Adipocytic Differentiation and Liver X Receptor Pathways Regulate the Accumulation of Triacylglycerols in Human Vascular Smooth Muscle Cells*

Received for publication, September 1, 2004, and in revised form, November 3, 2004
Published, JBC Papers in Press, November 16, 2004, DOI 10.1074/jbc.M410075200

John D. Davies‡, Keri L. H. Carpenter‡, Iain R. Challis‡, Nikki L. Figg‡, Rosamund McNaïr‡, Diane Proudfoot‡, Peter L. Weissberg‡, and Catherine M. Shanahan‡

From the ‡Department of Medicine, University of Cambridge, ACCI, Box 110, Addenbrooke’s Hospital, Hills Road, Cambridge, CB2 2QQ, United Kingdom and ¶Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1PQ, United Kingdom

Lipid accumulation by vascular smooth muscle cells (VSMC) is a feature of atherosclerotic plaques. In this study we describe two mechanisms whereby human VSMC foam cell formation is driven by de novo synthesis of fatty acids leading to triacylglycerol accumulation in intracellular vacuoles, a process distinct from serum lipoprotein uptake. VSMC cultured in adipogenic differentiation medium accumulated lipids and were induced to express the adipocyte marker genes adipin, adipocyte fatty acid-binding protein, C/EBPα, PPARγ, and leptin. However, complete adipocyte differentiation was not observed as numerous genes present in mature adipocytes were not detected, and the phenotype was reversible. The rate of lipid accumulation was not affected by PPARγ agonists, but screening for the effects of other nuclear receptor agonists showed that activation of the liver X receptors (LXR) dramatically promoted lipid accumulation in VSMC. Both LXRα and LXRβ were present in VSMC, and their activation with T0901317 resulted in induction of the lipogenic genes fatty acid synthetase, sterol regulatory element binding protein (SREBP1c), and stearoyl-CoA desaturase. 27-Hydroxycholesterol, an abundant oxysterol synthesized by VSMC acted as an LXR antagonist and, therefore, may have a protective role in preventing foam cell formation. Immunohistochemistry showed that VSMC within atherosclerotic plaques express adipogenic and lipogenic markers, suggesting these pathways are present in vivo. Moreover, the development of an adipogenic phenotype in VSMC is consistent with their known phenotypic plasticity and may contribute to their dysfunction in atherosclerotic plaques and, thus, impinge on plaque growth and stability.

Atherosclerosis is the principle cause of coronary artery disease in the Western world with plaque rupture leading to an acute coronary event (1). Fatty plaques are late stage atherosclerotic lesions consisting of a lipid and matrix core confined within the vessel wall by a fibrous cap. Stability of these lesions depends on the abundance and reparative capacity of the VSMC in the fibrous cap (2). Lipid-filled VSMC are the dominant foam cell type in early lesions and are present at later stages of atherosclerosis (3, 4); such a perturbation in VSMC biology could promote lesion development and contribute to plaque instability. Therefore, an analysis of the mechanisms of lipid accretion in VSMC may provide insights into the process of atherosclerosis and other conditions of vascular dysfunction. Lipid-filled “foam cells” within atherosclerotic lesions are derived from both VSMC and monocytes. Macrophage foam cells appear in juvenile fatty streaks and become most abundant in the fatty plaque. Infiltrating monocytes differentiate in the atherogenic environment of the vessel wall. This is accompanied by expression of receptors such as CD36 and scavenger receptor type A and lipoprotein uptake, resulting in the formation of foam cells rich in cholesterol and cholesterol esters (CE) (5, 6). Lipoprotein uptake by VSMC has been reported (7–9). However, unlike the macrophage situation, establishment of a scavenger receptor phenotype is not essential for VSMC lipid uptake. VSMC do not express high levels of scavenger receptors in atherosclerotic tissue and are resistant to lipid accumulation after exposure to normal LDL and oxidized LDL in vitro (10–14). The absence of macrophage scavenger receptors in VSMC indicates that other mechanisms of lipid accumulation may occur. These could include the utilization of LDL and VLDL receptors in the uptake of aggregated LDL and β-VLDL, a process that occurs in vitro (15, 16), or the uptake of non-esterified fatty acids from serum (17, 18). This mechanism of VSMC foam cell formation is of particular interest due to the elevated levels of FA in diabetes and the association of this condition with atherosclerosis (17). An alternative mechanism would be de novo synthesis of lipid, and it has long been known that VSMC, human arterial tissue, and foam cells incubated with radioactive acetate synthesize phospholipids, triacylglycerols (TG), and CE (19, 20), the type of lipid produced being dependent on VSMC phenotype and effected by oxysterols (21, 22). Excessive de novo synthesis could result in lipid accumulation and foam cell formation. Furthermore, the lipid in VSMC.
eased tissue is thought to be derived from both cellular and serum lipoprotein sources. CE and TG that accumulate in fatty streaks and advanced lesions are rich in oleic acid, this may in part result from cellular synthesis (23–25).

De novo lipogenesis and TG storage are specialized functions of white adipose tissue. The capacity of adipocytes to regulate lipid metabolism is associated with terminal differentiation of preadipocytes, a process generally linked with expression of the transcription factors PPARγ, C/EBPα, SREBP1/ADD1 (26–28). VSMC are known to have mesenchymal characteristics; they are not terminally differentiated and show considerable phenotypic plasticity in normal and diseased tissues (29–31). We, therefore, hypothesized that VSMC maintain the propensity to develop an adipocyte phenotype if exposed to adipogenic conditions and that this would result in lipid accumulation. Enhanced de novo lipogenesis is also observed in non-adipocytic cells, in pancreatic β-cell lipotoxicity in type 1 diabetes, renal cell lipotoxicity, and alcohol induced fatty liver (32–35). In these situations SREBP1 plays a central role in promoting cell lipotoxicity, and alcohol induced fatty liver (32–35).

Lipogenesis results from either adipocytic differentiation or direct promotion of lipogenesis as the result of LXR/SREBP1c activation. In addition we identify VSMC expressing high levels of FASE, transcription factors PPAR and SREBP1, LXRx, and adipin within human atherosclerotic plaques, suggesting that de novo lipogenesis may play a role in VSMC lipid accumulation in vivo.

EXPERIMENTAL PROCEDURES

VSMC and Culture Reagents—Explant cultures of human VSMC were grown from aortic vessels in 20% FCS/Medium 199, as described previously (31). Adipogenic differentiation medium (DM) consisted of Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (1:1, by volume) or Medium 199, containing HEPES (15 mM), biotin (33 μM), pantothenate (17 μM), and antibiotics. Immediately before use this was supplemented with human insulin (1.2 μU/ml), dexamethasone (100 nM), triiodothyronine (1 nM), and 3-isobutyl-1-methylxanthine (0.25 mM). For the reverse transcription total RNA from human VSMC cultures was subjected to “Super-RT” avian myeloblastosis virus reverse transcriptase (RT, Promega, Madison, WI). Experimental conditions for the RT-PCR were described previously (31) except that random hexamers (Promega, Madison, WI) were added to the RT step as well as oligo-dT. β-Microglobulin was used to control for RT-PCR (n = 3). Most of the PCR primer pairs were designed to be functionally active at 58 °C annealing temperature and to have a product size between 200 and 600 bases. In the binary experiments with a range of cycle numbers, primers were performed to ensure that PCR amplification was within the “log phase” of the reaction. The functional activity of the primers and size of positive control amplicons were determined using RNA from pre-adipocytes, adipocytes, and if necessary atherosclerotic tissue (data not shown). Primer sequences are available on request. The PCR products were subjected to electrophoresis on a 1% agarose gel in the presence of ethidium bromide and quantified by electronic digitized imaging using a MultiImage light unit with Alphamager 1200 software (Alpha Innotech Corp, San Leandro, CA). The SREBP1 and PPAR primers do not distinguish between SREBP1a/c or PPAR-β/γ.

Western Blotting—Cell extracts were prepared by lysis in non-reducing Laemmli sample buffer minus bromphenol blue, and protein was quantified by the BCA assay (Pierce). Dye and reducing agent was added to the samples, and the proteins were separated by PAGE and transferred to Immobilon-P membrane (Millipore, Billerica, MA). Equal protein loading and transfer were confirmed by Coomassie and Ponceau S staining. The membrane was blocked with 10% milk, phosphate-buffered saline, 0.1% Tween 20 for 1 h and then incubated with diluted primary antibody in 3% milk, phosphate-buffered saline, and 0.05% Tween 20. Antibodies reactive toward LRXα (H114, sc13068), LRXβ (H20, sc13081), and LRXβ (H160, sc12071) were kindly provided by Dr. H. Shimano (42). Both SREBP1c and LXRXα plasmids have the oxysterol-inducible region that contains two LRX binding sites (LRXα and β). LRXα and LRXβ were mutated to non-binding sites using the following oligonucleotides: mutant LRXα, −265 to −306, tggcgccgtcgcgctggaAtTctggcggAcTctgtcgtcc; mutant LRXβ, −264 to −223, gacgcggttaaaggcggaAgTccgctagAaTccccggcccca. The oligonucleotides were annealed to the wild type plasmids, and the DNA was filled in by two temperature step PCR using Pfu polymerase. After extension the ends of the PCR products were treated with kinase, ligated, and transfected into Escherichia coli. The resulting plasmids were sequenced to ensure mutation of both LRXαa/b and a correct DNA sequence at the 5′ juncture of the two oligonucleotide binding sites. The mutated SREBP1c promoter fragments were cut out and cloned into the parental pGL2-basic plasmid to remove the possibility of mutations in the luciferase gene. pRL-SV40 control plasmid (Promega) mixed with 25 μl of Superfect for each dish as described by the manufacturer (Stratagene, La Jolla, CA). Cells were transfected for 2 h, washed to remove plasmid, and left overnight in growth medium to recover. New culture medium with agonists was added for 48–72 h. Six dishes were analyzed for each culture condition. Cells were scraped off, spun down, and lysed in 50 μl of cell culture lysis reagent (Promega). 20 μl of lystate was analyzed using the dual luciferase reporter assay system according to 0.05% ORO in isopropanol and water (3:2 by volume). Cells were then washed with water and viewed by microscopy.

RNA Extraction and RT-PCR—RNA was isolated from VSMC using RNA STAT-60 as described (36). VSMC were plated at a density of 1–5 × 10^4 per dish, and cultured for 24 h before plasmid transfection. A total of 5 μg of luciferase reporter plasmid and 25 μg of empty pRL-SV40 control plasmid (Promega) was mixed with 25 μl of Superfect for each dish as described by the manufacturer (Stratagene, La Jolla, CA). Cells were transfected for 2 h, washed to remove plasmid, and left overnight in growth medium to recover. New culture medium with agonists was added for 48–72 h. Six dishes were analyzed for each culture condition. Cells were scraped off, spun down, and lysed in 50 μl of cell culture lysis reagent (Promega). 20 μl of lystate was analyzed using the dual luciferase reporter assay system according to
to manufacturer's instructions (Promega), and luciferase activity was detected using a luminometer (EC&G Berthold, Bad Wildbad, Germany). Transfection efficiency variations within each assay run were normalized for Renilla luciferase emission. The data were reported as relative luciferase units, representing the mean of the recorded firefly luciferase emission values minus the low background from non-transfected VSMC.

**Immunohistochemistry—** Human carotid endarterectomy specimens ($n = 9$) and normal aortic specimens ($n = 2$) were formalin-fixed and paraffin-embedded. Adjacent 5-μm sections were cut, and immunohistochemistry was performed as standard using antibodies recognizing the following: anti-α-SM actin (clone 1A4, Sigma), fatty acid synthetase (905–069, Assay Designs Inc.), SREBP1 (sc8984, Santa Cruz), adipsin (sc12402, Santa Cruz), and LXRα (sc13068, Santa Cruz). Anti-mouse horseradish peroxidase and anti-rabbit alkaline phosphatase secondary antibodies (Vector laboratory, Burlingame, CA) were used for dual detection of markers. Cell nuclei were counterstained with hematoxylin-eosin. ORO staining was performed as standard.

**RESULTS**

**Accumulation of ORO Staining in VSMC Cultured in Adipocyte Differentiation Medium**—ORO staining was low or absent from VSMC cultured in growth medium. In contrast some VSMC isolates cultured in adipocyte DM accumulated considerable amounts of ORO lipid. Analysis of a responsive VSMC isolate show that the abundance of cells containing lipid and the amount of lipid per cell was enhanced with increased periods of culture in adipogenic conditions; this was not observed when the cells were grown in 20% FCS (Fig. 1, A and B). Small clear vacuoles were observed in VSMC cultured in growth medium and DM. Lightly ORO-stained, small highly refractive intracellular droplets would then develop, often associated with stress fibers. The abundance, size, and staining of the vacuoles increased in cells cultured with DM, with the result that some cells were heavily loaded with lipid after 3 weeks of culture. The large globular lipid vacuoles were morphologically indistinguishable from those observed in true adipocyte cultures. The extent of lipid accumulation was dependent on the VSMC isolate, some of which did not accumulate lipid when cultured in DM. The variability of VSMC lipid accumulation is demonstrated by the photographs in Fig. 1C; the three VSMC isolates were cultured for 21 days in DM and stained with ORO.

TLC analysis revealed that TG was the major lipid type accumulated in VSMC cultured in DM. Fig. 1D shows the fatty acid composition of the TG from cells cultured in growth medium or DM, with the proportion of oleic acid (C18:1) greater in the TG-derived from VSMC cultured in DM. VSMC cultured in DM accumulated TG enriched with oleate, the same lipid type that is synthesized and stored in white adipose tissue and 3T3 cells induced to undergo adipocyte differentiation (43–45).

**Adipocyte Marker Genes Are Up-regulated in VSMCs Cultured in DM**—Semi quantitative RT-PCR was used to investigate expression in VSMC of a number of gene markers known to be up-regulated during adipocyte differentiation. Fig. 2A shows that expression of adipsin, adipocyte fatty acid-binding protein, C/EBPα, and PPARγ were elevated in VSMC cultured...
lipid accumulation was observed (as measured by ORO stain-
free conditions, or growth medium. No change in the rate of
cially increased the rate of lipid accumulation. Cells treated
the VSMC isolates with the LXR agonist TO901317 dramati-
ORO accumulation in cells cultured in DM (not shown).
treatment with the GW610742 slightly enhanced the rate of
increase the rate of lipid accumulation in VSMC, whereas
tested under the above culture conditions. WY14643 did not
function and PCR product size (fourth and fifth lanes).
all members, LXR
promoters via the LXRE—
were not detected in VSMC under any of the culture conditions
HSL, glycerol-3-phosphate dehydrogenase (G3PDH),
aPM1 (adipose most abundant gene tran-
Junctional nuclear hormone receptors could
in DM; gene expression was low or not detectable from cells
cultured in growth medium. The phenotype was reversible, as
shown by the reduction in marker gene expression after re-
placement of DM with medium containing 20% FCS (DM/FCS).
ORO staining of the cells also decreased as a result of changing
culture conditions from DM (21 days) to growth medium (data
not shown). Expression of FASE, the key gene involved in FA
synthesis, SREBP1, and leptin was also enhanced as the result of
VSMC culture in DM (Fig. 2B). The early adipocyte marker
adipose RNA was less than for the VSMC to prevent saturation of the RT-PCR
Expression of hormone-sensitive lipase (HSL), glycerol-3-phosphate dehydrogenase (G3PDH),
aPM1 (adipose most abundant gene transcript 1 (adiponectin)), and perilipin genes were detected by RT-PCR in the control mature adipocyte RNA; they were not readily detectable in RNA derived from the VSMC cultured in normal or differentiation conditions. Extremely low levels of hormone-sensitive lipase and perilipin were noted in a few reactions for differentiated VSMC.

Fig. 2. Expression of adipocytic marker genes in human VSMC. A, RNA derived from VSMC cultured in growth or differentiation
conditions was used for quantitative RT-PCR using primers specific for adipin, adipocyte fatty acid-binding protein (AFBP), C/EBPα, and PPARγ. Expression levels of the β-microglobulin demonstrate that the amount of VSMC RNA was the same between the three culture conditions. Cells were cultured for 21 days in growth medium containing 20% FCS (FC5), for 21 days in DM followed by 3 days culture in growth medium (DM/FCS), or for 21 days in adipocyte differentiation medium (DM). RNA from human pre-adipocytes and differentiated adipocytes act as controls for primer

in DM; gene expression was low or not detectable from cells
cultured in growth medium. The phenotype was reversible, as
shown by the reduction in marker gene expression after re-
placement of DM with medium containing 20% FCS (DM/FCS).
ORO staining of the cells also decreased as a result of changing
culture conditions from DM (21 days) to growth medium (data
not shown). Expression of FASE, the key gene involved in FA
synthesis, SREBP1, and leptin was also enhanced as the result of
VSMC culture in DM (Fig. 2B). The early adipocyte marker
adipose differentiation-regulated protein (adipophilin) and the
leptin receptor were expressed at equivalent levels in VSMC
cultured under normal or DM conditions; they both reportedly
increase during true adipocyte differentiation (46, 47). Finally,
certain genes known to be up-regulated in mature adipocytes
were not detected in VSMC under any of the culture conditions
tested. Hormone-sensitive lipase, glycerol-3-phosphate dehydro-
genase (G3PDH), aPM1 (adipose most abundant gene tran-
scription of VSMC culture in DM (Fig. 3)
Effect of Nuclear Receptor Agonists on Lipid Accumulation in
VSMCs—The above experiments indicated that VSMC could
adopt an adipocyte phenotype in response to DM; therefore, we
investigated whether specific agonists for members of the
PPAR and LXR families of nuclear hormone receptors could
hance this differentiation. VSMC were treated with 5 or 10
μM PPARγ agonist (BRL49653/rosiglitazone) in DM, serum-
free conditions, or growth medium. No change in the rate of
lipid accumulation was observed (as measured by ORO stain-
ing) between cells treated with the vehicle Me3SO or
BRL49653. Treatment of VSMC with ciglitazone also had no
effect on promoting lipid accumulation (not shown). Agonists
for PPARα (WY14643) and PPARδ/β (GW610742) were also
tested under the above culture conditions. WY14643 did not
increase the rate of lipid accumulation in VSMC, whereas
treatment with the GW610742 slightly enhanced the rate of
ORO accumulation in cells cultured in DM (not shown).
In contrast to the effect of the PPAR agonists, treatment of the
VSMC isolates with the LXR agonist TO901317 dramati-
cally increased the rate of lipid accumulation. Cells treated
with 0.5–10 μM TO901317 in serum-free medium, DM, or me-
dium containing 2.5% FCS, 5% FCS, 10% lipoprotein-deficient
chloroform stripped bovine serum stained positively for ORO.
TO901317 also promoted lipid accumulation in the VSMC iso-
late 99.2.5.12A (Fig. 3A); this isolate had otherwise been shown
to be resistant to lipid accumulation even when cultured in
DM. Lipid accumulation was clearly detectable after 5 days of
culture with TO901317. TLC showed that TG was increased in
VSMC treated with TO901317, with elevations in content of palmitic acid (C16:0) and, more markedly, of oleic acid (C18:1)

LXRXα and LXRXβ Are Expressed by VSMC and Can Activate
Promoters via the LXRE—The LXRX nuclear receptor subfamily
consists of two members, LXRXα (NR1H3) and LXRXβ (NR1H2)
(48). Western blot analysis of cell lysates from four VSMC
isolates with an antibody specific for LXRXα detected a 55-kDa
protein, consistent with the size of LXRXα in other cells types
(Fig. 4A). Using an LXRXβ antibody, two bands, a minor band at
75-kDa and a 105-kDa protein, were detected (Fig. 4B). Specific
antibody binding for both bands was confirmed by competition
with peptide (not shown).

The SREBP1c promoter is known to be activated by LXRX (42,
49–51). Western blot analysis of VSMCs cultured with or with-
out LXRX agonist indicated that SREBP1 promoter levels
were elevated after TO901317 treatment, the increase being greatest when the cells were cultured in low serum (2.5% FCS) or in
DM (Fig. 4C). A slight increase in SREBP1 expression was
observed in TO901317 treated cells cultured in growth medium
(20% FCS).

To confirm that LXRX activated genes in VSMCs via the
LXRE, SREBP1c promoter-luciferase reporter constructs with intact or mutated LXRE binding sites (LXREα/b) were tested for
responsiveness to TO901317. The 350-bp and 2.6-kb murine
SREBP1c promoters were activated by TO901317 but not by
vehicle Me3SO (Fig. 4D). Mutation of both LXRE sites elimi-
nated TO901317-dependent activation in the 350-bp promoter
and dramatically reduced activation of the larger 2.6-kb
promoter.

TO901317 Treatment Results in the Activation of the FASE,
SREBP1c, and ABC-A1 in VSMCs—The level of expression and
activity of FASE regulate the rate of FA synthesis. Western
blotting demonstrated that FASE was dramatically increased
in VSMC after TO901317 treatment, as was expression of the 150kDa SREBP1c band. Levels of the 160-kDa protein, probably the SREBP1a isoform, were not enhanced by TO901317 (Fig. 5B). This would be consistent with reports that LXR binds and activates the SREBP1c promoter but not the SREBP1a promoter (42, 49) and that FASE gene transcription is activated by both LXR and SREBP1 (52). Low molecular weight cleavage products of SREBP1 were also enhanced by TO901317. Protein levels of LXRβ were not influenced by treatment of VSMC with TO901317 or the other nuclear receptor agonists (Fig. 5C).

We next examined by RT-PCR the expression of SCD-1, the enzyme involved in converting stearate (C18:0) to monounsaturated oleate (Δ9–18:1). SCD-1 expression was enhanced by TO901317 (Fig. 5D). ABC-A1, a well characterized LXR-responsive gene, was also up-regulated in VSMC after TO901317 treatment. RNA levels for SREBP1 and FASE were also increased by TO901317. Expression of the adipocyte marker genes adipsin, C/EBPα, and adipocyte fatty acid-binding protein/αP2 were not elevated in VSMC isolate 99.2.5M.12A after culture with LXR agonist TO901317 (not shown).

Activation of the FASE and SREBP1c promoters by TO901317 are shown in Fig. 5E. VSMC transfected with the FASE or SREBP1c promoter luciferase reporters were cultured with vehicle Me2SO or 5 μM TO901317. There was significant activation of both the FASE promoter and SREBP1c after TO901317 treatment.

**Influence of Oxysterols 27-Hydroxycholesterol and 24(S)25-Epoxycholesterol on LXR Function in VSMC**—Specific natural oxysterols have been shown to be physiological ligands for LXR (53–57). We were interested in whether 27-hydroxycholesterol (27-HC), an oxysterol synthesized by normal VSMC and also present in diseased vessels (58), was an LXR agonist. 27-HC did not significantly activate the SREBP1c luciferase reporter plasmid pBP1c357-Luc (SREBP1c-360luc) and pBP1c2600-Luc (SREBP1c-2.6luc) contained 357-bp and 2.6-kb fragments of the SREBP1c promoter. VSMC transfected with plasmid were harvested 72 h after treatment, and the cell lysate was assayed for luciferase activity. RLU, relative luciferase units.

**Fig. 3.** Accumulation of triacylglycerol rich in oleate in human VSMC cultured with the LXR agonist TO901317. A is a photograph of VSMC isolate 99.2.5M.12A cultured for 5 days in DM containing 10 μM TO901317. The cells were stained with Oil Red O. This isolate did not accumulate Oil Red O staining lipid after extended culture in DM containing vehicle or BRL49653. TG accumulated rapidly after LXR activation. B shows the amounts of individual fatty acid species in the TG lipid fraction purified from VSMC isolate 99.2.5M.12A; the cells were cultured for 7 days in the presence of vehicle (ethanol), 10 μM BRL49653, or 10 μM TO901317. Cells cultured with the LXR agonist TO901317 had high levels of palmitate (C16:0) and especially of oleate (C18:1) when compared with cells treated with vehicle or BRL49653. Fatty acids were determined by GC. Quantitative analysis in (C18:1) when compared with cells treated with vehicle Me2SO or 5 μM TO901317 was achieved by the addition of an internal standard, triheptadecanoin, to the samples before preparative TLC.

**Fig. 4.** Human VSMCs express LXRα and LXRβ. A and B are Western blots of four different VSMC lysates with antibodies recognizing LXRα or LXRβ, respectively. The 55-kDa protein was consistent with the reported size for transcription factor LXRα. The LXRβ antibody specifically recognized two protein bands, a dominant band at 105 kDa and a lower band of 75 kDa. Blot C shows SREBP1 antibody reactivity to SMC protein lysates obtained from cells treated with the vehicle Me2SO (−) or various concentrations of the LXR agonist TO901317 (0.5–10 μM). The cells were cultured in growth medium containing 20% FCS, medium with 2.5% FCS, or adipocyte differentiation medium. D is the luciferase activity of the wild type (wt) or LXRE-mutated SREBP1c promoters in human VSMC; the transfected cells were treated with vehicle Me2SO (−) or 10 μM TO901317 (+). The luciferase reporter plasmid pBP1c357-Luc (SREBP1c-360luc) and pBP1c2600-Luc (SREBP1c-2.6luc) contained 357-bp and 2.6-kb fragments of the SREBP1c promoter. VSMC transfected with plasmid were harvested 72 h after treatment, and the cell lysate was assayed for luciferase activity. RLU, relative luciferase units.
tigated whether 27-HC could influence the ability of TO901317 or 24(S)/25-EC to activate the SREBP1c luciferase reporter plasmid pBP1c357-Luc. The addition of 27-HC resulted in a reduction in TO901317-induced SREBP1c promoter activity (Fig. 6C). 27-HC also repressed promoter activation by 24(S)/25-EC. There was a slight increase in promoter activity when VSMC were treated with 1, 5, or 10 μM 27-HC in the presence of the Me2SO vehicle (Fig. 6C).

Oleic Acid Was Enriched within the Triacylglycerol Purified from Atherosclerotic Lesions—GC was used to determine the fatty acid composition of TG from advanced lesions (plaques) and adjacent macroscopically normal arterial tissue from three carotid arteries. Palmitate (C16:0) and oleate (C18:1) were the dominant FA species within the TG from both normal and plaque tissue. There was significant enrichment for oleate within plaque TG (Fig. 7). The fatty acid composition of the TG isolated from normal vessel (Fig. 7) was similar to that of VSMC cultured in growth medium (Fig. 1D), whereas the fatty acid composition of TG from advanced lesions (Fig. 7) resembled those of VSMC cultured in DM or with TO901317 (Figs. 1D and 3B). The CE content of the arterial samples was dramatically lower than for the cultured VSMC. In the vessel CE was far more abundant than TG and characterized by a high proportion of the essential FA linoleate (C18:2).

VSMCs within Atherosclerotic Plaques Express High Levels of Adipsin, FASE, SREBP1, and LXRA—Immunohistochemistry was used to determine expression levels of phenotypic markers and transcription factors in normal and diseased human arteries (Fig. 8). Medial VSMCs in normal aortic vessel showed no reactivity to antibodies recognizing adipsin and the lipogenesis enzyme FASE (a and b). Similarly, the transcription factor SREBP1 was not detected, whereas LXRs was observed at low levels (c and d). The expression of these markers and transcription factors was markedly up-regulated in VSMC from diseased vessel. Medial VSMCs located beneath regions of atherosclerotic plaque were positive for adipsin (e), FASE (f), SREBP1 (g), and LXRs (h). The medial cells were located in a region of lesion that weakly stained with ORO (n). Although some of these cells were lipid-filled (8e’), others were not (f–h). In addition, intimal VSMCs deep within the lipid-rich ORO-positive regions of the plaque stained positively for adipsin, FASE, SREBP1, and LXRs (i–l). Many of these cells were morphologically foam cells with obvious cytoplasmic lipid vesicles. Foam cells of VSMC and macrophage origin were present in the intimal region, and both stain for SREBP1 (k). e shows extensive ORO staining in the lipid-rich intimal region of the plaque. Normal vessel was negative for ORO staining (not shown).

**DISCUSSION**

Lipid-containing VSMC within atherosclerotic plaques express elevated levels of the adipocyte marker adipsin and genes...
Liver X Receptor and SREBP1 Pathway—Direct ligand activation of LXRα in VSMC was of particular interest because it was previously thought to be restricted to cells with high lipid metabolism (48, 67). LXRα self-regulates its expression in sterol-loaded macrophages and was characterized by an abundance of linoleic acid (C18:2). Liver X Receptor and SREBP1 Pathway—Direct ligand activation of LXRα in VSMC was of particular interest because it was previously thought to be restricted to cells with high lipid metabolism (48, 67). LXRα self-regulates its expression in sterol-loaded macrophages and is more effective than the ubiquitously expressed LXRβ at involved with lipogenesis, including FASE, SREBP1, and LXRα. In vitro, elevated de novo fatty acid synthesis in VSMC leads to TG accumulation, suggesting that synthesized lipid may contribute to foam cell formation in vivo. In support of this, TG levels increase in vessels with age and atherosclerosis, albeit to a lesser degree than CE. Also noteworthy is that CE derived from intimal regions with abundant foam cells are oleate-rich, further supporting that lipid is derived at least partly from a cellular origin rather than exclusively from plasma lipoproteins (23–25). The preferential synthesis in vitro of TG rather than CE may reflect a characteristic function of VSMC, as suggested by TG accumulation after VSMC uptake of non-esterified FA (17, 18). Alternatively, the serum-free or low serum culture conditions may restrict ACAT1 activity by limiting cholesterol substrate.

Adipocyte Differentiation of VSMC—In vitro certain VSMC isolates were predisposed to accumulate TG and develop an adipocyte phenotype when incubated in DM, with some expressing the late differentiation marker CEBPα (26, 28, 59). However, VSMC were generally unable to express the full range of adipocyte markers, and the phenotype was reversed when cells were transferred back to growth medium.

The conversion of mesenchymal stem cells, mice embryonic fibroblasts, and bone marrow stromal cells among mesenchymal phenotypes, in particular the osteoblastic and adipocytic lineages, is well established and regulated in part by expression of the lineage-specific transcription factors Cbfα-1 (core binding factor β (Runx2)) and PPARγ (60–62). VSMC also exhibit phenotypic plasticity, being convertible to an osteochondrocytic phenotype in vivo and in vitro (29–31). Rosiglitazone did not promote adipocyte differentiation in VSMC. This suggests that PPARγ is not the central regulator for adipocytic conversion of VSMCs, similar to omental preadipocytes and certain murine stromal cell lines that are also refractory to PPARγ agonists (39, 40, 61–63). Events promoting VSMC adipogenesis in vivo are unknown but presumably involve responses to factors in the diseased vessel wall. These could include deregulated transforming growth factor β/bone morphogenic proteins signaling, atherogenic diet, modified LDL, FA, and aging, all of which can promote lipid accumulation or adipocytic characteristics in bone marrow stromal cells (60, 64, 65).

Liver X Receptor and SREBP1 Pathway—Direct ligand activation of LXRα in VSMC up-regulated FASE, SREBP1c, and SCD-1, thus promoting TG accumulation via de novo FA synthesis and SCD-1-mediated Δ⁹ desaturation. The increase in mature and processed SREBP1c together with SCD-1 expression indicated direct SREBP1c involvement in promoting TG synthesis (51, 66). The identification of LXRα in VSMC was of particular interest because it was previously thought to be restricted to cells with high lipid metabolism (48, 67). LXRα self-regulates its expression in sterol-loaded macrophages and is more effective than the ubiquitously expressed LXRβ at
promoting lipogenesis in adipocytes and hepatocytes (68–73). LXR activation by T0901317 promoted expression of genes necessary for lipogenesis, but not UCP1, indicating that LXR activation alone was insufficient to promote the transcriptional cascade responsible for adipocyte differentiation. Indeed, there are conflicting reports of the ability of LXR agonists to promote adipogenesis in 3T3 cells and preadipocytes, although treatment resulted in lipid accumulation (74–76). Overexpression of SREBP1c also inhibits adipogenesis in mice (77, 78). Although the etiology of adipocyte differentiation and LXR activation pathways are distinct, they intersect at the point of SREBP1-mediated activation of lipogenesis genes. Thus, within atherosclerotic lesions, factors that influence SREBP1 gene expression and cleavage might dramatically influence rates of fatty acid synthesis and lipid accumulation, known risk factors for atherosclerosis, reportedly up-regulate the expression of SREBP-responsive genes in liver and VSMC (33, 79). Hyperinsulinemia also elevates SREBP-1c activity, resulting in TG deposition in non-adipose tissues and lipotoxicity and, by analogy, may also play a role in vascular dysfunction and enhanced susceptibility to atherosclerosis in diabetes (34).

Oysterol Involvement in Foam Cell Formation—Certain oysterol derivatives are natural LXR ligands. 27-HC, an abundant oysterol, is a natural LXR ligand. Reports of the LXR-activating ability of 27-HC in other cell types are conflicting, whereas in a cell-free system 27-HC acted as a partial agonist (53, 56, 57). The inability of 27-HC to act as an agonist in the present study may stem from factors such as the transcriptional co-regulator composition of VSMC or that very weak LXR agonists are not detected in our system. 27-HC was reportedly excluded from liver nuclei, unlike 24(S)/25-EC and 24-EC (80). Should this be the situation in VSMC, then 27-HC would not be available to activate nuclear LXR, and the antagonist action observed would be independent of LXR binding.

In human advanced atherosclerotic lesions, mean levels of 27-HC are ~0.3 μg/ml of wet weight or 0.7 mm, well above the concentrations that antagonized LXR in vitro (58, 81–83). Macrophages within advanced lesions express high levels of sterol 27-hydroxylase and are the primary source of 27-HC and derivatives (84–86). The influence of 27-HC on lesion VSMCs would, therefore, be dependent on transfer of free 27-HC from macrophages to VSMC. The availability of 27-HC may also be modified by esterification and by the presence of cholesterol or other lipids in the lesion. 27-HC can be further metabolized by sterol 27-hydroxylase to form water-soluble cholestenoic acid (3β-hydroxy-5-cholestenoic acid). Cholestenoic acid has been reported as an LXR agonist (55) or, conversely, an insignificant (56). In lesions cholestenoic acid levels are less than 3% of that of 27-HC (87). The physiological relevance of cholestenoic acid as an LXR agonist needs to be determined.

Interestingly, sterol 27-hydroxylase expression by VSMC in the normal artery is greater than in VSMC in advanced lesions (84). 27-HC synthesis by VSMC in the normal vessel wall may exert an anti-atherogenic effect by restricting superfluous fatty acid synthesis as well as aiding cholesterol clearance. In humans, sterol 27-hydroxylase deficiency (a condition termed cerebroretinodis xanthomatosi) is invariably associated with premature atherosclerosis; this is thought to be due to diminished removal of cholesterol from macrophages (85). The role of VSMC in this accelerated atherosclerosis has not been studied.

Lipogenesis in VSMCs—This study provides evidence that lipid accumulation in VSMC in vivo can involve de novo lipogenesis, a process distinct from the cellular uptake of lipoproteins and FA from plasma. Identifying the factors in the plaque that induces the adipocytic/lipogenic phenotype in VSMCs and determining whether this phenotypic change occurs early in atherogenesis are important areas for further investigation. LXR agonists, as yet unidentified, may be present in atherosclerotic lesions, whereas factors that influence SREBP1 activity may include oxidative stress, cell senescence, and aging. Finally, the detrimental effects of enhanced VSMC lipogenesis and lipid accumulation should be considered when developing nuclear receptor agonists as therapeutic agents for atherosclerosis (88, 89).

Acknowledgments—We thank Stephen O’Rahilly, Vivion Crowley, and Ciaran Sewter for the human preadipocyte and adipocyte RNA used in this study, Dr. Hitoshi Shimano for providing us with the wild type 2.6-kb and 357-bp murine SREBP1c promoter luciferase reporter vectors, and Jeremy Tavare and Dr. Kenny Webster for the mouse fatty acid synthetase promoter luciferase plasmid. We are also grateful to Drs. Tim Willson, Kelly Halliday, and Elliott Bailis for providing the agonists BRL49653 and GW610742X. We thank Peter J. Kirkpatrick for the carotid endarterectomy specimens.

REFERENCES
1. Ross, R. (1986) N. Engl. J. Med. 314, 488–500
2. Weissberg, P. L. (2000) Heart 83, 247–252
3. Balis, J. U., Haust, M. D., and More, R. H. (1964) Exp. Mol. Pathol. 3, 511–525
4. Katsuda, S., Boyd, H. C., Fligner, C., Ross, R., and Gown, A. M. (1992) Am. J. Pathol. 140, 907–914
5. Greaves, D. R., Gough, P. J., and Gordon, S. (1998) Curr. Opin. Lipidol. 9, 425–432
6. Kunjathoor, V. V., Febrario, M., Podrez, E. A., Moore, K. J., Andersson, L., Koen, S., Rhee, J. S., Silverstein, R., Hoff, H. F., and Freeman, M. W. (2002) J. Biol. Chem. 277, 49982–49988
Adipocytic Differentiation and Liver X Receptor Pathways Regulate the Accumulation of Triacylglycerols in Human Vascular Smooth Muscle Cells
John D. Davies, Keri L. H. Carpenter, Iain R. Challis, Nikki L. Figg, Rosamund McNair, Diane Proudfoot, Peter L. Weissberg and Catherine M. Shanahan

J. Biol. Chem. 2005, 280:3911-3919.
doi: 10.1074/jbc.M410075200 originally published online November 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410075200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 87 references, 42 of which can be accessed free at http://www.jbc.org/content/280/5/3911.full.html#ref-list-1