

# Pemafibrate, A Novel Selective Peroxisome Proliferator-Activated Receptor $\alpha$ Modulator, Reduces Plasma Eicosanoid Levels and Ameliorates Endothelial Dysfunction in Diabetic Mice

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**Aims:** Various pathological processes related to diabetes cause endothelial dysfunction. Eicosanoids derived from arachidonic acid (AA) have roles in vascular regulation. Fibrates have recently been shown to attenuate vascular complications in diabetics. Here we examined the effects of pemafibrate, a selective peroxisome proliferator-activated receptor  $\alpha$  modulator, on plasma eicosanoid levels and endothelial function in diabetic mice.

**Methods:** Diabetes was induced in 7-week-old male wild-type mice by a single injection of streptozotocin (150 mg/kg). Pemafibrate (0.3 mg/kg/day) was administered orally for 3 weeks. Untreated mice received vehicle. Circulating levels of eicosanoids and free fatty acids were measured using both gas and liquid chromatography-mass spectrometry. Endothelium-dependent and endothelium-independent vascular responses to acetylcholine and sodium nitroprusside, respectively, were analyzed.

**Results:** Pemafibrate reduced both triglyceride and non-high-density lipoprotein -cholesterol levels ( $P < 0.01$ ), without affecting body weight. It also decreased circulating levels of AA ( $P < 0.001$ ), thromboxane B<sub>2</sub> ( $P < 0.001$ ), prostaglandin E<sub>2</sub>, leukotriene B<sub>4</sub> ( $P < 0.05$ ), and 5-hydroxyeicosatetraenoic acid ( $P < 0.001$ ), all of which were elevated by the induction of diabetes. In contrast, the plasma levels of 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>, which declined following diabetes induction, remained unaffected by pemafibrate treatment. In diabetic mice, pemafibrate decreased palmitic acid (PA) and stearic acid concentrations ( $P < 0.05$ ). Diabetes induction impaired endothelial function, whereas pemafibrate ameliorated it ( $P < 0.001$ ). The results of ex vivo experiments indicated that eicosanoids or PA impaired endothelial function.

**Conclusion:** Pemafibrate diminished the levels of vasoconstrictive eicosanoids and free fatty acids accompanied by a reduction of triglyceride. These effects may be associated with the improvement of endothelial function by pemafibrate in diabetic mice.

**Key words:** Pemafibrate, Eicosanoid, Diabetes, Endothelial function

**Abbreviation:** AA: arachidonic acid, Ach: acetylcholine, ANOVA: analysis of variance, COX: cyclooxygenase, GC-MS: gas chromatography-mass spectrometry, HDL: high-density lipoprotein, HETE: hydroxyeicosatetraenoic acid, LC-MS/MS: liquid chromatography-tandem mass spectrometry, LOX: lipoxygenase, LTB<sub>4</sub>: leukotriene B<sub>4</sub>, PA: palmitic acid, 15d-PGJ<sub>2</sub>: 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>, PGE<sub>2</sub>: prostaglandin E<sub>2</sub>, PPAR $\alpha$ : peroxisome proliferator-activated receptor  $\alpha$ , SNP: sodium nitroprusside, SPPAR $\alpha$ : selective peroxisome proliferator-activated receptor  $\alpha$  modulator, STZ: streptozotocin, TXB<sub>2</sub>: thromboxane B<sub>2</sub>

## Introduction

Vascular complications associated with atherosclerosis are the most serious consequences in diabetic patients. Endothelial dysfunction is an initiation step of their development<sup>1)</sup>. Various diabetes-related pathological processes cause endothelial dysfunction. Eicosanoids derived from arachidonic acid (AA) through the action of cyclooxygenases (COXs) and lipoxygenases (LOXs) play numerous roles in vascular regulation<sup>2-4)</sup>. Recent studies have shown that diabetic patients have higher enzymatic activity related to the AA cascade and therefore elevated levels of AA-derived eicosanoids<sup>5-7)</sup> and that these lipid mediators play critical roles in the development of vascular complications in diabetics<sup>8-11)</sup>.

Previous studies have suggested that fibrates, peroxisome proliferator-activated receptor (PPAR)  $\alpha$  agonists, attenuate the development of vascular complications such as endothelial dysfunction, retinopathy, and nephropathy in a diabetic condition<sup>12-16)</sup>. Fibrates are widely prescribed lipid-lowering drugs for the treatment of hypertriglyceridemia. Plasma triglyceride levels have recently garnered considerable attention as a residual risk for cardiovascular events<sup>17)</sup>. Clinical studies have demonstrated that treatment with fibrates reduces cardiovascular events associated with hypertriglyceridemia<sup>18-21)</sup>. Yet there are several limitations concerning the use of fibrates<sup>22)</sup>; for example, their activity on PPAR $\alpha$  is relatively weak, and their efficacy depends on the targeted population. However, previous studies have suggested that fibrates possess pleiotropic effects, including anti-inflammatory effects, in addition to beneficial effects on lipid levels<sup>23)</sup>. Pemaifibrate, which is a selective PPAR $\alpha$  modulator (SPPARM $\alpha$ ), which has very high potency and selectivity for PPAR $\alpha$ , has recently been introduced into clinical practice<sup>24, 25)</sup>. Both clinical and basic studies have indicated that pemaifibrate occupies a substantial role in lowering triglyceride levels and attenuating atherogenesis<sup>26-31)</sup>.

Nevertheless, the effects of pemaifibrate on eicosanoids and endothelial function, especially in diabetes, have not been fully investigated. Therefore, in this study, we examined the effects of pemaifibrate on plasma lipid concentrations and AA-derived eicosanoid levels as well as on endothelial function in diabetic mice.

## 2. Methods

### 2-1. Animal Model

Male C57BL/6 mice were purchased from Japan SLC, Inc. All mice were fed with normal chow throughout the study. The mice were randomly divided into three groups, namely, non-diabetic control, untreated diabetic, and pemaifibrate-treated diabetic. Diabetes was induced in 7-week-old male mice by a single injection of streptozotocin (STZ, 150 mg/kg). Pemaifibrate (0.3 mg/kg/day), provided by Kowa Company, Ltd. (Nagoya, Japan), was administered orally to the diabetic mice for 3 weeks; untreated mice received vehicle only. All animal care and experimental procedures conformed to the animal experimentation guidelines of Tokushima University. The protocol was reviewed and approved by the Animal Care and Use Committee of Tokushima University.

### 2-2. Blood Glucose Levels and Laboratory Data

Glucose levels in tail vein blood were measured using Startstrip XP2 (NIPRO) at three time points: before fasting, 3 days after injection (to confirm a diabetic condition), and at the time of harvest. During harvest, blood was collected from the left ventricle into EDTA-containing tubes. Plasma was separated by centrifugation at 9000 rpm for 15 min at 4°C and stored at -80°C until required. The plasma levels of lipids—e.g., total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglycerides—were measured at SanritsuZelkova (Japan). Non-HDL-cholesterol levels were calculated by subtracting HDL-cholesterol levels from total cholesterol levels.

### 2-3. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) and Gas Chromatography-Mass Spectrometry (GC-MS)

The concentrations of lipid mediators and free fatty acids in plasma were measured by LC-MS/MS and GC-MS, respectively, at Kobe University Graduate School of Medicine as previously described<sup>32, 33)</sup>. In this study, AA and AA-derived eicosanoids such as prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), 15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>), thromboxane B<sub>2</sub> (TXB<sub>2</sub>), leukotriene B<sub>4</sub> (LTB<sub>4</sub>), and 5-hydroxyeicosatetraenoic acid (5-HETE) were measured in plasma. The system consisted of a Q-Trap 6500 (Sciex) equipped with a Shimadzu LC-30AD HPLC system. A ZORBAX Eclipse Plus

C18 column (100 mm×4.6 mm, 3.5 μm; Agilent Technologies) was used with a gradient of methanol/water/acetic acid from 55:45:0.01 to 98:2:0.01 (v/v/v) at a flow rate of 0.4 mL/min. For monitoring and quantifying the levels of eicosanoids, a multiple reaction monitoring (MRM) method was developed with signature ion pairs of Q1 (parent ion)/Q3 (characteristic fragment ion) for each molecule. Identification was conducted according to published criteria by using the LC retention time, specific fragmentation patterns, and at least 6 diagnostic fragmentation ions. Quantification was carried out on the basis of the peak area of the MRM chromatograph, and linear calibration curves were obtained with authentic standards for each compound.

To measure plasma free fatty acid concentrations, nonadecanoic acid (C19:0) was used as an internal standard. After total lipids were extracted by methanol, free fatty acids were converted to methyl esters by derivatization with trimethylsilyldiazomethane. Fatty acid methyl esters were then analyzed using GC-MS QP2010 Plus (Shimadzu, Kyoto, Japan). The capillary column used for fatty acid separation was SP-2650 (100 m, an inner diameter of 0.25 mm, membrane thickness of 0.20 μm; Sigma-Aldrich). The column temperature was maintained at 140°C for 5 min and then increased gradually by 4°C/min to 240°C and held there for 20 min. Afterward, the samples were injected in split mode with a split ratio of 1:5. Each fatty acid methyl ester was detected in the selected ion-monitoring mode. All results were normalized to the peak height of the C19:0 internal standard.

#### 2-4. Vascular Reactivity Assay

At 3 weeks after the initiation of pemafibrate administration, the mice were sacrificed, and the whole aorta was immediately isolated after perfusion with 0.9% sodium chloride solution. After the fat and connective tissue around the aorta were carefully removed, aortic rings of 1.5–2 mm thickness were cut from the thoracic aorta for vascular reactivity analysis as described previously<sup>34</sup>. Briefly, vascular rings handled carefully to avoid damage to the inner surface were mounted on wires in the chambers of a multivessel myograph filled with modified Krebs–Henseleit buffer (118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, and 11.1 mM glucose), which was aerated (95% O<sub>2</sub>, 5% CO<sub>2</sub>) and warmed (37°C). Changes in isometric tension were recorded on a polygraph (LabChart). The viability of aortic segments was tested with 31.4 mM KCl. To determine the

relaxation response, the aortic rings were contracted by adding phenylephrine (10<sup>-9</sup> to 10<sup>-4</sup> M) to submaximal tensions (60% of the maximum relaxation response). After a stable contraction was determined, the rings were exposed to increasing concentrations of acetylcholine (ACh; 10<sup>-9</sup> to 10<sup>-4</sup> M) or sodium nitroprusside (SNP; 10<sup>-9</sup> to 10<sup>-4</sup> M) to obtain a cumulative concentration–response curve. Endothelium-dependent and endothelium-independent vascular reactivity was analyzed in response to ACh and SNP, respectively. In some experiments, aortic segments obtained from non-diabetic wild-type mice were incubated with 5 μM PGE<sub>2</sub>, 20 μM TXB<sub>2</sub> (Cayman Chemical), and 200 μM palmitic acid (PA; Chem Service, Inc.) for 4 h before examining vascular reactivity.

#### 2-5. Western Blotting

Protein was isolated from the liver and separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as described previously. The following primary antibodies were used: anti-thromboxane synthase, anti-PGE synthase (Abcam), and anti-α-tubulin (MBL). β-actin was used as an internal control. The protein expression level was analyzed with ECL-plus reagent (GE Healthcare) using a luminescent image analyzer (LAS-4000 mini, Fuji Film).

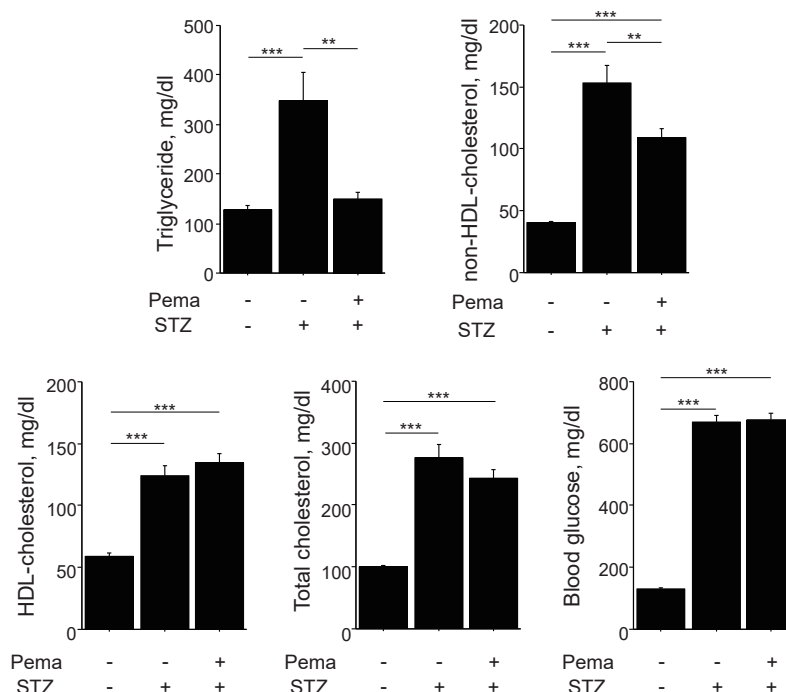
#### 2-6. Statistical Analysis

All results were expressed as mean±standard error of the mean (SEM). Comparison of parameters between two groups was performed with unpaired Student's *t*-test. Comparisons of multiple groups were performed using one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test. Dose–response curves were compared by two-factor repeated-measures ANOVA, followed by Scheffe's post hoc test for between-group comparisons. The correlation between the levels of eicosanoids and triglyceride concentration was examined by univariate analysis. A *P* value of <0.05 was considered significant.

### 3. Results

#### 3-1. Effect of Pemafibrate on Lipid Profile

Diabetes induction by STZ injection significantly increased plasma lipid levels, including triglyceride concentrations (*P*<0.001), as described previously<sup>34, 35</sup>. Pemafibrate significantly reduced both triglyceride and non-HDL-cholesterol levels in diabetic mice, without affecting the levels of total cholesterol, HDL-cholesterol, and blood glucose



**Fig. 1.** Effect of pemaifibrate on lipid profile

Induction of diabetes with STZ significantly elevated plasma lipid concentrations, whereas pemaifibrate reduced the levels of triglycerides and non-HDL-cholesterol in diabetic mice. Pemaifibrate did not affect the levels of HDL-cholesterol, total cholesterol, and blood glucose ( $n=16-18$  per group). \*\* $P<0.01$  and \*\*\* $P<0.001$ . All values are mean  $\pm$  SEM.

(**Fig. 1**). Pemaifibrate did not affect body weight in diabetic mice (untreated [ $19.7 \pm 2.6$  g] vs. treated [ $19.7 \pm 2.1$  g]).

### 3-2. Pemaifibrate Reduced Plasma Levels of AA-Derived Eicosanoids

We examined the effects of pemaifibrate on eicosanoids derived from AA. Induction of diabetes increased the plasma levels of AA and eicosanoids produced by the COX2 pathway, such as TXB<sub>2</sub> ( $P<0.001$ ) and PGE<sub>2</sub>. Treatment with pemaifibrate significantly diminished AA and TXB<sub>2</sub> levels ( $P<0.001$ , respectively) and tended to decrease PGE<sub>2</sub> in diabetic mice. In contrast, induction of diabetes reduced 15d-PGJ<sub>2</sub>, which remained unaffected by pemaifibrate (**Fig. 2**). We also explored the effect of pemaifibrate on the expression of enzymes synthesizing these vasoconstrictive eicosanoids in the liver. Thromboxane synthase, which was significantly elevated following diabetes induction by STZ ( $P<0.05$ ), tended to decline after pemaifibrate treatment. Furthermore, the levels of PGE synthase, which tended to increase with STZ-induced diabetes, were significantly lowered by pemaifibrate ( $P<0.05$ ; **Fig. 3**).

Induction of diabetes also increased the plasma levels of eicosanoids produced by the 5-LOX pathway,

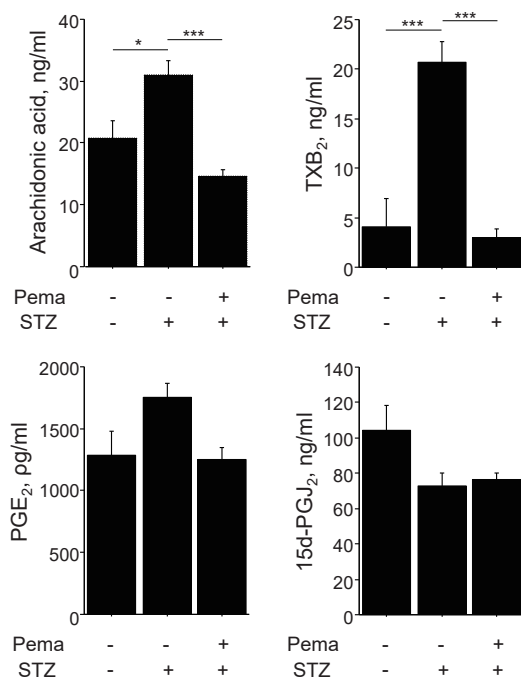
such as LTB<sub>4</sub> ( $P<0.01$ ) and 5-HETE ( $P<0.01$ ), whereas pemaifibrate attenuated these levels ( $P<0.05$  and  $P<0.001$ , respectively) in diabetic mice (**Fig. 4**). Moreover, these eicosanoid levels were found to positively correlate with triglyceride levels (**Fig. 5**).

### 3-3. Pemaifibrate Reduced Plasma Levels of Saturated Free Fatty Acids

We also examined the effects of pemaifibrate on the plasma levels of saturated free fatty acids. Induction of diabetes mildly increased plasma PA and stearic acid levels, which were significantly decreased following treatment with pemaifibrate ( $P<0.05$ , respectively), as shown in **Fig. 6**.

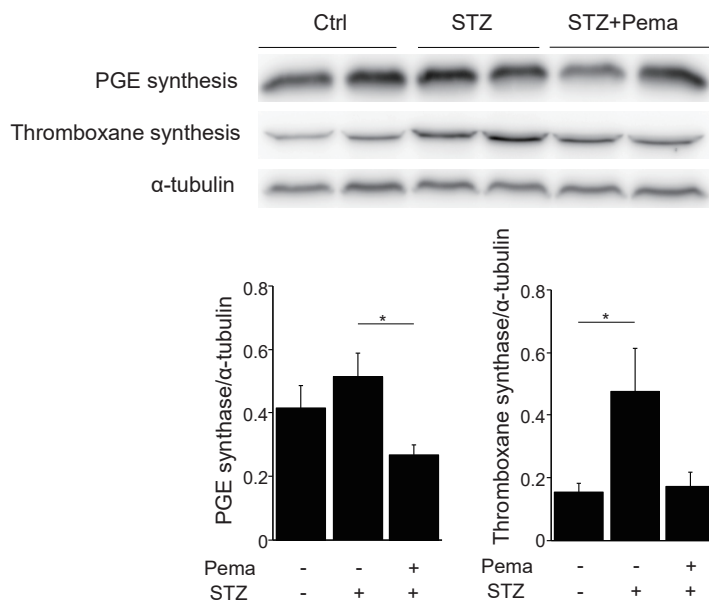
### 3-4. Pemaifibrate Ameliorated Endothelial Dysfunction in Diabetic mice

We examined whether pemaifibrate ameliorated endothelial dysfunction in diabetic mice. Induction of diabetes impaired endothelium-dependent vasodilation as determined in response to Ach ( $P<0.001$ ), whereas pemaifibrate ameliorated endothelial dysfunction in diabetic mice ( $P<0.001$ ), as compared with vehicle. On the other hand, endothelium-independent vascular reactivity in response to SNP did not differ across the three groups (**Fig. 7**). These



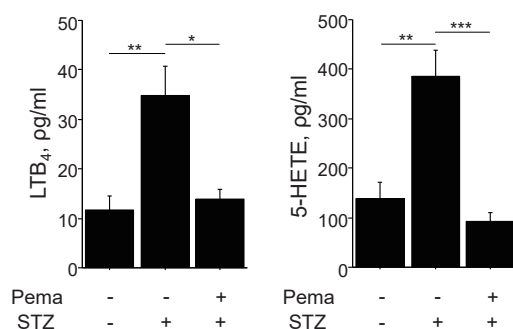
**Fig. 2.** Pemafibrate reduced eicosanoids produced by the COX2 pathway

Induction of diabetes elevated the levels of AA, TXB<sub>2</sub>, and PGE<sub>2</sub>, whereas pemafibrate decreased their expression levels. Induction of diabetes reduced 15d-PGJ<sub>2</sub> levels, which remained unaffected by pemafibrate treatment (*n*=5 per group). \**P*<0.05 and \*\*\**P*<0.001. All values are mean ± SEM.



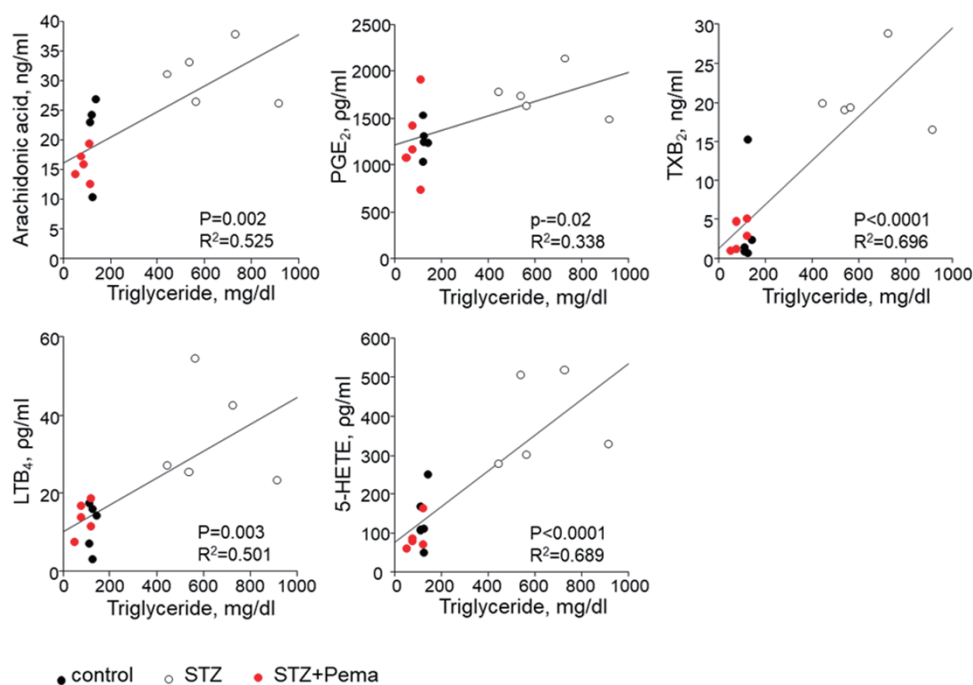
**Fig. 3.** Pemafibrate reduced the expression of PGE synthase and thromboxane synthase

Induction of diabetes by STZ significantly promoted the levels of thromboxane synthase, which tended to decline after treatment with pemafibrate. Furthermore, STZ-induced diabetes tended to increase PGE synthase levels, which were significantly lowered by pemafibrate (*n*=8 per group). All values are mean ± SEM. \**P*<0.05.



**Fig. 4.** Pema fibrate reduced eicosanoids produced by the LOX pathway

Induction of diabetes elevated LTB<sub>4</sub> and 5-HETE levels, whereas pema fibrate reduced their expressions ( $n=5$  per group). \* $P<0.05$ , \*\* $P<0.01$ , and \*\*\* $P<0.001$ . All values are mean  $\pm$  SEM.



**Fig. 5.** Eicosanoid levels positively correlated with triglyceride levels

The levels of AA and AA-derived eicosanoids positively correlated with triglyceride levels ( $n=5$ ).

results indicated that pema fibrate ameliorated endothelial dysfunction in diabetic mice.

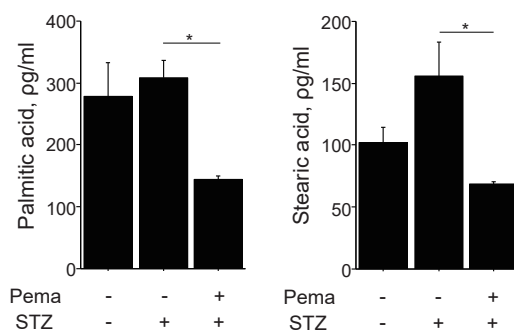
### 3-5. Eicosanoids and PA Impaired Endothelial Function

Finally, we examined whether eicosanoids or PA impaired endothelial function. PGE<sub>2</sub> impaired endothelium-dependent vasodilation, as determined by the response to Ach ( $P<0.05$ ). TXB<sub>2</sub> tended to impair endothelium-dependent vascular function. In addition, PA resulted in a significant impairment of endothelium-dependent vascular function (Fig. 8).

Nonetheless, PGE<sub>2</sub>, TXB<sub>2</sub>, and PA did not affect endothelium-independent vascular function, which was measured in response to SNP (Fig. 8). These results suggested that reduced levels of eicosanoids and PA were associated with improved endothelial function following pema fibrate treatment.

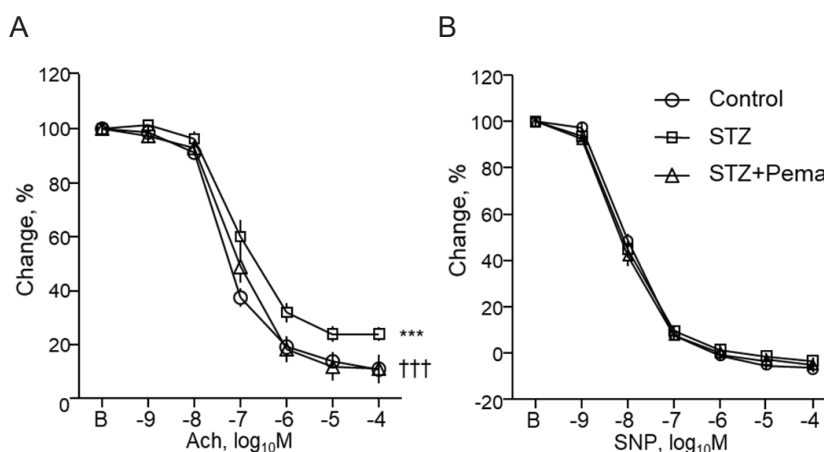
## 4. Discussion

Various pathological processes related to diabetes cause endothelial dysfunction, an initial step in atherosclerosis. Eicosanoids generated from AA have



**Fig. 6.** Pemafrate reduced saturated free fatty acids

Pemafrate reduced palmitic acid and stearic acid in diabetic mice ( $n=5$  per group). \* $P<0.05$ . All values are mean  $\pm$  SEM.



**Fig. 7.** Pemafrate attenuated endothelial dysfunction in diabetic mice

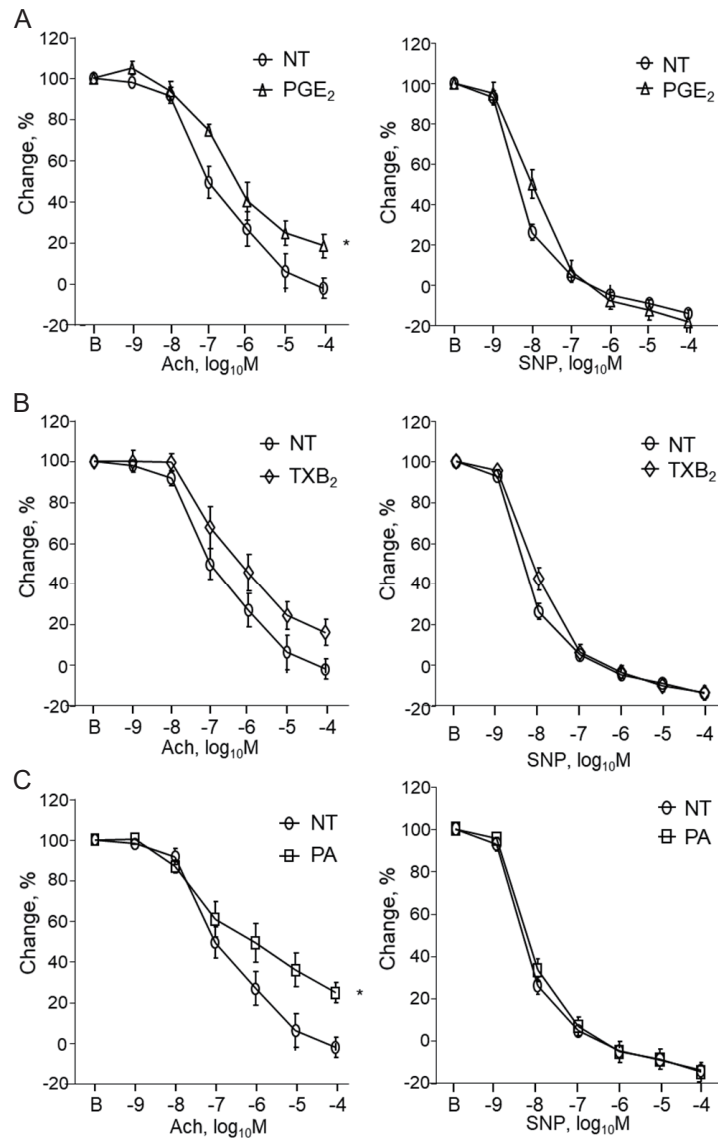
Vascular reactivity to Ach or SNP was determined using aortic rings obtained from our mice. (A) Induction of diabetes impaired endothelium-dependent vasodilation in response to Ach. Pemafrate ameliorated endothelial dysfunction, as compared with vehicle. (B) Endothelium-independent vascular response did not differ among the three groups ( $n=13-18$  per group). \*\*\* $P<0.001$  vs. control, ††† $P<0.001$  vs. STZ + vehicle. All values are mean  $\pm$  SEM.

attracted much interest as regulators of vascular homeostasis. In this study, we examined the effects of pemafrate, a newly developed SPPARM $\alpha$ , on the plasma levels of AA-derived eicosanoids in STZ-induced diabetic mice. Induction of diabetes increased plasma levels of TXB<sub>2</sub>, PGE<sub>2</sub>, LTB<sub>4</sub>, and 5-HETE, all of which potentially impair endothelial function; pemafrate, however, reduced these levels. In addition, pemafrate reduced plasma saturated free fatty acid levels in diabetic mice. We also found that pemafrate ameliorated endothelial dysfunction in diabetic mice. The results of our present study suggested that a reduction in the levels of vasoconstrictive eicosanoids and saturated fatty acids was associated with an improvement in the endothelial function of pemafrate-treated diabetic mice.

In addition to ameliorating dyslipidemia, fibrates exert various beneficial effects such as anti-

inflammatory, antioxidative, and antiapoptotic effects through activating PPAR $\alpha$ <sup>15</sup>). Therefore, previous studies have suggested that PPAR $\alpha$  agonists have vascular protective effects. Besides, several studies have demonstrated that fibrates attenuate the development of vascular complications (e.g., endothelial dysfunction, retinopathy, and nephropathy) in a diabetic condition<sup>12-16</sup>). Endothelial damage interferes with vascular homeostasis and initiates pathological processes related to vascular disease<sup>36</sup>). Thus, impaired endothelial function represents an early hallmark of and a potential therapeutic target for vascular disorders<sup>37</sup>). However, the effect of pemafrate on endothelial function remains obscure. Hence, we investigated the effects of pemafrate on endothelial function in diabetic mice and explored the underlying mechanisms.

Eicosanoids derived from AA have various roles



**Fig. 8.** Eicosanoids impaired endothelial function in non-diabetic mice

(A) Incubation with PGE<sub>2</sub> significantly impaired endothelium-dependent vasodilation, as determined by the response to Ach. (B) TXB<sub>2</sub> tended to impair endothelial function although it did not reach statistical significance. (C) PA significantly impaired endothelial function. Endothelium-independent vascular function, as measured in response to SNP, did not differ across these groups ( $n=8-9$  per group). \* $P<0.05$  vs. NT (non-treatment). All values are mean  $\pm$  SEM.

in vascular regulation<sup>2-4</sup>). In this study, we found that diabetes induction increased the plasma levels of AA, TXB<sub>2</sub>, PGE<sub>2</sub>, LTB<sub>4</sub>, and 5-HETE, whereas pemafibrate decreased these levels. The COX2 pathway produces TXB<sub>2</sub>, which is a metabolite of TXA<sub>2</sub>, and PGE<sub>2</sub> in the AA cascade. TXA<sub>2</sub> is one of the major vasoconstrictive eicosanoids. A previous study showed that TXA<sub>2</sub> plays a pivotal role in the development of endothelial dysfunction in diabetic mice<sup>38</sup>). In addition, TXA<sub>2</sub> stimulates platelet activation and leukocyte-endothelial cell interaction,

thereby contributing to the initiation and progression of atherosclerosis<sup>39</sup>). PGE<sub>2</sub>, too, performs a variety of roles in vascular regulation. It contributes to the production of cytokines and chemokines and induces leukocyte infiltration through an EP receptor. On the other hand, several studies have demonstrated that PGE<sub>2</sub> induces vasodilatation, resulting in increased local blood flow and vascular hyperpermeability<sup>40</sup>). Thus, PGE<sub>2</sub> may have distinct effects on vascular regulation<sup>41</sup>); however, previous studies have reported that PGE<sub>2</sub> promotes diabetic retinopathy in rats<sup>10</sup>). In



our present study, pemafibrate reduced PGE<sub>2</sub> levels, which was associated with the improvement of endothelial function. Besides, the results of our ex vivo experiment revealed that PGE<sub>2</sub> significantly impaired endothelial function in the aortic rings. These findings indicate that PGE<sub>2</sub> has vasoconstrictive effects, at least in our experimental conditions. On the contrary, 15d-PGJ<sub>2</sub> plasma levels, which declined following diabetes induction, were not affected by pemafibrate treatment. 15d-PGJ<sub>2</sub> is a member of the PGJ<sub>2</sub> family and is known as an endogenous PPAR $\gamma$  agonist<sup>42</sup>). Previous studies have proven that PGJ<sub>2</sub> family members have protective effects against vascular disease and may therefore be incorporated into preventive and therapeutic strategies for these conditions<sup>43-46</sup>). Both LTB<sub>4</sub> and 5-HETE, which are generated from AA by the 5-LOX pathway, stimulate inflammatory responses by activating neutrophils<sup>47</sup>). In addition, their effects on endothelial cell function have been documented in numerous studies<sup>48</sup>). Previous studies comparing patients with and without diabetes showed an association between diabetes and marked upregulation of 5-LOX activity as well as increased levels of 5-HETE and LTB<sub>4</sub><sup>11</sup>). Furthermore, recent studies have suggested that 5-HETE and LTB<sub>4</sub> can be used as biomarkers of diabetic vascular complications<sup>49, 50</sup>), with the latter also reported to be associated with the onset of acute coronary syndrome<sup>51</sup>).

Several papers have shown that STZ injection increases the levels of AA and eicosanoids such as PGE<sub>2</sub> and TXA<sub>2</sub>. Diabetes-induced activation of the protein kinase C pathway stimulates phospholipase A<sub>2</sub>, hence promoting the release of AA<sup>52</sup>). In fact, in this study, induction of diabetes by STZ injection increased AA levels, which were subsequently reduced by pemafibrate in line with the reduction in triglyceride levels. Since triglycerides serve as a source of AA, it can be postulated that the reduction in their levels might have been associated with the decrease in AA<sup>53, 54</sup>). In the current study, pemafibrate also lowered the levels of AA-derived eicosanoids in line with the reduction in triglyceride levels. These results suggest that triglyceride reduction by pemafibrate may, at least partially, have contributed to the decreased levels of AA and its eicosanoid metabolites. In addition, pemafibrate reduced the expression of thromboxane synthase and PGE synthase, which might have affected eicosanoid levels. Considering these data, various mechanisms were probably involved in the reduction of eicosanoids by pemafibrate treatment. Additionally, the results of our ex vivo experiment demonstrated that PGE<sub>2</sub> and TXB<sub>2</sub> impaired endothelial function. These findings led us to consider post-treatment decreases in the

levels of vasoconstrictive eicosanoids, which were elevated following diabetes induction, as one of the possible underlying mechanisms responsible for the amelioration of endothelial function; however, further research is warranted to elucidate underlying mechanisms.

We also observed that pemafibrate decreased the levels of saturated free fatty acids, such as PA and stearic acid, in diabetic mice. In this study, diabetes induction led to a relatively modest increase in PA and stearic acid levels; however, previous studies have reported that elevated plasma free fatty acid concentrations are associated with endothelial dysfunction in diabetic and non-diabetic subjects<sup>55</sup>). In fact, a clinical study reported diminished plasma fatty acid levels after pemafibrate treatment<sup>56</sup>), and the results of the present study demonstrated the impairment of endothelial function by PA. The reduction in these saturated fatty acid levels after treatment with pemafibrate might also have played a role in the improvement of endothelial function in our mice.

One of the major limitations of the present study was that we used STZ to induce diabetes. Thus, it is unclear whether our results can be generalized to type 2 diabetes, which represents the most common type of diabetes in humans. Second, the levels of lipids, including triglycerides, in STZ-induced diabetic mice were higher than those in non-diabetic mice. Other studies have shown elevated lipid levels in STZ-induced diabetic mice<sup>34, 35</sup>). Decreased peripheral lipolysis and/or increased hepatic lipogenesis are suggested as the underlying mechanisms. The activation of PPAR $\alpha$  reduced triglyceride levels through various mechanisms, including the promotion of LPL activity<sup>57</sup>). However, STZ-induced diabetic mice have some unique lipid metabolisms. Therefore, further research is required to clarify the mechanism by which pemafibrate reduced triglyceride levels in this study. Moreover, it is recommended that additional studies using different animal models be conducted to confirm the effects of pemafibrate on AA-derived eicosanoids and endothelial function, especially in a diabetic condition.

In summary, pemafibrate decreased the levels of vasoconstrictive eicosanoids, which were elevated following diabetes induction. This might be associated with the amelioration of endothelial dysfunction in pemafibrate-treated diabetic mice. The results of our study suggest that pemafibrate may prevent cardiovascular complications in patients with diabetes mellitus.

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## Competing Interests

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