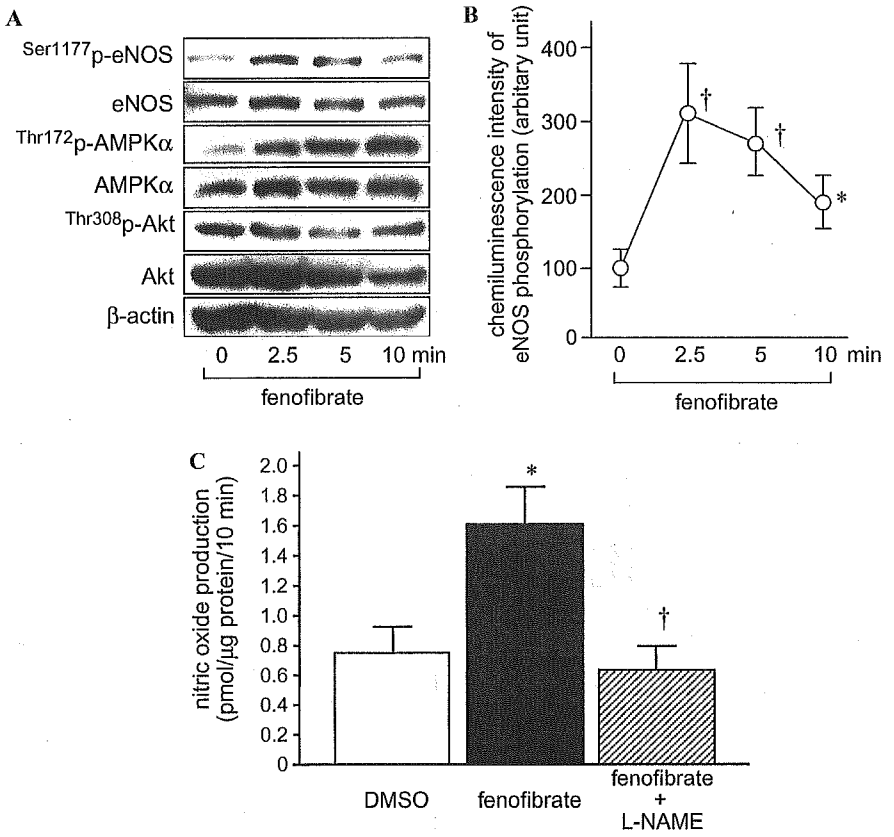

Fig. 1. Fenofibrate increased phosphorylation of eNOS and NO production in HUVEC. (A) HUVEC were incubated with 30 μM fenofibrate for indicated duration. The lysates were analyzed by SDS–PAGE and blotted with specific antibodies for anti-phosphorylated eNOS (Ser-1177), phosphorylated AMPKα (Thr-172), phosphorylated Akt (Thr-308), and β-actin antibodies. The membranes were re-blotted for anti-eNOS, AMPKα, and Akt antibodies. Representative blots were shown. (B) The chemiluminescence intensity on the phosphorylated eNOS from four independent experiments was quantified and shown. The values of phosphorylated eNOS were corrected by those of re-blotted eNOS. The results are represented as mean values ± SD (*$p < 0.05$ vs. control value, †$p < 0.01$ vs. control value). (C) Nitric oxide productions of HUVEC from six independent experiments were assessed using DAF-2/DA as written in Materials and methods. Quantitative results corrected by protein concentrations were shown. The results are represented as mean values ± SEM (*$p < 0.05$ vs. vehicle group, †$p < 0.05$ vs. fenofibrate group).

dose-dependent manner (Figs. 2A and B). In parallel with the increase in phosphorylated AMPK, the phosphorylation of a well-characterized substrate of AMPK, namely ACC, was significantly increased (Figs. 2A and B). Fenofibrate at a concentration of 25 μM, which is within the clinical treatment range, was sufficient to increase AMPK and ACC phosphorylation. Although baseline phosphorylation of AMPK gradually increased with increasing duration of the serum starvation, the significant difference between vehicle and fenofibrate treatment was maintained for at least 6 h after treatment (Figs. 2C and D). Fenofibrate did not affect the total AMPK protein expression over this period of time.

The effects of protein kinase inhibitors on fenofibrate-induced eNOS phosphorylation

The phosphorylation of eNOS has been shown to be regulated by various kinases, such as protein kinase A (PKA), protein kinase B (PKB, also known as Akt), and AMPK [9]. For the purpose of investigating whether fenofibrate-induced eNOS phosphorylation involves either the PKA or Akt pathway, we examined the effects of inhibitors of these kinases. As shown in Fig. 3, a PKA inhibitor (H89) failed to suppress fenofibrate-induced eNOS phosphorylation at 2.5 and 5 min. As for Akt, we confirmed in a preliminary study that a phosphatidylinositol-3-kinase (PI3K) inhibitor (wortmannin) effectively decreased phosphorylation of Akt even with fenofibrate (data not shown). We therefore examined the effect of wortmannin as a substitute for an Akt inhibitor. Wortmannin decreased the basal level of eNOS phosphorylation. However, co-administration of fenofibrate and wortmannin increased eNOS phosphorylation (Fig. 3).

The activation of AMPK by fenofibrate is independent of PPARα activity

To elucidate whether fenofibrate activates AMPK via transcriptional activation of PPARα, we investigated the effects of other PPARα agonists on AMPK activity. Neither bezafibrate nor WY-14643 increased AMPK and

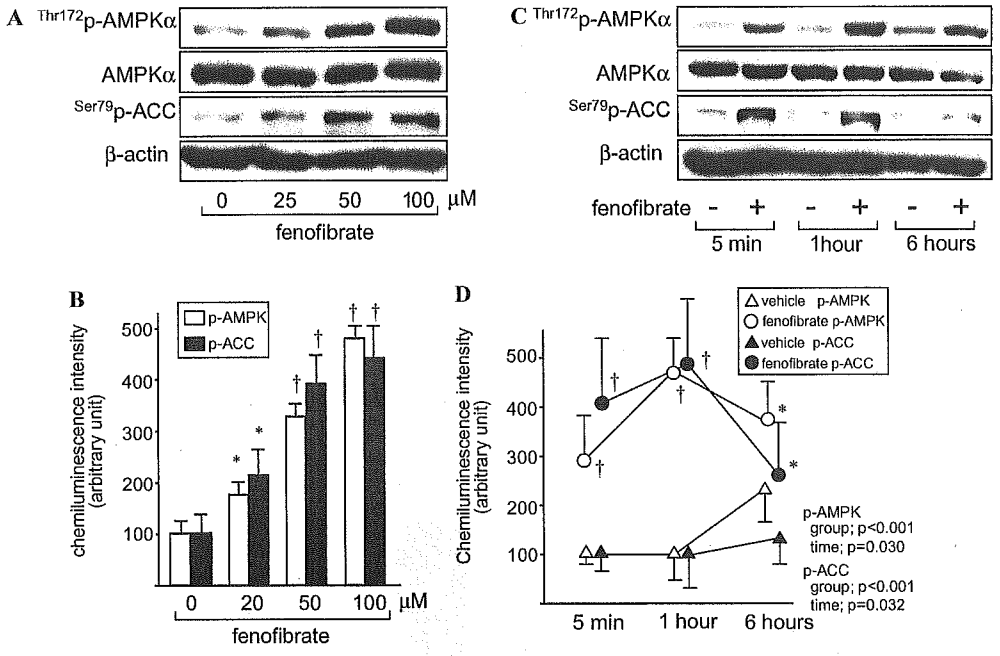

Fig. 2. Fenofibrate increased the phosphorylation of AMPK and ACC in HUVEC. (A) HUVEC were incubated with indicated concentration of fenofibrate for 30 min and immunoblotted for anti-phosphorylated AMPKα (Thr-172), phosphorylated ACC (Ser-79), and β-actin antibodies. The membranes were re-blotted for anti-AMPKα antibody. Representative blots were shown. (B) The chemiluminescence intensity for phosphorylated AMPK (open columns) and ACC (closed columns) from four independent experiments was quantified and shown. The values of phosphorylated AMPK and phosphorylated ACC were corrected by those of re-blotted AMPKα and β-actin, respectively. The results are represented as mean values ± SD (*$p < 0.05$ vs. vehicle, †$p < 0.01$ vs. vehicle). (C) HUVEC were incubated with 30 μM fenofibrate for indicated duration and immunoblotted. Representative blots were shown. (D) The chemiluminescence intensity for phosphorylated AMPK (open marks) and ACC (closed marks) from four independent experiments was quantified and shown. The values of vehicle (triangle) and fenofibrate (circle) treatment are shown, respectively. The results are represented as mean values ± SD (*$p < 0.05$ vs. vehicle, †$p < 0.01$ vs. vehicle).

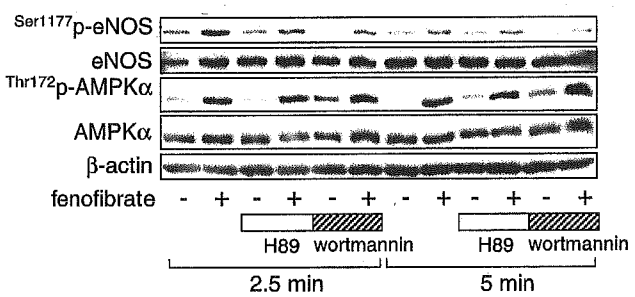

Fig. 3. Effects of protein kinase inhibitors on fenofibrate-induced phosphorylation of eNOS. HUVEC were pre-treated with 1 μM H-89 or 1 μM wortmannin for 30 min, and then cells were incubated with 30 μM fenofibrate for indicated duration and immunoblotted for anti-phosphorylated eNOS, phosphorylated AMPKα, and β-actin antibodies. The membranes were re-blotted for anti-eNOS and AMPKα antibodies, respectively. Representative blots from three independent experiments are shown.

ACC phosphorylation (Fig. 4A). We further investigated the effects of both RNA polymerase inhibitor and translational inhibitor on fenofibrate-induced AMPK phosphorylation. Neither actinomycin D nor cycloheximide inhibited the fenofibrate-induced AMPK phosphorylation (Fig. 4B). These results indicated that fenofibrate-induced AMPK activation is independent of PPARα activity.

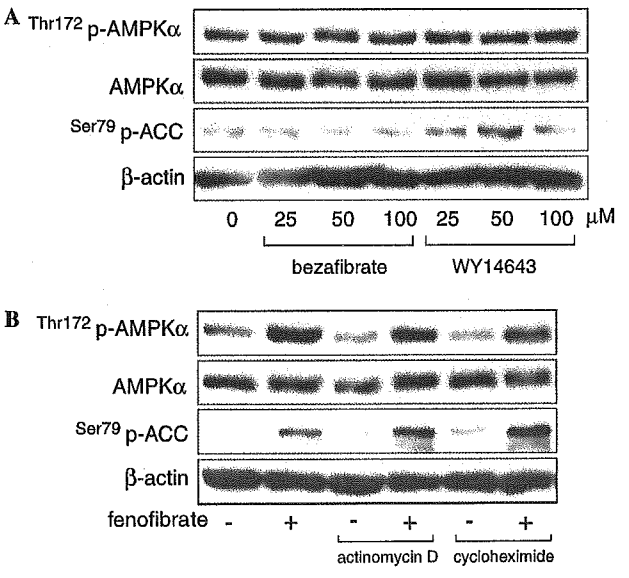

Fig. 4. (A) Effects of other PPARα agonists on AMPK activity. HUVEC were incubated with bezafibrate or WY-14643 at different concentrations for 30 min and immunoblotted for each antibody. Representative blots of three independent experiments are shown. (B) Effects of RNA polymerase or translational inhibitor on fenofibrate-induced phosphorylation of AMPK. After pre-treatment with 1 μg/mL actinomycin D or 10 μg/mL cycloheximide for 45 min, HUVEC were incubated with 30 μM fenofibrate for 30 min and immunoblotted for each antibody. Representative blots of three independent experiments are shown.

Discussion

In the present study, we demonstrated for the first time that fenofibrate can activate AMPK by increasing AMPK phosphorylation in HUVEC. Although we did not perform a strict kinase assay of AMPK, we are certain that fenofibrate indeed activates this kinase for following reasons. (1) The extent of AMPK phosphorylation at Thr-172 strongly reflects its activity [12]. (2) Fenofibrate indeed increased phosphorylation of its consensus substrate, ACC, at Ser-79. The ability of fenofibrate to activate AMPK is an attractive characteristic, because AMPK has been shown to mediate beneficial and bio-protective effects of several drugs and adipocytokines, such as metformin [18], thiazolidine derivatives [19], adiponectin [20], and leptin [21]. In this sense, our finding of fenofibrate-induced AMPK activation should be of significant value.

In regard to the effects of fenofibrate on eNOS, it has previously been reported that fenofibrate increases eNOS expression in endothelial cells [7], whereas the present study is the first to report fenofibrate-induced phosphorylation of eNOS. We consider AMPK to be a direct upstream kinase of fenofibrate-induced eNOS phosphorylation for the following reasons. (1) Pharmacological inhibition of PKA and PI3K, an upstream kinase of Akt, failed to decrease fenofibrate-induced eNOS phosphorylation. (2) Ser-1177 of eNOS is known to be the consensus phosphorylation site of AMPK [10], and thus AMPK is the most feasible among the upstream kinases of eNOS, except for PKA and Akt. (3) Fenofibrate failed to enhance Akt phosphorylation (Fig. 1A).

Fenofibrate has been reported to improve the progression of coronary artery disease and endothelial function [2–4], although the underlying mechanism is not fully understood. The ability of fenofibrate to correct lipoprotein abnormalities is, of course, an important characteristic for the prevention of atherosclerosis. In addition, fenofibrate has been demonstrated to inhibit the tumor necrosis factor-α-induced expression of interleukin-6 and vascular cell adhesion molecule-1 [5,6], which plays an important role in mediating mononuclear leukocyte-selective adhesion to the vascular endothelium and the progression of atherogenesis. The present study showed that fenofibrate could stimulate the production of endothelium-derived NO in HUVEC. This finding agrees with an earlier report that fenofibrate upregulates eNOS activity as estimated by the conversion of radiolabeled L-arginine to L-citrulline [7]. This stimulation of NO production may also contribute to the beneficial effects of fenofibrate on endothelial function. We speculate that the ability of fenofibrate to phosphorylate eNOS may also further enhance NO production in the vascular endothelium.

The mechanisms by which fenofibrate activates AMPK are still unknown. A direct or indirect action of PPARα could be among the possible explanations. However, we infer that the effect of fenofibrate on AMPK is independent of PPARα activity from the following facts. (1) Two other PPARα agonists, bezafibrate and WY-14643, had no effect on AMPK activity. So this effect would seem to be a unique characteristic of fenofibrate. (2) Pharmacological inhibition of both transcription and translation failed to prevent fenofibrate-induced AMPK activation, and therefore transcriptional activation of PPARα was not indispensable to this reaction. Although an investigation targeting selective deletion or silencing of the PPARα gene might be required in order to strictly negate the participation of PPARα on fenofibrate-induced AMPK activation, we performed all the necessary experiments in vitro, so that we were able to exclude at least secondary effects of PPARα, such as reduction of circulating lipids.

Another conceivable explanation is that fenofibrate activates AMPK by interfering with mitochondrial function. Fenofibrate has been reported to impair the mitochondrial function by inhibition of respiratory complex I [22]. This inhibitory effect of fenofibrate may have reduced mitochondrial ATP generation, leading to fenofibrate-induced AMPK activation, because AMPK is known to be activated by any stress that depletes cellular ATP and increases AMP/ATP ratio, such as metabolic poisoning, hypoxia, exercise, or nutrient deprivation in mammalian cells [12]. However, we confirmed that fenofibrate increased AMPK phosphorylation but did not increase the intracellular AMP/ATP ratio in C2C12 myoblasts (unpublished observations). Finally, we know that AMPK is phosphorylated by upstream kinases such as LKB1 [23] or calcium/calmodulin-dependent protein kinase kinase [24]. Further experiments, which include investigations of alteration of these kinase activities by fenofibrate, will be needed to elucidate the precise pathway of AMPK activation by fenofibrate.

In conclusion, we demonstrated that fenofibrate activates AMPK in endothelial cells, leading to increases in eNOS phosphorylation and NO production. These findings suggest the possibility that the beneficial effects of fenofibrate on endothelial function and atherosclerosis result from not only lipid-lowering and anti-inflammatory actions but also NO production through activation of the AMPK pathway.

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