Peroxisome proliferator-activated receptor α controls cellular cholesterol trafficking in macrophages

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Abstract   The mobilization of cholesterol from intracellular pools to the plasma membrane is a determinant that governs its availability for efflux to extracellular acceptors. NPC1 and NPC2 are proteins localized in the late endosome and control cholesterol transport from the lysosome to the plasma membrane. Here, we report that NPC1 and NPC2 gene expression is induced by oxidized LDL (OxLDL) in human macrophages. Because OxLDLs contain natural activators of peroxisome proliferator-activated receptor α (PPARα), a fatty acid-activated nuclear receptor, the regulation of NPC1 and NPC2 by PPARα and the consequences on cholesterol trafficking were further studied. NPC1 and NPC2 expression is induced by synthetic PPARα ligands in human macrophages. Furthermore, PPARα activation leads to an enrichment of cholesterol in the plasma membrane. By contrast, incubation with progesterone, which blocks postlysosomal cholesterol trafficking, as well as NPC1 and NPC2 mRNA depletion using small interfering RNA, abolished ABCA1-dependent cholesterol efflux induced by PPARα activators.■ These observations identify a novel regulatory role for PPARα in the control of cholesterol availability for efflux that, associated with its ability to inhibit cholesterol esterification and to stimulate ABCA1 and scavenger receptor class B type I expression, may contribute to the stimulation of reverse cholesterol transport.—Chinetti-Gbaguidi, G., E. Rigamonti, L. Helin, A. L. Mutka, M. Lepore, J. C. Fruchart, V. Clavey, E. Ikonen, S. Lestavel, and B. Staels. **Peroxisome proliferator-activated receptor α controls cellular cholesterol trafficking in macrophages.** *J. Lipid Res.* **2005.** 46: **2717–2725.**

Supplementary key words   nuclear receptors • gene regulation • atherosclerosis • cholesterol homeostasis

Macrophages play a pivotal role in the development of atherosclerosis. After recruitment in the subendothelial space, monocytes differentiate into macrophages and accumulate lipids, thus forming foam cells. The mobilization of cellular cholesterol to the plasma membrane and

its efflux to extracellular acceptors is an important mechanism in the regulation of cellular cholesterol levels. This constitutes the first step of reverse cholesterol transport (RCT), a pathway of cholesterol transport from peripheral tissues to the liver. In addition to the interaction of the HDL particle with its membrane receptors, such as scavenger receptor class B type I (SR-BI), the availability of cholesterol in the plasma membrane is an important determinant for efficient cholesterol efflux. Within the cells, modified LDL-derived cholesteryl esters (CEs) are hydrolyzed in lysosomes to free cholesterol (FC). This FC is probably initially transported to the plasma membrane, where it integrates the cell membrane (1). Excess cholesterol is transported back to the endoplasmic reticulum, where it is reesterified by ACAT1and stored as CEs in lipid droplets (2). The plasma membrane contains the highest percentage of cellular cholesterol (3). It is likely that maintenance of this cellular cholesterol equilibrium depends on specific intracellular transport processes.

Trafficking of cholesterol from the late endosome/lysosome to the plasma membrane is a process controlled by a network of proteins that includes at least two components, namely NPC1 and NPC2 (4). NPC1 is a transmembrane protein containing a sterol-sensing domain localized in the late endosomal compartment, and NPC2 is a soluble endosomal/lysosomal cholesterol binding protein (5, 6). Mutations in the NPC1 and NPC2 genes cause Niemann Pick type C (NPC) disease, a fatal recessive disorder characterized by the accumulation of LDL-derived cholesterol in lysosomes. In the brain, this leads to neuronal degeneration due to a defective movement of sterols out of the expanding pools of lysosomal cholesterol to other locations,

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in particular, the plasma membrane (7). Mutations in NPC1 cause the majority of cases of NPC disease, whereas mutations in the NPC2 gene account for less than 10% of the cases (4).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate the expression of genes controlling lipid and glucose metabolism. PPARα, which is activated by fibrates, fatty acids, and eicosanoids, is highly expressed in liver, heart, muscle, and kidney, and is also present in cells of the arterial wall, including monocytes and macrophages (8). PPARα controls macrophage lipid homeostasis and cholesterol efflux, the first step of the RCT pathway. PPARα activators enhance the expression of the HDL receptor CLA-1/SR-BI (9) and the ABC transporter ABCA1 (10). In addition, PPARα activation inhibits cellular CE formation activity, thus limiting CE accumulation in vascular macrophages and preventing foam cell formation (11). Along with the induction of ABCA1 expression, a decrease in the CE:FC ratio may contribute to an enhanced liberation and efflux of FC to extracellular acceptors, as observed upon PPARα activation (10, 11).

Here, we report that the expression of NPC1 and NPC2 is strongly induced by oxidized LDL (OxLDL) and to a lesser extent by acetylated LDL (AcLDL) in cultured human macrophages in vitro. Because OxLDLs contain natural ligands for PPARα (12), these observations prompted us to study whether PPARα also regulates processes controlling cholesterol mobilization upstream of the efflux step. Our results demonstrate that PPARα activation stimulates the postlysosomal mobilization of cholesterol and induces NPC1 and NPC2 gene and protein expression. This results in an enrichment of cholesterol in the plasma membrane and a redistribution of cholesterol in the external cell surface domains. These results identify a new role for PPARα in the control of macrophage cholesterol trafficking, resulting in an enhanced availability of cholesterol at the cell membrane, where it is presented for efflux.

MATERIALS AND METHODS

Tissue and cell culture

Mononuclear cells isolated by Ficoll gradient centrifugation from blood of healthy normolipidemic donors were suspended in RPMI 1640 medium containing gentamycin (40 mg/ml), glutamine (0.05%), and 10% pooled human serum (13). Differentiation of monocytes into macrophages occurs spontaneously by adhesion of cells to the culture dishes. Mature monocyte-derived macrophages were used for experiments after 10 days of culture. For experiments, medium was changed to medium without serum but supplemented with 1% Nutridoma HU (Boehringer Mannheim).

RNA extraction and analysis

Total cellular RNA was extracted from 10-day-old primary human macrophages treated or not with OxLDL or AcLDL (50 μg/ml) or the PPARα ligands Wy14643 (10, 25, and 50 μmol/l), fenofibric acid (50 μmol/l), ciprofibrate (50 μmol/l), bezafibrate (50 μmol/l), and GW7647 (600 nmol/l) for 24 h using Trizol (Life Technologies, France). For quantitative PCR, total RNA

was reverse transcribed using random hexameric primers and Superscript reverse transcriptase (Life Technologies). cDNA was quantified by real-time PCR on a MX 4000 apparatus (Stratagene), using specific primers for: NPC1, 5′-CTT AGT GCA GGA ACT CTG TCC AGG-3′ and 5′-TCC ACA TCA CGG CAG GCA TTG TAC-3′; NPC2, 5′-GGT TTG TCT TGT GAC CGC-3′ and 5′-AGG AAT GTA GCT GCC AGG-3′; ABCA1, 5′-GTG AAC AGC TCC AGC TCC TCC AC-3′ and 5′-AAG GTC TTG TTC ACC TCA GCC ATG AC-3′; and cyclophilin, 5′-GCA TAC GGG TCC TGG CAT CTT GTC C-3′ and 5′-ATG GTG ATC TTC TTG CTG GTC TTG C-3′. PCR amplification was performed in a volume of 25 μl containing 100 nmol/l of each primer, 4 mmol/l MgCl$_2$, the Brillant Quantitative PCR Core Reagent Kit mix as recommended by the manufacturer (Stratagene), and SYBR Green 0.33× (Sigma-Aldrich). The conditions were 95°C for 10 min, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. NPC1, NPC2, and ABCA1 mRNA levels were subsequently normalized to 28S or cyclophilin mRNA.

Protein extraction and Western blot analysis

Cells were harvested in ice-cold lysis buffer containing PBS, 1% NP-40, and protease inhibitor mixture. Equal amounts of proteins (20 μg) were separated by SDS-PAGE (6% gels for NPC1 detection and 12.5% for NPC2), and transferred to Hybond-C Extra membrane (Amersham). Equal protein loading of the gels was verified by Ponceau's red staining. Membranes were blocked with 5% milk (for NPC1 blots) or 3% fatty acid-free BSA (for NPC2 blots) in Tris-buffered saline and 0.1% Tween 20, and immunoblotted using antibodies against NPC1 (14), NPC2 (15), or α-calnexin (kindly provided by Dr. Ari Helenius). Western blots were scanned, and the intensity of the bands was quantified using TINA 2.1 software.

Labeling of membrane FC with filipin

Human macrophages were pretreated for 24 h and thereafter every 24 h with the PPARα activators bezafibrate (50 μmol/l) or GW7647 (600 nmol/l), and cholesterol-loaded by incubation for 48 h with AcLDL (50 μg/ml) in the presence or absence of progesterone (33 μmol/l) in RPMI 1640 medium supplemented with 1% Nutridoma. After incubation, cells were rinsed twice with ice-cold PBS and fixed with paraformaldehyde (4% in PBS) for 30 min at 4°C before incubation with filipin (50 μg/ml in PBS) for 30 min in the dark at room temperature. Labeling was then visualized by a fluorescence microscope (Axiovert 135 TV Zeiss), and fluorescent signals were quantified using Jimage 1.31v software.

Measurement of cholesterol in the outer plasma membrane layer

Cholesterol content in the outer layer of the plasma membrane was assessed by measuring specific oxidation using exogenous cholesterol oxidase from *Pseudomonas fluorescens* (Sigma, France) (16). Macrophages were pretreated for 24 h and thereafter every 24 h with the PPARα activators Wy14643 (50 μmol/l), bezafibrate (50 μmol/l), ciprofibrate (50 μmol/l), fenofibric acid (50 μmol/l), or GW7647 (600 nmol/l), and cholesterol-loaded by incubation for 48 h with [^{3}H]cholesterol-AcLDL (50 μg/ml) in RPMI 1640 medium supplemented with 1% Nutridoma. Macrophage cultures were then rinsed twice with ice-cold PBS and fixed with paraformaldehyde (4% in PBS) for 30 min at 4°C before incubation for 1 h at 37°C in PBS with or without cholesterol oxidase (1 U/ml). Thereafter, cells were rinsed twice with PBS, and intracellular lipids were extracted by hexane-isopropanol (3:2; v/v) and separated by TLC in petroleum ether-diethyl ether-acetic acid (160:40:2; v/v/v). Spots corresponding to FC and 4-cholestenone were scraped, and radioactivity was measured by scin-

tillation counting. Accessibility of the membrane cholesterol pool to cholesterol oxidase is expressed as the 4-cholestenone:FC ratio.

Cholesterol efflux

Human macrophages were pretreated for 24 h and thereafter every 24 h with the PPARα activator GW7647 (600 nmol/l), and cholesterol-loaded by incubation for 48 h with [³H]cholesterol-AcLDL (50 μg/ml) in RPMI 1640 medium supplemented with 1% Nutridoma. After this incubation period, cells were washed twice in PBS, and cholesterol efflux studies mediated by apolipoprotein A-I (apoA-I) were immediately performed by adding fresh RPMI medium without Nutridoma, containing or not 10 μg/ml of apoA-I for 24 h. [³H]cholesterol radioactivity was measured by scintillation counting in centrifuged medium and in cells after extraction of lipids with hexane-isopropanol. Where indicated, progesterone (33 μmol/l) was added during cholesterol-loading and cholesterol efflux periods.

Small interfering RNA (siRNA)-mediated knock-down of NPC1 and NPC2

Complementary mRNA oligonucleotides derived from the human NPC1 (siRNA ID #8092) and NPC2 (siRNA ID #18116) sequences (Ambion Europe) were used to downregulate NPC1 and NPC2 expression in primary human macrophages. GAPDH siRNA oligonucleotides used as control were also obtained from Ambion. For the cholesterol efflux assays, 10-day-old macrophages were transfected with siRNA using jetSI (Polyplus Transfection, France) according to the manufacturer's instructions and cholesterol-loaded by incubation for 48 h with AcLDL (50 μg/ml, containing [³H]cholesterol) in RPMI 1640 supplemented with 1% Nutridoma in the presence or absence of GW7647 (600 nmol/l). [³H]cholesterol efflux was measured as described above. NPC1 and NPC2 mRNA levels were determined by real-time PCR and normalized to cyclophilin mRNA.

Statistical analysis

Statistically significant differences between groups were analyzed by Student's *t*-test and considered significant at $P \leq 0.05$.

RESULTS

NPC1 and NPC2 mRNA levels are induced by OxLDL in differentiated human monocyte-derived macrophages

NPC1 and NPC2 belong to a network of proteins mediating postlysosomal cholesterol trafficking to the plasma membrane, a crucial process governing the balance between macrophage cholesterol import and export, with potential consequences in atherogenesis. To determine whether foam cell formation is accompanied by induction of NPC1 and NPC2 gene expression, differentiated hu-
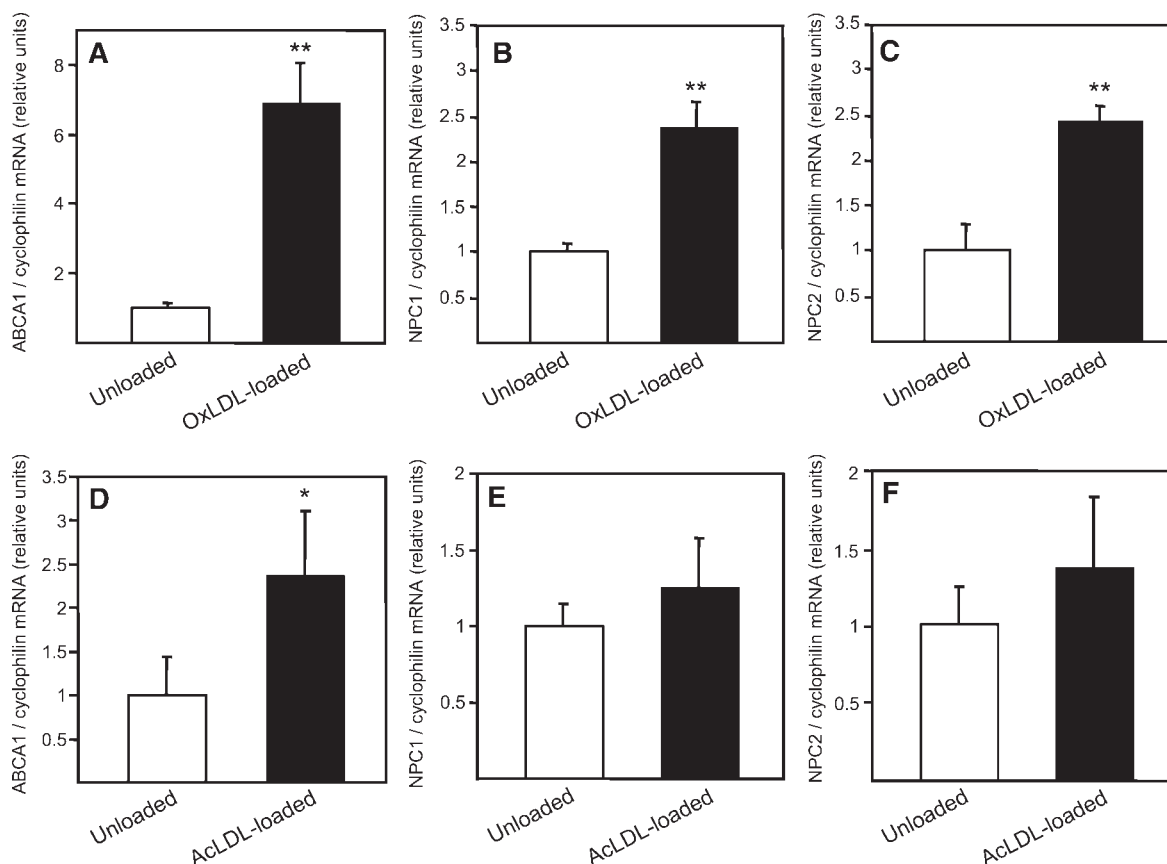

Fig. 1. NPC1 and NPC2 are induced by oxidized LDL (OxLDL) loading but not by acetylated LDL (AcLDL) loading in human macrophages. Quantitative PCR analysis of NPC1 and NPC2 mRNA in primary macrophages treated or not with OxLDL (50 μg/ml) (B, C) and AcLDL (50 μg/ml) (E, F). mRNA levels were normalized to cyclophilin mRNA and are expressed relative to the levels in untreated cells set as 1. Results are the mean ± SD of triplicate determinations, representative of three independent experiments. ABCA1 gene expression was measured as positive target gene for OxLDL and AcLDL (A, D). Statistically significant differences between treatments are indicated (Student's *t*-test; *$P < 0.05$, **$P < 0.01$).
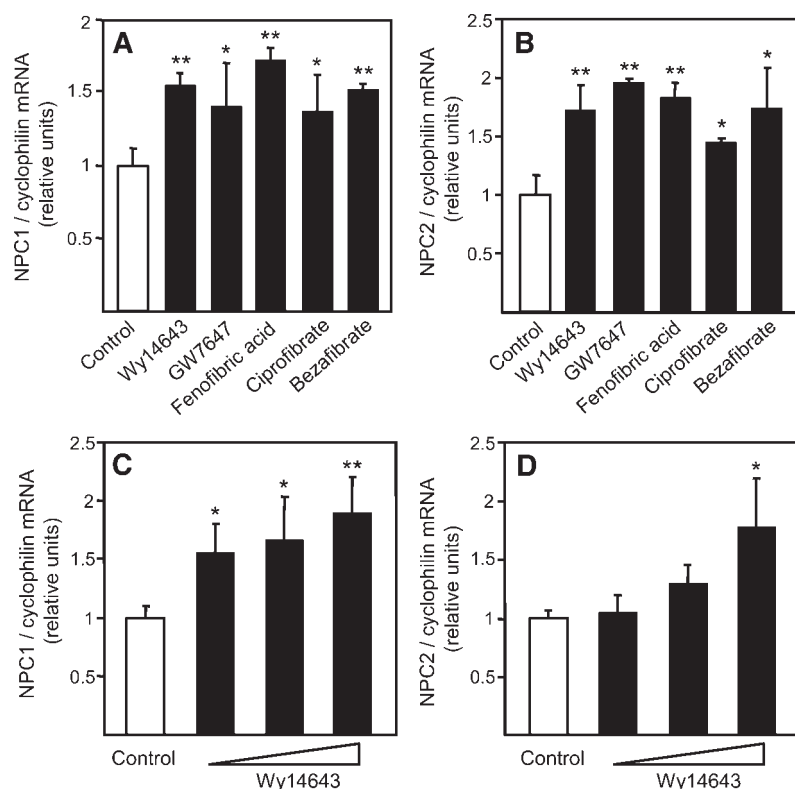
Fig. 2. NPC1 and NPC2 gene expression is regulated by peroxisome proliferator-activated receptor α (PPARα) agonists in human macrophages in a dose-dependent manner. Quantitative PCR analysis of NPC1 (A) and NPC2 (B) was performed on RNA isolated from primary human macrophages treated or not with Wy14643 (50 μM), GW7647 (600 nmol/l), fenofibric acid (50 μmol/l), ciprofibrate (50 μM), or bezafibrate (50 μM) for 24 h. Induction of NPC1 (C) and NPC2 (D) expression in human macrophages treated for 24 h with increasing concentrations of Wy14643 (10, 25, and 50 μmol/l). NPC1 and NPC2 mRNA levels were normalized to cyclophilin mRNA and are expressed relative to the levels in untreated cells set as 1. Results are expressed as mean $\pm$ SD. Statistically significant differences between treatments are indicated (ANOVA followed by Student's t-test; *$P <$ 0.05, **$P <$ 0.01).

man macrophages were incubated with OxLDL or AcLDL (50 μg/ml) and gene expression was analyzed by quantitative PCR. Our results demonstrate that OxLDL, but not AcLDL, strongly induces NPC1 and NPC2 mRNA levels (**Fig. 1B**, **C**, **E**, **F**). As a control, ABCA1 gene expression, whose mRNA levels are positively regulated by OxLDL and AcLDL, was measured (Fig. 1A, D).

PPARα activation increases NPC1 and NPC2 gene and protein expression in primary human macrophages

Because OxLDLs, and the oxidized phospholipids contained therein, are natural ligands for PPARα (12), a nuclear receptor expressed in macrophages controlling cholesterol metabolism (8), we investigated whether the expression of the NPC1 and NPC2 genes is also con-
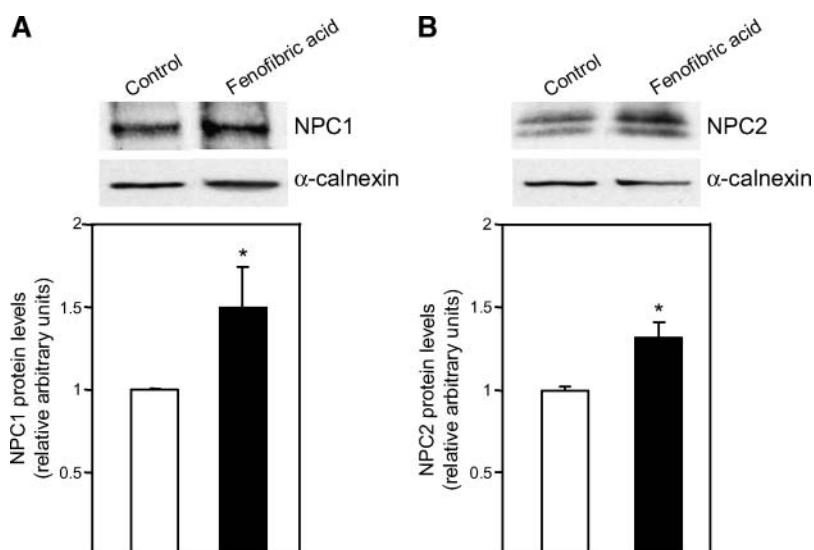

Fig. 3. PPARα activation induces NPC1 and NPC2 protein expression. Cell proteins were isolated from differentiated human macrophages treated or not with fenofibric acid (50 μmol/l) for 24 h, and NPC1 (A) and NPC2 (B) protein levels were measured by Western blot analysis, as described in Materials and Methods. Specific signals were quantified by densitometry. NPC1 and NPC2 protein levels in fenofibric acid-treated macrophages were normalized to those in untreated control cells set as 1. Results are expressed as mean $\pm$ SD of results obtained from three independent cell preparations. Statistically significant differences between treatments are indicated (Student's t-test; *$P <$ 0.05).
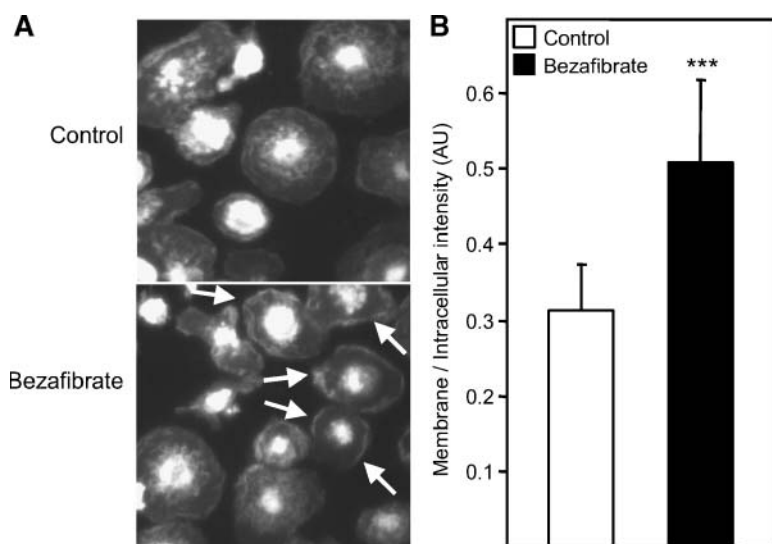
Fig. 4. PPARα activation induces enrichment of cholesterol in the plasma membrane. A: Primary human macrophages were cholesterol-loaded by incubation with 50 μg/ml AcLDL for 24 h. Bezafibrate (50 μmol/l) was added 24 h before and during cholesterol loading. Cells were fixed with paraformaldehyde (4% in PBS for 30 min at 4°C) and stained with filipin (50 μg/ml) for 30 min at room temperature. The figure is representative of two independent experiments. Original magnification, ×40. Arrows indicate cholesterol membrane enrichment in treated cells. B: Fluorescence intensity of filipin signals was quantified using Jimage 1.31v in the perinuclear regions and the plasma membrane. Results are expressed as mean ± SD. Statistically significant differences between treatments are indicated (Student's *t*-test; ***P < 0.001). AU, arbitrary units.

trolled by PPARα. Treatment of primary human macrophages with different PPARα activators [Wy14643 (50 μmol/l), GW7647 (600 nmol/l), fenofibric acid (50 μmol/l), bezafibrate (50 μmol/l), and ciprofibrate (50 μmol/l)] resulted in the induction of NPC1 and NPC2 gene expression, an effect that was concentration dependent (**Fig. 2**).

In addition, PPARα activation using fenofibric acid (50 μmol/l) led to a significant increase in NPC1 and NPC2 protein levels in primary macrophages (**Fig. 3**).

PPARα activation leads to an enrichment of cholesterol in the plasma membrane in primary human macrophages

To determine whether the regulation of genes involved in cholesterol trafficking from the lysosome by PPARα is accompanied by an accumulation of cholesterol in the plasma membrane, filipin staining experiments were performed on differentiated macrophages loaded with AcLDL (50 μg/ml) for 48 h and treated with PPARα ligands. PPARα activation by bezafibrate increased the amount of FC present in the plasma membrane, as demonstrated by an increase in filipin staining (**Fig. 4A**). Quantitative determination of the relative filipin fluorescent signal between the perinuclear and plasma membrane subcellular domains was performed using computer-assisted analysis (Fig. 4B). PPARα activation significantly increased the fluorescence at the plasma membrane level, with a concomitant reduction of the signal intensity in the perinuclear region.

To investigate whether PPARα activation results in a change in cholesterol distribution within the plasma membrane, a cholesterol accessibility test using cholesterol oxidase was performed on [³H]cholesterol-AcLDL-loaded macrophages treated with different synthetic PPARα ligands. Treatment with all PPARα ligands tested increased the amount of FC in the outer layer of the plasma membrane, as indicated by an enhanced accessibility of this cholesterol pool to cholesterol oxidase (**Fig. 5**).

Progesterone blocks the enrichment of plasma membrane cholesterol and cholesterol efflux by PPARα activators

To determine the role of the NPC1 and NPC2 pathway on the induction of lysosomal cholesterol mobilization to the plasma membrane by PPARα, the influence of progesterone (33 μmol/l), an inhibitor of postlysosomal choles-
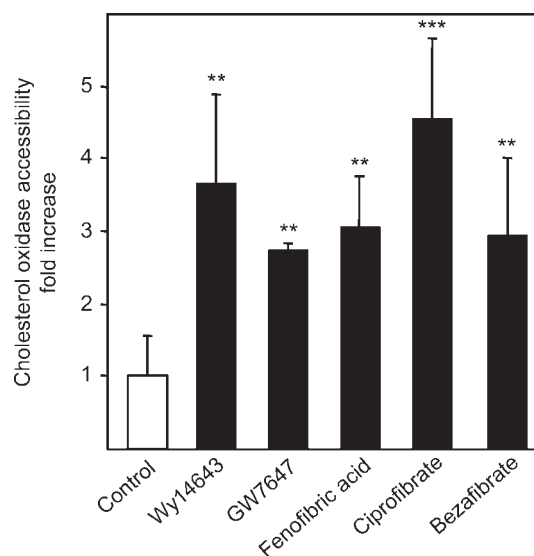

Fig. 5. PPARα agonists increase outer-layer plasma membrane cholesterol content. Human macrophages were pretreated for 24 h and thereafter every 24 h with Wy14643 (50 μmol/l), GW7647 (600 nmol/l), fenofibric acid (50 μmol/l), ciprofibrate (50 μmol/l), and bezafibrate (50 μmol/l) and cholesterol-loaded by incubation with [³H]cholesterol-AcLDL (50 μg/ml) for 48 h. Cells were then fixed with paraformaldehyde before incubation for 1 h at 37°C in PBS with or without cholesterol oxidase from *Pseudomonas fluorescens* (1 U/ml). Cellular lipids were then extracted by hexane-isopropanol and separated by TLC. Spots corresponding to free cholesterol and 4-cholestenone were scraped, and radioactivity was measured by scintillation counting. Results are expressed relative to untreated cells set as 1. Statistically significant differences between treatments are indicated (Student's *t*-test; **P < 0.01, ***P < 0.001). Error bars indicate mean and SD.

terol transport (17), on PPARα activator-induced cholesterol trafficking was assessed in AcLDL-loaded macrophages. As previously shown with bezafibrate (Fig. 4A), treatment with the PPARα activator GW7647 induced an enrichment of cholesterol in the plasma membrane. By contrast, when cells were treated with GW7647 in the presence of progesterone, the inductive effect of the PPARα ligand was lost and filipin staining localized mostly in the perinuclear region, suggesting cholesterol accumulation in this cellular compartment (**Fig. 6A**).

To further investigate the impact of the regulation of cholesterol trafficking by PPARα on the induction of cholesterol efflux, primary human macrophages were loaded with AcLDL (50 μg/ml) for 24 h in the presence or absence of progesterone (33 μmol/l), treated with GW7647 (600 nmol/l), and subsequently exposed to apoA-I for 24 h to induce cholesterol efflux. In the absence of progesterone, apoA-I-specific cholesterol efflux was induced approximately 2-fold in macrophages treated with GW7647 compared with control untreated cells. The presence of progesterone significantly reduced basal efflux and blocked GW7647-stimulated apoA-I-specific efflux (Fig. 6B). These data suggest that the control of cellular cholesterol transport at the lysosomal level by PPARα is a critical intermediate in the induction of cholesterol efflux by its ligands.

Suppression of NPC1 and NPC2 expression by siRNA blocks PPARα-induced cholesterol efflux in human macrophages

To firmly establish the contribution of NPC1 and NPC2 in the regulation of cholesterol efflux by PPARα, an siRNA approach was used to reduce NPC1 or NPC2 gene expression. Quantitative PCR analysis indicated that the specific siRNAs significantly suppressed NPC1 and NPC2 gene expression, by 90% and 70%, respectively, whereas control GAPDH siRNA did not influence their expression (**Fig. 7A**, **B**). Reduction of NPC1 or NPC2 expression led to a significant decrease of basal cholesterol efflux to apoA-I (Fig. 7C). In the absence of PPARα ligand, cholesterol efflux in NPC1 or NPC2 siRNA-transfected macrophages was reduced by 40% and 30%, respectively. Transfection with GAPDH siRNA did not affect cholesterol efflux when compared with untransfected cells (data not shown). However, induction of cholesterol efflux upon stimulation with GW7647 (600 nmol/l) was completely abolished in macrophages transfected with NPC1 or NPC2 siRNA, compared with GAPDH siRNA-transfected macrophages. These observations indicate that the regulation of the NPC1 and NPC2 genes, both involved in the postlysosomal cholesterol mobilization to the plasma membrane, participates in the stimulation of cholesterol efflux by PPARα.

DISCUSSION

Cellular cholesterol homeostasis is a balance of influx, catabolism, endogenous synthesis, and efflux. Cholesterol accumulation in macrophages in the form of cytoplasmic lipid droplets is an early step in the formation of atherosclerotic lesions. Thus, a proper understanding of the mechanisms controlling the uptake and intracellular transport
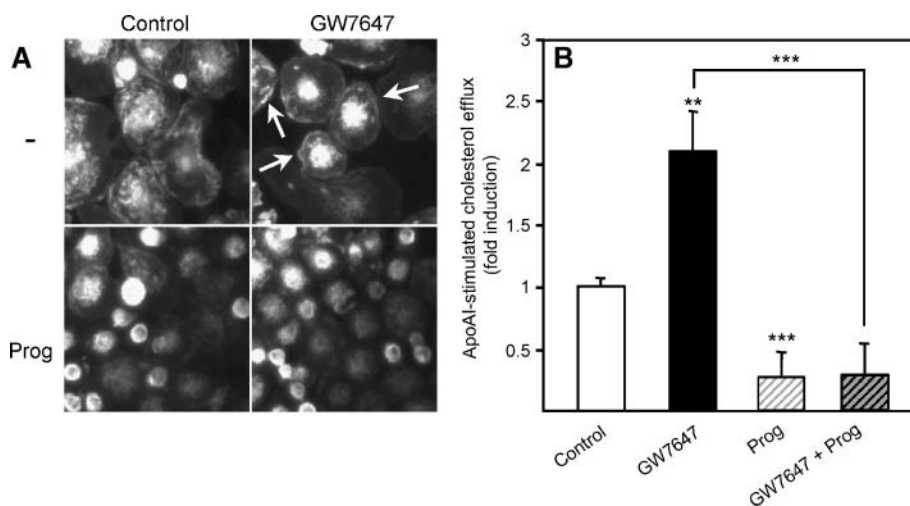

Fig. 6. Induction of plasma membrane cholesterol enrichment and efflux by PPARα activation is blocked by progesterone. A: Primary human macrophages were cholesterol-loaded by incubation with 50 μg/ml AcLDL for 24 h in the presence (Prog) or absence (−) of progesterone (33 μmol/l). GW7647 (600 nmol/l) was added 24 h before and during cholesterol loading. Cells were fixed with paraformaldehyde and stained with filipin (50 μg/ml for 30 min at room temperature). The figure is representative of two independent experiments. Original magnification, ×40. Arrows indicate cholesterol membrane enrichment in treated cells. B: [³H]cholesterol-loaded macrophages were treated or not with GW7647 (600 nmol/l) in the presence or absence of progesterone (33 μmol/l) and subsequently incubated with RPMI 1640 medium with or without apolipoprotein A-I (apoA-I) (10 μg/ml). ApoA-I-induced [³H]cholesterol efflux was measured as described. Values are expressed relative to the untreated controls set as 1. Statistically significant differences between treatments are indicated (Student's *t*-test; **$P < 0.01$, ***$P < 0.001$). Prog, progesterone. Error bars indicate mean and SD.
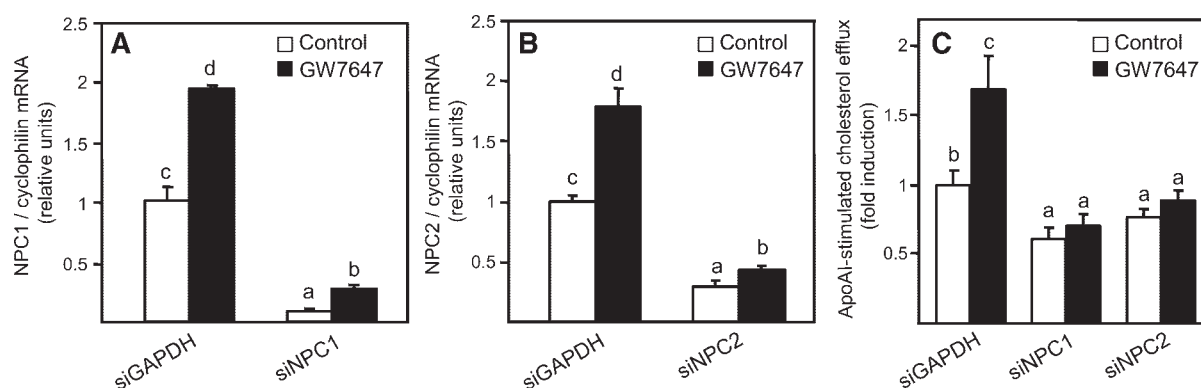
Fig. 7. Suppression of NPC1 and NPC2 expression by small interfering RNA (siRNA) blocks PPARα induction of cholesterol efflux in human macrophages. mRNA levels of NPC1 (A) and NPC2 (B) were determined by quantitative PCR analysis on RNA from siRNA-transfected primary human macrophages treated or not with GW7647 (600 nmol/l) for 24 h. Results are normalized to cyclophilin mRNA and expressed relative to the levels in GAPDH siRNA-transfected cells set as 1. Values followed by different letters are statistically significantly different from each other ($P < 0.05$). C: Primary human macrophages were transfected with control GAPDH siRNA or with NPC1 or NPC2 siRNA and cholesterol-loaded by incubation with 50 µg/ml [^{3}H]cholesterol-containing AcLDL for 48 h. GW7647 (600 nmol/l) was added 24 h before and during cholesterol loading. Cells were then incubated in the presence or absence of apo-AI, and [^{3}H]cholesterol efflux was measured as described. Results are expressed relative to the siGAPDH-transfected cells set as 1 (mean ± SD of two independent experiments). Values followed by different letters are statistically significantly different from each other ($P < 0.05$).

of cholesterol and its efflux in macrophages is of great importance.

The maintenance of the cellular cholesterol equilibrium depends on balanced intracellular transport processes. Within the cells, modified LDL-derived CEs are hydrolyzed in lysosomes to generate FC, which is then transported to the plasma membrane, where it integrates with cell membrane cholesterol. Trafficking of cholesterol from the late endosome/lysosome to the plasma membrane is a protein-mediated process controlled by NPC1 and NPC2 (4).

These proteins could represent interesting targets in the control of processes upstream of cholesterol efflux in macrophages.

In the present study, we show that NPC1 and NPC2 are expressed in differentiated human macrophages and are regulated by OxLDL. By contrast, AcLDL loading appears not to regulate these genes. Because OxLDLs contain natural PPARα ligands, such as 9- and 13-HODE (12), and certain of their effects could be mediated by activation of the PPAR transcription factors, we studied whether the ex-
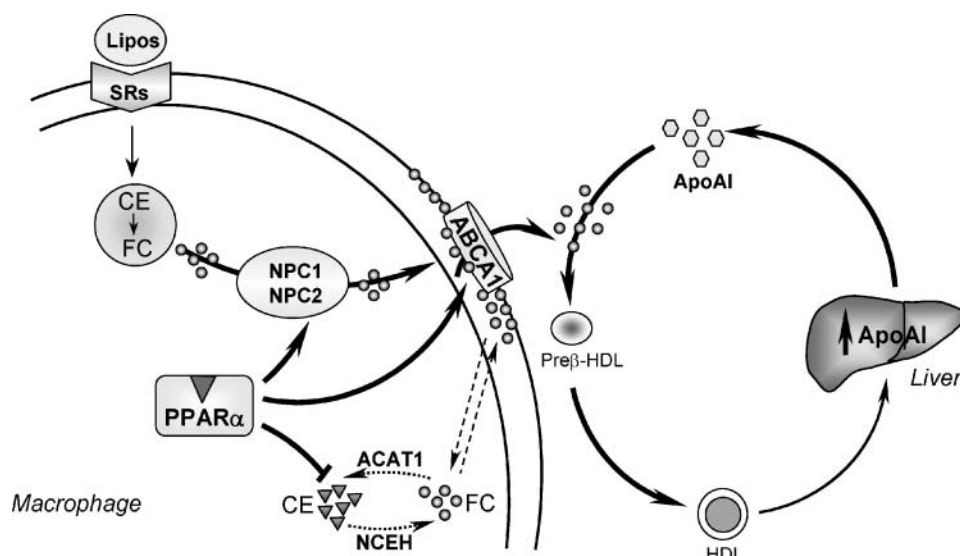

Fig. 8. Schematic representation of PPARα actions on cholesterol trafficking in macrophages. PPARα stimulates the postlysosomal mobilization of cholesterol through the regulation of NPC1 and NPC2 expression. This results in an enrichment of cholesterol in the plasma membrane and a redistribution of cholesterol in external cell surface domains, where it is more available for efflux through the ABCA1 pathway. PPARα activation also decreases the cellular amount of cholesteryl esters. Pathways controlled by PPARα are depicted in bold arrows. SR, scavenger receptor; CE, cholesteryl ester; FC, free cholesterol; NCEH, neutral cholesteryl ester hydrolase.

pression of these genes is controlled by PPARα. For this purpose, different synthetic activators at concentrations that specifically activate human PPARα (18) were used. PPARα activation resulted in the induction of NPC1 and NPC2 gene and protein expression in human macrophages. Because NPC1 and NPC2 are involved in cholesterol trafficking, we studied the effects of PPARα activation on cholesterol mobilization to the plasma membrane. Our results show that PPARα activation leads to an enrichment of cholesterol in the plasma membrane accompanied by a redistribution of cholesterol within the plasma membrane to the outer layer. The enrichment of plasma membrane cholesterol as well as the induction of apoA-I-specific cholesterol efflux by PPARα was abolished in the presence of progesterone used at concentrations known to block cholesterol mobilization from the late endosome/lysosome and to mimic a phenotype comparable to the one observed in NPC-deficient cells (17, 19). To further investigate the mechanism by which cholesterol trafficking could contribute to cholesterol efflux, an siRNA approach to knock-down NPC1 or NPC2 expression was used. Interestingly, repression of NPC1 and NPC2 expression led to a drastic reduction of basal cholesterol efflux as well as to an abolishment of PPARα-induced cholesterol efflux. Our observations indicate that stimulation of postlysosomal cholesterol mobilization to the plasma membrane by PPARα activation via NPC1 and NPC2 induction is a crucial step upstream of the stimulation of its efflux through the ABCA1 pathway.

One determinant governing the efflux rate of cholesterol is the availability of cholesterol in the plasma membrane. It has already been reported that PPARα activation in human macrophages leads to an induction of ABCA1 gene expression and, as such, to an increased efflux of cholesterol to an extracellular acceptor (10). Thus, our data reporting a positive effect of PPARα ligands in promoting cholesterol transport to the plasma membrane demonstrate that PPARα not only controls cholesterol efflux from macrophages but also regulates upstream processes. The observation that these effects are blocked by progesterone suggests that proteins controlling postlysosomal cholesterol trafficking are mediating PPARα actions. Our results from the cholesterol oxidase accessibility test demonstrate that PPARα activation also leads to a redistribution of cholesterol within the plasma membrane, with an enrichment of cholesterol in the outer layer, leading to more availability for the interaction with an extracellular acceptor such as apoA-I, an effect that would accelerate its efflux. Our results on the induction of cholesterol oxidase accessibility by PPARα ligands are in line with a previous report indicating that activation of PPARγ has the same effects on MA-10 Leyding tumor cells by inducing the plasma membrane FC pool (20).

Recently, it has been demonstrated that fibroblasts isolated from NPC subjects are characterized by a reduced accessibility to cholesterol oxidase (21), resulting in an impaired cholesterol efflux to apoA-I (22). In line with this observation, it has been reported that NPC1-deficient subjects have decreased plasma HDL cholesterol levels

(22). Thus, regulation of NPC1 and proteins associated in its pathway appears to be important in the control of plasma HDL levels. The stimulatory role of PPARα activators in hepatic HDL production and apoA-I expression is well documented (23). The results of this study, demonstrating that PPARα activators induce NPC1 and NPC2 expression in macrophages, resulting in an enhanced availability of plasma membrane cholesterol for cholesterol efflux, provide a mechanism that may contribute to the observed clinical effects of PPARα activators on RCT and HDL metabolism (**Fig. 8**) (24).

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