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Pioglitazone suppresses macrophage proliferation in apolipoprotein-E deficient mice by activating PPARγ

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HIGHLIGHTS

- Pioglitazone suppressed atherosclerosis progression in *Apoe*$^{-/-}$ mice.
- Pioglitazone reduced macrophage proliferation in atherosclerotic plaques.
- Pioglitazone inhibited macrophage proliferation through PPARγ activation.
- Low-dose pioglitazone suppressed macrophage proliferation without apoptosis.

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ABSTRACT

Background and aims: Local macrophage proliferation is linked to enhanced atherosclerosis progression. Our previous study found that troglitazone, a thiazolidinedione (TZD), suppressed oxidized low-density lipoprotein (Ox-LDL)-induced macrophage proliferation. However, its effects and mechanisms are unclear. Therefore, we investigated the effects of pioglitazone, another TZD, on macrophage proliferation.
Methods: Normal chow (NC)- or high-fat diet (HFD)-fed apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice were treated orally with pioglitazone (10 mg/kg/day) or vehicle (water) as a control. Mouse peritoneal macrophages were used in *in vitro* assays.
Results: Atherosclerosis progression was suppressed in aortic sinuses of pioglitazone-treated *Apoe*$^{-/-}$ mice, which showed fewer proliferating macrophages in plaques. Pioglitazone suppressed Ox-LDL-induced macrophage proliferation in a dose-dependent manner. However, treatment with peroxisome proliferator-activated receptor-γ (*PPARγ*) siRNA ameliorated pioglitazone-induced suppression of macrophage proliferation. Low concentrations (less than 100 μmol/L) of pioglitazone, which can suppress macrophage proliferation, activated PPARγ in macrophages, but did not induce macrophage apoptosis. Pioglitazone treatment did not induce TUNEL-positive cells in atherosclerotic plaques of aortic sinuses in *Apoe*$^{-/-}$ mice.
Conclusions: Pioglitazone suppressed macrophage proliferation through PPARγ without inducing macrophage apoptosis. These findings imply that pioglitazone could prevent macrovascular complications in diabetic individuals.

1. Introduction

Patients with type-2 diabetes mellitus (T2DM) have increased risk of cardiovascular disease (CVD) [1]. In fact, patients with T2DM and no history of myocardial infarction have the same risk of coronary artery disease (CAD) as nondiabetics with histories of myocardial infarction [2]. As CVDs are a leading cause of mortality and morbidity for diabetics, clarifying the protective effects of antidiabetic agents against

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several atherosclerotic diseases is important.

Thiazolidinediones (TZDs), which are pharmacological ligands of peroxisome proliferator-activated receptor γ (PPARγ), are now commonly used as insulin-sensitizing agents to achieve glycemic control in patients with T2DM. The PROactive Study established a specific role for pioglitazone, a TZD, in secondary prevention of macrovascular events in patients with T2DM [3]. The PERISCOPE Trial showed that pioglitazone treatment more significantly reduced coronary plaque volume than glimepiride [4]. Thus, pioglitazone may have a beneficial effect on atherosclerosis suppression. Moreover, TZDs and other PPARγ activators reportedly have direct anti-atherosclerotic effects in atherosclerosis mouse models and various cell types, such as macrophages, that exist in atherosclerotic lesions [5].

Macrophage and macrophage-derived foam cells play important roles in atherosclerosis development and progression [6]. Macrophage-derived foam cells have been shown to proliferate in atherosclerotic lesions in several non-diabetic and diabetic animal models [7–9], as well as in human coronary arteries [10]. We previously reported that oxidized low-density lipoprotein (Ox-LDL) can induce macrophage proliferation *in vitro* [11,12]. However, a previous study indicated that macrophage accumulation at atherosclerotic lesions reflects both monocyte accumulation and macrophage proliferation [13]. Moreover, our recent study, which used transgenic mice in which macrophage proliferation was specifically suppressed, provided direct evidence that local macrophage proliferation contributes to the formation and progression of atherosclerotic plaques and plaque stability [14]. These findings suggest that local macrophage proliferation increases atherosclerosis progression.

Our previous *in vitro* study showed that another TZD, troglitazone, suppressed Ox-LDL-induced macrophage proliferation by inhibiting the generation of reactive oxygen species (ROS) [15]. However, whether TZDs actually suppress macrophage proliferation during atherosclerosis generation and progression, and whether TZD-induced suppression of macrophage proliferation is mediated by PPARγ activation, are unclear. Therefore, we investigated the inhibitory effect of pioglitazone on macrophage proliferation in atherosclerotic plaques of apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice, and the involvement of PPARγ activation on pioglitazone-mediated suppression of macrophage proliferation.

2. Materials and methods

2.1. Materials

Pioglitazone was generously provided by Takeda Pharmaceutical Company Limited (Tokyo, Japan). We acquired 15-deoxy-$\Delta^{12,14}$-15d-prostaglandin J_2 (15d-PGJ_2) from Calbiochem (San Diego, CA). Mouse monoclonal anti-Iba-1 antibody (Catalog No. 019–19741) was purchased from Wako (Tokyo, Japan). A rabbit monoclonal anti-Ki67 antibody (Catalog No. 418071) was purchased from Nichirei Biosciences Inc. (Tokyo, Japan). A rabbit monoclonal anti-malondialdehyde (MDA) antibody (Catalog No. ab6463) was purchased from Abcam (Cambridge, UK). A normal rabbit IgG antibody (Catalog No. sc-2027) was purchased from Santa Cruz Biotechnology (Dallas, TX). All other chemicals were of the highest grade available from commercial sources.

2.2. Lipoprotein preparation

Human LDL (d = 1.019–1.063 g/mL) was isolated by ultracentrifugation from plasma obtained from consenting normolipidemic subjects after overnight fasting [12], and dialyzed against 0.15 mol NaCl and 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 7.4. Ox-LDL was prepared by incubating the obtained LDL with 5 μmol/L $CuSO_4$ for 20 h at 37 °C, followed by the addition of 1 mmol/L EDTA and cooling [12]. Protein concentration was determined using a BCA protein assay (Pierce Chemical, Rockford, IL). The endotoxin level of Ox-LDL was < 1 pg/μg protein, as evaluated using the Toxicolor system (Seikagaku Corp, Tokyo, Japan). We confirmed that the proliferation and viability of mouse resident macrophages were unaffected by endotoxins at concentrations of < 1 ng/mL under our experimental conditions [12].

2.3. Animals

We obtained *Apoe*$^{-/-}$ mice (C57BL/6 background) from Jackson Laboratories (Bar Harbor, ME). Mice were housed at the Animal Resource Facility of Kumamoto University under specific pathogen-free conditions and provided with free access to food and water. Animal procedures were approved by the Animal Research Committee at Kumamoto University (Permit No. A27-040R1). All procedures conformed to the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources. Mice were given a normal rodent chow diet (NC) (CE-2; CLEA, Tokyo, Japan; protein 25.5%, fat 4.6%, carbohydrate 48.6% of total calories) developed for mice. At 12 weeks of age, all mice were given NC or high-fat diet (HFD) (HFD32; CLEA; protein 25.5%, fat 32.0%, carbohydrate 29.4% of total calories), and treated with 10 mg/kg/day pioglitazone (NC group: n = 5, HFD group: n = 5) or placebo (control) (NC group: n = 5, HFD group: n = 7) by oral administration. Body weight, amount of food intake, and casual blood glucose levels were measured at least once a week. After 8 weeks, mice were given intraperitoneal glucose tolerance tests (injected glucose: 1.5 g/kg) and intraperitoneal insulin tolerance tests (injected insulin: 1 U/kg), then sacrificed under general anesthesia with isoflurane to collect aortas. Whole aortas or 6-μm-thick frozen sections of aortic sinuses obtained from *Apoe*$^{-/-}$ mice were stained with Oil Red O, as described previously [16]. Digital microphotographs of the aortic sinus were analyzed for lesion sizes in specific regions by measuring the stained surface area using a BZ-X700 microscope (Keyence, Tokyo, Japan). Plasma total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) concentrations were measured at Skylight Biotech Inc. (Akita, Japan) [16].

2.4. Immunohistochemistry

The aortic valve throughout the aortic sinus was fixed with neutral-buffered 10% formalin and embedded in paraffin. To identify macrophage proliferation, we performed Ki67/Iba-1 double immunohistochemical staining. Tissues were incubated for 1.5 h at room temperature with an anti-Ki67 antibody and visualized by 3,3′-diaminobenzidine staining (Nichirei Bioscience). After sections were rinsed with 100 mmol/L glycine–HCl buffer (pH 2.2) to remove the first and second antibodies, sections were incubated with an anti-Iba-1 antibody overnight at 4 °C. Visualization of the reaction was performed using HistoGreen (Cosmobio, Tokyo, Japan) in 50 mmol/L Tris–HCl buffer (pH 8.5), which produced a green color. Apoptotic nuclei were determined by direct immunoperoxidase detection of digoxigenin-labeled 3′ DNA strand breaks using a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method. In situ end-labeling was performed using an Apoptosis In Situ Detection Kit (Wako) according to the manufacturer's instructions. Immunostaining was histologically analyzed under a BZ-X700 confocal fluorescence microscope [14].

2.5. Cell culture

Peritoneal macrophages were collected from anesthetized male C57BL/6 mice (25–30 g) by peritoneal lavage with 8 mL PBS; centrifuged at 200 × g for 5 min; resuspended in medium A (RPMI 1640; Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal calf serum (Nichirei Biosciences), 0.1 mg/mL streptomycin, and 100 U/mL penicillin; and then incubated in tissue culture plates for 90 min [12]. More than 98% of adherent cells were considered to be macrophages based

on four criteria: (i) adherence to culture plates, (ii) morphological features resembling mononuclear cells after Giemsa staining, (iii) capacity to take up carbon particles, and (iv) immunohistochemically positive for CD68, as described previously [17,18].

RAW264.7 cells were cultured in medium A in appropriate tissue culture plates as previously described [19], and used from the fourth to eighth passages.

2.6. Cell proliferation assay

Macrophage proliferation was estimated using a Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Japan), according to the manufacturer's instructions. In brief, mouse peritoneal macrophages (1×10^4 cells/well) were cultured in medium A, with or without appropriate effectors, for 96 h. After incubation, 10 μL of working solution containing WST-8 was added to each well. After 4 h of incubation, the absorbance of each well was measured at 450 nm using an xMark™ Microplate Absorbance Spectrophotometer (Bio-Rad, Hercules, CA) [14].

2.7. GAL4 chimera assay for PPARα and PPARγ

The fusion protein expression vectors pM-PPARα and pM-PPARγ, which contain residues 1–147 of the *GAL4* DNA-binding domain and either residues 167–467 of the human PPARα ligand-binding domain or residues 204–505 of the human PPARγ ligand-binding domain, are described elsewhere [20]. The reporter plasmid p4xUASg-tk-luc, containing four copies of a 17-mer upstream activating sequence for the *GAL4* DNA-binding domain, is also described elsewhere [20]. To measure PPARα or PPARγ ligand-binding activity, RAW264.7 cells (2×10^6 cells/well) or mouse peritoneal macrophages (2×10^6 cells/well) were transfected with p4xUASg-tk-luc and pM-*PPARα* or pM-*PPARγ*, and luciferase assays were performed as described below.

2.8. Luciferase assays

Luciferase reporter plasmids were transfected into RAW264.7 cells (2×10^6 cells/well) or mouse peritoneal macrophages (2×10^6 cells/well) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were also co-transfected with a Renilla luciferase plasmid (pRL-SV40; Promega, Madison, WI) as an internal control. After transfection, cells were cultured for 5 h, and compounds were added to the medium at appropriate concentrations. After an additional 24 h of incubation, cells were lysed and subjected to luciferase assays using a Dual-Luciferase Reporter Gene Assay system (Promega) according to the manufacturer's instructions [20].

2.9. Transfection of siRNA

siRNA against *PPARγ* and a random 21-nucleotide siRNA duplex (control) were purchased from Santa Cruz Biotechnology. Mouse peritoneal macrophages (2×10^6 cells/well) were transfected with siRNA against *PPARγ* or control using Lipofectamine 2000 [21]. After 4 h incubation, the medium was changed to medium A, and real-time RT-PCR was performed as described below. Inhibitory effects of *PPARγ* siRNA against PPARγ expression were tested using a cell proliferation assay, as described above.

2.10. Real-time RT-PCR analysis

Total RNA was extracted with TRIzol (Invitrogen). First-strand cDNA synthesis of 1 μg of total RNA was primed with oligo(dT). PCR amplifications were performed in a LightCycler System (Roche Molecular Biochemicals, Mannheim, Germany) using SYBR Green I master mix and specific primers for mouse *PPARγ* and *β-actin*: *PPARγ*, forward primer, 5′-GCCCTTTGGTGACTTTATGG-3′ and reverse primer, 5′-CAGCAGGTTGTCTTGGATGT-3′; *β-actin*, forward primer, 5′-AACAC CCCAGCCATGTACG-3′ and reverse primer, 5′-ATACCCAAGAAGGAAG GCTG-3′ [20]. Quantitative results for *PPARγ* were normalized to levels of *β-actin* mRNA. To assess the specificity of amplified PCR products, a melting curve analysis was performed after the last cycle [20].

2.11. Apoptosis assay

Total DNA was extracted from mouse peritoneal macrophages (2×10^6 cells) treated with effectors for 48 h. Total DNA was incubated with RNase (DNase-free) for 60 min at 50 °C, and then incubated with proteinase K for 60 min at 50 °C. We electrophoresed 2 μg of DNA from each sample on a 2% agarose gel, which was stained with SYBR Green I (Wako). Cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) were quantified by a cell-death detection enzyme-linked immunosorbent assay (ELISA) kit (Roche Molecular Biochemicals). Briefly, mouse peritoneal macrophages (1×10^5 cells) were incubated with effectors for 48 h, and then DNA fragments in cells were determined according to the manufacturer's instructions.

2.12. Statistical analysis

All data are expressed as mean ± SEM. Statistical tests used included the unpaired Student *t*-test using Welch's correction for unequal variances, and one-way analysis of variance followed by Tukey's multiple comparison test. $p < 0.05$ was considered significant.

3. Results

3.1. Pioglitazone improved both glucose and insulin intolerance in NC- and HFD-fed *Apoe*$^{-/-}$ mice

First, we investigated the effect of pioglitazone on the food intake, body weight, glucose metabolism, and lipid profiles of NC- or HFD-fed *Apoe*$^{-/-}$ mice. We found that pioglitazone did not affect the amount of food intake (Supplemental Fig. IA and IB) or lipid profile (Supplemental Table). However, while pioglitazone treatment did not affect the body weight of NC-fed *Apoe*$^{-/-}$ mice (Supplemental Fig. IC), it increased the body weight of HFD-fed *Apoe*$^{-/-}$ mice (Supplemental Fig. ID). Moreover, although pioglitazone treatment did not affect casual glucose levels (Supplemental Fig. IE and IF), it decreased blood glucose levels in glucose tolerance tests (Supplemental Fig. IG and IH) and insulin tolerance tests (Supplemental Fig. II and IJ) in both mouse models. To confirm the anti-inflammatory effects of pioglitazone, we examined the effect of pioglitazone on cytokine expression in liver, adipose tissue, and blood serum. Our results demonstrated that treatment with pioglitazone in NC- or HFD-fed *Apoe*$^{-/-}$ mice decreased mRNA expression of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, and IL-6 in liver (Supplemental Fig. II). Moreover, it decreased mRNA expression of TNF-α, IL-1β, and monocyte chemoattractant protein 1 (MCP-1) in adipose tissue (Supplemental Fig. III), and TNF-α and IL-1β in blood serum (Supplemental Fig. IV). These results suggested that treatment with pioglitazone inhibited low grade inflammation in NC- or HFD-fed *Apoe*$^{-/-}$ mice.

3.2. Pioglitazone suppressed atherosclerosis progression in *Apoe*$^{-/-}$ mice and decreased proliferating macrophages in atherosclerotic plaques

We next investigated the effect of pioglitazone on atherosclerosis progression and the number of proliferated macrophages in aortic sinus plaques of NC- or HFD-fed *Apoe*$^{-/-}$ mice. Pioglitazone suppressed the progression of atherosclerotic lesions in aortic sinuses of both mouse models (Fig. 1A–1D). Moreover, immunohistochemical data showed that pioglitazone-treated NC-fed or HFD-fed *Apoe*$^{-/-}$ mice had fewer Iba1$^+$ cells per plaque compared with control mice (Fig. 2A–2D, Fig. 3A–3D). Pioglitazone also decreased the number of Ki67$^+$/Iba1$^+$ cells, which represent proliferating macrophages, in the atherosclerotic

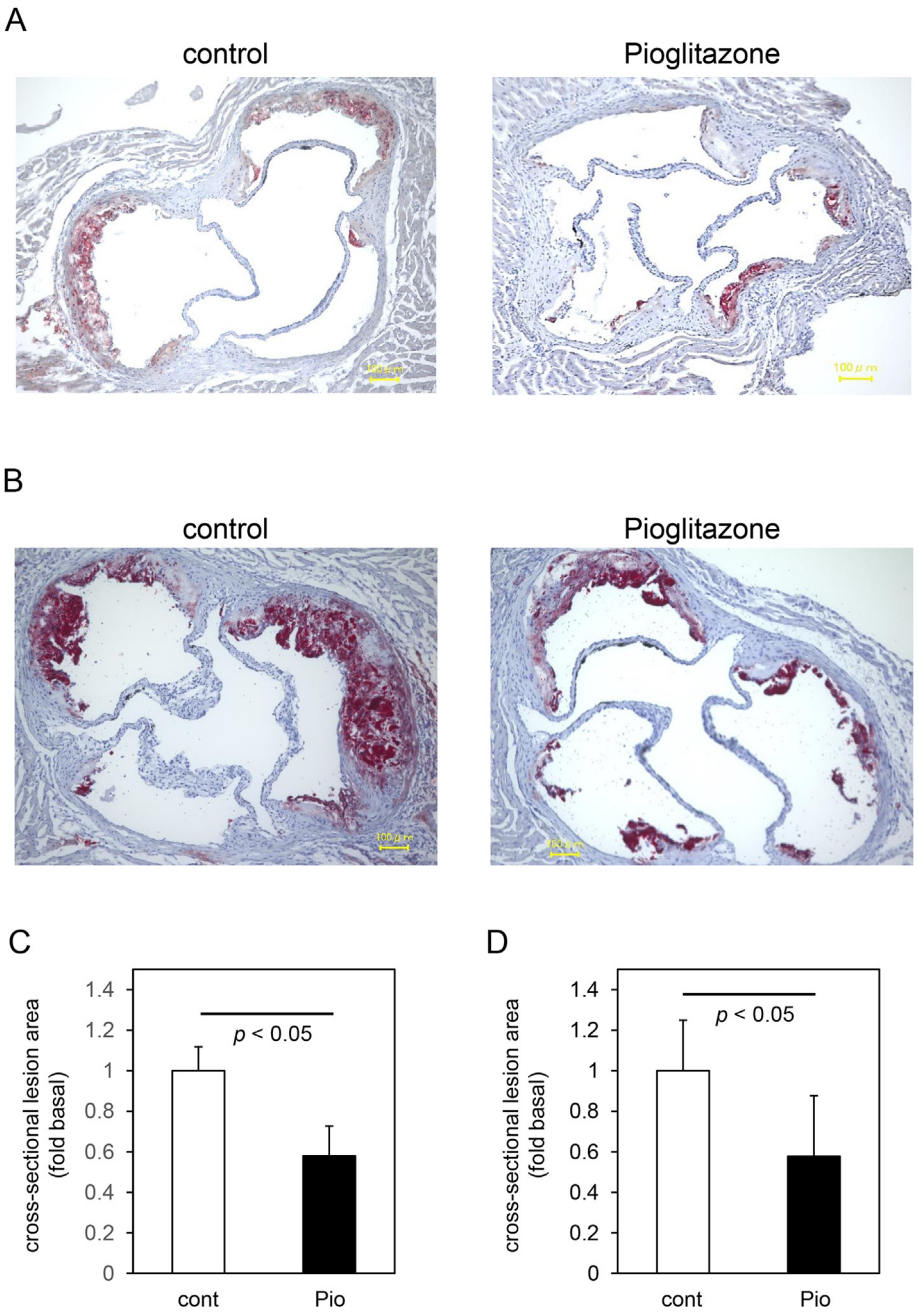

Fig. 1. Pioglitazone suppressed atherosclerosis progression in *Apoe*$^{-/-}$ mice.
Normal chow (NC)-fed (A) or high-fat-diet (HFD)-fed (B) *Apoe*$^{-/-}$ mice were treated with pioglitazone (Pio: $n = 5$ and $n = 5$, respectively) or placebo (control: $n = 5$ or $n = 7$, respectively) for 8 weeks. Representative photomicrographs of Oil Red O-stained fatty streaks (A and B) and quantitative analysis of atherosclerotic lesion size in cross-sections of aortic sinuses in NC-fed (C) or HFD-fed (D) *Apoe*$^{-/-}$ mice are shown. *$p < 0.05$ vs. control mice. Scale bars: 100 μm.

lesions of NC-fed or HFD-fed *Apoe*$^{-/-}$ mice (Fig. 2A–2D, Fig. 3A–3D, Supplemental Fig. V). To clarify the involvement of Ox-LDL in macrophage proliferation in atherosclerotic plaques, we examined the localization of malondialdehyde (MDA), an Ox-LDL marker [22], in atherosclerotic plaques in NC- or HFD-fed *Apoe*$^{-/-}$ mice. Our results demonstrated that MDA-positive areas (brown) co-localized with Iba-1-positive areas (green) (Fig. 2E, 2F, 3E and 3F), suggesting that Ox-LDL affect cell growth signals in local macrophages of atherosclerotic plaques in both mouse models.

3.3. Pioglitazone directly suppressed macrophage proliferation by activating PPARγ

Next, we investigated the effect of pioglitazone on macrophage proliferation using peritoneal macrophages obtained from C57BL/6 mice. We found that Ox-LDL significantly increased cell numbers, while pioglitazone suppressed this Ox-LDL-induced increase in cell numbers in a dose-dependent manner (Fig. 4A). In peritoneal macrophages from *Apoe*$^{-/-}$ mice, Ox-LDL-induced increases in cell numbers were also inhibited by pioglitazone (Supplemental Fig. VI). Moreover, different concentrations of glucose did not affect the inhibitory effect of pioglitazone on Ox-LDL-induced macrophage proliferation (Supplemental Fig. VII). Pre-treatment with PPARγ siRNA, which suppressed PPARγ

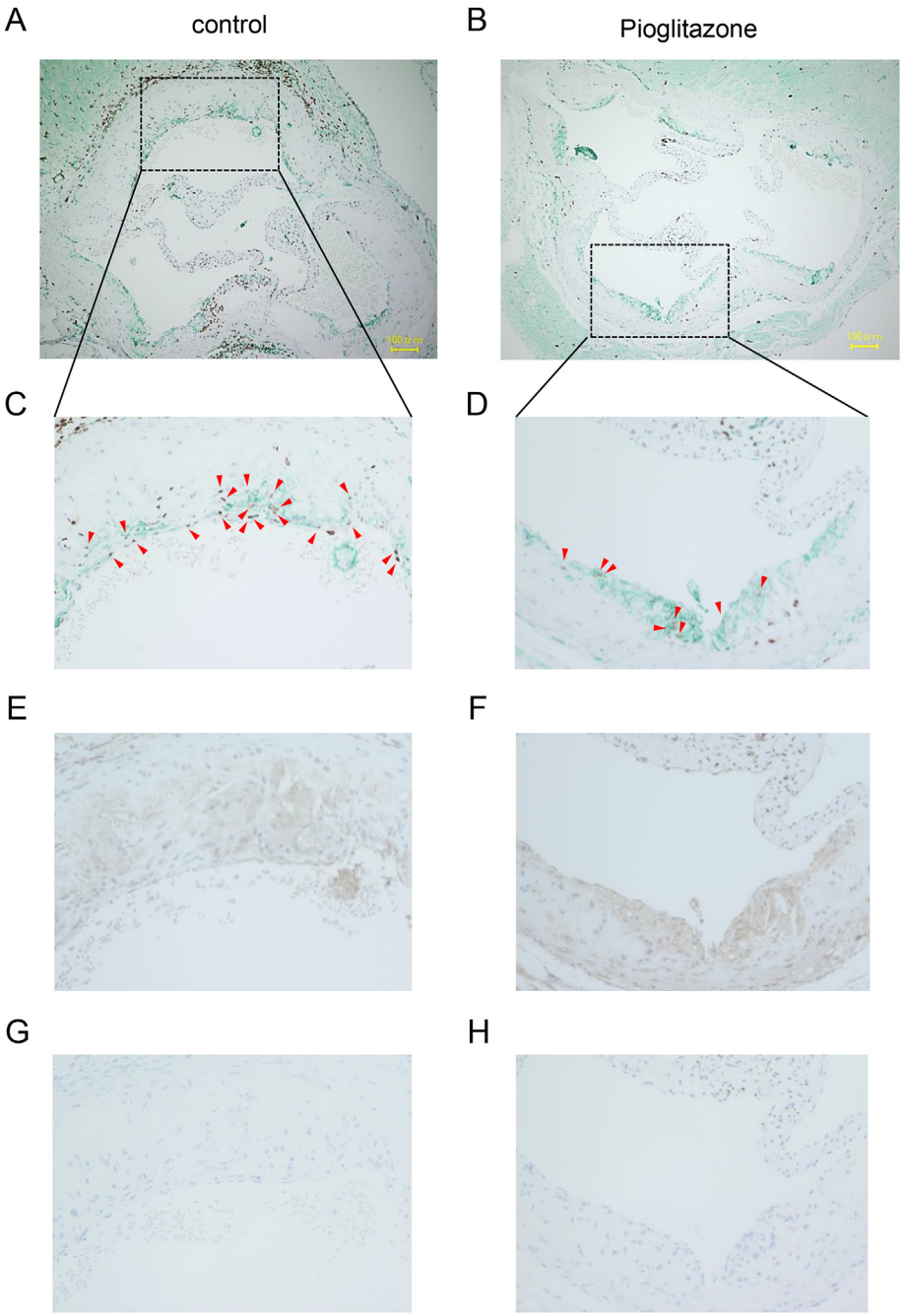

Fig. 2. Pioglitazone decreased numbers of proliferating macrophages in atherosclerotic plaques of normal chow (NC)-fed *Apoe*$^{-/-}$ mice.
Atherosclerotic lesions in the aortic sinus of placebo (control)- or pioglitazone (Pio)-treated NC-fed *Apoe*$^{-/-}$ mice were stained for Ki67 (brown) and Iba1 (green) (A–D), MDA (brown) (E and F), or normal IgG (G and H). Scale bars: 100 μm. Red arrowhead: Ki67-and Iba1-positive cells.

mRNA expression by 80% (Fig. 4B), restored pioglitazone-mediated suppression of macrophage proliferation (Fig. 4C). As such, we next investigated the effect of pioglitazone on PPARγ activation in mouse peritoneal macrophages. We found that 1 μmol/L of pioglitazone increased PPARγ ligand-binding activity in macrophages (Fig. 4D). Moreover, pioglitazone dose-dependently increased PPARγ ligand-binding activity in the macrophage cell line RAW264.7 (Fig. 4E). Interestingly, pioglitazone also dose-dependently increased PPARα ligand-binding activity (Fig. 4F). However, PPARα activation required a much higher concentration (~50 fold) of pioglitazone compared with PPARγ activation (Fig. 4F). These results suggest that pioglitazone suppressed macrophage proliferation by activating PPARγ.

3.4. Low-dose pioglitazone did not induce macrophage apoptosis

Reportedly, PPARγ agonists such as pioglitazone induce macrophage apoptosis [23–25]. Therefore, we next examined the effect of pioglitazone on macrophage apoptosis. A DNA ladder assay showed that treatment with 100 μmol/L of pioglitazone or 10 μmol/L of 15d-PGJ_2 induced DNA ladders (Fig. 5A); whereas, doses of ≤10 μmol/L of pioglitazone did not induce DNA ladders in macrophages (Fig. 5A). In ELISA assays, although 100 μmol/L of pioglitazone significantly increased the absorbance of macrophages, 1 or 10 μmol/L of pioglitazone did not (Fig. 5B). Therefore, only high (100 μmol/L) but not low (1 or 10 μmol/L) concentrations of pioglitazone induced macrophage apoptosis.

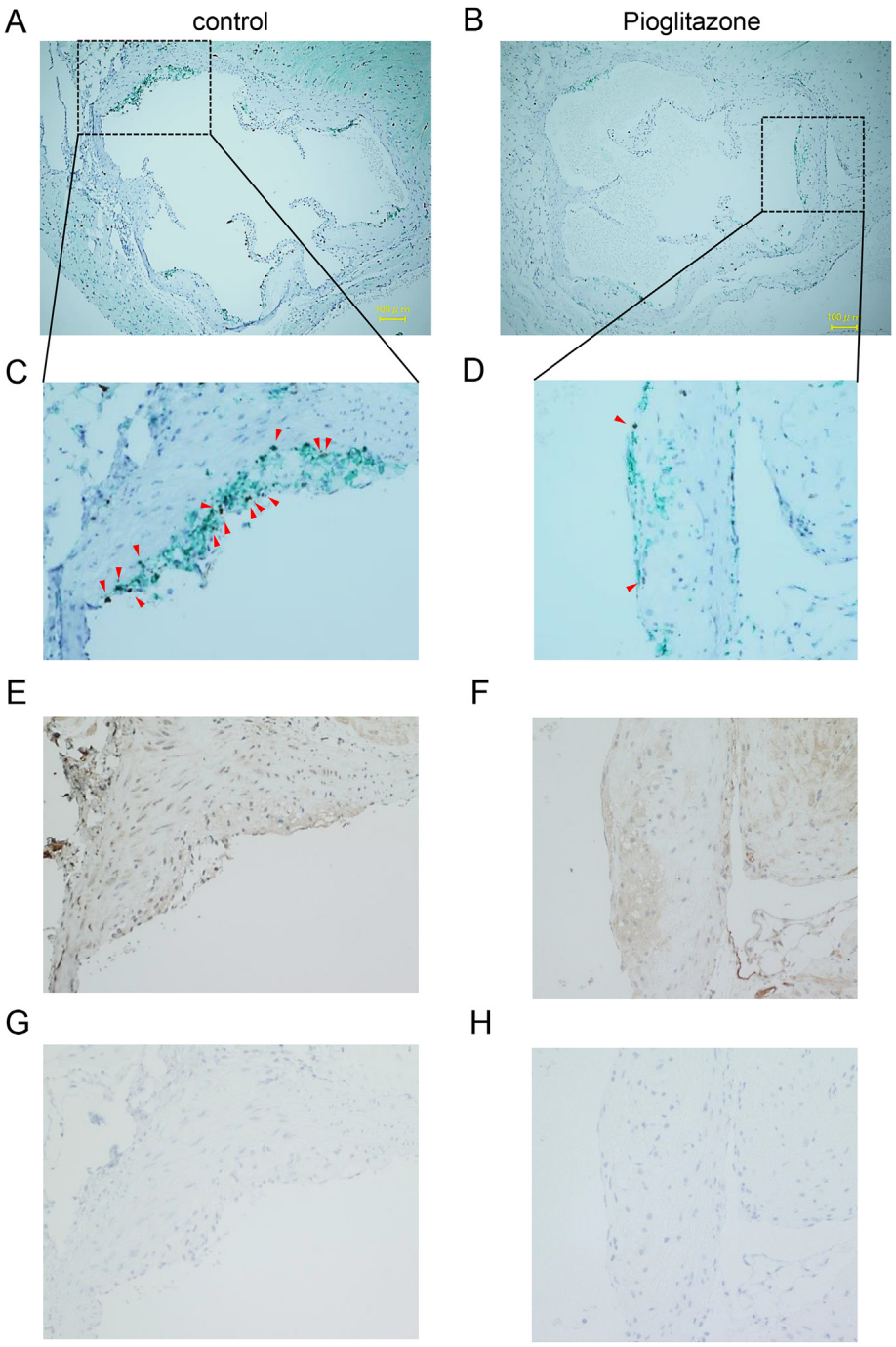

Fig. 3. Pioglitazone decreased numbers of proliferating macrophages in atherosclerotic plaques of high-fat diet (HFD)-fed *Apoe*$^{-/-}$ mice.
Atherosclerotic lesions in the aortic sinus of placebo (control)- or pioglitazone (Pio)-treated NC-fed *Apoe*$^{-/-}$ mice were stained for Ki67 (brown) and Iba1 (green) (A–D), MDA (brown) (E and F), or normal IgG (G and H). Scale bars: 100 μm. Red arrowhead: Ki67-and Iba1-positive cells.

Finally, we examined the effect of pioglitazone on apoptosis in atherosclerotic plaques of *Apoe*$^{-/-}$ mice aortas. A TUNEL assay showed that pioglitazone treatment did not increase TUNEL-positive cells in atherosclerotic plaques compared with control mice (Fig. 5C).

4. Discussion

Several clinical and basic investigations have shown that PPARγ activators, including TZDs, have anti-atherosclerotic effects in various pathological conditions (summarized by Matsumura et al. [5]). Notably, several *in vivo* studies showed that pioglitazone suppressed atherosclerosis progression in LDL receptor-deficient (*Ldlr*$^{-/-}$) mice [25–28]. However, Tian et al. reported that pioglitazone suppressed early-phase atherosclerotic lesion formation in *Apoe*$^{-/-}$ mice fed a western diet (containing 21% fat and 0.15% cholesterol) [29]. In the present study, we found that pioglitazone suppressed atherosclerosis progression in both NC- and HFD-fed *Apoe*$^{-/-}$ mice. Therefore, pioglitazone can suppress atherosclerotic lesion formation under several conditions in atherosclerotic mouse models.

Although treatment with pioglitazone did not affect body weights or lipid profiles in three studies using *Ldlr*$^{-/-}$ mice [26–28], Thorp et al. demonstrated that pioglitazone reduced serum TC levels and increased serum HDL-C levels in *Ldlr*$^{-/-}$ mice [25]. However, Tian et al. found that while early treatment with pioglitazone did not affect body weight

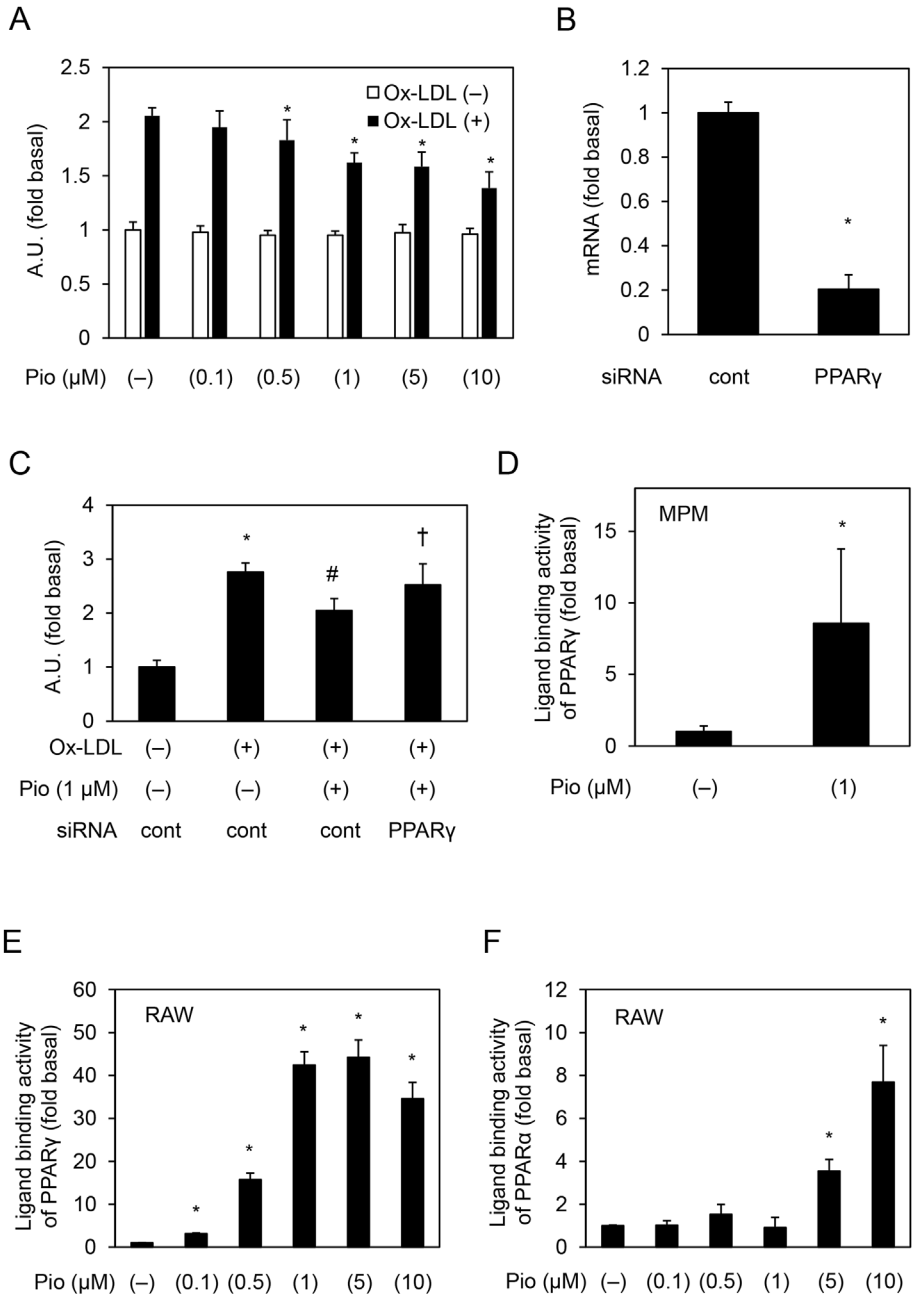

Fig. 4. Pioglitazone inhibited macrophage proliferation through PPARγ activation.
(A) Mouse peritoneal macrophages (MPM) were incubated with 20 μg/mL Ox-LDL in the absence or presence of the indicated concentration of pioglitazone (Pio). After 4 days of incubation, numbers of macrophages were measured using a CCK-8 assay. Data represent mean ± SEM of four separate experiments. *$p < 0.05$ *vs.* Ox-LDL alone. (B and C) Mouse peritoneal macrophages were transfected with siRNA against control or PPARγ. (B) PPARγ mRNA levels were determined by real-time RT-PCR. *$p < 0.05$ *vs.* control siRNA. (C) Mouse peritoneal macrophages transfected with or without siRNA against control or PPARγ were incubated with 20 μg/mL Ox-LDL in the absence or presence of the indicated concentrations of pioglitazone (Pio). After 4 days of incubation, numbers of macrophages were measured using a CCK-8 assay. Data represent mean ± SEM of four separate experiments. *$p < 0.05$ *vs.* control + control siRNA, $^{\#}p < 0.05$ *vs.* Ox-LDL + control siRNA. $^{\dagger}p < 0.05$ *vs.* Ox-LDL + pio + control siRNA. (D–F) Mouse peritoneal macrophages (MPM) (D) or RAW264.7 cells (RAW) (E and F) were incubated with or without the indicated concentration of pioglitazone for 24 h. PPARγ ligand-binding activity (D and E) and PPARα ligand-binding activity (F) were determined using a PPARγ GAL4 chimera assay system and PPARα GAL4 chimera assay system, respectively. Data represent mean ± SEM of five separate experiments. *$p < 0.05$ *vs.* control.

or lipid profile, delayed treatment with pioglitazone significantly increased serum HDL-C levels in *Apoe*$^{-/-}$ mice fed a western diet [29]. The present study showed that treatment with pioglitazone had no effect on lipid profile, but increased body weights of HFD-fed *Apoe*$^{-/-}$ mice. These differences in the effects of pioglitazone on lipid profile and body weight may depend on slight differences in mouse conditions, such as food type, mouse model, period of observation, and dose of pioglitazone.

Upon investigating the effect of pioglitazone on glucose metabolism, we found that treatment with pioglitazone had no effect on casual blood glucose level. However, it reduced serum glucose levels measured with a glucose tolerance test and improved insulin sensitivity, as indicated by an insulin tolerance test, in both NC- and HFD-fed *Apoe*$^{-/-}$ mice. Therefore, metabolic effects of PPARγ might also affect pioglitazone-mediated suppression of atherosclerotic lesion formation in NC- and HFD-fed *Apoe*$^{-/-}$ mice.

TZDs reportedly have several anti-atherogenic effects for blood-vessel constitutive cells, including smooth muscle cells, endothelial cells, and macrophages [5]. Most notably in macrophages, TZDs increased apolipoprotein-AI-dependent efflux of cholesterol [30–33] via PPAR activation [31]. PPARγ is a key regulator of M1/M2 macrophage polarization [34]; TZDs prime monocytes into M2 macrophages, and PPARγ expression is enhanced during differentiation of M2 macrophages [34,35]. Moreover, TZDs suppress expression of M1 macrophage-associated proinflammatory cytokines in monocytes (including TNF-α, IL-1β, and IL-6 [36]) and inhibit macrophage activation *in vitro* [37]. We previously reported that 15d-PGJ$_2$ (a high-affinity ligand for PPARγ) and troglitazone (a TZD) suppressed Ox-LDL-induced macrophage proliferation [15,38]. However, whether PPARγ activation is involved in PPARγ agonist-mediated suppression of macrophage proliferation was unclear. We previously reported that telmisartan (an antagonist of angiotensin type I receptor) activated PPARγ in macrophages and suppressed Ox-LDL-induced macrophage proliferation through PPARγ activation [16].

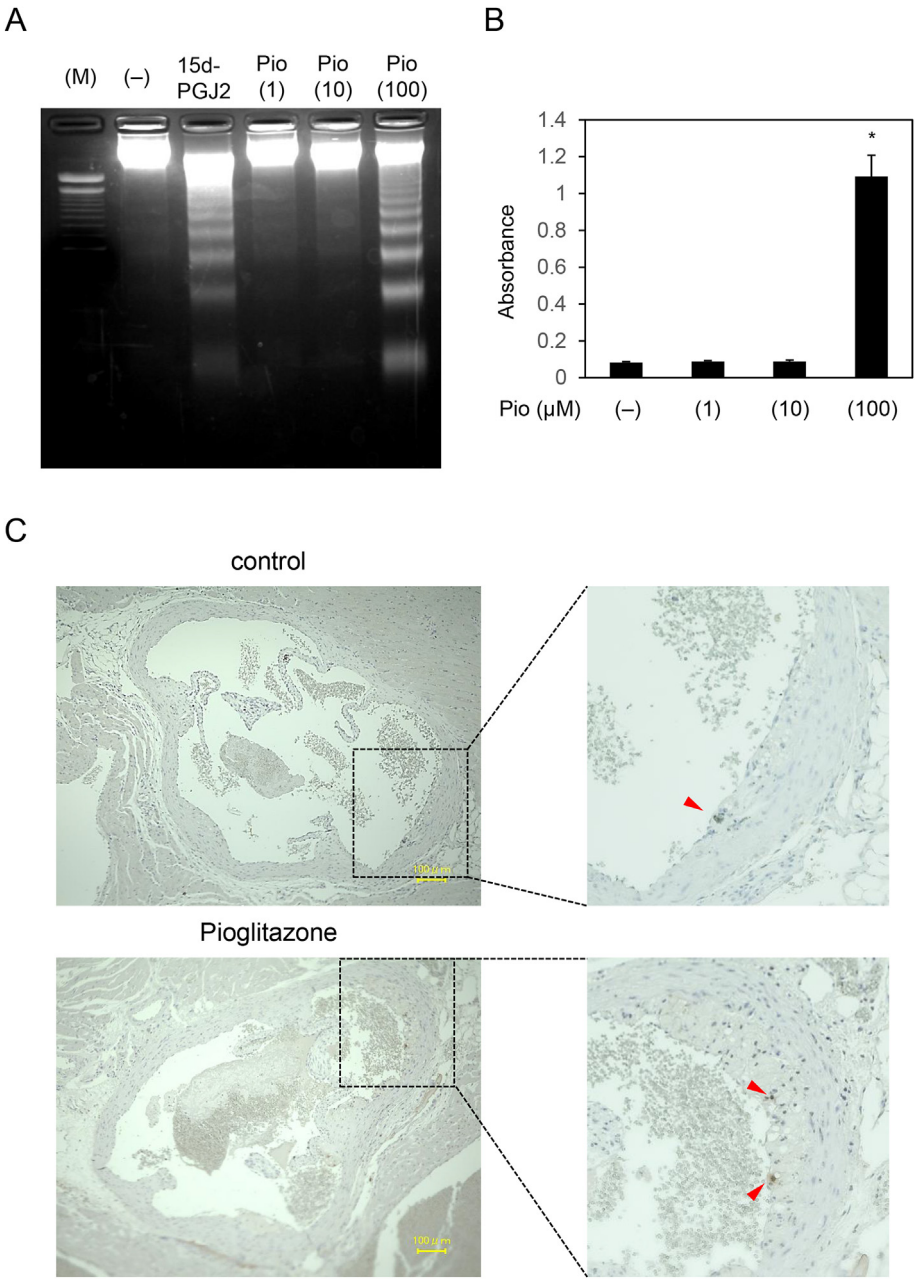

Fig. 5. Concentrations of pioglitazone ≤10 μmol/L did not induce apoptosis in macrophages.
(A and B) Mouse peritoneal macrophages were incubated with or without 10 μmol/L PGJ2 or indicated concentrations of pioglitazone. Macrophage apoptosis was analyzed by DNA ladder assay (A) and ELISA assay (B). Data represent mean ± SEM of four separate experiments. *$p < 0.05$ *vs.* control. (C) Presence of apoptotic cells was analyzed by TUNEL staining of atherosclerotic lesions in the aortic sinus of placebo (control)- or pioglitazone (Pio)-treated high-fat diet-fed *Apoe*$^{-/-}$ mice. Red arrowhead: TUNEL-positive cells.

To the best of our knowledge, the present study is the first to show that pioglitazone suppresses macrophage proliferation through PPARγ activation. We have shown that pioglitazone activated PPARγ in macrophages at a concentration similar to that required to effectively suppress macrophage proliferation. We have also shown that pioglitazone-mediated suppression of macrophage proliferation was abrogated by downregulation of PPARγ expression. Therefore, although the metabolic effect of PPARγ might affect pioglitazone-mediated decreases in plaque size, pioglitazone-induced PPARγ activation may suppress macrophage proliferation to subsequently decrease plaque size in NC- and HFD-fed *Apoe*$^{-/-}$ mice.

PPARγ agonists reportedly induce apoptosis in macrophages [23]. Notably, Tabas and colleagues reported that pioglitazone enhanced macrophage apoptosis *in vitro* and *in vivo* [25]. Therefore, apoptosis was speculated to be the mechanism through which pioglitazone suppressed macrophage proliferation. However, in the present study, low concentrations of pioglitazone (1 or 10 μmol/L) suppressed macrophage proliferation without inducing apoptosis. Thus, suppression of macrophage proliferation induced by low concentrations of pioglitazone (≤10 μmol/L) might be mediated by a mechanism other than macrophage apoptosis.

Although the present study demonstrated that pioglitazone suppresses macrophage proliferation through PPARγ activation without apoptosis, mechanistic details are still unclear. Previous studies reported that Ox-LDL-induced production of granulocyte/macrophage colony-stimulating factor (GM-CSF) plays an important role in Ox-LDL-

induced macrophage proliferation [39]. In addition, our previous studies demonstrated that troglitazone [15] and prostaglandin J_2 [38] suppress Ox-LDL-induced macrophage proliferation by inhibiting Ox-LDL-induced GM-CSF production. Therefore, pioglitazone-induced suppression of macrophage proliferation might reflect suppression of GM-CSF production. However, several lines of evidence indicate that TZDs inhibit proliferation of both cancer cells and non-cancer cells. In particular, pioglitazone reportedly suppressed proliferation through cell-growth arrest in lung cancer cells [40], renal carcinoma cell lines [41], a proximal tubular model of HK-2 cells [42], and human endothelial cells [43]. Moreover, pioglitazone was shown to inhibit hepatocyte proliferation by upregulating p27 (a cyclin-dependent kinase inhibitor) in rats after 70% partial hepatectomy [44]. Therefore, suppressed macrophage proliferation induced by low-concentration pioglitazone may be caused by upregulation of cyclin-dependent kinase inhibitors. Interestingly, Srivastava et al. reported that pioglitazone-mediated metabolic changes can rapidly and directly inhibit cell-cycle progression in cancer cells by altering ROS levels [40]. As our previous reports show that ROS generation affects Ox-LDL-induced macrophage proliferation [15,45], pioglitazone might suppress macrophage proliferation through metabolic changes in macrophages. Further studies are needed to clarify this mechanism.

In conclusion, treatment with pioglitazone suppressed atherosclerosis progression in NC-and HFD-fed *Apoe*$^{-/-}$ mice. Moreover, we showed for the first time that pioglitazone suppressed macrophage proliferation through PPARγ activation. As proliferation of vascular cells, including macrophages, is considered a key event in atherosclerosis development and progression, these findings suggest that pioglitazone could help prevent macrovascular complications in patients with T2DM.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

SMN, TM, TS, HM, TK, MT and EA conceived and designed the experiments. SMN, TM, TS, NI, HK, SN and YK performed the experiments. SMN, TM, TS and EA analyzed the data. TM, SY, YM and EA contributed reagents and materials. SMN, TM and EA wrote the manuscript with contributions from all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.04.229.

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