes de personas sanas y macrofagos diferenciandos y polarizados *ex vivo* a partir de estos monocitos (M0, macrófagos sin polarizar; M1, macrófagos polarizados hacia el fenotipo clásico; M2, macrófagos polarizados hacia el fenotipo alternativo), y así confirmar que el modelo de células THP-1 puede ser representativo de células monocíticas primarias. Observamos que las TRL postprandiales estimulan la expresión del gen apoB48R en los monocitos e inhiben dicha expresión en los macrófagos M0, M1, y M2. Se demuestra que, al menos en macrófagos M0, M1, y M2, PPARα, y PPARγ participan en la regulación de apoB48R inducida por las TRL postprandiales.

Triglyceride-rich lipoprotein regulates APOB48 receptor gene expression in human THP-1 monocytes and macrophages[1,2,3]

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Running title: Postprandial TRL on *APOB48R*

Abbreviations: ACAT (acyl-CoA:cholesterol acyltransferase), apoB48R (apoB48 receptor), As2O3 (arsenic trioxide), CE (cholesteryl esters), CHD (coronary heart disease), 9cRA (9-cis-retinoic acid), DR-1 (direct repeat type 1 element), FC (free cholesterol), HPRT (hypoxanthine phosphoribosyltransferase), PPRE (peroxisome proliferator responsive element), PMA (phorbol 12-myristate-13-acetate), RPLP0 (ribosomal protein large P0), RPMI (Roswell Park Memorial Institute), RXR (retinoid X receptor), siRNA (small interfering RNA), TRL (TG-rich lipoprotein).

Abstract

The postprandial metabolism of dietary fats implies that the production of TG-rich lipoproteins (TRL) contributes to the progression of plaque development. TRL and their remnants cause rapid receptor-mediated monocyte/macrophage lipid engorgement via the cell surface apoB48 receptor (apoB48R). However, the mechanistic basis for apoB48 receptor (*APOB48R*) regulation by postprandial TRL in monocytes and macrophages is not well established. In this study, we investigated the effects of postprandial TRL from healthy volunteers on the expression of *APOB48R*mRNA and lipid uptake in human THP-1 monocytes and THP-1–derived macrophages. The expression of *APOB48R* mRNA was upregulated in THP-1 monocytes, but downregulated in THP-1–derived macrophages when treated with postprandial TRL ($P < 0.05$), in a dose- and time-dependent manner. TG and free cholesterol were dramatically increased in THP-1–derived macrophages (140 and 50%, respectively; $P< 0.05$) and in THP-1 monocytes (160 and 95%, respectively; $P < 0.05$). This lipid accumulation was severely decreased (~50%; $P< 0.05$) in THP-1–derived macrophages by small interfering RNA (siRNA) targeting of *APOB48R*. Using PPAR and retinoid X receptor (RXR) agonists, antagonists, and siRNA, our data indicate that PPARα, PPARγ, and RXRα are involved in postprandial TRL-induced *APOB48R*transcriptional regulation. Co-incubation with acyl-CoA synthetase or acyl-CoA:cholesterol acyltransferase inhibitors potentiated the effects of postprandial TRL on the expression of *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages. Our findings collectively suggest that *APOB48R* represents a molecular target of postprandial TRL via PPAR-dependent pathways in human THP-1 monocytes and macrophages and advance a potentially important link between postprandial metabolism of dietary fats and atherogenesis.

1. INTRODUCTION

Growing evidence suggests that postprandial hypertriglyceridemia contributes to the pathogenesis of atherosclerosis, including coronary heart disease, stroke, and subsequent sudden death (1, 2). In the postprandial state, the lipid moieties in the circulation exist mainly in the form of TRL carrying one molecule of apoB48 protein, which has a proteoglycan-binding site in the amino-terminal region that confers atherogenicity to apoB48- and apoB100-containing lipoproteins (3). TRL and their remnants are the only native, nonmodified lipoproteins that cause rapid, receptor-mediated monocyte/macrophage lipid engorgement via the cell surface apoB48R (4). ApoB48R is an apoE-independent receptor that may function as a nutritional receptor to provide dietary fatty acids and lipid-soluble vitamins to cells, and this pathway is not regulated by sterol (5).

Several members of the human nuclear receptor superfamily have been studied as fatty acid receptors, including PPAR, RXR, liver X receptors, and, more recently, hepatocyte nuclear factor 4α (6). These nuclear receptors can regulate gene expression in response to FFA released from TG intracellularly. Binding of ligands to PPAR isoforms α (PPARα) and γ (PPARγ) leads to the suppression of *APOB48R* gene expression in mature macrophages (7), suggesting that dietary fatty acids can modulate the functional endpoint of the apoB48R pathway. The dominant role of apoB48R in atherogenesis has also been stressed by the effect of pitavastatin in suppressing macrophage-derived foam cell functions through the regulation of *APOB48R* (8).

Postprandial TRL circulate together with monocytes and can enter the arterial intima, even becoming trapped preferentially over LDL (9). However, studies on how postprandial TRL affect the molecular regulation and functional properties of *APOB48R* in monocytes and mature macrophages have not, to our knowledge, been conducted. Therefore, we set out to examine whether postprandial TRL could influence the transcriptional regulation of *APOB48R* in

THP-1 monocytes and THP-1–derived macrophages and whether this could mediate lipid uptake from postprandial TRL.

2. METHODS

2.1. Preparation and characterization of postprandial TRL and fasting VLDL from healthy volunteers

This study was conducted according to the guidelines of good clinical practice. Prior to the beginning of the study, all participants provided their informed consent using protocols approved by the Human Clinical Commission and Ethics Committee of University Hospital Virgen del Rocio, Seville. The investigation conformed with the principles outlined in the Helsinki Declaration of the World Medical Association.

Fasting blood samples from six healthy donors were taken 12 h after the evening meal. The participants were then administered a high-fat meal consisting of butter (50 g/m^2 body surface area) along with a portion of plain pasta, one slice of brown bread, and one skim-milk yogurt (10). Blood was drawn 2–3 h following the high-fat meal ingestion, a time point previously shown to correlate with the most recently (nascent) secreted particles by the intestine. ApoB48-containing TRL were isolated from plasma by ultracentrifugation (11), pooled, dialyzed against PBS, and immediately stored at –80°C. Only once-thawed, postprandial TRL samples were used. Plasma TG were measured on a Hitachi Modular Analytics D-2400 analyzer using commercially available reagents and an enzyme-based kit (TG GPO-PAP, Roche Diagnostics). Total TG and cholesterol in postprandial TRL were measured using enzyme-based colorimetric kits (TG GPO and Infinity Cholesterol, Thermo Trace). The means ratio of TG:cholesterol was 2.5. ApoB100 and apoB48 were separated by SDS-PAGE and scanned with a laser densitometer. Mean postprandial TRL apoB48 was 5-fold higher relative to apoB100. We therefore considered postprandial TRL as large, apoB48-rich lipoproteins. Lipid

oxidizability of postprandial TRL was checked (TBARS level) during isolation and storage, but oxidation of lipids was not detected (62 ± 27 nmol/mmol TG). VLDL were prepared by sequential ultracentrifugation (12).

2.2. Cell culture and treatments

The human monocytic THP-1 cell line, in suspension, was cultured in RPMI medium 1640 supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FBS. Monocytes were differentiated at a density of 7 × 108 cells/L with PMA (100 nmol/L, Sigma) for 4 d. Non-PMA–stimulated THP-1 cells (referred to as THP-1 monocytes) or PMA-stimulated THP-1 cells (referred to as THP-1–derived macrophages) were exposed to RPMI medium containing postprandial TRL, at different concentrations and times, along with or without PPARα antagonist MK886, PPARγ antagonist GW9662, PPARα agonist Wy14643, PPARγ agonist 15-*d*-PGJ2, RXR agonist 9cRA, RXRα-heterodimer transactivation inhibitor As_2O_3, acyl-CoA synthetase inhibitor Triacsin D (kindly provided by Dr. Satoshi Omura, The Kitasato Institute, Japan), or ACAT inhibitor 58–035 (a generous gift from Novartis). All reagents were from Sigma except as indicated.

2.3. Intracellular lipid analysis

Total cellular lipids were extracted by using a hexane/isopropyl alcohol (3:2) mixture, followed by cellular protein extraction with 0.2 mol/L NaOH. TG were determined using a TG GOP-PAP kit (Roche Diagnostics). FC and CE were determined by GC-flame ionization detector. Methyl heptadecanoic ester and α-cholestanol were used as internal standards. Cellular FFA levels were extracted (13) and quantified using a NEFA-HR kit (Wako Chemicals). Hematoxylin and Oil red O were used for nuclei and intracellular neutral lipid staining, and the stained cells were examined by using an Olympus model IX81

inverted phase microscope fitted with a DP71 Olympus digital camera. Cell R software (Olympus) was used for acquisition and further image analysis.

2.4. mRNA extraction and analysis of PCR products

Total RNA was extracted from THP-1 monocytes and THP-1–derived macrophages by using Trizol Reagent (Roche). RNA quality was assessed using the OD260:OD280 ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). RNA (2 μg) was subjected to RT at 42°C for 1 h. The reaction mixture contained 1× RT buffer, 1 mmol/L deoxyribonucleotide triphosphate mix, 20 units of ribonuclease inhibitor, and 200 units of revertAid M-MuLV reverse transcriptase (Fermentas). One-tenth (2 μL) of the resulting cDNA was used as a template for qRT-PCR. The mRNA levels for specific genes were determined in a MX3000P system (Stratagene). For each PCR, a cDNA template was added to Brilliant SYBR green QPCR Master mix (Stratagene) containing the primer pairs for *APOB48R*, *PPARα, PPARγ, RXRα*, or for *RPLP0* and *HPRT* as housekeeping genes. The sequence and information for primers used in this study are as follows: *APOB48R* (GenBank accession no. NM_182804): 5′-GCCAGTCACCTCTTCCTCTG-3′ (forward) and 5′-AGGATGCACAGACTGGCTCT-3′ (reverse); *PPARα* (NM_001001928): 5′-GTTTGAGGGGGTAACAGCAA-3′ and 5′-GCTAACTGCAGAGGGTGAGG-3′; *PPARγ* (NM_138712): 5′-GCTGTGCAGGAGATCACAGA-3′ and 5′-GGGCTCCATAAAGTCACCAA-3′; *RXRα* (NM_002957): 5′-GGGTTTTCTTCCCTTTCGAG-3′ and 5′-GCGTGTTCCTTTTCCACAAT-3′; *RPLP0* (NM_001002): 5′-TCGACAATGGCAGCATCTAC-3′ and 5′-ATCCGTCTCCACAGACAAGG-3′; and *HPRT* (NM_000194): 5′-ACCCCACGAAGTGTTGGATA-3′ and 5′-AAGCAGATGGCCACAGAACT-3′. All amplification reactions were performed in triplicate. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (*RPLP0* and *HPRT*) gene levels and expressed as percentage of control.

2.5. siRNA transfections

siRNA derived from human target sequences were synthesized by Dharmacon Research using SMARTpool selection to suppress the expression of *APOB48R*, *PPARα, PPARγ*, and *RXRα* genes. Cells were transfected with siRNA (100 nmol/L) by using Oligofectamine from Invitrogen. The efficiency for siRNA delivery was tested with si*TOX* Transfection Control (Dharmacon Research). We also used siCONTROL Non-Targeting siRNA Pool for negative control siRNA and si*CONTROL GAPDH* siRNA for positive control siRNA (Dharmacon Research). Two days after transfection, the cells were treated with postprandial TRL.

2.6. Statistical analysis

Data are presented as mean ± SD. The homogeneity of variance was tested with Bartlett's test. Comparisons of two means were performed by using Student's t test. Group statistical comparisons were performed by 1- or 2-way ANOVA with a post hoc Bonferroni test. A value of $P < 0.05$ was considered different. All experiments were performed at least three times in triplicate.

3. RESULTS

3.1. *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages

The expression of *APOB48R* mRNA was significantly increased in THP-1 monocytes (**Figure 1A**) but decreased in THP-1–derived macrophages (**Figure 1C**) relative to nonloaded control cells. These effects occurred in a dose- (**Figure 1A, C**) and time- (**Figure 1B, D**) dependent manner and were not altered by the presence of VLDL (**Figure 2**), indicating that apoB100-containing

lipoproteins are not involved in postprandial TRL-mediated expression of *APOB48R* mRNA.

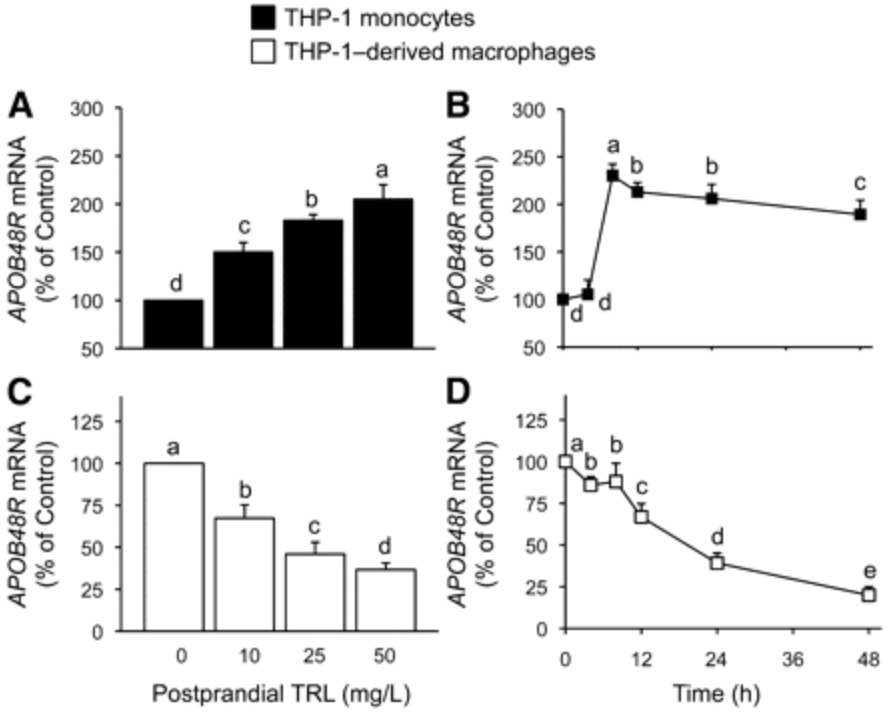


Figure 1. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL at concentrations of TG ranging from 0 to 50 mg/L for 24 h (A,C) or at fixed concentration of TG (50 mg/L) for times ranging from 0 to 48 h (B,D). Values are mean ± SD, *n* = 3. Means without a common letter differ, $P < 0.05$. apoB48R, apoB48 receptor; TRL, TG-rich lipoprotein.

3.2. *APOB48R* mRNA is increased in THP-1 monocytes but decreased in THP-1–derived macrophages by human postprandial TRL

The expression of *APOB48R* mRNA was significantly increased in THP-1 monocytes (**Figure 1A**) but decreased in THP-1–derived macrophages (**Figure 1C**) relative to nonloaded control cells. These effects occurred in a dose- (**Figure 1A, C**) and time- (**Figure 1B, D**) dependent manner and were not altered by the presence of VLDL (**Figure 2**), indicating that apoB100-containing

lipoproteins are not involved in postprandial TRL-mediated expression of *APOB48R* mRNA.

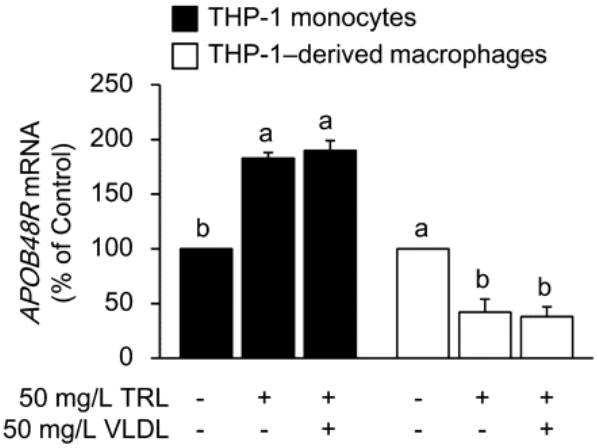


Figure 2. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL for 12 h in the absence or presence of VLDL. Values are mean ± SD, *n* = 3. Means without a common letter differ, *P* < 0.05. apoB48R, apoB48 receptor; TRL, TG-rich lipoprotein.

3.3. Foam cell formation is modulated in THP-1 monocytes and THP-1–derived macrophages in an *APOB48R*-dependent manner by human postprandial TRL

Oil Red O staining and quantitative lipid analysis showed a dramatic increase in neutral lipid droplets (mainly TG) (**Supplemental Figure 1; Figure 3A**) as well as a modest increase in CE (**Figure 3B**) and FC (**Figure 3C**) after the incubation of THP-1–derived macrophages with postprandial TRL. Interestingly, these lipids also accumulated, but to a lesser extent, in THP-1 monocytes when incubated with postprandial TRL. To determine the possible role of *APOB48R* in foam cell formation, siRNA-mediated knockdown studies were undertaken. The expression of *APOB48R* mRNA was reduced by the *APOB48R*-siRNA in THP-1 monocytes (–73 ± 3%) (P < 0.05) and THP-1–derived macrophages (–78 ± 5%) (P < 0.05) compared to the control siRNA. The efficiency of siRNA uptake was >90%. Under these conditions, we found substantial decreases of TG (**Figure**

3A), CE (**Figure 3B**), and FC (**Figure 3C**) in THP-1–derived macrophages, which was greater ($P < 0.05$) in the presence of postprandial TRL. Conversely, THP-1 monocytes with a similar reduction of expression of *APOB48R* mRNA (>70%) dramatically accumulated TG (**Figure 3A**), CE (**Figure 3B**), and FC (**Figure 3C**).

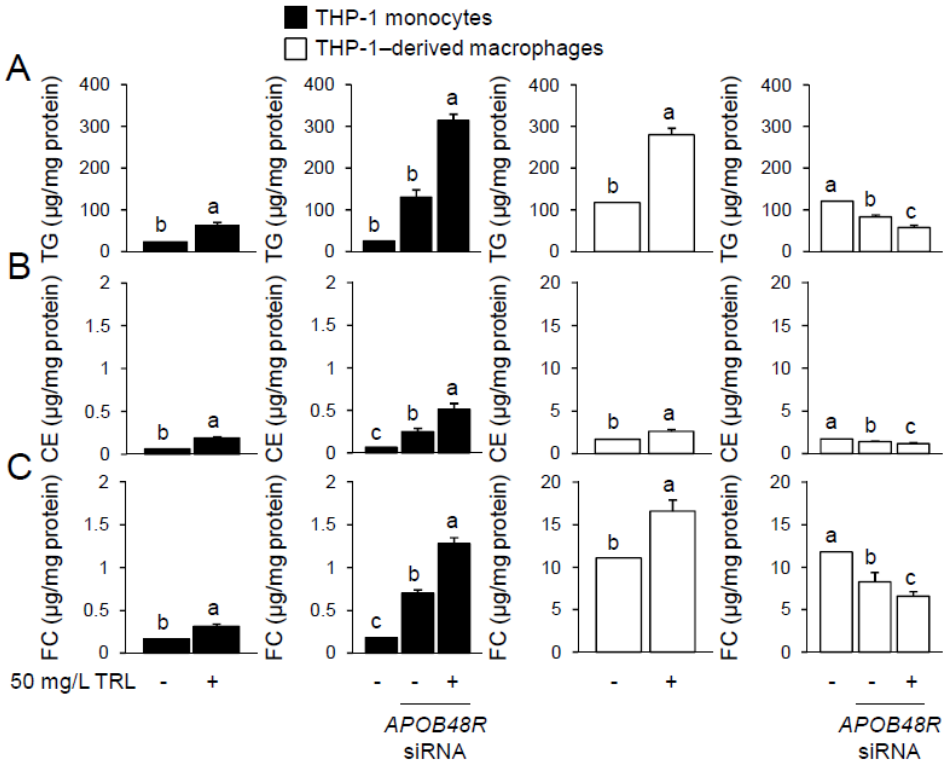
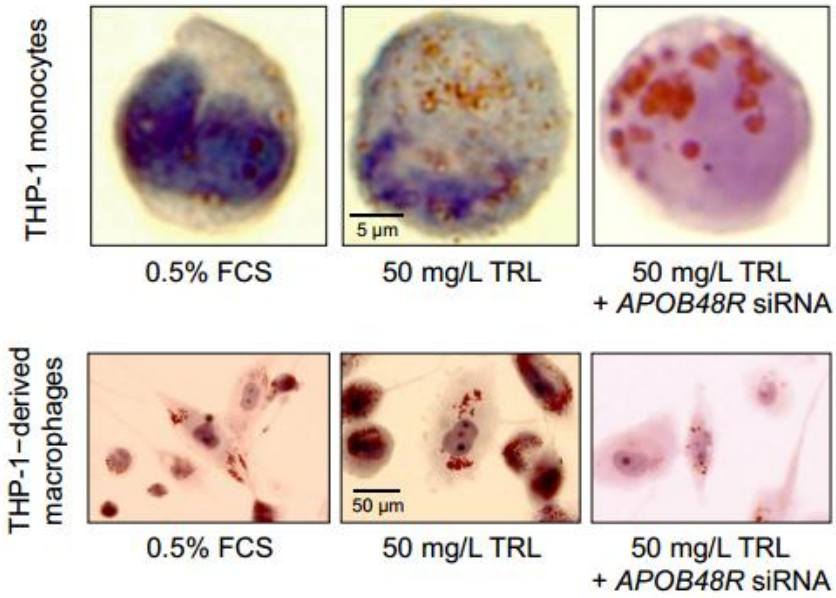


Figure 3. Lipid accumulation in THP-1 cells treated with postprandial TRL for 12 h in the absence or presence of *APOB48R*-siRNA (100 nmol/L). Cells were harvested to measure TG (A), CE (B), and FC (C). Values are mean ± SD, $n = 3$. Means without a common letter differ, $P < 0.05$. apoB48R, apoB48 receptor; CE, cholesteryl ester; FC, free cholesterol; siRNA, small interfering RNA; TRL, TG-rich lipoprotein.



Supplemental Figure. Lipid accumulation in THP-1 cells treated with postprandial TRL for 12 h in the absence or presence of *APOB48R*-siRNA. Cells were stained with hematoxylin and Oil red O.

3.4. Involvement of PPARα, PPARγ, and RXRα in human postprandial TRL-dependent *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages

Treatment of THP-1 monocytes and THP-1–derived macrophages with MK866 (PPARα antagonist) (14) or GW9662 (PPARγ antagonist) (15) resulted in dramatic changes in the expression of *APOB48R* mRNA after incubation with postprandial TRL (**Table 1**). The expression of *APOB48R* mRNA decreased in THP-1 monocytes and increased in THP-1–derived macrophages, which were the opposite directions from when cells were incubated with postprandial TRL alone. By contrast, Wy14643 (PPARα agonist) or 15-*d*-PGJ2 (PPARγ agonist) increased the expression of *APOB48R* mRNA in THP-1 monocytes co-incubated with postprandial TRL (**Table 1**). A contrary effect was observed in THP-1–derived macrophages (data not shown). To further explore the relevance of these observations, we inhibited the expression of *PPAR* mRNA by transfection

of THP-1 monocytes and THP-1–derived macrophages with *PPARα*-siRNA (–32 ± 8% and –37 ± 15%, respectively) ($P < 0.05$) or *PPARγ*-siRNA (–58 ± 17% and –90 ± 4%, respectively) ($P < 0.05$). The cells were then stimulated with postprandial TRL. Under these conditions, siRNA against *PPARα* or *PPARγ* had similar effects on the expression of *APOB48R* mRNA as the PPAR antagonists. Each siRNA in the presence of postprandial TRL suppressed *APOB48R* transcription in THP-1 monocytes, whereas it promoted *APOB48R* transcription in THP-1–derived macrophages (**Table 2**).

Table 1. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL (50 mg/L TG) for 12 h in the absence or presence of PPAR antagonists and agonists[1]

Cell type	Control	MK866	GW9662	Wy14643	15-*d*-PGJ_2
			%		
THP-1 monocytes	100[b]	53 ± 7[c]	50 ± 3c	283 ± 15a	251 ± 22a
THP-1-derived macrophages	100[b]	477 ± 55[a]	433 ± 65a	76 ± 8c	69 ± 9c

[1]Values are mean ± SD, = 3. Means in a row without a common letter differ, $P < 0.05$. MK866 (3 μmol/L), PPARα antagonist; GW9662 (20 μmol/L), PPARγ antagonist; Wy14643 (5 μmol/L), PPARα agonist; 15-d-PGJ2 (30 μmol/L), PPARγ agonist. apoB48R, apoB48 receptor; TRL, TG-rich lipoprotein.

Table 2. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL (50 mg/L TG) for 12 h in the absence or presence of PPAR-siRNA[1]

Cell type	Control	*PPARα*-siRNA	*PPARγ*-siRNA
		%	
THP-1 monocytes	100[a]	79 ± 6[b]	67 ± 1[b]
THP-1-derived macrophages	100[b]	152 ± 15[a]	173 ± 11[a]

[1]Values are mean ± SD, = 3. Means in a row without a common letter differ, $P < 0.05$. Cells were transfected with 100 nmol/L siRNA. apoB48R, apoB48 receptor; siRNA, small interfering RNA; TRL, TG-rich lipoprotein

Because RXRα is a known transcriptional partner of PPAR (16), we performed an additional series of experiments to evaluate the influence of RXRα on the expression of *APOB48R* mRNA induced by postprandial TRL in THP-1 monocytes and THP-1–derived macrophages. Treatment of cells with 9cRA strongly potentiated the effects of postprandial TRL on the expression of *APOB48R* mRNA (**Figure 4A**), a response that was reversed by As_2O_3 in THP-1 monocytes (**Figure 4B**) or *RXRα*-siRNA (–54 ± 14%) ($P < 0.05$) in THP-1–derived macrophages (**Figure 4C**). As expected, As_2O_3 in THP-1–derived macrophages and RXRα-siRNA in THP-1 monocytes also reversed the effects of postprandial TRL on the expression of *APOB48R* mRNA (data not shown).

3.5. Inhibition of intracellular FFA utilization potentiates the effects of human postprandial TRL on *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages

FFA intracellular pools from either dietary sources or endogenous production play a key role in the binding of PPAR/RXRα to peroxisome proliferator responsive element for target gene expression (17, 18). Triacsin D and Novartis 58–035, which are inhibitors of enzymes involved in FFA utilization, clearly potentiated the effects of postprandial TRL on the expression of *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages (**Figure 5**).

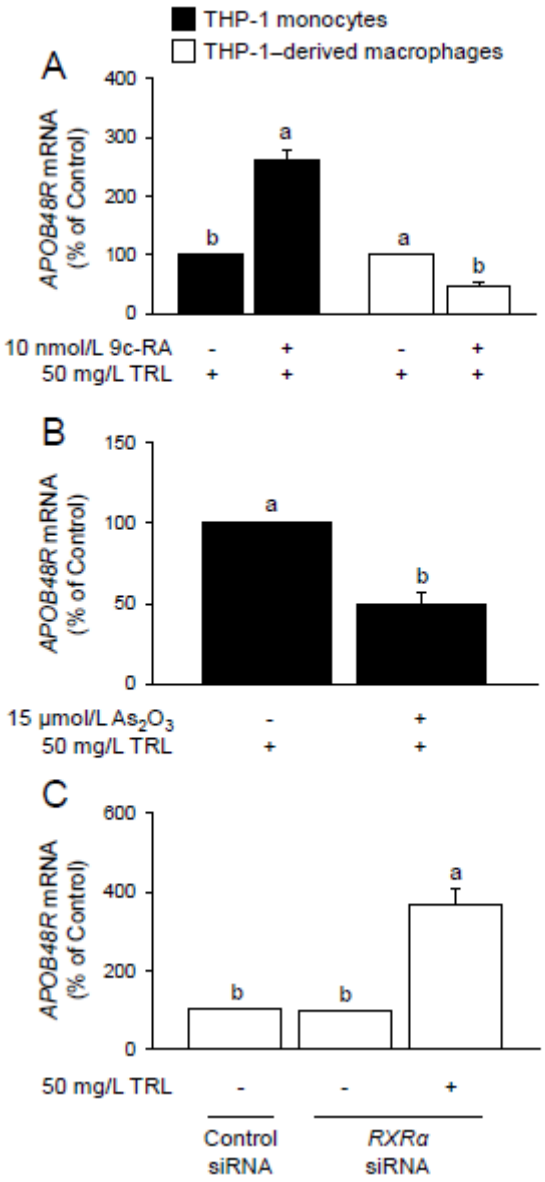


Figure 4. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL for 12 h in the absence or presence of RXRα agonist 9cRA (A), RXRα inhibitor As_2O_3 (B), or *RXRα*-siRNA (100 nmol/L) (C). Values are mean ± SD, $n = 3$. Means without a common letter differ, $P < 0.05$. apoB48R, apoB48 receptor; RXR, retinoid X receptor; siRNA, small interfering RNA; TRL, TG-rich lipoprotein.

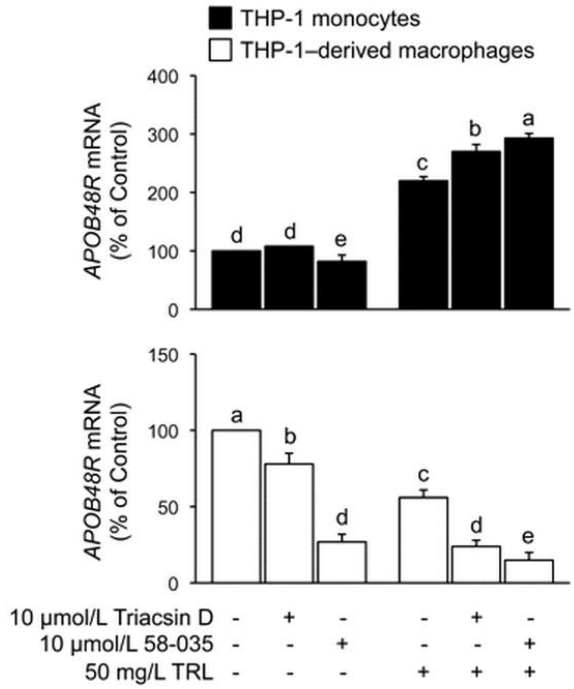


Figure 5. Transcriptional regulation of *APOB48R* in THP-1 cells treated with postprandial TRL for 12 h in the absence or presence of acyl-CoA synthetase inhibitor triacsin D or ACAT inhibitor 58–035. Values are mean ± SD, $n = 3$. Means without a common letter differ, $P < 0.05$. ACAT, acyl-CoA:cholesterol acyltransferase; apoB48R, apoB48 receptor; TRL, TG-rich lipoprotein.

4. DISCUSSION

Accumulating evidence concerning TG levels in the postprandial state as a predictor of higher coronary heart disease and stroke risk (1, 2) suggests atherogenesis as a phenomenon in which apoB48-containing TRL could play an important role. According to the current paradigm, the atherosclerotic process is primarily initiated in the vascular wall itself by: 1) recruitment of circulating monocytes into atherogenic foci; 2) retention of these monocytes as monocyte-derived macrophages; and 3) transformation of macrophages to foam cells by accumulating lipids. However, unprocessed monocytes represent a large pool of circulating precursors that coexist in the bloodstream with

postprandial TRL and may start accumulating lipids even prior to their migration to tissues and differentiation to macrophages. It is thought that apoB48R mediates the rapid, high-affinity uptake of postprandial TRL (4). However, the mechanism by which postprandial TRL influences the regulation and functional properties of *APOB48R* in monocytes and macrophages has not yet been studied.

Here, we show that postprandial TRL regulate *APOB48R* gene transcription in THP-1 monocytes and THP-1–derived macrophages. Our findings reveal that postprandial TRL have opposite effects, in a time- and dose-dependent manner, on the expression of *APOB48R* mRNA in THP-1 monocytes (upregulating) and THP-1–derived macrophages (downregulating). However, changes in *APOB48R* mRNA abundance might not reflect apoB48R protein abundance, because we could not analyze the protein levels of apoB48R. A positive-feedback loop that elevates *APOB48R* gene transcription could mediate postprandial TRL clearance by THP-1 monocytes, which is the mechanism that underlies the transcriptional activity of macrophage scavenger receptors in response to oxidized LDL (19). Thus, the expression of *APOB48R* mRNA in THP-1 monocytes may be perpetuated by a cycle in which postprandial TRL drives its own uptake. In contrast, a negative-feedback mechanism in which postprandial TRL repress *APOB48R* gene transcription could operate in THP-1–derived macrophages, as previously described for the monocyte LDL receptor in response to native LDL (20). However, the ability of postprandial TRL to promote cytoplasmic lipid accumulation was higher in THP-1–derived macrophages than in THP-1 monocytes, suggesting that apoB48R could be highly functional for binding and internalization of postprandial TRL at the monocyte differentiation stage.

Additionally, our results demonstrate that gene silencing of *APOB48R* by siRNA inhibits macrophage-derived formation of foam cells upon challenge with postprandial TRL, further implicating apoB48R as a potential major contributor in postprandial TRL-mediated atherosclerotic foam cell development. Our study also provides evidence that large apoB48-rich

lipoproteins, as well as small, apoB48-poor, remnant-like lipoproteins (8), could be involved in atherogenic processes via apoB48R. The observation that postprandial TRL cause dramatic increases of lipid accumulation in *APOB48R*-siRNA–transfected THP-1 monocytes is unexpected. This probably suggests that endogenous lipid biosynthesis compensates for the deficiency of apoB48R in THP-1 monocytes, contributing to restore the supply of ligands (FFA) for nuclear receptors (21) and channel the excess FFA toward lipid stores (22). It can also be speculated that *APOB48R* could directly or indirectly function as a molecular break or desensitizer of mechanisms involved in the regulation of monocyte lipid accumulation, a condition that would be bypassed in macrophages.

Activators (Wy14643 and 15-*d*-PGJ2) of PPARα and PPARγ decrease the expression of *APOB48R* and lipid uptake in human THP-1 and peripheral, blood-borne, mature macrophages (6). We obtained confirmatory findings in THP-1–derived macrophages by using antagonists of PPARα (MK866) and PPARγ (GW9662) and siRNA oligonucleotides targeting *PPARα* and *PPARγ* cosupplemented with postprandial TRL. In an identical set of experiments in THP-1 monocytes, we reciprocally observed a decrease in the expression of *APOB48R* mRNA in THP-1 monocytes, an effect that was reversed by Wy14643 and 15-*d*-PGJ2. These data anticipate that distal transcriptional effects of postprandial TRL on *APOB48R* are tightly regulated by PPARα and PPARγ. These PPAR do not act alone but as a heterodimer with their obligate partner, RXRα (23). We used three alternative methods to establish that RXRα is a key element involved in the expression of *APOB48R* mRNA. Comparable to other nuclear receptors, the binding of ligand to PPAR stimulates the recruitment of co-regulators, and subsequent binding of the ligand-bound heterodimeric PPAR-RXR complex to DNA response elements in the promoter or enhancer regions of target genes (so-called direct repeat type 1 element or peroxisome proliferator responsive element) alters co-activator/co-repressor dynamics for their transcription (6, 24). This dual transcriptional activity of PPAR enables them to both activate and inhibit gene expression. To the best of our knowledge, our study is the first to describe such effects on the *APOB48R* gene

in a cell differentiation-dependent manner. Further studies to characterize the molecular mechanisms whereby postprandial TRL mediate *APOB48R* gene transactivation in monocytes and transrepression in macrophages will be required.

FFA can be intracellularly generated from postprandial TRL by enzymatic and nonenzymatic mechanisms (25), and they are the best-known, bone fide PPAR activators (17). Inside the cells, FFA are likely transported to the nucleus in association with fatty acid-binding proteins, which may function as positive regulators of PPARα and PPARγ (18). We observed that preventive reesterification of FFA released from lipolysis of postprandial TRL by inhibiting acyl-CoA synthetase or ACAT potentiated the effects of postprandial TRL on the expression of *APOB48R* mRNA in THP-1 monocytes and THP-1–derived macrophages, which further suggests the ability of postprandial TRL to modulate *APOB48R* gene transcription by increasing intracellular FFA concentration. Recent data have also linked intracellular fatty acid biosynthesis to the generation of physiologically relevant endogenous ligands for PPAR in the murine liver (26) and hypothalamus (27). Therefore, our observations contribute to the understanding of this issue and extend the influence of FFA derived from postprandial TRL, as PPAR ligands, on *APOB48R* gene expression to human monocytes.

In conclusion, we have elucidated the previously uncharacterized ability of postprandial TRL, in concert with PPARα/PPARγ and RXRα, to modulate *APOB48R* gene expression in THP-1 monocytes and THP-1–derived macrophages. It is likely that apoB48R plays a primary role in postprandial TRL-mediated foam cell formation. Our experiments support the concept that postprandial TRL may function as a nutritional responsive entity that is chaperoned by apoB48R from the extracellular environment. Thus, *APOB48R* is potentially an intriguing target for the prevention of proatherogenic events during the postprandial state. This issue is especially relevant for the clinical outcomes of people who have pathologically exacerbated and delayed postprandial responses to the ingestion of dietary fats.

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Tablas y Figuras correspondientes al ANEXO a este capítulo:

Table 1S. Fatty acid composition of the meals.

	VEFO	ROO	HPSO	Butter
Fatty acid	% by weight of total fatty acids			
10:0				2.0
12:0				2.5
14:0				9.9
16:0	5.4	11.7	24.9	31.8
16:1ω-7	0.6	1.0	6.8	2.4
18:0	4.2	2.8	2.0	13.3
18:1ω-9	74.4	79.8	58.7	26.2
18:2ω-6	10.3	3. 6	6.9	3.0
20:5ω-3	1.0			
22:6ω-3	2.3			
18:1ω-9/16:0	13.81	6.83	2.36	0.82
MUFA/SFA	7.08	5.43	2.42	0.48

The proportion of major fatty acids [capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic (16:1ω-7), stearic (18:0), oleic (18:1ω-9), linoleic (18:2ω-6), eicosapentaenoic (20:5ω-3) and docosahexaenoic (22:6ω-3) acids] in the meals enriched in VEFO (mixture of vegetable and fish oils), ROO (olive oil), HPSO (high-palmitic sunflower oil) and butter was determined by GC. The ratios of oleic to palmitic acid and monounsaturated to saturated fatty acids (MUFA/SFA) were then calculated.

Table 2S. Fatty acid composition of postprandial TRL after different meals.

	VEFO	ROO	HPSO	Butter
Fatty acid	% by weight of total fatty acids			
14:0				3.7±0.7
16:0	10.6±0.7	14.4±1.2	23.6±2.6	27.7±2.3
16:1ω-7	1.1±0.2	1.2±0.3	5.3±0.9	2.2±0.2
18:0	6.2±0.6	5.4±0.2	4.8±0.5	12.6±1.1
18:1ω-9	59.7±2.8	64.5±2.6	51.4±1.4	35.5±5.2
18:2ω-6	15.0±1.6	9.6±0.5	9.7±0.7	12.1±1.0
20:5ω-3	1.6±0.3	0.6±0.2	0.4±0.3	0.6±0.4
22:6ω-3	2.3±0.4	0.8±0.3	0.7±0.4	0.9±0.3
18:1ω-9/16:0	5.63	4.48	2.18	1.28
MUFA/SFA	3.51	3.24	2.01	0.88

Postprandial TRL at 2-3 h after the ingestion of meals enriched in VEFO (mixture of vegetable and fish oils), ROO (olive oil), HPSO (high-palmitic sunflower oil) and butter were prepared from healthy donors. The proportion of major fatty acids [myristic (14:0), palmitic (16:0), palmitoleic (16:1ω-7), stearic (18:0), oleic (18:1ω-9), linoleic (18:2ω-6), eicosapentaenoic (20:5ω-3) and docosahexaenoic (22:6ω-3) acids] in postprandial TRL was determined by GC. The ratios of oleic to palmitic acid and monounsaturated to saturated fatty acids (MUFA/SFA) were then calculated. Data represent the average median of at least two determinations ± SD (*n* = 14).

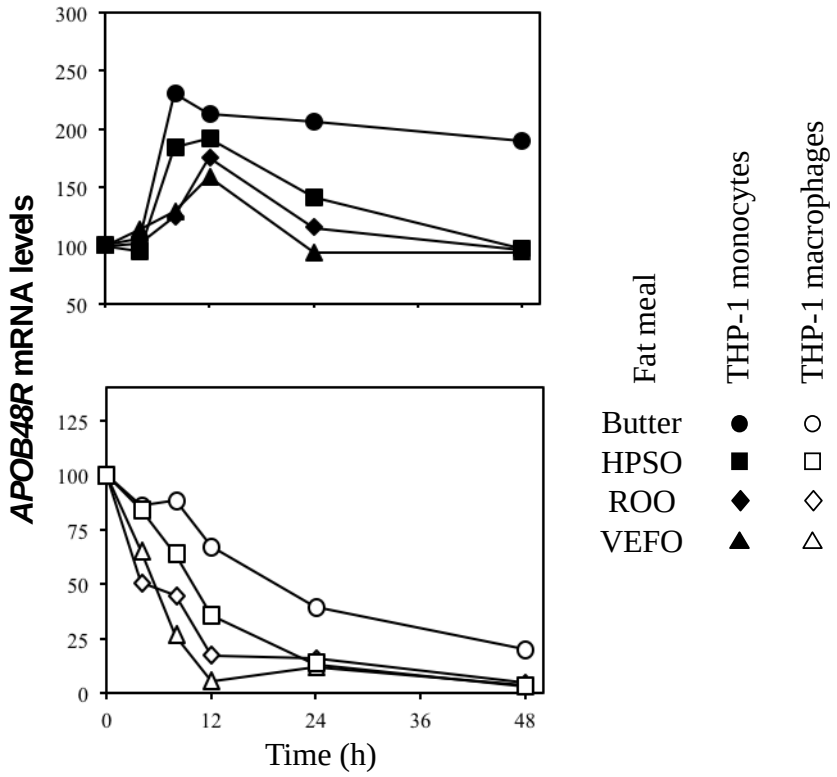

Figure 1S. Postprandial TRL from different fat-meals do not equally regulate the transcription of apoB48R mRNA in THP-1 monocytrs and macrophages. Quiescent non-PMA-stimulated and PMA-stimulated THP-1 cells were treated, for 0, 4, 8, 12, 24, and 48 h, with postprandial TRL (50 µg triglycerides/mL) from healthy donors. Postprandial TRL were isolated at 2-3 h after a fat-meal rich in butter, HPSO (high-palmitic sunflower oil), ROO (refined olive oil) or VEFO (mixture of ROO, high-oleic sunflower oil, and fish oil). ApoB48R mRNA was measured and normalized to housekeeping genes average. The data represent the average median of at least three independent experiments (by triplicate). Non-coincident data were significantly different ($P < 0.05$) from each other. Values for SD are not drawn for the sake of clarity.

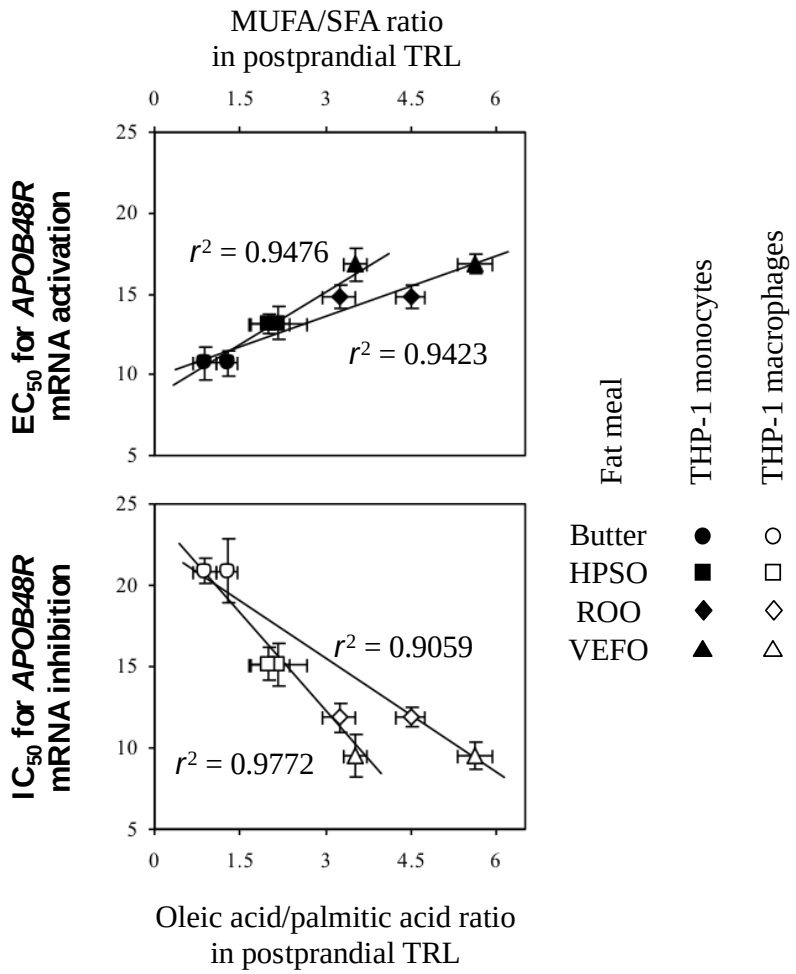

Figure 2S. Regulation of apoB48R mRNA in THP-1 monocytes and macrophages strongly correlates with the ratio of oleic to palmitic acid (MUFA/SFA) in postprandial TRL. Quiescent non-PMA-stimulated and PMA-stimulated THP-1 cells were treated with postprandial TRL (50 µg triglycerides/mL) from healthy donors at 2-3 h after a fat-meal rich in butter, HPSO (high-palmitic sunflower oil), ROO (refined olive oil) or VEFO (mixture of ROO, high-oleic sunflower oil, and fish oil) for 12 h. ApoB48R mRNA was measured and normalized to housekeeping genes average. Plots representing the activation in monocytes and inhibition in mature macrophages of apoB48R mRNA by postprandial TRL were constructed (data not shown) and EC50 and IC50 calculated. The ratios of oleic to palmitic acid and monounsaturated to saturated fatty acids (MUFA/SFA) were obtained from Table 2S (see above). Lines were drawn by linear-regression analysis and correlation coefficients are shown next to each line. Data represent the average median of at least three independent experiments ± SD (*n* = 9).

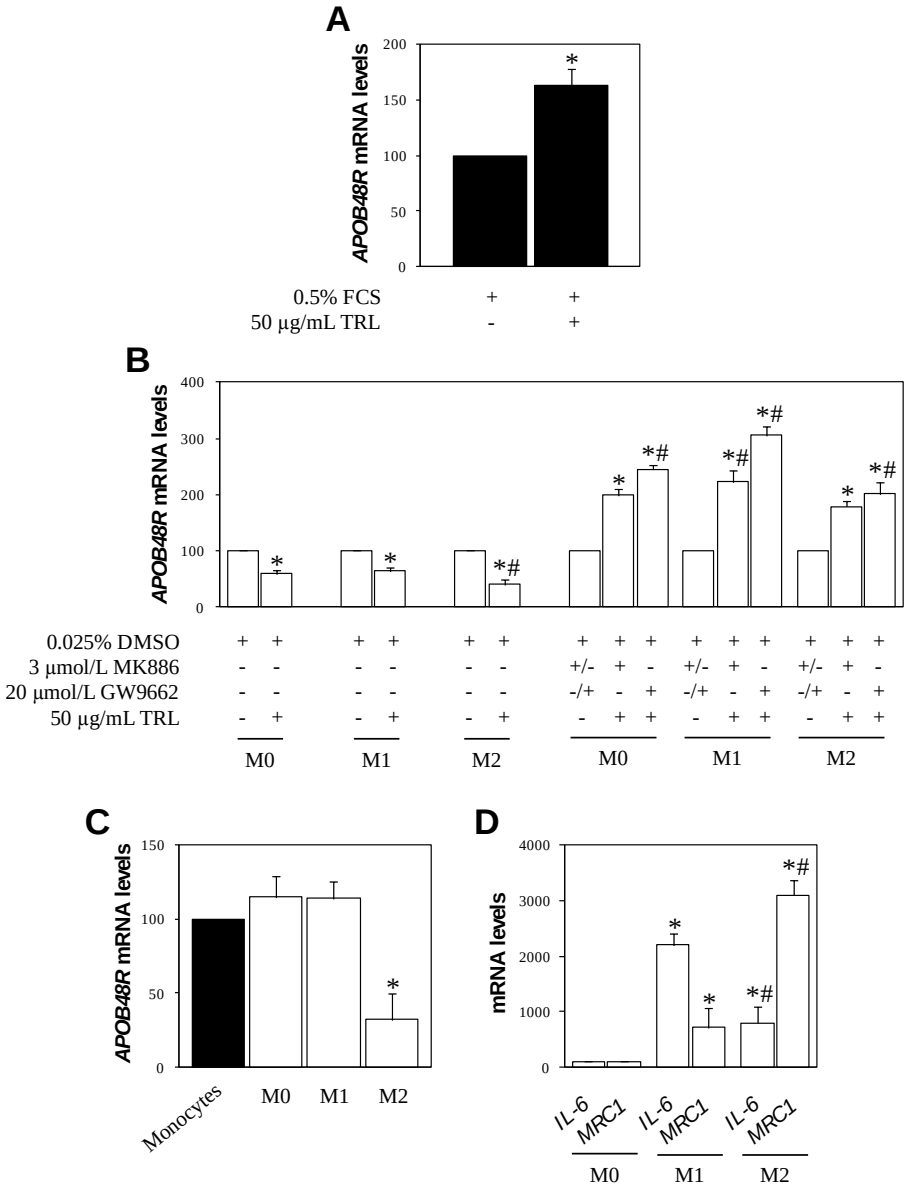

Figure 3S. Postprandial TRL regulate the transcription of apoB48R mRNA in human primary monocytes and macrophage subtypes. Monocytes from fasting healthy donors (black bars) were isolated by negative selection, and subjected to differentiation with M-CSF (M0) for 6 days. M-CSF–driven macrophages were subjected to polarization with LPS plus IFNγ (M1) or IL-4 (M2) (white bars) for an additional 18 h. Monocytes and macrophage subtypes were then treated with postprandial TRL, at 50 µg triglycerides/mL for 12 h and, in the case of macrophage subtypes, in the absence or presence of PPARα antagonist MK866 or PPARγ antagonist GW9662. (A) Transcript levels of apoB48R in monocytes. (B,C) Transcript levels of apoB48R in macrophage

subtypes. (D) Transcript levels of IL-6 (marker of M1, classically activated macrophages) and MRC1 (marker of M2, alternatively activated macrophages) in macrophage subtypes. All the transcript levels were set up as 100% for resting monocytes or untreated macrophage subtypes. **P*<0.05 vs control cells; #*P*<0.05 vs any other treatment or cell type. Independent experiments from 4 different subjects are expressed as the average median ± SD.

CAPÍTULO 5

"Postprandial triglyceride-rich lipoproteins modulate ADRP and TIP47 in macrophages"

Lourdes M. Varela, Sergio López, Almudena Ortega, Beatriz Bermúdez, Insa Buers, Horst Robenek, Francisco J.G. Muriana and Rocío Abia

En este trabajo se incluyen los estudios llevados a cabo para cumplir con el **Objetivo 3** de esta Tesis Doctoral: Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la expresión de ADRP y TIP47 en macrófagos y su transformación en células espumosas.

RESUMEN: La acumulación de lípidos (colesterol y triglicéridos) en células de la pared vascular tiene lugar en unos orgánulos especializados conocidos como gotas lipídicas, cuerpos lipídicos o adiposomas. En macrófagos, ADRP y TIP47 son las principales proteínas implicadas en la formación de dichas gotas lipídicas y por consiguiente en la transformación de los macrófagos en células espumosas. En este estudio se demuestra, *in vitro*, que las lipoproteínas postprandiales ricas en triglicéridos (TRL) aisladas de personas sanas pueden estimular la expresión génica y proteica de ADRP y modificar la localización espacial de TIP47 en las gotas lipídicas en macrófagos humanos THP-1. El patrón temporal de expresión génica de TIP47 precedió al de ADRP, concordante con el papel preponderante de TIP47 en la biogénesis y de ADRP en la estabilización de las gotas lipídicas. Además, se demuestra que la administración de una dieta enriquecida con mantequilla (SFA) a ratones deficientes de apoE (como modelo experimental de aterosclerosis inducida por la dieta) causa la acumulación intracelular de triglicéridos y el aumento de la expresión de ADRP y TIP47 en macrófagos de médula ósea. Estos efectos fueron menos intensos tras una dieta enriquecida con aceite de oliva (MUFA) o

inexistentes tras una dieta enriquecida con aceite de oliva suplementado con aceite de pescado (PUFA). Por primera vez se establece que las TRL postprandiales pueden influir en la formación de células espumosas derivadas de macrófagos mediante la regulación de TIP47 y ADRP.

Postprandial triglyceride-rich lipoproteins modulate ADRP and TIP47 in macrophages

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(pending submission)

Abstract

Lipid accumulation alters macrophage and contributes to the lipid retention within the atherosclerotic plaque. Within cells, lipids are stored in spherical organelles called lipid droplets (LDs), which consists of a neutral lipid core surrounded by a phospholipid monolayer that contains proteins of the PAT family [perilipin, adipocyte differentiation-related protein (ADRP) and the tail interacting protein of 47kDa, (TIP47)].

ADRP and TIP47 are the main PAT proteins involved in LDs formation in macrophage-derived foam cells. Macrophages take up postprandial triglyceride-rich lipoproteins (TRL) through the apoB48 receptor resulting in LDs accumulation and foam cell formation, however, the role of TRL on ADRP and TIP47 induction has not been investigated so far. Using THP-1 macrophages and bone-marrow derived macrophages (BMM) from an apoE-/- mice model of atherosclerosis, we have shown that ADRP and TIP47 are modulated by postprandial TRL. In THP1–derived macrophages, postprandial

TRL are able to induce ADRP protein expression and translocate TIP47 to the LDs surface. The regulation is dependent on the fatty acid composition of the postprandial TRL; TRL rich in monounsaturated fatty acids (TRL-MUFA) attenuate this effect, and TRL rich in polyunsaturated fatty acids (TRL-PUFA) blunt it, compare with TRL rich in saturated fatty acids (TRL-SFA). A novel finding of the present work is that the rate of ADRP and TIP47 production in macrophages and the concomitant triglyceride accumulation in response to an excess of triglycerides, as occurs after postprandial TRL stimulation, is highly dependent on the basal triglyceride content of the cells.

1. INTRODUCTION

Excessive lipid accumulation in macrophages and their conversion to foam cells is a hallmark of atherosclerosis through all stages of lesion development. Within cells, lipids are stored in spherical organelles called lipid droplets (LDs), which have been reported to play a very important role in the control of lipid homeostasis in healthy cells. LDs formation has been proposed to be protective against cellular lipotoxicity (Mei et al., 2011). Moreover, LDs formation in various nonadipose tissues has been correlated with certain human diseases such as atherosclerosis, steatosis, diabetes, obesity and cancer (Bozza & Viola, 2010; Murphy, 2001; Yuan et al., 2012). Structurally, LDs consists of a neutral lipid core surrounded by a phospholipid monolayer that contains a diverse array of embebbed proteins (Li et al., 2010). The major proteins of mammalian LDs are those of the PAT family (the collective term for perilipin, ADRP, and TIP47, which are thought to play a role in the assembly of the organelle and the regulation of lipolysis of the lipids stored in these particles (Ducharme & Bickel, 2008). ADRP and TIP47 are ubiquitously expressed in a variety of cells, while perilipin is almost specific from adipocytes. ADRP is the most prominent LD-associated protein in macrophages; it plays a key role in foam cell formation and in the pathogenesis of atherosclerosis (Paul et al., 2008). ADRP has been localized in lipid-rich macrophages in atherosclerotic plaques of human carotid endarterectomised

specimens and coronary arteries (Wang et al., 1999). Importantly, ADRP gene inactivation in apolipoprotein E-deficient mice protects mice against atherosclerosis (Paul et al., 2008). Macrophage expression of ADRP is upregulated by synthetic agonists of the peroxisome proliferator-activated nuclear receptor β/δ (PPARβ/δ) (Chawla et al., 2003) and γ (PPARγ) (Hodgkinson, 2003). TIP47 is fundamental to formation and functions of LDs (Buers et al., 2011). TIP47 act as a carrier protein of free fatty acids and participates in the conversion of macrophages into foam cells (Buers et al., 2009). Toll-like-receptor-mediated pathway stimulates TIP47 expression leading to LDs formation mainly by promoting triglyceride accumulation (Gu et al., 2010). In fact, knocking down TIP47 by siRNA reduces triglycerides but not cholesterol accumulation (Gu et al., 2010). This data suggests that TIP47 may be a key protein regulating LDs formation when cells are challenged with large amounts of triglycerides.

Postprandial hypertriglyceridemia is characterized by an increase in triglyceride-rich lipoproteins (TRL) in the circulating blood after the ingestion of a high-fat meal. Postprandial TRL along with other lipoproteins such as low-density lipoproteins (LDL) are entrapped in the intima of the arterial wall, since human atherosclerotic plaques contain intact TRL (Rapp et al., 1994). The atherogenic role of postprandial TRL has been well established, in fact, TRL appear to promote atherogenesis independently of LDL (Cohn, 2006). Macrophages take up postprandial TRL through the apoB48 receptor, resulting in LDs accumulation and foam cell formation (Bermudez et al., 2012). However, the effects of postprandial TRL on PAT proteins (ADRP and TIP47) have not been investigated so far. Since the kind of fatty acids that are secreted in form of TRL after the ingestion of a high-fat meal is dependent of the type of fat consumed, we postulated that TRL obtained after the ingestion of a meal rich in saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) could differently modulate these effects. We also studied if ADRP and TIP47 had different roles in early response to excess lipids and long-term lipid storage. In the present study, using the monocytic cell line THP-1 and macrophages from an apoE-/- mice model of

atherosclerosis, we have shown that ADRP and TIP47 are modulated by postprandial TRL in a lipid-dependent fashion. A novel finding of the present work is that the rate of ADRP and TIP47 production after postprandial TRL stimulation is highly dependent on the metabolic state of the macrophages.

2. METHODS

2.1. Preparation of postprandial TRL from healthy volunteers

Human postprandial TRL were isolated by ultracentrifugation from pooled sera of fourteen healthy non-smokers men; lipoproteins were identified as described (Abia et al., 1999). Subjects were given, in three different occasions, a fat-rich meal containing refined olive oil (MUFA), refined olive oil plus fish oil (PUFA), or butter (SFA). Total triglycerides and cholesterol in postprandial TRL were measured using the GPO/PAP (Axiom) and CHOD/PAP method (ProDiagnostica) according to the manufacturer´s instructions. For fatty acid composition of postprandial TRL, we used a simple and rapid 1-step lipid extraction and fatty acid methyl esters procedure according to the method described previously (Garces & Mancha, 1993) with some modifications. The lipid composition of the triglyceride rich lipoproteins used in the study is shown in **Table 1**. Postprandial TRL from the individuals in each group were mixed and the pools used for cell culture studies. All protocols were approved before the start of the study by the Human Clinical Commission and the Ethics Committee of Hospitales Universitarios Virgen del Rocío (SAS, Seville) and informed consent was obtained from each subject. The study was carried out in accordance with the principles outlined in the Helsinki Declaration. Details of the study design have been reported previously (Pacheco et al., 2006).

2.2. Cell culture conditions and antibodies

Human monocytic THP-1 cells were cultured in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated FBS. THP-1–derived macrophages were obtained after incubating THP-1 monocytes at a density of 2.5 x 10^5 cells per cm^2 with PMA (100 nmol/L, Sigma) to the medium for 72 hours. PMA-stimulated THP-1 cells (referred to as THP-1–derived macrophages) were exposed to RPMI medium containing 50 mg/L of postprandial TRL for 48 hours (foam-THP-1). A guinea pig polyclonal antibody against a synthetic polypeptide corresponding to the N-terminus (amino acids 1-16) of human TIP47 (GP30; Progen Biotechnik) was used to detect TIP47. ADRP was detected using a mouse monoclonal antibody against a synthetic peptide representing the N-terminus (amino acids 5-27) of human ADRP (AP125; Progen Biotechnik). Secondary peroxidase-conjugated anti-guinea pig or anti-mouse antibodies (Dianova) were used for detection of TIP47 and ADRP proteins in western blots. For fluorescence microscopy, a Cy3-conjugated anti-guinea pig antibody (AP193C; Millipore) and a Cy2-conjugated anti-mouse (AP192C; Chemicon International) secondary antibodies were used for detection of TIP47 and ADRP proteins, respectively. For electron microscopy, a donkey anti-guinea pig or a goat anti-mouse coupled to 12 nm or 18 nm colloidal gold (Jackson Immunoresearch) were used as secondary antibodies for detection of TIP47 and ADRP proteins, respectively.

2.3. Animals

Male apoE−/− mice (The Jackson Laboratory), on a C57Bl/6 background, were housed 6 per cage and maintained in a pathogen-free facility with a 12-h light/dark cycle. Mice were randomly divided into 4 groups (*n* = 12 per group): one group allocated to a control low-cholesterol chow diet (CHOW, 3.3% fats, 46.1% carbohydrates, and 19% proteins), and three groups allocated to high-triglyceride low-cholesterol diets (HTGD, 20% fat, 50 % carbohydrates, 19.5% proteins, and 0.01% cholesterol) for 16 weeks. Three different HTGD were

studied either enriched in butter (HTGD-SFA), refined olive oil (HTGD-MUFA), or refined olive oil plus 3% fish oil (HTGD-PUFA). The fats used were the same as the ones for the human studies. Drinking water and food were provided *ad libitum*.

Experiments were performed at the animal facility of the Pablo de Olavide University (UPO). All animal protocols received appropriate institutional approval (Animal Care and Use Committee of the UPO) and were performed according to official rules formulated in the Spanish law on care and use of experimental animals.

2.4. Bone-marrow-derived macrophages (BMM) isolation

BMM cells were isolated and pooled from three 20-week-old mice in each group of mice. Mice were euthanized with an overdose of penthobarbital (1:10 in PBS, 150 mg/kg ~200 µL). Femoral and tibiae were aseptically removed and dissected free of adherent soft tissue. The bone ends were cut, and the marrow cavity was flushed out into a petri dish by slowly injecting ice-cold phosphate-buffered saline (PBS) solution at one end of the bone using a sterile 21-gauge needle. The bone marrow suspension was carefully agitated with a plastic Pasteur pipette to obtain a single cell suspension. Bone marrow cells were washed and depleted of red blood cells (RBC) by hypotonic lysis using RBC lysing buffer. After washing twice with PBS, cells were cultured and differentiated in 12-well dishes containing RPMI-1640 medium supplemented with 50 units/mL penicillin G, 50 mg/L streptomycin, 2 mM glutamine, 10% FBS and 15% (v/v) L929-cell conditioned medium for 7 days at 37°C under a humidified atmosphere containing 5% CO_2. Then BMM were stimulated for 24 hours with postprandial TRL (50 mg/L) from the same type of fat that mice were fed for 16 weeks, whereas BMM isolated from mice fed with CHOW diet were incubated with TRL-SFA, TRL-MUFA or TRL-PUFA. A total of four pooled samples were evaluated in triplicate from each group of mice.

2.5. Measurement of triglyceride and cholesterol content

Cells were washed twice with PBS and scraped in 400 µL 0.9% NaCl. After sonication the protein content of the lysate was measured by using the Bradford protein assay (Bio-Rad). Triglyceride and cholesterol content was measured using the GPO/PAP (Axiom) and CHOD/PAP method (ProDiagnostica) according to the manufacturer´s instructions.

2.6. Real Time RT-PCR

The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene, La Jolla, USA). Total RNA was extracted from cells by using Trisure^{TM} Reagent (Bioline, BIO-38032). RNA quality was assessed using the OD_{260}:OD_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Reverse transcription was performed using 1 µg RNA and RevertAid^{TM} (Fermentas, EP0441) according to the manufacturer's instructions. The cDNA template was added to Brilliant SYBR green QPCR Master mix (Agilent Technologies, Stratagene 600828) containing the primer pairs for TIP47 or ADRP and subjected to quantitative real-time PCR analysis. RPLP0 and HPRT, or 18S and GADPH, were used as housekeeping genes for THP-1–derived macrophages and BMM, respectively. The sequences of the primers used in this study are shown in **Table 2**. Reactions were performed in triplicate and the change in mRNA expression was calculated by using the $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (RPLP0 and HPRT or 18S and GADPH) gene levels and expressed as the change with respect to the control.

2.7. SDS-PAGE and Western blot analysis

Cells were washed twice with PBS and lysed in HIDE buffer (0.5% Nonidet P40, 50 mM Tris-HCl pH 8.0, 250 mM NaCl, 5 mM EDTA, and 50 mM NaF). 10 µg of total protein was loaded on 10% SDS-polyacrylamide gels. Proteins were

transferred to Immobilon-P polyvinylidene fluoride membranes (Millipore). Membranes were incubated with anti-human TIP47 antibody or anti-human ADRP antibody followed by incubation with peroxidase conjugated anti-guinea pig or anti-mouse antibodies (Dianova). Densitometric analysis (ImageJ software) was performed to measure the amount of TIP47 and ADRP protein. For total protein extracts, TIP47 and ADRP proteins were normalized to their tubulin content. For LDs protein extracts, TIP47 and ADRP proteins were normalized to the total protein amount determined by Ponceau S staining.

2.8. LDs isolation

THP-1–derived macrophages were washed twice with PBS and scraped into extraction buffer (200 mM KH_2PO_4, 200 mM Na_2HPO_4, 0.25 M sucrose, 80 mM KCl, 5 mM 2-mercaptoethanol) containing protease inhibitor cocktail (Roche). Cells were homogenized by sonication and then transferred to a centrifuge tube. After centrifugation at 10,000 rcf for 60 min the LDs fraction was transferred to acetone (1:6) for protein precipitation. The precipitate of the LDs was centrifuged at 10,000 rcf for 10 min and dissolved in HIDE lysis buffer containing protease inhibitor cocktail (Roche). 10 µg of LDs-derived protein extracts were used for western blot analysis.

2.9. Immunofluorescence microscopy

THP-1–derived macrophages were plated on coverslips for fluorescence microscopy. After the treatments, the cells were washed twice with PBS and fixed with 4% paraformaldehyde. Then the cells were incubated with PBS containing 1% BSA to block nonspecific binding, and 0.05% Tween 20 for permeabilization for 60 min. Cells were immuno-labeled for detecting TIP47 and ADRP proteins for 60 min, followed by incubation with secondary antibodies for another for 60 min. To visualize LDs, the fluorophore BODIPY 493/503 (Invitrogen), which specifically stains neutral lipids, was dissolved in

ethanol at 1 g/L and added to a final concentration of 20 mg/L. The preparations were mounted in fluorescence mounting medium (Dako) and examined in a fluorescence microscope (Zeiss).

2.10. Freeze-fracture replication

THP-1–derived macrophages were scraped from the culture vessels, centrifuged to remove excess medium, re-centrifuged briefly in 30% glycerol for 30 sec, frozen in Freon 22 cooled with liquid nitrogen, and freeze-fractured in a BA310 freeze-fracture unit (BAL-TEC AG) at -100°C. Replicas of the freshly fractured cells were immediately made by electron beam evaporation of platinum-carbon and carbon at angles of 38° and 90° and to thicknesses of 2 and 20 nm, respectively. Replicas were incubated overnight in 5% SDS to remove cellular material except for those molecules 3 adhering directly to the replicas. Replicas were then washed in distilled water and incubated briefly in 5% BSA before immunolabeling. Labeling of the SDS-treated freeze-fracture replicas was carried out using the primary antibodies against TIP47 and ADRP described above followed by matching secondary antibodies coupled to 12 nm or 18 nm colloidal gold. The freeze-fracture replicas were viewed under an electron microscope. The electron-dense gold particles (black dots) clearly mark the positions of these proteins.

2.11. Freeze-fracture nomenclature and interpretation of freeze-fracture replica immunolabeling data

When frozen membranes are fractured, the fracture preferentially splits membranes into their two constituent half-membrane leaflets along a plane between the hydrophobic tails of the phospholipids in the bilayer. In the case of the plasma membrane, one leaflet remains attached to the extracellular space (E-half), whereas the other leaflet remains attached to the cytoplasm or protoplasm (P-half). The view of the E-half revealed by freeze-fracturing is

termed the E- face, and that of the P-half is termed the P-face. A corresponding nomenclature is applied to intracellular membranes. The fracture faces of the cytoplasmic leaflets of the ER and nuclear membranes are designated as P-face views, and those of the endoplasmic leaflets adjacent to the ER lumen and perinuclear space are the complementary E-face views. In this way, a consistent terminology describes structurally and functionally equivalent portions of the different membrane systems of the cell. The envelope surrounding the LDs presents a special case, because it is not part of a classic bilayer, but a phospholipid monolayer apposed against the lipids of the core. The fracture plane often exposes the interface between the hydrophobic aspect of the monolayer and the core. The fracture face of the monolayer revealed in concavely fractured LDs is considered to be the P-face view, and the complementary aspect seen in convexly fractured LDs (which actually represents a view of the outermost layer of the neutral lipid core) is the E-face equivalent. Correct identification of the fracture faces of membranes relies on multiple cues, such as membrane curvature, specific structural features of the membrane type or fracture face, the distribution of metal evaporation, and labelling specificity. Rather than removing all cellular material from the replicas with strong oxidants (the standard procedure in freeze-fracture replica preparation), replicas for immunolabeling are washed with SDS. This preserves molecules adhering directly to the replicas, while the remaining cellular material is flushed away. Integral membrane proteins may then be labelled using immunocytochemical techniques. Labelling of the SDS-treated freeze-fracture replicas was carried out using the primary antibodies against TIP47 and DGAT described above followed by matching secondary antibodies coupled to 12 nm or 18 nm colloidal gold. Viewed in the electron microscope, the electron-dense gold particles clearly mark the positions of these proteins, superimposed upon the en face views of the membranes or LDs phospholipid monolayers (Robenek & Severs, 2008; Robenek et al., 2011).

2.12. Statistical analysis

For *in vitro* studies, the experimental results are expressed as mean ± SD of three independent experiments that were performed in triplicate. For *ex vivo* studies, the BMM isolated from the animals were randomly pooled into four distinct fractions. Statistical significances were assessed by the analysis of the variance (ANOVA) with Tukey post-hoc for multiple comparisons. For differences among postprandial TRL-treatments in BMM fed with the distinct HTGD a two-way ANOVA was used. A *P*-value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1 Triglyceride and cholesterol accumulation in THP-1–derived macrophages

The amount of triglycerides content in THP-1–derived macrophages was determined after 48 hours of treatment with TRL-SFA, TRL-MUFA or TRL-PUFA. Intracellular triglycerides accumulation occurred in the following order: TRL-SFA > TRL-MUFA stimulation. Not significant differences were found after the TRL-PUFA treatment compared with the control (**Figure 1A**). THP-1–derived macrophages accumulated cholesterol after TRL-SFA stimulation compared to control, TRL-MUFA, and TRL-PUFA (**Figure 1B**).

Bodipy staining of the neutral LDs (**Figure 1C**) confirmed these results and showed that TRL-SFA treated cells showed LDs of bigger size than control cells or TRL-PUFA treated cells. THP-1–derived macrophages from control cells spontaneously formed few LDs.

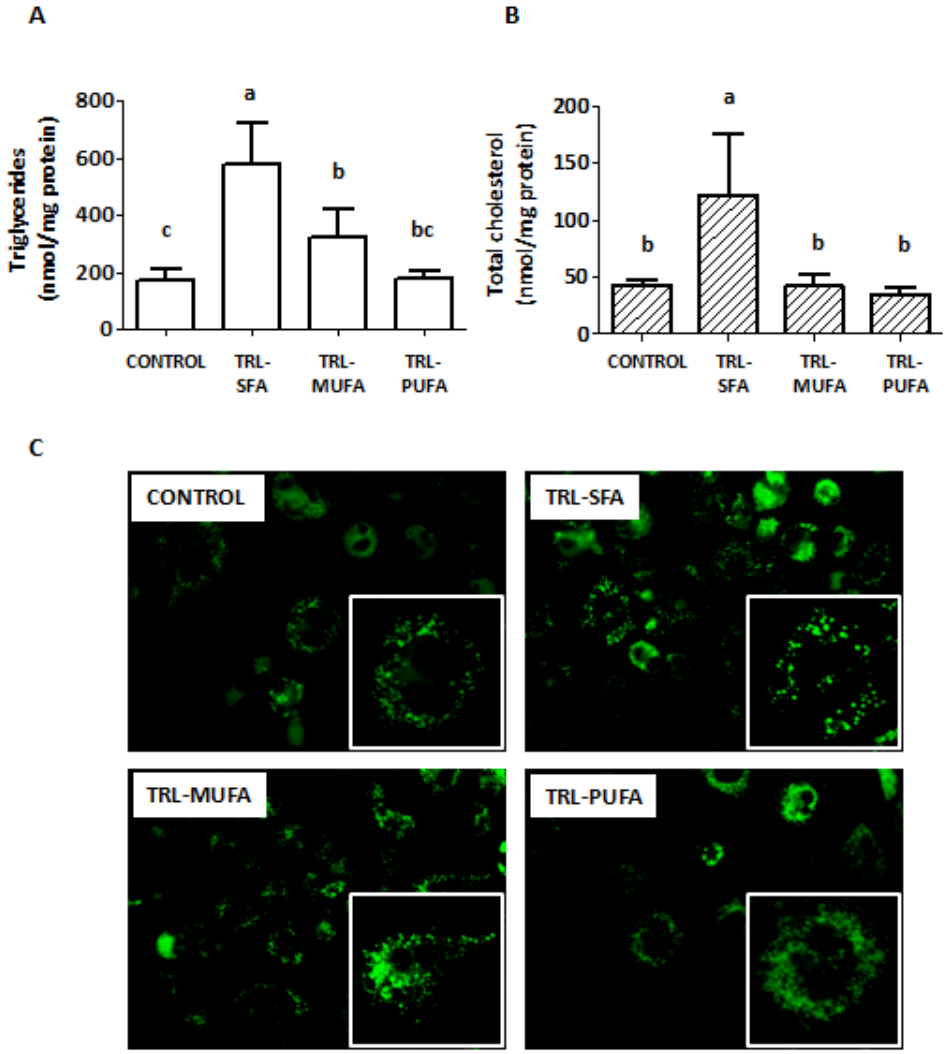

Figure 1. Lipid accumulation in THP-1–derived macrophages after 48 hours of treatment (foam-THP1) with postprandial triglyceride-rich lipoproteins rich in saturated fatty acids (TRL-SFA), monounsaturated fatty acids (TRL-MUFA) or polyunsaturated fatty acids (TRL-PUFA). A-B) Measures of intracellular triglycerides (left, empty columns) and cholesterol (right, dashed columns) after TRL-SFA, TRL-MUFA or TRL-PUFA stimulation. B) Representative images of BODIPY staining of neutral lipids in lipid droplets (LDs) after TRL-SFA incubation. Values are mean ± SD relative to control (n=3). Columns with a different letter are significantly different (P < 0.05).

3.2. Determination of ADRP and TIP47 in THP-1–derived macrophages

Expression of ADRP and TIP47 mRNA was quantified after incubation with TRL-SFA at different times. TIP47 transcript was upregulated early (6 hours) after exposure to TRL-SFA (3.7-fold induction with respect to the control), followed by a return to control values at 48 hours of exposure. ADRP transcript increased significantly with respect to control at longer times (24 hours) (52.3-fold induction with respect to the control), values did not decrease to baseline after 48 hours of stimulation (**Figure 2A**).

Treatment of THP-1–derived macrophages with TRL-SFA and TRL-MUFA but not with TRL-PUFA increased the expression of ADRP mRNA, compared to control cells. This increment was higher after TRL-SFA than TRL-MUFA (**Figure 2B**). Contrary, the expression of TIP47 mRNA was not significantly changed with any of the treatments (**Figure 2C**). Densitometric analysis showed that total ADRP protein expression levels increased after all the TRL treatments in the following order: TRL-SFA = TRL-MUF A> TRL-PUFA (**Figures 2D and 2E**). No significant differences were found in the amount of total TIP47 protein expression with any of the treatments (**Figures 2D and 2F**).

3.3. Localization of ADRP and TIP47 in THP-1–derived macrophages

Immunofluorescence images of ADRP, TIP47, and neutral lipids (BODIPY counterstaining) (**Figure 3**), demonstrated that ADPF and TIP47 were localised in the LDs surface in THP-1–derived macrophages treated with TRL-SFA. We then investigated the ADPF and TIP47 distribution pattern. The information obtained from these experiments showed that non-treated control cells (**Figures 4A, 4B, and 4C**) showed ADRP at the LDs (green fluorescence) and TIP47 diffusely localized in the cytoplasm (red fluorescence) with only occasional co-localization of the proteins at the LD surface (yellow fluorescence). TRL stimulation induced a prominent increase of ADRP in the LDs (**Figures 4D, 4G, and 4J**). Interestingly, TRL-SFA (**Figure 4E**) and TRL-MUFA

(**Figure 4H**) but not TRL-PUFA (**Figure 4K**) stimulation induced a redistribution of TIP47 to the LDs. Accordingly, both, ADRP and TIP47 co-localized mainly at the LDs surface of TRL-SFA (**Figure 4F**) and TRL-MUFA (**Figure 4I**) treated THP-1−derived macrophages.

We then aimed to determine the exact location of ADRP and TIP47 in the LDs by freeze-fracturing and immunogold labelling in THP-1−derived macrophages stimulated with TRL-SFA. Freeze-fracture images of control cells showed that ADRP was mainly localized on the membrane and to a less extent in the core of the LDs (**Figure 5A**) (P-face). Conversely, TIP47 was mainly found in the cytoplasm of the cells (**Figure 5B**) (circles). Induction of LDs formation by exposure of THP-1−derived macrophages with TRL-SFA showed that ADRP was markedly increased and mainly localized on the membrane of the LDs of the cells (**Figures 5C and 5E**) (P-face and E-face), and less in the lipid core (**Figure 5E**) (cross-fracture face). Freeze-fracture immunogold labelling confirmed the increase of TIP47 at the membrane of the LDs (E-face and P-face) and a decrease in the cytoplasm (circle) (**Figures 5D and 5F**).

A mobilization of TIP47 from the cytoplasm to the LDs should be detected from compartmentalized protein cell extracts. To verify these results, we isolated LDs from control and from TRL-SFA, TRL-MUFA or TRL-PUFA treated THP-1−derived macrophages. Densitometric analysis of the LDs protein content showed that TRL-SFA and TRL-MUFA stimulation increased the amount of TIP47 in the LDs compared to the control (**Figures 6A and 6B**).

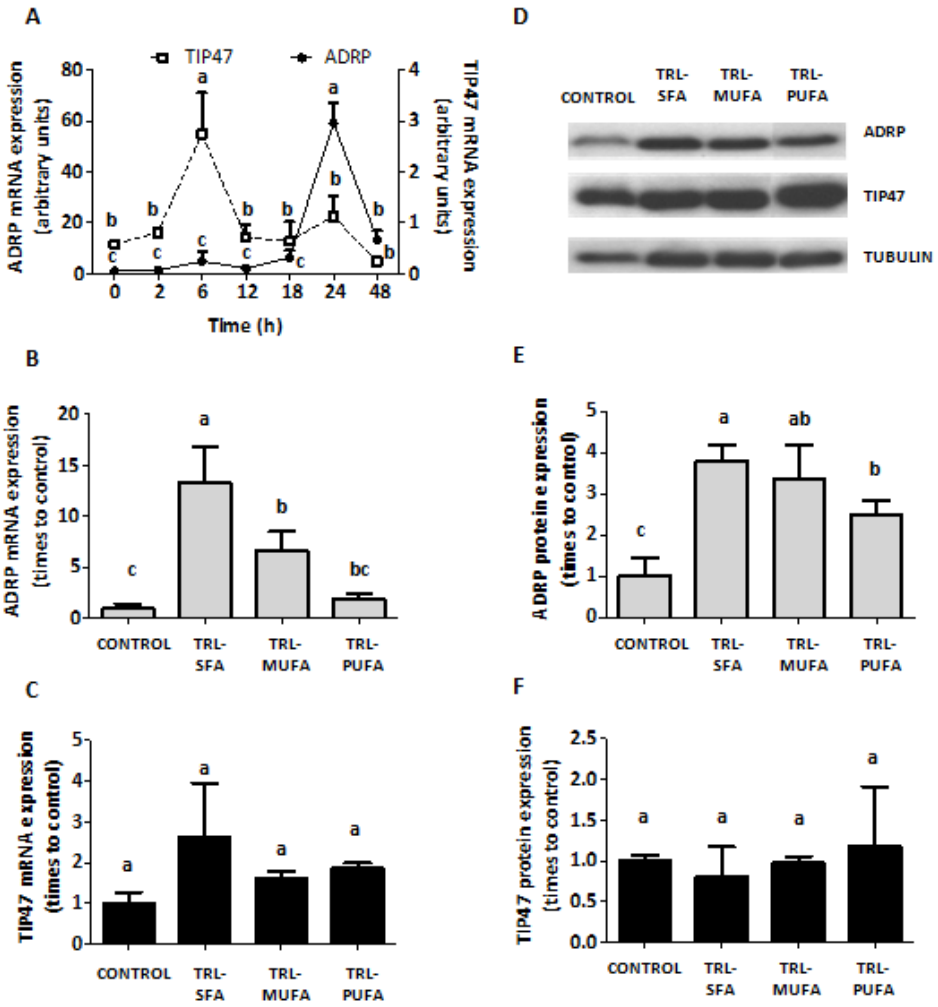

Figure 2. ADRP and TIP47 mRNA and protein levels in THP-1–derived macrophages. A) Time course of ADRP (circles) and TIP47 (squares) mRNA levels in THP-1–derived macrophages, 2, 6, 12, 18, 24 and 48 hours after TRL-SFA treatment. B-C) ADRP and TIP47 mRNA expression, respectively, after the treatment of THP-1–derived macrophages with TRL-SFA, TRL-MUFA or TRL-PUFA. D) Representative image of the western blot for ADRP and TIP47. E-F) ADRP and TIP47 protein expression in THP-1–derived macrophages after TRL-SFA, TRL-MUFA or TRL-PUFA stimulation. Data (mean ± SD, *n*=3) are expressed as fold induction with respect to the corresponding controls after normalization for RPLP0 and HPRT for mRNA or for GAPDH for protein. Columns with a different letter are significantly different ($P < 0.05$).

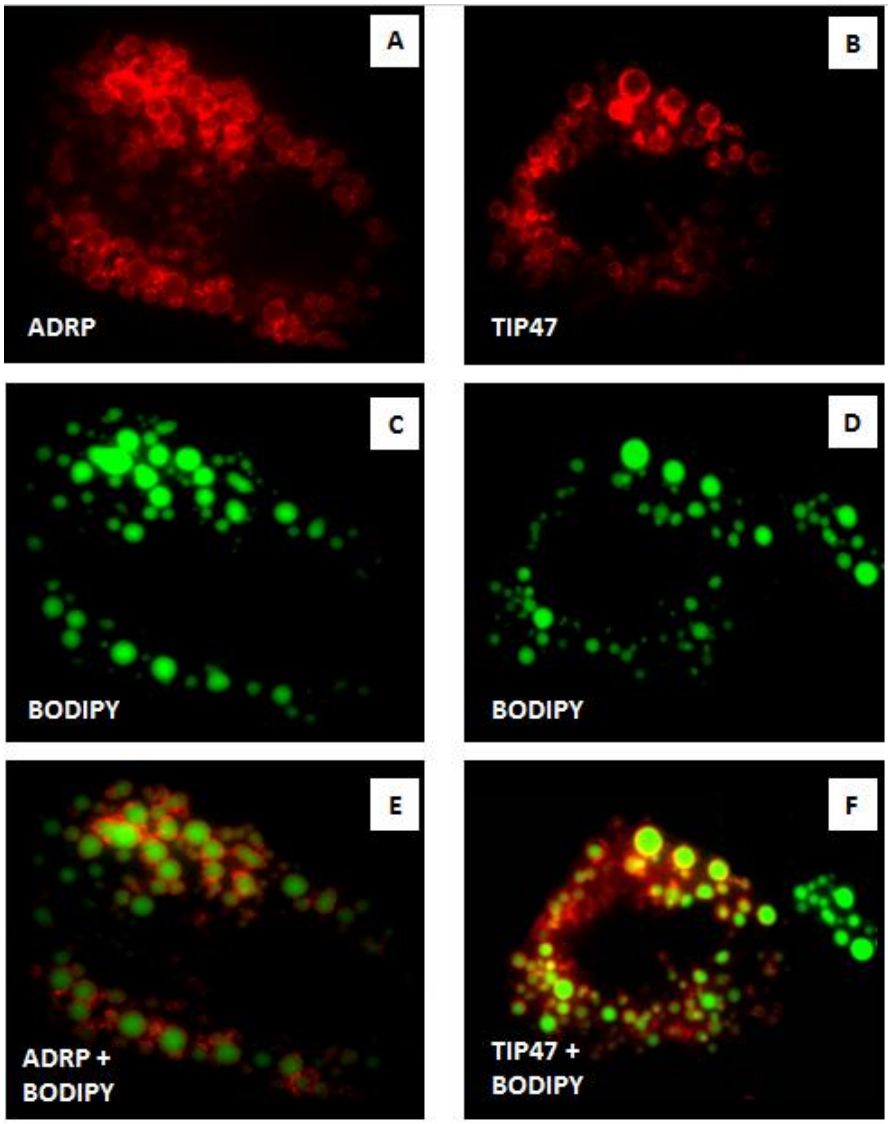

Figure 3. Localizacion of ADRP and TIP47 proteins in LDs in THP-1–derived macrophages after the treatment with TRL-SFA. A-B) Inmunofluorescence for ADRP and TIP47 (red); C-D) Localization of neutral lipids in LDs with BODIPY (green); E) Localization of ADRP in LDs; F) Localization of TIP47 in LDs.

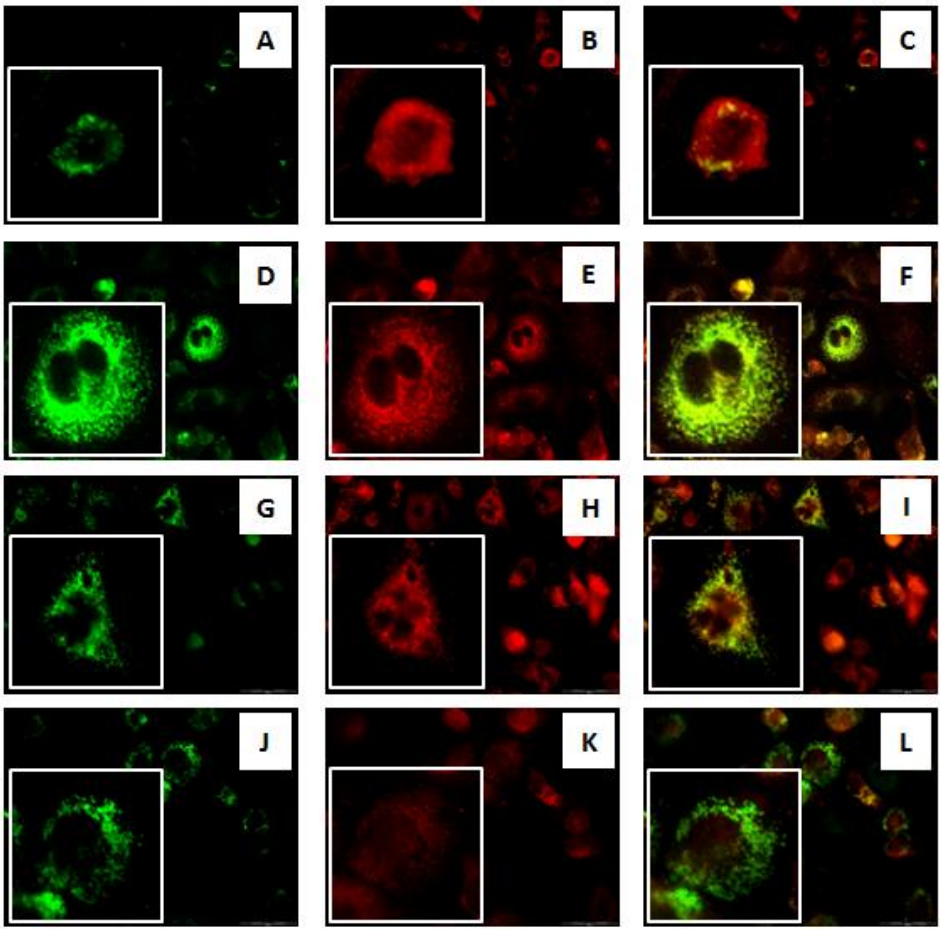

Figure 4. Immunofluorescence localizacion of ADRP (green) and TIP47 (red) proteins in THP-1–derived macrophages after the treatment with TRL-SFA, TRL-MUFA or TRL-PUFA. A-C) control cells; D-F) cells treated with TRL-SFA; G-I) Cells treated with TRL-MUFA; J-L) Cells treated with TRL-PUFA; C, F, I and L) Co-localization of ADRP and TIP47 is shown in yellow.

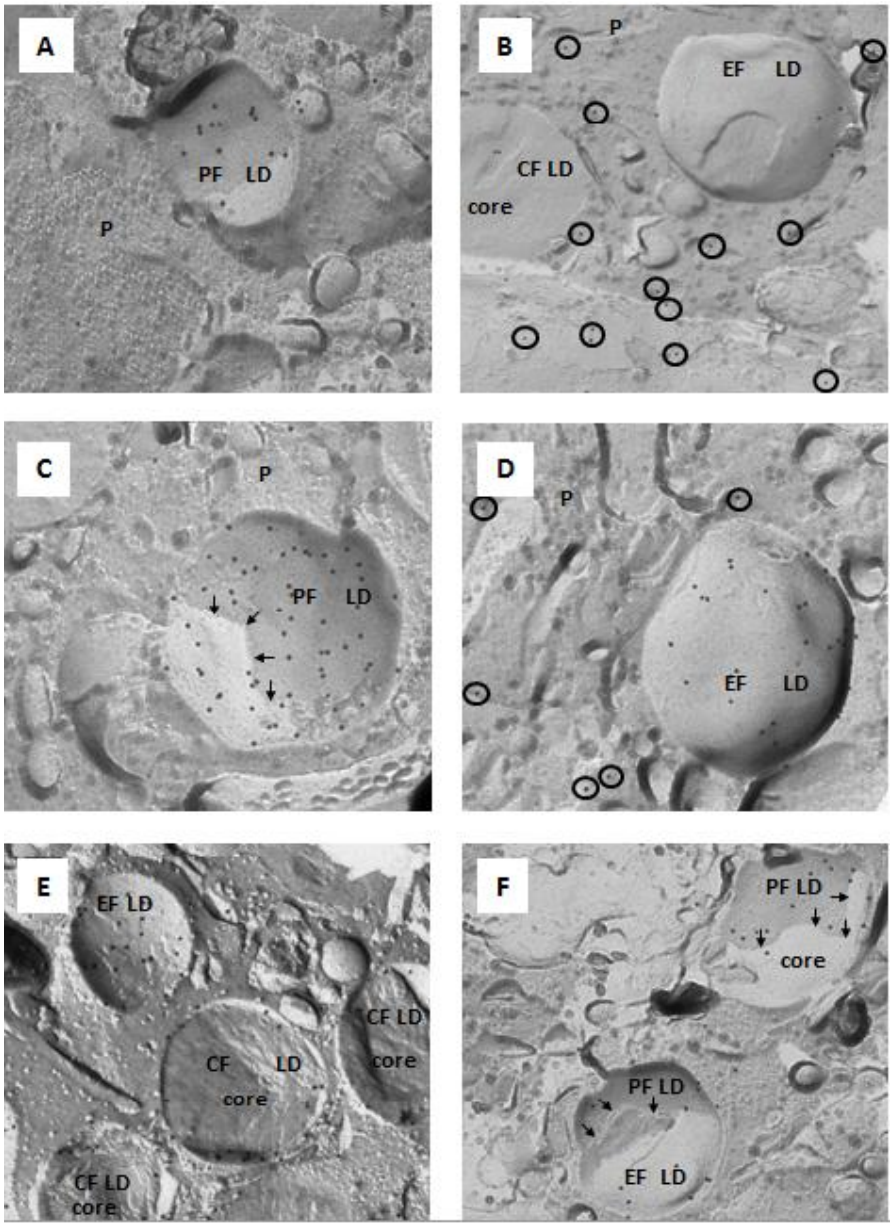

Figure 5. Electron microscopic comparison of cryosection and FRIL imaging of ADRP and TIP47 immunogold labeled in LDs of THP-1–derived macrophages after the treatment with TRL-SFA. A) In control cells ADRP is localized to the phospholipid monolayer of the LDs seen in P-face (PF); B) In control cells, TIP47 is mainly localized in the cytosol (circles); C) TRL-SFA treatment increases ADRP in the PF, arrows indicate fractures of the LDs membrane; D) TRL-SFA treatment shows an

increase of TIP47 in LDs and a decrease of cytosolic TIP47 (circles); E and F) ADRP and TIP47 are localized at the LDs phospholipid monolayer PF and also on the E-face (EF) equivalent of an underlying convexely fracture LDs, cross-fracture (CF) LDs shows little protein in the LDs core. P, protoplasm.

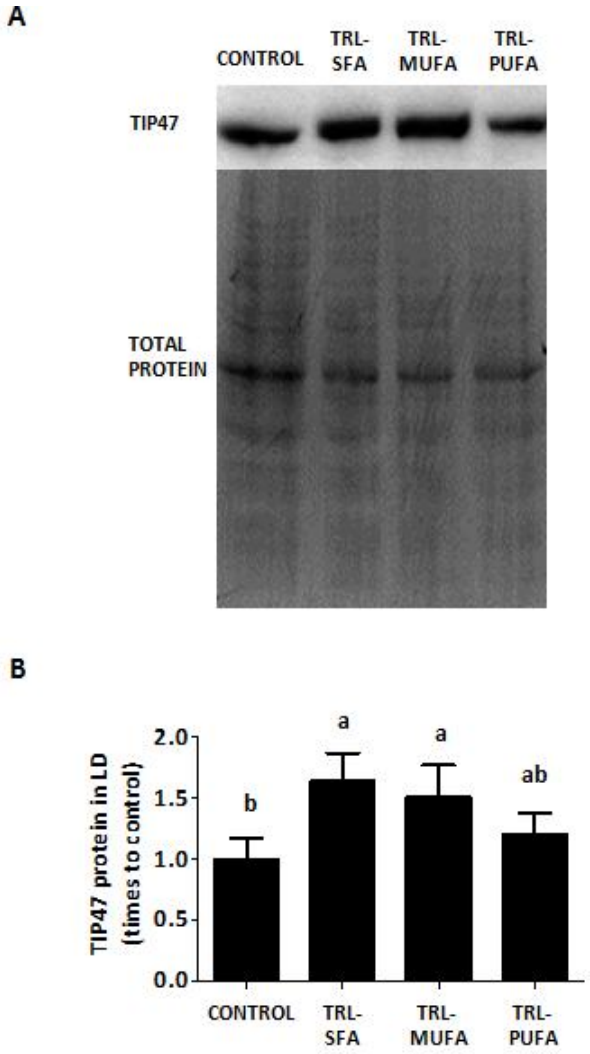

Figure 6. TIP47 protein expression in LDs isolated from THP-1–derived macrophages after the treatment with TRL-SFA, TRL-MUFA or TRL-PUFA. A) Representative image of the western blot for TIP47. B) Densitometric analysis of TIP47 protein. Values are mean ± SD normalized to total protein and relative to control (n=3). Columns with a different letter are significantly different ($P < 0.05$).

3.4. *In vivo* and *ex-vivo* determination of triglyceride accumulation and ADRP and TIP47 expression in BMM isolated from ApoE knockout mice

Sixteen-weeks feeding with the hipertriglyceridemic diets (HTGD) rich in SFA, MUFA, and PUFA, increased triglycerides, ADPR and TIP47 protein values in BMM in a fatty acid-dependent manner. Triglycerides accumulation in BMM increased after the HTGD rich in SFA and MUFA and remained similar to control values after the HTGD rich in PUFA (**Figure 7A**). ADRP and TIP47 mRNA values decreased below control levels for TRL-PUFA (**Figure 7B and 7C**). ADRP was the most prominent protein produced in the cells. Not significant differences were found in ADRP and TIP47 between the HTGD rich in SFA (4.6 and 1.5-fold times to control) and MUFA (3.9 and 1.5-fold times to control), respectively. The HTGD rich in PUFA attenuated ADRP values (2.9-fold times to control) and completely blunted TIP47 (**Figures 7D, 7E and 7F**).

In *ex vivo* experiments, BMM obtained after treatment with the HTGD (high-triglyceride macrophages) were treated with their respective TRL-SFA, TRL-MUFA, or TRL-PUFA for 24 hours. Not significant differences were found in intracellular triglyceride increase among treatments (40-50%) compared to their respective controls (**Figure 8A**). ADRP protein expression levels increased equally after all the postprandial TRL (7.5-8.3-fold to control) (**Figures 8D and 8E**). We could detect only a small increase of TIP47 expression (1.2-1.4-fold to control) for all the postprandial TRL (**Figures 8D and 8F**). The mRNA expression of ADRP and TIP47 was reduced after TRL-MUFA and TRL-PUFA stimulation compared to TRL-SFA for ADRP (**Figure 8B**) and even compared to control for TIP47 (**Figure 8C**). Challenging the low-triglyceride macrophages with postprandial TRL induced an increment in triglyceride accumulation in these cells (87-99%), which was not significant among the postprandial TRL (**Figure 9A**). ADRP protein expression increment (5.9-6.5-fold to control) was lower than the one found in the high-triglyceride macrophages (**Figures 9D and 9E**). Contrary, TIP47 induction (2.0-2.2 fold to control) was higher in the low-triglyceride macrophages (**Figures 9D and 9F**). We could not detect differences in protein expression among the postprandial TRL after 24 hours of incubation.

However, ADRP (**Figure 9B**) and TIP47 (**Figure 9C**) transcripts showed significant down-regulation with TRL-MUFA and PUFA

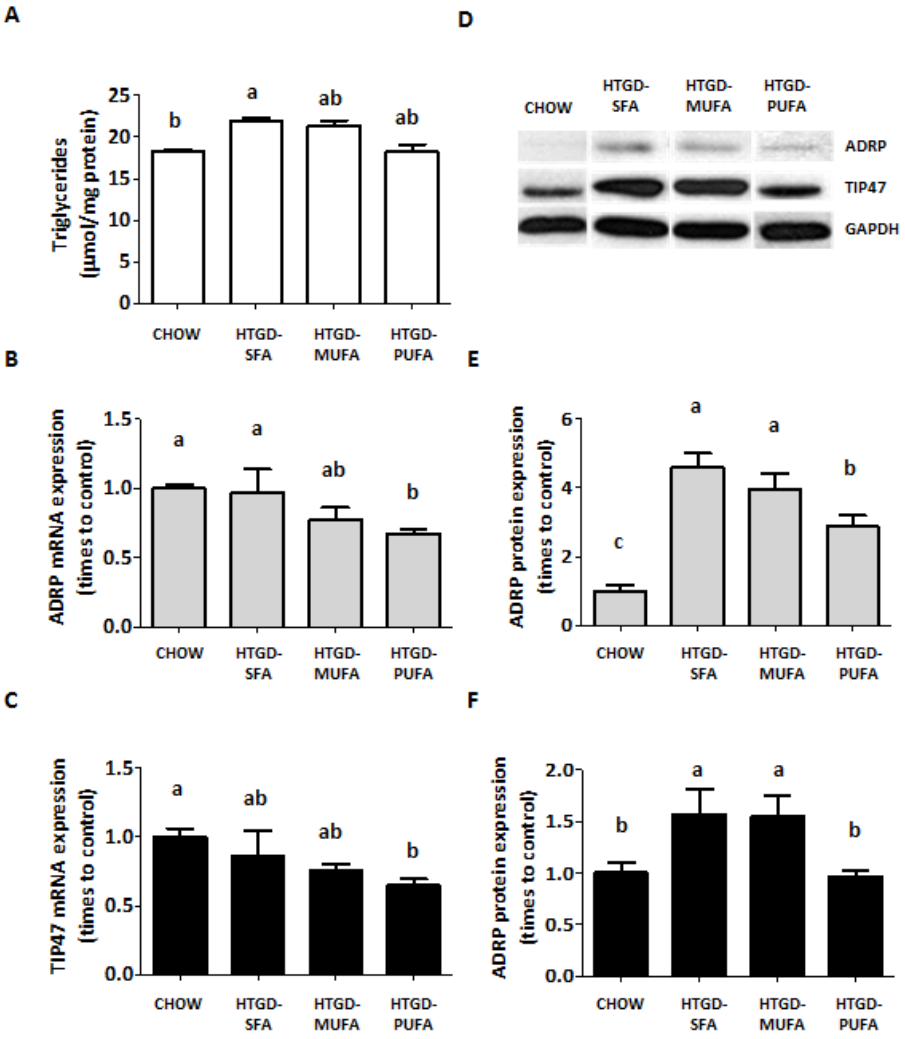

Figure 7. Triglyceride, ADRP, and TIP47 values in bone-marrow-derived macrophages (BMM) isolated from apoE-/- knockout mice after 4 months under a low-fat diet (CHOW), a diet rich in SFA (HTGD-SFA), MUFA (HTGD-MUFA) or PUFA (HTGD-PUFA). A) Intracellular triglyceride accumulation. B-C) mRNA levels of ADRP (grey) and TIP47 (black). D) Western blot of ADRP and TIP47. E-F) Protein levels of ADRP and TIP47. Data (mean ± SD, *n*=4) are expressed as fold induction with respect to the corresponding controls after normalization for 18S and GAPDH for

mRNA or for GAPDH for protein. Columns with a different letter are significantly different ($P < 0.05$).

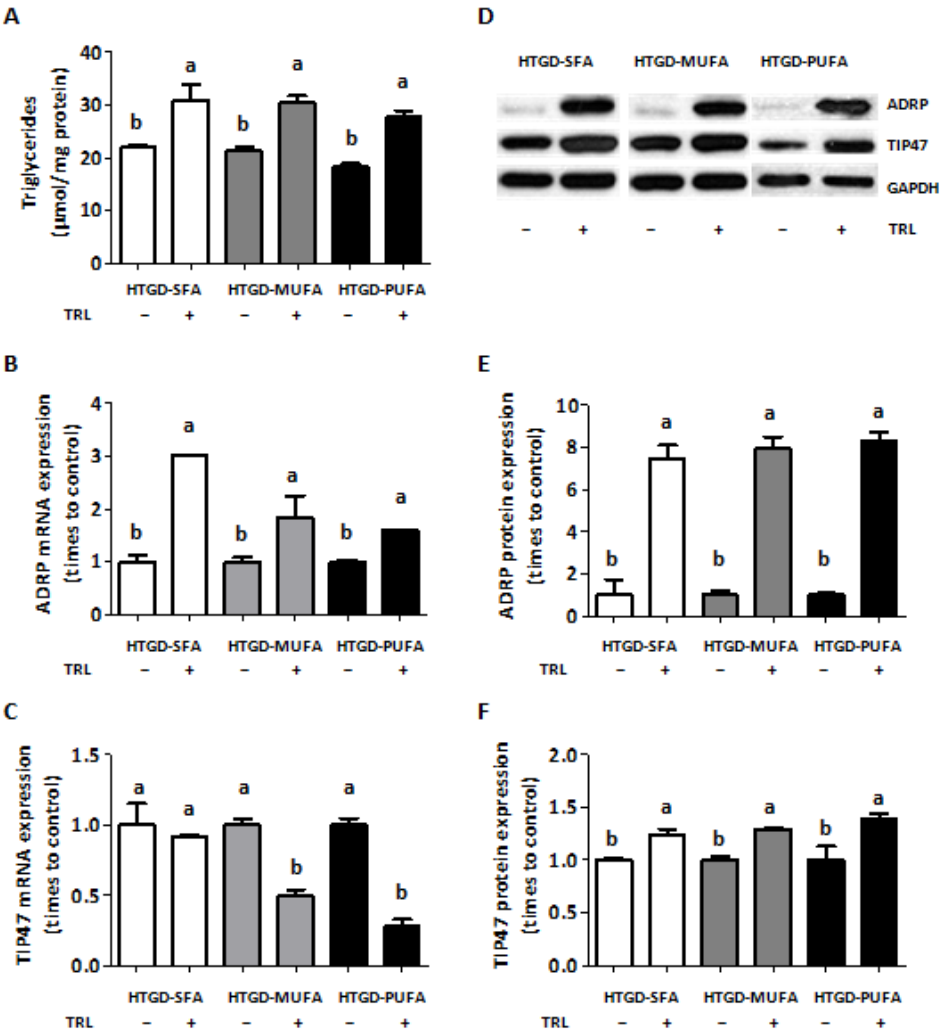

Figure 8. Triglyceride, ADRP, and TIP47 values in bone-marrow-derived macrophages (BMM) isolated from apoE-/- mice after 4 months under a low-fat diet (CHOW), a diet rich in SFA (HTGD-SFA), MUFA (HTGD-MUFA) or PUFA (HTGD-PUFA) (high-triglyceride BMM) after stimulation with the corresponding TRL-SFA, TRL-MUFA or TRL-PUFA. A) Intracellular triglyceride accumulation. B-C) mRNA levels of ADRP (grey) and TIP47 (black). D) Western blot of ADRP and TIP47. E-F) Protein levels of ADRP and TIP47. Data (mean ± SD, *n*=4) are expressed as fold induction with

respect to the corresponding controls after normalization for 18S and GAPDH for mRNA or for GAPDH for protein. Columns with a different letter are significantly different ($P < 0.05$).

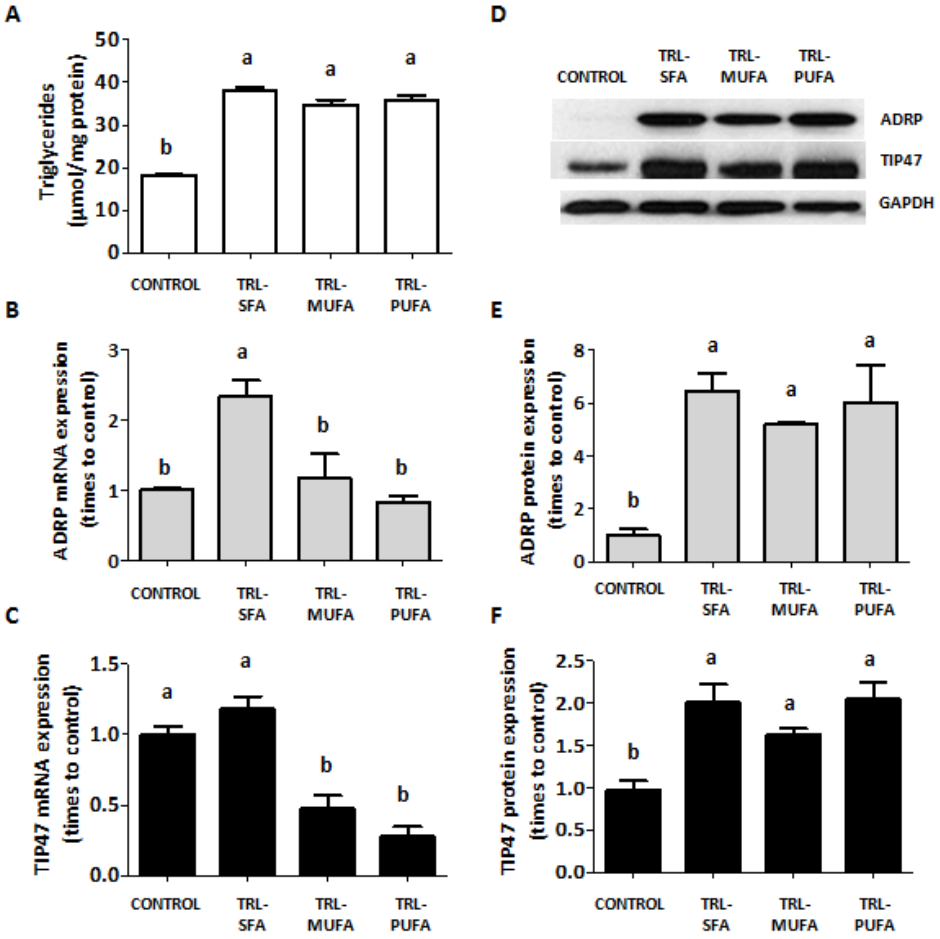

Figure 9. Triglyceride, ADRP and TIP47 values in bone –marrow-derived macrophages (BMM) isolated from apoE-/- mice after 4 months under the chow diet (low triglyceride BMM) after stimulation with TRL-SFA, TRL-MUFA or TRL-PUFA. A) Intracellular triglyceride accumulation. B-C) mRNA levels of ADRP (grey) and TIP47 (black). D) Western blot of ADRP and TIP47. E-F) Protein levels of ADRP and TIP47. Data (mean ± SD, n=4) are expressed as fold induction with respect to the corresponding controls after normalization for 18S and GAPDH for mRNA or for GAPDH for protein. Columns with a different letter are significantly different ($P < 0.05$).

Table 1. Quantification of the triglycerides, cholesterol and % of SFA, MUFA and PUFA in TRL isolated from blood after the consumption of a meal enriched in SFA, MUFA or PUFA in healthy volunteers.

TRL type		Triglycerides (μg/mL)	Cholesterol (μg/mL)	SFA (%)	MUFA (%)	PUFA (%)
TRL-SFA	*mean*	1151.5[b]	189.5[a]	53.4[b]	35.9[a]	10.7[a]
	SD	141.7	100.5	1.7	1.6	0.1
TRL-MUFA	*mean*	1293.2[b]	270.4[b]	18.1[a]	74.1[c]	7.8[a]
	SD	177.1	61.9	2.1	3.7	1.6
TRL-PUFA	*mean*	841.5[a]	279.5[b]	20.2[a]	62.5[b]	17.3[b]
	SD	194.9	61.9	0.4	0.2	0.6

Data are presented as mean ± SD (*n*=3). Mean values in a column with different letters are significantly different ($P < 0.05$).

Table 2. Sequences of the primers used for quantitative real-time PCR analysis.

Human		Mouse	
Name	**Sequence (5´to 3´)**	**Name**	**Sequence (5´to 3´)**
ADRP	Forward: AGGGGCTAGACAGGATTGAGGAGA Reverse: ACGGGAGTGAAGCTTGGTAGAC	**ADRP**	Forward: GATTGAATTCGCCAGGAAGA Reverse: TGGCATGTAGTCTGGAGCTG
TIP47	Forward: CCCCTGGAATCACTGAGAAA Reverse: AGAGGCTGAGAGATGGGTCA	**TIP47**	Forward: CTGAGAAAGGCGTCAAGACC Reverse: TTTCTTGAGCCCCAGACACT
RPLP0	Forward: TCGACAATGGCAGCATCTAC Reverse: ATCCGTCTCCACAGACAAGG	**18S**	Forward: CGCGGTTCTATTTTGTTGGT Reverse: AGTCGGCATCGTTTATGGTC
HPRT	Forward: ACCCCACGAAGTGTTGGATA Reverse: AAGCAGATGGCCACAGAACT	**GAPDH**	Forward: CACATGGCCTCCAAGGAGTAAG Reverse: CCAGCAGTGAGGGTCTCTCT

4. DISCUSSION

Atherosclerosis is the main underlying pathology of cardiovascular disease. Atherosclerosis has its origins in the response of the arteries to subendothelial accumulation of lipoproteins. Macrophages contribute to the formation of arterial lesions by accumulating excessive amount of lipids via interaction with

lipoproteins such as oxidized LDL (oxLDL), acetylated LDL (acLDL) and TRL. Macrophages take up postprandial TRL through the apoB48 receptor resulting in LDs accumulation and foam cell formation (Bermudez et al., 2012). Lipid storage is facilitated by the production of large amounts of LDs coating proteins such as ADRP and TIP47; however, the effects of TRL on these proteins have not been investigated so far. In this study, we have shown for the first time that postprandial TRL induce ADRP and TIP47 protein expression in a time- and fatty-acid-dependent manner in THP-1 macrophages. TIP47 is synthesised earlier, increasing the TIP47 pool for further translocation to the LDs surface. A novel finding of the present work is that the rate of ADRP and TIP47 production in BMM to an excess of triglycerides, as occurs after postprandial TRL stimulation, is highly dependent on the basal triglyceride content of these cells.

LDs exist of a core of neutral lipids, surrounded by a phospholipid monolayer (Martin & Parton, 2006). In most cells, the neutral lipid store in the LDs consists of triglycerides and cholesterol esters. LDs vary in size and abundance according to cellular needs. Physiological conditions that promote lipid storage rapidly and markedly increase LDs volume and surface. In this study, the macrophages derived from THP-1 were incubated for 48 hours with TRL to induce foam-THP-1. We used the monocytic cell line THP-1 because it is a well-characterized model system for studying the transformation of macrophages into foam cells. Our data demonstrate that lipid accumulation and LDs size in foam-THP-1 was dependent on the fatty acid composition of the TRL. After incubation with TRL-SFA, cells accumulated large amounts of triglycerides and less of cholesterol. Foam-THP-1 did not show cholesterol accumulation when THP-1–derived macrophages were incubated with TRL-MUFA and TRL-PUFA, despite of the fact that both postprandial TRL contained larger amounts of cholesterol than TRL-SFA. This indicates that TRL-SFA were more effectively up-taken by THP-1–derived macrophages; accordingly, LDs size in foam-THP1 was bigger. TRL elevated ADRP mRNA and protein expression levels in a fatty acid-dependent manner. ADRP can be also increased in response to different forms of lipid loading in primary human

monocytes (Buechler et al., 2001), in thioglycollate-elicited mouse peritoneal macrophages (Paul et al., 2008), and in several macrophage/monocytic cell lines. Similarly to postprandial TRL, macrophage expression of adipophilin can be upregulated by oxLDL (Li et al., 2010), acLDL (Larigauderie et al., 2004), enzymatically modify LDL (Buechler et al., 2001), and VLDL (Larigauderie et al., 2006). In our studies, TRL-SFA induced higher ADRP expression than TRL-MUFA and TRL-PUFA, which is consistent with the same increase in triglyceride accumulation. In FAO hepatoma cells, unsaturated fatty acids from VLDL hydrolysis can induce canonical peroxisome proliferator-activated-nuclear receptor β/δ (PPARβ/δ) dependent transcriptional responses, increasing ADRP mRNA levels which have functional effects on cellular LDs formation (Brown et al., 2011). Fatty acid-mediated PPARβ/δ regulation have been also reported in murine macrophages (Chawla et al., 2003). The amount of triglycerides and their fatty acid composition that are secreted in form of postprandial TRL after the ingestion of a high-fat meal is dependent of the type of fat consumed. Thus, it is plausible that, in our study, the fatty acids generated from postprandial TRL may have differently regulated the expression of ADRP in macrophages via PPARβ/δ transcriptional regulation. It is known that the activation of PPARβ/δ is regulated by the degree of unsaturation of the fatty acids. PUFA demonstrate minimal PPAR transcriptional activity (Brown et al., 2011), which agree with the lower amounts of ADRP found in our study after stimulation of THP-1–derived macrophages with TRL-PUFA. In RAW264.7 macrophages, the ETS-1/AP-1 element in the mouse ADRP promoter regulates the expression of the gene. In our previous work we have shown that TRL induction of DNA binding of AP-1 in smooth muscle cells is affected by the fatty acid composition of the postprandial TRL (Bermudez et al., 2008), therefore it is possible that postprandial TRL could have induced ADPR protein expression via AP-1 in macrophages. A number of reports have also demonstrated that specific ligands for PPARα and PPARγ upregulate the expression of ADRP (Hodgkinson & Ye, 2003).

The effect of TIP47 in foam cell formation is poorly defined. TIP47 has been found to function in the biogenesis of LDs (Bulankina et al., 2009) and may act

as a carrier protein for free fatty acids participating in conversion of macrophages into foam cells (Buers et al., 2009). Macrophage expression of TIP47 is down-regulated by oxLDL (Li et al., 2010) and up-regulated by VLDL in cells, after adipophilin suppression (Ducharme & Bickel, 2008). We did not found changes in both, mRNA and protein TIP47 expression after incubation with each of the postprandial TRL. To strengthen these results we investigated the presence of ADRP and TIP47 in the LDs using immunofluorescence microscopy. ADRP and TIP47 co-localized in THP-1–derived macrophages after incubation with TRL-SFA and TL-MUFA, but not after TRL-PUFA. BODIPY counterstaining in TRL-SFA-induced foam-THP1 confirmed the presence of ADRP surrounding almost all sizes of LDs; surprisingly, we could also detect TIP47 surrounding LDs despite of the fact that mRNA and protein TIP47 levels did not increased in TRL-induced foam-THP-1. Further studies using freeze fracturing and immunogold labelling demonstrated that the presence of TIP47 in LDs was caused by a major translocation of TIP47 from the cytosol to the LDs surface. Similar results have been found in human macrophages in response to oleic acid loading (Ducharme & Bickel, 2008). The ability of TIP47 to regulate triglyceride accumulation is consistent with studies in other tissues; thus, when adipocytes are provided with long-chain fatty acids, they rapidly synthesize triglycerides and a set of proteins, such as TIP47, are rapidly mobilized from a pre-existing cytosolic pool to coat the nascent triglyceride droplets (Wolins et al., 2006; Wolins et al., 2005). Herein, the recruitment of TIP47 in the LDs in response to TRL-SFA and TRL-MUFA and their inhibition by TRL-PUFA stimulation was further confirmed by western blot of isolated LDs. The mechanisms by which MUFA and, in a higher degree PUFA, could have reduced TIP47 translocation is unknown, but we can speculate that since TIP47 may promote the transport of free fatty acids activated by acyl-coenzyme A synthetase from the cytosol site of the plasma membrane or from the cytoplasm to LDs, different kind of fatty acids could specifically intervene in their union with the 4 helix hydrophobic domain of the protein (Buers et al., 2009). After releasing the lipids, TIP47 may dissociate from the LDs and become available for new lipid binding. TIP47 has two predicted binding structures that may mediate the reversible binding to the LDs. One of this lipid

binding structures resides in the amino terminal sequence of the protein comprising an elongated helix formed by 11-mer repeats (Bulankina et al., 2009). The other resides in the carboxyl-terminal half, and its crystal structure has revealed a 4-helix bundle (Hickenbottom et al., 2004). Dilglyceride, the immediate precursor of triglyceride and its first hydrolytic product is also involved in the recruitment of TIP47 to LDs (Skinner et al., 2009).

Migration of TIP47 from a cytosolic pool to the LDs has been also demonstrated in THP-1 macrophages after VLDL stimulation when ADRP is lacking (Buers et al., 2009). We show for the first time that postprandial TRL are able to induce TIP47 translocation to the LDs surface even in the presence of ADRP. Furthermore, mRNA expression of TIP47 and ADRP in THP-1–derived macrophages peaked at 6 and 24 hours respectively, after postprandial TRL stimulation. In both cases, levels decreased after 48 hours of incubation but only reached basal levels for mRNA TIP47 expression. The distinct time courses observed for TIP47 and ADRP during postprandial TRL exposure might reflect the different roles played by each protein in lipid metabolism in macrophages. TIP47 may represent an early response to excess of triglycerides in order to rapidly coat nascent triglyceride droplets. On the contrary, ADRP alters the cellular content of different lipids and enhances the size of LDs by inhibition of β-oxidation and stimulation of long-chain fatty acid incorporation into triglycerides as a massive increase in triglyceride content (Larigauderie et al., 2006). Similar to our results, in primary rat hepatocytes, TIP47 is increased rapidly by fatty acids followed by a slow decline, while ADRP is increased steadily during fatty acids exposure (Li et al., 2010). Taken together, our data suggest that after postprandial TRL stimulation, macrophages tend to eliminate the excess of triglycerides (fatty acids) through the induction of TIP47, once the cell is overloaded with lipids, an upregulation of ADRP would then address lipids towards storage inside LDs. In THP-1 macrophages, stimulation of ADRP expression by modified LDL promotes triglycerides and cholesterol storage and reduces cholesterol efflux (Larigauderie et al., 2004).

Interestingly, distinct roles for TIP47 and ADRP have been suggested in enterocytes during dietary fat absorption. TIP47, but not ADRP, coats LDs in enterocytes after an acute high-fat challenge, suggesting that TIP47 plays a role in the synthesis of LDs from newly synthesized triglycerides at the beginning of the process of dietary fat absorption in enterocytes. On the other hand, ADRP coats LDs only in enterocytes of chronic high-fat fed mice suggesting that ADRP may play a role in the stabilization of triglycerides stored in LDs in longer term (Lee et al., 2009). It is therefore possible that TIP47 and ADRP have different roles in the early response to excess lipids and long-term lipid storage in macrophages. We then investigated how a HTGD rich in SFA, MUFA or PUFA for 16 weeks could affect triglyceride accumulation and protein levels of TIP47 and ADRP in macrophages by using an apoE-/- mice model of atherosclerosis. Chronic high-fat feeding increased levels of ADRP and TIP47 in BMM. Mice preferentially stimulated ADRP after all diets, interestingly, the ingestion of a PUFA-rich diet attenuated ADRP and did not increase TIP47 protein levels. Concomitantly, triglycerides increased in macrophages after the HTGD rich in SFA and MUFA, suggesting that macrophage foam cell formation after a chronic diet is regulated preferentially by ADRP and probably to a lesser extent by TIP47. Mice fed with a high-fat diet develop fatty acid liver characterized by excessive accumulation of lipid droplets, ablation of ADRP and reduction of TIP47 improves hepatic steatosis in mice (Chan et al., 2010; Carr et al., 2012) suggesting that both proteins affect hepatic lipid metabolism.

Further, we investigated if postprandial TRL stimulation of low-triglyceride and high-triglyceride BMM could modulate a different TIP47 and ADRP response. In our study, the basal content of triglycerides in BMM did affect the production of ADRP and TIP47 after exposure of the macrophages to an excess of lipids. Treatment of high-triglyceride BMM with postprandial TRL resembled a situation of chronic hipertrygliceridemic feeding, showing low production of TIP47. Challenging low-triglyceride BMM with postprandial TRL induced a higher TIP47 protein response, which was concomitant with a higher increment in triglyceride accumulation in these cells. We could not detect differences in protein expression among the postprandial TRL after 24 hours of incubation; mRNA transcripts, however, showed significant down-regulation of

ADRP and TIP47 with TRL-MUFA and TRL-PUFA, which may suggest a decrease in protein concentration at latter times. Altogether, these data indicate that the response of TIP47 and ADPR to an excess of triglycerides, as occurs after postprandial TRL stimulation, is highly dependent on the basal triglycerides content in BMM. After exposure to postprandial TRL, macrophages with low basal levels of triglycerides would initially activate catabolic pathways though the induction of TIP47 in order to eliminate excess of fatty acids. Contrary, BMM with high basal levels of triglycerides would primarily induce ADRP, which would stimulate the storage of triglyceride in the LDs to avoid excess stimulation of catabolic pathways that would result in oxidative stress conditions. Our data also suggest that TIP47 may be a key regulator of triglyceride accumulation after exposure of macrophages to postprandial TRL. Further understanding of the molecular mechanisms of TIP47 actuation will provide new insights into the role of postprandial lipemia in the pathogenesis of atherosclerosis.

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CAPITULO 6

"Postprandial triglyceride-rich lipoproteins induce migration in human coronary artery smooth muscle cells via regulation of Rac1 activation and MMP2"

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En este trabajo se incluyen los estudios llevados a cabo para cumplir con el **Objetivo 4** de esta Tesis Doctoral: Evaluar el efecto diferencial de los ácidos grasos de la dieta incorporados en las lipoproteínas postprandiales ricas en triglicéridos sobre la migración, incluyendo las rutas intracelulares implicadas, en células de musculatura lisa de arteria coronaria humana.

RESUMEN: Los cambios fenotípicos de las células de la musculatura lisa en el seno de las lesiones de la pared vascular representan el inicio de alteraciones anatómicas más profundas en el desarrollo de la aterosclerosis. En una arteria sana, el fenotipo de las células de la musculatura lisa es contráctil; en una arteria con lesión, este fenotipo se transforma a sintético o proliferativo, y las células de la musculatura lisa adquieren la capacidad de migrar desde la media hasta la íntima. Este avance de la lesión y el cambio de comportamiento de estas células también se relacionan con el aumento de la inestabilidad de la placa de ateroma. En este estudio se demuestra *in vitro* que las lipoproteínas postprandiales ricas en triglicéridos y su composición en ácidos grasos influyen de forma significativa en la migración de células de la musculatura lisa de arterias coronarias humanas. En este proceso, por primera vez asociado a la ingesta de comidas ricas en grasas, al menos puede producirse la sobre-expresión de MMP2, la activación de la GTPasa Rac1, y la fosforilación de las AKT, ERK1/2, y JNK en respuesta a la interacción de las células de la musculatura lisa con las TRL postprandiales. En relación al contenido de ácidos

grasos en las lipoproteínas postprandiales ricas en triglicéridos, se observa el siguiente orden en relación al efecto sobre la migración de las células de la musculature lisa: ácidos grasos saturados (mantequilla) > ácidos grasos monoinsaturados (aceite de oliva) > ácidos grasos poliinsaturados de cadena larga de la familia omega-3 (aceite de pescado). Mediante técnicas de inmunofluorescencia también se ha comprobado que las lipoproteínas postprandiales ricas en triglicéridos, a través de Rac1, intervienen en la organización de las fibras de actina del citoesqueleto y en los consiguientes cambios morfológicos característicos de la migración de las células de la musculatura lisa.

Postprandial triglyceride-rich lipoproteins induce migration in human coronary artery smooth muscle cells via regulation of Rac1 activation and MMP2

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(pending submission)

Running tittle: Smooth muscle cells migration is induced postprandially via Rac1 activation.

Abstract

Objective: Vascular smooth muscle cells may contribute to pathological changes in the vasculature such as atherosclerosis and neointima hyperplasia when they are inappropriately stimulated to proliferate and migrate. Postprandial triglyceride-rich lipoproteins (TRLs) may enter the artery wall and have a direct effect on vascular cells; it has been suggested that the quality rather than the quantity of postprandial TRLs may determine the initiation and progression of the atherosclerotic plaque. The aim of the present study was to

investigate the effect of postprandial TRLs with different fatty acid composition on human coronary artery smooth muscle cells (hCASMCs) migration.

Methods and Results: Postprandial TRLs were isolated from plasma of healthy volunteers after the ingestion of single meals enriched in refined olive oil (ROO), butter (BUT) or a mixture of refined olive oil and fish oil (VEFO). hCASMCs migration was performed in transwell cell culture chambers containing a Matrigel solution. Statistical analysis showed that TRLs-BUT provoked the highest migration followed by TRLs-ROO and TRLs-VEFO. Pull-down assays showed that postprandial TRLs were able to increase Rac1 and transiently decreased RhoA activities. Phosphorylation of AKT, JNK, and ERK was detected by western blot analysis and an increase in MMP2 mRNA expression by RT-PCR. NSC23766, an inhibitor of Rac1 and dominant negative Rac1, showed that Rac1 induced lamellipodia formation and played a major role in postprandial TRLs-induced migration. Y27632 (PI3K inhibitor), SP600125 (JNK inhibitor), PD98059 (ERK inhibitor,) and OA-Hy (MMP2 inhibitor) reversed postprandial TRLs-induced hCASMCs migration. PI3K was found upstream of Rac1. LY294002 treatment (RhoA inhibitor) suggests that increased Rac1 activity upon TRLs stimulation may be induced by inhibition of RhoA-dependent signalling.

Conclusion: Our data demonstrate, for the first time to our knowledge, that postprandial TRLs induce hCASMCs migration in a fatty-acid-dependent manner. Furthermore, the migratory effects of postprandial TRLs are mediated by Rac1, PI3K, JNK, and MMP2 pathways.

1. INTRODUCTION

Vascular smooth muscle cells (VSMCs) exist in a differentiated and contractile phenotype under physiological conditions. Normally, they are surrounded by and embedded in an extracellular matrix (ECM) scaffold that acts as a barrier to VSMCs migration. In response to pathological stimuli, these contractile cells may be switched to a proliferative and migratory phenotype, they secrete several proteinases, which degrade the ECM and in turn facilitate VSMCs proliferation and migration. When they are inappropriately stimulated to proliferate and migrate, VSMCs may contribute to pathological changes in the vasculature such as atherosclerosis and neointima hyperplasia. One of the critical factors implicated in the development of atherosclerosis is the accumulation of lipoproteins within large arteries. Postprandial triglyceridemia, in which triglyceride-rich lipoproteins (TRLs) and their remnants accumulate in the circulating blood, has emerged as a key contributor to the progression of coronary artery disease (CAD) (Ginsberg, 2002). Postprandial TRLs can enter the arterial wall (Nakano et al., 2008; Rapp et al., 1994) and be retained in the sub-endothelial space where they may exacerbate inflammation and foam cell formation (Fujioka & Ishikawa, 2009; Schwartz & Reaven, 2012).

Very little is known regarding the effects of postprandial TRLs on VSMCs despite the fact that VSMCs and their ECM products comprise the major structural components of atherosclerotic plaques. Postprandial TRLs induce VSMCs proliferation by activation of the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) that belong to the mitogen-activated protein kinase (MAPK) family (Pacheco et al., 2002), and the sphingomyelin-ceramide pathway (Pacheco et al., 2003). Additionally, proliferation of human coronary artery smooth muscle cells (hCASMCs) is affected by the fatty acid composition of postprandial TRLs (Bermudez et al., 2008), suggesting that the quality rather than the quantity of postprandial TRLs may determine the initiation and progression of the atherosclerotic plaque. Lipoproteins are also involved in VSMCs migration and vascular remodeling in different degrees. Oxidized LDL

(oxLDL) induces matrix-metalloproteinases (MMPs) activity, migration and proliferation of VSMCs (Zhao et al., 2011). However, aggregated LDL impairs VSMCs attachment, migration, and hinder wound repair after injury (Padro et al., 2008). Postprandial lipoproteins, such as remnant lipoproteins induce migration in VSMCs via lectin-like oxLDL receptor-1 (LOX-1) (Aramaki et al., 2008).

Members of the Rho family of small GTPases are key regulators of cell movement through their actions on actin assembly, actomyosin contractility, and microtubes (Ridley, 2012). Rac1, Cdc42, and RhoA are the best-characterized Rho GTPases. Rac1 and Cdc42 are required for lamellipodia actin polymerization and filopodia formation, respectively, while RhoA regulates actin bundling into contractile stress fibers and modulates myosin-dependent tail retraction (Raftopoulou & Hall, 2004). Previous studies suggest that the function of Rho GTPases in shape control, actin organization, and focal contacts is highly cell-type dependent. In Swiss 3T3 fibroblasts, it has been demonstrated that activated Rac1 leads to the activation of RhoA resulting in stress fibers formation (Nobes & Hall, 1995). However, in neuronal cells, cell spreading and neurite outgrowth is determined by a balance between Rac1- and Rho-mediated pathways, and activation of Rac1 antagonizes Rho signaling (Leeuwen et al., 1997). Equally, Rac1 activation mediates inhibition of RhoA in endothelial responses to oxidative stress (Wojciak-Stothard et al., 2005).

Thus, in this study, we hypothesized that postprandial TRLs with different fatty acid composition exhibit distinct hCASMCs migration, in addition to its proliferative effect, and that these differences may have implications in fibrous cap composition and, therefore, in atherosclerotic plaque formation. Furthermore, we studied the signal transduction pathways involved in these processes.

2. MATERIALS AND METHODS

2.1. Lipoprotein isolation

Human postprandial TRLs were isolated by ultracentrifugation from pooled sera of fourteen healthy non-smokers men; lipoproteins were identified as described (Abia et al., 1999). Subjects were given, in three different occasions, a fat-rich meal containing refined olive oil (TRLs-ROO), a mixture of refined olive oil (TRLs-VEFO) or butter (TRLs-BUT). Postprandial TRLs from the individuals in each group were mixed and the pools used for cell culture studies. Fatty acid composition, triglycerides, and endotoxin concentration in postprandial TRLs were determined as described (Bermudez et al., 2008). The study was carried out in accordance with the principles outlined in the Helsinki Declaration. Details of the study design have been reported previously (Pacheco et al., 2006).

2.2. Cell culture

hCASMCs (Clonetics, Walkersville, ML) were used up to the eighth passage. hCASMCs were grown as indicated (Bermudez et al., 2008). Cells were arrested when subconfluent by maintaining them in serum-free medium (control) for 48 hours, before they were stimulated with different concentrations of postprandial TRLs at different time periods.

2.3. Transwell invasion assay

The quantification of cell invasion was performed in transwell cell culture chambers containing fluorescence-blocking polycarbonate porous membrane inserts (8 µm pore size HTS FluoroBlokTM insert, BD Biosciences, 351152). 100 µL of a 1 mg/mL Matrigel solution (BD MartigelTM Basement Matrix, BD Biosciences, 354234) was added in the transwells. Cells were starved overnight

in assay media (0.5% FCS) then trysinised and counted. Cells (4 x 10^4) were plated in postprandial TRLs-containing medium (50 µg TRLs/mL) on top of the layer of the Matrigel in the upper chamber of the transwell. The lower chamber was filled with postprandial TRLs containing medium (100 µg TRL/ mL) to establish a soluble gradient of chemoattractant that permits cell invasion through the matrigel. For negative and positive controls, the upper chambers were filled with 0% FCS and 2% FCS and the lower chambers with 0% FCS and 10% FCS, respectively. Cells were allowed to invade through the gel at 37°C and 5% CO_2 for 18 hours before fixing in 3.7% formaldehyde. Cells that had invaded trough the matrigel were detected on the lower side of the filter by using an inverted microscope (Carl Zeiss). The whole surface of the well was scanned as a mosaic of 100 images and cell number counted using MetaMorph 6 software (Molecular Devices) (Gadea et al., 2007; Vinot et al., 2008). For assays with chemical inhibitors, cells were pre-incubated for 2 hours before plating with Rho inhibitor Y27632 (10 µM) (Calbiochem 688000), with Rac1 inhibitor NSC23766 (100 µM) (Calbiochem 553502), with MMP2 inhibitor cis-9-octadecenoyl-N-hydroxylamide (OA-Hy) (5 µM) (Calbiochem 444244), with an extracellular signal-regulated kinase-(ERK)-specific inhibitor PD98059 (20 µM) (Sigma-Aldrich P215), with a p38 MAP kinase-specific inhibitor SB203580 (20 µM) (Sigma-Aldrich S8307), with a PI 3´-kinase/AKT-specific inhibitor LY294002 (5 µM) (Sigma-Aldrich L9908), and with a c-Jun NH_2-terminal kinase (JNK)-specific inhibitor SP600125 (20 µM) (Sigma-Aldrich S5567). Inhibitors were also added into both upper and bottom chambers and into the matrigel at the aforementioned concentrations. Each essay was performed in triplicate.

2.4. Quantitative real-time polymerase chain reaction

The mRNA levels for specific genes were determined by real-time PCR in a MX3000P system (Stratagene, La Jolla, USA). Total RNA was extracted from hCASMCs by using TrisureTM Reagent (Bioline, BIO-38032). RNA quality was assessed using the OD_{260}:OD_{280} ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Reverse transcription was performed

using 1 µg RNA and RevertAidTM (Fermentas, EP0441), according to the manufacturer's instructions. The cDNA template was added to Brilliant SYBR green QPCR Master mix (Agilent Technologies, Stratagene 600828) containing the primer pairs for MMP1, MMP2, MMP3, MMP9 or for RPLP0 and HPRT as housekeeping genes. The sequence and information for primers used in this study are as follows: MMP1 (Gene bank accession no. NM_002421): 5´ATGCTGAAACCCTGAAGGTG-3´ (forward) and 5´-CTGCTTGACCCTCAGAGACC-3´ (reverse); MMP2 (Gene bank no. NM_004530): 5´-ATGACAGCTGCACCACT-GAG-3´ (forward) and 5´-ATTTGTTGCCCAGGAAAGTG-3´ (reverse); MMP3 (Gene bank no. NM_002422): 5´-GCAGTTTGCTCAGCCTATCC-3´ (forward) and 5´-GAGTGTCGGAGTCCAGCTTC-3´ (reverse); MMP9 (Gene bank no. NM_004994): 5´-TTGACAGCGACAAGAAGTGG-3´ (forward) and 5´-GCCATTCACGTCGTCCTTAT-3´ (reverse); RPLP0 (Gene bank no. NM_001002): 5´-TCGACAATGGCAGCATCTAC-3´ (forward) and 5´-ATCCGTCTCCACAGACAAGG-3´ (reverse); and HPRT (Gene bank no. NM_000194): 5´-ACCCCACGAAGTGTTGGATA-3´ (forward) and 5´-AAGCAGATGGCCACAGAACT-3´ (reverse). Reactions were performed in triplicate and the change in mRNA expression was calculated by using the $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (RPLP0 and HPRT) gene levels and expressed as the change with respect to the control.

2.5. Rho GTPases activity assays

hCASMCs were treated with postprandial TRLs (100 µg/mL) for 2, 5, and 20 min, and washed with ice-cold PBS before scrapping with lysis buffer on ice. For RhoA assay, the cells were lysed in 1x Lysis buffer [50 mM TRIS, pH 7.5, 500 mM NaCl, 10 mM $MgCl_2$, 0.5% sodium deoxycholate, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethyl sulphonyl fluoride (PMSF), 0.5 mM Na_3VO_4] containing protease inhibitor cocktail. For Rac1/Cdc42 assay, the cells were lysed in 1x Lysis buffer (25 mM HEPES, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1% NP40, 5% Glycerol, 5 mM NaF, 1 mM PMSF, 1 mM Na_3VO_4) with the protease inhibitor cocktail. Cellular debris was removed by centrifugation and cell lysate

was immunoprecipitated with either 100 µL Rhotekin-RBD Protein GST Beads (for RhoA) (Cytoskeleton Inc RT02) or 15 µl PAK-GST Protein Beads (for Rac1 and Cdc42) (Cytoskeleton Inc PAK02) at 4°C for 1 hour. Eluted samples were run on a 12% separating gel on PAGE and subjected to western blot analysis using antibodies against Rac1 (BD Biosciences 61065), Cdc42 (BD Biosciences 610928) or RhoA (anti-RhoA 26C4) (Santa Cruz Biotechnology sc-418). Total cell lysates were also directly immunoblotted for the levels of total Rac1, Cdc42 and RhoA for normalization (Gadea et al., 2002).

2.6. Immunofluorescence

hCASMCs were treated with postprandial TRLs (100µg/mL) on coverslips at a confluence of ~30%. After 18 hours cells were fixed for 10 min in 3.7% formaline in PBS followed by a 5 min permeabilization in 0.1% Triton X-100 in PBS and incubation in PBS containing 0.1% bovine serum albumin. Rac1 was labelled with primary anti-Rac1 (BD Biosciences 61065) followed with Alexa Fluor 444-conjugated antibody (Invitrogen A11017). Hoechst 33342 (Invitrogen H3570) was used for DNA staining. Cells were washed in PBS and mounted in Prolong-Gold (Invitrogen P36930). Visualization of actin filaments was achieved by labelling with Rhodamine Phalloidine (Sigma Aldrich P1951). For experiments with inhibitors, cells were pre-incubated with Y27632 or NSC23766 for 2 hours before addition of postprandial TRLs. Cells were observed using a DMB B microscope with a PL APO 40x objective (Leica, Germany).

2.7. Immunoblotting

hCASMCs grown to subconfluence were stimulated with postprandial TRLs for various times (5, 10, 15, 30, and 60 min). The method of immunoblotting has been described previously (Pacheco et al., 2002). Briefly, cells were lysed in RIPA buffer containing 1 mmol/L Na_3VO_4, 1 mmol/L NaF, 1 µg/ mL aprotinin, 1

µg/mL leupeptin, 1 µg/mL pepstatine, 1 µmol/L PMSF. Protein concentrations were determined by the Lowry method, were separated by electrophoresis through a 10% sodium dodecyl sulphate-polyacrylamide gel and transferred to nitrocellulose membranes. Protein loading equivalence was controlled after electrotransfer to nitrocellulose membranes by staining with ponceau S and by immunoblotting for actin. Membranes were immunoblotted with mouse anti p-AKT (Ser473) (Cell Signalling 4051), p-JNK (G-7) (Santa Cruz Biotechnology sc-6254), and p-p38 (D-8) (Santa Cruz Biotechnology, sc-7973); anti-phospho-MAPK (Thr202/Tyr204) polyclonal antibody was also used to detect ERK1 (p44mapk) and ERK2 (p42mapk), specific antigen-antibody complexes were detected with the ECL Western blot detection kit (Supersignal West Pico Chemiluminescent Substrate, Thermo Scientific MB153729).

2.8. Plasmids and transfection

The plasmids encoding Rac1Q61L (constitutively active, Addgene plasmid 13720) and Rac1T17N (dominant negative mutant, Addgene plasmid 13721) were kindly provided by Dr. K. Hahn (Kraynov et al., 2000). pcDNA3EGFP plasmid was obtained after cutting one of the above. Addgene plasmid with EcoRI-XhoI and removing the cDNA insert, then plasmid ends were blunted with Klenow and religated with T4 DNA Ligase. All the plasmids were verified by DNA sequencing and isolated from transformed DH5α E. coli cells with JETSTAR 2.0 plasmid midiprep kit (GENOMED GmbH) as transfection-ready DNA. Transfection of hCASMCs cells was performed using an Amaxa Nucleofector II Device (Lonza, Germany) and manufacturer´s protocol with minor modifications. In each transfection, 1 x 10^6 cells were resuspended in 100 µL of Nucleofector Solution of Amaxa Basic Nucleofector Kit for Primary Mammalian Smooth Muscle Cells (Amaxa Inc. VPI-1004) containing 2 µg of either the pcDNA3EGFP vector (mock) or the corresponding Addgene plasmid DNA. Electrical setting given by program A-033 was chosen for the experiments providing the best results in terms of transfection efficiency, which was greater

than 67%. Transfected cells were serum depleted for 24 hours, and used for transwell invasion assay.

2.9. Statistics

Data are presented as mean ± SD. The homogeneity of variance was tested with Bartlett's test. Comparisons of two means were performed by using Student's t test. Group statistical comparisons were performed by 1- or 2-way ANOVA with a post hoc Bonferroni test. A value of $P < 0.05$ was considered statistically significant. All experiments were performed at least three times in triplicate.

3. RESULTS

3.1. Postprandial TRLs induced hCASMCs migration and MMPs expression in a fatty-acid--dependent manner

Cell migration of VSMCs from the media to the intima is a multistep process that is crucial in atherogenesis. Since cell motility on two-dimensional (2D) culture studies is not necessarily applicable to the study of the movement of cells in the vessel wall, we used a three-dimensional (3D) *in vitro* model in which cell shape, motility, and cell environment closely match those of an *in vivo* model. To determine whether postprandial TRLs regulate migration in hCASMCs, firstly we treated cells with different concentrations (50 and 100 µg triglycerides/mL) of the lipoprotein. Cell exposure to postprandial TRLs resulted in a dose-dependent migration of hCASMCs 18 hours after stimulation (**Figure 1A**). The concentration of 100 µg triglycerides/mL of postprandial TRLs induced the highest percentage of migration ($P < 0.005$). This concentration did not negatively affect viability of hCASMCs as assessed by trypan blue exclusion (data not shown) and was used for all subsequent experiments.

Previously, we have found that hCASMCs proliferation depends on the fatty acid composition of the postprandial TRLs (Bermudez et al., 2008). Next, we wanted to know whether postprandial TRLs-induced migration was also dependent on the fatty acid composition of the lipoproteins. The fatty acid composition of postprandial TRLs is shown in **Table 1**. The butter meal provided SFA,, resulting in the enrichment of TRLs-BUT with palmitic and stearic acids. The ROO meal provided MUFA, and TRLs-ROO had high amounts of oleic acid. The percentage of MUFA in TRLs-VEFO was also high; in addition, there was enrichment in PUFA of the *n-3* family, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids. **Figure 1B** shows that TRLs-BUT induced a 3-fold increase migration compared to the control ($P < 0.005$). Meanwhile, only a 2-fold increase was observed when cells were stimulated with TRLs-ROO ($P < 0.05$) and 1.5-fold increase after stimulation with TRLs-VEFO. Significant differences were found between TRLs-ROO ($P < 0.05$) and TRLs-BUT, and between TRLs-VEFO ($P <0.005$) and TRLs-BUT, but not between TRLs-ROO and TRLs-VEFO. These data suggest that postprandial TRLs induce migration in hCASMCs in a fatty-acid-dependent manner.

VSMCs migration and proliferation requires the secretion of MMPs (Galis and Khatri, 2002). Postprandial TRLs induced the mRNA expression of MMP1, MMP2, and MMP3 but no of MMP9, 18 hours after hCASMCs stimulation, compared to the control (serum-starved cells) (**Table 2**). This induction was dependent on the kind of postprandial TRLs used in the assay. TRLs-BUT increased mRNA levels of MMP1, MMP2 and MMP3; however TRLs-ROO and TRLs-VEFO only showed increase in MMP2 mRNA expression. Level of MMP2 mRNA were above the ones detected for the other MMPs after hCASMCs stimulation with all the postprandial TRLs. TRLs-BUT showed the highest expression ($P < 0.05$), however, not significant differences were found after TRLs-ROO and TRLs-VEFO stimulation. All the postprandial TRLs induced MMP2 mRNA expression at a higher degree than oxLDL ($P < 0.05$), used as a positive control.

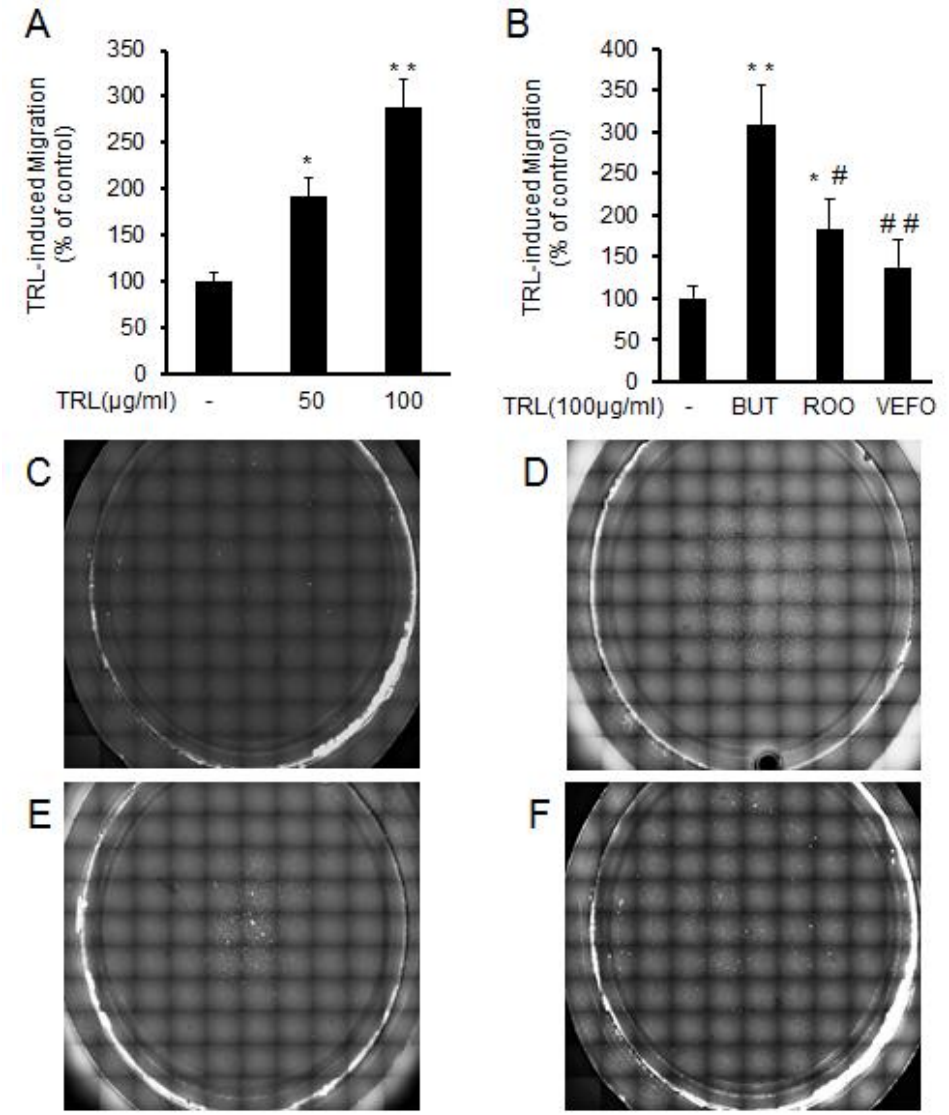

Figure 1. Postprandial TRLs induce migration of human coronary artery smooth muscle cells (hCASMCs) in a dose and fatty-acid-dependent manner. (A) Migration of hCASMCs was examined using transwells with Matrigel after stimulation with serum-free SmBM, and different doses of triglycerides in postprandial TRLs for 18 hours. (B) Quantification of migration after stimulation with serum-free SmBM and postprandial TRLs with different fatty acid composition (TRLs-BUT, TRLs-ROO and TRLs-VEFO) for 18 hours. Reconstituted photographs of the mosaic of 100 images representing the entire surface of the well. Cells are visualized as white points. (C) Cells treated with serum-free SmBM. (D) Cells stimulated with TRLs-BUT. (E) Cells stimulated

with TRLs-ROO. (F) Cells stimulated with TRLs-VEFO. The data represent the mean ± SD of triplicate samples repeated in three separate experiments. **P* < 0.05, ** *P* < 0.005, compared to controls and #*P* < 0.05, ## *P* < 0.005 compared between the postprandial TRLs.

Table 1. Fatty acid composition of TRLs-BUT, TRLs-ROO, and TRLs-VEFO.

Fatty acids	**TRLs-BUT**	**TRLs-ROO (%)**	**TRLs-VEFO**
Miristyc (14:0)	7.33 ± 0.30^{c}	0.20 ± 0.09^{a}	1.61 ±0.08^{b}
Palmitic (16:0)	34.06 ± 1.07^{b}	13.00 ± 0.73^{a}	14.73 ±0.51^{a}
Stearic (18:0)	11.39 ± 0.26^{b}	3.96 ± 0.96^{a}	3.37 ± 0.16^{a}
Arachiric (22:0)	0.51 ± 0.06^{b}	0.22 ± 0.04^{a}	0.21 ± 0.01^{a}
Lignoceric (24:0)	0.13 ± 0.00^{a}	0.71 ± 0.40^{c}	0.48 ± 0.01^{b}
Palmitoleic (16:1*n*-7)	2.43 ± 0.06^{b}	0.77 ± 0.06^{a}	2.52 ± 0.09^{b}
Oleic (18:1*n*-9)	31.70 ± 0.47^{a}	72.40 ± 3.44^{c}	58.17 ± 0.33^{b}
Eicosenoic (20:1*n*-9)	0.41 ± 0.17^{a,b}	0.30 ± 0.00^{a}	0.56 ± 0.04^{b}
Linoleic (18:2*n*-6)	8.41 ± 0.22^{b}	5.47 ± 1.07^{a}	6.89 ± 0.21^{a}
α-Linolenic (18:3*n*-3)	0.42 ± 0.01^{a}	0.46 ± 0.01^{a}	0.62 ± 0.02^{b}
Eicosatetraenoic (20:4*n*-3)	0.09 ± 0.04^{a}	0.37 ± 0.13^{b}	0.37 ± 0.04^{b}
Arachidonic (20:4*n*-6)	1.40 ± 0.02^{b}	0.92 ± 0.52^{a}	1.11 ± 0.01^{a}
Eicosapentaenoic (20:5*n*-3)	0.10 ± 0.00^{a}	0.15 ± 0.02^{a}	5.78 ± 0.14^{b}
Docosapentaenoic (22:5*n*-3)	0.14 ± 0.02^{a}	0.18 ± 0.04^{a}	0.49 ± 0.03^{b}
Docosahexaenoic (22:6*n*-3)	0.25 ± 0.01^{a}	0.27 ± 0.16^{a}	1.68 ± 0.06^{b}

Data are the mean ± SD, *n*=3

P < 0.05, different letters denote significantly different among the postprandial TRLs.

Table 2. MMPs mRNA expression in hCASMCs after TRLs-BUT, TRLs-ROO, and TRLs-VEFO stimulation.

	MMP1	**MMP2**	**MMP3**	**MMP9**
Control	1.00 ± 0.86^{a}	1.00 ± 0.56^{a}	1.00 ± 0.08^{a}	1.00 ± 0.16^{a}
oxLDL	4.01 ± 5.76^{c}	4.71 ± 1.75^{b}	6.70 ± 0.38^{c}	5.28 ± 1.23^{b}
TRLs-BUT	2.23 ± 0.22^{b}	14.71 ± 1.13^{d}	2.62 ± 0.27^{b}	0.88 ± 0.26^{a}
TRLs-ROO	1.26 ± 2.09^{a}	6.48 ± 1.84^{c}	1.42 ± 0.58^{a}	1.09 ± 0.64^{a}
TRLs-VEFO	1.11 ± 1.08^{a}	7.14 ± 2.90^{c}	0.77 ± 0.44^{a}	1.29 ± 0.47^{a}

Data are the mean ± SD, values are normalized respect to control (serum-free SmBM), *n*=3

P < 0.05, different letters denote significantly different.

3.2. Postprandial TRLs-induced an increased in Rac1 activity and transient decreased in RhoA activity

The Rho GTPase family members (RhoA, Rac1, Cdc42) are very early elements in signalling pathways that promote cell migration. To study the role of Rho GTPase family members in TRLs-induced migration, we measure RhoA, Rac1, and Cdc42 activities by pull-down assays in hCASMCs after stimulation with TRLs-BUT. We have used TRLs-BUT at 100 µg triglycerides/mL, a concentration that has been shown in our present study to have maximal stimulation of migration without exhibiting any effect on cell survival, for further studies. As shown in **Figure 2A**, GTP-Rac1 values were high and sustained along the assay period as compared to the control. The amounts of GTP-Rac1 increased rapidly within 3 min of TRLs-BUT stimulation, peaked at 10 min (5-fold), then decreased but remained above the control levels. RhoA was not activated by TRLs-BUT, furthermore, pull-down assay showed a transient inhibition of the basal levels (2-fold) after 10 min of stimulation. The amounts of GTP-Cdc42 peaked at 3 min, but the level of activation was modest compared to GTP-Rac1. After 10 min of stimulation, activity dropped dramatically to levels even lower than control (3-fold). Because it is generally believed that the majority of active GTPases are membrane bound and these membrane-bound active enzymes are involved in cytoskeletal changes required for cell migration, we also determined the presence of Rac1 on the plasma membrane. Counterstaining of hCASMCs with DAPI and Rac1 showed increased staining of Rac1 in the cytosol and in the leading edge of broad membrane lamellipodia protrusions (arrows) in cells challenged with postprandial TRLs compared to control (serum starved cells) (**Figure 2B**). This result suggests that postprandial TRLs can translocate cytosolic Rac1 to the plasma membrane.

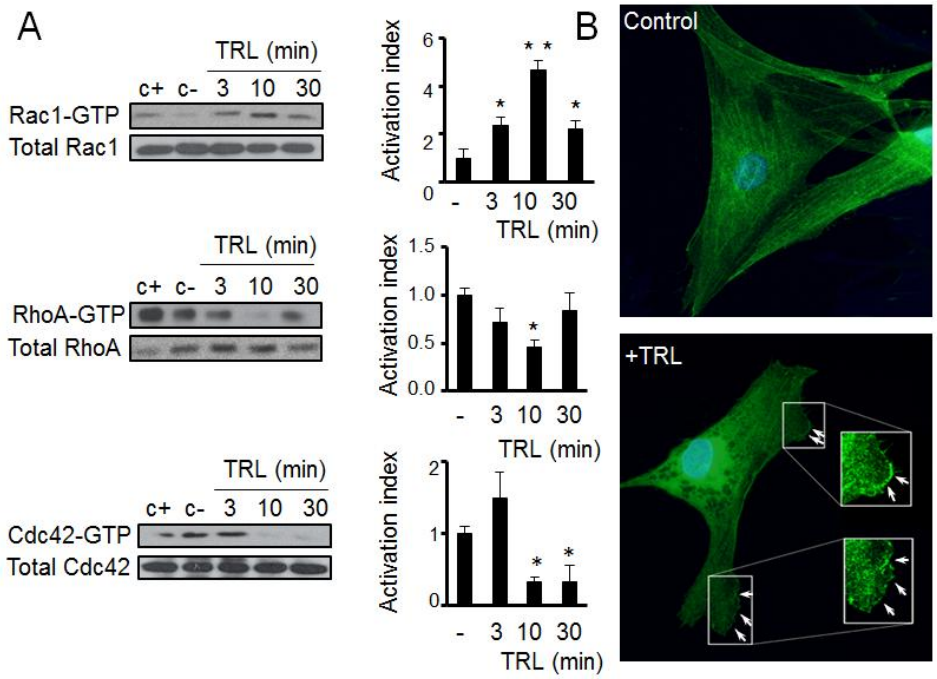

Figure 2. Activation of Rho GTPases in hCASMCs. (A) Cells were stimulated with serum (c+) o with postprandial TRLs for the indicated time intervals, and Rac1, RhoA and Cdc42 activation was determined by pull down assays. Activated GTPases levels were normalized to the amount of total GTPases in cell lysates as analysed by western blotting. The intensity of the bands was quantified and the values are the mean ± SD of three independent experiments normalized to the response of untreated cells (c-). **P* < 0.05, ** *P* < 0.005, compared to control. (B) hCASMCs immunostained with Rac1 antibody in serum starved cells and after treatment with 100 µg/ mL of TRLs-BUT. Arrows indicate Rac1 localization on the plasma membrane of treated cells as compared to controls.

3.3. Postprandial TRLs causes morphological changes in hCASMCs accompanied by reorganization of the actin cytoskeleton

Actin cytoskeleton reorganization is required for cell shape modification and cell migration. We therefore investigated the ability of postprandial TRLs to induce changes in F-actin reorganization in hCASMCs using rhodamine phalloidin, a dye that specifically binds to polymerized F-actin. Serum-starved hCASMCs (control) displayed a flat nonpolarized shape. Cells stimulated with

TRLs-BUT adopted well-defined characteristics of migrating cells including elongated morphology and cell polarization, exhibited an overall decrease in staining intensity, a marked reduction in intact stress fibers, and the formation of broad membrane lamellipodia protrusions (arrows in figure) compared to the control (**Figure 3A**). Because Rac1 activation stimulated the formation of distinct actin-rich lamellipodia, and because TRLs-BUT transiently inactivated RhoA, we examined the effects of RhoA and Rac1 on hCASMCs cytoskeleton organization. Cells exposed to Y27632, stimulated a reorganization of actin filaments including a great reduction of intact actin stress fibers and a strong actin staining at the edges of the cells when compared to the control, consistent with blockade of Rho isoforms activity. Surprisingly, 30% of cells showed the appearance of a ruffled morphology, as indicated by the formation of some actin bundles in the periphery of Y27632-treated cells (arrows). Quantification showed that 60-65% of postprandial TRLs-stimulated cells, previously incubated with Y27632, increased lamellipodia formation (arrows), which is characteristic of Rac1 activation. In contrast, cells pre-incubated with NSC23766, an inhibitor of Rac1 activation, retained their original polygonal morphology, as seen in the untreated control cells, since addition of TRLs-BUT had no effect on stress fibers architecture or lamellipodia formation. Taken together, these data indicate that, postprandial TRLs are able to induce lamellipodia formation in Y-27632-treated cells, and that Rac1 signalling is essential for postprandial TRLs-induced cytoskeleton modification.

3.4. Postprandial TRLs-induced migration is Rac1- and MMP2- dependent

To examine the role of MMP2 and Rac1 on postprandial TRLs-induced migration, MMP2 and Rac1 activities were blocked using specific inhibitors, OA-Hy and NSC23766, respectively. OA-Hy and NSC23766, showed not effect on basal migration at 18 hours as compared to the control. Cells treated with NSC23766 were then challenged with TRLs-BUT, but their migration could not be rescued. MMP2 inhibitor prevented cell migration by 74% after postprandial TRLs stimulation (**Figure 3B**). These results suggest an important

role for Rac1 and MMP2 in hCASMCs postprandial TRLs-induced migration. NSC23766 is a reversible Rac1 inhibitor that competitively inhibits interaction between Rac1 and Rac-specific guanine nucleotide exchange factors (GEFs) and has no effect on Cdc42 and RhoA activation, however it is not a Rac1 selective inhibitor. Therefore, we transfected hCASMCs with dominant negative Rac1 (Rac1N17) and examined the effect of its expression on postprandial TRLs-induced migration. To determine whether activation of Rac1 signalling is sufficient to induce migration, we expressed a constitutively active mutant of Rac1 protein (Rac1Q61L) in hCASMCs. Migration was analysed in serum-free SMC basal medium (SmBM), and after TRLs-BUT stimulation (**Figure 3C**), dominant negative Rac1N17 lead to a significant reduction (70%) in cell migration, indicating that Rac1 is essential for basal hCASMCs migration. TRLs-BUT were not able to recover the migration inhibitory effect of Rac1N17. Constitutively active Rac1Q61L showed increased basal migration. These data confirm the fact that Rac1 mediates postprandial TRLs-induced migration and that Rac1 is essential for hCASMCs migration.

3.5. Postprandial TRLs-induced migration is associated to an increased Rac1 activity and decreased RhoA activity

It has been shown that Rac1 and RhoA reciprocally regulate each other in a variety of cell types to regulate their motility. Data discussed earlier have shown that postprandial TRLs transiently inhibit RhoA activation while increasing Rac1 activation in hCASMCs, and that this inhibition is not sustained along the assay period. We extended our observations of the effects of RhoA inhibition in postprandial TRLs-induced migration, by treating cells with Y27632, an inhibitor of the downstream Rho effector Rho-kinase/ROCK (**Figure 3D**). Migration assays in serum-free conditions showed that the basal migration of hCASMCs treated with Y27632 (10 µmol/L) for 1 hour was increased (43%) compared to control ($P < 0.05$). Postprandial TRLs stimulation enhanced this effect (70%), showing that postprandial TRLs induce migration when ROCK is inhibited in the cells. Additionally, analysis of Rac1 activity, by

using a Rac1 pull-down assay in Y27632 treated cells upon TRLs addition, demonstrated that activity of Rac1 increased with increasing concentrations of Y27632 treatment (**Figure 3E**). This result suggests that increased Rac1 activity upon postprandial TRLs stimulation may be induced by inhibition of Rho-dependent signalling.

3.6. Postprandial TRLs-induced migration is mediated by PI3K, JNK and ERK

VSMCs migration may be involved in multiple signalling pathways. MAPKs, ERK1/2, JNK, and p38MAPK, are a family of central signalling molecules that respond to numerous stimuli and participate in cell proliferation and migration (Duru et al., 2011; Sun et al., 2011). TRLs-BUT significantly activated the p38MAPK, ERK1/2, and JNK signalling transduction pathways in hCASMCs, with a maximum activation at 15, 10, and 10 min, respectively. TRLs-BUT also induced AKT at 5 min after stimulation, which is known to be downstream of PI3K in this pathway (**Figure 4A**). To further investigate the signalling pathways involved in postprandial TRLs-induced hCASMCs migration, we assessed the effect of PD98059, an extracellular signal-regulated kinase (ERK)-specific inhibitor; SB203580, a p38 MAP kinase-specific inhibitor; LY294002, a PI 3′-kinase/AKT-specific inhibitor; and SP600125, a c-Jun NH_2-terminal kinase (JNK)-specific inhibitor. The optimal concentrations for these inhibitors were identified in preliminary dose response experiments (data not shown). Pre-treatment with specific inhibitor of JNK, PI3K, and ERK suppressed postprandial TRLs-induced cell migration by 60%, 48%, and 33%, respectively. On the other hand, p38 inhibitor did not affect postprandial TRLs-induced cell migration (**Figure 4B**). We then assessed whether PI3K is involved in postprandial-TRLs-mediated activation of Rac1 signalling pathways in hCASMCs. Postprandial TRLs-induced activation of Rac1 signalling was monitored by measuring the amount of GTP-bound Rac1 in the presence of the inhibitor LY294002. Pull-down assay and western blot analysis showed that postprandial TRLs-induced Rac1 activation was inhibited by LY294002. These results indicate that

activation of Rac1 by postprandial TRLs-stimulation is a PI3K-dependent process and that JNK was not found to be upstream of Rac1 (**Figure 4C**).

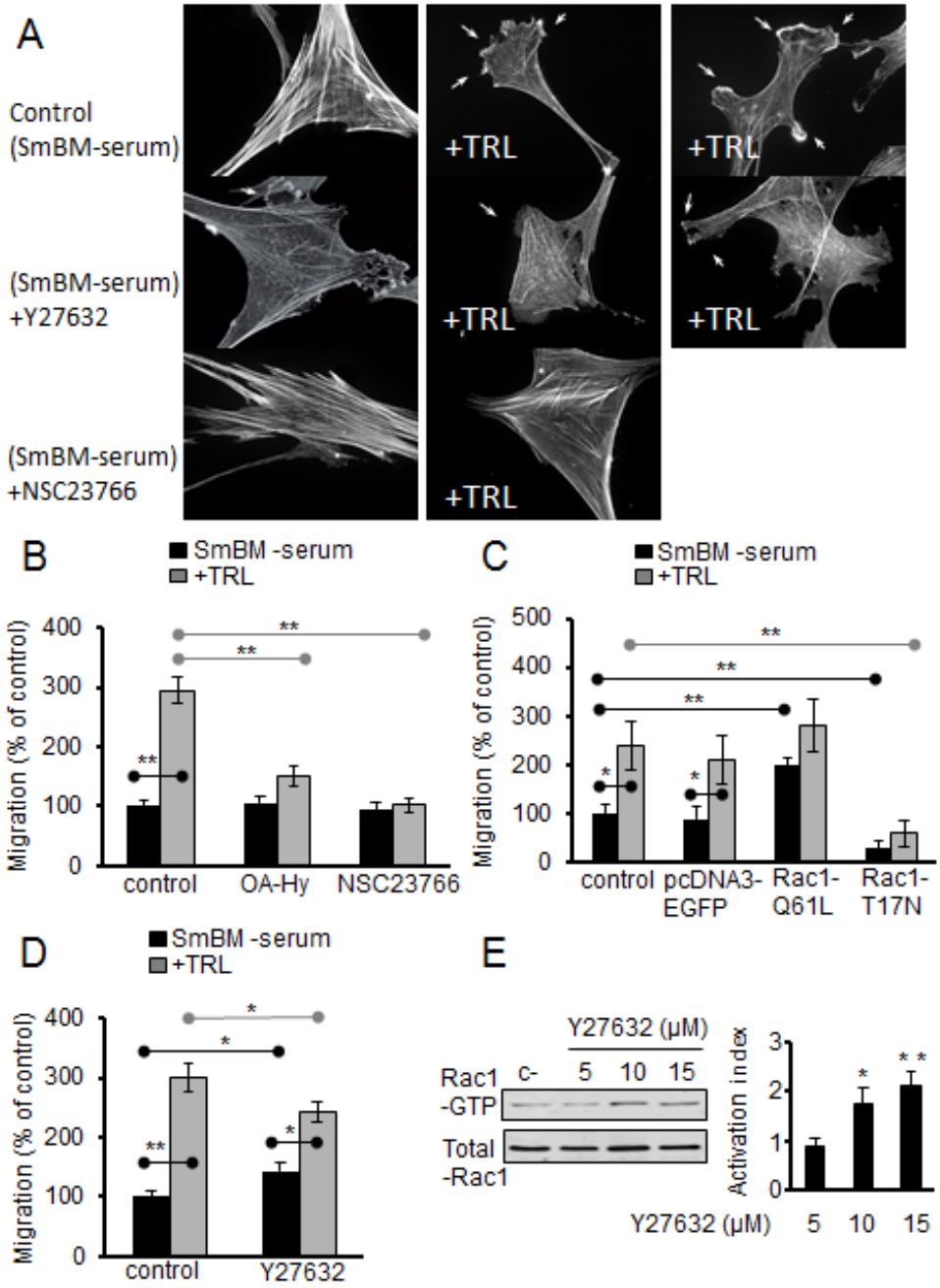

Figure 3. Regulation of migration by Rac1, RhoA, and MMP2. (A) Effects of TRLs-BUT, RhoA inhibitor (Y27632), and Rac1 inhibitor (NSC23766) on the morphology and actin cytoskeleton of hCASMCs as shown by inmunofluorescence. First row shows a representative phalloidin labelled

F-actin staining of hCASMCs in the absence of postprandial TRLs. Second and third rows show hCASMCs treated with 100 µg triglycerides/mL of TRLs-BUT for 18 hours. TRLs-BUT stimulates actin cytoskeleton organization leading to loss of stress fibers and lamellipodia formation (arrowheads) compared with untreated controls. Cells treated with Y27632 showed a loss of actin stress fibers and the appearance of some membrane ruffling, addition of TRLs-BUT increased lamellipodia (arrowheads). hCASMCs treated with NSC23766 remain predominantly polygonal in shape and showed no modification in stress fibers, addition of TRLs-BUT did not abolished these characteristics. (B) PostprandialTRLs TRLs-induced migration after pre-incubating cells with the MMP2 inhibitor OA-Hy (5 µM for 2 hours) and Rac1 inhibitor NSC23766 (100 µmol/L, for 2 hours). (C) Effects of postprandial-TRLs induced migration after hCASMCs transfection with dominant negative Rac1 (Rac1N17) and constitutively active mutant of Rac1 protein (Rac1Q61L). (D) Postprandial-TRLs-induced migration after pre-incubating cells with the RhoA inhibitor Y27632 (10 µmol/L, 2 hours) (D) Rac1 pulldown assays after postprandial-TRLs-stimulation of cells pre-treated with Y27632 (10 µmol/L, 2 hour). The levels of total Rac1 and active Rac1, after pull-down assay, were analysed by western blotting. Total Rac1 protein served as loading control. Bars indicate the SD as calculated from three different experiments $*P < 0.05$, $** P < 0.005$.

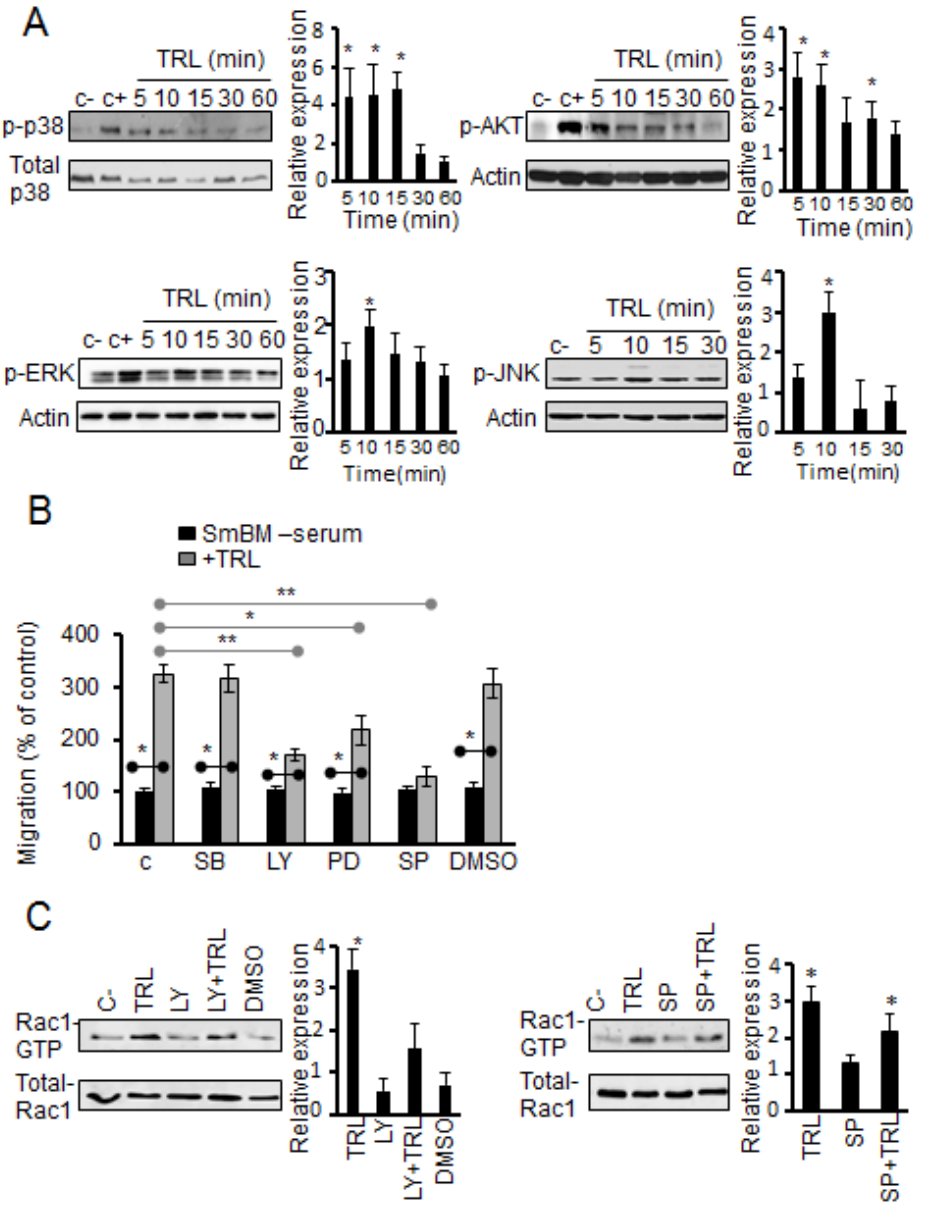

Figure 4. Signalling pathways involved in postprandial TRLs-induced migration. (A) p-38, ERK, JNK and AKT phosphorylation in hCASMCs after postprandial-TRLs-stimulation. Cells were incubated with serum (c+) or with 100 µg triglycerides/mL of postprandial TRLs at different times. The values are expressed as the change with respect to protein expression in controls (SmBM-serum, c-). Autoradiographs are representative of three different experiments. (B) Involvement of p-38,

ERK, JNK, and AKT in postprandial TRLs-induced migration. hCASMCs were pre-incubated during 2 hours with 20 μM PD98059 (an ERK inhibitor), SB202190 (a p38 inhibitor), SP600125 (a JNK inhibitor), and with 10 μM LY294002 (a PI3K inhibitor) for 2 hours before TRLs-BUT-induced migration determination. Values are referred to the controls (SmBM-serum, c). (C) Rac1 pull-down assays after postprandial-TRLs-stimulation of cells pre-treated with LY294002 and SP600125. The levels of total Rac1 and active Rac1, after pull-down assay, were analysed by western blotting. Total Rac1 protein served as loading control. Bars indicate the SD as calculated from three different experiments *P <0.05, ** P < 0.005.

4. DISCUSSION

Postprandial lipemia is characterized by a rise of TRLs after a fat-rich meal. There is evidence that higher levels of postprandial TRLs or their remnants predict progression of CAD (Ginsberg, 2002). The pathogenesis of the relationship between postprandial TRLs and CAD remains unclear, however, several studies have demonstrated that postprandial TRLs can penetrate the artery wall (Nakano et al., 2008; Rapp et al., 1994) and may have a direct atherogenicity by interacting with components of the developing atherosclerotic lesion in the subintimal space. Studies design to assess this question has mainly focused on characterising their interaction with the arterial endothelium and with macrophages. However, the effects of postprandial TRLs on VSMCs, the major cell type in atheroma, are poorly understood. In our current study, we demonstrated that first, postprandial TRLs induce migration of hCASMCs; secondly, postprandial-TRLs-induced migration is fatty-acid-dependent; thirdly, ERK, PI3K, JNK, Rac1, and MMP2 are involved in the signalling pathways of postprandial-TRLs-induced migration; and fourthly, an antagonistic effect between RhoA and Rac1 pathways does exist in hCASMCs to regulate their motility in response to postprandial TRLs.

Cellular migration is a fundamental process linked to diverse pathological states, such as diabetes and its complications, atherosclerosis, inflammation and cancer (Ross, 1999). Lipoproteins such as native LDL, oxLDL and remnant-like lipoprotein particles promote the growth and migration of SMC (Lim & Ryoo, 2011; Sun et al., 2011; Aramaki et al., 2008). The present study shows for

the first time that postprandial TRLs induces *in vitro* 3D hCASMCs migration in a fatty-acid-dependent manner. Postprandial TRLs enriched in SFA (TRLs-BUT) induce significantly higher migration than TRLs enriched in MUFA (TRLs-ROO) and *n*-3 PUFA (TRLs-VEFO). A variety of lipid products, such as free fatty acids, may be generated during TRLs lipolysis that occurs directly within the artery wall lesions. Lipolysis products have a wide contribution to vascular function; they may cause dysfunction of human aortic endothelial cell cytoskeletal organization, and can increase lipid droplet accumulation in the endoplasmic reticulum of monocytes (Schwartz & Reaven, 2012). Postprandial TRLs used in the study have different fatty acid composition; therefore it is possible that the postprandial TRLs lipolysis products may have resulted in different hCASMCs migration. In that sense, it is known that free fatty acids regulate VSMCs proliferation and migration and the underlying signalling pathways. Oleic acid induces migration (Greene et al., 2001), while *n*-3 PUFA have an inhibitory effect (Mizutani et al., 1997). In line with these results, we have previously shown that the mitogenic effects of postprandial TRLs on hCASMCs also depend on their fatty acid composition; TRLs-BUT had the strongest effect on proliferation, while the presence of MUFA (TRLs-ROO) and the presence of moderate amounts of *n*-3 PUFA (TRLs-VEFO) were able to attenuate proliferation (Bermudez et al., 2008). VSMCs migration and proliferation observed during restenosis and atherosclerosis involve the degradation of the ECM and require the secretion of MMPs (Galis & Khatri, 2002). We show here that postprandial TRLs induce MMPs mRNA expression in hCASMCs in a fatty-acid-dependent manner. TRLs-BUT increased MMP1, MMP2 and MMP3 mRNA expression, which is in line with the fact that TRLs-BUT highly stimulated migration. TRLs-ROO and TRLs-VEFO only increased the expression of MMP2; furthermore, this expression was smaller than the one induced after TRLs-BUT stimulation. These differences may be attributed to the presence of different fatty acids in the postprandial TRLs since oleic acid, the main fatty acid in TRLs-ROO and DHA, present in TRLs-VEFO, are associated with reduced MMP2 and MMP9 activity in carcinoma cells (Suzuki et al., 1997). In fact, inhibition of MMP2 by oleic acid is driven by binding of the fatty acid to a hydrophobic region located within the fibronectin type II–like domains of the enzyme

(Emonard et al., 1999). TRLs-ROO and TRLs-VEFO induced similar MMP2 mRNA expression, which agree with the fact that both lipoproteins induced equal rate of migration. In spite of these differences, all the postprandial TRLs stimulated higher MMP2 mRNA expression than oxLDL, which is a well established chemoattractant directing the migration of VSMCs toward the vessel intima during atherosclerosis and neointimal hyperplasia (Ishigaki et al., 2009). MMP2 is a major MMP derived from VSMCs that degrades various ECM proteins leading to vascular remodelling via local SMC migration and proliferation (Pauly et al., 1994). In fact, postprandial TRLs prevented cell migration in MMP2 inhibitor-treated cells, demonstrating that postprandial TRLs-induced migration is highly regulated by MMP2.

It is known that Rho proteins (RhoA, Rac1, and Cdc42) play major roles in regulating cell function through their ability to coordinate the actin cytoskeleton. The precise temporal and spatial coordination of Rho proteins is an important determinant for a large range of cellular activities and cell phenotypes. In the present study, postprandial TRLs treatment caused a transient decrease in RhoA activity, while at the same time it increased the activity of Rac1 in hCASMCs. The process of F-actin reorganization, which plays important roles in cell migration, is under tight regulation of RhoA and Rac1. In fact, studies have shown that activation of one of these proteins can abolish the actin organization governed by the other. We investigated that ability of TRLs-BUT to induce changes in reorganization of F-actin on hCASMCs cells in the presence of Y27632 and NSC23766. Postprandial-TRLs-stimulated hCASMCs adopted an elongated morphology, cell polarization, loss of intact actin stress fibers, and the formation of membrane ruffles (lamellipodia), which are well-defined characteristics of cell motility. Surprisingly, cells exposed to Y27632 exhibited the induction of some actin-positive membrane ruffles. Interestingly, further exposure of these cells to TRLs-BUT increased the extent of membrane ruffles (lamellipodia), suggesting activation of Rac1. In this line, membrane-ruffling formation after ROCK inhibition has also been found in different cell lines including Swiss 3T3 cells and human glioma cell lines (Salhia et al., 2005; Tsuji et al., 2002). We show that Rac1 activation is

involved in elongated morphology and lamellipodia formation in TRLs-stimulated hCASMCs, since cell pre-incubation with NSC23766 retained their original polygonal morphology even after the treatment with TRLs-BUT. The significance of the morphological changes in cells, lies in their implications for cell motility; these data therefore indicate that Rac1 may be essential for TRLs-induced hCASMCs migration and suggest an association between Rac1 activation and RhoA inhibition. Postprandial TRLs, by tilting the balance between these two GTPases in favour of Rac1, may cause the actin organization governed by Rac1 to become dominant. In fact, it has been shown that Rac1 and Rho activities are mutually antagonistic on different modes of tumour cell motility. Active Rac represses Rho activity and *vice versa*, and by changing the activity of one signal pathway, tumour cells may switch between mesenchymal (dependent on Rac activation) and amoeboid (driven by RhoA and its effector proteins ROCKI and ROCKII) types of movement (Sanz-Moreno et al., 2008). Postprandial-TRLs-induced hCASMCs migration *in vitro* requires Rac1 activation, since NSC23766, a Rac1 inhibitor and the constitutively inactive Rac1TN17 expressed on hCASMCs, inhibited the stimulatory effect of postprandial TRLs. Additionally, Rac1 was localized in the cytosol and on membrane ruffles after TRLs-BUT stimulation, which indicates a translocation from the cytosol to the cell periphery that is a requirement for Rac1 activation in motile cells. Additionally, treatment of cells with Y27632 followed by TRLs-BUT led to increased motility of cells, suggesting that a delicate balance between the RhoA and Rac1 pathways does exist in postprandial TRLs-induced hCASMCs. Further analysis of Rac1 activity in Y27632-treated hCASMCs by using a Rac1 pull-down assay demonstrated that activity of Rac1 increased with increasing concentrations of Y27632 treatment in the presence of TRLs-BUT. Taken together, these data may indicate that inhibition of ROCK enhances postprandial-TRLs-induced cell migration by increasing Rac1 activity. An intricate balance between RhoA and Rac1 signalling has been observed in a variety of cell types. Thus, Y27632 treatment of Swiss 3T3 cells can enhance Rac1 activation, leading to increased cell motility. This effect is likely to be mediated by inhibition of a suppressive effect of ROCK on Rac1 activity (Tsuji et al., 2002). Furthermore, it has been demonstrated that RhoA phosphorylation,

which leads to RhoA-Rho kinase pathway inhibition, accelerates VSMCs migration and adhesion via Rac1 activation (Rolli-Derkinderen et al., 2010).

MAPKs are known to participate in cell proliferation, apoptosis, migration, and differentiation. Previous studies have demonstrated that activation of JNK, PI3K, and ERK1/2 are involved in VSMCs migration (Zhan et al., 2003; Seo et al., 2011; Kang et al., 2012). The results of the present study suggest that postprandial-TRLs-induced migration is mediated by PI3K, JNK, and ERK. In addition, Rac1 stimulates the motility of hCASMCs though PI3K activation, which is in agreement with the fact that active PI3K induces actin reorganization in the form of Rac-mediated lamellipodia and focal complexes (Reif et al., 1996). JNK is a critical molecule in SMC proliferation and migration (Wei et al., 2010); furthermore, PI3-kinase-Rac1-JNK pathway promotes migration in several cell lines (Kanazawa et al., 2010). Several studies have also shown that phosphorylation of ERK is involved in cell adhesion and migration, and that pERK is necessary for MMP2 motility in aortic SMCs (Sundberg-Smith et al., 2005; Kenagy et al., 1997).

In conclusion, our data demonstrate that postprandial TRLs induce migration in hCASMCs. The rate of migration highly depends on the fatty acid composition of postprandial TRLs, in that sense TRLs enriched in MUFA and *n*-3 PUFA reversed migration when compared to TRLs enriched in SFA. Finally, Rac1, PI3K, JNK, ERK, and MMP2 pathways mediate postprandial-TRLs-induced migration. These data suggest that the fatty acid composition of dietary fats may determine the initiation and progression of the atherosclerotic plaque.

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DISCUSIÓN GENERAL

Dietary fat promotes lipid accumulation in circulating monocytes

Los monocitos y macrófagos son células que desempeñan un papel clave en todas las etapas del desarrollo de la aterosclerosis (Gerszten & Tager, 2012; Gui et al., 2012). Uno de los fenómenos más tempranos en esta patología es la adhesión de monocitos circulantes al endotelio y su infiltración hacia la íntima de la arteria. Allí, se diferencian a macrófagos y comienzan a acumular lípidos hasta transformarse en células espumosas (Mei et al., 2012). En este sentido, muchos estudios se han centrado en el papel de las LDL oxidadas y su captación a través de receptores residuales, como SR-A y CD36 (Riazy et al., 2011). Sin embargo, se ha prestado menos atención a la acumulación de lípidos en los monocitos circulantes y a las implicaciones en este proceso de las TRL postprandiales. En el **capítulo 3** de esta Tesis Doctoral se demuestra que, durante el periodo postprandial, los monocitos circulantes pueden acumular gotas lipídicas. Esta acumulación es independiente de la concentración plasmática de ácidos grasos libres, pero dependiente de la concentración de TRL postprandiales que contienen apoB48, que regula positivamente la expresión del receptor apoB48R, lo cual aumentaría la captación de TRL postprandiales. Este proceso, además, implica la activación transitoria de los monocitos circulantes.

En principio, conforme al diseño experimental indicado en dicho capítulo, se suministraron dos tipos de desayunos, uno sin grasa y otro enriquecido con mantequilla, a voluntarios sanos. Se procedió al aislamiento de monocitos de sangre periférica en ayunas y en el pico hipertrigliceridémico postprandial; una porción de células se utilizó para teñirlas con hematoxilina y *Oil Red O*. También se aislaron TRL en el pico hipertrigliceridémico postprandial, que se incubaron con monocitos aislados de sangre periférica en ayunas. Además, se extrajeron los lípidos y el ARN de monocitos en ayunas, en el pico hipertrigliceridémico postprandial, e incubados con las TRL postprandiales. Cada voluntario actuó como su propio control.

Como era de esperar, sólo hubo un aumento de la concentración postprandial de triglicéridos tras la ingesta del desayuno enriquecido con grasa, cuyo valor máximo se alcanzó a las 3-4 horas; el aclaramiento de las TRL postprandiales no fue suficiente para que la concentración de triglicéridos retornara hasta los valores basales a las 8 horas; la respuesta hipertrigliceridémica postprandial fue monofásica (un pico postprandial), todo ello en concordancia con los resultados previos (López et al., 2008). Otros estudios han descrito curvas postprandiales bifásicas, e incluso trifásicas (Cohn et al., 1988). Es más frecuente la descripción de curvas monofásicas o bifásicas, aunque la variabilidad entre individuos tiende a ser mayor en personas de avanzada edad y en mujeres. Esta variabilidad también puede depender de la concentración de triglicéridos en ayunas (Xiao et al., 2011) y de la naturaleza de las grasas de la dieta previa a la intervención postprandial (Zampelas et al., 1998). Entre los factores que influyen en la concentración postprandial de triglicéridos están la entrada en el torrente circulatorio de TRL procedentes del intestino, la secreción de VLDL por el hígado, la actividad de la LPL, y la captación de las TRL postprandiales y sus remanentes por procesos dependientes y no-dependientes de receptores. La magnitud del primer pico hipertrigliceridémico postprandial es siempre muy superior a los demás. La presencia de otros picos hipertrigliceridémicos postprandiales se asocian preferentemente con un estímulo de la secreción hepática de VLDL (Cohn et al., 1989). Puesto que las extracciones de sangre se realizaron cada 2 horas (0, 2, 4, 6, y 8 horas) tras la ingesta del desayuno, no podemos descartar la posibilidad de que un segundo pico hipertrigliceridémico postprandial pudiera haber existido entre las horas 6 y 8, e incluso un tercer pico tras la hora 8 postprandial. La variabilidad de la respuesta hipertrigliceridémica postprandial en los voluntarios que participaron en nuestro estudio fue minimizada mediante la selección de varones jóvenes con valores similares de concentración de triglicéridos en ayunas al comienzo de cada intervención y previo periodo de una semana de adaptación a la grasa de estudio.

Con respecto a los ácidos grasos libres, su concentración disminuyó tras el pico hiperinsulinémico postprandial, que precedió al aumento de triglicéridos

tras la ingesta del desayuno enriquecido con grasa. Es bien conocido que esta disminución postprandial de la concentración de ácidos grasos libres en plasma se debe al efecto de la insulina como supresor de la lipolisis de los triglicéridos almacenados en el tejido adiposo (Adiels et al., 2012). La producción y secreción de VLDL también se inhibe mientras persista la hiperinsulinemia (Scherer & Buettner, 2011). De hecho, nuestros resultados indicaron que la normalización de la concentración de insulina fue coincidente con la recuperación de la concentración de ácidos grasos libres aproximadamente a partir de las 4 horas, dicha concentración aumentó y se mantuvo por encima de los valores basales a las 8 horas tras el desayuno enriquecido con grasa.

Por lo tanto, en relación a los lípidos, nuestro estudio fue capaz de distinguir dos fases postprandiales entre 0-4 horas y 4-8 horas. La primera fase se caracterizó por el aumento de triglicéridos (+75%) asociados a lipoproteínas con índice de flotación de Svedberg (Sf) mayor de 400 y particularmente ricas en apoB48 (equivalentes a CM nacientes), pues no hubo cambios en los triglicéridos procedentes de lipoproteínas con Sf entre 400 y 20 (entre las que se encuentran las VLDL), ni en el colesterol total en plasma. En esta fase, la respuesta postprandial de los ácidos grasos libres fue negativa. La segunda fase se caracterizó por la disminución de los triglicéridos y el aumento relativo de la concentración de ácidos grasos libres. Fue interesante observar que los monocitos circulantes aislados en la primera fase postprandial acumularan gotas lipídicas que fueron identificadas como triglicéridos. De hecho, esta acumulación sólo se correlacionó (R +0,99) con la concentración de triglicéridos en plasma y en las lipoproteínas con Sf > 400; por el contrario, la concentración de triglicéridos disminuyó en los monocitos aislados en la segunda fase postprandial. Estudios recientes han descrito, *in vitro*, que los ácidos grasos libres generados tras la lipolisis de VLDL pueden inducir la acumulación de gotas lipídicas en células THP-1 (línea monocítica de leucemia humana) y que esa podría ser la causa de la acumulación de lípidos *in vivo* (den Hartig et al., 2010). Sin embargo, en nuestro estudio no se observaron coincidencias temporales entre la acumulación de triglicéridos en los monocitos circulantes con la concentración de ácidos grasos libres durante el

periodo postprandial, ni tras el desayuno enriquecido con grasa o el desayuno sin grasa (donde también hubo cambios en la concentración de ácidos grasos libres en plasma). Este hecho concuerda con la interpretación de que la disminución de la concentración de ácidos grasos libres en la primera fase postprandial se debe realmente a la acción anti-lipolítica de la insulina y no al posible aclaramiento por captación de ácidos grasos libres por parte de los monocitos circulantes.

La acumulación de triglicéridos evolucionó de forma paralela a la transcripción del gen apoB48R en los monocitos circulantes. Es la primera vez que se detecta la expresión de apoB48R en dichas células. Así, hubo un aumento de la expresión del ARNm de apoB48R que correlacionó (R +0,98) con el aumento de la concentración de triglicéridos en los monocitos circulantes en la primera fase del periodo postprandial; y el descenso de dicha expresión que correlacionó (R -0,97) con la disminución de triglicéridos en la segunda fase del periodo postprandial. No se observó un aumento en la expresión de otros receptores de lipoproteínas, como VLDLR (que apenas se expresó), LRP-1 ó SR-BI (que disminuyeron); por lo que, probablemente, estos receptores no estén implicados en la captación de las TRL postprandiales por parte de estas células. No se descarta la posibilidad de que pudieran participar otros receptores. Además, se realizaron estudios de incubación de monocitos aislados en ayunas con TRL postprandiales aisladas en el pico hipertrigliceridémico postprandial, que demostraron, también *ex vivo*, un aumento de la expresión del ARNm de apoB48R y de la acumulación de triglicéridos. Nuestros resultados sugieren que, durante el periodo postprandial, la afluencia transitoria pero masiva de TRL postprandiales en sangre puede iniciar un programa adaptativo de sobre-expresión de apoB48R en los monocitos circulantes; el cual puede estar destinado a la predisposición de dichos monocitos para el aclaramiento de las TRL postprandiales y la consiguiente acumulación de triglicéridos. Estudios previos han descrito que los monocitos de personas con hipercolesterolemia familiar pueden captar LDL y LDL oxidadas, sobre-expresar receptores implicados en esta captación, y acumular lípidos (ésteres de colesterol) (Mosig et al., 2008).

La mayoría de los estudios de captación de lipoproteínas se han documentado en macrófagos (Woollard & Geissmann, 2010). Todavía desconocemos el significado fisiopatológico de este proceso de aclaramiento de los triglicéridos postprandiales por los monocitos circulantes. A diferencia de otros leucocitos directamente implicados en la respuesta inflamatoria asociada a la acumulación de lípidos, como neutrófilos y eosinófilos (Bozza et al., 2009), los monocitos de personas sanas en ayunas carecen de gotas lipídicas (den Hartig et al., 2010). Sin embargo, se ha descrito que la incubación *ex vivo* de monocitos y TRL postprandiales o emulsiones lipídicas pueden causar una activación de los monocitos incluso mayor que el péptido quimiotáctico sintético fMLP (N-formil-metionil-leucil-fenilalanina) (Alipour et al., 2008b). Nuestros resultados también sugieren que las TRL postprandiales pueden activar (*in vivo*) los monocitos circulantes durante el periodo postprandial, pues observamos un aumento de la expresión del ARNm de ITGAM/CD11b en la primera fase y una disminución en la segunda fase postprandial, pudiendo existir una relación causal entre la acumulación de triglicéridos y la activación de los monocitos.

Con este estudio, se puede concluir que las grasas de la dieta tienen un papel determinante en la acumulación de triglicéridos, en la regulación del gen apoB48R, y en la activación de monocitos circulantes durante el periodo postprandial. Esto sugiere que las TRL postprandiales (preferentemente CM) podrían estar implicadas, mucho antes de lo que se pensaba, en procesos relacionados con la aterosclerosis.

Triglyceride-rich lipoproteins and *APOB48R* in THP-1 cells

Cada vez existen más evidencias respecto a las implicaciones del metabolismo postprandial de las grasas en las ECV (Nakatani et al., 2011). En la actualidad, todavía se considera que el proceso aterosclerótico comienza en la propia pared de los vasos sanguíneos afectados; mediante la adhesión de los monocitos circulantes a la pared endotelial, la retención subendotelial de

macrófagos que se han diferenciado a partir de estos monocitos, y la transformación de los macrófagos en células espumosas tras acumular lípidos (Mei et al., 2012). El mecanismo por el cual las TRL postprandiales regulan la expresión y las propiedades funcionales de apoB48R en monocitos y macrófagos aún se desconoce. Los resultados del capítulo anterior sugieren que, en monocitos circulantes, la captación de las TRL postprandiales a través de apoB48R fomenta la actividad transcripcional precisamente del gen que codifica dicho receptor, como un bucle metabólico cuya función puede ser la de facilitar el aclaramiento de los triglicéridos por parte de los monocitos circulantes tras la ingesta de comidas ricas en grasas. Según estudios previos (Haraguchi et al., 2003), activadores sintéticos de las isoformas α y γ de los PPAR son capaces de suprimir la expresión génica de apoB48R en macrófagos THP-1. También se ha descrito que la pitavastatina (inhibidor de la enzima HMG-CoA reductasa) impide la transformación de macrófagos a células espumosas mediante la supresión de la expresión de apoB48R (Kawakami et al., 2005). Se conoce que los ácidos grasos son ligandos naturales de los PPAR (Plutzky, 2011). Por lo tanto, es posible que las TRL postprandiales puedan regular la transcripción del gen apoB48R mediante la interacción de los ácidos grasos que transportan (y que se liberan extra- o intracelularmente por la acción de lipasas) con los PPAR. En el **capítulo 4** de esta Tesis Doctoral se demuestra, *in vitro*, que PPARα, PPARγ, y RXRα son intermediarios en la regulación por las TRL postprandiales de la transcripción del gen apoB48R, estimulando la expresión de dicho gen en monocitos THP-1 y suprimiéndola en macrófagos diferenciados a partir de estos monocitos. El estado de diferenciación celular también afecta la funcionalidad de apoB48R en respuesta a las TRL postprandiales, y estos efectos se potencian cuando aumenta la disponibilidad intracelular de ácidos grasos libres.

Siguiendo el diseño experimental de este capítulo (idéntico al del capítulo anterior respecto a la intervención), se procedió al aislamiento de TRL de voluntarios sanos en el pico hipertrigliceridémico postprandial tras la ingesta de un desayuno enriquecido con mantequilla. También se aislaron VLDL en ayunas. Las células THP-1 (modelo de monocitos humanos) se obtuvieron de la

ATCC (TIB-202^{TM}); y crecieron en suspensión. Para diferenciar estos monocitos a macrófagos, se incubaron con PMA (forbol-12-miristato-13-acetato) durante 4 días; y crecieron adheridos en el fondo del frasco/placa/pocillo en forma de monocapa. Se utilizaron diferentes procedimientos para activar y bloquear la activación de los PPAR. Antes y después de la incubación con las TRL postprandiales, los monocitos y macrófagos THP-1 se tiñeron con hematoxilina y *Oil Red O*; también se extrajeron los lípidos y el ARN.

Nuestros resultados indicaron que los monocitos THP-1 expresan un número de copias de ARNm de apoB48R similar a los macrófagos THP-1; es decir, la diferenciación de monocitos THP-1 no altera la capacidad transcripcional del gen apoB48R. Sin embargo, las TRL postprandiales aumentaron la expresión del ARNm de apoB48R en los monocitos y disminuyeron dicha expresión en los macrófagos THP-1. Estos efectos fueron dependientes de la concentración de triglicéridos en las TRL postprandiales y del tiempo de incubación, y fueron específicos de los CM en las TRL postprandiales, pues no se modificaron por la presencia de VLDL en el medio de cultivo. Estos resultados de los monocitos THP-1 son concordantes con el estudio *in vivo* del capítulo anterior. Es posible que las TRL postprandiales activen un mecanismo de retroalimentación positiva en los monocitos, como se ha descrito para algunos receptores residuales en macrófagos tras la captación de LDL oxidadas (Nicholson & Hajjar, 2004); y que activen un mecanismo de retroalimentación negativa en los macrófagos, como se ha descrito para LDLR en monocitos tras la captación de LDL nativas (Kong et al., 2006). En relación a los receptores conocidos capaces de captar lipoproteínas, nuestro estudio es el primero en describir una regulación diferente del mismo gen según el estado de diferenciación celular y, además, dependiente de la composición de los ácidos grasos de la dieta; destacando el papel relevante de la proporcionalidad entre el ácido oleico (MUFA) y el ácido palmítico (SFA). El modelo de monocitos y macrófagos THP-1 tuvo un comportamiento parecido, respecto a la regulación de la expresión del gen apoB48R por las TRL postprandiales, a los monocitos y macrófagos primarios (polarizados o no). El nivel de expresión de apoB48R en los macrófagos M2 (anti-inflamatorios) fue

menor que en los M1 (pro-inflamatorios). Estos resultados son concordantes con la capacidad de transformación de los macrófagos M1 en células espumosas (Prieur et al., 2011) y sugieren que apoB48R podría ser clave en la homeostasis de macrófagos M1 y M2 en respuesta a la ingesta de grasas, puesto que los macrófagos M2 son más sensibles a la lipotoxicidad inducida por la acumulación de lípidos (Isa et al., 2011).

Así, hemos sido capaces de demostrar que los cambios en la expresión del gen apoB48R en monocitos y macrófagos THP-1 están vinculados a la acumulación de lípidos, aunque se aprecian aparentes contradicciones que a continuación explicamos.

En primer lugar, los macrófagos acumularon mayor cantidad de lípidos (4 veces más) que los monocitos THP-1 tras la incubación con las TRL postprandiales, lo cual no es extraño, pues los macrófagos son células más especializadas en la captación de lipoproteínas y acumulación de lípidos (McLaren et al., 2011; Ley et al., 2011). En monocitos THP-1, el aumento de la expresión del ARNm de apoB48R inducido por las TRL postprandiales fue concomitante con el aumento de la acumulación de ésteres de colesterol, colesterol libre, y triglicéridos (+95%); sin embargo, los macrófagos THP-1 también acumularon lípidos (+85% triglicéridos) tras la incubación con las TRL postprandiales, a pesar de la disminución de la expresión del ARNm de apoB48R. Creemos que apoB48R debe ser particularmente eficiente en macrófagos, teniendo aumentada su capacidad de captar e internalizar las TRL postprandiales respecto al mismo receptor en monocitos. Estudios previos han descrito que la diferenciación de monocitos causa cambios profundos en el fenotipo de estas células, incluyendo la organización de la membrana citoplasmática que modifica el microambiente en el entorno de los receptores localizados en la superficie de membrana, la expresión de receptores residuales que favorece la captación de las LDL oxidadas, y la activación de UPR (la respuesta a la desnaturalización de proteínas) que estimula la síntesis *de novo* de proteínas e incrementa la supervivencia de los macrófagos (Dickhout et al., 2011). Estudios recientes también han descrito que si bien los niveles de

expresión del ARNm de algunos receptores de lípidos, como FF2/ALX (receptor de la lipoxina A4), son similares en monocitos sin diferenciar y diferenciados, la expresión y las propiedades funcionales de este receptor en dichas células puede ser distinta (Waechter et al., 2012). Hubiera sido interesante contrastar los resultados de expresión génica con los de expresión proteica en los monocitos y macrófagos THP-1, pero el anticuerpo anti-apoB48R humano no está comercializado.

La acumulación de lípidos inducida por las TRL postprandiales en los macrófagos THP-1 disminuyó cuando se silenció la expresión del gen apoB48R mediante técnicas de ARN interferente, indicativo del papel clave de este receptor en la captación de las TRL postprandiales por parte de estas células. Sin embargo, el silenciamiento del gen apoB48R en los monocitos THP-1 y la posterior incubación con las TRL postprandiales determinaron un aumento, inesperado, de la acumulación de lípidos; creemos que ello puede deberse a diferentes motivos: 1) biosíntesis endógena que compensaría la deficiencia de la captación a través de apoB48R y que mantendría las necesidades intracelulares de ácidos grasos libres (Ayala-Sumuano et al., 2011), cuyo exceso se canalizaría hacia la acumulación intracelular de triglicéridos (Listenberger et al., 2003); 2) captación de las TRL postprandiales y/o de los productos de su hidrólisis mediante mecanismos dependientes de receptores distintos de apoB48R y/o no-dependientes de receptores (Kruth, 2011).

En estudios previos, se ha descrito que agonistas de PPARα (Wy14643) y PPARγ (15-d-PGJ_2) disminuyen la expresión de apoB48R y la captación de lípidos en macrófagos (Marx et al., 2004). Por ello, realizamos distintas experiencias co-incubando los monocitos y macrófagos THP-1 con las TRL postprandiales y con agonistas de PPARα (Wy14643) y PPARγ (15-d-PGJ_2), antagonistas de PPARα (MK866) y PPARγ (GW9662), y ARN interferente de PPARα y PPARγ. Los agonistas potenciaron los efectos de las TRL postprandiales en las células, mientras que los antagonistas o el silenciamiento de PPARα y PPARγ impidieron dichos efectos. Por lo tanto, nuestros resultados confirmaron los hallazgos previos y demostraron que ambos receptores

nucleares están implicados en la regulación transcripcional del gen apoB48R en respuesta a las TRL postprandiales en monocitos y macrófagos THP-1. Durante su activación, los PPAR heterodimerizan con RXRα y este complejo interactúa con los elementos de respuesta ubicados en la regiones promotoras de los genes diana (Kota et al., 2005). Utilizamos tres métodos [co-incubación de las células con las TRL postprandiales y un agonista de RXRα (ácido 9 *cis*-retinoico), un inhibidor de RXRα (As_2O_3), y ARN interferente de RXRα] que también demostraron la participación de RXRα en la regulación transcripcional del gen apoB48R en respuesta a las TRL postprandiales en monocitos y macrófagos THP-1.

La estructura molecular de los PPAR se caracteriza por diferentes dominios: de unión a ADN, en su extremo amino-terminal; de unión a ligando, en su extremo carboxilo; y de unión a co-activadores o co-represores que facilitan o inhiben la transcripción génica (Oyekan, 2011; Ricote et al., 2007; Marx et al., 2004). En ausencia de ligando, los PPAR, formando heterodímeros con RXRα, están unidos al ADN y pueden estar formando complejos con proteínas co-represoras; la unión al ligando produce la disociación del co-represor y atrae proteínas co-activadoras que promueven la síntesis del ARNm. Los agonistas inducen un cambio de conformación en los PPAR que les permite la unión de proteínas co-activadoras, las cuales tienen una actividad intrínseca de acetilación de histonas que modifica la estructura de la cromatina, debilitando la asociación de estas proteínas con el ADN y en consecuencia quedan expuestos residuos específicos que promueven la transcripción génica; en contraste, la unión de antagonistas a PPAR induce un cambio de conformación que facilita la unión de proteínas co-represoras, las cuales atraen desacetilasas de histonas que fortalecen la asociación de histonas al ADN y reprimen la transcripción génica (Montagner et al., 2011). Esta complejidad reguladora de genes entre monocitos y macrófagos también se asocia con interferencias en las regiones no-promotoras de los genes (Waechter et al., 2012). Así, se han descrito programas matemáticos cuyos algoritmos permiten predecir un número considerable de micro ARN (miRNA) con capacidad de regular regiones no-promotoras del gen apoB48R (Betel et

al., 2010). Los miRNA suelen mostrar complementariedad imperfecta con la región 3'-UTR y generalmente inhiben la traducción del ARNm (Pritchard et al., 2012). Por lo tanto, el efecto dual en la regulación transcripcional del gen apoB48R (de estimulación en monocitos y de inhibición en macrófagos THP-1) en respuesta a las TRL postprandiales puede relacionarse, de forma combinada o no, con la presencia de proteínas co-reguladoras (activadoras o represoras) y miRNA cuya naturaleza y/o afinidad dependerán del estado de diferenciación celular y probablemente sensibles a la presencia intracelular de los ácidos oleico (MUFA) y palmítico (SFA).

Puesto que los ácidos grasos son ligandos naturales de los PPAR, resulta tentativo proponer que las TRL postprandiales pueden ser captadas (e internalizadas) mediante apoB48R e inducir un aumento de la concentración intracelular de ácidos grasos libres en monocitos y macrófagos THP-1; estos ácidos grasos libres interaccionarían directamente con los PPAR y se modularía la expresión del gen apoB48R. En concordancia con esta idea, nuestros resultados demostraron que el bloqueo de enzimas implicadas en la utilización intracelular de ácidos grasos libres [con inhibidores de las enzimas acil-CoA sintetasa (triacsina D) y acil-CoA colesterol-aciltransferasa (58-035)] potencia los efectos de las TRL postprandiales en los monocitos y macrófagos THP-1. Otros estudios han relacionado la importancia de los ácidos grasos libres en la regulación de los PPAR en el hígado (Chakravarthy et al., 2009) e hipotálamo (Chakravarthy et al., 2007) de animales de experimentación. Nuestro estudio es el primero en describir a los ácidos grasos de la dieta, que se incorporan en las TRL postprandiales, como ligandos de PPAR y reguladores de la expresión del gen apoB48R y de la acumulación de lípidos en monocitos y macrófagos humanos.

Postprandial TRL modulate the expression of ADRP and TIP47 in human THP-1 and mice bone marrow macrophages

La acumulación intracelular de lípidos constituye un proceso esencial en la biología de las células del tejido adiposo, especializadas en mantener las reservas en forma de triglicéridos y satisfacer las necesidades energéticas de los tejidos periféricos (preferentemente el músculo esquelético) mediante la movilización de ácidos grasos libres al torrente circulatorio tras la lipolisis dependiente de hormona (Lass et al., 2011). La acumulación excesiva de lípidos, no solo en el tejido adiposo sino también en tejidos no adiposos, se relaciona con diferentes condiciones patológicas, incluyendo la resistencia a la insulina, la diabetes tipo 2, las ECV, y el hígado graso o esteatohepatitis no alcohólica (van Herpen et al., 2008). En los adipocitos, la biogénesis, estabilidad, y degradación de las gotas lipídicas requieren de la participación de proteínas especializadas de la familia PAT que forman parte de la propia estructura de dichas gotas lipídicas. Entre estas proteínas, las de mayor interés son PLIN, ADRP, y TIP47 (Ducharme & Bickel, 2008). En otros tipos celulares, como en macrófagos, ADRP y TIP47 son las principales proteínas PAT implicadas en la transformación de estos macrófagos en células espumosas (Buers et al., 2011). En la actualidad, se desconoce si la captación de las TRL postprandiales, y el tipo de ácido graso que transportan, pueden afectar la expresión de ADRP y TIP47 en macrófagos y, por lo tanto, la expansión de las células espumosas que tiene lugar durante la progresión y las etapas más avanzadas de las lesiones ateroscleróticas. En el **capítulo 5** de esta Tesis Doctoral se demuestra, *in vitro*, que las TRL postprandiales pueden estimular la expresión génica y proteica de ADRP y modificar la localización espacial de TIP47 en las gotas lipídicas en macrófagos THP-1. También se demuestra que la incubación de macrófagos de médula ósea de ratones deficientes en apoE con las TRL postprandiales estimula la expresión proteica de ADRP y TIP47. Estos efectos están preferentemente asociados a los SFA de las TRL postprandiales.

El diseño experimental de este capítulo incluyó un estudio en humanos y otro en animales de experimentación. Se suministraron tres tipos de desayuno

(enriquecido con mantequilla, aceite de oliva refinado, y aceite de oliva refinado suplementado con aceite de pescado azul) a voluntarios sanos. Las técnicas de intervención y la obtención de las TRL postprandiales en el pico hipertrigliceridémico postprandial fueron idénticas a las de capítulos anteriores. Igualmente, las células THP-1 se obtuvieron de la ATCC (TIB-202^{TM}) y se diferenciaron a macrófagos con PMA. Antes y después de la incubación con las TRL postprandiales, los macrófagos THP-1 se visualizaron mediante tinción con BODIPY e inmunofluorescencia, y microscopía electrónica; también se extrajeron las proteínas y el ARN. En el modelo experimental animal, los macrófagos se obtuvieron mediante diferenciación de células de la médula ósea de ratones apoE-/- (The Jackson Laboratory) con medio de células L929 como fuente de M-CSF. Estos mismos ratones fueron previamente alimentados con cuatro tipos de dieta (la habitual de ratones, y enriquecida con mantequilla, aceite de oliva refinado, y aceite de oliva refinado suplementado con aceite de pescado azul). Antes y después de la incubación de los macrófagos de la médula ósea con las TRL postprandiales, se extrajeron las proteínas y el ARN.

Nuestros resultados demostraron que las TRL postprandiales inducen la transformación de macrófagos THP-1 en células espumosas y el aumento de la expresión proteica de ADRP. Este efecto fue dependiente del tipo de ácido graso en las TRL postprandiales, coincidiendo una mayor acumulación de lípidos (colesterol total y sobretodo triglicéridos) y de expresión génica y proteica de ADRP con las TRL postprandiales más saturadas. Tanto ADRP como TIP47 co-localizaron en la superficie de las gotas lipídicas tras la incubación de las células con TRL postprandiales aisladas tras los desayunos enriquecidos con mantequilla y aceite de oliva, aunque TIP47 preferentemente permaneció en el citoplasma tras la incubación con las TRL postprandiales aisladas tras la ingesta del desayuno enriquecido con aceite de oliva y aceite de pescado. El patrón temporal de expresión génica de TIP47 precedió al de ADRP, concordante con el papel preponderante de TIP47 en la biogénesis (Grasselli et al., 2010) y de ADRP en la estabilización (Lee et al., 2009) de las gotas lipídicas. En hepatocitos y macrófagos, estudios previos han descrito que la región promotora del gen

que codifica ADRP contiene PPRE (elementos de respuesta a los PPAR) (Chawla et al., 2003; Targett-Adams et al., 2005); por lo tanto, es posible que las TRL postprandiales puedan regular la transcripción del gen ADRP mediante la interacción de los ácidos grasos que transportan (y que se liberan extra- o intracelularmente por la acción de lipasas) con los PPAR. En células hepáticas NMuLi, este mecanismo requiere la formación de un complejo entre PPRE y elementos de respuesta a ETS-1/AP-1 en sitios adyacentes del promotor de ADRP, con el que pueden interaccionar los ácidos grasos e incluso co-regular la estabilidad del ARNm de ADRP (Fan et al., 2009). De hecho, la β-oxidación de los ácidos grasos reduce la acumulación intracelular de triglicéridos y la expresión de ADRP asociadas a la captación de VLDL en macrófagos THP-1 (Larigauderie et al., 2006). En estas células, la expresión de ADRP también se estimula por LDL oxidadas (Li et al., 2010; Liu et al., 2010), LDL acetiladas y agonistas sintéticos de PPARγ (Chen et al., 2010) y PPARβ/δ (Chawla et al., 2003). ADRP incluso modula positivamente la expresión y secreción de TNFα, MCP-1, e IL-6 (Chen et al., 2010). Otros autores han señalado que LPS (lipopolisacáridos), mediante TLR (*Toll-like receptors*), estimula la acumulación de lípidos y la expresión de ADRP, pero no de TIP47, en macrófagos RAW 264.7 y J774 (Feingold et al., 2010). Recientemente, se ha descrito que el aumento de la expresión de FABP4/aP2 (proteína 4 de unión a los ácidos grasos) correlaciona con la sobre-expresión de ADRP y de otros marcadores pro-inflamatorios en macrófagos de placas inestables humanas (Agardh et al., 2011). Todo ello en concordancia con la idea de que la naturaleza de los ácidos grasos de la dieta, a través de las TRL postprandiales, puede influir en la formación de células espumosas derivadas de macrófagos mediante la regulación de TIP47 y ADRP.

Como modelo experimental de aterosclerosis inducida por la dieta, utilizamos ratones apoE-/- que fueron alimentados con diferentes dietas enriquecidas con grasas durante 4 semanas. Nuestros resultados demostraron una acumulación de triglicéridos en los macrófagos de la médula ósea de estos animales, pero sólo tras las dietas enriquecidas con mantequilla y aceite de oliva. Todas las dietas enriquecidas con grasas (20% de grasa), respecto a la

dieta habitual (*chow*) (4% de grasa), aumentaron la expresión proteica de ADRP en los macrófagos de médula ósea, aunque con mayor intensidad tras las dietas enriquecidas con mantequilla y aceite de oliva que con aceite de oliva suplementado con aceite de pescado. A diferencia de los estudios *in vitro* con las células THP-1, la expresión proteica de TIP47 aumentó en los macrófagos de médula ósea, pero sólo en aquellos animales alimentados con las dietas enriquecidas con mantequilla y aceite de oliva. Como en humanos, animales alimentados con dietas enriquecidas con grasas desarrollan acúmulos de lípidos en tejidos no adiposos (Unger & Scherer, 2010) y sobre-expresan ADRP en placas ateroscleróticas (Paul et al., 2008). De hecho, las células de médula ósea de ratón expresan marcadores adipogénicos, como ADRP y PPARγ (Karaöz et al., 2011). Sin embargo, no existen estudios previos que comparen estos efectos dependiendo del tipo de ácido graso en la dieta, ni siquiera se había descrito la presencia de TIP47 en macrófagos de médula ósea. Se conoce que las hipelipemias experimentales inducen el aumento de la expresión de ADRP y TIP47 en la esteatosis hepática, reduciéndose la acumulación de triglicéridos en el hígado de los animales tratados con ADRP y TIP47 ASO (oligonucleótidos antisentido de ADRP y TIP47) (Carr et al., 2012). En relación a la esteatosis hepática, estudios previos han descrito que dietas enriquecidas con mantequilla, respecto a dietas enriquecidas con aceite de cártamo (~70% de ácido linoleico), aumentan la acumulación de triglicéridos y la expresión de ADRP y PPARγ en el hígado de los animales de experimentación (Yamazaki et al., 2011); en este mismo estudio, la inyección intravenosa de un adenovirus portador de ARN interferente de PPARγ causó la reducción de la expresión de ADRP, indicativo de la posible regulación selectiva de ADRP por los ácidos grasos de la dieta (SFA > PUFA de la familia ω-6) mediante su interacción con, al menos, PPARγ (Yamazaki et al., 2011). En músculo esquelético, dietas ricas en MUFA y PUFA (mezcla de aceites de oliva y cártamo) producen mayor sobre-expresión de ADRP que dietas ricas en SFA (aceite de palma), aunque este hecho no desfavorece la mejora de la sensibilidad del músculo esquelético a la insulina en los animales de experimentación alimentados con la dieta enriquecida con la mezcla de aceites de oliva y cártamo (de Wilde et al., 2010).

Los macrófagos de médula ósea obtenidos de los ratones apoE-/- alimentados con las dietas enriquecidas con mantequilla, aceite de oliva, y aceite de oliva suplementado con aceite de pescado, se incubaron con las TRL postprandiales aisladas de los voluntarios sanos tras la ingesta del desayuno enriquecido con mantequilla, aceite de oliva, y aceite de oliva suplementado con aceite de pescado, respectivamente. Partiendo de la base que son células de roedores incubadas con lipoproteínas humanas, nuestros resultados demostraron que todas las TRL postprandiales, independientemente de su composición en ácidos grasos, inducen una acumulación de triglicéridos y expresión proteica de ADRP y TIP47 en los macrófagos de médula ósea. Sin embargo, la estimulación de la expresión génica de ADRP fue mayor con las TRL postprandiales ricas en SFA (mantequilla) que con las TRL postprandiales ricas en MUFA (aceite de oliva) o PUFA (aceite de oliva suplementado con aceite de pescado); mientras que la expresión génica de TIP47 fue inhibida con estas últimas lipoproteínas (las menos saturadas). Cuando los macrófagos de médula ósea obtenidos de los ratones apoE-/- alimentados con la dieta habitual se incubaron con las TRL postprandiales aisladas de los voluntarios sanos tras la ingesta del desayuno enriquecido con mantequilla, aceite de oliva, y aceite de oliva suplementado con aceite de pescado, se observó un patrón de expresión génica y proteica de ADRP y TIP47 caracterizado por el aumento de la expresión del ARNm de ADRP con las TRL ricas en SFA y la inhibición de la expresión del ARNm de TIP47 con las TRL ricas en MUFA o PUFA; y por el aumento de la expresión proteica de ADRP y TIP47, sin diferencias entre las distintas TRL postprandiales. La acumulación de triglicéridos en los macrófagos de médula ósea de los ratones tras la dieta habitual fue menor que la de los macrófagos de médula ósea de los ratones tras la dieta enriquecida con las grasas, y ello fue determinante del mayor aumento proporcional de la expresión proteica de TIP47 en los primeros y de ADRP en los segundos inducido por las TRL postprandiales, que concuerda con un posible inicio de la biogénesis (Grasselli et al., 2010) y de la estabilización (Lee et al., 2009) de las gotas lipídicas, respectivamente. Se puede concluir que las TRL postprandiales pueden promover la transformación de macrófagos de médula ósea en células espumosas; y que el tipo de ácido graso en las TRL

postprandiales puede modular la actividad transcripcional de ADRP y TIP47, aunque sin entidad suficiente como para inducir diferencias en la acumulación de lípidos y el contenido proteico de ADRP y TIP47. Es probable que la pertenencia de las células y las lipoproteínas a distintas especies pueda tener influencia en los resultados de nuestro estudio, relacionada con la eficiencia en la captación de las TRL postprandiales y/o los productos de su lipolisis por los macrófagos de la médula ósea, e incluso con las rutas intracelulares de regulación de ADRP y TIP47, que en su conjunto minimizarían las posibles diferencias entre los efectos inducidos por las TRL postprandiales de diferente composición en ácidos grasos. Otro aspecto importante, y que no descartamos, es que los ácidos grasos puedan sensibilizar de forma selectiva la estabilidad del ARNm de ADRP y TIP47 en la médula ósea (Fan et al., 2009).

Smooth muscle cell migration is postprandially induced via Rac1 activation and MMP2

En el desarrollo de la lesión aterosclerótica, los monocitos/macrófagos, las células endoteliales, plaquetas, los linfocitos T, y las SMC liberan diversas sustancias que estimulan la proliferación y migración de estas SMC desde la media hacia el lumen arterial; este proceso se facilita tras el aumento de la actividad proteolítica inducida por las MMP que se liberan por los monocitos/macrófagos y las propias SMC (Allahverdian et al., 2012). La progresiva acumulación de SMC contribuye a la formación de la capa neoíntima y al engrosamiento de la pared vascular. A diferencia de las LDL, las TRL postprandiales pueden entrar irreversiblemente en el espacio subendotelial (Nakano et al., 2008); donde interaccionan con las células de la pared vascular, incluidas las SMC (Fujioka et al., 2009; Schwartz & Reaven, 2012). Así, las TRL postprandiales estimulan la proliferación de las SMC (Pacheco et al., 2003; Bermudez et al., 2008). Sin embargo, se desconoce si las TRL postprandiales, y el tipo de ácido graso que transportan, desempeñan algún papel en la migración de las SMC. En el **capítulo 6** de esta Tesis Doctoral se demuestra, *in vitro*, que las TRL postprandiales pueden estimular la

migración de las SMC de arteria coronaria humana (hCASMCs). Estos efectos están preferentemente asociados a los SFA de las TRL postprandiales y al aumento de la expresión génica de diferentes MMP y de la actividad Rac1 en las hCASMCs.

El diseño experimental de este capítulo incluyó el suministro de tres tipos de desayuno (enriquecido con mantequilla, aceite de oliva refinado, y aceite de oliva refinado suplementado con aceite de pescado azul) a voluntarios sanos. Este procedimiento fue idéntico al de los capítulos anteriores. hCASMCs de donantes humanos y obtenidas de Clonetics^{TM} (cc-2583) se incubaron con TRL aisladas en el pico hipertrigliceridémico postprandial. Se utilizaron técnicas para establecer el potencial invasor (migratorio) 3D de las células incubadas con las TRL postprandiales. También se utilizaron inhibidores para bloquear diferentes rutas de señalización relacionadas con las familias de GTPasas Rho, MAPK (quinasas activadas por proliferadores peroxisomales), y MMP. Antes y después de la incubación con las TRL postprandiales, las células se visualizaron mediante inmunofluorescencia; también se extrajeron las proteínas y el ARN.

Nuestros resultados demostraron que las TRL postprandiales inducen migración en las hCASMCs. Este efecto fue dependiente de la concentración de triglicéridos y del tipo de ácido graso en las TRL postprandiales, cuanto mayor concentración de triglicéridos y menor saturación de los ácidos grasos en dichos triglicéridos, mayor potencial invasor de las hCASMCs; es decir, a una misma concentración de triglicéridos, las TRL postprandiales aisladas tras la ingesta del desayuno enriquecido con mantequilla tuvieron un mayor impacto sobre la migración que las TRL postprandiales tras la ingesta del desayuno enriquecido con aceite de oliva o aceite de oliva suplementado con aceite de pescado. Estudios previos han descrito que las LDL (Lim & Ryoo, 2011), LDL oxidadas (Sun et al., 2011), y VLDL remanentes (Aramaki et al., 2008) promueven la migración (y proliferación) de las SMC mediante mecanismos relacionados con el estrés oxidativo, probablemente a través del factor de transcripción ETS-1 (de la familia *E-twenty six*) (Mohseny et al., 2012; Ni et al., 2007). Aunque en nuestro estudio no hemos determinado el nivel de expresión

de ETS-1, no descartamos que este factor de transcripción pueda ser un punto de regulación común de la migración celular inducida por las lipoproteínas, incluidas las TRL postprandiales, pues los ácidos grasos que transportan (y que se liberan extra- o intracelularmente por la acción de lipasas) pueden regular la actividad de PPARγ (Plutzky, 2011) y se ha descrito que PPARγ puede regular la expresión y funcionalidad de ETS-1 en las SMC (Meredith et al., 2009). Disponemos de evidencias según las cuales las TRL postprandiales aisladas tras el desayuno enriquecido con mantequilla, respecto a las TRL postprandiales aisladas tras desayunos enriquecidos con aceite de oliva con y sin suplemento de aceite de pescado, estimulan la expresión de genes regulados por ETS-1 en hCASMCs (Bermúdez et al., 2008). Parece muy importante el papel de PPARγ en la plasticidad del fenotipo de las SMC; de hecho, estudios recientes han descrito el efecto inhibidor de PPARγ sobre la migración de las SMC y la hiperplasia de la íntima en modelos de experimentación animal (Zhang et al., 2011). Puesto que el ácido oleico es uno de los ácidos grasos libres mayoritarios en plasma (Fusconi et al., 2003; Anderson et al., 2009), también se han publicado diferentes estudios *in vitro* que, en general, describen un efecto pro-migratorio del ácido oleico en las SMC, mediante rutas de señalización asociadas al sistema renina-angiotensina-aldosterona (Greene et al., 2001), PGC-1α (coactivador-1 alfa de PPARγ) (Zhang et al., 2007), y S-X-PR (receptores de esfingosina-X-fosfato acoplados a proteínas G) (Duru et al., 2012a). Por el contrario, productos metabólicos del ácido oleico (como su derivado nitrado, que también es un ligando de PPARγ) inhiben la migración de las SMC inducida por PDGF y la hiperplasia de la íntima inducida por angioplastia, mediante el aumento de la expresión de HO-1 (hemo oxigenasa-1) (Cole et al., 2009). Asimismo, estudios recientes indican que los PUFA de cadena larga de la familia ω-3 reducen *in vitro* la migración de las SMC, mediante la activación de Notch (Delbosc et al., 2008), la producción de resolvinas (Ho et al., 2010), y la disminución de uPAR (receptor activador del plasminógeno tipo-uroquinasa) (Whyte et al., 2012). Estos estudios explican, en parte, los resultados obtenidos en el nuestro; aunque es difícil establecer paralelismos entre ellos, al existir importantes diferencias en los diseños

experimentales, en los modelos de SMC, y sobretodo en las fuentes de ácidos grasos, que en nuestro caso fueron las TRL postprandiales.

La proliferación y migración de las SMC requieren la secreción de MMP por las propias SMC, que constitutivamente expresan y secretan MMP2, siendo MMP9 inducible por NFκB (Lacolley et al., 2012). Por ello, estudiamos la expresión de los genes que codifican MMP2 y MMP9 (gelatinasas), y otras MMP (MMP1, colagenasa; y MMP3, estromalisina) en las hCASMCs incubadas con las TRL postprandiales. Nuestros resultados demostraron que las TRL postprandiales aisladas tras la ingesta del desayuno enriquecido con mantequilla estimulan la expresión del ARNm de MMP2, MMP1, y MMP3; y que las TRL postprandiales aisladas tras la ingesta del desayuno enriquecido con aceite de oliva, suplementado o no con aceite de pescado, sólo estimulan la expresión del ARNm de MMP2 en las hCASMCs. Comparando las grasas, esta sobre-expresión de MMP2 fue mayor con las TRL ricas en SFA (mantequilla) que con las TRL ricas en MUFA (aceite de oliva), con ó sin PUFA de cadena larga de la familia ω-3 (aceite de pescado). Las TRL postprandiales tuvieron un menor impacto sobre MMP1 y MMP3, pero un mayor impacto sobre MMP2, que las LDL oxidadas; aunque estas últimas también estimularon la expresión del ARNm de MMP9. Nuestro estudio es el primero en analizar el efecto de los SFA, MUFA, y PUFA (en las TRL postprandiales) en la expresión de MMP en SMC. Tan sólo dos estudios previos han descrito que el ácido docosahexaenoico (22:6ω-3), a través de su interacción con PPARα/PPARγ, puede prevenir la actividad de factores de transcripción (NFκB, AP-1, y STAT1) (Blaschke et al., 2006) y la expresión y secreción de componentes del sistema fibrinolítico (t-PA y PAI-1) (Delbosc et al., 2008) implicados en la transcripción y actividad de las MMP. Los ácidos eicosapentaenoico (20:5ω-3) y docosahexaenoico también inhiben la producción de MMP9 en leucocitos circulantes de personas sanas y en células Jurkat (Shinto et al., 2011). En otros estudios donde comparan dietas enriquecidas con mantequilla y aceite de oliva, se ha descrito que el aceite de oliva puede inducir una menor expresión de NFκB y MMP9 en leucocitos circulantes (Camargo et al., 2011) y una disminución de la concentración plasmática de t-PA y PAI-1 (Pacheco et al.,

2008) en el periodo postprandial. Aunque orientativos, estos estudios concuerdan con el nuestro, en el que las TRL postprandiales representan un nuevo factor regulador de la producción de MMP en las hCASMCs, observándose efectos dependientes del tipo de grasa de la dieta y diferentes a los efectos inducidos por las LDL oxidadas.

En la migración de las SMC es crítica la activación de proteínas monoméricas de la familia de GTPasas Rho; entre las cuales, RhoA (Lacolley et al., 2012), Cdc42 (Lengfeld et al., 2012), y Rac1 (Cheng et al., 2012) han sido las más estudiadas por su papel relevante en la aterosclerosis (Rolfe et al., 2005; Rathinam et al., 2011). La activación coordinada de estas proteínas permite a las SMC desarrollar proyecciones de la membrana citoplasmática (lamelipodios), remodelar los microtúbulos (fibras de estrés) y microfilamentos (F-actina), y generar el movimiento hacia el estímulo quimiotáctico (Oelz et al., 2008). Nuestros resultados demostraron que las TRL postprandiales estimulan (temporal y espacialmente) la activación de Rac1, mientras inhiben la activación de RhoA y Cdc42 en las hCASMCs; las cuales adoptaron una morfología típica de células en elongación y polarizadas, con menos fibras de estrés, y con numerosas protrusiones de F-actina en forma de lamelipodios. Rac1 se acumuló en la fracción de membranas y en los límites de dichos lamelipodios. Estos experimentos sólo se realizaron con las TRL aisladas tras la ingesta del desayuno enriquecido con mantequilla, por lo que no fue posible distinguir los posibles efectos entre las diferentes grasas. La co-incubación de las hCASMCs con las TRL postprandiales y el inhibidor Y-27632, específico de p160ROCK (proteína efectora de RhoA), aumentó la activación de Rac1, la formación de lamelipodios, y la migración. Por el contrario, la co-incubación de las hCASMCs con las TRL postprandiales y el inhibidor NSC23766, específico de la interacción de Rac1 con GEF (factor de intercambio de nucleótidos de guanina), disminuyó la formación de lamelipodios y la migración. Estos resultados implican efectos mutuamente antagónicos entre Rac1 y RhoA, y sugieren que Rac1 puede ejercer como un interruptor molecular en la regulación de la reorganización estructural del citoesqueleto de las hCASMCs y su migración en respuesta a las TRL postprandiales. De hecho, la transfección

de las células con un dominante negativo de Rac1 (Rac1-T17N) inhibió la migración, mientras que la transfección con el mutante constitutivamente activo de Rac1 (Rac1-Q61L) estimuló la migración inducida por las TRL postprandiales. Estudios recientes han incidido en la importancia de Rac1 en la migración de las SMC, al describir que la deficiencia del gen que codifica Rac1 específicamente en las SMC (Li et al., 2010) o la inhibición de Rac1 mediante la administración oral de 8-OHdG (8-hidroxi-2-deoxiguanosina) (Young et al., 2012) impide la formación de la neoíntima en la aorta de animales de experimentación. Comportamientos parecidos a los observados en nuestro estudio, de activación relativa de Rac1 cuando disminuye la actividad de RhoA, han sido descritos en células tumorales invasivas (Sanz-Moreno et al., 2008) y en neuronas (Picard et al., 2009). No existen estudios previos sobre la influencia de los ácidos grasos en la regulación de las GTPasas Rho en las SMC, aunque se sabe que estas proteínas pueden sufrir modificaciones postranslacionales que son esenciales para su localización celular y actividad biológica, mediante la adición de isoprenoides (prenilación) y ácidos grasos (S-palmitoilación) en el tetrapéptido (en el extremo carboxilo) *CAAX* (donde *C* representa a un residuo de cisteína; *A* es un aminoácido alifático; y *X* es cualquier aminoácido) (Mitin et al., 2012). En células β pancreáticas (Syed et al., 2010) y hepatocitos (Sharma et al., 2012), el ácido palmítico induce la activación de Rac1. En cardiomiocitos, la translocación de LPL a la superficie de membrana y el aumento de su actividad (promoviendo la captación de ácidos grasos) se correlacionan con la activación de RhoA, la fosforilación de su efector ROCK, y la formación de fibras de estrés (Ganguly et al., 2011); efectos similares sobre RhoA y ROCK han sido observados por el ácido araquidónico (20:4ω-6) en células de piel (Garcia et al., 2009) y por el ácido linoleico (18:2ω-6) en células endoteliales (Jung et al., 2012). Otros estudios, incluso han descrito que las apolipoproteínas pueden modular la actividad de las GTPasas Rho y la migración de las SMC: apo(a) estimula la migración dependiente de TGFβ (O'Neil et al., 2004); apoD promueve la activación de Rac1 y potencia los efectos pro-migratorios de PDGF (Leung et al., 2004); y apoE inhibe la migración mediante la inducción de AMPc (Hui & Basford 2005).

Las rutas de señalización de las MAPK son determinantes en la migración de las SMC (Seo et al., 2011; Kang et al., 2012). Por ello, estudiamos si las TRL postprandiales (aisladas tras la ingesta del desayuno enriquecido con mantequilla) fueron capaces de promover la fosforilación (activación) de $p38^{MAPK}$, AKT, ERK1/2, JNK, y la migración en las hCASMCs. Nuestros resultados demostraron que las TRL postprandiales inducen la fosforilación de dichas MAPK y la migración se reduce cuando se co-incuban las células con las TRL postprandiales y con inhibidores de ERK1/2 (-33%), PI3K/AKT (-48%), y JNK (-60%). Los inhibidores de PI3K/AKT y JNK también bloquearon la activación de Rac1. Por lo tanto, se puede deducir: 1) que aunque p38, AKT, ERK1/2, y JNK pueden ser activadas en las hCASMCs por las TRL postprandiales, solo AKT, ERK1/2, y JNK están implicadas en la migración inducida por dichas lipoproteínas; 2) que Rac1 representa un efector (y no al contrario) de las rutas de señalización de AKT y JNK inducidas por las TRL postprandiales en las hCASMCs. En estudios previos demostramos que las TRL postprandiales pueden inducir la acumulación intracelular de S-1-P (esfingosina-1-fosfato) y la fosforilación de ERK1/2 en SMC de aorta (Pacheco et al., 2003). Si bien no determinamos S-1-P en las hCASMCs incubadas con las TRL postprandiales, estudios recientes han descrito que el principal mecanismo por el que PDGF induce la migración en las SMC es la producción intracelular de S-1-P (por estimulación de la esfingosina quinasa), iniciando rutas de señalización a través de S-1-PR que conducen a la activación de $p38^{MAPK}$, JNK, y a la extensión de lamelipodios (Duru et al., 2012a; Duru et al., 2012b). PDGF regula la fosforilación de JNK previa activación de Rac1 en SMC de aorta de roedores (Liu et al., 2011), nuestro estudio indica que las TRL postprandiales regulan la activación de Rac1 a través de AKT y JNK en hCASMCs de humanos. La estimulación de receptores AGE (productos finales avanzados de la glucosilación) también conduce a la activación de Rac1, AKT, y a la migración en las SMC (Touré et al., 2012); dicha migración y la expresión de MMP2 se reducen cuando se bloquea la activación de AKT (Chan et al., 2012).

Estos resultados sugieren que las TRL postprandiales podrían estar implicadas en la migración de las células de la musculatura lisa en el seno de la

pared vascular y, por lo tanto, en el inicio y la progresión de las placas ateroscleróticas. En este proceso, por primera vez asociado a la ingesta de comidas ricas en grasas, al menos puede producirse la sobre-expresión de MMP2, la activación de la GTPasa Rac1, y la fosforilación de las MAPK AKT, ERK1/2, y JNK en respuesta a la interacción de las células de la musculatura lisa con las TRL postprandiales.

CONCLUSIONES

1. **Durante el metabolismo postprandial de las grasas en personas sanas:**
   a. Los monocitos circulantes acumulan lípidos a través del receptor apoB48R de forma independiente a la concentración plasmática de ácidos grasos libres.
   b. Esta acumulación de lípidos implica la activación de los monocitos circulantes y, por lo tanto, el aumento del riesgo de la adhesión al endotelio vascular.

2. **Las lipoproteínas postprandiales ricas en triglicéridos, aisladas de personas sanas, y dependiendo del tipo de ácido graso que transportan (según el siguiente orden: ácidos grasos saturados > ácidos grasos monoinsaturados > ácidos grasos poliinsaturados):**
   c. Son capaces *in vitro* de estimular en monocitos y reprimir en macrófagos humanos la expresión del gen que codifica el receptor apoB48R, mediante un mecanismo de retroalimentación.
   d. Son capaces *in vitro* de inducir la acumulación de lípidos según la expresión relativa del gen que codifica el receptor apoB48R en monocitos y macrófagos humanos. Esta acumulación de lípidos implica, al menos, la liberación intracelular de los ácidos grasos de las lipoproteínas postprandiales ricas en triglicéridos que han sido internalizadas.
   e. Son capaces *in vitro* de inducir la activación de los factores de transcripción PPARα, PPARγ, y RXRα, los cuales regulan la expresión del gen que codifica apoB48R.
   f. Son capaces *in vitro* de estimular la expresión génica y proteica de ADRP y modificar la localización espacial de TIP47 en las gotas lipídicas en macrófagos.
   g. Son capaces *in vitro* de estimular la migración de células de la musculatura lisa de arteria coronaria humana a través de la activación de GTPasa Rac1, MMP2, JNK, PI3K, y ERK, dependiendo del tipo de ácidos grasos mayoritarios que se transportan en dichas lipoproteínas.

3. En el modelo experimental de aterosclerosis inducida por la dieta:

h. Los ácidos grasos insaturados de la dieta tienen menor impacto en la formación de células espumosas derivadas de macrófagos mediante la regulación de TIP47 y ADRP.

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Becas de investigación disfrutadas

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