Fenofibrate promotes ischemia-induced revascularization through the adiponectin-dependent pathway

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Fenofibrate is a peroxisome proliferator–activated receptor-α (PPARα) ligand that is widely used for the treatment of dyslipidemia (3). The clinical trial in the FIELD (The Fenofibrate Intervention and Event Lowering in Diabetes) study demonstrated that fenofibrate reduces cardiovascular events in patients with type 2 diabetes (15). Recent clinical trials have indicated that, in addition to its lipid-lowering effect, fenofibrate has beneficial effects on the vascular wall, including improvement of endothelial function and revascularization process in the mouse model of hindlimb ischemia. Treatment with fenofibrate led to acceleration of revascularization of ischemic hindlimb relative to the contralateral limb in wild-type (WT) mice, as measured by laser Doppler blood flow and capillary density analyses. Treatment of WT mice with fenofibrate increased the serum levels of adiponectin, which has protective actions on the vasculature. Of importance, fenofibrate had no effects on the revascularization in ischemic limbs of adiponectin-deficient (APN-KO) mice. Fenofibrate stimulated the phosphorylation of AMPK and eNOS in the ischemic muscles in WT mice but not in APN-KO mice. AMPK inhibitor compound C suppressed fenofibrate-induced increase in limb perfusion and AMPK phosphorylation in ischemic muscle in WT mice without affecting adiponectin levels. NOS inhibitor L-NAME also blocked the increased blood flow of ischemic limbs in fenofibrate-treated WT mice. Our observations suggest that fenofibrate could promote revascularization in response to ischemia through adiponectin-dependent AMPK signaling.

MATERIALS AND METHODS

Animals and experimental protocol. Male adiponectin-deficient (APN-KO) mice and their wild-type (WT) littermates in a C57BL/6J background at the age of 8–10 wk were used in this study. The study protocol was approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. We used a mouse model of revascularization, in which the entire left femoral artery and vein were removed surgically, as described previously (22, 33). WT and APN-KO mice were randomly divided into two groups and were treated with or without fenofibrate as food admixture at a concentration of 0.1% (11). One week later, mice were subjected to unilateral hindlimb ischemia. In some experiments, AMPK inhibitor compound C (20 mg/kg, 3 times/wk) dissolved in dimethyl sulfoxide (DMSO) or vehicle (DMSO) was injected intraperitoneally into the abdomen of WT mice 1 day before the operation until euthanization, as described previously (18, 21, 35). In some experiments, we intraperitoneally injected NOS inhibitor L-NAME (20 mg·kg−1·day−1)

Endothelial nitric oxide synthase (eNOS) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-eNOS (Ser1177), phospho-AMP-activated protein kinase (AMPK; Thr172), and pan-AMPKα antibodies were purchased from Cell Signaling Technology (Beverly, MA). GAPDH antibody was purchased from Biogenes. Compound C was purchased from Calbiochem, Merck KGaA (Darmstadt, Germany). N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Sigma Chemical (St. Louis, MO). Fenofibrate was provided as a generous gift by Kaken Pharmaceutical (Tokyo, Japan).

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dissolved in PBS or vehicle (PBS) into WT mice from 1 day prior to surgery for 3 or 14 consecutive days, as described previously (18, 19).

Laser Doppler blood flow analysis. We measured hindlimb blood flow using a laser Doppler blood flowmetry (LDBF; MoorLDI, Moor Instrument, Devon, UK), as described previously (22, 33). Before and on postoperative days 0, 3, 7, 14, 21, and 28, we performed LDBF analysis over the legs and feet. After scanning, storage images were analyzed to quantify blood flow, and mean LDBF values of the ischemic and nonischemic limbs were calculated. To avoid data variations because of ambient light and temperature, hindlimb blood flow was expressed as the ratio of the left (ischemic) to right (nonischemic) hindlimb LDBF.

Capillary density analysis. Capillary density in adductor muscle was analyzed to obtain specific evidence of vascularity at the level of microcirculation. Tissue samples were obtained from the ischemic thigh adductor skeletal muscles on postoperative day 28. Frozen tissue sections with 5-µm thickness were prepared from each sample. Capillary endothelial cells were identified by immunohistochemical staining with CD31 monoclonal antibody (BD Biosciences). Fifteen random microscopic fields from two different sections in each tissue block were examined for the presence of capillary endothelial cells, and capillary muscle fiber ratio was expressed as the ratio of number of capillaries to the number of myobers per high-power field (×400).

Measurement of plasma parameters. Blood samples were obtained in WT mice treated with or without fenofibrate for 28 days. Total cholesterol (TC) and triglyceride (TG) levels were measured with enzymatic kits (Wako Chemicals). Adiponectin levels were determined using ELISA kits (Otsuka Pharmaceutical, Tokyo, Japan).

Western blot analysis. Tissue samples obtained on postoperative day 3 were homogenized in lysis buffer containing 20 mM Tris·HCl (pH 8.0), 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma Chemical). Protein content was determined by the Bradford method. The same amounts of protein (60 µg) were separated with denaturing SDS 10% polyacrylamide gels. The membranes were immunoblotted with the primary antibodies at a 1:1,000 dilution followed by secondary antibody at a 1:5,000 dilution. Bands were visualized using an ECL Western blotting detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

Statistical analysis. Data are presented as means ± SE. All of the data were subjected to one-way ANOVA followed by Scheff’s analysis. P values <0.05 were considered to be statistically significant.

RESULTS

Fenofibrate promotes blood flow recovery and capillary formation in ischemic tissue. To assess the effect of fenofibrate on the revascularization process in response to ischemia, WT mice treated with or without fenofibrate were subjected to unilateral hindlimb ischemia. All mice survived after surgery and appeared healthy during the followup period. Figure 1A shows representative LDBF images of hindlimb blood flow before surgery and at different time points after surgery. Blood flow recovery in ischemic hindlimb appeared to be accelerated in the fenofibrate-treated WT mice compared with untreated mice. Quantitative analysis of hindlimb perfusion showed that treatment with fenofibrate significantly increased the limb flow of ischemic muscle in WT mice on postoperative days 3, 7, 14, 21, and 28 (Fig. 1B).

To assess the extent of revascularization at the microcirculatory level, we measured capillary density in a histological section harvested from the ischemic muscle. Figure 1C shows representative photomicrographs of muscle tissue stained with endothelial cell marker CD31. Quantitative analysis of CD31-positive cells revealed that, on postoperative day 28, the capillary density in the ischemic hindlimb was significantly greater in WT mice treated with fenofibrate than in nontreated WT mice (Fig. 1D).

Effect of fenofibrate on adiponectin level in WT mice. Treatment of WT mice with fenofibrate for 2 wk has been reported to increase circulating adiponectin (11). Therefore, we assessed serum adiponectin levels in WT mice treated with or without fenofibrate for 28 days. Treatment with fenofibrate led to a 1.6-fold increase in serum adiponectin levels in WT mice (Table 1). There was no significant difference in body weight between the two groups. Consistent with previous reports (10, 36), there was a significant reduction in TG and a significant increase in TC in WT mice following treatment with fenofibrate (Table 1).

Adiponectin is required for fenofibrate-regulated revascularization. To analyze the potential involvement of adiponectin in fenofibrate-mediated improvement of vascular response, we examined the impact of fenofibrate treatment on revascularization of ischemic muscles in APN-KO mice. APN-KO mice had a reduced blood flow of ischemic limbs, in agreement with our previous works (18, 33). Treatment with fenofibrate did not affect ischemic limb perfusion of APN-KO mice (Fig. 1, A and B). Fenofibrate also had no effect on the capillary density of ischemic muscle in APN-KO mice (Fig. 1, C and D). Collectively, these data suggest that the effect of fenofibrate on ischemia-induced revascularization is dependent on its ability to upregulate adiponectin.

Effect of fenofibrate on AMPK and eNOS activation in muscle. eNOS is an important regulator of revascularization in response to tissue ischemia (22). To examine the participation of eNOS in fenofibrate-mediated improvement of ischemia-induced revascularization, the expression and phosphorylation of eNOS in ischemic adductor muscle on day 3 after surgery were assessed by Western blot analysis. Although the expression of total eNOS protein in ischemic muscles did not differ between fenofibrate-treated and nontreated WT mice, the phosphorylation of eNOS at Ser1177 in ischemic muscle was significantly greater in fenofibrate-treated WT mice than in nontreated WT mice (Fig. 2A). APN-KO mice exhibited reduced phosphorylation of eNOS, and the stimulatory effects of fenofibrate on eNOS phosphorylation were abolished in APN-KO mice (Fig. 2A).

AMPK is reported to directly phosphorylate eNOS at Ser1177 (8). It has also been shown that adiponectin stimulates eNOS phosphorylation through its ability to activate AMPK (25). To examine the involvement of AMPK in revascularization induced by fenofibrate treatment, the expression and phosphorylation of AMPK in ischemic adductor muscle were assessed by Western blot analysis. Fenofibrate significantly stimulated the phosphorylation of AMPK in ischemic muscle in WT mice (Fig. 2B). Decreased phosphorylation of AMPK in ischemic muscle was observed in APN-KO mice, and treatment of APN-KO mice with fenofibrate did not lead to appreciable changes in AMPK phosphorylation (Fig. 2B).

Role of AMPK/eNOS signaling in regulation of fenofibrate-induced revascularization. To further analyze the involvement of the AMPK/eNOS regulatory axis in enhancement of revascularization by fenofibrate, we examined the effect of the AMPK inhibitor C or the NOS inhibitor l-NAME on blood flow recovery of ischemic
Fig. 1. Fenofibrate promotes revascularization following hindlimb ischemia. A: representative images of laser Doppler blood flowmetry (LDBF) for both wild-type (WT) and adiponectin-deficient (APN-KO) mice treated with or without fenofibrate before surgery and at different time points after surgery. A low-perfusion signal (dark blue) was observed in the ischemic hindlimb of WT and APN-KO mice, whereas a high-perfusion signal (white to red) was detected in WT mice treated with fenofibrate on postoperative days 3, 7, 14, 21, and 28. B: quantitative analysis of ischemic/normal LDBF ratio in WT mice (n = 10 in each group) or APN-KO mice (n = 5 in each group) treated with or without fenofibrate (*P < 0.05, **P < 0.01 vs. WT mice). C: immunostaining of ischemic tissues with anti-CD31 monoclonal antibody (brown) on postoperative day 28 (×400). D: quantitative analysis of capillary density in WT (n = 10 in each group) and APN-KO mice (n = 5 in each group) treated with or without fenofibrate.

muscles in WT mice receiving fenofibrate. Treatment of WT mice with compound C blocked increased limb perfusion caused by fenofibrate (Fig. 3A). Compound C treatment did not change increased circulating adiponectin levels by fenofibrate (Table 1). Similarly, l-NAME suppressed the fenofibrate-mediated increase in limb blood flow of WT mice without altering adiponectin levels (Fig. 3A and Table 1). The fenofibrate-induced increase in AMPK phosphorylation in ischemic muscle was diminished by compound C but not by l-NAME (Fig. 3B). Collectively, these data suggest that the AMPK/eNOS signaling axis is involved in fenofibrate-induced enhancement of revascularization.
DISCUSSION

The present study demonstrates that systemic administration of fenofibrate stimulates revascularization in response to ischemia in a mouse model of vascular insufficiency. Treatment of WT mice with fenofibrate resulted in a more rapid recovery of limb perfusion and enhanced capillary density compared with untreated mice, which was accompanied by an increase in circulating adiponectin. The beneficial actions of fenofibrate on revascularization were abrogated in APN-KO mice. Adiponectin has been shown to promote ischemia-induced revascularization in muscle in a model of hindlimb ischemia (18, 33). Therefore, fenofibrate exerts the beneficial actions on revascularization under conditions of ischemia through upregulation of adiponectin.

The beneficial actions of adiponectin on vascular cells are mediated, at least in part, by its ability to activate AMPK signaling (9, 16, 25). It has been shown that adiponectin stimulates phosphorylation of eNOS in endothelial cells and promotes endothelial function through the AMPK pathway (7, 25). We have reported previously that adiponectin-mediated activation of the AMPK/eNOS pathway enhances angiogenic repairs of ischemic muscle in vivo (18, 33). Our observations from experiments with chemical inhibitors show that fenofibrate stimulated blood flow recovery following ischemia

Table 1. Characteristics of WT and APN-KO mice

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<th>WT</th>
<th>APN-KO</th>
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<tr>
<td></td>
<td>Control (n = 10)</td>
<td>Feno (n = 10)</td>
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<tr>
<td>BW, g</td>
<td>24.6 ± 0.4</td>
<td>23.9 ± 0.2</td>
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<tr>
<td>TG, mg/dl</td>
<td>79.2 ± 9.8</td>
<td>20.6 ± 4.5**</td>
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<tr>
<td>TC, mg/dl</td>
<td>54.5 ± 1.4</td>
<td>82.6 ± 3.5</td>
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<tr>
<td>Adiponectin, μg/ml</td>
<td>9.5 ± 0.6</td>
<td>15.3 ± 0.5**</td>
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Results are presented as means ± SE. WT, wild-type mice; APN-KO, adiponectin-deficient knockout mice; Feno, fenofibrate; L-NAME, N^G^-nitro-L-arginine methyl ester; BW, body weight; TG, triglyceride; TC, total cholesterol. Blood samples were obtained from WT mice and APN-KO mice treated with or without Feno for 28 days. **P < 0.01 vs. WT control; *P < 0.05 vs. WT control; †P < 0.05 vs. APN-KO control.

Fig. 2. Effect of fenofibrate on phosphorylation of AMP-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS) in ischemic muscle in WT and APN-KO mice. The representative immunoblots and quantitative analysis of relative changes in phosphorylated eNOS and AMPK, respectively. Phosphorylation of eNOS and AMPK was normalized to the GAPDH signal and expressed as percentage of the signal intensity of untreated WT mice (n = 5).
through the AMPK/eNOS-dependent pathway. Our findings also show the fenofibrate-mediated increase in perfusion recovery, and AMPK/eNOS activation in ischemic muscle is dependent on the ability of this reagent to upregulate adiponectin. These findings suggest that the increased production of adiponectin by fenofibrate exerts a protective action on the process of revascularization under conditions of ischemia via an AMPK/eNOS regulatory signaling.

The in vivo findings to elucidate the role of fenofibrate in regulating angiogenesis provide conflicting results. In agreement with our data, fenofibrate treatment leads to an increase in revascularization in ischemic hindlimbs (14). Likewise, another PPARα agonist, WY-14643, promotes neovascular growth in a model of angiogenesis in the mouse cornea (5). In contrast, it has been reported that fenofibrate inhibits tumor growth in vivo by suppression of neovascularization (27). The possible reason for this discrepancy is the differences in utilized assay systems for angiogenesis. It is also possible that fenofibrate differentially regulates pathological and physiological angiogenesis, as has been proposed for the effects of statins on vascularization (32). Similarly, adiponectin enhances angiogenic responses in mouse ischemic hindlimb and rabbit cornea angiogenesis assays (18, 25, 33), whereas adiponectin suppresses tumor angiogenesis in vivo (6, 20). Of importance, it is established that activation of AMPK/eNOS signaling confers a proangiogenic phenotype in ischemic hindlimb (18, 22, 23, 26, 33). Taken together, these observations suggest that the induction of AMPK/eNOS signaling by fenofibrate therapy can facilitate revascularization response during muscle ischemia.

Obesity-related complications such as type 2 diabetes are closely associated with microvascular rarefaction and reduced collateralization in ischemic tissues (1, 39, 40). These circulatory changes can accelerate vulnerability to ischemic injury and impair wound healing, ultimately leading to an occurrence of lower limb amputation. The FIELD study showed that fenofibrate therapy is associated with lower risk of limb amputations in patients with type 2 diabetes (28). In the present study, fenofibrate could promote angiogenic repair in ischemic limbs by its ability to upregulate the vascular protective adipocytokine adiponectin. These results may provide important basic data explaining the efficacy of the FIELD study.

ACKNOWLEDGMENTS

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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