PPAR-α and PPAR-γ activators induce cholesterol removal from human macrophage foam cells through stimulation of the ABCA1 pathway

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Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate lipid and glucose metabolism and cellular differentiation. PPAR-α and PPAR-γ are both expressed in human macrophages where they exert anti-inflammatory effects. The activation of PPAR-γ may promote foam-cell formation by inducing expression of the macrophage scavenger receptor CD36. This prompted us to investigate the influence of different PPAR- activators on cholesterol metabolism and foam-cell formation of human primary and THP-1 macrophages. Here we show that PPAR-α and PPAR-γ activators do not influence acetylated low density lipoprotein-induced foam-cell formation of human macrophages. In contrast, PPAR-α and PPAR-γ activators induce the expression of the gene encoding ABCA1, a transporter that controls apoAI-mediated cholesterol efflux from macrophages. These effects are likely due to enhanced expression of liver-x-receptor α, an oxysterol-activated nuclear receptor which induces ABCA1- promoter transcription. Moreover, PPAR-α and PPAR-γ activators increase apoAI-induced cholesterol efflux from normal macrophages. In contrast, PPAR-α or PPAR-γ activation does not influence cholesterol efflux from macrophages isolated from patients with Tangier disease, which is due to a genetic defect in ABCA1. Here we identify a regulatory role for PPAR-α and PPAR-γ in the first steps of the reverse-cholesterol-transport pathway through the activation of ABCA1-mediated cholesterol efflux in human macrophages.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that, upon heterodimerization with the retinoid X receptor (RXR), function as ligand-activated transcriptional regulators of genes controlling lipid and glucose metabolism[1]. PPAR-α is highly expressed in liver, heart, muscle, kidney and cells of the arterial wall (such as monocyte-derived macrophages, smooth muscle and endothelial cells), and it is activated by fibrates, fatty acids and eicosanoids. 15-deoxy-Δ^{12-14}prostaglandin J_2 (15-d PGJ_2), oxidized fatty acids and the antidiabetic glitazones are ligands for PPAR-γ, which is expressed at high levels in white adipose tissue where it triggers adipocyte differentiation[2]. Both PPAR-α and PPAR-γ are expressed in fully-differentiated human macrophages where they regulate genes implicated in the inflammatory response, modulate macrophage differentiation and promote TNF-α/IFN-γ–induced apoptosis[3–5]. Moreover, PPAR-γ activation results in the transcriptional induction of CD36 expression in macrophages[6], whereas the transcriptional activation of scavenger-receptor A by proinflammatory stimuli is inhibited[4]. In primary human macrophages, both PPAR-α and PPAR-γ activators induce the expression of the scavenger-receptor CLA-1/SR-BI, which binds high-density lipoproteins (HDL) with high affinity[7]. These data indicate that, through their action on macrophage scavenger receptors, both PPAR-α and PPAR-γ are important modulators of macrophage lipid metabolism and may influence development of atherosclerosis. Presently, however, it is unclear whether activation of PPAR-α and PPAR-γ in macrophages promotes or inhibits atherogenesis.

ABCA1 is a member of the ATP-binding cassette-transporter family, which is involved in the control of apoAI-mediated cholesterol efflux from macrophages[8,9]. ABCA1 deficiency causes both Tangier disease and familial HDL deficiency—two pathologies characterized by very low levels of plasma HDL (refs. 8,10,11). Tangier patients exhibit an accumulation of cholesteryl esters in various tissues such as tonsils, liver, spleen and intestinal mucosa. Macrophages, present as foam cells in affected tissues, are the principal cell type that accumulate this excess tissue cholesterol[12]. ABCA1 is implicated in the first steps of the reverse-cholesterol-transport pathway and in the control of plasma levels of HDL, a major protective factor against atherosclerosis. Although fibrate PPAR-α activators increase HDL-cholesterol and its major apolipoproteins (apoAI and apoAII) by induction of their genes in liver[1], they increase HDL-cholesterol more than apoAI levels and also modify HDL composition. In addition, glitazone PPAR-γ acti-

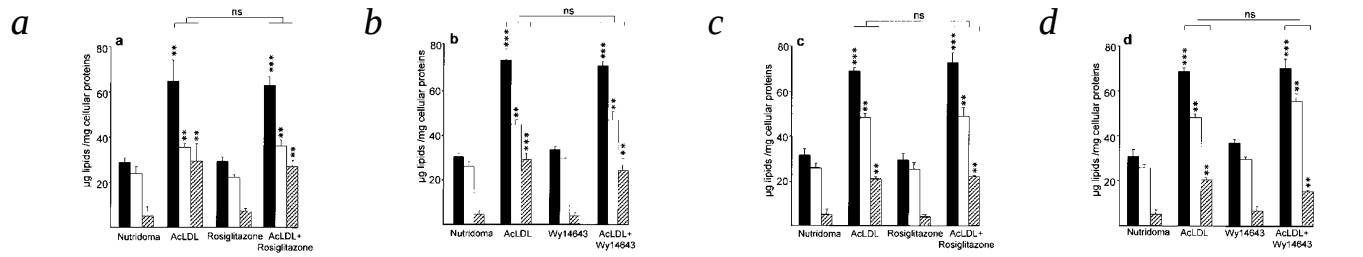

Fig. 1 PPAR-α and PPAR-γ activators do not influence foam-cell transformation of human macrophages. Cholesterol-loaded human primary (***a*** and ***b)*** or THP-1 (***c*** and ***d)*** macrophages were treated with rosiglitazone (100 nM; *a* and *c*) or Wy14643 (50 μM, *b* and *d*). Intracellular total cholesterol (■), free cholesterol (□) and esterified cholesterol(▨) were enzymatically determined. Results are the mean ± s.e. of triplicate determinations, representative of 4 independent experiments. Statistically significant differences between treatments are indicated (ANOVA followed by Mann-Whitney's test; **, $P < 0.01$, ***, $P < 0.001$, ns: not significant).

vators induce HDL-cholesterol, but not apoAI levels, by an unknown mechanism[13].

Given the widespread use of fibrates and glitazones in, respectively, the treatment of dyslipidemia and type II diabetes (two metabolic disorders predisposing to atherosclerosis) and given the suggestion that PPAR-γ activation may promote foam-cell formation through its action on CD36, we investigated the effects of these PPAR-activators on macrophage foam-cell formation. We also assessed a potential role of these transcription factors in the regulation of ABCA1-gene expression and the first steps of the reverse-cholesterol-transport pathway. Given the well-established, species-specific differences in the regulation of lipid and lipoprotein metabolism by PPAR-activators[14], we used primary human monocyte-derived macrophages as our model of human physiology. We demonstrate that neither PPAR-α nor PPAR-γ activation induces foam-cell formation of human macrophages. We also show that both PPAR-α and PPAR-γ activators induce ABCA1 expression, possibly through their inductive effects on expression of LXR-α, a nuclear receptor activated by oxysterols[15], which mediates ABCA1-promoter transcription induction[16]. In addition, we demonstrate that both PPAR-α and PPAR-γ ligands promote apoAI-mediated cholesterol efflux. These data reveal a molecular mechanism that contributes to the increase in HDL-cholesterol levels observed in patients treated with fibrates and glitazones, as well as to the cardioprotective activity of these drugs in clinical studies[17–20].

PPAR-activation does not influence foam-cell formation

PPARs regulate macrophage scavenger receptor expression[4,6,7], and this led to the suggestion that PPAR-activators may modulate foam-cell formation, a known atherogenic process characterized by cholesteryl-ester-droplet accumulation in the cytoplasm of macrophages[21]. We investigated the influence of both PPAR-α and PPAR-γ activators on acetylated low density lipoprotein (AcLDL)-induced transformation of human macrophages into foam cells. We loaded primary human monocyte-derived macrophages as well as THP-1–differentiated macrophages with AcLDL for 48 hours and treated them with specific synthetic PPAR-activators. We treated these cells with either the PPAR-α ligand Wy14643 or the PPAR-γ ligand rosiglitazone at concentrations of 2–3 times their EC_{50} values (Wy14643 EC_{50} for human PPAR-α = 25 μM and rosiglitazone EC_{50} for human PPAR-γ = 43 nM)[22] This did not affect cholesterol accumulation from AcLDL in these cells (Fig. 1*a*, *b*, *c* and *d*). Moreover, PPAR-α or PPAR-γ activators did not influence cholesterol accumulation in primary human macrophages incubated and treated under the same conditions with oxidized low-density lipoproteins (OxLDL) (data not shown). The observation that PPAR-α or PPAR-γ activators do not stimulate esterified- or free-cholesterol accumulation (Fig. 1) indicates that these PPARs are not implicated in foam-cell formation.

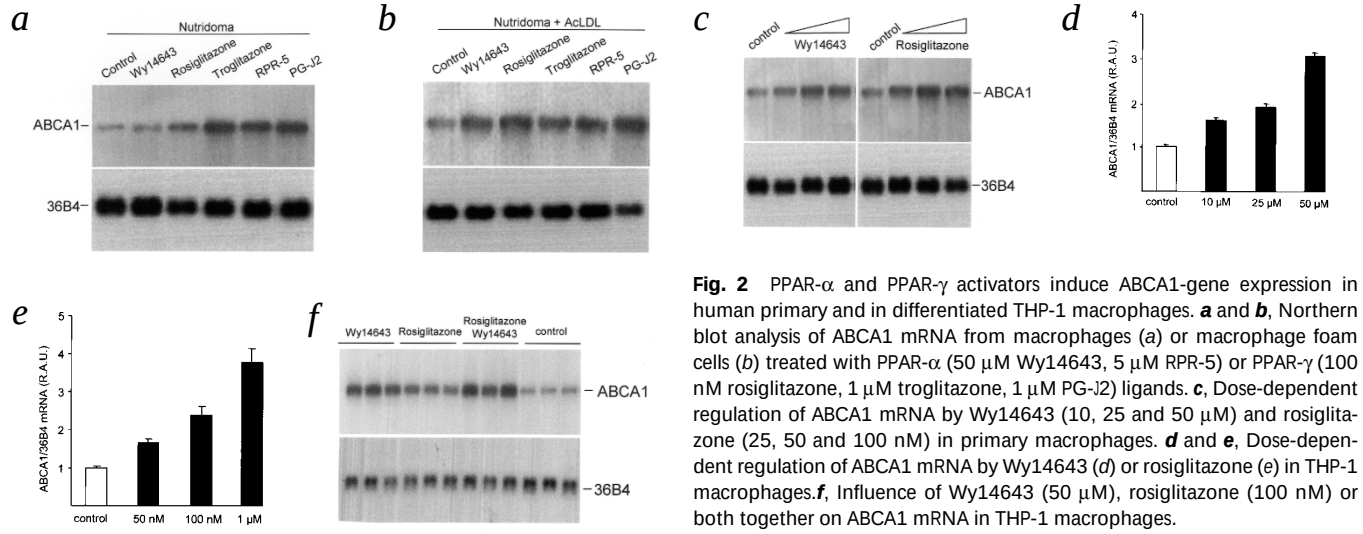

Fig. 2 PPAR-α and PPAR-γ activators induce ABCA1-gene expression in human primary and in differentiated THP-1 macrophages. ***a*** and ***b***, Northern blot analysis of ABCA1 mRNA from macrophages (*a*) or macrophage foam cells (*b*) treated with PPAR-α (50 μM Wy14643, 5 μM RPR-5) or PPAR-γ (100 nM rosiglitazone, 1 μM troglitazone, 1 μM PG-J2) ligands. ***c***, Dose-dependent regulation of ABCA1 mRNA by Wy14643 (10, 25 and 50 μM) and rosiglitazone (25, 50 and 100 nM) in primary macrophages. ***d*** and ***e***, Dose-dependent regulation of ABCA1 mRNA by Wy14643 (*d*) or rosiglitazone (*e*) in THP-1 macrophages.***f***, Influence of Wy14643 (50 μM), rosiglitazone (100 nM) or both together on ABCA1 mRNA in THP-1 macrophages.

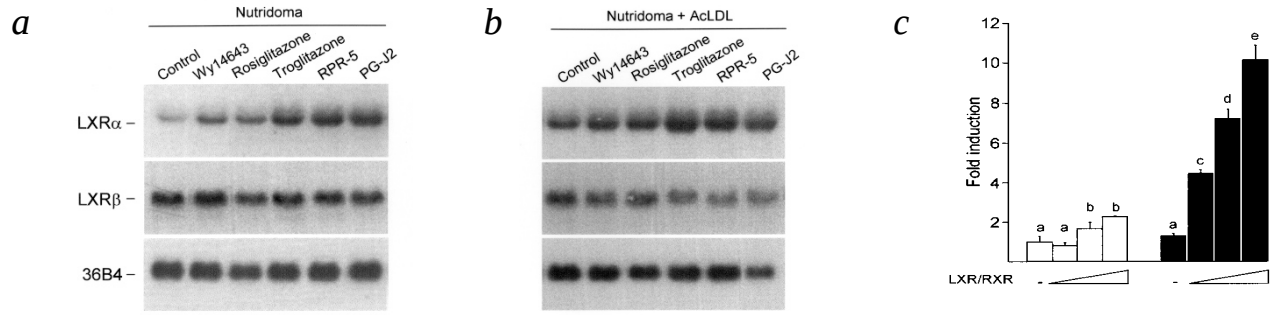

Fig. 3 PPAR-α and PPAR-γ activators induce LXR-α gene expression in primary human macrophages. ***a*** and ***b,*** Northern blot analysis of LXR-α and LXR-β mRNA from macrophages (*a*) or foam cells (*b*) treated with PPAR-α (50 μM Wy14643, 5 μM RPR-5) or PPAR-γ (100 nM rosiglitazone, 1 μM troglitazone, 1 μM PG-J2) ligands. ***c***, COS cells were transfected with a LXR-E-driven reporter vector and increasing amounts of the LXR-α/RXR-α expression plasmids in the absence (□) or in the presence of 10 μM 22-(R)-OH-cholesterol (■). Values followed by different letters are statistically significantly different (ANOVA followed by Mann-Whitney's test; $P < 0.05$).

PPAR-activation induces ABCA1 expression

To determine whether PPARs may influence the anti-atherogenic reverse-cholesterol-transport pathway, we studied the effects of different PPAR- activators in human macrophages on the gene expressing ABCA1, which controls the first steps of apoAI-mediated cholesterol efflux and the reverse-cholesterol-transport pathway. Northern blot analysis showed that PPAR-α (Wy14643 and RPR-5 EC_{50} for human PPAR-α = 0.13 μM) or PPAR-γ (rosiglitazone and troglitazone EC_{50} for human PPAR-γ = 0.55 μM; PG-J2 EC_{50} for human PPAR-γ = 2 μM) activators induce the expression of ABCA1 in primary human monocyte-derived macrophages (Fig. 2*a*) and in macrophage-derived foam cells (Fig. 2*b*) at concentrations within the range of their EC_{50} values. Moreover, in primary human macrophages Wy14643 and rosiglitazone induce ABCA1 mRNA levels in a dose-dependent manner (Fig. 2*c*). Wy14643 and rosiglitazone also increase ABCA1 expression in THP-1 macrophages in a dose-dependent manner (Fig. 2*d* and *e*), an effect which was additive in the presence of both activators together (Fig. 2*f*).

PPAR-activators induce LXR-α expression

ABCA1 mRNA levels are induced in human THP-1 macrophages after treatment with oxysterols and human ABCA1-promoter activity is positively regulated by activation of the oxysterol receptor LXR-α[16]. To investigate the potential mechanism by which PPAR-α and PPAR-γ activators induce ABCA1 mRNA expression, we studied the effect of these compounds on LXR-α and LXR-β expression. Northern-blot analysis demonstrated that Wy14643 (50 μM), RPR-5 (5 μM), rosiglitazone (100 nM), troglitazone (1 μM) and PG-J2 (1 μM) induce LXR-α but not LXR-β mRNA expression in primary human monocyte-derived macrophages and in macrophage-derived foam cells (Fig. 3*a* and *b*). Since LXR-α has been shown to display constitutive activity in the absence of added exogenous ligands[23,24], changes in expression levels may lead to transcriptional changes of target genes, such as that encoding ABCA1.

These observations[24,25] were confirmed by results of co-transfection experiments. Co-transfection of increasing amounts of LXR-α expression vector resulted in a dose-dependent induction of the activity of a LXR-E-driven reporter vector in the absence of added exogenous ligand (Fig. 3*c*). Moreover, in the presence of a fixed amount of the LXR-ligand, 22-OH-cholesterol, transcriptional activity of the reporter correlated positively with the quantity of co-transfected LXR-α (Fig. 3*c*).

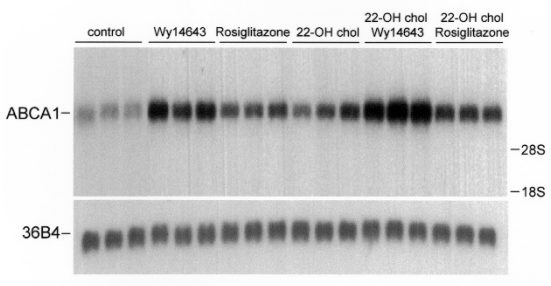

Fig. 4 PPAR- and LXR-agonists cooperatively induce ABCA1-gene expression in human macrophages. Northern blot analysis of ABCA1 mRNA from THP-1 macrophages treated with Wy14643 (50 μM), rosiglitazone (100 nM), 22-(R)-OH-cholesterol (1μM) alone or combined.

PPAR- and LXR-activators additively induce ABCA1 expression

To demonstrate cross-talk between PPAR- and LXR-α activation on ABCA1 gene regulation, we performed northern blot analysis using RNA of THP-1 cells treated with the different PPAR-ligands with and without the presence of 22 OH-cholesterol (Fig. 4). The results confirm that ABCA1 gene expression is induced by both PPAR- or LXR-α agonists alone and that combination treatment with both PPAR- and LXR-agonists results in more induction, thus indicating a functional positive cross-talk between both receptor pathways.

PPAR- activators induce cholesterol efflux

As both PPAR-α and PPAR-γ regulate ABCA1 expression in macrophages, we next investigated their role in the control of apoAI-mediated cholesterol efflux from macrophages. We incubated human monocyte-derived macrophages with AcLDL to induce cholesteryl-ester accumulation, treated 24 hours before cholesterol loading and thereafter every 24 hours with Wy14643 (50 μM) or rosiglitazone (100 nM) at concentrations within the range of their EC_{50} values for PPAR-α and PPAR-γ respectively, and subsequently exposed the cells to apoAI to induce cholesterol efflux. After 24 hours, apoAI treatment induced cholesterol efflux, as measured by the change in cellular cholesterol levels, from human cholesterol-loaded macrophages (Fig. 5). Both Wy14643 and rosiglitazone treat-

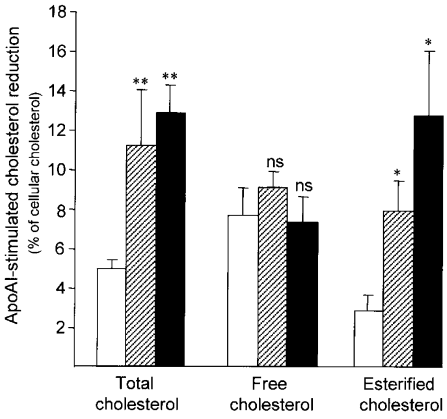

Fig. 5 PPAR-activators induce apoAI-mediated cholesterol efflux from human macrophages. Cholesterol-loaded human macrophages were treated with rosiglitazone (100 nM, ▨), Wy14643 (50 μM, ■) or vehicle (□) and subsequently incubated with RPMI 1640 medium with or without apoAI (100 μg/ml). Intracellular lipids were determined. Results are the mean ± s.e.m. of triplicate determinations of 5 experiments and are expressed as the percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium, calculated as described. Statistically significant differences between treatments are indicated (ANOVA followed by Mann-Whitney's test; *, $P < 0.05$, **, $P < 0.01$, ns: not significant).

ment reduced intracellular cholesterol concentrations approximately 2.5-fold relative to vehicle treatment (Fig. 5). Under these conditions, both PPAR-α and PPAR-γ activators reduced the esterified cholesterol pool, whereas free cholesterol levels did not change substantially (Fig. 5). Moreover, apoAI-mediated phospholipid efflux, measured as mass of phospholipid released in the culture medium, increased after treatment with both Wy14643 and rosiglitazone (control cells: 1.27 ± 0.13 μg phospholipid/mg cellular protein; Wy14643: 2.16 ± 0.65 μg/mg; rosiglitazone: 4.60 ± 0.47 μg/mg). Thus both PPAR-α and PPAR-γ agonists stimulate the removal of cholesterol and phospholipids from cells to their acceptor in the medium, apoAI.

To demonstrate that the variation of intracellular lipids was not due to the action of these PPAR- ligands on *de novo* cholesterol synthesis, we loaded human macrophages with [^{3}H]cholesterol-AcLDL (50 μg/ml) for 48 hours and determined apoAI-mediated efflux of cholesterol by measuring the appearance of tritiated cholesterol in the medium. Wy14643 (50 μM) and rosiglitazone (100 nM), added 24 hours before cholesterol loading and thereafter every 24 hours, increased [^{3}H]cholesterol release by approximately 3-fold and 2-fold, respectively, compared with solvent-treated cells (Fig. 6*a*). These results indicate that PPAR-α and PPAR-γ agonists increase apoAI-mediated cholesterol efflux from human macrophages.

ABCA1 inhibition prevents PPAR—induced cholesterol efflux

To determine whether these effects of PPAR-α and PPAR-γ agonists are mediated by the ABCA1 transporter pathway, we performed efflux studies in the presence of DIDS, an inhibitor of the anion transport activities of ABCA1(ref 26,27) and blocker of apoAI-mediated cholesterol efflux from human fibroblasts[9]. The presence of DIDS reduced the apoAI-mediated efflux of tritiated cholesterol by approximately 50% and completely abolished the positive effects of Wy14643 and rosiglitazone on apoAI-mediated efflux (Fig. 6*a*). Moreover, DIDS treatment also abolished both the rosiglitazone-induced (Fig. 6*b*) and Wy14643-induced (Fig. 6*c*) reduction of cellular-cholesterol mass after apoAI exposure.

ABCA1 deficiency prevents PPAR—induced cholesterol efflux

Finally, we performed efflux studies on macrophages from two Tangier patients, which lack functional ABCA1 and are defective in cholesterol efflux[28]. To induce cholesteryl ester accumulation, we incubated cells with AcLDL treated 24 hours before cholesterol loading and thereafter every 24 hours with Wy14643 (50 μM) or rosiglitazone (100 nM), and we subsequently exposed them to apoAI to induce cholesterol efflux. We determined intracellular cholesterol levels by an enzymatic assay. In contrast with wild-type cells, apoAI did not induce cholesterol efflux from Tangier macrophages (Fig. 7). In fact, a slight increase in cholesterol levels was observed in these cells, which may be due to a combination of both endogenous ongoing cholesterol biosynthesis and the absence of cholesterol efflux in Tangier cells[8,11,28]. Moreover, neither PPAR-α nor PPAR-γ activators influenced the apoAI-mediated cholesterol efflux in Tangier macrophages, but they induced cholesterol removal from normal wild-type cells (Fig. 7). These data from Tangier macrophages patients, along with the results obtained in the presence of DIDS, indicate that the induction of cholesterol efflux by PPAR-α and PPAR-γ ligands from macrophages requires functional ABCA1 expression.

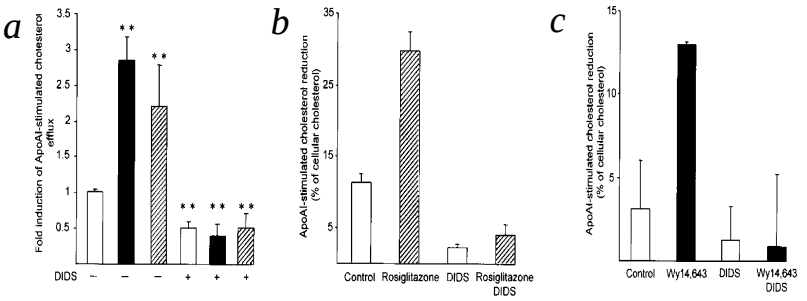

Fig. 6 The ABCA1 transporter inhibitor DIDS inhibits the apoAI-mediated induction of cholesterol efflux by PPAR-activators from human macrophages. ***a***, [^{3}H]cholesterol-loaded primary macrophages were treated with rosiglitazone (100 nM, ▨), Wy14643 (50 μM, ■) or vehicle (□) and subsequently incubated with RPMI 1640 medium with or without apoAI (100 μg/ml) in the presence of DIDS (400 μM) where indicated. ApoAI-induced [^{3}H]cholesterol efflux was measured as described. Values are expressed relative to the untreated controls, set as 1. Results are the mean ± s.e. of triplicate determinations, representative of 3 independent experiments. Statistically significant differences from control are indicated (ANOVA followed by Mann-Whitney's test; **, $P < 0.01$). ***b*** and ***c,*** Cholesterol-loaded macrophages were treated with rosiglitazone (100 nM, ▨) (*b*), Wy14643 (50 μM, ■) (*c*) or vehicle (Control, □) and subsequently incubated with RPMI 1640 medium with or without apoAI (100 μg/ml) in the presence of DIDS (400 μM) where indicated. Results are the mean ± s.e.m. of triplicate determinations of 2 independent experiments and are expressed as the percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium, as in Fig. 5.

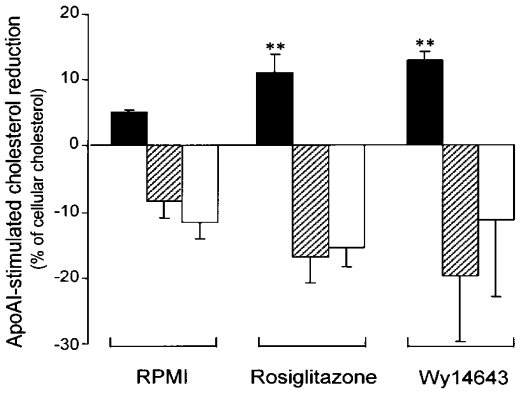

Fig. 7 PPAR-activators do not influence cholesterol efflux from Tangier macrophages. Cholesterol loaded-macrophages of two Tangier patients (□, ▨) and a healthy donor (■) were treated with rosiglitazone (100 nM) or Wy14643 (50 μM) and incubated with RPMI 1640 medium with or without apoAI (100 μg/ml) and changes in intracellular cholesterol were measured and expressed as in Fig. 5. Total cholesterol efflux is expressed as under Fig. 5. Results are the mean ± s.e.m. of triplicate determinations. Statistically significant differences from controls are indicated (ANOVA followed by Mann-Whitney's test; **, $P < 0.01$).

Discussion

PPAR-α and PPAR-γ are lipid-activated transcription factors, which—upon activation by their respective ligands, fibrates and glitazones—regulate genes involved in lipid and glucose metabolism, cellular differentiation and inflammation control[1]. PPARs have an important role in vascular pathophysiopathology: both PPAR-α and PPAR-γ are present in human endothelial, smooth muscle cells and macrophages *in vitro* as well as in *in vivo* atherosclerotic lesion macrophages[7,29]. The fact that PPAR-γ ligands inhibit the transcriptional activation of scavenger receptor A (SRA) (ref. 4) and enhance SR-BI and CD36 expression[6,7], raised the question of a potential role of PPARs in the control of cholesterol homeostasis in macrophages. Here we studied the influence of PPAR-α and PPAR-γ activation on foam-cell formation and cholesterol removal in human macrophages.

PPAR-γ activation has been suggested to promote foam-cell formation through the transcriptional induction of CD36 in human macrophages leading to enhanced oxidized LDL uptake[6]. These experiments, however, were done in the presence of RXR agonists on transformed monocytic cell lines. Moreover, intracellular cholesterol was not measured[6], thus, these studies are not conclusive about the role of PPAR-γ in foam-cell formation. Here we show that neither PPAR-α nor PPAR-γ activators induce lipid accumulation after AcLDL loading, indicating that PPAR-α or PPAR-γ activation does not promote foam-cell formation of primary human macrophages or THP-1 macrophages.

We demonstrate that in human macrophages and macrophage-derived foam cells, PPAR-α and PPAR-γ activators induce expression of the gene encoding ABCA1, which is involved in apoAI-mediated cholesterol efflux from macrophages. These effects of PPAR-α and PPAR-γ activators might be mediated by their stimulatory action on LXR-α expression and activity, as ABCA1 mRNA and promoter transcription are induced by oxysterols acting via LXR-α (ref 16). In addition, unsaturated fatty acids and synthetic PPAR-α ligands such as Wy14643 induce LXR-α, but not LXR-β mRNA or protein levels *in vitro* in cultured rat hepatocytes and *in vivo* in liver. This is due to a direct induction of LXR-α gene transcription via PPAR-α interacting with several PPREs in the LXR-α promoter[30]. We show that in primary human monocyte-derived macrophages and in macrophage-derived foam cells, both PPAR-α and PPAR-γ ligands induce the expression of LXR-α, but not of LXR-β. These data provide a plausible mechanism explaining the action of PPAR-agonists on ABCA1 gene expression.

PPAR-α and PPAR-γ agonists induce macrophage expression of CLA-1/SR-BI (ref. 7), a receptor implicated in HDL-dependent cholesterol removal from macrophages[31]. Together with this observation, the induction of ABCA1 expression indicates that both PPAR-α and PPAR-γ control the initial steps of the reverse-cholesterol-transport pathway. These effects of PPAR-α and PPAR-γ activators occur at concentrations within the ranges of their EC_{50} values and circulating plasma levels in humans (a single dose of rosiglitazone at 2 mg results in peak plasma concentration of 255 nM)[32]. In human macrophage-foam cells, PPAR-activators induce apoAI-mediated cholesterol efflux through the ABCA1 pathway, as no influence on cholesterol efflux is observed when ABCA1 is not functional due to either a genetic deficiency as in Tangier cells or to chemical inhibition by the ABCA1 inhibitor DIDS. The stimulatory role of PPAR-α activators on hepatic HDL production and apoAI expression is well-documented[33]. Moreover, treatment of type II diabetic patients with rosiglitazone and pioglitazone results in increased plasma HDL cholesterol levels, whereas apoAI levels appear unaffected[13]. Here we show that PPAR-α and PPAR-γ activators induce ABCA1 expression in macrophages resulting in an enhanced apoAI-mediated cholesterol efflux, and we provide a mechanism that may contribute to the observed clinical effects of both PPAR-α and PPAR-γ activators on HDL metabolism[13,20].

Methods

Transactivation assays of the RPR-5 compound. We performed transactivation assays with the RPR-5 compound in A10 cells (a rat smooth-muscle-cell line) using full-length human PPAR-α and PPAR-γ and the pGL3-J_3-TK reporter vector[34]. After 24 h, luciferase activity was measured with LucLite (Packard, Meridan, Connecticut) according to manufacturer's instructions. EC_{50} values were calculated after curve-fitting with XLFit (human PPAR-α EC_{50} = 0.13 μM; human PPAR-γ EC_{50} = 5 μM).

Cell Culture. We isolated mononuclear cells from blood of healthy normolipidemic donors or from Tangier patients by Ficoll gradient centrifugation and cultured them as described[5]. Mature monocyte-derived macrophages were used for experiments after 10 d of culture. For treatment with the different activators, medium was changed to medium without human serum but supplemented with 1% Nutridoma HU (Boehringer). Efflux studies were performed in the absence of any serum. Human monocytic THP-1 cells (ATCC, Rockville, Maryland) were maintained in RPMI 1640 medium containing 10% of FCS and differentiated 48h with 100 nM PMA.

RNA extraction and analysis. After incubation with the indicated PPAR-activators, we washed cells with PBS and used them for RNA extraction. Total cellular RNA was extracted after 10 d from differentiated macrophages and THP-1 cells treated for 24 hours with the indicated compounds using Trizol (Life Technologies, France). For northern-blot analysis, we hybridized membranes containing 10 μg of total RNA with radiolabeled ABCA1 or 36B4 control cDNA probes. An *Eco*RI LXR-β and a *Hin*dIII–*Hin*cII LXR-α cDNA fragment were used as probes. For the human ABCA1 probe, a 1.1 kb cDNA fragment derived from PCR amplification with the following primers, 5′-GGCAATGGCACTGAGGAAGATGCTGAAA-3′ and 5′-GCACTGCAGGATTGTCACCACAGCAAA-3′, was cloned into a pCR4-4-TOPO vector (Invitrogen).

Transient transfection assay. 24 hours before transfection, we plated COS1 cells in 24-well plates at a density of 5×10^4 cells/well and cultured them in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal-calf serum. Cells were transfected by lipofection using ExGen 500 (Euromedex) in OPTIMEM 1 medium, with 50 ng of reporter plasmid and increasing quantities of LXR-α and RXR expression plasmids (10, 25 and 50 ng) in the presence of 50 ng of the internal control β-gal expression vector, for 3 h in serum-free medium. Fresh medium containing 0.2% fetal calf serum and 10 μM 22(R)-hydroxycholesterol (Sigma) or its solvent was subsequently added and cells were further incubated for 48 h. Cell extracts were prepared and assayed for luciferase activity as described[35] and results were normalized to internal control β-gal activity.

Cholesterol loading and efflux. We pretreated 10-day-old human macrophages for 24 h and thereafter every 24 h with the indicated PPAR-activators (rosiglitazone, 100 nM; Wy14643, 50 μM) and cholesterol loaded by incubation with AcLDL (50 μg/ml, containing or not [^{3}H]cholesterol)[36] in RPMI 1640 medium supplemented with 1% Nutridoma (Boehringer) for 48 h. After this incubation period, cells were washed twice in PBS and apoAI-mediated cholesterol efflux studies were immediately performed by adding fresh RPMI medium without Nutridoma with or without 100 μg/ml of apoAI for 24 h. Because, in macrophages, the equilibrium between esterified and free cholesterol is not obtained even after a 24 h additional incubation period[37], the experiments were performed in the absence of equilibrium. At the end of this incubation, intracellular lipids were extracted in hexane/isopropanol, dried under nitrogen and free cholesterol, and total cholesterol and phospholipids were measured by enzymatic assays (Boehringer). Esterified cholesterol was measured as the difference between total and free cholesterol. Cellular proteins were collected by digestion in NaOH and measured by Bradford assay (BioRad). The percent change of intracellular cholesterol amounts in the presence of apoAI relative to apoAI-free medium was expressed according to the following equation:

$$\text{percent decrease in cellular cholesterol} = \{[(\text{cellular cholesterol})_{RPMI} - (\text{cellular cholesterol})_{ApoAI}] \div [\text{cellular cholesterol}]_{RPMI}\} \times 100.$$

In the experiments with [^{3}H]cholesterol, we measured radioactivity by scintillation counting in centrifugated medium and in cellular lipids extracted with hexane/isopropanol. ApoAI-induced [^{3}H]cholesterol efflux was measured as the fraction of total radiolabeled cholesterol appearing in the medium in the presence of apoAI after substraction of values for apoAI-free medium. For the phospholipid efflux study, lipids were isolated from culture medium by chloroform/methanol extraction as described[38]. Extracted lipids were subsequently dried under nitrogen pressure and phospholipid mass determined by an enzymatic assay (Boehringer). Where indicated, the ABCA1-inhibitor DIDS (4,4-diisothiocyanostilbene-2,2'-disulfonic acid) was added at concentration of 400 μM simultaneously with apoAI.

Acknowledgments

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