Liver X Receptor Activation Induces the Uptake of Cholesteryl Esters From High Density Lipoproteins in Primary Human Macrophages

Stephanie Bultel, Lionel Helin, Veronique Clavey, Giulia Chinetti-Gbaguidi, Elena Rigamonti, Morvane Colin, Jean-Charles Fruchtart, Bart Staels, Sophie Lestavel

Objective—Liver X receptors (LXRs) are oxysterol-activated nuclear receptors regulating reverse cholesterol transport, in part by modulating cholesterol efflux from macrophages to apoAI and HDL via the ABCA1 and ABCG1/ABCG4 pathways. Moreover, LXR activation increases intracellular cholesterol trafficking via the induction of NPC1 and NPC2 expression. However, implication of LXRs in the selective uptake of cholesteryl esters from lipoproteins in human macrophages has never been reported.

Methods and Results—Our results show that (1) selective CE uptake from HDL₃ is highly efficient in human monocyte-derived macrophages; (2) surprisingly, HDL₃-CE uptake is strongly increased by LXR activation despite antiatherogenic effects of LXRs; (3) HDL₃-CE uptake increase is not linked to SR-BI expression modulation but it is dependent of proteoglycan interactions; (4) HDL₃-CE uptake increase is associated with increased expression and secretion of apoE and LPL, two proteins interacting with proteoglycans; (5) HDL₃-CE uptake increase depends on the integrity of raft domains and is associated with an increased caveolin-1 expression.

Conclusions—Our study identifies a new role for LXRs in the control of cholesterol homeostasis in human macrophages. LXR activation results in enhanced dynamic intracellular cholesterol fluxes through an increased CE uptake from HDL and leads to an increased cholesterol availability to efflux to apoAI and HDL. (Arterioscler Thromb Vasc Biol. 2008;28:2288-2295.)

Key Words: liver X receptors ▪ cholesteryl ester uptake ▪ primary human macrophages

Macrophages play a pivotal role in the development of atherosclerosis.¹ During the initial stages of the disease, monocytes migrate into the subendothelial space of blood vessels and differentiate into macrophages. Macrophage scavenger receptors, CD36 and SR-A, which lack negative feedback regulation by cholesterol, mediate the uptake of oxidized lipids in the subendothelial space. Under physiological conditions, the net accumulation of lipids in macrophages is the result of a complex balance and reflects the rate of cholesterol endocytosis and its removal via the reverse cholesterol transport pathway. Caveolae are free cholesterol rich, invaginated microdomains (50 to 100 nm in diameter) at the surface of most peripheral cells. Caveolin-1, the main structural protein of caveolae, is involved in the regulation of cellular cholesterol metabolism and lipid uptake, as well as efflux.² The entrance of excessive amounts of lipoprotein-derived lipids into macrophages leads to their conversion into foam cells.

Selective cholesteryl ester (CE) uptake is a mechanism where CE are taken up by the cells independently from the entry of the lipoparticles. It mainly occurs from HDL in liver and steroidogenic tissues and involves principally SR-BI, a transmembrane glycoprotein receptor.³⁴ In macrophages, it has been mainly reported in cell lines like THP-1 and J774 but may also occur in primary human cells.⁵

Liver X receptors (LXRs) are oxysterol-activated transcription factors which after heterodimerization with the 9-cis-retinoic acid receptor (RXR), bind to specific LXR response elements (LXREs), thus regulating the expression of target genes involved in intra- and extracellular lipid metabolism. LXRβ is ubiquitously expressed,⁶ whereas the expression of LXRα is predominantly restricted to tissues known to play important roles in lipid metabolism, such as liver, adrenal glands, kidney, macrophages, intestine, and adipose tissue.⁷ In the last few years LXRs have emerged as key regulators of macrophage cholesterol homeostasis. LXR ac-
tivators promote apoAI-mediated cholesterol efflux through the increase of cholesterol trafficking to the plasma membrane and the induction of ABCA1, which is a direct target gene of LXR in human and murine macrophages. Other members of the ATP binding cassette family, ABCG1 and ABCG4, which are involved in lipid efflux to HDL, were also identified as LXR target genes in macrophages. Although LXRs do not promote cholesterol accumulation via the scavenger receptor pathway, it would be important to know whether LXRs control other pathways to provide cells with cholesterol, namely selective CE uptake.

The goal of this study was thus to investigate the role of LXR agonists in selective CE uptake from HDL₃ (HDL₃-CE uptake) in differentiated human macrophages. We demonstrate that HDL₃-CE uptake is highly efficient in these cells and strongly increased by LXR activators in an LXR-dependent manner. Modulation of SR-BI protein levels does not explain this increase. In the presence of heparin, which inhibits protein interactions with proteoglycans or of xyloside, a general inhibitor of proteoglycan synthesis, basal and LXR-activated HDL₃-CE uptake were also identified as LXR target genes in macrophages. Although LXRs do not promote cholesterol accumulation via the scavenger receptor pathway, it would be important to know whether LXRs control other pathways to provide cells with cholesterol, namely selective CE uptake.

The goal of this study was thus to investigate the role of LXR agonists in selective CE uptake from HDL₃ (HDL₃-CE uptake) in differentiated human macrophages. We demonstrate that HDL₃-CE uptake is highly efficient in these cells and strongly increased by LXR activators in an LXR-dependent manner. Modulation of SR-BI protein levels does not explain this increase. In the presence of heparin, which inhibits protein interactions with proteoglycans or of β-D-xyloside, a general inhibitor of proteoglycan synthesis, basal and LXR-activated HDL₃-CE uptake are strongly decreased. HDL₃-CE uptake increase is associated with the increase of gene expression, synthesis, and secretion of apoE and LPL, two proteins interacting with proteoglycans. Moreover, methyl-β-cyclodextrin (MBCD), a reagent which destabilizes raft domains, reduces HDL₃-CE uptake and its induction by LXRs. Finally, caveolin-1 and apoE are both strongly induced in raft fractions on LXR activation which could suggest a potential role of caveolin-1 in apoE effects on HDL₃-CE uptake.

In conclusion, these results suggest that LXR activation increases HDL₃-CE uptake through upregulation of secreted proteins interacting with proteoglycans, namely apoE and LPL, and this regulation requires raft domain integrity and may involve caveolae.

### Materials and Methods

An expanded Materials and Methods section is available online (please see http://atvb.ahajournals.org).

#### Isolation, Culture, and Activation of Human Monocyte-Derived Macrophages

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient centrifugation of healthy normolipemic donor blood. On day 12 of the primary culture, human monocyte-derived macrophages (HMDMs) were incubated in serum-free RPMI medium supplemented with 1% Nutridoma HU (Roche). HMDMs were activated for 48 hours with synthetic LXR ligands T0901317 (1 μmol/L) or GW3965 (1 μmol/L) or with the natural ligand 22-R-Hydroxycholesterol (1 μmol/L) or DMSO (control cells).

#### Lipoprotein Interaction With Cells

Selective uptake of [³H]-cholesterol esters from HDL₃ and cell-association of [¹²⁵I]-labeled HDL₃ were studied on HMDMs.

#### Lipid Analysis

Total lipids were extracted by chloroform/methanol (2/1, vol/vol). Free cholesterol (FC) and CE of each sample were separated by thin layer chromatography (TLC). Corresponding spots were scraped and radioactivity counted.

#### mRNA Analysis

RNA were extracted by Trizol and SR-BI, apoE, LPL, and caveolin-1 mRNA expression levels were analyzed by quantitative polymerase chain reaction (PCR).

#### Raft Domain Isolation

Raft domain isolation was performed by discontinuous sucrose gradient method and GM1 was used as raft domain marker.

#### Immunoblot Analysis

On postnuclear supernatants, on chloroform/methanol precipitated cell proteins from each fraction of the sucrose gradients and on trichloroacetic acid (TCA) precipitated proteins from culture supernatants, immunoblot was performed followed by SR-BI, apoE, LPL, and caveolin-1 protein analysis.
Immunofluorescence Staining

Primary human monocytes were grown on BD Falcon CultureSlides (1.10^6 cells per well) in presence of M-colony stimulating factor (CSF) to promote their differentiation to macrophages. Caveolin-1 and apoE were immunolocalized by confocal microscopy.

Statistical Analysis

Statistically differences between control and activated conditions were analyzed by Student t test and were considered significant when P≤0.05 (*P<0.05; **P<0.01).

Results

LXR Activation Increases Selective CE Uptake From HDL3 but Does Not Induce CE Accumulation

To determine whether human macrophages display a selective CE uptake from HDL3, HMDMs were incubated with [3H]-CE-HDL3 or [125I]-HDL3 for 4 hours at 37°C. As previously described,5 HDL3-CE uptake level is much more important than HDL3 or [125I]-HDL3 for 4 hours at 37°C. As previously described,5 HDL3-CE uptake level is much more important than HDL3 or [125I]-HDL3 for 4 hours at 37°C. As previously described,5 HDL3-CE uptake level is much more important than HDL3 particle accumulation.

Induction of HDL3-CE Uptake After LXR Activation Is Not Linked to SR-BI Modulation but Is Dependent on Proteoglycan-Protein Interactions

First, we verified whether SR-BI protein modulation could explain HDL3-CE uptake regulation by LXRs. HMDMs were incubated with LXR agonists for 48 hours and SR-BI expression measured. Although SR-BI mRNA level was slightly increased, SR-BI protein expression in cellular post nuclear extracts was not modified (supplemental Figure II). Although a role of SR-BI in basal selective uptake in HMDMs cannot be excluded, HDL3-CE uptake induction by LXRs is not attributable to change in SR-BI protein expression.

It was reported that selective CE uptake mechanisms could be independent on SR-BI.21,22 Two complementary approaches were used to assign a role of proteoglycans: (1) HDL3-CE uptake was measured with or without heparin which removes proteins interacting with proteoglycans,14 (2) caveolin-1 and apoE were immunolocalized by confocal microscopy.

HDL3-CE uptake was measured in the presence or the absence of β-d-xyloside which inhibits proteoglycan synthesis.15 CE uptake from HDL3 in control conditions and its upregulation by T0901317 or GW3965 were completely abolished on heparin incubation (Figure 2A). In the presence of β-d-xyloside, basal HDL3-CE uptake was strongly decreased and no longer induced after LXR activation with the natural ligand 22-R-Hydroxycholesterol (Figure 2B). LXR activation by T0901317 still enhanced HDL3-CE uptake but to a lesser extent than in the absence of β-d-xyloside.

Hence, HDL3-CE uptake appears to be dependent not only on proteoglycans but also on interacting proteins both in basal and LXR activated conditions. Thus, induction of expression of proteins which interact with proteoglycans likely plays a role in the upregulation of HDL3-CE uptake after LXR activation.

LXR Activation Increases Intracellular Pools and Secretion of ApoE and LPL

ApoE and LPL are proteins that interact with proteoglycans and are involved in the mechanism of CE uptake.17,22
Because LPL and apoE are also LXR target genes in certain cell lines,\textsuperscript{23,24} the regulation of expression of apoE and LPL by LXR was studied in HMDMs. Incubation of HMDMs with synthetic LXR ligands for 48 hours resulted in a strong increase of apoE and LPL gene expression and a tendency with the natural ligand (Figure 3A and Figure 3B). A 48-hour LXR activation with T0901317 led to a strong induction of LPL and apoE secretion in the culture medium, which was paralleled with an induction of intracellular apoE.

To determine the relevance of a potential role of secreted apoE and LPL in the induction of HDL\textsubscript{3}-CE uptake, HMDMs were incubated with \textsuperscript{3}H\textendash{}CE\textendash{}HDL\textsubscript{3} for 4 hours at 37°C with or without exogenous free human apoE (10 \textmu g/mL) or exogenous bovine LPL (100 ng/mL). Our results show that free apoE and LPL induce an increase of HDL\textsubscript{3}-CE uptake (Figure 3C), moreover heparin treatment decreases this induction.

In the human adrenal cell line NCI-H295R which does not secrete apoE, the LXR ligand T0901317 did not influence HDL\textsubscript{3}-CE uptake (1.542±0.113 \textmu g TC/mg cellular proteins in control cell versus 1.608±0.122 \textmu g TC/mg cellular proteins in T0901317 activated cells). Additionally, we have previously shown that exogenous apoE strongly increases HDL\textsubscript{3}-CE uptake in this cell line.\textsuperscript{17} Taken together these data support the hypothesis that apoE is important in the upregulation of the CE uptake through LXR activation in HMDM.

Figure 3. LXR activation induces apoE and LPL expression and secretion in HMDMs. A and B, HMDMs were activated with LXR ligands. Normalized ApoE and LPL mRNA levels were expressed relative to levels in untreated cells set at 1. ApoE and LPL were immunodetected in postnuclear extracts and culture medium. C, HMDMs treated or not with heparin were incubated with \textsuperscript{3}H\textendash{}CE\textendash{}HDL\textsubscript{3} in the presence of free apoE or LPL. *\textit{P}<0.05; **\textit{P}<0.01.

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Therefore, the increase in HDL₃-CE uptake by LXR ligands could be mediated through upregulation of apoE and LPL which interact with proteoglycans.

**Basal and LXR-Induced CE Uptake Involve Raft Domains and Correlate With Caveolin-1 Expression**

Lipid rafts are specialized membrane domains involved in selective CE uptake in certain cell types. Methy]-β-cyclodextrin (MBCD) is a cholesterol-binding reagent used to deplete plasma membranes from cholesterol, thus disrupting structural integrity of raft domains. Incubation of cells with MBCD led to a decrease of HDL₃-CE uptake in control and LXR-activated cells (Figure 4A).

Because regulation of HDL₃-CE uptake by LXR was sensible to disruption of raft domains by methyl-β-cyclodextrin, it was analyzed whether LXR modulates expression of caveolin-1, the major protein involved in caveolae formation.

Caveolin-1 mRNA and protein were detected in HMDM cells in basal condition (Figure 4B and 4C). In LXR activated cells caveolin-1 mRNA (Figure 4B) and protein (Figure 4C) were strongly increased compared to control cells suggesting that LXR activation could participate in lipid homeostasis via the modulation of caveolin-1 expression.

**SR-BI, ApoE, LPL, and Caveolin-1 Are Located in Raft Domains and LXR Induces Caveolin-1 and ApoE in These Domains**

SR-BI-mediated HDL₃-CE uptake may be related to its localization in raft domains. Analysis of GM1 allows locating raft domains among different fractions isolated by a discontinuous sucrose gradient (see Materials and Methods; Figure 5A). Presence of SR-BI in raft (fraction 3 represents 57±3% of total SR-BI) and in nonraft fractions (fractions from 4 to 11 represent 43±4% of total SR-BI) was not significantly different after LXR activation. A significant amount of apoE or LPL was found in rafts (apoE and LPL respectively represent approximately 25% and 37% of total protein). On LXR activation, apoE increased to the same extent both in raft and in nonraft fractions whereas LPL was induced in both fractions but to a higher extent in the nonraft fractions. LXR activation increased caveolin-1 protein expression in the raft fraction.

**Discussion**

LXRs are nuclear receptors involved in lipogenesis and lipoprotein metabolism. LXRs have emerged as key regulators of macropage cholesterol homeostasis, and particularly in the process of lipid efflux to extracellular acceptors. Although LXRs have no effect on the cholesterol entry via the scavenger receptor pathway, LXR regulation of selective CE uptake from lipoproteins has not yet been reported. In vitro and in vivo experiments demonstrated that SR-BI is a physiologically relevant HDL₃ receptor that can mediate selective CE uptake in the liver and in steroidogenic tissues as well as in primary human macrophages. In this study we confirm that, in HMDMs, selective CE uptake from HDL₃ is highly efficient but with high interindividual variations. This uptake is independent of HDL₃ internalization and degradation. CE uptake from HDL₃ by LXR ligands is highly stimulated. This increase is found for each macrophage preparation with a variable factor from 1.7 to 7 (22 donors) and is LXR-dependent as it is lost after LXRα/β mRNA silencing. Although SR-BI contributes to basal HDL₃-CE uptake, it appears not to account for the stimulation of CE uptake on LXR activation.

After a 4-hour incubation with [³H]-CE-HDL₃, analysis of the radioactivity in cells revealed a paralleled increase in intracellular CE and FC in LXR activated HMDMs without any modification of the CE/TC ratio. Thus, despite the increase of CE uptake by LXR activation, there is no CE accumulation and, hence, no stimulation of foam cell formation. LXR ligands activate efflux to apoAI and to HDL by inducing ABC transporters A1, G1, and G4. We thus hypothesize that LXR activation leads to a dynamic recycling of cholesterol which was evidenced by the fact that LXR...
activation simultaneously upregulates CE uptake and free cholesterol efflux to HDL under the studied experimental conditions (supplemental Figure III).

Our results demonstrated that proteins interacting with proteoglycans are probably involved in the HDL₃-CE uptake regulation by LXR activation because preincubation of HMDMs with β-D-xyloside or heparin led to a significant decrease of basal selective CE uptake and attenuated induction after LXR activation. Assessment of the relationship between these functional results and the modulation of expression of two LXR targets known to play a role in CE uptake and to interact with proteoglycans, ie, apoE and LPL,¹⁷,²²,²³,²⁴ showed that LXR activation increased apoE and LPL expression and secretion. Moreover, in HMDMs, free exogenous apoE and LPL were able to induce HDL₃-CE uptake, an effect that was inhibited by heparin. The effect of exogenous apoE and LPL on HDL₃-CE uptake thus appears to be dependent on proteoglycans.

Figure 5. Caveolin-1, SR-BI, LPL, and apoE are located in the raft fraction and LXR activation induces upregulation and colocalization of apoE and caveolin-1. HMDMs were activated by LXR ligands. A, Caveolin-1, SR-BI, LPL, and ApoE were immunodetected in cell lysates fractioned with a discontinuous sucrose gradient. B, Caveolin-1 and apoE were immunolocalized in fixed and permeabilized cells. Microscopy images are representative of 2 donors.
Raft domains have been shown to be involved in CE uptake.25 Both basal CE uptake and its upregulation by LXR ligands are highly dependent on raft integrity because methyl-β-cyclodextrin decreases HDL₃-CE uptake in both conditions. It has been demonstrated that caveolin-1 protein expression is sufficient for caveolae formation at the cell surface.33–35 Caveolin-1 protein is expressed at low levels in primary human macrophages but LXR activation strongly enhances its expression, which could result in a modification of cell surface microdomain composition. Such changes could influence lipid uptake and efflux properties.36 Moreover, we observed that, on LXR activation, apoE expression increased in the different intracellular pools, in particular in the raft fractions, and is colocalized with caveolin-1. Thus the increase of apoE in raft domains and subsequent colocalization with other proteins (especially caveolin-1 and SR-BI) could be involved in the induction of CE uptake by LXR ligands.

In conclusion, selective CE uptake from HDL₃ in HMDM is a specific phenomenon which is upregulated by natural and synthetic LXR ligands in an LXR-dependent manner. This regulation may implicate several partner proteins, i.e., apoE, LPL, and caveolin-1. Although the function of each protein is not clearly elucidated, this regulation is highly dependent on proteoglycans and specialized membrane domains, namely raft domains and caveolae. Interestingly LXR activation induces a dynamic intracellular phenomenon because the increase of HDL₃-CE uptake leads to cellular cholesterol mobilization associated with an increase in cholesterol efflux, avoiding a cholesterol accumulation in macrophages. We previously showed that LXR activation in HMDMs does not affect neutral CE hydrolase activity, nor acyl-coenzyme A (CoA):cholesterol acyltransferase 1 (ACAT) expression, 2 enzymes involved in cellular cholesterol homeostasis, but rather upregulates NPC proteins implicated in transport of cholesterol from intracellular pools to the plasma membrane.8 Our results suggest that LXR activation could accelerate intracellular dynamics of cholesterol resulting, despite the increase in CE uptake, in an increase of free cholesterol available for efflux to apoAl or HDL. Subsequently, the remodeled HDL with less cholesterol could have improved activity in reverse cholesterol transport as previously suggested.37

Acknowledgments
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Disclosures
None.

References

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Selective CE uptake from HDL₃ by HMDM. T0901317 does not influence cellular association and degradation of HDL₃.

A: HMDM were preincubated with RPMI alone for 1 hour and were subsequently incubated for 4 hours at 37°C with [³H]-CE-HDL₃ (7.5 µg TC/ml) for CE uptake measurement (white column) or with [¹²⁵I]-HDL₃ (7.5 µg TC/ml) for protein cell-association (striped column) and degradation (grey column) evaluation.

B and C: HMDM were activated for 48 hours without or with T0901317 (1 µM), followed by preincubation with RPMI alone for 30 min. Cells were then incubated for 4 hours at 37°C with [¹²⁵I]-HDL₃ (7.5 µg TC/ml) to measure cell-association (B) and degradation (C).

Results are expressed in equivalent µg TC/mg cellular proteins as means +/- S.D. of two independent experiments (performed with cells from two different donors) and performed in triplicate.
**Additionnal figure II**

**A**

SR-BI/cyclophilin mRNA level

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**B**

SR-BI/α-Actin protein level

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**T0901317 does not modify SR-BI protein expression in HMDM.**

HMDM were activated or not with the synthetic LXR ligands T0901317 (1 µM) or GW3965 (1 µM) or with the natural LXR ligand 22-R-Hydroxycholesterol (22OHChol 1 µM) for 48 hours.

A : Quantitative PCR analysis of SR-BI was performed on RNA isolated from control and LXR activated HMDM, normalized to cyclophilin mRNA and expressed relative to levels in untreated cells set at 1. Results are from a representative experiment performed in triplicate.

B : 50 µg of postnuclear extracts were separated by reducing SDS-PAGE, electro-transfered onto nitrocellulose and subsequently incubated with polyclonal anti-SR-BI antibodies. SR-BI protein abundance was normalized to actin and expressed relative to levels in untreated cells set at 1. Immunoblots are representative of results obtained with four donors.
T0901317 up-regulates both HDL$_3$-CE uptake and cholesterol efflux to HDL.

HMDM were cholesterol loaded by a 48 hour incubation with acetylated LDL (either $[^3]$H-cholesterol labeled (B) or not (A)) and activated or not with T0901317 (1 µM).

A : After 1 hour in RPMI alone, HMDM were incubated for 4 hours at 37°C with $[^3]$H-CE-HDL$_3$ (7.5 µg TC/ml) for CE uptake measurement.

B : HMDM were incubated for 4 hours with unlabelled HDL$_3$ (7.5 µg/ml) and specific cholesterol efflux was measured by counting radioactivity in cells and culture media. Specific efflux for control cells was set at 1.
Expanded materials and methods

**Materials**

[^125I]-iodine was purchased from PerkinElmer and [³H]-cholesteryl esters from Amersham. RPMI-1640/glutamax medium, PBS, Gentamycine (10 mg/ml) and Prolong Gold antifade reagent were supplied by Invitrogen Life Technologies, pool of human sera by Promo Cell (Heidelberg, Germany), Nutridoma HU by Roche Diagnostics, and Primaria 6-well plates by Becton-Dickinson. Short-interfering (si)RNA specific for human LXRα and LXRβ (SMARTpool siRNA) and non silencing control siRNA were purchased from Dhharmacon. Antibodies against human apoE were mouse monoclonal antibodies (homemade (1)). Antibodies against SR-BI were rabbit polyclonal antibodies raised against the 470-509 COOH terminal part of SR-BI (2). Antibodies against actin were goat polyclonal antibodies purchased from Santa Cruz Biotechnology, Inc. Antibodies against LPL were mouse monoclonal antibodies purchased from Abcam. Antibodies against caveolin-1 were rabbit polyclonal antibodies purchased from BD Transduction Laboratories. Peroxidase labeled goat anti-mouse, rabbit anti-goat and goat anti-rabbit antibodies were from Chemicon International. Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG were from Molecular Probes. P-nitrophenyl-β-D-xylopyranoside, methyl-β-cyclodextrin, molecular mass standards, purified bovine LP, HRP-conjugated cholera toxin β sub-unity (CTXB), 4-Morpholine-EthaneSulfonic Acid (MES) and SYBR Green 0.33X were supplied by Sigma-Aldrich, heparin by Elkins-Skinn INC (Cherry Hill NJ), ECL (Enhanced ChemiLuminescent substrat for detection of HRP) by Pierce, protease inhibitors “Complete” by Roche (Mannheim, Germany), Trizol by Life Technologies
(France), Brillant Quantitative PCR Core Reagent Kit mix by Stratagene. LXR ligands were stored in dimethylsulfoxide at 10 mM. Free apoE3 is a generous gift of G. Siest (3).

Isolation, culture and activation of human monocyte-derived macrophages

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient centrifugation from blood of healthy normolipemic donors and cultured on Primaria in RPMI-1640/glutamax medium containing 10% human serum (4). On day 8, human monocyte-derived macrophages (HMDM) were changed. On day 12 of the primary culture, HMDM were incubated in a medium without serum supplemented with 1% Nutridoma HU. HMDM were activated with the LXR ligands T0901317 or GW3965 or 22-R-Hydroxycholesterol (1µM) for 48 hours.

To silence LXRα and LXRβ, HMDM were transfected with short-interfering siRNA specific for human LXRα and LXRβ (SMARTpool siRNA) using the transfection reagent DharmaFECT Reagent 4 at day 12. Non-silencing siRNA was used as control. Twenty four hours after transfection, HMDM were activated with LXR ligands for 48 hours and HDL₃-CE uptake was then measured.

To inhibit proteoglycan synthesis, cells were incubated for 20 h with 1 mmol/l p-nitrophenyl-β-D-xylopyranoside (β-D-xyloside), a general inhibitor of proteoglycan synthesis (5) during the second day of cell activation by LXR ligands and during the lipoprotein uptake assay.

To inhibit interactions of proteins with proteoglycans, cells were incubated for 1 h with 30 U/ml of heparin and during the lipoprotein uptake assay.

To disrupt integrity of raft domains, HMDM were incubated with methyl β-cyclodextrin (5 mM) for 1 hour and during the lipoprotein uptake assay.
For microscopy studies, PBMC were grown on BD Falcon CultureSlides in presence of M-CSF (50 ng/ml) to promote their interaction on wells and their differentiation to macrophages. On day 8, HMDM were changed for RPMI medium containing 10 % human serum and free of M-CSF.

**Lipoprotein isolation and labelling**

High Density Lipoprotein fraction 3 (HDL₃) were prepared from human plasma by sequential ultracentrifugation at density 1.12 g/ml < d < 1.21 g/ml (6). Lack of apoE in HDL₃ subfraction was tested by ELISA (1). The ratio of protein/cholesterol (w/w) in the HDL₃ fractions used in the course of this study is as expected 4.0 +/- 0.2 SD, measured by enzymatic assay for cholesterol (Cholesterol FS, Diasys, Diagnostic Systems International) and Bradford protein assay (7). Native HDL₃ were labelled with [³H]-CE as previously described (3). The final specific activities varied between 45 and 400 dpm per ng of cholesterol. HDL₃ were labelled in protein moiety with [¹²⁵I]-iodine using the iodine monochloride method (8). The final specific activities varied between 400 and 1500 dpm per ng of protein.

**Cell-association of [¹²⁵I]-labeled lipoproteins**

HMDM were washed with PBS, preincubated for 1 hour at 37°C in serum free medium containing 0.5 % Bovine Serum Albumin. Then, cells were incubated for 4 hours at 37°C with [¹²⁵I]-HDL₃ at 7.5 µg total cholesterol (TC)/ml (equivalent to 30 µg protein/ml) without (total cell-association) or with a 40-fold excess of unlabeled HDL₃ (non specific cell-association). Specific cell-association was calculated as the difference between total and non-specific binding. Cells were washed, dissolved with 1mol/l
NaOH and cell associated radioactivity was counted. An aliquot was used to quantify cellular proteins (7). Apolipoprotein degradation of lipoproteins was measured in cell medium after incubation at 37°C and corresponds to the non TCA precipitable material (9). Results were expressed as µg of cell-associated or degraded HDL₃ per mg of cellular proteins. Results in µg HDL₃ protein per mg cellular proteins are recalculated and expressed as µg HDL₃ TC per mg cellular protein by using the protein/cholesterol ratio (w/w, 4/1).

**Selective uptake of [³H]-cholesteryl esters from HDL₃**

HMDM were washed and preincubated for 1 hour at 37°C in serum free medium and incubated for 4 h with [³H]-CE-HDL₃ at 7.5 µg TC/ml without (total uptake) or with a 40-fold excess of unlabeled HDL₃ (non specific uptake). Cells were then washed, dissolved with 1 mol/l NaOH and cell-associated radioactivity was counted. An aliquot was used to quantify cell proteins (7). Results are expressed as µg HDL₃-CE uptake per mg cellular proteins.

**Analysis of free cholesterol and cholesteryl ester by Thin Layer Chromatography**

HMDM, previously incubated with [³H]-CE-HDL₃, were rinsed twice with PBS and intracellular lipids were extracted by hexane/isopropanol (3:2, vol/vol) and separated by thin layer chromatography. Spots corresponding to free cholesterol (FC) and cholesteryl esters (CE) were scraped and radioactivity measured by scintillation counting. Results expressed as number of cpm (count per minute) associated to CE and FC and percentage of FC/(CE+FC) and CE/(CE+FC) are mean +/- S.D. of two independent experiments performed in triplicate.
**RNA extraction and analysis**

Total cellular RNA were extracted from HMDM using Trizol. For quantitative PCR, total RNA were reverse transcribed (RT) using random hexameric primers and Superscript reverse transcriptase (Life Technologies, France). cDNA were quantified by real-time PCR on a MX 4000 apparatus (Stratagene), using specific primers (apoE : 5’– TCA GCT CCC AGG TCA CCC AG -3’ and 5’–GCG CCG CCT GCA GCT CCT TG –3’ ; SR-BI : 5’- CCT CGG AAA ACA ATG GAG TGA GCA –3’and 5’– CAC AGG TTT GCC CCA GGG TCC A –3’ ; LPL : 5’– CCG CCG CCG ACC AAA GAA GAG ATT –3’ and 5’– CCC GTG ACA GCC AGT CCA CCA CAA T –3’ ; caveolin-1 : 5’- CCA AGG AGA TCG ACC TGG ACC TCA ACC -3’ and 5’- AAA GAG GGC AGA CAG CAA GCG G -3’ ; cyclophilin : 5’– GCA TAC GGG TCC TGG CAT CTT GTC C –3’ and 5’– ATG GTG ATC TTC T TG CTG GTC TTG C –3’). PCR amplification was performed in a volume of 20 µl containing 1 µl of RT product, 100 nmol/L of each primer, 4 mmol/l MgCl₂, the Brillant Quantitative PCR Core Reagent Kit mix and SYBR Green 0.33X. The conditions were 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 30 seconds at 72°C. SR-BI, apoE, LPL and caveolin-1 mRNA levels were subsequently normalized to cyclophilin mRNA.

**Isolation of raft domains by discontinuous sucrose gradient**

HMDM were washed three times then lysed and fractioned according to a method previously described by Smart *et al.* and modified by Song *et al.* (10), (11). Briefly, cells were washed, lysed on ice with 500 mM Na₂CO₃ (pH 11), with a protease inhibitor cocktail and kept on ice for 30 min. Lysates were homogenized, then sonicated (3*10s) using an ultrasonic processor. The lysates were adjusted to 45% sucrose by adding an equal volume of 90% sucrose in MES-buffer saline (MBS) (150 mM NaCl, 25 mM MES, 5 mM EDTA, pH 8.2).
The samples were placed in ultracentrifuge tubes then overlaid with a gradient of 5 ml of 30% sucrose in the buffer MBS/500 mM Na$_2$CO$_3$ (v/v) followed by 3 ml of 5% sucrose in the buffer MBS/500 mM Na$_2$CO$_3$ (v/v). The gradients were centrifuged at 39,000 rpm at 4°C for 17 hours in a SW41 rotor (Beckman Coulter, Palo Alto, CA). Then 1 ml fractions were harvested from the top of the gradients. We obtained 10 fractions of 1 ml where fraction 1 is the lightest fraction, and fraction 10 the heaviest one.

**IMMUNODETECTION**

**Analysis of Postnuclear supernatant**

HMDM were washed three times with PBS at 4°C. Then cells were lysed in 50 µl of buffer containing protease inhibitors, vortexed for 10 seconds and were kept for 10 minutes on ice. Lysates were centrifugated at 10000 rpm (Eppenforf Centrifuge 5417R – ROTOR GE 040) at 4°C for 15 minutes, supernatants collected and proteins quantified (7). Postnuclear supernatants (50 µg) were resuspended in Laemmli buffer then boiled at 100°C for 5 min and separated by SDS PAGE (12%) under non reducing condition for SR-BI and caveolin-1 analysis and under reducing condition for apoE and LPL analysis.

**Analysis of cell culture medium**

250 µl from culture supernatant were collected, precipitated by trichloracetic acid (10%) and resuspended in Laemmli buffer then boiled at 100°C for 5 min and separated by SDS PAGE (12%) under reducing conditions for apoE and LPL analysis.
**Analysis of fractions from discontinuous sucrose gradients**

For Dot Blot analysis, 50 µl from each fraction of discontinuous sucrose gradient were dotted onto nitrocellulose sheet (12). For Western immunoblotting, 200 µl of each fraction of discontinuous sucrose gradient were precipitated by the chloroform/methanol method (13) and resuspended in Laemmli buffer, boiled at 100°C for 5 min and separated by SDS PAGE (12%) under non-reducing conditions. Equicharge of the gel was verified by cellular protein measurement.

After transfer onto nitro-cellulose sheets, immunoblots were revealed with anti-SR-BI (1/1000), anti-apoE (1/1000), anti-LPL (1/250), anti-caveolin-1 (1/250) or anti-actin (1/1000) antibodies. After incubation with primary antibodies, sheets were incubated with peroxidase labeled goat anti-mouse or goat-anti rabbit antibodies both at 1/1000 and revealed by enhanced chemiluminescence detection (ECL). Ponceau staining of membranes was used to detect molecular mass standards. Amounts of protein were determining using Quantity One software (Biorad). Postnuclear supernatant proteins were normalized by actin determination.

Dot Blots were revealed either with peroxydase labeled toxin B to locate GM1 marker of rafts, or with monoclonal anti apoE or monoclonal anti LPL and next with goat anti mouse antibodies.

**Immunofluorescence staining**

After a 48 hour activation by T0901317 (1 µM), cells were briefly washed once with culture medium then fixed with 4% paraformaldehyde at 4°C during 30 minutes. Cells were washed three times with TN buffer (Tris 20 mM pH 7.4, NaCl 0.5 M) and incubated one hour
with lysine 0.1 M. Cells were then permeabilized with saponin 0.05% for 5 min and were washed two times with TN buffer. Cells were blocked by incubation in TNO buffer (TN buffer + 0.5% ovalbumin) and the following primary antibodies were used in an overnight incubation at 4°C at a dilution in TNO buffer of 1:20 for rabbit polyclonal anti-caveolin-1 and 1:40 for mouse monoclonal anti-apoE. The cells were washed three times for 5 min with TNO buffer and were incubated with a mixture of Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 568-conjugated goat anti-mouse IgG in TNO at a dilution of 1:200 at room temperature for 30 min. After washing, the slides were incubated with RNase A (1 mg/ml) and TO-PRO-3 (1.5 µM) to visualize the nuclei. Samples that were incubated without a primary antibody served as controls. The cells were washed three times for 5 min with TN buffer and were mounted in prolong Gold antifade reagent. Laser scanning microscopy was performed using a confocal microscope TCS SP Leica (Lasertechnichk GmbH), at the magnitude of X 63. Image processing and image analysis were performed with the software ImageJ and co-localisation was evidenced with the algorithms previously described (14, 15).

**Statistical analysis**

Statistically differences between control and activated conditions were analysed by Student’s test and were considered significant when $p \leq 0.05$ (*: $p<0.05$) (**: $p<0.01$).


Figure legends on line

**Figure 1**: LXR activation increases selective CE uptake from HDL₃ but not CE accumulation

A: HMDM were activated for 48 hours with T0901317, GW3965, 22-R-Hydroxycholesterol (1 µM) or DMSO, followed by preincubation with RPMI alone for 30 min. Cells were incubated with [³H]-CE-HDL₃ (7.5 µg TC/ml) to measure selective CE uptake. Results from a representative experiment out of 22 performed in triplicate (expressed in µg of TC uptake for 4 hours per mg of cellular proteins) are shown.

B: HMDM were transfected with scrambled or synthetic siRNA against LXRα and LXRβ (siRNA LXRα/β). 24 hours later, cells were treated or not with GW3965 (1 µM) for 48 hours and HDL₃-CE uptake was then measured.

C: HMDM activated or not for 48 hours with T0901317 (1 µM) were incubated with [³H]-CE-HDL₃ (7.5 µg TC/ml) for 4 hours. Thereafter, cells were rinsed twice with PBS and intracellular lipids were extracted with hexane/isopropanol and separated by TLC. Results are expressed as number of cpm (count per minute) associated to CE and FC and percentages of CE and FC are indicated (mean +/- S.D. of two independent experiments (two donors) performed in triplicate). NS: not significant ; *: p<0.05 ; **: p<0.01.

**Figure 2**: Heparin and β-D-xyloside inhibit basal and LXR-activated selective [³H]-CE-HDL₃ uptake

A: HMDM were activated for 48 hours with T0901317, GW3965 (1 µM) or DMSO. HMDM were pre-incubated with RPMI with or without heparin (30 U/ml) for 1 hour. Cells were incubated with [³H]-CE-HDL₃ (7.5 µg TC/ml) for 4 hours in the presence or in the absence of heparin (30 U/ml) and selective CE uptake was measured. Results are expressed relative to
levels in untreated cells (control without heparin) set at 1 (mean +/- SD of three independent experiments performed in triplicate).

B: HMDM were activated for 48 hours without or with T0901317 or 22-R-Hydroxycholesterol (1µM). After 24 hours, they were incubated or not with β-D-xyloside (1 mM) for 20 hours. Cells were subsequently incubated with RPMI alone for 1 hour then incubated with [$^3$H]-CE-HDL$_3$ (7.5 µg TC/ml) for 4 hours in the presence or in the absence of β-D-xyloside and selective CE uptake was measured. Results are expressed relative to levels in untreated cells (control without β-D-xyloside) set at 1 (mean +/- SD of two independent experiments performed in triplicate). NS: not significant; *: p<0.05; **: p<0.01.

**Figure 3: LXR activation induces apoE and LPL expression and secretion in HMDM**

A and B: HMDM were activated with T0901317, GW3965, 22-R-Hydroxycholesterol (1 µM) or DMSO for 48 hours. Quantitative PCR analysis of apoE or LPL was performed on RNA isolated from control and LXR activated HMDM. apoE or LPL mRNA levels were normalized to cyclophilin mRNA and expressed relative to levels in untreated cells set at 1. Results are from a representative experiment out of 3 performed in triplicate.

Postnuclear extracts (50 µg) or TCA precipitated proteins (250 µl of culture medium) were separated by reducing SDS-PAGE and electro-transferred onto nitrocellulose and subsequently incubated with monoclonal anti-apoE or monoclonal anti-LPL antibodies. Immunoblots are representative of results obtained with HMDM from four donors.

C: HMDM were preincubated with RPMI with or without heparin (30 U/ml) for 1 hour. Cells were incubated with [$^3$H]-CE-HDL$_3$ (7.5 µg TC/ml) for 4 hours in the presence or in the absence of heparin (30 U/ml) with or without free human apoE (10 µ/ml) or bovine LPL (100 ng/ml) to measure selective CE uptake. Results are expressed in µg of TC uptake per mg of
cellular proteins (mean +/- S.D. of one experiment performed in triplicate and representative of two experiments). *: p<0.05; **: p<0.01.

**Figure 4:** Basal HDL₃-CE uptake and its increase by LXR ligands depend on the integrity of raft domains and correlates with caveolin-1 expression

HMDM were activated with T0901317, GW3965, 22-R-Hydroxycholesterol (1 µM) or DMSO for 48 hours.

A: HMDM were preincubated with RPMI with or without methyl-β-cyclodextrin (MBCD) (5 mM) for 1 hour. Cells were incubated with [³H]-CE-HDL₃ (7.5 µg TC/ml) for 4 hours in the presence or in the absence of MBCD (5 mM) and selective CE uptake was measured. Results are expressed in µg of TC uptake per mg of cellular proteins (mean +/- S.D. of one experiment performed in triplicate and representative of experiments performed on HMDM from three donors).

B: Quantitative PCR analysis of caveolin-1 was performed on RNA isolated from control and activated HMDM. Caveolin-1 mRNA levels were normalized to cyclophilin mRNA and expressed relative to levels in untreated cells set at 1. Results are from a representative experiment performed in triplicate with the cells of three donors.

C: Postnuclear extracts (50 µg) were separated by reducing SDS-PAGE, electro-transferred onto nitrocellulose and subsequently incubated with polyclonal anti-caveolin-1 antibody. Immunoblots are representative of results obtained with HMDM from four donors. NS : non significant ; *: p<0.05; **: p<0.01.

**Figure 5:** Caveolin-1, SR-BI, LPL and apoE are located in the raft fraction and LXR activation induces up-regulation and co-localization of ApoE and Caveolin-1

HMDM were activated by T0901317 (1 µM) or DMSO for 48 hours.
A : Cell lysates were fractioned with a discontinuous sucrose gradient as described under “Materials and Methods”. Raft fractions were located by a dot blot analysis of Ganglioside GM1: 50 µl of each fraction were dotted onto nitrocellulose sheet then incubated with peroxidase-labeled cholera toxin B and revealed by enhanced chemiluminescence detection. For caveolin-1 and SR-BI protein expression, 200 µl of each fraction were precipitated by chloroform/methanol method and separated by non-reducing SDS-PAGE and electro-transferred onto nitro-cellulose, which was incubated with polyclonal anti-SR-BI or anti-caveolin-1. Immunoblots were representative of three donors. For ApoE and LPL protein expression, 100 µL of each fraction from discontinuous sucrose gradient were dotted onto nitrocellulose sheet then incubated with monoclonal anti-apoE or monoclonal anti-LPL. Dot blots are representative out of two donors.

B : Cells were fixed, permeabilised and incubated with rabbit polyclonal anti-caveolin-1 antibody and mouse monoclonal anti-apoE antibody. After washing, they were then incubated with alexa fluor 488-conjugated goat anti-rabbit IgG (green) and alexa fluor 568-conjugated goat anti-mouse IgG (red). Nuclei were visualized with TO-PRO-3 (blue). Microscopy images are representative out of two donors.