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CLA-1/SR-BI Is Expressed in Atherosclerotic Lesion Macrophages and Regulated by Activators of Peroxisome Proliferator-Activated Receptors

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Background—The scavenger receptors are cell-surface receptors for native and modified lipoproteins that play a critical role in the accumulation of lipids by macrophages. CLA-1/SR-BI binds HDL with high affinity and is involved in the cholesterol reverse-transport pathway. Peroxisome proliferator–activated receptors (PPARs) are transcription factors regulating the expression of genes implicated in lipid metabolism, cellular differentiation, and inflammation. Here, we investigated the expression of CLA-1/SR-BI in macrophages and its regulation by PPARs.

Methods and Results—CLA-1 is undetectable in human monocytes and is induced upon differentiation into macrophages. Immunohistological analysis on human atherosclerotic lesions showed high expression of CLA-1 in macrophages of the lipid core colocalizing with PPARα and PPARγ staining. Activation of PPARα and PPARγ resulted in the induction of CLA-1 protein expression in monocytes and in differentiated macrophages. Finally, SR-BI expression is increased in atherosclerotic lesions of apoE-null mice treated with either PPARγ or PPARα ligands.

Conclusions—Our data demonstrate that CLA-1/SR-BI is expressed in atherosclerotic lesion macrophages and induced by PPAR activation, identifying a potential role for PPARs in cholesterol homeostasis in atherosclerotic lesion macrophages. (Circulation. 2000;101:2411-2417.)

Key Words: receptors ■ atherosclerosis ■ plaque ■ lipoproteins ■ immunohistochemistry

Lipid-laden foam cells originating principally from monocyte-derived macrophages are characteristic of the fatty streak, the first defined atherosclerotic lesion.1,2 The accumulation of cholesteryl esters in macrophages is essentially due to the internalization of oxidatively modified lipoproteins via scavenger receptors, a family of cell surface membrane proteins.3 The first of the scavenger receptors to be characterized were the types I and II of class A (SR-AI and SR-AII).4 These SR-As bind acetylated and oxidized LDL and may play a role in host defense and cell adhesion, as well as in atherosclerosis.5,6 In addition, a class B of scavenger receptors has been identified, which includes CD36,7 LIMPII,8 the murine scavenger receptor class B type I (SR-BI),9 and its human homologue CD36- and LIMPII-analogous 1 (CLA-1).10

SR-BI, which is highly expressed in liver, adrenal gland, ovary,11 and atherosclerotic lesions of apoE-deficient mice,12 binds HDL with high affinity and mediates the selective uptake of cholesteryl esters from HDL in liver and steroidogenic tissues.13 As such, SR-BI may play a role in the transport of cholesterol by HDL from peripheral tissues to the liver, known as the reverse cholesterol transport pathway. Overexpression of SR-BI in liver reduces HDL levels, increases reverse cholesterol transport,14,15 and decreases susceptibility to atherosclerosis.16 By contrast, genetic suppression of SR-BI activity in apoE-null mice accelerates the onset of atherosclerosis.17 Studies demonstrating that the rate of cholesterol efflux mediated by HDL or serum is correlated with cellular SR-BI expression levels13 suggest that SR-BI may promote cholesterol removal from peripheral cells, such as cultured macrophages. Taken together, these data indicate that SR-BI plays an important role in HDL metabolism.

CLA-1 binds HDL with an affinity similar to that of SR-BI,10 and its expression is mostly restricted to tissues involved in cholesterol metabolism, such as liver or steroidogenic tissues.18,19 Like CD36, CLA-1 recognizes anionic phospholipids, including those confined to the outer layer of the plasma membrane of apoptotic cells.20 Peroxisome proliferator–activated receptors (PPARs) are nuclear receptors21,22 that, upon heterodimerization with the...
retinoid X receptor, function as ligand-activated transcriptional regulators of genes controlling lipid and glucose metabolism. Two different PPARs, α, β, and γ, have been identified so far. PPARα, highly expressed in liver, heart, muscle, and kidney and in cells of the arterial wall, is activated by fibrates, fatty acids, and eicosanoids such as leukotriene B4 and 8(S)-hydroxyeicosatetraenoic acid. Fatty acid derivatives and eicosanoids such as 15-deoxy-D12,14-prostaglandin J2 and the antidiabetic glitazones are ligands for PPARα, which is expressed at high levels in white adipose tissue, where it triggers adipocyte differentiation. Both PPARα and PPARγ are expressed in differentiated human macrophages, where they regulate genes implicated in the inflammatory response (such as nuclear factor-κB and inducible nitric oxide synthase) and modulate macrophage differentiation and apoptosis induction. Furthermore, PPARγ activation results in the transcriptional induction of CD36 expression, whereas the transcriptional activation of scavenger receptor A by proinflammatory stimuli is inhibited. Together, these data suggest that PPARs are important regulators of lipid metabolism and atherosclerosis development. The goal of this study was to investigate the expression of CLA-1/SR-BI in human monocytes and macrophages and its regulation by PPAR activators. Furthermore, we also studied the effects of PPAR ligands on SR-BI expression in atherosclerotic lesions of apoE-null mice. Our results indicate that PPARs regulate CLA-1/SR-BI expression, identifying a possible modulatory role for PPARs in cholesterol homeostasis in atherosclerotic lesion macrophages.

Methods

Cell Culture

Mononuclear cells, isolated from blood by Ficoll gradient centrifugation, were suspended in medium containing 10% pooled human serum. Differentiation into macrophages occurred spontaneously by adhesion to the culture dishes. Mature monocytes-derived macrophages were used for experiments after 12 days of culture, whereas monocytes were used after 45 minutes of adherence to the plastic dish. For treatment with different activators, medium was changed to RPMI 1640 without serum but supplemented with 1% Nutridoma (Boehringer Mannheim).

Protein Extraction and Western Blot Analysis

Cells were harvested in lysis buffer containing PBS, 1% Triton X-100, 1 mmol/L PMSF, and a protease inhibitor cocktail (ICN). Western blot analysis was performed with a rabbit polyclonal antibody raised against amino acids 470 to 509 of human CLA-1 or a goat polyclonal antibody against β-actin (Santa Cruz). Specific signals of CLA-1/SR-BI were quantified on a Biorad GS670 densitometer and normalized to internal control β-actin.

Mice and Diets

Eleven-week-old female C57BL/6J apoE-null mice (n=12) were fed for 2 weeks with a standard chow diet (control animals) or with the same diet containing 0.2% (wt/wt) fenofibrate (Sigma) or 1.4% (wt/wt) troglitazone (Rezulin, Sankyo Parke-Davis).

Tissue Preparation and Immunohistological Analysis

The basal portion of the heart and proximal aorta of apoE-null mice were embedded in OCT compound (Tissue-Tek) and frozen in liquid nitrogen. Serial 5-μm-thick cryosections of the heart tissue, covering the area between the appearance and the disappearance of the mitral valves, were collected on poly-L-lysine–coated slides (Polylabo). Sections were saturated with 3% skim milk powder, 0.1% Tween 20 in PBS for 20 minutes at room temperature. Rabbit antibodies against CLA-1/SR-BI, PPARα, and PPARγ or rat anti–MOMA-2 (Biosource) were added overnight at 4°C. Signals were visualized with secondary fluorescein isothiocyanate–conjugated antibodies.

Immunohistochemical analysis was performed on atherosclerotic plaques removed from patients during carotid endarterectomy as described with anti–CLA-1, anti-PPARα, anti-PPARγ, or mouse anti-CD68 (DAKO-CD68, KP1) primary antibodies.

Tissue Protein Extraction

Aortas were homogenized in lysis buffer (mmol/L: sucrose 250, HEPES 10, KCl 10, EDTA 2, EGTA 1, and PMSF 0.5, and the protease inhibitor cocktail). Proteins were collected by centrifugation at 10 000 rpm for 25 minutes at 4°C. Then 20 μg of total proteins was loaded onto 10% SDS-PAGE, and Western blot analysis was performed.

Results

CLA-1 Is Expressed on Human Monocyte/Macrophage Differentiation

Western blot analysis was performed with a specific CLA-1 antibody on proteins from freshly isolated human monocytes and macrophages at different stages of differentiation. CLA-1 was not detectable in undifferentiated human monocytes after 45 minutes of adherence to culture dishes but was induced during the first stages of differentiation (after 3 days of culture) (Figure 1). CLA-1 expression increased strongly between days 4 and 6 and rose further in differentiated macrophages after 12 days of culture.

CLA-1 Is Expressed in Human Atherosclerotic Lesion Macrophages

Immunohistological analysis was performed on human carotid atherosclerotic plaques. High levels of CLA-1 expression were detected mainly in the region of the lipid core of the plaque (Figure 2A) but also in subendothelial macrophages (Figure 2D). Immunoreactive CLA-1 colocalized with macrophages (positive CD68 staining) (Figure 2B), whereas only weak CLA-1 signals were detectable in other cell types, such as smooth muscle cells (not shown). In these plaques, CLA-1 was observed within the same regions presenting PPARα (Figure 2C) and PPARγ (Figure 2E).

CLA-1 Expression Is Regulated by PPAR Activators in Human Monocytes

To study the effects of PPAR activators on CLA-1 expression in human monocytes, Western blot analysis was performed on extracts from mononuclear cells, isolated by 45 minutes of adhesion to culture dishes, incubated for 24 hours with activa-
tors. CLA-1 protein was induced ≈2-fold in the presence of the PPARα ligand Wy14,643 (10 μmol/L) (Figure 3). A similar induction was observed with GW2331 (200 nmol/L), a combined PPARα and PPARγ ligand (EC50 = 0.028 μmol/L for PPARα and 0.114 μmol/L for PPARγ). Treatment with the highly specific PPARγ ligand BRL49653 (20 nmol/L) was without major effect on CLA-1 protein levels, most likely because of the low amounts of PPARγ present in monocytes.26 By contrast, PG-J2 (1 μmol/L) strongly induced CLA-1 expression, which may be a result of the lower selectivity of this ligand for the different PPARs compared with BRL49653.36 β-Actin protein levels did not change upon incubation with the PPAR activators (Figure 3).

CLA-1 Expression Is Regulated by PPAR Activators in Differentiated Human Macrophages

The effects of PPAR activation on CLA-1 expression in differentiated human macrophages were studied by Western blot analysis using protein extracts isolated from 12-day-old macrophages incubated for 24 hours with different PPAR activators. In contrast to monocytes (Figure 3), the amount of CLA-1 protein increased ≈3-fold in differentiated macrophages incubated with BRL49653 (20 nmol/L), which is in accordance with the high level of expression of PPARγ in mature human macrophages.26 Treatment with the PPARα activator Wy14,643 (10 μmol/L) resulted in a 2-fold induction, whereas expression of CLA-1 was enhanced ≈5- and 2.3-fold after incubation with GW9820 (1 μmol/L) (EC50 = 0.37 μmol/L for PPARα and 0.288 μmol/L for PPARγ) and GW2331 (200 nmol/L) compounds, respectively (Figure 4). β-Actin protein content did not change upon treatment with any of the activators. Furthermore, incubation with PPARα (Wy14,643), PPARγ (BRL49653 and troglitazone), or combined PPARα and PPARγ (GW2331) ligands induced CLA-1 expression in a dose-dependent manner in differentiated macrophages (Figure 5).

In Vivo Regulation of CLA-1/SR-BI Protein Expression

To determine whether SR-BI regulation by PPAR activators also occurred in vivo, apoE-null mice were treated with chow diet or a diet enriched with either the PPARγ ligand troglita-
tazone or the PPARα ligand fenofibrate. As in human atherosclerotic lesions, substantial SR-BI staining was observed in the aortic atherosclerotic lesions of control (Figure 6A) and fenofibrate-treated (Figure 6B) and troglitazone-treated (Figure 6C) apoE-null mice, colocalizing with resident macrophages as determined by use of an antibody against the macrophage antigen MOMA-2 (not shown). The intensity and frequency of SR-BI signals were higher in troglitazone- and fenofibrate-treated mice than in control animals. This induction was not due to augmentation of resident macrophages in atherosclerotic lesions, because MOMA-2 staining remained constant (not shown). Moreover, PPARγ (Figure 6, G, H, and I) and PPARα (Figure 6, D, E, and F) were detected in all lesions at levels that were similar between control and treated animals.

Because clinical studies indicated that troglitazone may induce liver toxicity, the effects of troglitazone on animal hepatotoxicity were analyzed. Even used at concentrations 5 to 10 times higher than those used as an insulin sensitizer, troglitazone did not induce any hepatotoxicity in animals, as determined by analysis of serum alkaline phosphatase (control, 250 ± 36 IU and troglitazone, 284 ± 35 IU) and LDH (control, 459 ± 244 IU and troglitazone, 323 ± 146 IU) activities. The induction of SR-BI expression was quantified by Western blot analysis using protein extracts from descending aortas of these mice. Fenofibrate induced SR-BI expression 2.3-fold, whereas troglitazone increased its levels 4.5-fold (Figure 7). PPAR expression remained unchanged upon treatment (data not shown). The present data indicate that activation of PPARα and PPARγ induces the expression of SR-BI protein in vivo.

Discussion

PPARs are transcription factors regulating genes involved in lipid and glucose metabolism and cellular differentiation that mediate the action of fibrates and glitazones. PPARs play an important role in vascular physiopathology: both PPARα and PPARγ are present in human endothelial cells, smooth muscle cells, and macrophages in vitro, whereas only PPARγ has been described in resident atherosclerotic lesion macrophages. Here, we show that in addition to PPARγ, PPARα also is highly expressed in resident macrophages (Figure 2B) of human (Figure 2C) and mouse (Figure 6, D, E, and F) atherosclerotic lesions. In macrophages, PPARs are implicated in differentiation, foam cell formation, apoptosis, and inflammation control.
The fact that PPARγ ligands inhibit the transcriptional activation of SR-A and enhance CD36 expression and oxidized LDL–derived fatty acid uptake in macrophages after PPARγ/retinoid X receptor activation raised the question of a potential role for PPARs in the control of the expression of another member of the scavenger receptor family, the human HDL receptor CLA-1/SR-BI. Here, we demonstrate that CLA-1 is undetectable in monocytes but is strongly induced upon differentiation into macrophages. Therefore, CLA-1, like other members of the scavenger receptor family (such as SR-A, CD36, and macrosialin/CD68), is a marker for monocyte/macrophage differentiation. A previous report showed that CLA-1 expression is significantly downregulated when THP-1 cells are differentiated into macrophages upon treatment with phorbol ester. These differences between primary macrophages and THP-1 cells may be due either to a specific characteristic of the transformed THP-1 cell line or to the fact that phorbol ester downregulates CLA-1 independently of its differentiation-inducing effect. The observation that CLA-1 is expressed in differentiated macrophages in vitro raised the question whether CLA-1 is also expressed in vivo in macrophages resident in atherosclerotic plaques. In human carotid atherosclerotic lesions, positive CLA-1 staining is observed in the subendothelial region and the lipid core, colocalizing with specific macrophage markers. Therefore, high levels of CLA-1/SR-BI are present in both human and mouse atherosclerotic lesions, thus suggesting a possible role for this scavenger receptor in the pathogenesis of atherosclerosis in humans as well.

In human monocytes and macrophages, PPARs control CLA-1/SR-BI expression. PPARα activation by Wy14,643 results in a pronounced induction of CLA-1 expression. By contrast, addition of the PPARγ ligand BRL49653 at a concentration within the range of its \( K_d \) for PPARγ does not influence CLA-1 expression. This result is in line with our previous observations that human monocytes express low levels of PPARγ but higher levels of PPARα. However, treatment with PG-J2 resulted in a strong inductive effect on CLA-1, which might be a result of its lesser specificity for PPARγ compared with BRL49653 or of an effect indepen-
dent of the nuclear receptor PPARγ. In macrophages, both PPARα and PPARγ activators induce CLA-1 expression in fully differentiated macrophages (Figure 5), an observation in line with the presence of high levels of PPARα and PPARγ protein in these cells. Because the currently used fibrates are low-affinity and low-specificity PPARα ligands, the effects of 2 compounds with higher activity and specificity for PPARα were tested in macrophages. GW2331 and GW9820 at concentrations that activated both PPARα and PPARγ (200 nmol/L and 1 μmol/L, respectively) induce CLA-1 protein to a higher extent, suggesting that both PPARs can cooperate to induce CLA-1 expression.

The induction of CLA-1/SR-BI expression by PPAR activation occurs not only in vitro but also in vivo. In apoE-null mice, both fenofibrate and troglitazone (at a nonhepatotoxic dose) significantly increase aortic SR-BI protein content together with increased CLA-1/SR-BI levels in macrophages of atherosclerotic lesions. To the best of our knowledge, this is the first demonstration of SR-BI protein regulation in atherosclerotic lesions. Furthermore, the observation that CLA-1/SR-BI protein expression is upregulated after treatment with PPAR activators provides direct evidence for a role of PPARs in the in vivo regulation of genes of the arterial wall and further confirms a role for these nuclear receptors in vascular function and physiopathology.

Taken together with the previously reported induction of CD36 in macrophages by PPAR activators, our results indicate that PPARα and PPARγ positively regulate members of the class B scavenger receptors (CD36 and CLA-1/SR-BI). By contrast, SR-A transcription may be inhibited by PPARγ activators, suggesting that PPARs act as negative regulators of the class A scavenger receptors. Further studies are necessary to investigate the molecular mechanisms by which PPARs modulate the expression of CLA-1/SR-BI in vitro and in vivo.

It is tempting to speculate that CLA-1/SR-BI upregulation by PPARs affects arterial wall functions and atherosclerosis development. Several experimental studies implicate the scavenger receptor SR-BI, which binds HDL with high affinity, in the selective uptake of esterified and free cholesterol from HDL in liver and steroidogenic tissues, thereby supplying a substrate for steroid hormone synthesis. In addition to its role in cholesterol delivery in peripheral tissues, such as the arterial wall, SR-BI may be involved in cellular cholesterol efflux, leading to the removal and transport of cholesterol from peripheral tissues to the liver for elimination. Indeed, in several cell lines, including macrophages, the rate of cholesterol efflux mediated by HDL correlates well with SR-BI expression levels. Thus, in atherosclerotic lesion macrophages, an increase of CLA-1/SR-BI expression could enhance the removal by HDL of unesterified cholesterol from foam cells, resulting in the regression of the fatty streak, leading to a beneficial action of PPAR activators in the control of lipid content and cholesterol homeostasis in macrophages. However, like other members of the scavenger receptor family, CLA-1/SR-BI also binds other native and modified lipoproteins, such as VLDL, LDL, and oxidized LDL, which may lead to the accumulation of cholesterol esters within cells of the arterial wall. It is tempting to speculate that the balance between cholesterol influx and efflux from cells is controlled by the intracellular amounts of cholesterol and that influx and efflux of cholesterol ultimately depends on the cholesterol gradient between cells and their environment. If such a hypothesis is true, it is conceivable that SR-BI mediates influx in normal macrophages, whereas efflux occurs from lipid-laden foam cells. Finally, CLA-1/SR-BI, like other scavenger receptors, may participate in physiological processes other than lipoprotein and lipid metabolism, such as the recognition of cell-surface-exposed phosphatidylserine on apoptotic cells. Overall, the observation that CLA-1/SR-BI is upregulated by PPAR activators supports the hypothesis of a possible role of PPARs not only in macrophage apoptosis induction but also in tissue remodeling during development and aging by recognizing damaged or apoptotic cells. Further studies are necessary to explore an involvement of CLA-1/SR-BI in any of these or other processes and to determine the influence of induction of CLA-1/SR-BI by PPARs on atherosclerosis development.

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