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Statins meditate anti-atherosclerotic action in smooth muscle cells by peroxisome proliferator-activated receptor-γ activation

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ABSTRACT

The peroxisome proliferator-activated receptor-γ (PPARγ) is an important regulator of lipid and glucose metabolism, and its activation is reported to suppress the progression of atherosclerosis. We have reported that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) activate PPARγ in macrophages. However, it is not yet known whether statins activate PPARγ in other vascular cells. In the present study, we investigated whether statins activate PPARγ in smooth muscle cells (SMCs) and endothelial cells (ECs) and thus mediate anti-atherosclerotic effects. Human aortic SMCs (HASMCs) and human umbilical vein ECs (HUVECs) were used in this study. Fluvastatin and pitavastatin activated PPARγ in HASMCs, but not in HUVECs. Statins induced cyclooxygenase-2 (COX-2) expression in HASMCs, but not in HUVECs. Moreover, treatment with COX-2-siRNA abrogated statin-mediated PPARγ activation in HASMCs. Statins suppressed migration and proliferation of HASMCs, and inhibited lipopolysaccharide-induced expression of monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) in HASMCs. These effects of statins were abrogated by treatment with PPARγ-siRNA. Treatment with statins suppressed atherosclerotic lesion formation in *Apoe*$^{-/-}$ mice. In addition, transcriptional activity of PPARγ and CD36 expression were increased, and the expression of MCP-1 and TNF-α was decreased, in the aorta of statin-treated *Apoe*$^{-/-}$ mice. In conclusion, statins mediate anti-atherogenic effects through PPARγ activation in SMCs. These effects of statins on SMCs may be beneficial for the prevention of atherosclerosis.

1. Introduction

The 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) are potent inhibitors of cholesterol biosynthesis, and are utilized worldwide for the treatment of hypercholesterolemia. Clinical trials have provided clear evidence that cholesterol-lowering therapy with statins decreases the incidence of coronary heart disease [1]. However, the clinical efficiencies observed with statin therapy are greater than what might be expected from changes in lipid profile alone. Therefore, the beneficial effects of statins may extend beyond their effects on serum cholesterol levels [1–3]. Several lines of evidences indicate the following cholesterol-independent effects of statins: (i) endothelial normalization of nitric oxide production [4]; (ii) inhibition of monocyte/endothelial cell (EC) adhesion [5]; (iii) anti-inflammatory effects and cell growth arrest in macrophages [6]; (iv) strengthening of the fibrous cap [7,8]; (v) inhibition of platelet thrombus formation/reduction of thrombotic response [9]; and (vi) inhibition of smooth muscle cell (SMC) proliferation and migration [10].

Peroxisome proliferator-activated receptor-γ (PPARγ) is a transcription factor belonging to the nuclear receptor superfamily that heterodimerizes with the retinoid X receptor and binds to PPAR response elements in target gene promoters [11]. PPARγ is highly expressed in adipose tissue where it plays a key role in adipocyte differentiation and regulates genes involved in lipid metabolism. PPARγ also regulates gene expression in atherosclerotic lesions and mediates several anti-atherogenic effects in vascular cells [12]. Because PPARγ agonists have been shown to inhibit the

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development of atherosclerosis in a mouse model of atherosclerosis [13], it has been suggested that activation of PPARγ is one of the beneficial means for the prevention of atherosclerosis.

Our previous report demonstrated that statins activate PPARγ in macrophages [14]. In this report, we show that statin-induced PPARγ activation is mediated by cyclooxygenase-2 (COX-2)-dependent 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) production. Moreover, we previously demonstrated that statins inhibited lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) mRNA expression through PPARγ activation [14]. Thus, it is possible that statin-mediated anti-atherogenic effects are caused, at least in part, by PPARγ activation in macrophages. However, there is not yet any evidence to show that statins induce anti-atherogenic effects in other vascular cells, such as ECs and SMCs, through PPARγ activation. Therefore, in the present study, we examined whether statins activate PPARγ and induce PPARγ-mediated antiatherogenic effects in vascular cells, especially VSMCs.

We reveal that statins activate PPARγ in human aortic SMCs (HASMCs), but not in human umbilical vein ECs (HUVECs). Statin-induced PPARγ activation was mediated by COX-2-dependent 15d-PGJ_2 production in HASMCs, as well as in macrophages. Statins suppressed LPS-induced MCP-1 and TNF-α expression, cell migration and cell proliferation in HASMCs. Moreover, statins suppressed the progression of atherosclerosis in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice, increasing PPARγ activity and CD36 expression, and inhibiting MCP-1 and TNFα mRNA expression, in the atheromatous plaques.

2. Materials and methods

2.1. Materials

Fluvastatin and pitavastatin were generously provided by Tanabe Seiyaku Co. Ltd. and Kowa Co. Ltd., respectively. LPS (*Escherichia coli* O111:B4) was purchased from Sigma. Rabbit polyclonal anti-murine COX-2 (catalog #: 160107) antibody was purchased from Cayman Chemicals. Goat polyclonal anti-β-actin (catalog #: sc-1616) antibody was purchased from Santa Cruz Biotechnology Inc. All other chemicals were of the highest grade available from commercial sources.

2.2. Cell culture

HASMCs were purchased from Clonetics (Walkersville, MD, USA). The cells were cultured and propagated in Smooth Muscle Cell Growth Medium (Cell Applications Inc., San Diego, CA, USA) supplemented with 20% fetal bovine serum, 0.1 mg/mL streptomycin and 100 U/mL penicillin (medium A), and used in passages 4–7. Immunofluorescence for SMC α-actin confirmed SMC identity [15]. HUVECs were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). The cells were grown in M199 medium supplemented with 10% fetal bovine serum, 0.1 mg/mL streptomycin and 100 U/mL penicillin, amphotericin B, 1 mM glutamine, 5 IU/mL heparin, and 50 μg/mL endothelial cell growth supplement (medium B), and used in passages 2–5 [16]. Cultured cells were identified as endothelial cells based on their morphology and the presence of von Willebrand factor using indirect immunofluorescence microscopy.

2.3. Animals

Apoe$^{-/-}$ mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) 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 (permit number: B25-169R1), 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-four 6-week-old male mice were treated orally with fluvastatin (5 mg/kg) or a placebo (control). After 10 weeks of treatment, mice were sacrificed under anesthesia, and cross-sectional lesions of the aortic sinus were used for real-time reverse-transcription polymerase chain reaction (RT-PCR) assays, and for a PPARγ transcription activity assay, which was performed as described below. The whole aorta or 6-μm-thick frozen sections of the aortic sinus obtained from *Apoe*$^{-/-}$ mice were stained with oil red O as previously described [17,18]. Digital microphotographs of the aortic sinus were analyzed for lesion size in specific regions by measuring the stained surface area using ImageJ software (NIH, Bethesda, MD, USA). Plasma total cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride and high-density lipoprotein (HDL) cholesterol concentrations were measured at Skylight Biotech Inc. (Akita, Japan) [17,18].

2.4. Real-time RT-PCR analysis

Cells (2×10^6 cells/well in 6-well plates) were incubated with the indicated effectors. Total RNA was extracted with TRIzol (Invitrogen Co.). First-strand cDNA synthesis containing 1 μg of total RNA was primed with oligo (dT). PCR amplifications were performed in a LightCycler System (Roche Molecular Biochemicals) using SYBR Green I master mix. Specific primers were presented in Supplemental Table S1. Quantitative results for human COX-2 (*Cox2*), human MCP-1 (*Mcp1*), human PPARγ (*Pparg*), mouse CD36 (*Cd36*), and human and mouse TNF-α (*Tnfa*) were normalized to the levels of 18S ribosomal RNA (18S). Specific primers for 18S were as follows: forward primer, 5′-CGA TCC GAG GGC CTC ACT A-3′ and reverse primer, 5′-AGT CCC TGC CCT TTG TAC ACA-3′.

2.5. Enzyme-linked immunosorbent assay (ELISA) for MCP-1 and TNF-α

HASMCs (2×10^6 cells) were cultured with the indicated effectors for 1 h, then 1 μg/mL of LPS was added. After 3 h incubation, the media was collected and protein levels of MCP-1 and TNF-α were determined using ELISA assay kits of human MCP-1 and TNF-α (Life Technology).

2.6. Thymidine incorporation assay

For the thymidine incorporation assay, 18 h before the termination of the experiments, 1 μCi/mL [^{3}H] thymidine was added to each well and incubated. The medium was discarded, and the cells were dissolved in 0.1 mL of 0.5% sodium dodecyl sulfate and subsequently precipitated with 0.1 mL of ice-cold 10% trichloroacetic acid. The resulting trichloroacetic acid-insoluble materials were collected on filters using a Labomash LM-101 semiautomatic cell harvester (Labo Science, Tokyo, Japan). The filters were dried, and radioactivity was counted in a liquid scintillation spectrophotometer.

2.7. Cell migration assay

Cell migration was assessed using the QCM Chemotaxis 8-μm cell migration assay system (Millipore, Bedford, MA). HASMCs (3×10^4 cells) were seeded into the migration chamber, and DMEM containing the indicated effectors was placed in the lower chamber. After allowing cell migration for 12 h, HASMCs that had

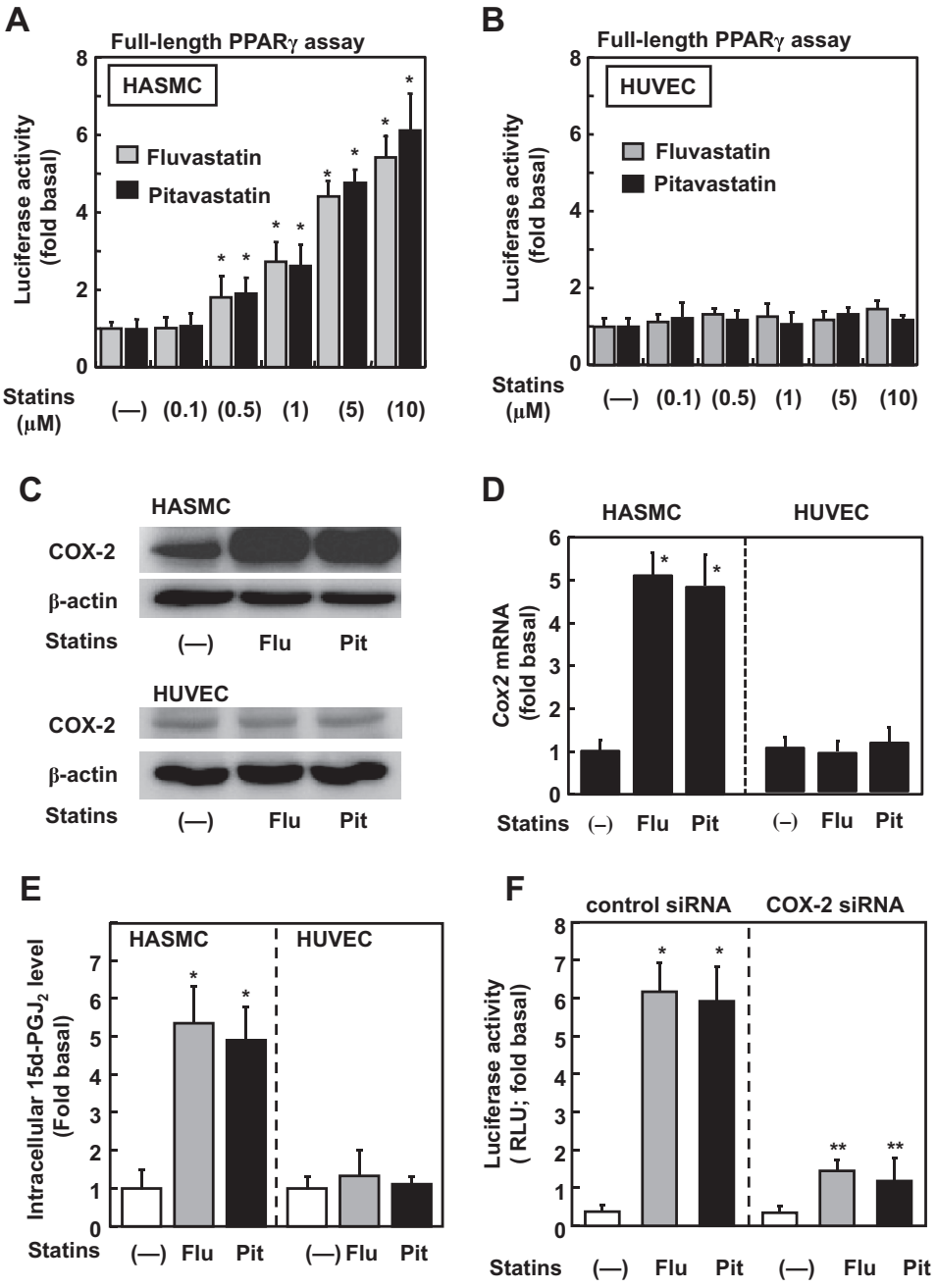

Fig. 1. Statins activate PPARγ in HASMCs but not in HUVECs. Cells were incubated for 24 h with the indicated concentrations of fluvastatin or pitavastatin. (A and B) A luciferase activity was undertaken using a full-length PPARγ luciferase assay system (A and B). Values are mean ± SD of five separate experiments. $^{*}P < 0.01$ vs. control. (C and D) COX-2 expression at the protein level (C) and the mRNA level (D) were determined by western blotting and real-time RT-PCR, respectively. (E) Concentrations of 15d-PGJ_2 were determined using EIA. (F) HASMCs were transfected with COX-2 siRNA or control siRNA, and incubated with 10 μM fluvastatin (Flu) or 10 μM pitavastatin (Pit) for 24 h. PPARγ ligand-binding activity was determined using the GAL4 chimera system. All data represent the mean ± SD of four separate experiments. $^{*}P < 0.01$ vs. the control cells or cells treated with control siRNA alone. $^{**}P < 0.01$ vs. cells treated with control siRNA plus statins.

migrated through the membrane were stained, lysed, and quantified on a microplate at 520 nm.

2.8. Statistical analysis

All data are expressed as the mean ± SD. Statistical differences between groups were examined using one-factor analysis of variance. A value of $P < 0.01$ was considered statistically significant.

Full-length PPARγ luciferase assay system, GAL4 chimera assay for PPARγ, Luciferase assays, Transfection of a siRNA against COX-2 and PPARγ, assessment of PPARγ, Enzyme immunoassay (EIA) for 15d-PGJ_2, western blot analysis and transcription activity of PPARγ are described in the online supplementary data available at http://www.sciencedirect.com.

3. Results

3.1. Statins activate PPARγ in HASMCs through over-production of COX-2

First, we investigated the effect of fluvastatin and pitavastatin on PPARγ activation in HASMCs and HUVECs using the full-length PPARγ assay system. Both statins increased luciferase activity in HASMCs in a dose-dependent manner (Fig. 1A). PPARγ ligand-

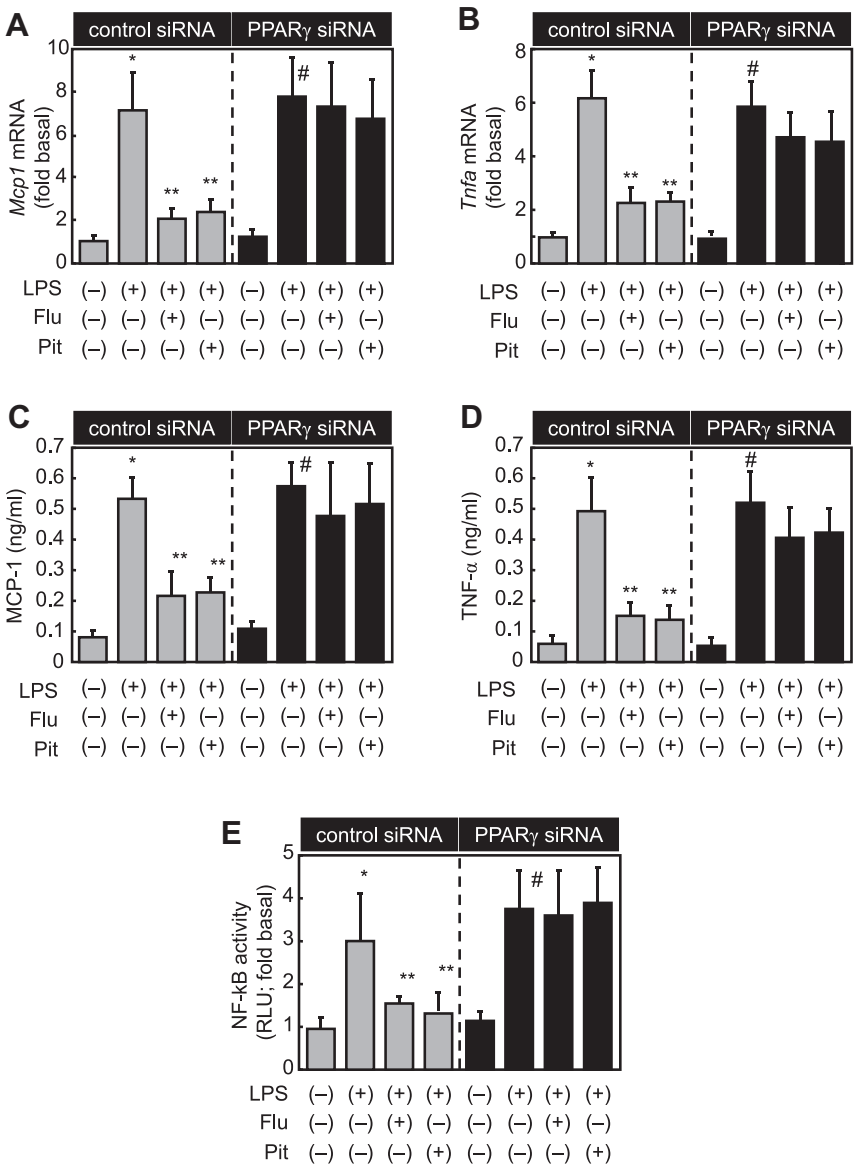

Fig. 2. Statins suppress MCP-1 and TNF-α expression through PPARγ activation. (A and B) HASMCs were transfected with control or PPARγ siRNA, and incubated with combinations of 10 μM fluvastatin (Flu) or pitavastatin (Pit), as indicated by the + symbols. The cells were then incubated with 1 μg/mL LPS for 3 h (A–D) or 24 h (E). The mRNA levels of *Mcp1* and *Tnfa* (A and B), the protein levels of MCP-1 and TNF-α (C and D), and NF-κB activity (C) were determined by real-time RT-PCR, western blotting and a NF-κB luciferase assay, respectively. Data are mean ± SD of four separate experiments. $^{*}P < 0.01$ vs. cells treated with control siRNA alone. $^{**}P < 0.01$ vs. cells treated with control siRNA plus LPS. $^{\#}P < 0.01$ vs. cells treated with PPARγ siRNA alone.

binding assay (GAL4 chimera assay) also revealed that statins increased luciferase activity in HASMCs in a dose-dependent manner (Supplemental Fig. S1A). In contrast, in HUVECs statins did not increase luciferase activity in either assay (Fig. 1C and Supplemental Fig. S1B). We previously reported that statin-induced PPARγ activation in macrophages was mediated not by over-production of PPARγ but by COX-2-dependent 15d-PGJ_2 production [14]. Similarly to macrophages, 10 μM of fluvastatin and pitavastatin did not increase PPARγ mRNA level in HASMCs and HUVECs (Supplemental Fig. S2). On the other hand, statins increased COX-2 expression at both the protein and mRNA level in HASMCs (Fig. 1C and D). However, these phenomena were not observed in HUVECs (Fig. 1C and D). Both statins increased intracellular 15d-PGJ_2 level in HASMCs but not in HUVECs (Fig. 1E). Treatment with COX-2 siRNA decreased the expression of PPARγ in HASMCs by 96% (Supplemental Fig. S3), and the statin-induced PPARγ activation was suppressed by treatment with COX-2 siRNA (Fig. 1F).

3.2. Statins suppress LPS-induced production of MCP-1 and TNF-α, and activation of NF-κB by activating PPARγ

It is well known that inflammatory chemokines, including MCP-1 and TNF-α (which are produced by macrophages, ECs and SMCs), are involved in the progression of atherosclerosis [19]. Most of the production of inflammatory molecules is mediated by activation of NF-κB. Moreover, it has been reported that the LPS-induced transcriptional regulation of MCP-1 is mediated by NF-κB activation in SMCs [20]. We therefore examined the effects of statins on LPS-induced expression of MCP-1 and TNF-α in HASMCs. LPS induced MCP-1 and TNF-α expression at the mRNA and protein level, and treatment with fluvastatin or pitavastatin suppressed LPS-induced MCP-1 and TNF-α expression (Fig. 2A and B). Moreover, treatment with PPARγ siRNA down-regulated PPARγ expression (Supplemental Fig. 3S), and the fluvastatin- or pitavastatin-induced suppression of MCP-1 and TNF-α expression was restored by treatment with the

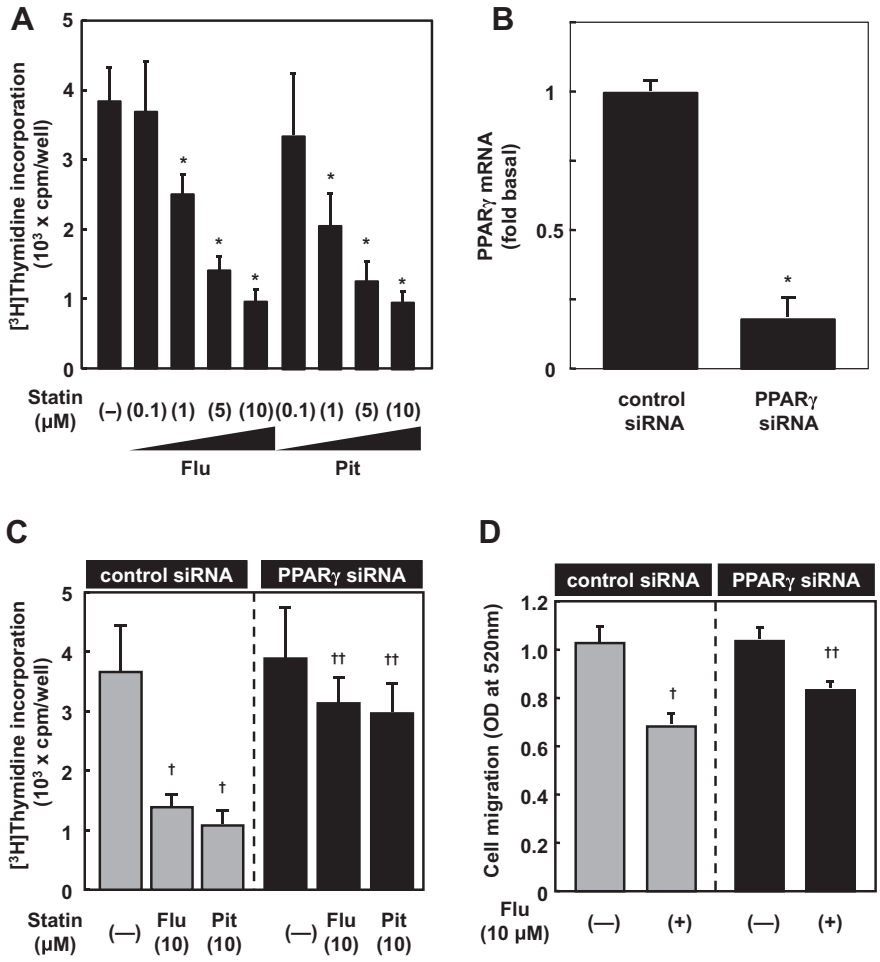

Fig. 3. Statins suppress HASMC proliferation and migration through PPARγ activation. HASMCs were either not transfected (A) or transfected with control or PPARγ siRNA (B–D), and incubated with the indicated concentrations of fluvastatin (Flu) or pitavastatin (Pit). (A and C) After 48 h incubation, [^{3}H]-thymidine incorporation was assayed. (B) After 24 h incubation, PPARγ mRNA expression was determined by real-time RT-PCR. D, after 72 h incubation, a cell migration assay was performed. $^*P < 0.01$ vs. control cells. $^\dagger P < 0.01$ vs. cells treated with control siRNA alone. $^{\dagger\dagger}P < 0.01$ vs. cells treated with control siRNA plus statins.

PPARγ siRNA (Fig. 2A and B). We next examined the effect of statins on NF-κB activation in HASMCs using an NF-κB luciferase reporter system. Both statins suppressed LPS-induced NF-κB activation, and this effect was restored by the PPARγ siRNA (Fig. 2C).

3.3. Statins suppress cell proliferation and cell migration in HASMCs via PPARγ activation

We next examined the effects of statins on cell proliferation in HASMCs. The cell proliferation assay demonstrated that treatment with fluvastatin and pitavastatin suppressed cell proliferation of HASMCs in a dose-dependent manner (Fig. 3A). The cell counting assay demonstrated similar results (Supplemental Table S2). Treatment of HASMCs with siRNA for PPARγ reduced the expression of PPARγ mRNA by 82% (Fig. 3B), and restored the statin-mediated suppression of cell proliferation (Fig. 3C). We further investigated the effects of statins on cell migration in HASMCs. The cell migration assay revealed that treatment with fluvastatin suppressed cell migration of HASMCs, and treatment with the siRNA for PPARγ restored statin-mediated suppression (Fig. 3D).

3.4. Statins suppress the progression of atherosclerosis in Apoe$^{-/-}$ mice by activating PPARγ

Treatment with fluvastatin did not affect body weight, systolic BP, diastolic BP, serum total cholesterol, triglyceride, or HDL-cholesterol levels in *Apoe*$^{-/-}$ mice (Supplemental Table S3). However, fluvastatin decreased the size of atherosclerotic lesions in the aorta, as determined by oil red O staining (Fig. 4A–C). Fluvastatin increased transcriptional activity of PPARγ and mRNA expression of CD36, which was a marker of PPARγ activation, in the aortic sinus of *Apoe*$^{-/-}$ mice (Fig. 4D and E). Moreover, fluvastatin suppressed mRNA expression of MCP-1 and TNF-α in the aorta (Fig. 4F and G).

4. Discussion

Vascular SMCs are the most abundant cells in blood vessels, and are the main cell type that contributes to atherosclerotic plaque formation [21]. There is a large body of evidence showing that statins prevent atherosclerotic vascular diseases, and they have been reported to show several direct anti-atherogenic effects in SMCs [2,22]. In this study, we have shown for the first time that statins activate PPARγ in HASMCs. We have also demonstrated that statin-induced PPARγ activation is mediated by COX-2-dependent 15d-PGJ$_2$ production in HASMCs, as we have previously shown in macrophages [14]. In contrast, we observed that statins do not activate PPARγ in HUVECs. Furthermore, unlike in HASMCs and macrophages, statins did not induce COX-2 expression and 15d-PGJ$_2$ production in HUVECs. This difference in the induction of COX-2 expression is one of the key factors that may explain the difference in the ability of statins to active PPARγ in different cell types.

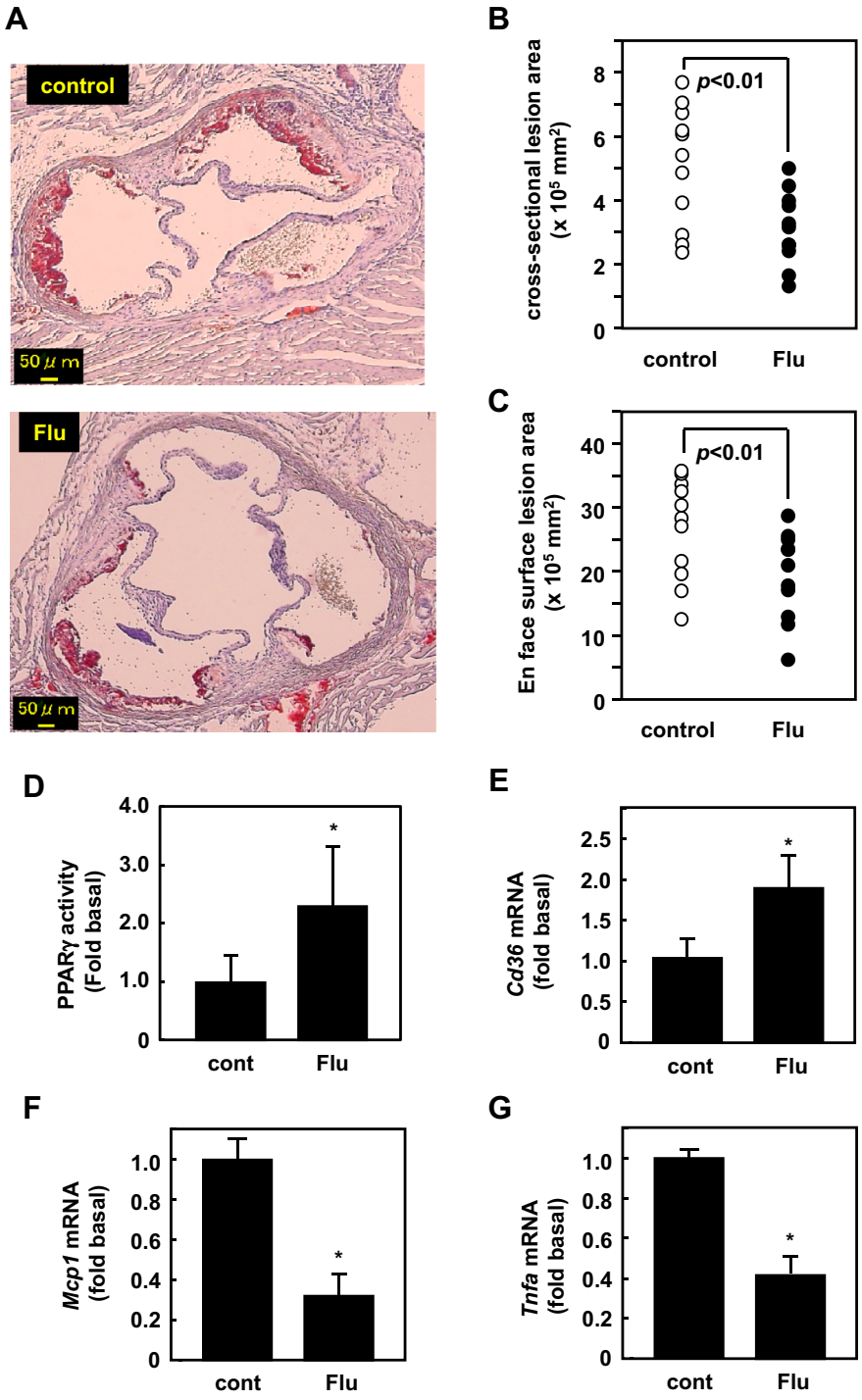

Fig. 4. Fluvastatin suppresses the progression of atherosclerosis in *Apoe*$^{-/-}$ mice. *Apoe*$^{-/-}$ mice were either untreated, or treated with fluvastatin (5 mg/kg/day) for 10 weeks. (A–C) Representative photomicrographs (A) of oil red O-stained fatty streaks (original magnification, ×5) and quantitative analysis of the atherosclerotic lesion size in cross-sections of the aortic sinus (B) or the whole aorta (C) in control or fluvastatin-treated (Flu) *Apoe*$^{-/-}$ mice ($n = 11$ per group). (D–G) Sample mRNA (E–G) or proteins (D) were obtained from control (cont) or fluvastatin-treated (Flu) *Apoe*$^{-/-}$ mice aorta. The mRNA levels of *Cd36*, *Mcp1* and *Tnfa* (E–G), and PPARγ activity (D) were determined by real-time RT-PCR and a PPARγ transcriptional activity assay, respectively. Data are the mean ± SD of five separate experiments. $^{*}P < 0.01$ vs. control mice.

The production of inflammatory mediators, including TNF-α and MCP-1, is involved in the acceleration of atherosclerosis [2]. Especially, MCP-1 plays a critical role in recruiting monocytes into early atherosclerotic lesions [19]. In fact, overexpression of MCP-1 in vessel wall macrophages led to increased foam cell formation and increased atherosclerosis [23], while a lack of MCP-1 decreased the progression of atherosclerosis in atherosclerotic mouse models [24]. Several studies have shown that LPS induces MCP-1 expression in vascular SMCs [21]. We have previously demonstrated that pitavastatin suppresses LPS-induced MCP-1 expression in macrophages [14]. Our present study has shown that fluvastatin and pitavastatin suppress LPS-induced MCP-1 expression and NF-κB activation in HASMCs through PPARγ activation. Moreover, treatment with statins suppressed MCP-1 mRNA expression in the aortic sinus of *Apoe*$^{-/-}$ mice. These results suggest that statins provide an anti-atherogenic effect by inhibiting

excess recruitment of monocyte/macrophages through PPARγ activation.

It is well known that migration and proliferation of VSMCs contribute to the formation of the fibrous cap in atherosclerotic vessels [21]. Previous studies have shown that statins suppress SMC migration in *in vitro* experiments [25–27]. Several mechanisms have been reported to be involved in statin-mediated inhibition of SMC migration. For example, statin-mediated inhibition of SMC migration was mediated by suppression of oxidative stress [28], modulation of LR11 (which is a member of the LDL receptor family) and urokinase-type plasminogen activator receptor [27], and inhibition of matrix metalloproteinase-9 expression [27]. In addition to these studies, we show here that statin-mediated inhibition of SMC migration is caused, at least in part, by PPARγ activation, which is a novel observation. Several studies have been reported that statins suppress SMC proliferation by inhibiting geranylgeranylation [28,29], with subsequent Rho GTPase-induced down-regulation of $p27^{Kip1}$ [30]. Our results suggest that PPARγ activation is also involved in statin-mediated suppression of SMC proliferation. Interestingly, we previously reported that one of the mechanisms of statin-induced PPARγ activation was the suppression of geranylgeranylation of small-G proteins, including RhoA [14]. Moreover, PPARγ agonists suppressed SMC proliferation [31,32], and conversely, dominant-negative loss of PPARγ function enhances SMC proliferation [33]. Thus, it is possible that PPARγ activation is a major factor in statin-mediated suppression of SMC proliferation.

In the present study, we investigated whether statins activate PPARγ in atheromatous vessel walls in *Apoe*$^{-/-}$ mice. Similar to previous reports [34,35], treatment with fluvastatin significantly suppressed the progression of atherosclerosis. Moreover, our results show for the first time that fluvastatin treatment increased PPARγ activity and CD36 expression, and decreased mRNA expression of MCP-1 and TNF-α in the plaque of the aorta. Therefore, statins may contribute to the prevention of atheromatous plaque formation through the activation of PPARγ.

In conclusion, we have demonstrated that statins activate PPARγ in HASMCs, as well as in macrophages, and that expression of COX-2 and the subsequent production of intracellular 15d-PGJ_2 are involved in statin-induced PPARγ activation. Moreover, statin-mediated suppression of cell migration, cell proliferation and the expression of inflammatory mediators was attenuated by down-regulating PPARγ expression. Therefore, the present study indicate that some of the anti-atherosclerotic effects mediated by statins may be caused by activating PPARγ in SMCs and macrophages.

Disclosure statement

The authors disclose no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.063.

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