Pemafibrate, a PPAR alpha agonist, attenuates neointima formation after vascular injury in mice fed normal chow and a high-fat diet

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ARTICLE INFO
Keywords:
Diabetes mellitus
Vascular smooth muscle cell
Atherosclerosis
PPAR alpha
Neointima formation
Cell biology
Health sciences
Cardiology
Internal medicine
Endocrinology

ABSTRACT
Recently, the prevention of cardiovascular events has become one of the most important aims of diabetes care. Peroxisome proliferator-activated receptor (PPAR) agonists have been reported to have vascular protective effects. Here, we examined whether pemafibrate, a selective PPAR alpha agonist, attenuated neointima formation after vascular injury and vascular smooth muscle cell (VSMC) proliferation. We performed endothelial denudation injury in mice treated with a high-fat diet (HFD) or normal chow. Orally administered pemafibrate significantly attenuated neointima formation after vascular injury in HFD and normal chow mice. Interestingly, pemafibrate increased the serum fibroblast growth factor 21 concentration and decreased serum insulin concentrations in HFD mice. In addition, body weight was slightly but significantly decreased by pemafibrate in HFD mice. Pemafibrate, but not bezafibrate, attenuated VSMC proliferation in vitro. The knockdown of PPAR alpha abolished the anti-VSMC proliferation effect of pemafibrate. BrdU assay results revealed that pemafibrate dose-dependently inhibited DNA synthesis in VSMCs. Flow cytometry analysis demonstrated that G1-to-S phase cell cycle transition was significantly inhibited by pemafibrate. Pemafibrate attenuated serum-induced cyclin D1 expression in VSMCs. However, apoptosis was not induced by pemafibrate as assessed by the TUNEL assay. Similar to the in vitro data, VSMC proliferation was also decreased by pemafibrate in mice. These data suggest that pemafibrate attenuates neointima formation after vascular injury and VSMC proliferation by inhibiting cell cycle progression.

1. Introduction
Cardiovascular events (CVEs) are one of the most critical vascular complications in patients with type 2 diabetes mellitus (T2DM). Accordingly, large scale clinical trials investigating CVE reduction using novel anti-diabetic agents have provided profound results for diabetes care [1]. In addition to primary CVEs, restenosis after coronary angioplasty remains a critical problem for patients with T2DM [2]. Guidewire-induced endothelial denudation injury has been established as an experimental mouse model for restenosis and vascular thickening after coronary angioplasty or vascular injury [3]. Importantly, the pathogenesis of vascular thickening caused by wire injury reflects vascular smooth muscle cell (VSMC) proliferation after the phenotype switching of VSMCs [4]. Previously, we demonstrated that anti-diabetic agents, including glucagon-like peptide-1 (GLP-1) receptor agonist [5], dipeptidyl peptidase-4 (DPP-4) inhibitor [6], and sodium-glucose cotransporter 2 (SGLT2) inhibitor [7], attenuated neointima formation after vascular injury and VSMC proliferation. VSMC proliferation and phenotype switching are precisely regulated by nuclear receptors. We previously reported that nuclear orphan receptor NR4A neuron-derived orphan receptor1 (NOR1) plays an important role in VSMC proliferation and neointima formation after vascular injury [8, 9]. Among nuclear orphan receptors, drugs targeting peroxisome proliferator-activated receptor (PPAR) alpha are clinically available for the treatment of hypertriglyceridemia, which is a residual risk for CVEs in patients with obesity and T2DM [10]. Pemafibrate (K-877) is a PPAR alpha agonist with a highly selective binding affinity to PPAR alpha compared with other agonists [11,12]. In the present study, we examined whether pemafibrate attenuated neointima formation in mice fed normal chow and a high-fat diet (HFD) and VSMC proliferation in vitro.

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2. Materials and methods

2.1. Animals

All study protocol and experiments were reviewed and approved by the Animal Care and Use Committee of Fukuoka University (Approval number 1705050). The study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Four-week-old male C57BL/6 mice were purchased from Oriental Yeast (Tokyo, Japan). All mice were housed as previously described [7]. Water was available ad libitum. Mice were divided into the following treatment groups: normal Chow-control (n = 10), normal Chow-pemabrate (n = 10), HFD-control (n = 10), and HFD-pemabrate (n = 10). Pemabrate was kindly provided by the Kowa Company, Ltd. (Tokyo, Japan). Control mice were fed normal chow (22.6% protein, 53.8% carbohydrate, 5.6% fat, 6.6% minerals, a vitamin mixture, and 3.3% fiber; 356 kcal/100 g) or HFD (20% protein, 20% carbohydrate, 60.0% fat, D12492, Research Diet) with methyl cellulose (vehicle control) or pemabrate, at 6 weeks of age. Pemabrate was dissolved in methyl cellulose, diluted with water, and administered to the relevant experimental groups (0.1 mg/kg/day). The animal room had a 12-h light/dark cycle, constant temperature (22 ± 1 °C), and relative humidity of 55 ± 5% throughout the experimental period. Endothelial denudation injuries were induced in the left femoral artery at 8 weeks of age, followed by the evaluation of neointima formation at 12 weeks of age.

2.2. Guidewire-induced endothelial denudation injury

A femoral artery endothelial denudation injury was established in mice at 6 weeks of age as described previously [5, 6, 7, 9]. Mice were euthanized at 12 weeks of age, and femoral arteries were isolated for analysis.

2.3. Tissue preparation and morphometry

Following sacrifice, mice were perfused via a cannula in the left ventricle with phosphate-buffered saline for 5 min, followed by 4% paraformaldehyde for 30 min at 100 cm H2O. The femoral arteries were embedded in paraffin, cut into 5-μm sections, and prepared for Elastica van Gieson staining. Serial sections were evaluated using an Elastica van Gieson stain kit (HT25A-1KT; Sigma-Aldrich, Tokyo, Japan) to visualize the internal elastic lamina. Sections were viewed under a BZ9000 microscope (Keyence, Tokyo, Japan) to visualize the internal elastic lamina. The media area represents the area between external and internal elastic laminae. The intima-to-media ratio was calculated as a ratio of the control (untreated) cell proliferation.

2.4. Immunohistochemistry

Paraffin sections were incubated with a Cy-3-conjugated α-smooth muscle actin antibody (C6198, Sigma Aldrich). Serial sections were incubated with an anti-PCNA antibody (#sc-56, Santa Cruz) and subsequently incubated with an Alexa Fluor 540 goat anti-mouse IgG secondary antibody (#A-11030, Thermo Fisher Scientific). Sections were counterstained with DAPI and visualized by confocal microscopy.

2.5. Laboratory data

Blood samples were collected at euthanasia. The plasma glucose concentration was measured by a Glueste Neo Super device (Sanwa Chemical Co., Kanagawa, Japan). Insulin concentrations in mouse serum were measured using the Ultra Sensitive Mouse Insulin ELISA Kit (Morinaga Institute of Biological Science, Inc. Kanagawa, Japan) according to the manufacturer’s protocol. Fibroblast growth factor 21 (FGF21) concentrations in mouse serum were measured using an FGF21 Mouse/Rat ELISA Kit (RD291108200R, BioVendor, Karasek, Czech Republic) according to the manufacturer’s protocol. Lipid profiles were determined by a local laboratory (Skylight Biotech, Tokyo, Japan).

2.6. Cell culture

Rat aortic smooth muscle cells (RASMCs) were purchased from Lonza (Allendale, NJ, USA) and maintained in DMEM/Ham’s F-12 (042–3055S, Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Passages three and six cells were used between for experiments, and individual experiments were repeated at least three times with different cell preparations.

2.7. Proliferation assay

Cell proliferation assays were performed as described previously [6, 7] with minor modifications. Briefly, RASMCs were seeded in 12-well tissue culture plates and maintained in complete media with or without 1–100 nM pemabrate or 100–500 μM bezafibrate (#022-16091, Fujifilm Wako Chemicals, Osaka, Japan). Cell proliferation was analyzed from 0 to 72 h by cell counting using a hemocytometer.

2.8. Small interfering RNA (siRNA) knockdown of PPAR alpha expression and cell proliferation assay

To knockdown PPAR alpha, we used an order-designed siRNA (Therio Science, Tokyo, Japan) targeting rat PPAR alpha. We used negative control siRNA from the same company, as a control. For transfection, RASMCs were plated at 2 × 104 cells/well in six-well plates and transfected with 10 nmol/l of siRNA targeting PPAR alpha or negative control siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). Seventy-two hours after transfection, cells were subjected to the cell proliferation assay. Briefly, cells were detached and re-plated in 12-well tissue culture plates in complete media with or without 100 nM pemabrate, or 100 μM bezafibrate. At 0–72 h after treatment, cells were collected and counted using a hemocytometer. The siRNA knockdown efficiency was confirmed by RT-PCR.

2.9. Bromodeoxyuridine (BrdU) assay

For evaluation of the cell proliferation of RASMCs, a BrdU incorporation assay was demonstrated using a Cell Proliferation ELISA kit (1647229; Roche Applied Science, Mannheim, Germany). RASMCs were cultured at 4000 cells/well in 96-well culture plates in complete media (n = 5). At 60%–70% confluence, cells were maintained in serum-free media for 24 h. At 12 h before serum stimulation, cells were treated with 0–1000 nM pemabrate, or 0–1000 μM bezafibrate and subsequently stimulated with 10% FBS for 48 h. A BrdU (10 μM) solution was added during the last 2 h of incubation. Subsequently, the cells were dried and fixed, and cellular DNA was denatured with FixDenat solution (Roche Applied Science) for 30 min at room temperature. A peroxidase-conjugated mouse anti-BrdU monoclonal antibody (Roche Applied Science) was added to the culture plates, followed by incubation for 90 min at room temperature. Finally, a tetramethylbenzidine substrate was applied for 15 min at room temperature, and the absorbance of samples was measured using a microplate reader at 450-620 nm. Mean data were expressed as a ratio of the control (untreated) cell proliferation.

2.10. Cell cycle analysis using flow cytometry

RASMCs were seeded in 60-mm dishes at a density of 1 × 105 cells/ml. After growth to 60%–70% confluence and serum deprivation for 24 h, the cells were pretreated with 100 nM pemabrate or dimethyl...
sulfoxide (DMSO) for 12 h and then stimulated with FBS for 24 h. Cell cycle analysis was performed using a CycleTest™ Plus DNA reagent kit (BD Biosciences, Franklin Lakes, NJ) following the manufacturer's instructions and BD FACSVerse (BD Biosciences, Franklin Lakes, NJ). Obtained data were analyzed by FlowJo (Tree Star, Inc., Ashland, OR, USA).

2.11. Apoptosis assay

To label the nuclei of cells going into apoptosis, 1 × 10⁵ RSMCs were plated on glass coverslips in Lab-Tek Chamber Slides (177380; Nunc, Thermo Scientific, Waltham, MA, USA) and maintained in complete media with 100 nM pemafibrate for 48 h. The cells were fixed in 4% paraformaldehyde for 25 min. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was demonstrated using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) in accordance with the manufacturer's protocol. Cells treated with 1 U/100 μl RQ1 RNase-Free DNase (M6101; Promega) for 10 min were used as a positive control.

2.12. Western blot analysis

Western blotting was performed as described previously [13]. The following primary antibodies were used: Cyclin D1 (#2978; Cell Signaling Technology, Danvers, MA) and β-actin (sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

2.13. Reverse transcription (RT) and quantitative real-time PCR

RT and quantitative real-time PCR were performed as described previously [13]. Each sample was analyzed in triplicate and normalized against hypoxanthine phosphoribosyltransferase (HPRT) mRNA expression. The primer sequences were as follows: rat HPRT,

Figure 1. Pemafibrate attenuates neo-intima formation after vascular injury in mice with or without a HFD. Endothelial denudation injuries were induced in the left femoral artery of mice maintained with normal chow (A, B) or a HFD (C, D). (A, C) The left femoral artery with injury was evaluated by Elastica von Gieson staining to visualize the internal elastic lamina (magnification, ×200). (B, D) Intima areas and the intima/media ratio (I/M ratio) were calculated for each group. Data are the mean ± SEM. Unpaired t-test was performed to calculate statistical significance (*P < 0.05 vs control).
5′-AGGACCTCTCGAAGTGTTGG-3′ (forward), 5′-GATCCAACCTGGCGCTGTCT-3′ (reverse); rat Ppar alpha, 5′-TGGCAATGCACTGAACATCG-3′ (forward), 5′-GCAACAATGGGGTTGTC-3′ (reverse); and rat Ccnd1, 5′-ATGCTGTGTGTTGTGCTTG-3′ (forward), 5′-AGCTAGCTGACCAAAGTGC-3′ (reverse).

2.14. Statistical analysis

Results are expressed as the mean ± SEM. All statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software, La Jolla, CA, USA). In experiments comparing multiple groups, differences were analyzed by an unpaired t-test or one-way or two-way ANOVA, followed by Sidak’s post-hoc test as appropriate. *P < 0.05 was considered statistically significant.

3. Results

3.1. Pemafibrate attenuates neointima formation in mice fed both normal chow and a HFD

To evaluate neointima formation after vascular injury by endothelial denudation, we performed elastic staining of the left femoral artery with injury extracted from mice maintained on a normal chow diet (Figure 1A). In the control vessel, obviously thickened neointima formation was observed. However, a much smaller neointima was observed in the vessels from pemafibrate-treated mice. Intima area and intima/media ratio measurements revealed that pemafibrate significantly reduced the intima/media ratio (Figure 1B). In the vascular injury of mice maintained on a HFD, pemafibrate reduced neointima formation (Figure 1C) and significantly reduced the intima/media ratio (Figure 1D).

3.2. Pemafibrate attenuates VSMC proliferation in mice fed both normal chow and a HFD

To confirm that pemafibrate attenuated neointima formation by inhibiting VSMC proliferation, we stained and counted VSMC numbers localized in the neointima and media. As shown in Figure 2A, the neo-intima and media were occupied by VSMCs, and cell counting revealed that pemafibrate significantly attenuated VSMC numbers in the neo-intima and the intima/media ratio (Figure 2B). The same result was observed in HFD mice (Figure 2C, D). Laboratory data of mice are shown in Table 1. Compared with the normal chow group, body weight, serum insulin levels, LDL cholesterol, and HDL cholesterol were significantly increased, and VLDL cholesterol was significantly decreased in HFD mice. In HFD mice, the body weight and serum insulin level were

Figure 2. Pemafibrate attenuates VSMC proliferation and migration after vascular injury in mice with or without a HFD. Endothelial denudation injuries were induced in the left femoral artery of mice maintained on normal chow (A, B) or a HFD (C, D). (A, C) Sections were subjected to immunohistochemistry for α-SMA (red) and counterstained with DAPI to visualize nuclei (blue). Magnification, 630×. (B, D) Total DAPI count of the intima and the intima/media ratio (I/M ratio) were calculated for each group. Data are the mean ± SEM. Unpaired t-test was performed to calculate statistical significance (*P < 0.05 vs control).
significantly decreased by pefamibrate. Triglyceride and VLDL cholesterol were significantly decreased by pefamibrate in both groups. Interestingly, FGF21 was only detected in diabetic HFD mice and was significantly increased by pefamibrate.

3.3. Pefamibrate attenuates VSMC proliferation in vitro

To confirm that pefamibrate directly attenuated VSMC proliferation, we performed in vitro experiments using RASMCs. As shown in Figure 3A, pefamibrate significantly attenuated VSMC proliferation in a dose-dependent manner. However, the widely-used clinically available PPAR alpha agonist bezafibrate did not attenuate RASMC proliferation, and long-term incubation with bezafibrate increased RASMC numbers, whereas pefamibrate decreased RASMC numbers (Figure 3B). The BrdU assay results revealed that pefamibrate but not bezafibrate attenuated RASMC proliferation by inhibiting DNA duplication dose-dependently (Figure 3C, D).

3.4. Pefambrate attenuates VSMC proliferation via PPAR alpha activation

To confirm that pefamibrate attenuated RASMC proliferation via PPAR alpha activation, we knocked down PPAR alpha in RASMCs. As shown in Figure 4A, a significant reduction in cell proliferation induced by pefamibrate was observed in RASMCs treated with control siRNA, but the anti-proliferative effect of pefambrate was completely abolished by knocking down PPAR alpha. However, bezafibrate did not decrease the number of RASCs treated by both the control and PPAR alpha siRNA (Figure 4B). To confirm the knockdown efficiency of PPAR alpha, we

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**Table 1. Laboratory data of mice after treatment with or without pefamibrate.**

|                      | Normal Chow (n = 8) | Pefambrate (n = 6) | High-Fat Diet (n = 9) | Pefambrate (n = 6) |
|----------------------|---------------------|--------------------|----------------------|--------------------|
| **Body Weight (g)**  | 25.0 ± 0.4          | 25.6 ± 0.3         | 35.3 ± 0.8**         | 31.5 ± 0.7*        |
| **Blood Glucose (mg/dl)** | 176.2 ± 4.2       | 193.2 ± 5.3       | 182.4 ± 8.0          | 184.4 ± 9.0        |
| **Insulin (ng/ml)**  | 0.49 ± 0.10         | 0.45 ± 0.08        | 1.21 ± 0.14**        | 0.63 ± 0.10*       |
| **FGF21 (ng/ml)**    | ND                  | ND                 | 1.21 ± 0.14**        | 0.63 ± 0.10*       |
| **Triglyceride (mg/dl)** | 75.5 ± 12.3        | 42.0 ± 6.8*        | 28.8 ± 2.9           | 12.6 ± 1.9**       |
| **LDL cholesterol (mg/dl)** | 11.4 ± 0.4       | 19.4 ± 2.3*        | 31.8 ± 3.9**         | 53.8 ± 5.5         |
| **VLDL cholesterol (mg/dl)** | 6.3 ± 0.7         | 4.0 ± 0.4*         | 2.5 ± 0.2**          | 1.1 ± 0.3**        |
| **HDL cholesterol (mg/dl)** | 77.6 ± 4.8       | 95.9 ± 3.4*        | 115.3 ± 5.4**        | 123.4 ± 3.8        |

One-way ANOVA was performed to calculate statistical significance (*P < 0.05, **P < 0.01 vs control, ***P < 0.01 vs normal chow control).
performed quantitative RT-PCR of PPARalpha. As shown in Figure 4C, PPARalpha expression was almost completely abolished in cells transfected with siRNA targeting PPARalpha.

3.5. Pemafibrate attenuates VSMC proliferation by inhibiting cyclin D1 expression and cell cycle progression but does not induce apoptosis

VSMC proliferation is mainly regulated by cell cycle progression, and therefore we performed flow cytometry analysis to determine cell cycle distribution. As shown in Figure 5A, pemafibrate attenuated the G0/G1-to-S phase transition induced by serum stimulation in RASMCs. However, pemafibrate did not induce apoptosis in RASMCs (Figure 5B). Cyclin D1 is an important cell cycle regulator in the G0/G1-to-S phase transition and is a target gene of PPAR alpha required for VSMC proliferation [14]. Next, we examined cyclin D1 expression. As shown in Figure 5C, cyclin D1 protein expression induced by serum stimulation was significantly decreased by pemafibrate. In addition, cyclin D1 gene expression was significantly decreased by pemafibrate (Figure 5D).

3.6. Pemafibrate attenuates VSMC proliferation in vivo

To confirm the mechanism by which pemafibrate attenuates neointima formation and connect the data obtained from in vitro and in vivo experiments, we performed the immunohistochemistry of proliferation cell nuclear antigen (PCNA) using injured mice vessels. As shown in Figure 6, the proportion of PCNA-positive proliferating VSMCs was significantly decreased by pemafibrate in both mice fed normal chow (Figure 6A, B) and a HFD (Figure 6C, D).

4. Discussion

Currently, the aim of treating patients with diabetes includes glucose-lowering and decreasing CVEs [1]. In addition to anti-diabetic agents, the vascular protective effects of lipid-lowering drugs, such as statins [15] and eicosapentaenoic acid [16], were demonstrated in patients with T2DM. However, the vascular protective effect of fibrates is not well understood either in the clinic or basic science research. In the present study, we investigated whether pemafibrate attenuated neointima formation in both mice fed normal chow and a HFD. Cell counting revealed that the inhibition of neointima formation by pemafibrate was related to the attenuation of VSMC proliferation. In HFD mice, body weight and serum insulin levels were significantly decreased by pemafibrate, suggesting pemafibrate improved insulin resistance and hyperinsulinemia. Because insulin is a proliferation stimulus for VSMCs [17], the reduction in serum insulin levels may be a mechanism by which pemafibrate decreased neointima formation after vascular injury. However, an insulin-independent mechanism may also exist because pemafibrate decreased neointima formation in non-diabetic and non-hyperinsulinemic mice on a normal chow diet. Interestingly, serum FGF21 levels were increased by pemafibrate in HFD mice. In a previous study, pemafibrate increased serum FGF21 levels in humans [18] and FGF21 gene expression in mice [19]. In addition, pemafibrate attenuated...
neovascularization by increasing FGF21 [20]. However, FGF21 was not part of the mechanism by which pemafibrate attenuated neointima formation because we did not observe an anti-proliferative effect of FGF21 in VSMC in vitro experiments (data not shown). Further studies of the vascular protective effect of FGF21 induced by pemafibrate are required. In the previous report, Lee et al. reported that the PPAR alpha agonist fenofibrate also reduced neointima formation after vascular injury [21]. In this report, they observed a 30%-40% reduction in neointima formation after vascular injury, which was greater than our present data. However, the animal model and drug dose were substantially different. Therefore, further experiments directly comparing the reduction in neointima formation by PPAR alpha agonists are required.
We also observed that pemafibrate attenuated VSMC proliferation in vitro, suggesting it directly attenuates VSMC proliferation independent of body weight and serum insulin level reduction. Interestingly, another PPAR alpha agonist, bezafibrate, which is clinically available as a drug for hypertriglyceridemia, did not inhibit VSMC proliferation even at a higher dose compared with pemafibrate. This suggests that the vascular protective effect of pemafibrate is not a class effect of fibrates but a specific drug effect of pemafibrate. This might be a feature of the selective PPAR alpha modulator [19]. Furthermore, knocking down PPAR alpha confirmed that the molecular target of pemafibrate that attenuated VSMC proliferation was PPAR alpha. In the BrdU assay, >10 nM pemafibrate decreased DNA synthesis; however, the serum concentration of pemafibrate administered orally at a clinical dose is almost 3 nM (unpublished data by Kowa Company Ltd.), suggesting that our data might mirror clinical conditions. Compatible with our previous reports regarding the inhibition of VSMC proliferation [9, 22], pemafibrate attenuated VSMC proliferation by inhibiting G0/G1-to-S phase cell cycle transition. Furthermore, cyclin D was the target molecule and gene, similar to a previous report [14]. However, other reports suggested that the target of PPAR alpha in VSMCs was p16INKa [23] and that p16 and cyclin D1 correlated with VSMC biology [24]. Further experiments to elucidate the precise molecular mechanism by which pemafibrate attenuates VSMC proliferation are required. In the present study, we observed that pemafibrate attenuated VSMC proliferation both in vitro and in vivo. These data suggested that the anti-proliferative effect could be a confirmative direct mechanism by which pemafibrate attenuates vascular diseases. There are some limitations to our VSMC experiments. We used only VSMC cultures because we focused on VSMC proliferation. However, the micro-artery does not include VSMCs. Accordingly, our experiments demonstrated the pathophysiology of the muscular artery. In addition, we cultured VSMCs in high glucose medium following the supplier’s instruction and cell culture protocol. This did not precisely reproduce normal glucose tolerance conditions, and single-cell culture does not enable cell-cell interactions. Therefore, further elucidation is required. In addition to our study, previous reports demonstrated a vascular protective effect of pemafibrate using other vascular cells, such as endothelial cells [25] and macrophages [26], suggesting that this effect of pemafibrate is not cell-type specific.

In a previously reported clinical study, fenofibrate did not reduce CVEs in patients with T2DM and metabolic syndrome [27]. In addition, it was reported that fenofibrate was not associated with beneficial changes in carotid intima-media thickness in patients with T2DM [28]. Unfortunately, these data suggested that the reduction in hypertriglyceridemia induced by fibrates did not exhibit a vascular protective effect, even if hypertriglyceridemia is the most critical risk factor for CVEs in Japanese patients with T2DM [29]. However, a clinical study investigating pemafibrate is currently ongoing [30]. Hopefully, we will observe CVE reduction by pemafibrate in patients with T2DM.

In conclusion, we report that the PPAR alpha agonist pemafibrate attenuates neointima formation in both mice fed by normal chow and HFD, and reduced VSMC proliferation by inhibiting cyclin D1 expression and cell cycle progression.

Figure 6. Pemafibrate attenuates VSMC proliferation in vivo. Endothelial denudation injuries were induced in the left femoral artery of mice maintained on normal chow (A, B) or a HFD (C, D). (A, C) Sections were subjected to immunohistochemistry for PCNA (red) and counterstained with DAPI to visualize nuclei (blue). Magnification, 630×. (B, D) PCNA-positive cells were quantified by analyzing the fraction of stained cells to the total number of nuclei (n = 5). Data are the mean ± SEM. An unpaired t-test was performed to calculate statistical significance (*P < 0.05 vs control).
Declaration of interests statement

TN has received lecture fees from Ono Pharmaceutical Co. Ltd., Sumitomo Dainippon Pharma Co. Ltd., Nippon Boehringer Ingelheim Pharma Co., MSD K.K., and Eli Lilly Japan K.K. TY has received an endowed chair from MSD K.K., Takeda Pharmaceutical Co. Ltd., and lecture fees from Eli Lilly Japan K.K., Takeda Pharmaceutical Co., Ono Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Co., Novo Nordisk Pharma Co., MSD K.K., and Eli Lilly Japan K.K. TY has received an endowed chair from MSD K.K., Takeda Pharmaceutical Co. Ltd., and lecture fees from Eli Lilly Japan K.K., Takeda Pharmaceutical Co., Ono Pharmaceutical Co., Ltd., Mitsubishi Tanabe Pharma Co., Novo Nordisk Pharma Co., MSD K.K., and Eli Lilly Japan K.K.

Acknowledgements

Pemafibrate was kindly provided by Kowa Company, Ltd. (Tokyo, Japan). We thank Edanz Group (https://en-author-services.edanzgrou p.com/) for editing the English text of a draft of this manuscript.

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