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A PPARγ-LXR-ABCA1 Pathway in Macrophages Is Involved in Cholesterol Efflux and Atherogenesis

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Summary

Previous work has implicated PPARγ in the regulation of CD36 expression and macrophage uptake of oxidized LDL (oxLDL). We provide evidence here that in addition to lipid uptake, PPARγ regulates a pathway of cholesterol efflux. PPARγ induces ABCA1 expression and cholesterol removal from macrophages through a transcriptional cascade mediated by the nuclear receptor LXRα. Ligand activation of PPARγ leads to primary induction of LXRα and to coupled induction of ABCA1. Transplantation of PPARγ null bone marrow into LDLR −/− mice results in a significant increase in atherosclerosis, consistent with the hypothesis that regulation of LXRα and ABCA1 expression is protective in vivo. Thus, we propose that PPARγ coordinates a complex physiologic response to oxLDL that involves particle uptake, processing, and cholesterol removal through ABCA1.

Introduction

Oxidized lipids play a causal role in the development of atherosclerosis (Ross, 1995; Steinberg, 1997). Oxidized lipids initiate and help to maintain a chronic inflammatory reaction in the artery wall that ultimately evolves into atherosclerotic plaque. LDL acquires several novel biological activities as a result of oxidative modification, including the ability to induce specific changes in macrophage gene expression. Although the mechanisms by which oxidized LDL (oxLDL) regulates cellular gene expression are still poorly understood, recent work suggests that transcriptional pathways involving nuclear receptors mediate many biological effects of oxidized lipids.

PPARγ is a member of the nuclear receptor superfamily that functions as a key transcriptional regulator of cell differentiation and lipid metabolism (Spiegelman, 1997; Tontonoz and Nagy, 1999). In addition, PPARγ is now recognized to be the biological receptor for prostaglandin J_2 and the thiazolidinedione class of antidiabetic drugs (Forman et al., 1995; Lehmann et al., 1995). Recent evidence indicates that PPARγ is expressed at high levels in macrophages, including the foam cells of atherosclerotic lesions (Ricote et al., 1998b; Tontonoz et al., 1998). Moreover, the oxLDL itself can activate PPARγ by providing the cell with oxidized fatty acid ligands of the receptor (Nagy et al., 1998). The elucidation of a PPARγ signaling pathway in macrophages provides a mechanism by which oxidized lipids may directly regulate gene expression in the context of the atherosclerotic lesion. To date, the only macrophage gene known to be a direct target for PPARγ regulation is the class B scavenger receptor CD36 (Tontonoz et al., 1998). The identification of CD36 as a PPARγ target gene suggested a role for PPARγ in the scavenging of oxLDL and control of lipid uptake in multiple cell types, including macrophages and adipocytes. Accordingly, in undifferentiated monocytes or fibroblasts that do not express scavenger receptors, induction of CD36 expression by PPARγ ligands promotes the uptake of oxLDL. However, in PPARγ null macrophages, the resulting loss of CD36 regulation does not significantly compromise lipid uptake, suggesting that other pathways must also be involved (Chawla et al., 2001).

Recent work has begun to define a pathway for cholesterol efflux from lipid-loaded cells. ABCA1 (also called ABC-1) and ABCG1 (also called ABC-8 or white), two members of the ABC family of transporter proteins, have been shown to be highly induced in lipid-loaded macrophages (Langmann et al., 1999; Klucken et al., 2000; Venkateswaran et al., 2000b). Loss-of-function mutations in the ABCA1 gene result in Tangier disease, a disease characterized by marked cholesterol accumulation in macrophages and other reticuloendothelial cells (Bodzioch et al., 1999; Brooks-Wilson et al., 1999; Rust et al., 1999). This phenotype, along with the observation that fibroblasts from Tangier patients are impaired in their ability to donate cholesterol to apolipoprotein AI (apoAI), suggests that ABCA1 plays a pivotal role in cellular cholesterol efflux. While the function of ABCG1 is less well understood, this transporter may also be involved in lipid efflux (Klucken et al., 2000). Recent studies have provided evidence that the nuclear receptors LXRα and LXRβ mediate the lipid induction of both ABCG1 and ABCA1 (Costet et al., 2000; Repa et al., 2000; Venkateswaran et al., 2000a; Venkateswaran et al., 2000b).

We provide evidence here for convergence of PPARγ

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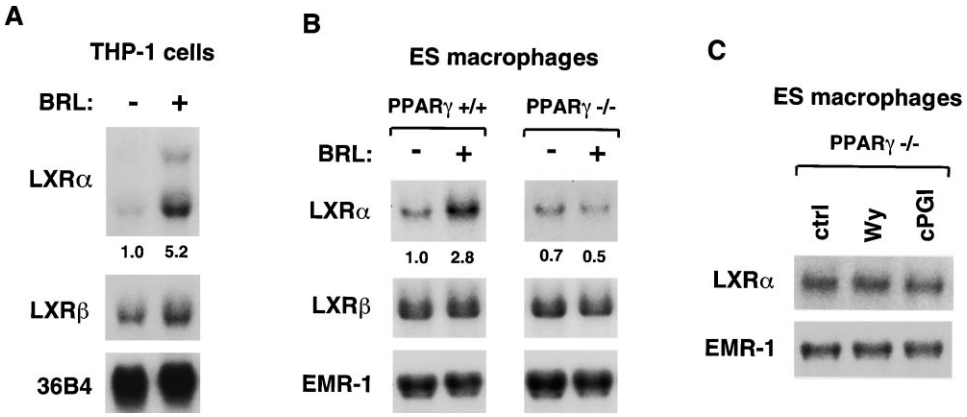

Figure 1. LXRα Is a PPARγ-Responsive Gene in Macrophages

(A) LXRα mRNA is induced by PPARγ ligands in differentiated THP-1 cells. THP-1 cells were treated with 40 ng/ml of TPA for 48 hr to induce differentiation. Subsequently, differentiated THP-1 cells were treated with BRL (5 μM) for 24 hr. Total RNA (10 μg/lane) was analyzed by Northern blotting using labeled cDNA probes specific for mLXRα, mLXRβ, and 36B4.

(B) Induction of macrophage LXRα expression by thiazolidinediones requires PPARγ.

(C) Ligands for PPARα and PPARδ fail to induce LXRα expression in PPARγ −/− macrophages. ES cells were differentiated into macrophages as previously described. PPARγ +/+ and −/− ES cell–derived macrophages were treated with rosiglitazone (1 μM), Wy 14,563 (5 μM), or carbaprostacyclin (cPGI, 1 μM) for 24 hr. Northern analysis was performed as in (A). EMR-1, the cDNA encoding the macrophage-specific F4/80 antigen, was used as a differentiation and loading control. Fold induction of mRNA normalized to the control is shown for LXRα.

and LXRα signaling pathways on a macrophage response to oxidized lipid loading: a pathway for cholesterol efflux. We show that the LXRα gene is itself a direct target of PPARγ, and that these nuclear receptors cooperate in the regulation of macrophage ABCA1 expression and the control of cholesterol efflux. Lastly, we show that deletion of the PPARγ gene in macrophages worsens atherosclerosis in LDLR −/− mice, thereby confirming the importance of this pathway in modulating lesion development. This unexpected link between PPARγ and LXRα creates a potentially unique opportunity for the pharmacological manipulation of cellular cholesterol metabolism.

Results

As part of a systematic effort to identify macrophage target genes for PPARγ, we discovered that PPARγ ligands could induce expression of the gene encoding LXRα in THP-1 cells. As shown in Figure 1A, treatment of TPA-differentiated THP-1 macrophages for 24 hr with the PPARγ ligand rosiglitazone (BRL, 5 μM) resulted in an approximately 5-fold induction of LXRα mRNA expression. This induction was specific for LXRα and was not observed with the closely related family member LXRβ. To confirm that the induction of LXRα mRNA in response to BRL was mediated by PPARγ, we analyzed LXR expression in macrophages derived from either PPARγ +/+ or PPARγ −/− ES cells. BRL treatment resulted in similar induction of LXRα mRNA that was completely dependent on PPARγ expression (Figure 1B). Interestingly, the ability to regulate macrophage LXRα expression appears to be specific to PPARγ because treatment of PPARγ −/− macrophages with Wy 14,563 and cPGI, ligands for PPARα and PPARδ respectively, did not affect LXRα expression (Figure 1C). As in THP-1 cells, there was no difference in expression of LXRβ between wild-type and PPARγ −/− ES macrophages.

The above data suggested that the LXRα gene might be a direct target for the PPARγ/RXR heterodimer. To test this possibility, the promoter region of both mouse LXRα and LXRβ were analyzed in transient transfection experiments. A reporter construct containing 1.8 kb of the proximal promoter of LXRα was activated by the liganded PPARγ/RXRα heterodimer (data not shown), whereas the proximal 2.4 kb of the LXRβ promoter was unresponsive (Figure 2A). Deletion studies further localized the PPARγ-responsive region to the proximal 933 bp of the LXRα promoter (Figure 2A). Surprisingly, the –933 LXRα promoter construct was activated by liganded PPARγ but not PPARα or PPARδ. This observation is consistent with our finding that expression of LXRα mRNA in macrophages is induced by ligands for PPARγ but not PPARα or PPARδ (Figure 1).

Analysis of this 933 bp region revealed a DR-1 element, the preferred binding site for PPARγ/RXR heterodimers, between –722 and –710 bp (Figure 2A). Gel mobility shift assays verified that only PPARγ/RXRα heterodimers bound efficiently to the DR-1 PPAR response element (PPRE) of the LXRα promoter, whereas all three subtypes of PPARs bound the PPRE from the acyl-CoA oxidase (AOx) promoter (Figure 2B). Specificity was further established by competition with excess unlabeled probe (Figure 2C, left panel). As expected, point mutations in the LXRα PPRE abolished binding of the PPARγ/RXRα heterodimer to the corresponding radiolabeled probes (Figure 2C, right panel).

To examine whether the identified LXRα PPRE could function as a PPARγ response element in vivo, transfection experiments were carried out with a reporter construct containing two copies of the LXRα PPRE linked to a heterologous promoter. As shown in Figure 2D, the LXRα $PPRE_2$ TK-Luc reporter was strongly activated by cotransfection of PPARγ and RXRα expression vectors

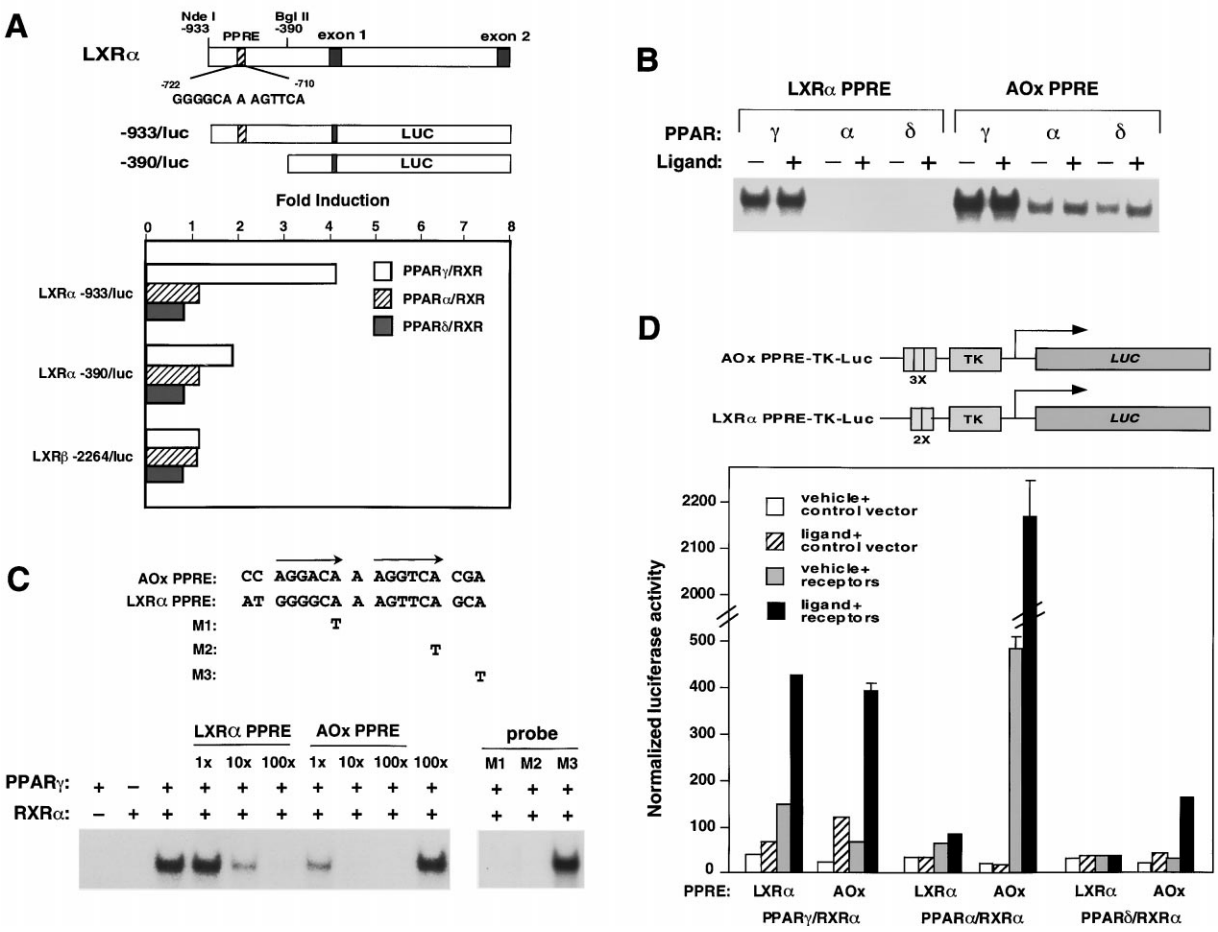

Figure 2. The LXRα Promoter Is a Direct Target for Regulation by the PPARγ/RXR Heterodimer

(A) PPARγ/RXR transactivates the LXRα promoter. The LXRα and β promoters were amplified by PCR and cloned upstream of the luciferase reporter gene. Transient transfection experiments were carried out in triplicate in CV1 cells using luciferase reporter constructs (0.1 μg) and CMX-PPAR expression vectors (10 ng) as described in Experimental Procedures. Cells were treated with ligands (PPARγ: BRL [1 μM]; PPARα: Wy 14,673 [15 μM]; and PPARδ: cPGI [5 μM]) for 24 hr and then collected for reporter gene analysis. Luciferase activity was normalized to an internal β-galactosidase control.

(B) Identification of a PPARγ-selective binding site in LXRα promoter. Gel mobility shift assays were performed using in vitro translated receptors and ^{32}P end-labeled LXR PPRE or acyl-CoA oxidase PPRE oligonucleotides. Specific ligands (PPARγ: BRL [1 μM]; PPARα: Wy 14,673 [15 μM]; and PPARδ: cPGI [5 μM]) were included in the binding reaction as indicated.

(C) Specific binding of the LXRα PPRE by PPARγ/RXR heterodimers. Competition assays for binding to the LXRα PPRE were performed using unlabeled PPRE oligonucleotides or nonspecific (NS) DNA at the indicated molar excess (left panel). A point mutation in either half site of the LXRα PPRE (M1 and M2) abolishes binding of the PPARγ/RXR heterodimer to corresponding ^{32}P-labeled probes (right panel). A similar mutation outside the binding site (M3) had no effect on binding.

(D) The LXRα PPRE functions as a PPARγ-selective response element. Two copies of the LXRα PPRE were cloned upstream of the tk-luciferase reporter. CV1 cells were cotransfected with receptor expression vectors (10 ng) and either LXRα or AOx PPRE-tk-luc reporter (0.1 μg), treated with PPAR ligands as in (A), and analyzed for reporter gene activity.

in a ligand-dependent manner. Consistent with the in vitro binding data presented above, cotransfection of PPARα or PPARδ had minimal effect on the activity of this reporter. By contrast, a control AOx_3 TK-Luc reporter was transactivated by all three PPARs and their respective ligands.

Since both PPARγ and LXRα are activated by lipid components of oxLDL, we hypothesized that these nuclear receptors may comprise a cascade that coordinates a macrophage response to oxLDL uptake. We therefore examined the ability of PPARγ and LXR ligands to modulate expression of ABCA1 and ABCG1 in THP-1 cells. As shown in Figure 3A, treatment of TPA-differentiated THP-1 macrophages with either BRL (5 μM) or the LXR ligand 20(S)-hydroxycholesterol (2.5 μM) resulted in a marked induction of ABCA1 and ABCG1 mRNA expression. The combination of both ligands had an additive effect. The quantitative analysis shown in Figure 3B illustrates that treatment of THP-1 cells with BRL shifted the dose response curve for induction of ABC expression by 20(S)-hydroxycholesterol. In contrast to ABCA1 and ABCG1, expression of CD36 mRNA was induced by PPARγ ligand but not by LXR ligand (Figure 3A). Thus, PPARγ and LXR combine to regulate a restricted overlapping set of macrophage genes.

The ability of PPARγ and LXR to regulate ABCA1 and

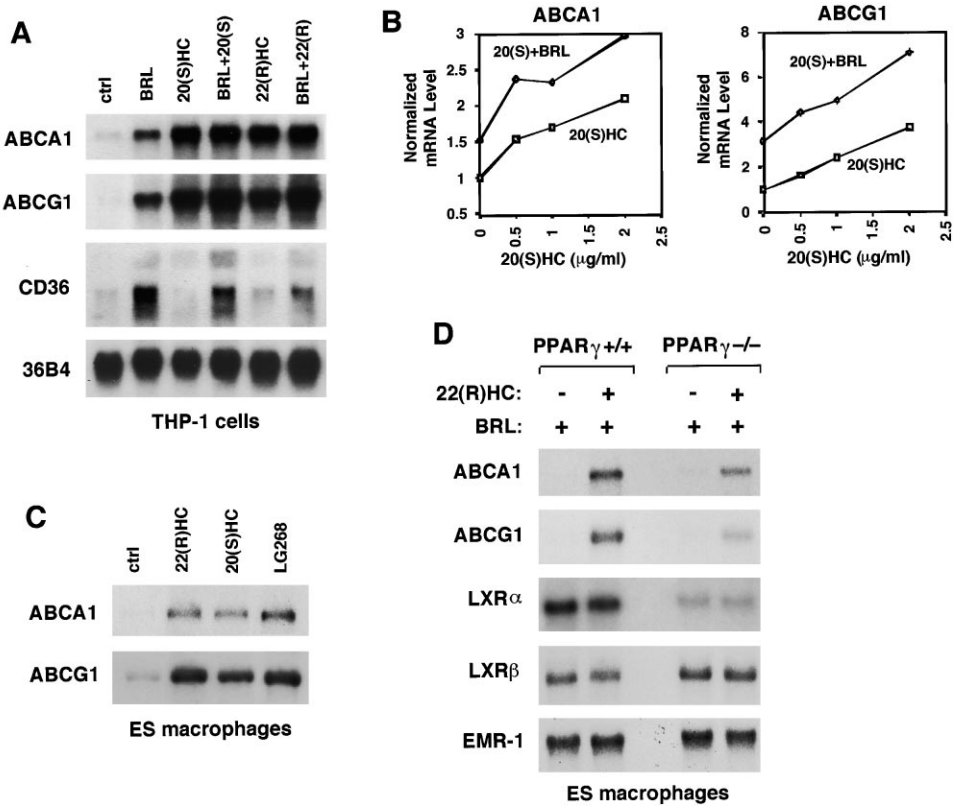

Figure 3. PPARγ and LXR Ligands Cooperate to Induce ABCA1 and ABCG1 Expression in THP-1 Cells and ES Cell–Derived Macrophages

Northern analysis was carried out as in Figure 1 using the indicated ^{32}P-labeled cDNA probes.

(A) Induction of ABCA1 and ABCG1 expression by PPAR and LXR ligands in THP-1 cells. THP-1 cells were differentiated as in Figure 1 and treated with various combinations of PPARγ (BRL, 5 μM) and LXR ligands (20[S]-hydroxycholesterol 2.5 μg/ml or 22[R]-hydroxycholesterol 2.5 μg/ml) for 24 hr.

(B) THP-1 cells were differentiated as in Figure 1 and treated with the indicated concentration of 20(S)-hydroxycholesterol in the presence or absence of 1 μM BRL. ABCA1 and ABCG1 mRNA levels were quantitated by phosphorimaging and normalized to 36B4.

(C) LXR and RXR ligands induce ABCA1 and ABCG1 expression in ES cell–derived macrophages. Wild-type ES cell–derived macrophages were treated with either LXR ligand (10 μM) or RXR ligand (LG 268, 0.1 μM) for 24 hr.

(D) Expression of ABCA1 and ABCG1 is impaired in PPARγ −/− macrophages. ES cell–derived macrophages were pretreated with BRL (1 μM), compactin (5 μM), and mevalonic acid (100 μM) for 24 hr. Cells were subsequently cultured in the presence or absence of the LXR ligand, 22(R)-hydroxycholesterol (5 μM), for 24 hr. Longer exposure time shows a low level of expression of ABCA1 and ABCG, which is reduced in the PPARγ null cells.

ABCG1 expression was further studied in ES cell–derived macrophages. Treatment of wild-type macrophages with either 22(R)-hydroxycholesterol or 20(S)-hydroxycholesterol resulted in a marked induction of ABCA1 and ABCG1 (Figure 3C), consistent with the results obtained in THP-1 cells. Furthermore, both LXR and RXR ligands were able to induce ABCA1 and ABCG1 gene expression in PPARγ −/− macrophages, thus confirming that the LXR signaling pathway is intact in PPARγ null macrophages (data not shown). To verify that PPARγ and LXR ligands could cooperate to induce ABCA1 and ABCG1 expression in these cells, PPARγ wild-type and null macrophages were pretreated with BRL (1 μM) and then cultured in the presence or absence of 22(R)-hydroxycholesterol (5 μM). Induction of ABCA1 and ABCG1 mRNA by LXR ligands was reduced in PPARγ null macrophages by 2- to 4-fold (Figure 3D). The residual ability of LXR ligands to induce expression of these genes is consistent with the observation that both PPARγ +/+ and PPARγ −/− macrophages abundantly express LXRβ (Figure 3D).

To complete the analysis of the regulatory loop, we investigated whether murine ABCA1 might be a direct target for nuclear receptors. Previous work has shown that the human ABCA1 gene is regulated by the LXR/RXR heterodimer (Costet et al., 2000; Repa et al., 2000; Schwartz et al., 2000; Venkateswaran et al., 2000a). We analyzed the murine ABCA1 promoter in transient transfection assays to identify *cis*-elements that might mediate regulation by PPARγ and LXR. Luciferase reporter constructs containing the proximal –2450 bp of the mABCA1 promoter were significantly activated by the LXRα/RXRα heterodimer, but not by PPARγ/RXRα (data available upon request). Activation of the murine promoter was enhanced by addition of either the LXR or RXR ligand, and an additive response was observed with the addition of both (data available upon request). Sequence analysis of the murine ABCA1 promoter revealed a well-conserved DR-4 element between –68 and –53 bp that was similar to the LXR/RXR binding site in the human ABCA1 promoter. Gel mobility shift assays using this sequence as a probe confirmed binding by

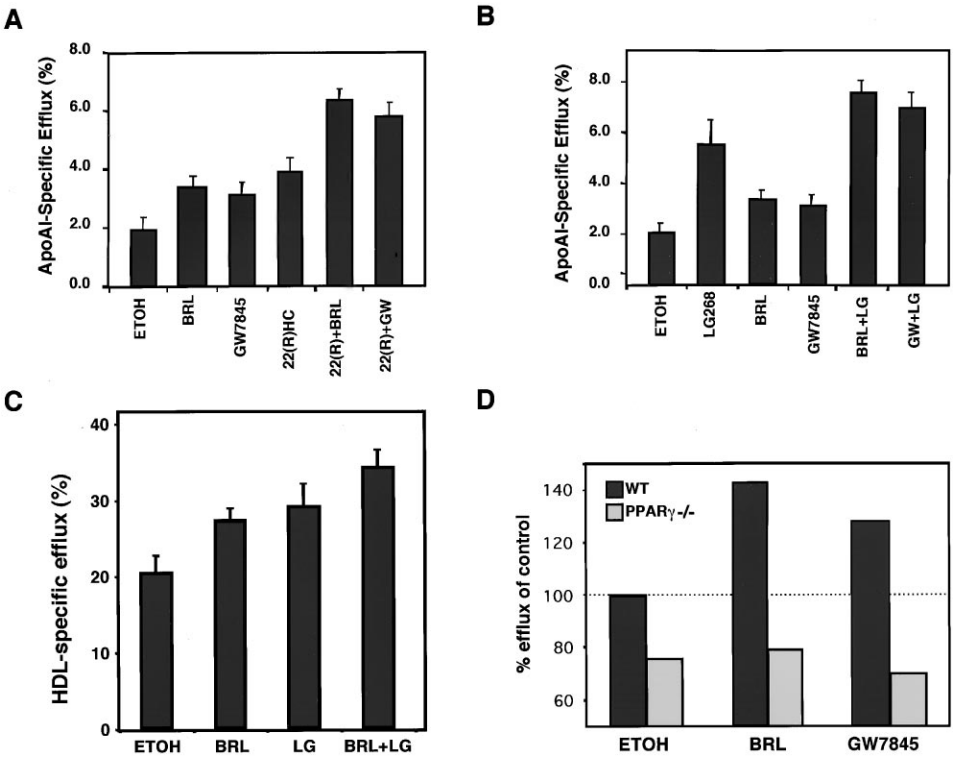

Figure 4. The PPARγ and LXR Pathways Cooperate to Stimulate Cholesterol Efflux from Macrophages

THP-1 cells (A–C) or ES cell–derived macrophages (D) were plated in 24-well plates and incubated for 24 hr in RPMI 1640 supplemented with 10% LPDS and [^{3}H]cholesterol in the absence or presence of the indicated receptor ligands (BRL [5 μM], GW7845 [5 μM], 22[R]-hydroxycholesterol [1 μg/ml], and/or LG268 [50 nM]) as indicated. ApoAI- or HDL-dependent cholesterol efflux to the medium was determined as described in Experimental Procedures. Data are presented as a percentage (+/− SE) of the total radioactivity in the cells and medium; each point is the numerical average of triplicate experiments.

(A) PPARγ and LXR ligands additively promote cholesterol efflux from THP-1 macrophages.

(B) PPARγ and RXR ligands additively promote cholesterol efflux from THP-1 macrophages.

(C) PPARγ and RXR ligands increase cholesterol efflux from acLDL-loaded THP-1 macrophages.

(D) Altered basal and ligand-inducible cholesterol efflux in PPARγ null macrophages.

both the LXRα/RXR and LXRβ/RXR heterodimers. As expected, mutations in the murine ABCA1 LXRE abolished binding and transcriptional activation by the LXR/RXR heterodimer (data available upon request). Thus, both the murine and human ABCA1 promoters are targets for regulation by the LXR/RXR heterodimer (Costet et al., 2000; Schwartz et al., 2000). By contrast, we have been unable to identify a functional PPARγ binding site in the ABCA1 promoter, suggesting that the effects of PPARγ ligands on ABCA1 expression are likely to be secondary to induction of LXRα expression.

The ability of PPARγ and LXRα to cooperate in the regulation of ABCA1 and ABCG1 expression suggested that these nuclear receptors might also cooperate to promote cholesterol removal from macrophages. To test this possibility, two types of cholesterol efflux assays were performed. TPA-differentiated THP-1 cells were labeled for 24 hr with [^{3}H]cholesterol in the presence or absence of an ACAT inhibitor (58-035). The ACAT inhibitor prevents esterification of cholesterol resulting in a free cholesterol pool with high specific activity. As shown in Figure 4A, under these conditions, treatment of THP-1 macrophages with either PPARγ (BRL or GW7845) or LXR ligand (22[R]-hydroxycholesterol) resulted in a significant increase in cholesterol efflux to extracellular apoAI. Moreover, the combination of a PPARγ and an LXR ligand had an additive effect. We further observed that the RXR ligand LG268 (that can activate both PPAR/RXR and LXR/RXR heterodimers) was also an effective inducer of cholesterol efflux (Figure 4B), while the combination of a PPARγ ligand and LG268 was most efficacious. In a second series of studies, THP-1 cells were labeled with [^{3}H]cholesterol and acetylated LDL (acLDL) in the absence of an ACAT inhibitor. This protocol leads to a significant increase in cellular cholesteryl ester content, a condition similar to the lipid loading of macrophages during the formation of the atherosclerotic lesion. Although this experiment is complicated by the fact that acLDL loading itself activates both the LXR and PPARγ signaling pathways (Nagy et al., 1998; Venkateswaran et al., 2000a), treatment with PPARγ or RXR ligands nevertheless increased cholesterol efflux by at least 50% (Figure 4C). Thus, PPARγ ligands promote the removal of free cholesterol from lipid-loaded macrophages.

Finally, to establish the role of the PPARγ signaling pathway in controlling macrophage lipid efflux, we analyzed apoAI-dependent cholesterol efflux in wild-type

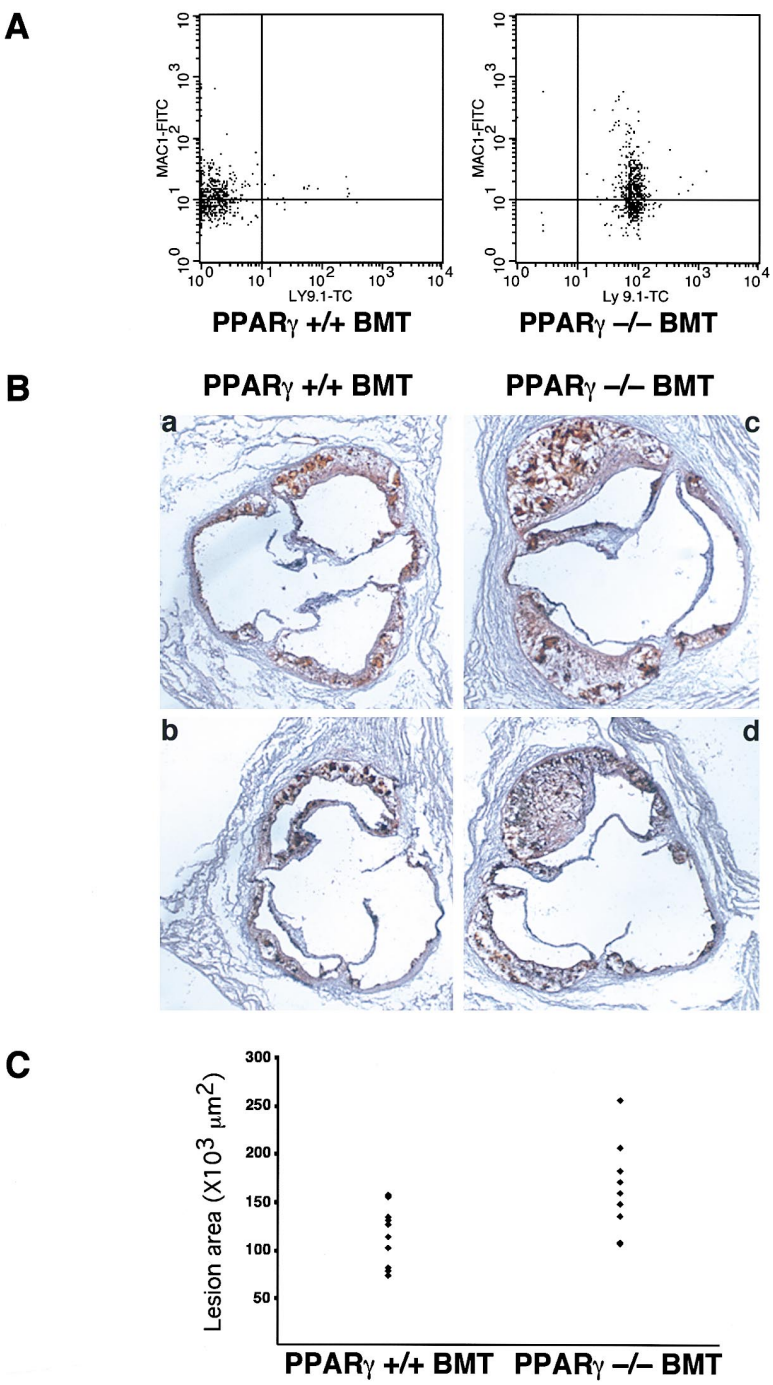

Figure 5. Bone Marrow Transplantation (BMT) with PPARγ −/− Bone Marrow Increases Atherosclerosis in LDLR −/− Mice

Bone marrows from high-percentage PPARγ −/− mice and wild-type mice were transplanted into γ-irradiated-LDLR −/− mice. After BMT, donor marrow was allowed to repopulate the recipient mice for 4 weeks. Mice were placed on an atherogenic diet at the end of 4 weeks, and lesion analysis was performed after 8 weeks of the atherogenic diet.

(A) Representative facs analysis of peripheral blood after BMT. Peripheral blood leukocytes were analyzed using Ly 9.1, Mac-1 (CD11b/CD18), and Gr-1 (Ly6-G). Monocytes, which are Mac-1 positive and Gr-1 negative, were analyzed for Ly 9.1 expression. PPARγ +/+ BMT recipients are Ly 9.1 negative, whereas PPARγ −/− BMT recipients are Ly 9.1 positive.

(B) Representative oil red O–stained sections of aortic valves from two mice in each BMT group. Bright red staining of atherosclerotic lesions is visualized by oil red O in the cryosections of aortic valves. (a) and (b) are from recipients of PPARγ +/+ BMT and are characterized by less advanced lesions, whereas (c) and (d) are from recipients of PPARγ −/− BMT and are characterized by more advanced, larger lesions.

(C) Quantification of the size of aortic valve lesion areas. Oil red O–stained lesions were quantified by a computer-assisted video-imaging system. Each symbol represents the mean lesion area of five sections taken every 40 μm through the aortic valve of a single animal. The mean ± SD lesion area of PPARγ +/+ BMT recipients was 119,628 ± 30,186 μm^2, whereas it was 155,768 ± 46,283 μm^2 for recipients of PPARγ −/− BMT.

and PPARγ −/− ES–derived macrophages. As shown in Figure 4D, the ability of rosiglitazone and GW7845 to stimulate cholesterol efflux was completely abolished in PPARγ −/− cells. Unexpectedly, basal cholesterol efflux in PPARγ −/− macrophages was decreased by 20%–25%, indicating a role for PPARγ signaling in the physiologic pathway for cholesterol removal from the cell. In aggregate, these data establish a functional molecular cascade between PPARγ and LXRα that serves to couple the process of cellular lipid loading to the activation of ABCA1-mediated lipid efflux.

To definitively evaluate the functional role of PPARγ signaling in atherogenesis, we performed transplantation with PPARγ null bone marrow into a murine model of atherosclerosis (Boisvert et al., 1995; Linton et al., 1995). PPARγ −/− ES cells were injected into the blastocysts from the C57/Bl6 mouse strain to generate PPARγ −/− chimeric mice. Since the PPARγ −/− ES cells are derived from the SV129 strain (Chawla et al., 2001), percent chimerism for the hematopoetic lineages could be readily assessed by differential expression of Ly 9 alloantigens. The Ly 9.1 antigen is present on hematopoetic cells derived from the SV129 strain, whereas it is absent on cells from the C57/Bl6 background (Mathieson et al.,

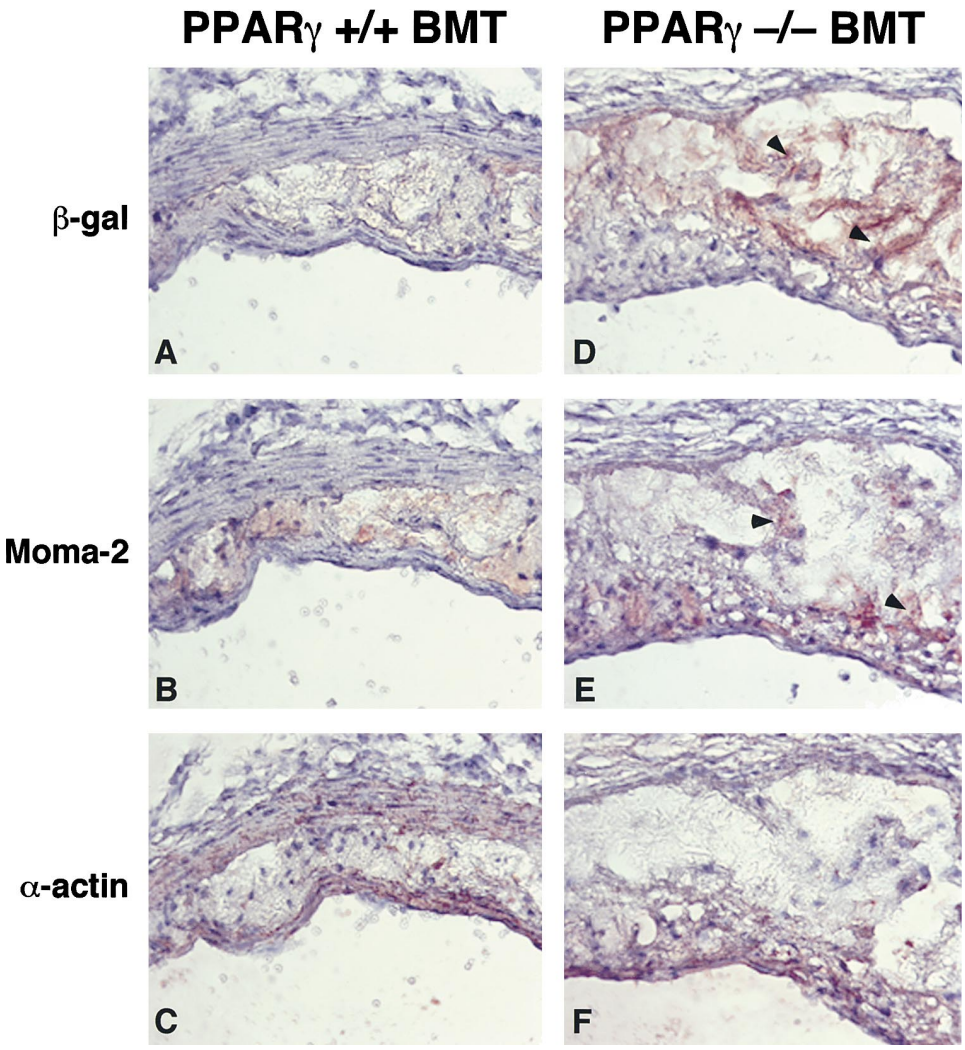

Figure 6. Immunohistochemistry of Aortic Valve Lesions with Antibodies for Wild-Type Macrophages, PPARγ −/− Macrophages, and Smooth Muscle Cells

β-galactosidase, MOMA-2, and α-actin. Aortic valve sections from either PPARγ +/+ BMT recipients (A–C, 125×) or PPARγ −/− BMT recipients (D–F, 125×) were stained with antisera to β-galactosidase (A and D), MOMA-2 (B and E), or α-actin (C and F). The β-galactosidase antibody is a surrogate marker for PPARγ −/− cells, while the MOMA-2 and α-actin antibodies are markers of macrophages and smooth muscle cells, respectively. The lipid-rich areas of aortic lesions in the PPARγ −/− BMT mice coexpress the MOMA-2 and β-galactosidase antigens (D and E, arrowheads).

1980). Facs analysis with Ly 9.1, CD11b/CD18, and F4/80 antibodies verified that greater than 90% of monocytes and elicited macrophages from high-percentage chimeras were derived from the SV129 strain, and were therefore null for the functional PPARγ gene (data not shown). Bone marrow from either wild-type or high-percentage PPARγ −/− chimeric mice was transplanted into γ-irradiated LDL receptor knockout (LDLR −/−) male mice (Boisvert et al., 1998). After 4 weeks, facs analyses were performed to verify the origin of the circulating monocytes. Figure 5A shows a representative analysis of peripheral blood from the PPARγ +/+ and PPARγ −/− bone marrow–transplanted mice. Circulating monocytes in the PPARγ +/+ BMT recipient mice were negative for Ly 9.1 expression, consistent with their wild-type origin, whereas those in PPARγ −/− BMT recipient mice were positive for the Ly 9.1 antigen, verifying that they were derived from PPARγ null cells (Figure 5A). At the end of 4 weeks, the transplanted mice were placed on an atherogenic diet for 8 weeks to induce a moderate degree of atherosclerosis. Plasma total cholesterol levels in both groups of transplanted mice were similar before (244 ± 24 mg/dl in PPARγ +/+ BMT mice and 258 ± 37 mg/dl in PPARγ −/− BMT mice) and after 8 weeks of an atherogenic diet (1065 ± 161 mg/dl in PPARγ +/+ BMT mice and 1173 ± 221 mg/dl in PPARγ −/− BMT mice). To evaluate the extent of atherosclerosis in the transplanted mice, aortic valves were sectioned, stained with oil red O, and digitally analyzed to quantify the aortic valve lesion areas. Remarkably, as shown in Figure 5B, the oil red O positive lesions were markedly larger in PPARγ −/− BMT recipients as compared to those in PPARγ +/+ BMT recipients. The mean aortic valve lesion areas ± SD were 119,628 ± 30,186

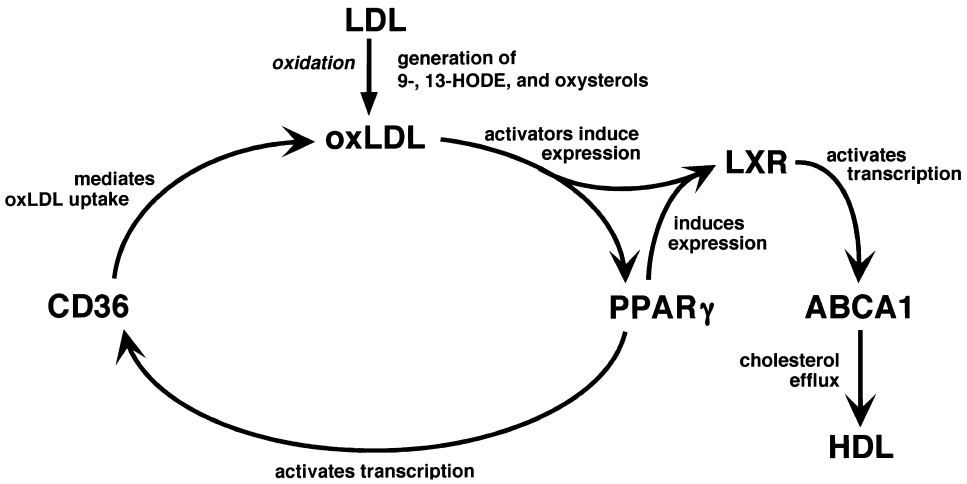

Figure 7. Model of PPARγ Action in Atherosclerosis

μm^2 (n = 12) and 155,768 ± 46,283 μm^2 (n = 11) for recipients of PPARγ +/+ and PPARγ −/− BMT, respectively (Figure 5C). This represents a statistically significant increase of 34% in the aortic valve lesion areas in the PPARγ −/− BMT recipient mice (p value = 0.04).

Finally, to verify that the PPARγ −/− macrophages were indeed present in the aortic valve lesions, we performed immunohistochemistry with antibodies directed against β-galactosidase, smooth muscle α-actin, and the macrophage-specific antigen MOMA-2. Since the mutation of the PPARγ gene was initially performed by an in-frame insertion of the *lacZ*-neomycin cassette (Barak et al., 1999), we could employ cytoplasmic β-galactosidase expression as a surrogate marker for PPARγ −/− macrophages. As shown in Figures 6D and 6E, foam cells in PPARγ −/− BMT mice coexpressed the β-galactosidase gene and MOMA-2 (arrowheads), whereas macrophages outside the lipid core stained positive for only the MOMA-2 antigen. This pattern of PPARγ expression in atherosclerotic lesions is consistent with what previously has been observed by others (Ricote et al., 1998a; Tontonoz et al., 1998). By contrast, there was no overlap in the expression pattern of the smooth muscle cell marker α-actin and β-galactosidase in these lesions (Figures 6D and 6F).

In summary, the sequential activation of the LXR-ABCA1 cholesterol efflux pathway by PPARγ in macrophages and the progression of atherosclerotic plaques in its absence in bone marrow–transplanted LDLR −/− mice collectively suggest that PPARγ and thiazolidinediones (TZDs) play an important antiatherogenic function in lesion macrophages.

Discussion

Macrophage uptake of oxLDL leads to profound changes in gene expression and lipid metabolism that are collectively thought to influence the development of the atherosclerotic lesion. Together with our previous work, this study supports a central role for the nuclear receptors PPARγ and LXRα in coordinating the macrophage response to lipid loading. Expression of both PPARγ and LXRα in macrophages is induced by exposure to oxLDL (Tontonoz et al., 1998; our unpublished data). Moreover, receptor-mediated uptake of the oxLDL particle provides the macrophage with a source of ligands for both receptors. PPARγ is activated by oxidized fatty acids such as 9- and 13-HODE (Nagy et al., 1998), while LXRα is activated by oxidized cholesterol metabolites such as 22(R)- and 25-hydroxycholesterol (Janowski et al., 1996; Lehmann et al., 1997; Janowski et al., 1999). Collectively, these observations suggest that many of the well-characterized effects of oxLDL on macrophage gene expression may be mediated by transcriptional activation of PPARγ and LXRα.

Alterations in the rates of lipid accumulation in or efflux from the artery wall each have the potential to affect the development of the atherosclerotic lesion. Previous work has suggested a role for the PPARγ signaling pathway in regulation of CD36 expression and lipid uptake in macrophages (Nagy et al., 1998; Tontonoz et al., 1998). Retroviral expression of PPARγ in cells is sufficient to induce both CD36 expression and oxLDL uptake (Chawla et al., 2001). While the macrophage PPARγ-CD36 pathway might be expected to be detrimental if unopposed, it could also provide a mechanism for lipid clearance from the artery wall if coupled to the reverse cholesterol transport pathway. The ability of PPARγ to influence macrophage cholesterol efflux, however, has not been examined. In this report, we have provided evidence that PPARγ participates in the control of macrophage cholesterol efflux through a transcriptional cascade involving LXRα and ABCA1. We have shown that the LXRα gene is a target of PPARγ, and along with previous studies on ABCA1 (see Discussion), that the ABCA1 gene is a target of LXRα. As a consequence of this regulatory loop, PPARγ and LXR ligands cooperate to promote ABCA1 expression and cholesterol efflux from macrophages. Finally, we provide in vivo evidence that loss of PPARγ function in macrophages accelerates atherogenesis in LDLR −/− mice.

During the preparation of this manuscript, several groups reported that the ABCA1 gene was a target for regulation by LXRs (Costet et al., 2000; Schwartz et al.,

2000; Venkateswaran et al., 2000a), and that intestinal cholesterol absorption and macrophage expression of ABCA1 is compromised in LXR null mice (Repa et al., 2000). In addition, Tobin et al. have recently reported that LXRα expression is responsive to fatty acids and suggested that LXRα may be a target for PPARα regulation in liver (Tobin et al., 2000). These authors identified several potential DR-1 sequence motifs in the LXRα promoter; however, the ability of PPAR/RXR to bind these sequences was not tested. In independent studies, we have found that, with the exception of the PPRE identified in Figure 2 (that preferentially binds PPARγ), none of the elements identified by Tobin et al. bind PPARα/RXR or PPARγ/RXR heterodimers in vitro. Furthermore, we have not observed regulation of LXRα expression by PPARα-specific ligands (Figure 1 and data not shown). Our results suggest that LXRα is a PPARγ-selective target gene in macrophages.

As depicted in the model in Figure 7, the regulation of the macrophage oxLDL uptake and cholesterol efflux pathways by PPARγ provides a mechanism by which these cells can efficiently deal with rising levels of oxLDL in the artery wall. Initially, uptake of oxLDL by macrophages results in induction of PPARγ, release of its ligands (such as 9- and 13-HODEs), and induction of its target genes, including CD36 and LXRα. Increased expression of CD36 facilitates the endocytosis of oxLDL, resulting in higher intracellular levels of oxysterols. These oxysterols then stimulate cellular cholesterol efflux by activating LXRs to upregulate ABCA1 and ABCG1 gene expression. Thus, PPARγ is proposed to couple a pathway of oxLDL uptake to a pathway of cholesterol and phospholipid efflux, thereby enhancing the ability of the macrophage to remove oxLDL from the vessel wall. In order for oxLDL cholesteryl ester to be effectively removed, it must be taken up by macrophages, hydrolyzed, and the free cholesterol shunted into the reverse cholesterol transport pathway. This model is supported by the BMT experiment showing that loss of the macrophage PPARγ-LXRα-ABCA1 pathway in vivo accelerates atherosclerosis.

These findings have potentially important implications for the treatment of human coronary artery disease. Several members of the thiazolidinedione class of PPARγ ligands are already in widespread clinical use for the treatment of Type II diabetes. From this and previous work, it is now clear that PPARγ contributes to the control of both macrophage oxLDL scavenging and cholesterol efflux, and that the net effect of these pathways is likely to be lipid removal from the artery wall. Thus, our results provide a potential explanation for the observation that TZDs actually improve atherosclerosis in vivo despite their ability to induce CD36 expression in macrophages (Minamikawa et al., 1998; Shiomi et al., 1999; Li et al., 2000). Moreover, our data provide compelling evidence that the observed antiatherogenic effects of PPARγ ligands result, at least in part, from direct effects on macrophages within the atherosclerotic lesion. Given that PPARγ is expressed at high levels in lipid-loaded macrophages in both mice and humans in vivo (Ricote et al., 1998b; Tontonoz et al., 1998), it may also be possible to accelerate the rate of reverse cholesterol transport from human atherosclerotic lesions through ligand activation of PPARγ. Finally, in light of the direct regulatory relationship between PPARγ and LXRα in macrophages, the combination of ligands for both PPARγ and LXR, or their common heterodimer partner RXR, would be expected to be maximally effective in reducing plaque burden.

Experimental Procedures

Reagents

Oxysterols (Sigma) were dissolved in ethanol prior to addition to cells (<1 μl/ml). GW7845 was provided by Dr. Tim Willson (Glaxo Wellcome). Apolipoprotein AI (apoAI, Intracel) was resuspended in phosphate buffered saline, quick frozen, and stored in aliquots at −80°C. LG268 was a gift from Dr. Richard Heyman (Ligand Pharmaceuticals). [^{3}H]cholesterol (45 Ci/mmol) was from NEN Life Science Products.

Cell Culture, Stable Cell Lines, and RNA Analysis

THP-1 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum or 10% lipoprotein-deficient fetal bovine serum (LPDS) as indicated. ES cells were cultured in the presence of rLIF (Gibco) as described (Keller et al., 1993). Macrophage differentiation was induced in ES cells in a two–stage process. Initially, ES cells were differentiated into embryoid bodies as described previously. Day 6 embryoid bodies were isolated, disaggregated, and replated in the presence of IL-3 and M-CSF-1 to induce differentiation of monocytes and macrophages, respectively. Total RNA was isolated using TRIzol reagent (Gibco), and Northern analyses were performed as described previously.

Transfection Assays

Murine LXRα and β promoters were amplified by PCR using the published genomic structure and sequence. Primers for PCR of LXR promoters were: LXRα: 5′ ATCCTGTCCCTTCTGTCC 3′ and 5′ CCTCCAGAGTCAGCGTTC 3′, and LXRβ: 5′ CAGTGAGCGCATA CAGGT 3′ and 5′ TCTCCGACTCTGTTGCCC 3′. To determine the transcription initiation site for mABCA1, 5′ RACE was performed using the 5′/3′ RACE kit (Roche) with RNA from the RAW 274 cell line and mouse liver, and the gene-specific 3′ primer (5′ CTGAGGCC AACAAGCCAT 3′). The extended RACE products were used to screen a mouse genomic library (Stratagene). Sequence analysis of the isolated clones by the Blast program revealed that the identified initiation site and 5′ regulatory region of ABCA1 were identical to the sequences deposited in GenBank (accession number AJ017356). The 2.5 kb genomic fragment of the mABCA1 promoter was cloned into a pGL3-basic vector. CV-1 cells were transfected by lipofection and assayed for reporter activity as described previously (Forman et al., 1995). Transfections were performed in triplicate and normalized to an internal CMX-βgal control. pCMX expression vectors for the PPARs and LXRs have been described (Kliewer et al., 1994; Willy et al., 1995).

Gel Mobility Shift Assays

Gel mobility shift assays were performed using in vitro translated receptors and ^{32}P end-labeled oligonucleotides in a buffer containing 20 mM HEPES (pH 7.4), 100 mM KCl, 1 mM β-mercaptoethanol, 10% glycerol, 100 μg/ml polydI-dC, and 5 mg/ml BSA. For competition studies, an excess of unlabeled oligonucleotide was added at the indicated concentration.

Cholesterol Efflux

Cholesterol efflux assays were performed as described (Venkateswaran et al., 2000a), with minor modifications. Cells were plated at 50% confluence. On day 2, cells were washed and incubated for 24 hr in RPMI 1640 supplemented with 10% LPDS. Cells were labeled with [^{3}H]cholesterol (1.0 μCi/ml), either in the presence of the ACAT inhibitor (58-035; 2 μg/ml) or with acLDL (50 μg/ml) in the absence of the ACAT inhibitor. Ligands for PPARγ (BRL; 5 μM), RXR (LG268; 50 nM), or LXR (22[R]-OHC; 2.0 μg/ml) were added to the cells as indicated in the figure. To equilibrate cholesterol pools, cells were washed twice with PBS and incubated for 8 hr in RPMI containing 0.2% BSA plus the indicated ligands, but lacking radiola-

beled cholesterol or acLDL. Cells were again washed with PBS and incubated in RPMI containing 0.2% BSA in the absence or presence of HDL (50 μg/ml) or apoAI (15 μg/ml), for 4 hr. An aliquot of the medium was removed and centrifuged at 14,000 × g for 2 min, and the radioactivity was determined by liquid scintillation counting. Total cell-associated radioactivity was determined by dissolving the cells in isopropanol. The data is presented as percent HDL-specific efflux or apoAI-specific efflux, which is efflux in the presence of acceptor minus efflux in the absence of acceptor. Each assay was performed in triplicate.

Bone Marrow Transplantation

LDL-R −/− mice backcrossed onto the C57BL/6 background were initially obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at the Scripps Research Institute Animal Facility. Mice were fed a chow diet (Harlan Teklad, Madison, WI) ad libitum until they were enrolled into the study. PPARγ −/− ES cells were injected into C57/Bl6 blastocysts to generate PPARγ −/− chimeric mice. The majority of the chimeric mice obtained were high-percentage chimeras. Peripheral blood, spleen, and bone marrow PPARγ −/− chimeras were extensively analyzed by facs analysis with antibodies directed against Ly 9.1, CD11b/CD18, F4/80, CD3, B220, and Ly 6.1 to quantify the extent of chimeraism (data not shown). Chimeras, which were greater than 90% null in their contribution to the monocytic/macrophage lineage, were used for bone marrow transplantation, along with wild-type control mice.

Twenty-four 6-week-old male LDL-R −/− mice were γ-irradiated with 1000 rads to eliminate the majority of their bone marrow cells. Each of the γ-irradiated mice were reconstituted via a tail vein injection with 2,000,000 bone marrow cells isolated from either PPARγ +/+ mice (designated PPARγ +/+ BMT) and PPARγ −/− mice (designated PPARγ −/− BMT). The mice were placed on a chow diet for 4 weeks while their marrow repopulated with the donor cells. For induction of atherosclerosis, the transplanted mice were placed on an atherogenic diet containing 15.8% (wt/wt) fat and 1.25% cholesterol (no cholate; diet 94059, Harlan Teklad) for 8 weeks. At weeks 0 (before BMT), 4, and 12, blood was drawn via the retroorbital plexus following an 8 hr fast, and total plasma cholesterol was measured via an enzymatic assay (Sigma, St. Louis, MO). All procedures were in accordance with institutional guidelines.

Lesion Analysis and Immunohistochemistry

Atherosclerotic lesions were quantified in the aortic valve of each mouse as described previously (Boisvert et al., 1998). Briefly, the OCT-embedded, frozen aortic valves were sectioned serially at 10 μm thickness for a total of 300 μm beginning at the base of the aortic valve, where all three leaflets are first visible. Every fourth section for a total of five sections from each animal was stained with oil red O to identify the lipid-rich lesions. The stained areas were quantified using a computer-assisted video imaging system, and the mean area of the five sections from each animal was used for comparison analysis. Statistical analysis was performed using the Mann-Whitney U test, and data are expressed as mean ± SD.

The mouse aortic valve lesions were analyzed immunohistochemically with the following antibodies: anti-MOMA-2 (Serotec) for detection of intimal macrophages; anti-β-galactosidase (Cappel) for detection of PPARγ −/− cells; and anti-α-actin (Dako) for detection of smooth muscle cells in the lesion. The frozen tissue sections were blocked with 5% normal sera and incubated for 2 hr at room temperature with the primary antibody (1–10 μg/ml). The sections were blocked for endogenous peroxidase activity with Peroxo-Block (Zymed, South San Francisco, CA), followed by incubation with the appropriate secondary antibody (5 μg/ml) for 1 hr. The washed sections were finally incubated for 30 min with Vectastain ABC Elite solution (Vector Laboratories, Burlingame, CA), developed with 9-amino-3-ethylene-carbazole (AEC; Vector Laboratories), and counterstained with hematoxylin.

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