Telmisartan Exerts Antiatherosclerotic Effects by Activating Peroxisome Proliferator-Activated Receptor-\(\gamma\) in Macrophages

Takeshi Matsumura, Hiroyuki Kinoshita, Norio Ishii, Kazuki Fukuda, Hiroyuki Motoshima, Takaumi Senokuchi, Kayo Taketa, Shuji Kawasaki, Tomoko Nishimaki-Mogami, Teruo Kawada, Takeshi Nishikawa, Eiichi Araki

Objective—Telmisartan, an angiotensin type I receptor blocker (ARB), protects against the progression of atherosclerosis. Here, we investigated the molecular basis of the antiatherosclerotic effects of telmisartan in macrophages and apolipoprotein E–deficient mice.

Methods and Results—In macrophages, telmisartan increased peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)) activity and PPAR ligand-binding activity. In contrast, 3 other ARBs, losartan, valsartan, and olmesartan, did not affect PPAR\(\gamma\) activity. Interestingly, high doses of telmisartan activated PPAR\(\alpha\) in macrophages. Telmisartan induced the mRNA expression of CD36 and ATP-binding cassette transporters A1 and G1 (ABCA1/G1), and these effects were abrogated by PPAR\(\gamma\) small interfering RNA. Telmisartan, but not other ARBs, inhibited lipopolysaccharide-induced mRNA expression of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-\(\alpha\), and these effects were abrogated by PPAR\(\gamma\) small interfering RNA. Moreover, telmisartan suppressed oxidized low-density lipoprotein-induced macrophage proliferation through PPAR\(\gamma\) activation. In apolipoprotein E\(^{-/-}\) mice, telmisartan increased the mRNA expression of ABCA1 and ABCG1, decreased atherosclerotic lesion size, decreased the number of proliferative macrophages in the lesion, and suppressed MCP-1 and tumor necrosis factor-\(\alpha\) mRNA expression in the aorta.

Conclusion—Telmisartan induced ABCA1/ABCG1 expression and suppressed MCP-1 expression and macrophage proliferation by activating PPAR\(\gamma\). These effects may induce antiatherogenic effects in hypertensive patients. (Arterioscler Thromb Vase Biol. 2011;31:1268-1275.)

Key Words: atherosclerosis ■ hypertension ■ macrophages ■ vascular biology ■ peroxisome proliferator-activated receptor

The renin-angiotensin system regulates electrolyte balance, body fluid volume and blood pressure.\(^1\) The renin-angiotensin system also plays an important role in the progression of atherosclerosis.\(^2\) In fact, inhibition of the renin-angiotensin system by either angiotensin-converting enzyme inhibitors or angiotensin type I receptor blockers (ARBs) reduced atherosclerotic lesion formation in experimental studies using various models of atherosclerosis.\(^3,4\) However, it is possible that the effects of each individual ARB on cardiovascular disease are variable, because there is a difference in pharmacological futures of each ARB, such as liposolubility, potency, stability, and other selective properties.

Macrophages are present in all stages of atherosclerosis and are considered to be fundamental to atherogenesis and the behavior of established plaques.\(^8\) Macrophages take up chemically modified low-density lipoprotein (LDL) through scavenger receptor pathways and transform into foam cells in vitro.\(^9\) In turn, foam cells produce various bioactive molecules, such as chemokines, cytokines, and growth factors, all of which play important roles in the development and progression of atherosclerosis.\(^8\) On the other hand, several in vivo studies have reported that macrophages and macrophage-derived foam cells proliferate in atherosclerotic lesions.\(^10-12\) We have shown,\(^13,14\) as have other groups,\(^15,16\) that oxidized LDL (Ox-LDL) enhances macrophage proliferation and survival in vitro. Therefore, it is possible that macrophage proliferation may promote the progression of atherosclerosis.

Telmisartan, an ARB, has also been identified as a ligand for peroxisome proliferator-activated receptor-\(\gamma\) (PPAR\(\gamma\)).\(^17,18\) PPAR\(\gamma\) is a transcription factor belonging to the nuclear receptor superfamily that heterodimerizes with the retinoid X receptor

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From the Department of Metabolic Medicine, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan (T.M., H.K., N.I., K.F., H.M., T.S., K.T., S.K., T.N., E.A.); Department of Biochemistry and Metabolism, National Institute of Health Sciences, Setagaya-ku, Tokyo, Japan (T.N.-M.); Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan (T.K.).
Drs Matsumura and Kinoshita contributed equally to this work.
Correspondence to Takeshi Matsumura, MD, PhD, Department of Metabolic Medicine, Faculty of Life Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan. E-mail takeshimi@gpo.kumamoto-u.ac.jp
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and binds to PPAR response elements in target gene promoters. PPARγ has many antiatherogenic effects in macrophages, endothelial cells, and smooth muscle cells. Furthermore, PPARγ agonists inhibit the development of atherosclerosis in various mouse models of atherosclerosis. Thus, PPARγ activation by telmisartan is beneficial for suppressing atherosclerosis. Indeed, telmisartan suppresses the progression of atherosclerosis in apolipoprotein E–deficient (apoE−/−) mice. However, the role of PPARγ activation in the protective effects of telmisartan against the progression of atherosclerosis is poorly understood.

Therefore, the aim of the present study was to clarify whether the antiatherogenic effects of telmisartan are mediated by the activation of PPARγ in macrophages. We found that telmisartan activated PPARγ, suppressed tumor necrosis factor-α (TNFα) and monocyte chemoattractant protein-1 (MCP-1) expression, and inhibited Ox-LDL-induced cell proliferation by activating PPARγ in macrophages. Telmisartan also activated PPARγ in the aorta of apoE−/− mice and suppressed the progression of atherosclerosis.

Methods

Cell Culture

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Kumamoto University, Japan. Peritoneal macrophages were collected from anesthetized male C3H/He mice (body weight, 25 to 30 g) by peritoneal lavage with 8 mL of phosphate-buffered saline (PBS), centrifuged at 200g for 5 minutes, resuspended in medium A (RPMI 1640 medium [Nissui Seiyaku, Tokyo, Japan] supplemented with 10% fetal calf serum [Invitrogen], 0.1 mg/mL streptomycin, and 100 U/mL penicillin), and incubated in tissue culture plates for 90 minutes.

More than 98% of the adherent cells were considered to be macrophages, based on criteria, as previously described.

RAW264.7 cells were cultured in medium A in tissue culture plates, as previously described, and were used at passages 4 to 8.

Lipoprotein Preparation

Human LDL (d = 1.019 to 1.063 g/mL) was isolated by ultracentrifugation from plasma samples obtained from consenting normolipidemic subjects after an overnight fast. LDL was dialyzed against 0.15 mol/L NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4. Ox-LDL was prepared by incubating LDL with 5 μmol/L CuSO4 for 20 hours at 37°C followed by the addition of 1 mmol/L EDTA and cooling.

The protein concentration was determined using a BCA protein assay reagent (Pierce Chemical Co, Rockford, IL). The endotoxin level of Ox-LDL was <1 pg/μg protein and was measured using a Toxicolor system (Seikagaku Corp, Tokyo, Japan).

Animals

ApoE−/− mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and were maintained on the C57BL/6 background strain. Mice were housed at the Animal Resource Facility at Kumamoto University under specific pathogen-free conditions and were given free access to food and water. All animal procedures were approved by the Animal Research Committee at Kumamoto University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources. The mice were given a normal rodent chow diet developed for mice (CLEA, Tokyo, Japan). Twenty male mice of 6 weeks of age were treated orally with telmisartan (3 mg/kg) or a placebo (control). After 10 weeks of treatment, they were euthanized. Cross-sectional lesions of the aortic sinus were used for real-time reverse transcription–polymerase chain reaction (RT-PCR) assays, and the PPARγ transcription activity assay was performed as described below.

Results

Telmisartan Activates PPARγ in Macrophages

First, we investigated the effect of telmisartan on PPARγ activation in RAW264.7 macrophages using a full-length PPARγ luciferase assay system. Telmisartan increased lu-
Telmisartan activates PPARγ in macrophages. RAW264.7 cells were incubated for 24 hours with medium A in the absence or presence of the indicated concentrations of telmisartan (Telm), pioglitazone (Pio), losartan (Los), valsartan (Val), or olmesartan (Olm). Luciferase activity was determined using a full-length PPARγ luciferase assay system (A), a PPARγ GAL4 chimera assay system (B and C), and a PPARα GAL4 chimera assay system (D). Values are means±SEM of 5 separate experiments. *P<0.01 vs control.

Telmisartan Induces PPARγ-Responsive Gene Transcription in Macrophages

We next examined whether telmisartan increased the expression of PPARγ-responsive genes, including CD36, ATP-binding cassette transporter A1 (ABCA1), and ABCG1, and induced the activation of liver X receptor, a member of the nuclear receptor family of transcription factors that also increased luciferase activity, and the increase in luciferase activity elicited by 10 μmol/L pioglitazone was approximately 2.7-fold higher than that at the same concentration of telmisartan (Figure 1C). Interestingly, telmisartan at ≥10 μmol/L increased luciferase activity using GAL4 chimera assay of PPARγ in macrophages (Figure 1D).

Figure 1. Telmisartan activates PPARγ in macrophages. RAW264.7 cells were incubated for 24 hours with medium A in the absence or presence of the indicated concentrations of telmisartan (Telm), pioglitazone (Pio), losartan (Los), valsartan (Val), or olmesartan (Olm). Luciferase activity was determined using a full-length PPARγ luciferase assay system (A), a PPARγ GAL4 chimera assay system (B and C), and a PPARα GAL4 chimera assay system (D). Values are means±SEM of 5 separate experiments. *P<0.01 vs control.

Figure 2. Telmisartan increases the mRNA expression of CD36, ABCA1, and ABCG1. Mouse peritoneal macrophages were transfected without (A to D) or with control (con) or PPARγ siRNA (E and F) and were incubated with medium A for 4 hours. A to D and F, Cells were incubated with medium A in the absence or presence of 10 μmol/L telmisartan (Telm) for 24 hours. The mRNA levels of CD36 (A), ABCA1 (B and F), and ABCG1 (C and F), and liver X receptor (LXR) activity were determined by real-time RT-PCR and an LXR luciferase assay, respectively. Data are means±SEM of 4 separate experiments. *P<0.01 vs cells treated with control siRNA alone; **P<0.01 vs cells treated with control siRNA plus telmisartan. E, Protein samples were immunoblotted with anti-PPARγ or β-actin antibodies. Data are representative of 4 separate experiments.
It is well known that inflammatory cytokines, including MCP-1 and TNF-α, are involved in the progression of atherosclerosis. Therefore, we examined the effects of telmisartan on lipopolysaccharide (LPS)-induced mRNA expression of MCP-1 and TNF-α in macrophages. Telmisartan suppressed LPS-induced mRNA expression of MCP-1 and TNF-α, whereas losartan, olmesartan, and valsartan did not (Figure 3A and 3B). Compared with treatment with control siRNA, the telmisartan-induced suppression of MCP-1 and TNF-α mRNA expression was restored by treatment with PPARγ siRNA (Figure 3C and 3D). Telmisartan also suppressed LPS-induced nuclear factor-κB (NF-κB) activation, and this effect was restored by PPARγ siRNA (Figure 3E).

**Telmisartan Suppresses Ox-LDL-Induced Macrophage Proliferation**

We next examined the effects of ARBs on Ox-LDL-induced macrophage proliferation. Twenty μg/mL of Ox-LDL increased [3H]thymidine incorporation into macrophages, as previously reported,13,14,25,26 and pretreatment with telmisartan suppressed Ox-LDL-induced [3H]thymidine incorporation in a dose-dependent manner (Figure 4A). However, losartan, valsartan, and olmesartan had no such effects.

**Figure 3.** Telmisartan suppresses mRNA expression of MCP-1 and TNF-α. A and B, Mouse peritoneal macrophages were transfected with control or PPARγ siRNA and were incubated with medium A for 4 hours. The cells were then incubated with medium A in the absence or presence of 10 μmol/L telmisartan (Telm), losartan (Los), valsartan (Val), or olmesartan (Olm). After incubation for 24 hours, the cells were incubated with 1 μg/mL LPS for 3 hours. MCP-1 (A and C) and TNFα (B and D) mRNA levels and NFκB activity (E) were determined by real-time RT-PCR and a NFκB luciferase assay, respectively. Data are means±SEM of 4 separate experiments. *P<0.01 vs control cells; **P<0.01 vs cells treated with Ox-LDL alone; ††P<0.01 vs cells treated with control siRNA alone; †††P<0.01 vs cells treated with control siRNA plus telmisartan; #P<0.01 vs cells treated with PPARγ siRNA alone.

**Figure 4.** Telmisartan suppresses Ox-LDL-induced macrophage proliferation. Mouse peritoneal macrophages were transfected without (A) or with control or PPARγ siRNA (B) and were incubated with medium A for 4 hours. The cells were then incubated with medium A in the absence or presence of the indicated concentration of telmisartan (Telm), losartan (Los), valsartan (Val), or olmesartan (Olm). After incubation for 24 hours, the cells were incubated with 20 μg/mL Ox-LDL for 6 days, and [3H]thymidine incorporation was assayed. Data are means±SEM of 4 separate experiments. *P<0.01 vs control cells; **P<0.01 vs cells treated with Ox-LDL alone; ††P<0.01 vs cells treated with control siRNA alone; †††P<0.01 vs cells treated with control siRNA plus Ox-LDL; #P<0.01 vs cells treated with PPARγ siRNA alone.

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regulates the gene expression of ABCA1 and ABCG1.33 Ten μmol/L telmisartan increased the mRNA expression of CD36, ABCA1 and ABCG1 (Figure 2A to 2C) and increased LXR activity (Figure 2D) in mouse peritoneal macrophages. Treatment with PPARγ siRNA decreased the expression of PPARγ by 71% in macrophages (Figure 2E). Compared with treatment with control siRNA, the telmisartan-induced increases in mRNA expression of ABCA1 and ABCG1 were suppressed by treatment with PPARγ siRNA (Figure 2F).

**Telmisartan Suppresses Lipopolysaccharide-Induced MCP-1 and TNFα Production by Activating PPARγ**

It is well known that inflammatory cytokines, including MCP-1 and TNFα, are involved in the progression of athero-
MCP-1 mRNA expression in the aortic sinus of apoE−/− mice. Moreover, telmisartan suppressed PPARγ activity of PPARγ (Figure 5D). Telmisartan increased transcriptional activity of PPARγ in the aorta (Figure 5A to 5C). Treatment with telmisartan did not affect body weight, systolic blood pressure, diastolic blood pressure, serum total cholesterol, triglyceride, or high-density lipoprotein cholesterol levels in apoE−/− mice (Supplemental Table). However, telmisartan decreased the size of atherosclerotic lesions in the aorta, as determined by Oil Red O staining (Figure 5A to 5C). Telmisartan increased transcriptional activity of PPARγ in the aorta (Figure 5D). Moreover, telmisartan suppressed MCP-1 mRNA expression in the aortic sinus of apoE−/− mice (Figure 5E) and increased mRNA expression of ABCA1 and ABCG1 (Figure 5F).

Finally, we investigated the effects of telmisartan on macrophage proliferation in atherosclerotic lesions in apoE−/− mice. PCNA-positive and CD11b-positive cells were detected in the atherosclerotic lesion of apoE−/− mice (Figure 6A, top) and the number of CD11b-positive cells was decreased in the atherosclerotic lesions in telmisartan-treated mice, as compared with control mice (Figure 6A, bottom, and 6B). Moreover, the ratio of PCNA-positive cells to all CD11b-positive cells was decreased in the atherosclerotic lesions of telmisartan-treated mice (Figure 6A and 6C).

Discussion

Angiotensin II activates the AT1 receptor to promote vasoconstriction, oxidative stress, inflammation, and atherosclerosis.34–37 Thus, it is reasonable that blockade of the AT1 receptor prevents the formation of atherosclerotic lesions. In fact, ARBs were reported to suppress inflammation, reduce neointimal formation and decrease smooth muscle cell proliferation.38–43 Because atherosclerosis is thought to be a chronic inflammatory disease, it is possible that treatment with ARBs prevents the progression of atherosclerosis. On the other hand, telmisartan is capable of not only blocking the AT1 receptor signal but also activating PPARγ. Because the activation of PPARγ also induces antiatherosclerotic effects, telmisartan may have an additive effect for the prevention of atherosclerotic lesion formation.

In the present study, we showed that telmisartan at concentrations ≥1 mmol/L activates PPARγ in macrophages, whereas the plasma concentration of telmisartan in hypertensive patients was reported to be 0.54 μmol/L.44 In fact, we revealed in the present study that PPARγ activity was increased in the aorta of telmisartan-treated apoE−/− mice. Therefore, it is possible that, in clinical use, telmisartan activates PPARγ in macrophages present in atherosclerotic
lesions. Interestingly, a novel finding is that telmisartan slightly but significantly activated PPARγ in macrophages. Because PPARγ induces several antiinflammatory effects and suppresses the progression of atherosclerosis, the telmisartan-mediated antiatherogenic effects may be mediated by activation of both PPARα and PPARγ. Additional studies are needed to explore the role of PPARα on the antiatherogenic effects of telmisartan.

In the present study, we demonstrated that telmisartan increased the expression of CD36, ABCA1 and ABCG1, 3 PPARγ-responsive genes, and the activation of LXR, a transcription factor that regulates ABCA1 and ABCG1 gene transcription, in macrophages. We also demonstrated that telmisartan increased the mRNA expression of ABCA1 and ABCG1 in atherosclerotic lesions of apoE⁻/⁻ mice. This finding is supported by a previous report that telmisartan inhibits the transcriptional activation of early growth response gene-1 and NF-κB, 2 proinflammatory transcription factors, in macrophages and in atherosclerotic lesions of apoE⁻/⁻ mice.24 In addition to these findings, our novel findings indicated that the number of macrophages in atherosclerotic lesions decreased in telmisartan-treated mice. Thus, telmisartan may suppress the expression of atherogenic molecules, such as TNFα and MCP-1, by activating PPARγ, and subsequently, it may suppress the recruitment of monocytes into the atherosclerotic lesions.

It has been reported that macrophages and macrophage-derived foam cells proliferate in atherosclerotic lesions in the rabbit aorta11,12 and in the coronary artery of humans.10 Our novel findings also revealed that 28.3% of the macrophages in atherosclerotic lesions of apoE⁻/⁻ mice were proliferative. Because macrophages are capable of expressing proatherogenic molecules, the increase in the number of macrophages by its proliferation may be involved in the progression of atherosclerosis. We revealed that telmisartan reduced the number of proliferative macrophages in these lesions and suppressed Ox-LDL-induced macrophage proliferation via PPARγ activation. Thus, the suppression of macrophage proliferation may represent one of the mechanisms underlying...
ing the antiatherogenic effects of telmisartan in apoE−/− mice.
It has been reported that the activator of PPARγ induces macrophage apoptosis.45 Thus, telmisartan may not only inhibit the MCP-1 expression and macrophage proliferation but also induce macrophage apoptosis, thereby decreasing the number of macrophages in atherosclerotic lesion. On the other hand, Arai et al reported that Ox-LDL induced AIM (apoptosis inhibitor expressed by macrophages), which can protect macrophage apoptosis, and that early atherosclerotic lesions in AIM-deficient (AIM−/−), LDL receptor–deficient double knockout mice are dramatically reduced compared with AIM+/+ LDL receptor−/− controls.46 Thus, telmisartan may inhibit the expression of AIM, thereby inducing macrophage apoptosis. Additional studies are needed to clarify the involvement of macrophage apoptosis in telmisartan-mediated antiatherosclerotic effects.

In the present study, we did not observe a significant decrease in blood pressure by telmisartan. A significant decrease in systolic blood pressure by telmisartan with concentration between 1 and 5 mg/kg per day was not observed in mice or rats with normal blood pressure in several studies.49–51 Because the dose of telmisartan in our present study was close to that of these previous studies and because the apoE−/− mice we used presented normal blood pressure, telmisartan might not have affected blood pressure.

We have previously reported that nifedipine, which is a L-type calcium channel blocker, activates PPARγ by down-regulating serine phosphorylation of PPARγ in macrophages.32 Moreover, we have reported that nifedipine, similar to telmisartan, suppressed MCP-1 expression and induced ABCA1 expression via PPARγ activation.32 However, there is no evidence whether any differences exist in the effects on the phenotypes of arteriosclerosis between telmisartan and nifedipine. It has been reported that the molecular mechanism of PPARγ activation seems to differ between telmisartan and thiazolidinediones.52,53 Moreover, although PPARγ is the target of both telmisartan and pioglitazone, the profiles of gene expression in 3T3-L1 adipocytes treated with telmisartan are different from those of pioglitazone.52 Because the mechanism of PPARγ activation was also different between nifedipine and telmisartan, antiatherosclerotic effects may be partially different between nifedipine and telmisartan, and an additive effect for the prevention of atherosclerosis may be observed by the combination therapy with these compounds in hypertensive patients. Additional studies are needed to clarify whether additive effects by nifedipine and telmisartan could exist in the antiatherosclerotic action.

In conclusion, we have demonstrated that telmisartan activated PPARγ and subsequently exerted antiatherogenic effects in macrophages. Moreover, telmisartan suppressed the progression of atherosclerosis in apoE−/− mice by decreasing the number of macrophages in atherosclerotic lesions. Collectively, these results could explain, at least in part, the effectiveness of telmisartan in hypertensive patients and in patients with diabetes and hyperlipidemia who are at high risk for atherosclerotic vascular diseases.

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Disclosures
None.

References
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Supplemental Table

Body weight (BW), blood pressure (BP) and plasma lipid profile in control or telmisartan-treated apoE<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>control (n=8)</th>
<th>telmisartan (n=8)</th>
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<tr>
<td>BW (g)</td>
<td>31.3 ± 4.5</td>
<td>30.5 ± 5.4</td>
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<tr>
<td>SBP (mmHg)</td>
<td>101 ± 6</td>
<td>103 ± 9</td>
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<tr>
<td>DBP (mmHg)</td>
<td>79 ± 5</td>
<td>81 ± 4</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>890 ± 26</td>
<td>910 ± 22</td>
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<tr>
<td>TG (mg/dl)</td>
<td>88 ± 7</td>
<td>90 ± 5</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>44 ± 8</td>
<td>45 ± 7</td>
</tr>
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SBP indicates systolic BP; DBP, diastolic BP; TC, total cholesterol; TG, triglycerides; and HDL-C, HDL cholesterol.
Supplemental methods

Materials

Telmisartan was purchased from Boehringer Ingelheim Inc. (Ingelheim, Germany). Valsartan, losartan, olmesartan and pioglitazone were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Lipopolysaccharide (LPS) (Escherichia coli. O111:B4) was purchased from Sigma (St. Louis, MO, USA). Rabbit polyclonal anti-PPARγ and anti-PCNA antibodies, and goat polyclonal anti-CD11b and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). All other chemicals were of the best grade available from commercial sources.

Luciferase assays

Nf-κB luciferase reporter plasmid was purchased from Genetech Inc. (USA). Liver X receptor (LXR) luciferase reporter plasmid (LXRE-tk-Luc) was described previously.1 Luciferase reporter plasmids were transfected into RAW264.7 cells and mouse peritoneal macrophages (2 × 10^6 cells/well) using Lipofectamine 2000. Cells were also co-transfected with a Renilla luciferase plasmid (pRL-SV40; Promega) as an internal control. After transfection, the cells were cultured for 5 h, and compounds were added to the medium at appropriate concentrations. After an additional 24 h of incubation, the cells were lysed and subjected to luciferase assays using a Dual-Luciferase Reporter Gene Assay system (Promega) according to the manufacturer's instructions.2

Transcription activity of PPARγ

Transcription activity of PPARγ was assayed using an enzyme-linked immunosorbent assay-based PPARα, δ, γ Complete Transcription Factor Assay Kit (Cayman Chemical). Sample proteins were extracted from aortas of apoE−/− mice according to the manufacturer's
Supplement Material

instructions, and were added to a 96-well plate that had been immunobilized by an oligonucleotide containing PPAR response element. After 1 h, the wells were incubated with diluted primary PPARγ antibody to recognize the accessible epitope on PPARγ protein upon DNA binding. The horseradish peroxidase-conjugated secondary antibody was added and incubation conducted for 1 h. At the end, the reaction was stopped, and absorbance was read at 450 nm on a spectrophotometer. This assay is specific for PPARγ activation, and there is no cross-reaction with other PPAR isoforms.2

Transfection of siRNA

The siRNA against PPARγ and an irrelevant 21-nucleotide siRNA duplex as a control were purchased from Santa Cruz Biotechnology. Mouse peritoneal macrophages (2 × 10^6 cells/well) were transfected with the siRNA of PPARγ or control using Lipofectamine 2000 (Invitrogen). After 4 h incubation, the medium was changed to medium A, and real-time RT-PCR was performed.2 Inhibitory effect of PPARγ siRNA against PPARγ expression was tested by Western blot analysis as described below.

Tritiated thymidine incorporation and cell-counting assays

Macrophage monolayers (2 × 10^6 cells/well) were cultured in 24-well tissue culture plates (15.5 mm in diameter, Corning Glass works, Corning, NY) in the presence of the indicated effectors for 6 days. For thymidine incorporation assay, 18 h before the termination of the experiments, 1 µCi/mL [³H]thymidine was added to each well and incubated. Tritiated thymidine incorporation assay was performed as described previously.2

Western blot analysis

Cells (2 × 10^6 cells/well in 6-well plate, Nunc) were lysed by the lysis buffer, and
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centrifuged (20,000 × g at 4°C for 10 min). Supernatants were used as sample proteins. Protein concentrations were determined by the Micro BCA Protein Assay Reagent (Pierce), according to the protocol recommended by the manufacturer. Samples were applied to 10% sodium dodecyl sulfate (SDS) gels and transferred to nitrocellulose membranes (Bio-Rad) by using semi-dry blotting. Membranes were incubated with the indicated antibodies at a dilution of 1:1000 for 2 h. After washing, the membranes were stained with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse antibodies (Santa Cruz, Inc.). Antigen detection was performed with ECL plus kit (GE Healthcare UK Ltd.).

Real-time RT-PCR analysis

Macrophages (2 × 10⁶ cells/well) were incubated with or without the indicated effectors. Total RNA was extracted with TRIzol (Life Technologies, Inc.). The first strand cDNA synthesis containing 1 μg of total RNA was primed with oligo dT. To quantify gene transcripts, the LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN) was used. PCRs were performed using SYBR Green I master mix and specific primers for mouse MCP-1, TNF-α, ABCA1, ABCG1 and 36B4, which were designed as follows: MCP-1, forward primer, 5’-GGTCCCTGTCATGCTTCT-3’ and reverse primer, 5’-CATCTTGCTGGTGAATGAGT-3’; TNF-α, forward primer, 5’-AAATGGCCTCCCTCTCATCA-3’, and reverse primer, 5’-AAATGGCCTCCCTCTCATCA-3’; ABCA1, forward primer, 5’-GCGAACCCTGCAGAAGGGAGA-3’, and reverse primer, 5’-GTCCACCGCTCTGGATGAG-3’; ABCG1, forward primer, 5’-GCCAACCTGCAGAAGGGAGA-3’, and reverse primer, 5’-GCTCAGGACTCTGCTTCTC-3; CD36, forward primer, 5’-TCCAGGCAATGCCTTTCGC and reverse primer, 5’-GCTCAGGACTCTGCTTCTC-3; and reverse primer, 5’-TGGAGATTACTTTTCAGTGCA;
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β-actin, forward primer, 5’-AACACCCAGCCATGTACG-3’ and reverse primer, 5’-ATACCCAAGAAGGAAGGCTG-3’. Quantitative result for MCP-1, TNF-α, ABCA1, ABCG1, CD36 was normalized by the levels of 36B4 mRNA. To assess the specificity of the amplified PCR products, after the last cycle, a melting curve analysis was performed.

Statistical analysis

All data were expressed as the mean±SEM. Differences between groups were examined for statistical significance by one-factor analysis of variance. *P*<0.01 was considered to indicate a statistically significant difference.

References


Supplemental Material