Oxidized Low Density Lipoprotein Activates Peroxisome Proliferator-activated Receptor-α (PPARα) and PPARγ through MAPK-dependent COX-2 Expression in Macrophages

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It has been reported that oxidized low density lipoprotein (Ox-LDL) can activate both peroxisome proliferator-activated receptor-α (PPARα) and PPARγ. However, the detailed mechanisms of Ox-LDL-induced PPARα and PPARγ activation are not fully understood. In the present study, we investigated the effect of Ox-LDL on PPARα and PPARγ activation in macrophages. Ox-LDL, but not LDL, induced PPARα and PPARγ activation in a dose-dependent manner. Ox-LDL transiently induced cyclooxygenase-2 (COX-2) mRNA and protein expression, and COX-2-specific inhibition by NS-398 or meloxicam or small interference RNA of COX-2 suppressed Ox-LDL-induced PPARα and PPARγ activation. Ox-LDL-induced phosphorylation of ERK1/2 and p38 MAPK, and ERK1/2-specific inhibition abrogated Ox-LDL-induced COX-2 expression and PPARα and PPARγ activation, whereas p38 MAPK-specific inhibition had no effect. Ox-LDL decreased the amounts of intracellular long chain fatty acids, such as arachidonic, linoleic, oleic, and docosahexaenoic acids. On the other hand, Ox-LDL increased intracellular 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) level through ERK1/2-dependent overexpression of COX-2. Moreover, 15d-PGJ2 induced both PPARα and PPARγ activation. Furthermore, COX-2 and 15d-PGJ2 expression and PPAR activity were increased in atherosclerotic lesions of apoE-deficient mice. Finally, we investigated the involvement of PPARα and PPARγ on Ox-LDL-induced mRNA expression of ATP-binding cassette transporter A1 and monocyte chemotactant protein-1. Interestingly, specific inhibition of PPARα and PPARγ suppressed Ox-LDL-induced ATP-binding cassette transporter A1 mRNA expression and enhanced Ox-LDL-induced monocyte chemotactant protein-1 mRNA expression. In conclusion, Ox-LDL-induced increase in 15d-PGJ2 level through ERK1/2-dependent COX-2 expression is one of the mechanisms of PPARα and PPARγ activation in macrophages. These effects of Ox-LDL may control atherogenic progression.

Accumulation of modified low density lipoprotein (LDL), such as oxidized LDL (Ox-LDL), and recruitment of monocytes in the arterial subendothelial spaces are early events in atherosclerosis (1). Macrophages, which are derived from monocytes in these areas, take up Ox-LDL through the scavenger receptor pathways and become foam cells (2). Foam cells are well known to play an important role in the development and progression of atherosclerosis, through the production of various bioactive molecules such as growth factors and cytokines (1).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor superfamily (3–5). Three distinct PPARs, termed α, δ, and γ, have been identified. PPARs are characterized by distinct tissue distribution patterns and metabolic functions. PPARα is highly expressed in tissues that demonstrate high catabolic rates for fatty acids, such as liver, heart, kidney, and muscle, whereas PPARγ is highly expressed in adipose tissue where it plays a major regulatory role in adipocyte differentiation, and the expression of genes involved in lipid metabolism (4–7). PPARδ shows a widespread tissue distribution, but its physiological role remains to be fully elucidated (8). Recently, agonists of PPARα and PPARγ have been reported to improve atherosclerosis in LDL receptor-deficient mice (9, 10), suggesting that activation of PPARα and/or PPARγ suppresses the development and progression of atherosclerosis.

Recently, we have reported that 3-hydroxyl-3-methylglutaryl-CoA reductase inhibitors (statins) induced activation of PPARα and PPARγ in macrophages (11). Moreover, we revealed that statin-induced activation of PPARα and PPARγ were mediated by cyclooxygenase-2 (COX-2)-dependent pathways and progression of atherosclerosis.

2 The abbreviations used are: LDL, low density lipoprotein; ABCA1, ATP-binding cassette transporter A1; COX-2, cyclooxygenase-2; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactant protein-1; 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; PPAR, peroxisome proliferator-activated receptor; 9-HODE, 9-hydroxyoctadecenoic acid; 13-HODE, 13-hydroxyoctadecenoic acid; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; WT, wild type; EIA, enzyme immunoassay; TG, triglyceride; m-Ox-LDL, mildly oxidized LDL; c-LDL, control LDL; Mm-Ox-LDL, macrophage-mediated Ox-LDL; C/E, CAA/T enhancer-binding protein; TBA, thiobarbituric acid-reactive substances; CMV, cytomegalovirus; siRNA, small interference RNA; RT, reverse transcription.
increase in intracellular 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) production (11). Therefore, several pathways that lead to the overexpression of COX-2 may also induce the activation of PPARα and PPARγ.

Ox-LDL has been reported to activate PPARα in endothelial cells (12, 13) and PPARγ in CV-1 monkey kidney fibroblasts (14), and this is possibly caused by the action of oxidized metabolites of linoleic acid, including 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), and oxidized phospholipids, which are included in Ox-LDL (12, 14). However, the detailed mechanism involved in Ox-LDL-induced activation of PPARs and the role of PPARs in Ox-LDL-mediated acceleration of atherosogenesis are not fully understood.

In the present study, we investigated a novel mechanism of Ox-LDL-induced activation of PPARs in macrophages. We demonstrated that Ox-LDL activated both PPARα and PPARγ through extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent COX-2 expression in macrophages. In addition, Ox-LDL-induced activation of PPARs mediated the induction of ATP-binding cassette transporter A1 (ABCA1) mRNA expression, as well as the suppression of monocyte chemoattractant protein-1 (MCP-1) mRNA expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—PD98059, SB203580, meloxicam, NS-398, T0070907, and 15d-PGJ2 were purchased from Calbiochem. Anti-PPARα and anti-PPARγ antibodies, and goat polyclonal anti-β-actin, anti-integrin αM, and anti-apolipoprotein-B (apoB) antibodies were obtained from Sigma. Rabbit polyclonal anti-phospho ERK1/2 and anti-phospho p38 MAPK antibodies were purchased from Cell Signaling Technology (Santa Cruz, CA). Rabbit polyclonal anti-phospho ERK1/2 and anti-phospho p38 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). A rabbit polyclonal anti-murine COX-2 antiserum was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were of the best grade available from commercial sources.

**Animals**—C57BL/6 mice and apolipoprotein E-deficient (apoE−/−) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice were maintained on the C57BL/6 background strain, and C57BL/6 mice were used as wild-type (WT) mice. Mice were given access to food and water ad libitum in the Animal Resource Facility at Kumamoto University under specific pathogen-free conditions. 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 diet was a normal rodent chow diet for mouse (CLEA, Tokyo, Japan). 40 male mice of 24 weeks of age (20 as C57BL/6 and 20 as apoE−/−) were sacrificed, and atherosclerotic lesions of aortic sinus were used for Western blot assay, enzyme immunoassay (EIA) for 15d-PGJ2 assay of transcription activity of PPARα and PPARγ, and immunohistochemistry as described below. Plasma total cholesterol, triglyceride (TG), and HDL cholesterol concentrations were performed commercially by Skyline Biotech Inc. (Akita, Japan).

**Lipoprotein Preparation and Modification of Native LDL**—Human LDL (d = 1.019–1.063 g/ml) was isolated by ultracentrifugation from the plasma of consenting normolipidemic subjects, obtained after overnight fasting (21). LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA, pH 7.4. Ox-LDL or mildly oxidized LDL (m-Ox-LDL) was prepared by incubation of LDL with 5 μM CuSO4 for 20 or for 5 h, respectively, at 37 °C, followed by the addition of 1 mM EDTA, and cooling (22). The concentration of proteins was determined by BCA protein assay reagent (Pierce). The endotoxin level of Ox-LDL was <1 pg/μg of protein measured by Toxicolor system (Seikagaku, Japan). Macrophage-mediated oxidation of LDL was performed by using Lindstedt’s methods (23). Briefly, 100 μM LDL and 100 μM CuSO4 were incubated at 37 °C with medium A in the absence (c-LDL) or presence (Mm-Ox-LDL) of mouse peritoneal macrophages (1 × 10⁶). After 20-h incubation, the supernatant was removed, and the extent of lipid peroxidation of LDL in the supernatant was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARSs) as described below.

**Analysis of Oxidation, Electrophoretic Mobility, and Degradation of apoB in LDL Modified with CuSO4**—Following oxidative modification of LDL with CuSO4 or macrophages, lipid peroxidation was assessed by the following procedures. The
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peroxides were quantified in terms of TBARS according to the method of Nagano et al. (24). Lipoproteins (20 μg of protein) were suspended in 0.2 ml of 150 mM NaCl. The suspension was mixed with 0.5 ml of 20% (w/v) trichloroacetic acid and 0.5 ml of the reagent (335 mg of 2-thiobarbituric acid in 100 ml of 50% (v/v) acetic acid). The mixture was boiled at 95 °C for 60 min. After being cooled, 2.0 ml of 1-butanol was added, shaken vigorously, and centrifuged at 4000 × g for 10 min. The fluorescence of supernatant was measured with excitation at 515 nm and emission at 550 nm. Tetramethoxypropane was used as a standard. The electrophoretic mobility of native or oxidized LDL was measured in barbital/sodium barbital, pH 8.0, on 1% agarose gel SPE (Beckman Coulter, Fullerton, CA) (25). The lipoprotein pattern was visualized by staining the film with a lipid-specific stain. Relative electrophoretic mobility was defined as ratio of the distances migrated from the origin by modified LDL versus native LDL. For analysis of the degradation of apoB, lipoproteins were delipidated with ethyl acetate: ethanol (1:1), solubilized with 10% SDS, and separated on 4% to 20% SDS gels (Invitrogen Japan K.K. Minato-ku, Tokyo, Japan.) at 30 mA for 1 h. After electrophoresis, samples were transferred onto nitrocellulose membranes (Bio-Rad) by using semi-dry blotting, and the membranes were incubated with polyclonal rabbit antibody to apoB at a dilution of 1:1000 for 2 h. After washing, the membranes were stained with horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). Antigen detection was performed with an ECL manufacturer’s instructions (29).

Reporter Gene Assays—The fusion protein expression vector, containing the mouse PPARα ligand-binding domain or 204–505 of the human PPARγ ligand-binding domain, driven by the SV40 promoter were described previously (28). The reporter plasmid p4xUASg-tk-luc, containing four copies of a 17-mer upstream activating sequence for the GAL4 DNA-binding domain and a thymidine kinase gene promoter (tk-promoter) in front of a luciferase cDNA, was also described previously (28). To measure the PPARα or PPARγ ligand binding activity, RAW264.7 cells (2 × 10^6 cells/well) were transfected with p4xUASg-tk-luc and pM-PPARα or pM-PPARγ, and subjected to luciferase assays as described below.

Luciferase Assays—The NF-κB luciferase reporter plasmid (pNF-κB-Luc) used for measuring NF-κB activity was purchased from Takara Bio, Inc. (Shiga, Japan). Luciferase reporter plasmids were transfected into RAW264.7 cells, mouse peritoneal macrophages, or THP-1 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 (29).

Transcription Activity of PPARs—Transcription activity of PPARα and PPARγ was assayed using an enzyme-linked immunosorbent assay-based PPARα, -δ, and -γ Complete Transcription Factor Assay Kit (Cayman Chemical). Sample proteins were extracted from aortas of apoE−/− or WT mice according to the manufacturer’s instructions and were added to a 96-well plate that had been immobilized by an oligonucleotide containing PPAR response element. After 1 h, the wells were incubated with diluted primary PPARα or PPARγ antibody to recognize the accessible epitope on PPARα or PPARγ protein upon DNA binding. The horseradish peroxidase-conjugated secondary antibody was added, and incubation was 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 PPARα or PPARγ activation, and there is no cross-reaction with other PPAR isofoms.

Transfection of siRNA—The siRNA against COX-2, PPARα, PPARγ, and an irrelevant 21-nucleotide siRNA duplex as a control were purchased from Santa Cruz Biotechnology. RAW264.7 cells (2 × 10^6 cells/well) or mouse peritoneal macrophages (2 × 10^6 cells/well) were transfected with the siRNA of COX-2, PPARα, PPARγ, or control, in the absence or presence of appropriate plasmids using Lipofectamine 2000 (Invitrogen). After 4-h incubation, the medium was changed to medium A, and luciferase assay or real-time RT-PCR was performed. In comparison to the control siRNA, the siRNA of COX-2, PPARα, or PPARγ suppressed the expression of these proteins by 86, 72, or 77%, respectively, according to Western blot analysis (11).

Adenoviral Vectors—Cells were infected with a recombinant replication-deficient adenovirus containing each of the following genes: dominant negative ERK (DN-ERK) and p38 MAPK (DN-p38 MAPK) for 48 h at the multiplicity of infection of ~50, as described previously (26, 30), and were allowed to recover in medium A for 3 h. This condition conferred expression of LacZ as a marker gene in nearly 100% of transfected cells (26, 30).
Western Blot Analysis—Cells (2 × 10⁶ cells/well in 6-well plate, Nunc) or aortic sinus tissues were lysed by the lysis buffer, and 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% 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-conjugated goat anti-rabbit or anti-mouse antibodies (Santa Cruz Biotechnology). Antigen detection was performed with ECL plus kit (GE Healthcare UK Ltd.). Immunoreactive bands were quantified by using NIH Image (26).

Real-time RT-PCR Analysis—Macrophages (2 × 10⁶ cells/well) were incubated with or without the indicated effectors. Total RNA was extracted with TRIZol (Invitrogen). 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 (29). PCRs were performed using SYBR Green I master mix and specific primers for mouse COX-2, ABCA1, MCP-1, and β-actin, which were designed as follows: COX-2, forward primer, 5′-CCAGAGCAGAGATGAA-3′ and reverse primer, 5′-GGTACAGTTCATGAGATC-3′; ABCA1, forward primer, 5′-ATTCGACGAGCAGCCTG-3′ and reverse primer, 5′-ATGGGAAAGGGTGCACA-3′; MCP-1, forward primer, 5′-GGTCCCCGTATCTGTCTT-3′ and reverse primer, 5′-CATCTTGGCTGTGAATGATGCT-3′; and β-actin, forward primer, 5′-AACCCCCGACCCATGTACG-3′ and reverse primer, 5′-ATACCCCAAAGGAAAGGCTG-3′. The quantitative results for COX-2, ABCA1, and MCP-1 were normalized by the levels of β-actin mRNA (11). To assess the specificity of the amplified PCR products, after the last cycle, a melting curve analysis was performed.

EIA for 15d-PGJ2, PGE2, and PGD2—The 96-well-based EIA kit of 15d-PGJ2, prostaglandin E2 (PGE2) or prostaglandin D2 (PGD2) was purchased from Cayman Chemical (Ann Arbor, MI). Cells cultured in 6-well plates or aortic sinus tissues were treated with the indicated effectors for 24 h, and then the cells were lysed with lysis buffer. To measure the amounts of 15d-PGJ2 in lipoproteins, 40 μg of LDL or Ox-LDL were also suspended with the same lysis buffer. The concentrations of intra-cellular 15d-PGJ2, PGE2 and PGD2 in cells or in lipoproteins were determined by each EIA kit according to the manufacturer’s instructions (11).

Gas Chromatography—Gas chromatography of long chain fatty acids was performed commercially by SRL (Tokyo, Japan) (11). Briefly, cells (2 × 10⁶ cells/well in 6-well plates) were incubated with the indicated effectors. After 24-h incubation, cells were counted using a Beckman Coulter Counter Z2. An aliquot (4 × 10⁶ cells) of each sample was re-suspended in a glass tube, and the total lipids were extracted using Folch buffer (31). After hydrolysis with KOH, methyl esters of the fatty acids were prepared using BF3 methanol. C23:0 was used as an internal standard. The esters were analyzed by gas chromatography using a Shimadzu GC-17A gas chromatograph equipped with a hydrogen flame ionization detector.

Statistical Analysis—All data were expressed as the mean ± S.E. Differences between groups were examined for statistical significance by one-factor analysis of variance. p < 0.05 was considered to indicate a statistically significant difference.

RESULTS

Ox-LDL Induces PPARα and PPARγ Activation in Macrophages—We first examined the effect of Ox-LDL, which was prepared with 5 μM CuSO4 for 20 h, on PPARα and PPARγ activation in RAW264.7 cells by full-length PPARα and PPARγ systems. As shown in Fig. 1A, Ox-LDL increased luciferase activity of both full-length PPARα and full-length PPARγ in a dose-dependent manner, whereas LDL had no effect.

We next examined the effect of Ox-LDL on PPARα and PPARγ ligand binding activity by the GAL4 chimera system. Ox-LDL also increased luciferase activity of both GAL4-PPARα and GAL4-PPARγ in a dose-dependent manner, whereas LDL had no effect (Fig. 1B). In similar experiments...
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TABLE 1
Modification of native LDL with CuSO₄ for 5 or 20 h

| Treatment | TBARS | Electrophoretic mobility (relative to LDL) | Intact apoB |
|-----------|-------|------------------------------------------|-------------|
| LDL       | 2.2 ± 0.2 | 1% of LDL | 67.5 ± 2.2% |
| m-Ox-LDL  | 8.3 ± 0.5* | 1.5 ± 0.1* | 34.5 ± 1.8* |
| Ox-LDL    | 22.8 ± 1.2* | 2.8 ± 0.2* | 43.5 ± 1.8* |

*p < 0.01 versus native LDL.

using mouse peritoneal macrophages and THP-1 macrophages, 50 μg/ml Ox-LDL increased PPARα ligand binding activity by 6.3- and 5.2-fold, and PPARγ ligand binding activity by 5.8- and 4.9-fold, respectively (p < 0.01, compared with control, data not shown).

Next, we prepared m-Ox-LDL, which was incubated with 5 μM CuSO₄ for 5 h, and investigated the effect of m-Ox-LDL on PPARα and PPARγ activation. As shown in Table 1, the modification of m-Ox-LDL estimated by TBARS, electrophoretic mobility, and apoprotein B degradation was lower than that of Ox-LDL incubated with 5 μM CuSO₄ for 20 h. Although the effects were slightly milder than that of Ox-LDL incubated for 20 h, m-Ox-LDL also significantly increased PPARα and PPARγ ligand binding activity (Fig. 1C).

To confirm the effect of cell-mediated oxidized LDL on PPARα and PPARγ activation, we prepared Mm-Ox-LDL. The oxidation of Mm-Ox-LDL, which was estimated by TBARS, was 7.1 ± 0.3 nmol of MDA/mg of LDL. On the other hand, we also prepared control LDL (c-LDL) by incubation with the same condition medium without macrophages. The TBARS of c-LDL was 2.4 ± 0.4 nmol of MDA/mg of LDL. As shown in Fig. 1D, Mm-Ox-LDL increased luciferase activities of both GAL4-PPARα and GAL4-PPARγ, whereas c-LDL had no effect.

COX-2 Is Involved in Ox-LDL-induced PPAR Activation—We next examined whether Ox-LDL-induced COX-2 expression by real-time RT-PCR and Western blot analysis. After treatment of RAW264.7 cells with 40 μg/ml Ox-LDL, both COX-2 mRNA and protein were increased at 1 h, and these increases remained for up to 6 h (Fig. 2, A and B).

We then examined the effect of COX-2 inhibitors NS-398 and meloxicam on Ox-LDL-induced PPAR activation. Ox-LDL-induced activation of PPARα and PPARγ was partially but significantly inhibited by NS-398 and meloxicam (Fig. 2C). We further investigated the effect of COX-2 siRNA on Ox-LDL-induced PPARα and PPARγ activation. Treatment with siRNA for COX-2 down-regulated COX-2 protein expression by 86% (Fig. 2D) and inhibited Ox-LDL-induced PPARα and PPARγ activation by 58.8% and 56.1%, respectively (Fig. 2E).

Ox-LDL-induced COX-2 Expression Was Mediated by ERK1/2 Signals—We have previously reported that Ox-LDL can activate ERK1/2 and p38 MAPK (32), which are known to be involved in COX-2 expression induced by several stimuli in macrophages. Therefore, we speculated on the involvement of MAPKs signals in Ox-LDL-induced COX-2 expression in macrophages. First, we performed time-course experiments of ERK1/2 and p38 MAPK activation in RAW264.7 cells. Ox-LDL (40 μg/ml) transiently increased phosphorylation of ERK1/2 and p38 MAPK at 1 h, which remained for up to 6 h (Fig. 3A).

We therefore examined the effect of ERK1/2-specific inhibitor PD98059 and p38 MAPK-specific inhibitor SB203580 on Ox-LDL-induced COX-2 mRNA expression. The incubation of RAW264.7 cells with Ox-LDL for 3 h increased COX-2 mRNA level by 3-fold (Fig. 3B), and this increase was blocked by PD98059, but not by SB203580 (Fig. 3B). Ox-LDL-induced COX-2 protein expression was also inhibited by PD98059, but not by SB203580 (Fig. 3D). Moreover, Ox-LDL-induced activation of PPARα and PPARγ was significantly inhibited by PD98059, but not by SB203580 (Fig. 3D).
induced activation of PPARα and PPARγ was also inhibited by overexpression of DN-ERK but not by that of DN-p38 MAPK (Fig. 3E). These results suggest that Ox-LDL-induced activation of PPARα and PPARγ activation is mediated by ERK1/2-dependent COX-2 expression.

**Ox-LDL Decreases the Amount of Intracellular Long Chain Fatty Acids, but Increases Intracellular 15d-PGJ2 Level**—Intracellular fatty acids have been reported to be ligands of PPARα and PPARγ; therefore, we examined the effect of Ox-LDL on the amount of arachidonic acid, oleic acid, linoleic acid, and docosahexaenoic acid. Interestingly, Ox-LDL, but not LDL, significantly decreased all of these fatty acids (Table 2).

It is possible that overexpression of COX-2 may increase the amount of intracellular 15d-PGJ2, which is a natural ligand for PPARγ. Therefore, we examined whether Ox-LDL was capable of increasing 15d-PGJ2 level in macrophages. Ox-LDL (40 μg/ml) significantly increased intracellular 15d-PGJ2 level (Fig. 4A), and this was inhibited by PD98059, NS-398, or meloxicam, whereas SB203580 had no such effect (Fig. 4A). Moreover, overexpression of DN-ERK (Fig. 4B) or transfection of COX-2 siRNA (Fig. 4C) also suppressed Ox-LDL-induced increase in intracellular 15d-PGJ2 level. On the other hand, Ox-LDL (40 μg/ml) significantly increased not only intracellular 15d-PGJ2 level but also intracellular PGE2 (Fig. 4D) and PGD2 (Fig. 4E).

PPARα activation induced by Ox-LDL was also suppressed by COX-2 inhibition, therefore, we speculated that 15d-PGJ2

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**TABLE 2**

Effect of Ox-LDL on intracellular fatty acids production

|        | Control | Ox-LDL | LDL |
|--------|---------|--------|-----|
| Arachidonic acid (μg/ml) | 0.77 ± 0.06 | 0.35 ± 0.03 | 0.81 ± 0.04 |
| Oleic acid (μg/ml) | 1.58 ± 0.11 | 0.79 ± 0.08 | 1.65 ± 0.12 |
| Linoleic acid (μg/ml) | 0.52 ± 0.06 | 0.23 ± 0.01 | 0.57 ± 0.04 |
| Docosahexaenoic acid (μg/ml) | 0.55 ± 0.08 | 0.22 ± 0.02 | 0.58 ± 0.05 |

* *p < 0.01 versus the controls.*
**TABLE 3**

| Plasma lipid profile on WT and apoe<sup>-/-</sup> mice | Body weight | TC<sup>a</sup> | TG | HDL-C |
|------------------------------------------------------|-------------|--------------|----|-------|
| WT (<i>n</i> = 10)                                    | 24.4 ± 3.8  | 65 ± 18      | 60 ± 22 | 46 ± 8 |
| apoE<sup>-/-</sup> (<i>n</i> = 9)                      | 25.8 ± 4.2  | 499 ± 82<sup>b</sup> | 55 ± 27 | 48 ± 6 |

<sup>a</sup> TC indicates total cholesterol; TG, triglycerides; and HDL-C, HDL cholesterol.

<sup>b</sup> <i>p</i> < 0.01 versus WT group.

would also induce PPAR<sub>α</sub> activation. As shown in Fig. 4F, 15d-PGJ<sub>2</sub> induced not only PPAR<sub>γ</sub> activation but also PPAR<sub>α</sub> activation in a dose-dependent manner. Therefore, it seems likely that changes in COX-2-dependent increase in 15d-PGJ<sub>2</sub> level rather than intracellular fatty acids are involved in Ox-LDL-induced activation of PPAR<sub>α</sub> and PPAR<sub>γ</sub>.

On the other hand, the amount of 15d-PGJ<sub>2</sub> in Ox-LDL (0.55 ng/mg of protein) was significantly (<i>p</i> < 0.01) lower than that in native LDL (2.3 ng/mg of protein), suggesting that 15d-PGJ<sub>2</sub> contained in Ox-LDL had no effect on PPAR<sub>α</sub> and PPAR<sub>γ</sub> activation.

Expression of COX-2 and 15d-PGJ<sub>2</sub> and Activation of PPAR<sub>α</sub> and PPAR<sub>γ</sub> Were Observed in Atherosclerotic Lesion of apoE<sup>-/-</sup> Mice—First, we investigated the differentiation of plasma lipid profile between WT and apoE<sup>-/-</sup> mice of 24 weeks of age fed the normal diet. Plasma TC level on apoE<sup>-/-</sup> was ~7-fold higher than WT mice, whereas plasma TG level and HDL-cholesterol level had no difference (Table 3). We next investigated atherosclerotic lesion formation in apoE<sup>-/-</sup> and WT mice. As shown in Fig. 5A, atherosclerotic lesions stained by oil red O were observed in apoE<sup>-/-</sup> mice, but not in WT mice. Immunohistochemistry revealed that Ox-LDL was detected in the atherosclerotic lesion of apoE<sup>-/-</sup> mice and that was co-localized with macrophages (Fig. 5B). We further examined the expression of COX-2 and 15d-PGJ<sub>2</sub>, and the activation of PPAR<sub>α</sub> and PPAR<sub>γ</sub> in aortic sinus of apoE<sup>-/-</sup> or WT mice. In comparison to the WT mice, the expression of COX-2 and 15d-PGJ<sub>2</sub> were increased in aortic sinus of apoE<sup>-/-</sup> mice (Fig. 5, C and D). Moreover, the transcription activities of PPAR<sub>α</sub> and PPAR<sub>γ</sub> were also increased in apoE<sup>-/-</sup> mice by 2.55- and 2.28-fold, respectively (Fig. 5E).

Blockade of Either PPAR<sub>α</sub> or PPAR<sub>γ</sub> Inhibits Ox-LDL-induced ABCA1 mRNA Expression—First, to determine the appropriate concentrations of PPAR<sub>α</sub> or PPAR<sub>γ</sub> antagonists, we examined the inhibitory effects of PPAR<sub>α</sub> antagonist GW6471 or PPAR<sub>γ</sub> antagonist T0070907 on Ox-LDL-induced PPAR<sub>α</sub> or PPAR<sub>γ</sub> activation. As shown in Fig. 6A, 0.5 µM GW6471 completely inhibited Ox-LDL-induced PPAR<sub>α</sub> ligand binding activity, without affecting PPAR<sub>γ</sub> ligand binding activity. However, 1 µM GW6471 inhibited both PPAR<sub>α</sub> and PPAR<sub>γ</sub> ligand binding activities (Fig. 6A). On the other hand, ≥0.01 µM T0070907 completely inhibited Ox-LDL-induced PPAR<sub>α</sub> ligand binding activity (Fig. 6B). Unexpectedly and surprisingly, 1 µM T0070907 increased PPAR<sub>γ</sub> ligand binding activity (Fig. 6B). These results indicated that the appropriate concentration of GW6471 for the specific inhibition of PPAR<sub>α</sub> was 0.5 µM and that of T0070907 for the specific inhibition of PPAR<sub>γ</sub> was 0.01 µM, therefore, we used these concentrations for the following experiments.

Next, we examined the role of ERK1/2 and COX-2 on Ox-LDL-induced ABCA1 expression. In real-time RT-PCR analysis, 40 µg/ml Ox-LDL significantly induced ABCA1 mRNA expression in mouse peritoneal macrophages (Fig. 6C), and the effect was suppressed by NS-398 and PD98059 (Fig. 6C). We then examined the role of PPAR<sub>α</sub> and PPAR<sub>γ</sub> activation in Ox-LDL-induced ABCA1 expression. Treatment with 0.5 µM GW6471 or 0.01 µM T0070907 partially, but significantly, inhibited Ox-LDL-induced ABCA1 mRNA expression (Fig. 6D). Treatment with both GW6471 and T0070907 showed an additive inhibitory effect on Ox-LDL-induced ABCA1 mRNA expression (Fig. 6D).

To confirm the involvement of PPAR<sub>α</sub> and PPAR<sub>γ</sub> in Ox-LDL-induced ABCA1 mRNA expression, the effect of PPAR<sub>α</sub> and/or PPAR<sub>γ</sub> siRNA was examined. As shown in Fig. 6E, treatment with siRNA for PPAR<sub>α</sub> and PPAR<sub>γ</sub>, the expression of PPAR<sub>α</sub> and PPAR<sub>γ</sub>, including PPARγ1 and PPARγ2, were suppressed by 72 and 77%, respectively. In comparison to control siRNA, siRNA for PPAR<sub>α</sub> or PPAR<sub>γ</sub> significantly suppressed Ox-LDL-induced ABCA1 mRNA expression (Fig. 6F), and an
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Additive effect of siRNA for PPARα and PPARγ was observed (Fig. 6F).

PPAR Activation Influences the Two Contrary Effects of Ox-LDL on MCP-1 mRNA Expression—We further examined the effects of PPAR antagonists on Ox-LDL-induced MCP-1 mRNA expression. Ox-LDL (40 μg/ml) increased MCP-1 mRNA expression by 4.7-fold in mouse peritoneal macrophages (Fig. 7A). GW6471 or T0070907 significantly enhanced Ox-LDL-induced MCP-1 mRNA expression (Fig. 7A), and an additive effect of GW6471 and T0070907 was observed (Fig. 7A). Moreover, Ox-LDL-induced MCP-1 mRNA expression was significantly enhanced by siRNA for PPARα or PPARγ (Fig. 7B), and an additive effect was observed with combination of siRNAs for PPARα and PPARγ (Fig. 7B).

Ox-LDL has also been reported to exert an anti-inflammatory effect in macrophages (33, 34). To clarify the role of PPARα and PPARγ on Ox-LDL-mediated anti-inflammatory effects, we examined the effects of PPARα- and PPARγ-specific inhibition on Ox-LDL-mediated suppression of MCP-1 mRNA expression induced by LPS.

LPS (1 μg/ml) increased MCP-1 mRNA expression by 10.5-fold, which was suppressed by Ox-LDL in mouse peritoneal macrophages (Fig. 7C). GW6471 or T0070907 significantly recovered the suppression of MCP-1 mRNA (Fig. 7C), and an additive effect of GW6471 and T0070907 was observed (Fig. 7C). In addition, in comparison to control siRNA, siRNA for PPARα or PPARγ significantly recovered Ox-LDL-mediated suppression of MCP-1 mRNA (Fig. 7D), and an additive effect of PPARα and PPARγ siRNA was observed (Fig. 7D).
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On the other hand, it has been reported that MCP-1 expression was mediated by the activation of NF-κB (35). Therefore, we examined the effect of Ox-LDL on NF-κB activation. Ox-LDL (40 μg/ml) increased NF-κB activity (Fig. 7E). Ox-LDL-induced NF-κB activation was enhanced by treatment of siRNA for PPARα or PPARγ, and an additive effect of PPARα and PPARγ siRNA was observed (Fig. 7F). Moreover, LPS (1 μg/ml) induced NF-κB activation, and this activation was inhibited by Ox-LDL (Fig. 7F). Treatment with siRNA for PPARα or PPARγ partially recovered Ox-LDL-mediated suppression of NF-κB activation (Fig. 7F), and an additive effect of PPARα and PPARγ siRNA was observed (Fig. 7F).

DISCUSSION

It has been reported that Ox-LDL induces PPARα and PPARγ activation, and that 9-HODE, 13-HODE, and oxidized phospholipids, which are components of Ox-LDL, are involved in Ox-LDL-induced PPARα and PPARγ activation (12, 14). However, we newly revealed that Ox-LDL-induced PPARα and PPARγ activation was also mediated by overexpression of COX-2. COX-2 is a rate-limiting enzyme of PG synthesis, which catalyzes the conversion of arachidonic acid to PGG2 and further to PGH₂ (36). It has been reported that Ox-LDL induces COX-2 expression in monocytes/macrophages (37, 38). However, the mechanisms of Ox-LDL-induced COX-2 expression are not clearly understood.

We demonstrated here, possibly for the first time, that Ox-LDL induced COX-2 expression in RAW264.7 macrophages, and that this was mediated by the activation of ERK1/2, but not p38 MAPK. In addition, Ox-LDL-induced activation of PPARα and PPARγ was inhibited by inhibition of COX-2 and ERK1/2, suggesting that Ox-LDL-induced activation of both PPARα and PPARγ is mediated by ERK1/2-dependent COX-2 expression in macrophages. Interestingly, our recent report revealed that statins activated PPARα and PPARγ, and this effect was also mediated by overexpression of COX-2 (11). Thus, several pathways that lead to the overexpression of COX-2 may also induce the activation of PPARα and PPARγ.

In the present study, we demonstrated that siRNA for COX-2 suppressed Ox-LDL-induced COX-2 expression by 86%. However, the siRNA for COX-2 inhibited Ox-LDL-induced PPARα and PPARγ activation by ~50%. Moreover, although inhibitors of COX-2 or ERK1/2 suppressed Ox-LDL-induced increase in 15d-PGJ₂ level by 80% or more, they suppressed PPARα and PPARγ activation by 50%. These results suggest that one or more other mechanisms than COX-2 are involved in Ox-LDL-induced PPARα activation. Because it is reported that 9-HODE, 13-HODE, and oxidized phospholipids, which are included in Ox-LDL, are capable to activate PPARα and PPARγ (12, 14), additive effects by these particles may be involved in Ox-LDL-induced PPARα and PPARγ activation (Fig. 8).

It has been reported that CCAAT/enhancer-binding protein-β (C/EBPβ) and AP-1 are involved in transcriptional activation of the COX-2 gene (39), and MAPK signaling pathways influence AP-1 and C/EBPβ (40, 41). Therefore, Ox-LDL-induced ERK1/2 activation may activate AP-1 and/or C/EBPβ, thereby resulting in transcriptional activation of the COX-2 gene. Further studies are needed to clarify the mechanisms by which Ox-LDL induces expression of COX-2 mRNA.

We found that the COX-2 expression induced by Ox-LDL increased the amount of intracellular 15d-PGJ₂. Moreover, Ox-LDL increased not only 15d-PGJ₂ level but also PGE₂ and PGD₂, suggesting that Ox-LDL-induced COX-2 expression mediated the downstream production of prostaglandins. It is well known that 15d-PGJ₂ is a strong PPARγ activator. However, Forman et al. (42) have reported that 15d-PGJ₂ is not a PPARα ligand but also activates PPARα. In fact, we demonstrated that 15d-PGJ₂ increased ligand binding activity of both PPARα and PPARγ. These results suggest that COX-2-mediated increase in 15d-PGJ₂ level is one of the mechanisms by which Ox-LDL induces activation of both PPARα and PPARγ.

It has been reported that selective COX-2 inhibition suppresses atherosclerotic lesion formation in LDL receptor-deficient mice (43), suggesting that COX-2 act as an inducer of atherosclerosis. On the other hand, recent studies have revealed that treatment with COX-2 inhibitors in humans does not decrease, but rather increases cardiovascular events (44–46). According to our findings, it is possible that several eicosanoids produced by COX-2 may play a protective role in controlling atherosclerotic progression, through the activation of PPARs.

In the present study, we demonstrated several research activities by in vitro. On the other hand, we revealed for the first time that the activities of PPARα and PPARγ were increased in atherosclerotic lesions of apoE⁻/⁻ mice. We confirm that Ox-LDL was present in these atherosclerotic lesions and that was co-localized with macrophages. Moreover, the expression of COX-2 was also increased in these lesions. In fact, the existence of Ox-LDL and the expression of COX-2 in atherosclerotic lesions have been reported, and these are co-localized with macrophages (43, 47, 48). In addition, we newly found that the
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15d-PGJ$_2$ level was also increased in these atherosclerotic lesions. These results suggested that Ox-LDL activates PPAR$_\alpha$ and PPAR$\gamma$ in vivo, and the effect may be induced by COX-2-dependent 15d-PGJ$_2$ expression. ABCA1, a member of the ATP-binding cassette transporter family, is involved in the control of high density lipoprotein and apolipoprotein A1-mediated cholesterol efflux from macrophages (49, 50). The activation of ABCA1 plays an important role by influencing cellular cholesterol transport (51). It has been reported that both PPAR$_\alpha$ and PPAR$\gamma$ activators induce ABCA1 gene expression in macrophages (52), suggesting that the activation of PPARs leads to anti-atherogenic activity, by the expression of ABCA1 in atherosclerotic lesions. Ox-LDL has been reported to promote uptake of Ox-LDL by inducing the scavenger receptor CD36, via activation of PPAR$\gamma$ in macrophages (14, 53). On the other hand, we demonstrated here, possibly for the first time, that Ox-LDL induced ABCA1 mRNA expression, and this effect was also mediated by PPAR activation. Thus, Ox-LDL-induced activation of PPARs fundamentally accelerates the uptake of Ox-LDL via CD36 expression, and beside this action, Ox-LDL may negatively control the excess accumulation of OX-LDL via ABCA-1 expression.

It has been reported that Ox-LDL induces pro-inflammatory cytokines that are associated with the progression of atherosclerosis (1). On the other hand, in macrophages activated by LPS, Ox-LDL suppresses production of pro-inflammatory cytokines (33, 34), suggesting that Ox-LDL has both inflammatory and anti-inflammatory actions. In fact, in unstimulated conditions, Ox-LDL induces MCP-1 mRNA expression, which is well known to play an early and important role in the recruitment of monocytes to atherosclerotic lesions (54). On the contrary, Ox-LDL inhibited MCP-1 mRNA expression in macrophages activated by LPS. Moreover, Ox-LDL-induced MCP-1 mRNA expression was enhanced by both PPAR-specific inhibitors, and Ox-LDL-mediated suppression of MCP-1 mRNA induced by LPS was recovered by both PPAR-specific inhibitors. Furthermore, like MCP-1, NF-\kappa B, which has been reported to be one of the important molecules in MCP-1 expression (35), was also controlled by Ox-LDL. These results suggest that Ox-LDL can induce MCP-1, whereas the activation of PPARs by Ox-LDL partially suppresses its induction via anti-inflammatory action, such as inactivation of NF-\kappa B, through PPARs.

To confirm whether the ability of Ox-LDL on PPARs activation depended on a degree of its oxidation, we investigated the effect of m-Ox-LDL on PPAR$\alpha$ and PPAR$\gamma$ activation. We found that m-Ox-LDL mildly but significantly induced PPAR$\alpha$ and PPAR$\gamma$ activation, suggesting that Ox-LDL-induced PPAR$\alpha$ and PPAR$\gamma$ activation was depended on its oxidation. Moreover, we found that macrophage-mediated Ox-LDL, whose existence was more realistic than CuSO$_4$-mediated Ox-LDL in atherosclerotic lesions, also activated PPAR$\alpha$ and PPAR$\gamma$. Therefore, it is possible that Ox-LDL activates PPAR$\alpha$ and PPAR$\gamma$ in these lesions in vivo.

In conclusion, we demonstrated that Ox-LDL-induced PPAR$\alpha$ and PPAR$\gamma$ activation was mediated by increase in 15d-PGJ$_2$, via ERK1/2-dependent COX-2 expression in macrophages. In addition, inhibition of PPARs suppressed Ox-LDL-induced ABCA1 mRNA expression and enhanced Ox-LDL-induced MCP-1 mRNA expression. These unique signals of macrophages induced by Ox-LDL may be one of the protective mechanisms to prevent progression of atherosclerosis. Considering the involvement of COX-2 expression on Ox-LDL-induced activation of PPARs, activation or expression, rather than inhibition of COX-2, may be a novel therapeutic approach for atherosclerosis.

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