Statins Activate Peroxisome Proliferator-Activated Receptor γ Through Extracellular Signal-Regulated Kinase 1/2 and p38 Mitogen-Activated Protein Kinase–Dependent Cyclooxygenase-2 Expression in Macrophages

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Abstract—Both statins and peroxisome proliferator-activated receptor (PPAR)γ ligands have been reported to protect against the progression of atherosclerosis. In the present study, we investigated the effects of statins on PPARγ activation in macrophages. Statins increased PPARγ activity, which was inhibited by mevalonate, farnesylpyrophosphate, or geranylgeranylpyrophosphate. Furthermore, a farnesyl transferase inhibitor and a geranylgeranyl transferase inhibitor mimicked the effects of statins. Statins inhibited the membrane translocations of Ras, RhoA, Rac, and Cdc42, and overexpression of dominant-negative mutants of RhoA (DN-RhoA) and Cdc42 (DN-Cdc42), but not of Ras or Rac, increased PPARγ activity. Statins induced extracellular signal-regulated kinase (ERK)1/2 and p38 mitogen-activated protein kinase (MAPK) activation. However, DN-RhoA and DN-Cdc42 activated p38 MAPK, but not ERK1/2. ERK1/2- or p38 MAPK–specific inhibitors abrogated statin-induced PPARγ activation. Statins induced cyclooxygenase (COX)-2 expression and increased intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) levels through ERK1/2- and p38 MAPK–dependent pathways, and inhibitors or small interfering RNA of COX-2 inhibited statin-induced PPARγ activation. Statins also activate PPARα via COX-2–dependent increases in 15d-PGJ_2 levels. We further demonstrated that statins inhibited lipopolysaccharide-induced tumor necrosis factor α or monocyte chemoattractant protein-1 mRNA expression, and these effects by statins were abrogated by the PPARγ antagonist T0070907 or by small interfering RNA of PPARγ or PPARα. Statins also induced ATP-binding cassette protein A1 or CD36 mRNA expression, and these effects were suppressed by small interfering RNAs of PPARγ or PPARα. In conclusion, statins induce COX-2–dependent increase in 15d-PGJ_2 level through a RhoA- and Cdc42-dependent p38 MAPK pathway and a RhoA- and Cdc42-independent ERK1/2 pathway, thereby activating PPARγ. Statins also activate PPARα via COX-2–dependent pathway. These effects of statins may explain their antiatherogenic actions. **(*Circ Res*. 2007;100:1442-1451.)**

Key Words: cyclooxygenase ■ MAPK ■ macrophages ■ PPAR ■ statins

3-Hydroxyl-3-methylglutaryl coenzyme A reductase inhibitors (statins) are known to reduce the incidence of cardiovascular events and death, and these benefits are mainly caused by their lipid-lowering effects.[1] However, recent evidence has suggested that the beneficial effects by statins are independent of their cholesterol-lowering effects.[2]

Cholesterol is synthesized via the isoprenoid biosynthetic pathway.[3] In this pathway, isopentenyl-PP is the basic isoprene unit used for synthesis of all subsequent isoprenoids.[3] Among the isoprenoids, farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) serve as important lipid attachments for several proteins, including the small GTP-binding protein Ras and Ras-like proteins, such as Rho, Rac, and Cdc42, whose proper membrane localization and function are dependent on isoprenylation.[3] The pleiotropic effects of statins are thought to be mediated by blocking the synthesis of FPP and GGPP, resulting the inhibition of small GTP-binding proteins,[3] and these include improving the function of endothelial cells, vascular smooth muscle cells, and macrophages.[3]

Mitogen-activated protein kinases (MAPKs) are serine/threonine protein kinases that can phosphorylate their target

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proteins.[4] Three major subfamilies have been: extracellular-signal regulated kinases 1/2 (ERK1/2), p38 MAPKs, and Jun N-terminal kinases/stress-activated protein kinases (JNKs/SAPKs).[4] MAPKs are activated by a family of MAPK kinases (MKKs)[4] and play an important role in regulating cell growth, migration, and differentiation and production of several inflammatory genes, including cyclooxygenase-2 (COX-2).[5] In particular, ERK1/2- and p38MAPK-dependent COX-2 production by several mediators induce the production of prostaglandins, such as prostaglandin E2 (PGE_2).[6]

Peroxisome proliferator-activated receptor (PPAR)γ, a member of the nuclear hormone receptor family of ligand-dependent transcription factors, has been well characterized as a regulator of adipogenesis and is abundant in fat cells.[7] However, PPARγ also mediates several antiatherogenic effects in atherosclerotic lesions.[8] In fact, PPARγ agonists have been shown to inhibit the development of atherosclerosis in vivo.[9] Thus, the activation of PPARγ may be beneficial for the suppression of atherosclerosis.

PPARγ agonists improve endothelial function, inhibit the proliferation and migration of vascular smooth muscle cells, and inhibit the production of inflammatory cytokines and MMPs in macrophages.[8] Because the antiatherogenic effects of PPARγ agonists and statins have some common characteristics, we hypothesized that a common mechanism may exist via crosstalk of their pathways. Indeed, statins have been reported to increase the DNA-binding activity of PPARγ to PPAR-response elements in monocytes.[10,11] However, the mechanisms of statin-induced PPARγ activation in macrophages are not yet fully understood. The aims of the present study were to clarify whether statins have the capacity to activate PPARγ in macrophages and to examine their underlying mechanisms. We demonstrate that statins induce COX-2–dependent increase in intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) levels through both a RhoA- and Cdc42-dependent p38 MAPK pathway and a RhoA- and Cdc42-independent ERK1/2 pathway, thereby activating PPARγ.

Materials and Methods

Cell culture, reagents, several plasmids and adenovirus vectors, cell transfection, luciferase assays, Western blot, RT-PCR and quantitative real-time PCR, enzyme immuno assay, and statistical analysis are described in the online data supplement, available at http://circres.ahajournals.org.

Results

Statins Activate PPARγ via Suppression of the Mevalonate Pathway in Macrophages

In the present study, we first examined the effects of fluvastatin, simvastatin, atorvastatin, pitavastatin, and cerivastatin on the activation of PPARγ in RAW264.7 cells using the full-length PPARγ system. All 5 statins increased luciferase activity in a concentration-dependent manner (Figure 1A). We next examined the effect of these statins on PPARγ ligand-binding activity using the GAL4 chimera system. Statins also increased luciferase activity in a concentration-dependent manner in this system (Figure 1B). Fluvastatin at 1 μmol/L increased PPARγ ligand-binding activity by 5.6-fold in mouse peritoneal macrophages ($P<0.01$ versus the control) and 4.5-fold in THP-1 macrophages ($P<0.01$ versus the control).

PPARγ activities induced by statins were lower than those induced by the same concentration of pioglitazone and were 58%, 31%, 29%, 15%, and 73% of that induced by pioglitazone for fluvastatin, pitavastatin, simvastatin, atorvastatin, and cerivastatin, respectively (Figure 1C).

We next examined the effects of mevalonate and its metabolites on PPARγ activation. The statin-induced increases in luciferase activity were completely prevented by mevalonate, but not by cholesterol (Figure 1D), and were partially prevented by FPP or GGPP (Figure 1D). Moreover, the farnesyl transferase inhibitor FTI-276 and the geranylgeranyl transferase inhibitor GGTI-286 increased luciferase activity (Figure 1D). On the other hand, statins had no effect on PPARγ mRNA or protein levels (Figure I in the online data supplement).

Suppression of RhoA- and Cdc42-Dependent Signaling Pathways Activate PPARγ

Statins inhibit the activation of small G proteins by suppressing their farnesylation or geranylgeranylation.[3] Indeed, we confirmed that fluvastatin, pitavastatin, and simvastatin suppressed the membrane translocation of Ras, RhoA, Rac, and Cdc42 (supplemental Figure II). Therefore, we next examined the effects of overexpression of dominant-negative (DN)-Ras, DN-RhoA, DN-Rac or DN-Cdc42 on PPARγ activation. Overexpression of DN-RhoA or DN-Cdc42 increased luciferase activity, whereas DN-Ras or DN-Rac had no effect (Figure 2A). Moreover, overexpression of both DN-RhoA and DN-Cdc42 showed an additive effect (Figure 2A).

Involvement of ERK1/2 and p38 MAPK in Statin-Induced PPARγ Activation

We next examined the involvement of ERK1/2 and p38 MAPK in statin-induced PPARγ activation. Statins induced phosphorylation of ERK1/2 and p38 MAPK in RAW264.7 cells (Figure 2B), and these effects were prevented by FPP or GGPP (Figure 2C). We next examined the time course of ERK1/2 and p38 MAPK phosphorylation by statins. Fluvastatin increased ERK1/2 phosphorylation after 12 hours of incubation, and this increase was maintained until 24 hours. p38 MAPK phosphorylation also was increased after 12 hours of incubation; however, this increase was attenuated by 28% after 24 hours of incubation (supplemental Figure III). On the other hand, DN-RhoA and DN-Cdc42 had no effect on ERK1/2 phosphorylation (Figure 2D) but induced MAPK phosphorylation (Figure 2E), and an additive effect was observed in cells infected with both DN-RhoA and DN-Cdc42 (Figure 2E). Furthermore, the fluvastatin-induced PPARγ activation was inhibited by the MAPK/ERK kinase-specific inhibitor PD98059 or the p38 MAPK-specific inhibitor SB203580, and the combination of both inhibitors further enhanced the suppression of PPARγ activation (Figure 2F). On the other hand, DN-RhoA- or DN-Cdc42-induced PPARγ activation was inhibited by SB203580, whereas PD98059 had no effect (Figure 2A).

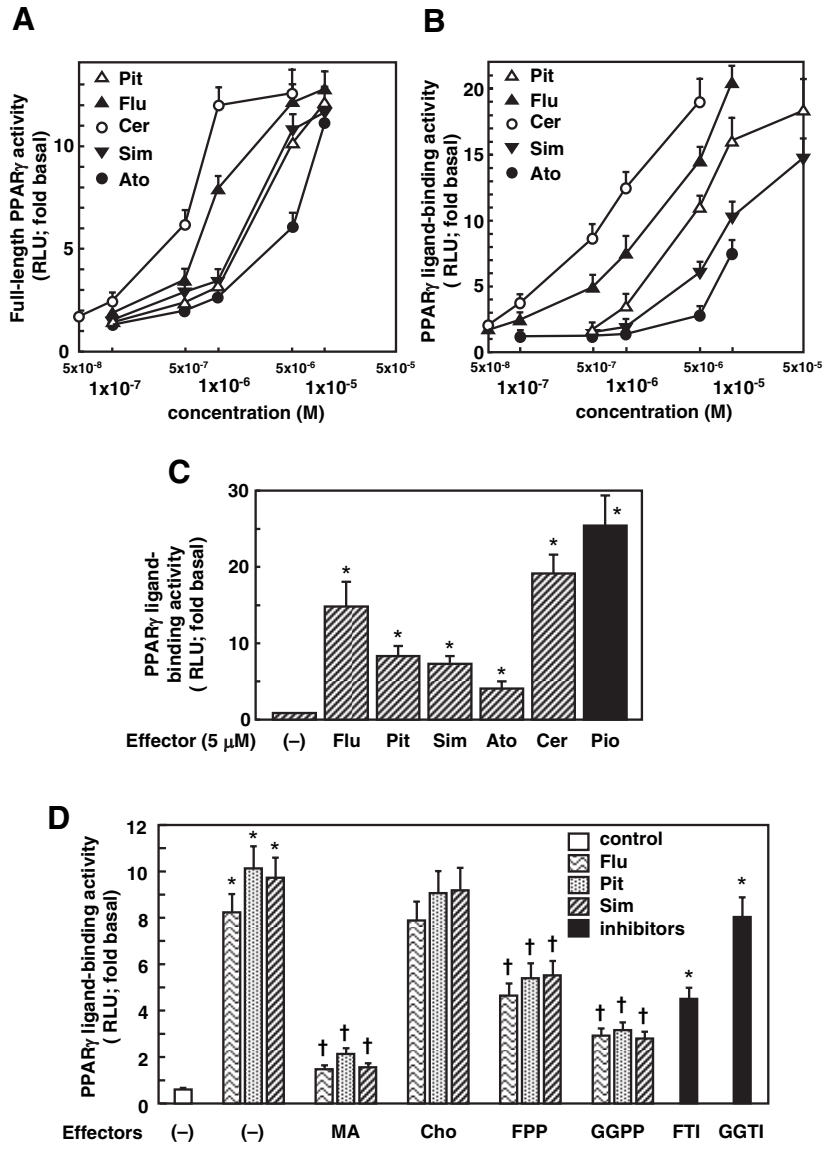

Figure 1. Statins activate PPARγ by inhibiting the mevalonate pathway. A through C, RAW264.7 cells were incubated with the indicated concentrations of fluvastatin (Flu), cerivastatin (Cer), simvastatin (Sim), pitavastatin (Pit), atorvastatin (Ato), or pioglitazone (Pio) for 24 hours. PPARγ activity (A) and PPARγ ligand-binding activity (B and C) were determined using the full-length PPARγ system and GAL4 chimera system, respectively. The data represent means±SD of 5 separate experiments. *$P<0.01$ vs the control. D, RAW264.7 cells were incubated for 24 hours with 10 μmol/L fluvastatin (Flu), 10 μmol/L pitavastatin (Pit), 10 μmol/L simvastatin (Sim), 10 μmol/L farnesyl transferase inhibitor (FTI), or 10 μmol/L geranylgeranyl transferase inhibitor (GGTI) with or without 50 μmol/L mevalonate (MA), 50 μmol/L cholesterol (Cho), 5 μmol/L FPP, or 5 μmol/L GGPP. PPARγ ligand-binding activity was determined using the GAL4 chimera system. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control; †$P<0.01$ vs cells with statins. RLU indicates relative light unit.

Statins Decreased the Intracellular Long-Chain Fatty Acid Level

Long-chain fatty acids (FAs), including arachidonic acid, linoleic acid, and docosahexaenoic acid, activate PPARγ.[12] Therefore, we next examined whether statins increased the intracellular FA level. Statins decreased the level of intracellular arachidonic acid, oleic acid, linoleic acid, and docosahexaenoic acid (supplemental Table I), and the fluvastatin-mediated decrease in arachidonic acid was blocked by a small interfering RNA (siRNA) against COX-2 (supplemental Table II).

Statins Induce COX-2 Expression

We next examined whether statins induced COX-2 expression. Statins induced COX-2 mRNA (Figure 3A) and protein (Figure 3C) expression. Time course experiments revealed that fluvastatin induced COX-2 protein expression after 12 hours of incubation, and this production was continued until 24 hours of incubation (supplemental Figure III). The statins also increased luciferase activity in cells transfected with pGL3-pCOX2 (Figure 3E). Overexpression of DN-RhoA or DN-Cdc42, but not DN-Ras or DN-Rac, induced COX-2 mRNA and protein expression as well as transcriptional activation of the COX-2 gene, and overexpression of both DN-RhoA and DN-Cdc42 showed an additive effect (Figure 3B, 3D, and 3F). On the other hand, the statins had no effect on the expression of COX-1 mRNA or protein (data not shown).

Furthermore, fluvastatin-induced COX-2 mRNA and protein expression were suppressed by PD98058 or SB203580, and the combination of both inhibitors further enhanced the suppression of COX-2 expression (Figure 3G and 3H).

Statins Increase Intracellular 15d-PGJ_2 Level

We speculated that overexpression of COX-2 may increase the intracellular 15d-PGJ_2 level, which is a natural ligand for PPARγ. Therefore, we next examined whether statins could increase the intracellular 15d-PGJ_2 level using an enzyme immunoassay detection system. Statins, as well as overexpression of DN-RhoA or DN-Cdc42, increased intracellu-

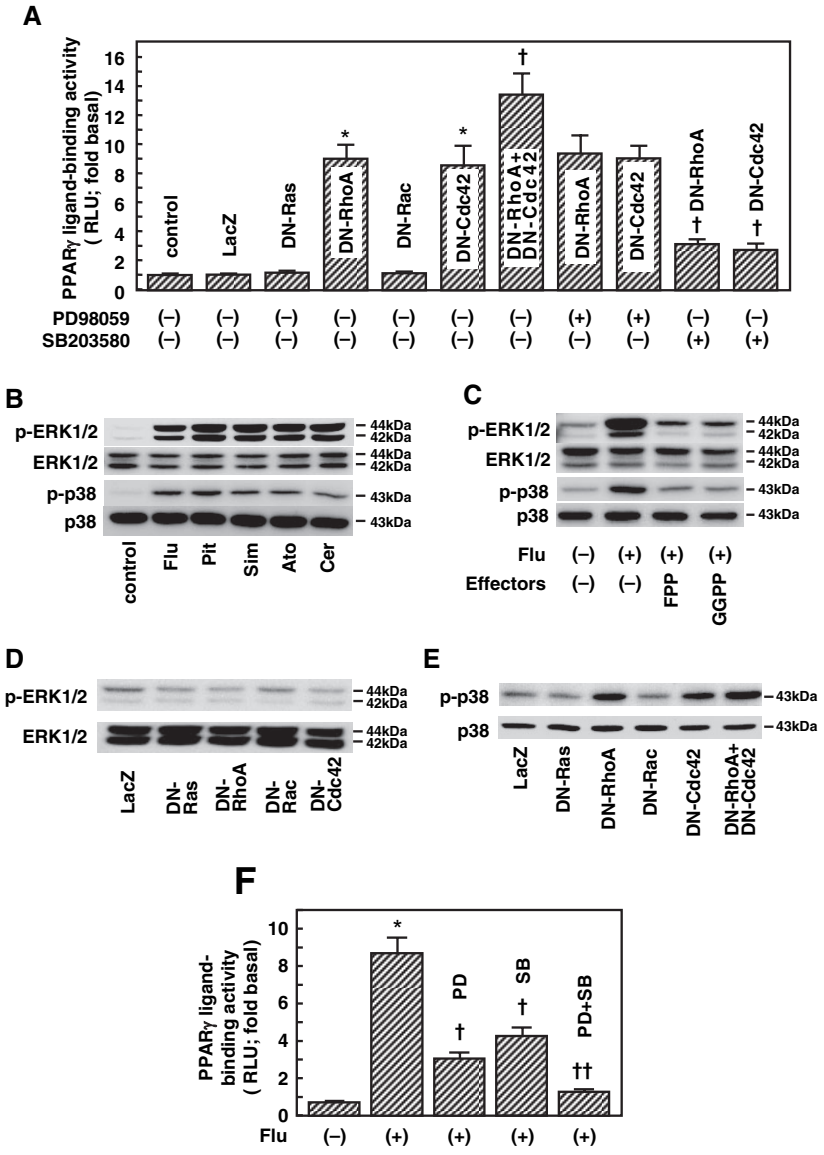

Figure 2. The roles of small G proteins and MAPK in statin-induced PPARγ activation. A, After infection with adenoviral vectors containing LacZ, DN-Ras, DN-RhoA, DN-Rac or DN-Cdc42, or both DN-RhoA and DN-Cdc42, cells were incubated for 24 hours in the absence or presence of 10 μmol/L the MAPK/ERK kinase-specific inhibitor PD98059 (PD) or the p38 MAPK-specific inhibitor SB203580 (SB). PPARγ ligand-binding activity was determined using the GAL4 chimera system. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, †$P<0.01$ vs cells with each adenoviral vector alone. B and C, RAW264.7 cells were incubated with 10 μmol/L fluvastatin (Flu), 10 μmol/L pitavastatin (Pit), 10 μmol/L simvastatin (Sim), 10 μmol/L atorvastatin (Ato), or 1 μmol/L cerivastatin (Cer) with or without 5 μmol/L FPP or 5 μmol/L GGPP. After 24 hours of incubation, protein samples were immunoblotted with anti–phospho-ERK1/2 (p-ERK1/2), anti-ERK1/2, anti–phospho–p38 MAPK (p-p38), or anti–p38 MAPK (p38) antibodies. The data are representative of 4 experiments with different cell preparations. D and E, After infection with adenoviral vectors containing LacZ, DN-Ras, DN-RhoA, DN-Rac or DN-Cdc42, or both DN-RhoA and DN-Cdc42, protein samples were immunoblotted with anti–phospho-ERK1/2 (p-ERK1/2), anti-ERK1/2, anti–phospho–p38 MAPK (p-p38), or anti–p38 MAPK (p38) antibodies. The data are representative of 4 experiments. F, RAW264.7 cells were incubated for 24 hours with 10 μmol/L fluvastatin (Flu) with or without 10 μmol/L PD98059 (PD) and/or 10 μmol/L SB203580 (SB). PPARγ ligand-binding activity was determined using the GAL4 chimera system. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, †$P<0.01$ vs cells with statin alone, ††$P<0.01$ vs cells with statin plus PD98059 or SB203580. RLU indicates relative light unit.

lar 15d-PGJ_2 level (Figure 4A and 4B), and overexpression of both DN-RhoA and DN-Cdc42 showed an additive effect (Figure 4B). Moreover, PD98059 or SB203580 suppressed the fluvastatin-induced increase in 15d-PGJ_2, and the combination of both inhibitors further suppressed it (Figure 4C).

Statins also increased intracellular PGE_2 levels (supplemental Figure IV). The PPARγ antagonist T0070907 did not inhibit fluvastatin-induced increases in 15d-PGJ_2 and PGE_2 (data not shown), suggesting that these effects by statins were not mediated by the activation of PPARγ.

Overexpression of COX-2 Is Involved in Statin-Induced PPARγ Activation

We next examined the effects of the COX-2 inhibitors NS-398 or meloxicam and COX-2 siRNA on statin-induced PPARγ activation. NS-398, meloxicam, and COX-2 siRNA suppressed basal luciferase activity in unstimulated conditions (Figure 4D and 4E). Fluvastatin-, pitavastatin-, and simvastatin-induced PPARγ activation were inhibited by NS-398 or meloxicam (Figure 4D). The COX-2 siRNA also inhibited statin-induced PPARγ activation (Figure 4E).

On the other hand, PPARγ siRNA did not inhibit the statin-induced expression of COX-2 protein (data not shown), suggesting that COX-2 expression was not a downstream signal but an upstream signal on PPARγ activation.

Statins Activate PPARα via Overexpression of COX-2

We next examined the effects of fluvastatin, simvastatin, atorvastatin, pitavastatin, and cerivastatin on PPARα ligand-binding activity using the GAL4 chimera system. Statins increased luciferase activity in a concentration-dependent manner (Figure 5A). Moreover, the COX-2 siRNA inhibited fluvastatin-induced PPARγ activation (Figure 5B). Furthermore, 15d-PGJ_2 activated both PPARα and PPARγ in a concentration-dependent manner (Figure 5C), suggesting that statins also activated PPARα via a COX-2–dependent increase in 15d-PGJ_2 level.

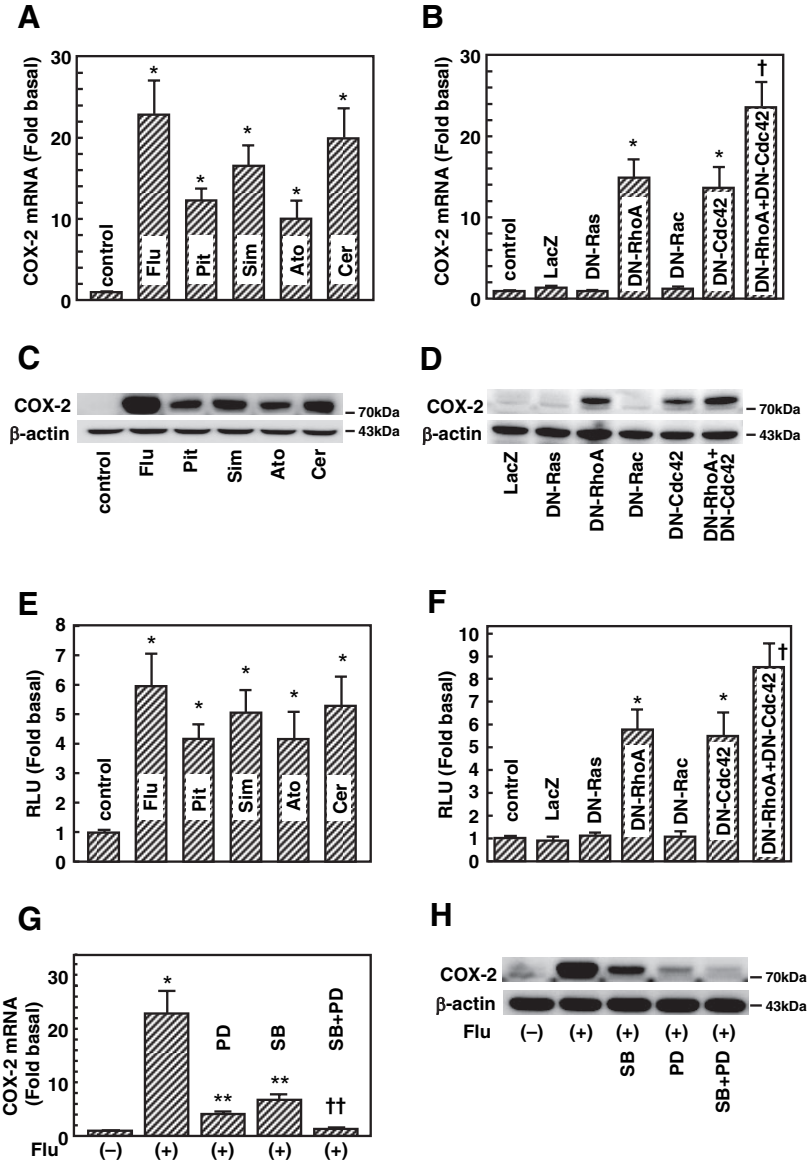

Figure 3. Statins induce COX-2 expression through ERK1/2 and p38 MAPK. RAW264.7 cells were incubated with 10 μmol/L fluvastatin (Flu), 10 μmol/L pitavastatin (Pit), 10 μmol/L simvastatin (Sim), 10 μmol/L atorvastatin (Ato), or 1 μmol/L cerivastatin (Cer) (A, C, E, G, and H) or were infected with adenoviral vectors containing LacZ, DN-Ras, DN-RhoA, DN-Rac or DN-Cdc42, or both DN-RhoA and DN-Cdc42 (B, D, F) with or without 10 μmol/L PD98059 (PD) and/or 10 μmol/L SB203580 (SB). A, B, and G, After 24 hours of incubation, the expression of COX-2 or β-actin mRNA was evaluated by real-time RT-PCR. The data represent means±SD of 4 separate experiments. $^*P<0.01$ vs the control, †$P<0.01$ vs cells with each adenoviral vector alone, $^{**}P<0.01$ vs cells with statin, ††$P<0.01$ vs cells with statin plus PD98059 or SB203580. C, D, and H, After 24 hours of incubation, protein samples were immunoblotted with anti–COX-2 or anti–β-actin antibodies. The data represent means±SD of 3 separate experiments. E and F, RAW264.7 cells were transfected with pGL3-pCOX2. After 24 hours of incubation, the transcriptional activity of COX-2 was determined by luciferase assays. The data represent means±SD of 4 separate experiments. $^*P<0.01$ vs the control, †$P<0.01$ vs cells with each adenoviral vector alone. RLU indicates relative light unit.

Statins Suppress Lipopolysaccharide-Induced Inflammatory Responses Through PPARα and PPARγ

To clarify the role of PPARs activation on statin-induced antiinflammatory effect, we next examined the effect of T0070907, PPARα siRNA, and PPARγ siRNA on statin-mediated suppression of tumor necrosis factor (TNF)α or monocyte chemoattractant protein (MCP)-1 mRNA expression, and suppression of nuclear factor (NF)-κB or activator protein (AP)-1 activation. First, we examined the appropriate concentration of T0070907 and found that 10 nmol/L T0070907 completely suppressed statin-induced PPARγ activation, and this concentration had no effect on PPARα activation (supplemental Figure V). Pitavastatin suppressed lipopolysaccharide (LPS)-induced TNFα and MCP-1 mRNA expression, and 10 nmol/L T0070907 abrogated the inhibitory effect of pitavastatin (Figure 6A and 6B). PPARα siRNA or PPARγ siRNA recovered pitavastatin-mediated suppression of TNFα and MCP-1 mRNA expression, and an additive effect of siRNAs for PPARα and PPARγ was observed (Figure 6C and 6D). Pitavastatin also suppressed LPS-induced NF-κB and AP-1 activation (Figure 6E and 6F). PPARα siRNA or PPARγ siRNA recovered pitavastatin-mediated suppression of NF-κB and AP-1 activation, and an additive effect of the siRNAs was observed (Figure 6E and 6F).

Statins Induce ATP-Binding Cassette Protein A1 and CD36 mRNA expression through PPARα and PPARγ

To clarify whether statins promote gene transcription in a PPAR-dependent manner, we examined the effect of pitavastatin on mRNA expression of ATP-binding cassette protein A1 (ABCA1) and CD36. Pitavastatin induced ABCA1 and CD36 mRNA expression (Figure 7A and 7B). PPARα siRNA or PPARγ siRNA suppressed pitavastatin-induced ABCA1 and CD36 mRNA expression, and an additive effect of the siRNAs was observed (Figure 7A and 7B).

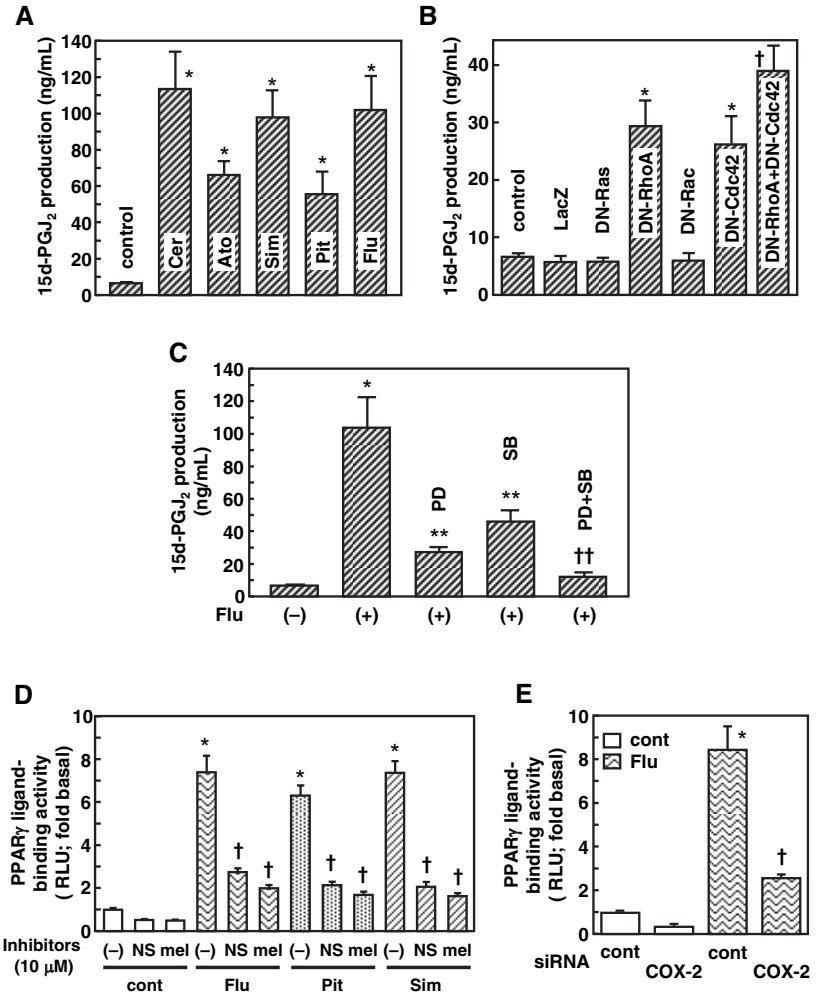

Figure 4. Overexpression of COX-2 is involved in PPARγ activation. RAW264.7 cells were incubated with 10 μmol/L fluvastatin (Flu), 10 μmol/L pitavastatin (Pit), 10 μmol/L simvastatin (Sim), 10 μmol/L atorvastatin (Ato), or 1 μmol/L cerivastatin (Cer) (A and C through E) or were infected with adenoviral vectors containing LacZ, DN-Ras, DN-RhoA, DN-Rac or DN-Cdc42, or both DN-RhoA and DN-Cdc42 (B) with or without 10 μmol/L PD98059 (PD) and/or 10 μmol/L SB203580 (SB) for 24 hours. A through C, The concentrations of 15d-PGJ_2 were determined by enzyme immunoassay. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, †$P<0.01$ vs cells with each adenoviral vector alone, **$P<0.01$ vs cells with statin alone, ††$P<0.01$ vs cells with statin plus PD98059 or SB203580. D and E, RAW264.7 cells were incubated with 10 μmol/L fluvastatin (Flu), 10 μmol/L pitavastatin (Pit), or 10 μmol/L simvastatin (Sim) for 24 hours in the absence or presence of 10 μmol/L NS-398 (NS) or 10 μmol/L meloxicam (mel) (D) or were transfected with COX-2 siRNA or control siRNA and incubated with 10 μmol/L fluvastatin (Flu) for 24 hours (E). PPARγ ligand-binding activity was determined using the GAL4 chimera system. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, †$P<0.01$ vs cells with statins alone. RLU indicates relative light unit.

Discussion

We demonstrated here that statins induced PPARγ activation and its mechanisms in macrophages. Atorvastatin and pravastatin were reported to activate PPARγ in human monocytes.[10,11] However, our preliminary data show that human monocytes do not express PPARγ, whereas human monocyte-derived macrophages do express PPARγ (T.M. and M.Y., unpublished data, 2006). Chinetti et al also reported similar results.[13] Thus, it is important to investigate the ability of statins to activate PPARγ and to investigate their mechanisms in macrophages. Moreover, Argmann et al reported that statins induced ABCA1 expression through PPARγ in macrophages.[14] However, they only used the PPARγ antagonist GW9662 and did not investigate PPARγ activity directly.[14] Thus, this is the first report of a direct effect of statins on PPARγ activation in macrophages.

Statins are classified into 2 groups: the first including hydrophilic compounds, the other including lipophilic compounds. The membrane permeability of lipophilic statins is higher than that of hydrophilic statins. In fact, pravastatin, among the hydrophilic statins, also activates PPARγ in macrophages. However, its action requires concentrations 100 times higher than those required for lipophilic statins to be active (T.M. and M.Y., unpublished data, 2006). Therefore, we used 5 lipophilic statins, which are currently being (or have previously been) used in clinical use. Among the 5 statins, the concentration of cerivastatin required for induction of PPARγ activation was the lowest. Among the other statins, the necessary concentration for PPARγ activation was lowest in fluvastatin, increasing in the following order: pitavastatin, simvastatin, and atorvastatin.

It was reported that cholesterol depletion induces the production of PPARγ and its ligands in HepG2 and 3T3-L1 cells.[15] However, we demonstrated that statins did not induce PPARγ expression in macrophages and that the addition of excess amounts of cholesterol did not inhibit statin-induced PPARγ activation. Therefore, we speculated other mechanisms may exist in statin-induced PPARγ activation.

We demonstrated here that mevalonate completely inhibited statin-induced PPARγ activation. We also demonstrated that statins increased the PPARγ ligand-binding activity, whereas mevalonate completely inhibited these effects. Thus, statins may be not direct ligands of PPARγ, and one of the mechanisms for the statin-induced PPARγ activation may be increases in the amounts of intracellular PPARγ ligands.

From our results using FTI and GGTI, inhibition of farnesylation and geranylgeranylation may be involved mainly in statin-induced PPARγ activation. Statins inhibit the activation of small G proteins by suppressing their farnesylation and geranylgeranylation.[3] In addition, statins have been

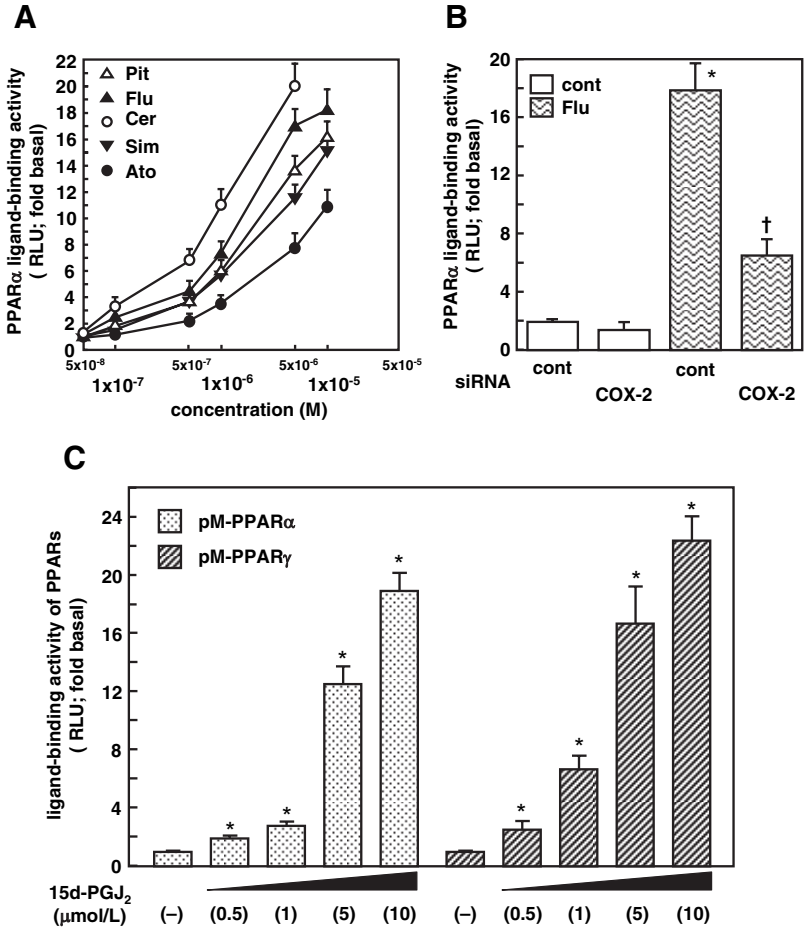

Figure 5. Statins activate PPARα via overexpression of COX-2. RAW264.7 cells were transfected with (B) or without (A and C) COX-2 siRNA or control siRNA for 4 hours, and then cells were incubated with the indicated concentrations of fluvastatin (Flu), cerivastatin (Cer), simvastatin (Sim), pitavastatin (Pit), atorvastatin (Ato) (A and B), or 15d-PGJ_2 (C) for 24 hours. Ligand-binding activity of PPARs was determined using the GAL4 chimera system. The data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, †$P<0.01$ vs cells with statins alone. RLU indicates relative light unit.

reported to activate PPARα through inhibition of the RhoA-signaling pathway.[16] We demonstrated that overexpression of DN-RhoA or DN-Cdc42 activate PPARγ, suggesting that not only RhoA but also Cdc42 negatively control the activation of PPARγ in the unstimulated state and that statins abrogate these negative effects, thereby activating PPARγ. Moreover, because an additive effect of DN-RhoA and DN-Cdc42 on PPARγ activation was observed, the mechanisms of PPARγ activation induced by the downstream signaling pathways of RhoA and Cdc42 may be different.

Members of the Rho protein family are targets of protein geranylgeranylation. However, the effects of statins were mediated by both farnesylation and geranylgeranylation. This may be explained by an alternative modification of small G proteins. Indeed, RhoB can be either geranylgeranylated or farnesylated by geranylgeranyl transferase I.[17] Thus, RhoA or Cdc42 may be prenylated by FPP.

The MAPK cascade is among the downstream signaling pathways of small G proteins, and PPARγ is negatively regulated by MAPK via its phosphorylation.[18] Therefore, we speculated that statins may suppress the MAPK cascade, thereby activating PPARγ. However, statins did not inhibit, but rather activated, ERK1/2 and p38 MAPK. In fact, atorvastatin does not induce serine phosphorylation of PPARγ in THP-1 cells.[14] Thus, statin-induced PPARγ activation cannot be mediated by inhibition of MAPK-dependent serine phosphorylation.

We demonstrated that ERK1/2 and p38 MAPK activation by statins was mediated by the suppression of farnesylation and geranylgeranylation. Moreover, statin-induced PPARγ activation was inhibited by PD98059 and SB203580, and an additive effect of the inhibitors was observed. Thus, statin-induced suppression of prenylation could induce ERK1/2 and p38 MAPK activation, although the mechanisms of PPARγ activation induced by the downstream signaling pathways of ERK1/2 and p38 MAPK may be different.

Our results suggest that statins activate ERK1/2 via a RhoA- and Cdc42-independent pathway and activate p38 MAPK via RhoA- and Cdc42-dependent pathway, thereby activating PPARγ. However, the mechanisms of prenylation-dependent ERK1/2 activation and RhoA- or Cdc42-dependent p38 MAPK activation are not fully understood. Further studies are necessary to clarify the mechanisms of statin-induced ERK1/2 and p38 MAPK activation.

Because COXs have the ability to produce prostaglandins, we speculated that statins may induce endogenous prostaglandin production via COXs expression. Our results demonstrated that statins did not induce COX-1 expression but induced COX-2 expression, suggesting that statin-induced COX-2 expression was selective. Interestingly, COX-2 expression was induced by both RhoA inhibition and Cdc42 inhibition, and statin-induced COX-2 expression was mediated by ERK or p38 MAPK. These results are supported by previous reports.[19,20] Moreover, COX-2 specific inhibition

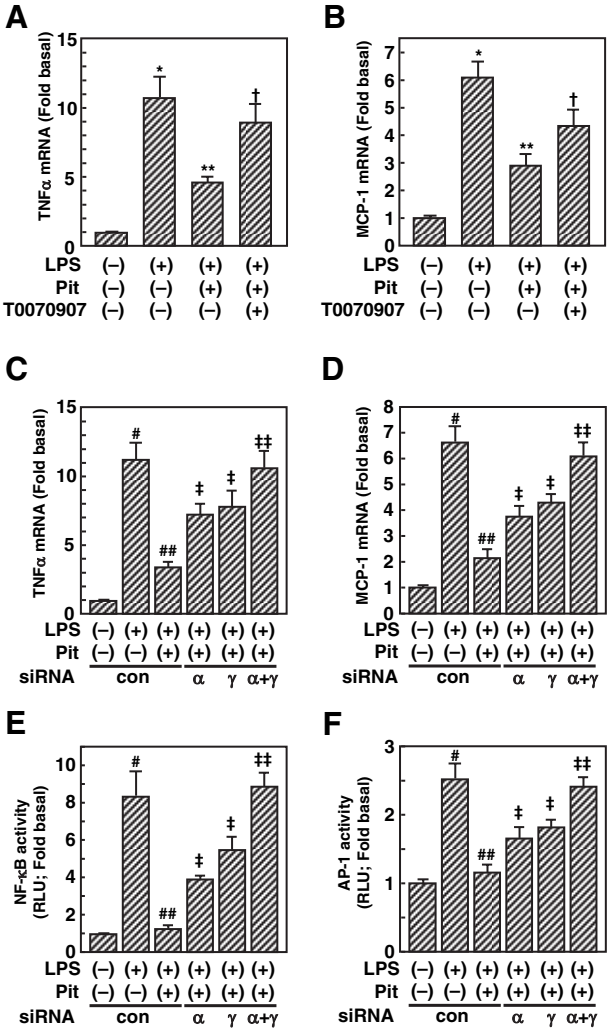

Figure 6. Involvement of PPARα and PPARγ on statin-mediated suppression of inflammatory responses. Mouse peritoneal macrophages were preincubated for 1 hour with 10 nmol/L T0070907, or cells were transfected with siRNA of control (con), or PPARα (α), and/or PPARγ (γ) in the absence (A through D) or presence of pNF-κB-Luc (E) or pAP-1-Luc (F) and cultured for 4 hours. Then, cells were incubated with 10 μmol/L pitavastatin. After 24 hours of incubation, cells were incubated with 1 μg/mL LPS for 3 hours. A through D, Total RNA was extracted, and the expression of mRNA for TNFα (A and C), MCP-1 (B and D), or β-actin was evaluated by real-time RT-PCR. Data represent means±SD of 4 separate experiments. E and F, The activities of NF-κB and AP-1 were determined by luciferase assays. Data represent means±SD of 4 separate experiments. *$P<0.01$ vs the control, **$P<0.01$ vs cells with LPS alone, †$P<0.01$ vs cells with LPS plus pitavastatin, #$P<0.01$ vs cells with control siRNA alone, ##$P<0.01$ vs cells with control siRNA and LPS alone, ‡$P<0.01$ vs cells with control siRNA and LPS plus pitavastatin, ‡‡$P<0.01$ vs cells with PPARα siRNA alone or PPARγ siRNA alone and with LPS plus pitavastatin. RLU indicates relative light unit.

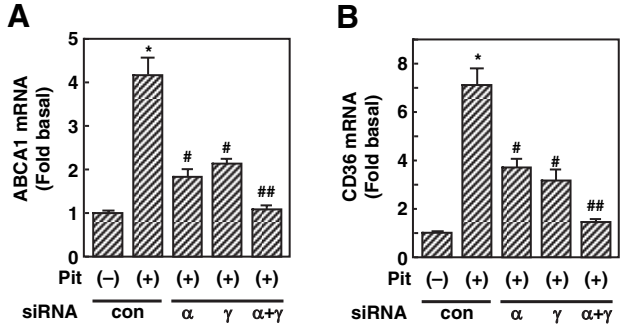

Figure 7. Involvement of PPARα and PPARγ on statin-induced mRNA expression of ABCA1 and CD36. Mouse peritoneal macrophages were transfected with siRNA of control (con) or PPARα (α) and/or PPARγ (γ). After 4 hours of incubation, cells were incubated with 10 μmol/L pitavastatin for 24 hours. Total RNA was extracted, and the expression of mRNA for ABCA1 (A), CD36 (B), or β-actin was evaluated by real-time RT-PCR. Data represent means±SD of 4 separate experiments. *$P<0.01$ vs cells with control siRNA alone, #$P<0.01$ vs cells with control siRNA and pitavastatin, ##$P<0.01$ vs cells with PPARα siRNA alone or PPARγ siRNA alone and with pitavastatin.

abrogated statin-induced PPARγ activation. Furthermore, statins increased intracellular 15d-PGJ_2, which is one of the natural PPARγ ligands, through ERK1/2 and p38 MAPK activation, and the same phenomenon was observed for DN-RhoA or DN-Cdc42. Therefore, statin-induced increase in 15d-PGJ_2 level mediated by overexpression of COX-2 through RhoA- or Cdc42-dependent p38 MAPK activation and RhoA- or Cdc42-independent ERK1/2 activation may be one of the important mechanisms in PPARγ activation.

We also found that COX-2 specific inhibition suppressed basal activity of PPARγ, suggesting that basal activity of PPARγ is mediated by COX-2–dependent production of prostaglandins in unstimulated conditions.

PGE_2, which has been reported to be an atherogenic molecule, is also induced by statins. However, the level of induction of PGE_2 was far lower than 15d-PGJ_2. Therefore, statins might be selective in eicosanoid production by unknown mechanism(s). Our present study is supported by a previous report that demonstrated that statins increased myocardial content of COX-2 expression and prostaglandin production.[21] Moreover, as PPARγ agonists are useful for treating myocardial damage,[22] statins might have protective effects against myocardial damage, at least in part through PPARγ activation.

Interestingly, statins decreased the intracellular FA levels, and statin-mediated decrease in arachidonic acid was blocked by COX-2 inhibition, suggesting that arachidonic acid was used for prostaglandin production by COX-2.

Statins was reported to activate PPARα via dephosphorylation of PPARα.[16] On the other hand, we demonstrated that statin-induced activation of PPARα was suppressed by COX-2 specific inhibition and that 15d-PGJ_2 activated both PPARα and PPARγ, suggesting that statin-induced PPARα activation is, at least in part, mediated by COX-2–dependent increase in 15d-PGJ_2 level.

The production of inflammatory molecules, which is involved in the acceleration of atherosclerosis,[3] is suppressed by statins in macrophages. Most of the production of inflammatory molecules are mediated by activation of NF-κB or AP-1. Paumelle et al reported that simvastatin has antiinflammatory effects trough PPARα.[23] Here, we further demonstrated that statins suppressed LPS-induced expression of TNFα and MCP-1 and activation of NF-κB and AP-1 through PPARα- and PPARγ-dependent pathways. Therefore, statins might suppress inflammatory responses via activation of both

PPARα and PPARγ, thereby inducing antiatherogenic actions.

ABCA1, which is involved in the control of apolipoprotein AI–mediated cholesterol efflux, and CD36, which is a scavenger receptor expressed in macrophages, are reported to be induced by agonists of PPARα and PPARγ.[24,25] We revealed that statin induced expression of ABCA1 and CD36 mRNA, and these effects were abrogated by siRNAs of PPARα and PPARγ, suggesting that statins induce the expression of ABCA1 and CD36 via both PPARα and PPARγ.

A statin concentration of 10 μmol/L, which we mainly used in the present study, might exceed the blood concentration in clinical use. However, our results demonstrated that PPARγ activation was observed following administration of statins at concentrations as low as 0.5 μmol/L; expression of COX-2 and an increase in 15d-PGJ_2 level are also induced by 0.5 μmol/L fluvastatin (T.M. and M.Y., unpublished data, 2006). Because these concentrations of statins, especially those for fluvastatin and pitavastatin, are close to their blood concentrations in clinical use, it is possible that PPARγ activation induced by some statins has physiological relevance for clinical use.

We previously demonstrated that statins, as well as PPARγ agonists, inhibit oxidized LDL-induced macrophage proliferation.[26,27] Therefore, statin-mediated suppression of macrophage proliferation might be caused by PPARγ activation. Further studies are needed to clarify the role of PPARγ activation on statin-mediated suppression of macrophage proliferation.

Because statins, as well as thiazolidinediones, have the capacity to activate PPARγ, treatment with statins may improve insulin sensitivity in patients. In fact, pravastatin reduces the incidence of type 2 diabetes mellitus,[28] and cerivastatin improves insulin sensitivity and insulin secretion in type 2 diabetes patients.[29] Our preliminary data also indicate that statins activate PPARγ in 3T3-L1 adipocytes and enhance adipocyte differentiation (T.M. and M.Y., unpublished data, 2006). Thus, it is possible that statins may act as anti-diabetic compounds.

In conclusion, we have demonstrated that statins activate PPARγ and that these effects are mediated by the suppression of FPP and GGPP. Statins induce p38 MAPK–dependent COX-2 expression by suppressing a RhoA- and Cdc42-dependent signaling pathway. Statin-induced COX-2 expression is also mediated by ERK1/2 activation through a RhoA- and Cdc42-independent signaling pathway. Finally, these signal increases in 15d-PGJ_2 levels, thereby activating PPARγ. These results could explain, at least in part, the antiatherogenic and antidiabetic effects of statins and may lead to the design of novel therapeutic approaches against atherosclerosis and diabetes.

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Disclosures

None.

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