Peroxisome Proliferator-Activated Receptor α Agonists Increase Nitric Oxide Synthase Expression in Vascular Endothelial Cells

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Objective—There has been accumulating evidence demonstrating that activators for peroxisome proliferator-activated receptor α (PPARα) have antiinflammatory, antiatherogenic, and vasodilatory effects. We hypothesized that PPARα activators can modulate endothelial nitric oxide synthase (eNOS) expression and its activity in cultured vascular endothelial cells.

Methods and Results—Bovine aortic endothelial cells were treated with the PPARα activator fenofibrate. The amount of eNOS activity and the expression of eNOS protein and its mRNA were determined. Our data show that treatment with fenofibrate for 48 hours resulted in an increase in eNOS activity. Fenofibrate failed to increase eNOS activity within 1 hour. Fenofibrate also increased eNOS protein as well as its mRNA levels. RU486, which has been shown to antagonize PPARα action, inhibited the fenofibrate-induced upregulation of eNOS protein expression. WY14643 and bezafibrate also increased eNOS protein levels, whereas rosiglitazone did not. Transient transfection experiments using human eNOS promoter construct showed that fenofibrate failed to enhance eNOS promoter activity. Actinomycin D studies demonstrated that the half-life of eNOS mRNA increased with fenofibrate treatment.

Conclusions—PPARα activators upregulate eNOS expression, mainly through mechanisms of stabilizing eNOS mRNA. This is a new observation to explain one of the mechanisms of PPARα-mediated cardiovascular protection. (Arterioscler Thromb Vasc Biol. 2004;24:1-6.)

Key Words: atherosclerosis ■ endothelium ■ nitric oxide ■ vascular biology ■ vasodilatation

Hypolipidemic fibrates are pharmacological compounds that activate peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor superfamily. These fibrates have been widely used as effective drugs lowering serum triglycerides and low-density lipoprotein cholesterol and raising high-density lipoprotein cholesterol. There has been accumulating evidence showing that fibrates have favorable effects of slowing the progression of atherosclerosis and reducing the number of events of coronary heart diseases in high-risk patients. PPARα is known to be expressed in the liver, which is mainly involved in lipid and lipoprotein metabolism exerted by fibrates. In addition, recent studies have shown that PPARα is also expressed in the cardiovascular system, including heart and vascular wall component cells such as vascular endothelial, vascular smooth muscle, and monocyte cells, and performs a direct antiatherogenic and antiinflammatory action. Staels et al have shown that PPARα ligands inhibit interleukin (IL)-1-induced expression of IL-6, prostaglandin, and cyclooxygenase 2 in aortic smooth muscle cells. These authors further showed that patients receiving fenofibrate, a potent fibrate, had lower plasma C-reactive protein, fibrinogen, and IL-6 concentrations. Furthermore, it has been demonstrated that PPARα activators inhibit cytokine-induced vascular cell adhesion molecule-1 (VCAM-1), IL-6 expression, and thrombin-induced endothelin-1 expression in vascular endothelial cells, and inhibit tissue factor expression and activity in monocytes.

Recent reports have shown that PPARα agonists improve vasodilator function. Playford et al clearly demonstrate that fenofibrate significantly improved brachial artery flow-mediated dilatation, but not nitroglycerin-mediated dilatation in patients with type 2 diabetes mellitus. Capell et al have also shown that forearm blood flow in response to acetylcholine, nitroprusside, or verapamil was significantly increased after fenofibrate treatment in subjects with hypertriglyceridemia. These clinical studies indicate that endothelial-dependent vasodilation is improved by the PPARα agonist.

Endothelial nitric oxide (NO) is an important mediator of cardiovascular protection. NO is produced from L-arginine by endothelial NO synthase (eNOS), which is shown to...
possess antiinflammatory and antiatherogenic properties.\(^\text{15}\) Endothelium-derived NO is mainly responsible for postischemic flow-mediated vasodilatation of peripheral conduit arteries.\(^\text{16}\) Taken together, we speculated that PPAR\(\alpha\) agonists act directly on NO production from vascular endothelium, and we thus aimed to investigate the effects of fenofibrate on eNOS activity and its expression in cultured vascular endothelial cells.

**Methods**

**Cell Culture and Materials**

Bovine aortic endothelial cells (BAECs; Cell Systems) were grown in DMEM with 10% FBS (JRH Biosciences). Cells from 3 to 5 passages were used in the experiments. Fenofibrate was purchased from Sigma Chemical. Bezafibrate was obtained from Wako Chemical, rosiglitazone from Mitsubishi Pharmaceutical, and WY14643 and RU486 from Biomol. Mouse anti-eNOS antibody and mouse anti-\(\beta\)-actin antibody were purchased from Biomedicals, respectively. Horseradish peroxidase-conjugated sheep anti-mouse IgG antibody and \(\text{[}^{14}\text{C}\text{]}\)-arginine were from Amersham.

**eNOS Activity Assay**

BAECs were grown in the growth medium on 35-mm collagen-coated dishes until subconfluency. After the culture medium was changed to DMEM with 2% FBS for 24 hours, the cells were treated with test compounds or vehicle (0.1% dimethylsulfoxide) for the indicated periods. The cells were harvested and homogenized in PBS containing 1 \(\text{mmol/L}\) EDTA. eNOS activity was measured as the conversion of radiolabeled L-arginine to L-citrulline using NOS Quantitative Assay Kit (Bioxynct, OXIS International). Incubation in the presence of the eNOS inhibitor \(N\text{-nitro-L-arginine methyl ester} (\text{L}-\text{NAME})\) (1 \(\text{mmol/L}\)) served as negative controls.

**Western Blot Analysis**

BAECs grown on 35-mm collagen-coated dishes were treated for test compounds or vehicle in DMEM with 2% FBS for the indicated periods. The cells were scraped in lysis buffer with protease inhibitor cocktail tablet (complete Mini; Roche), 200 \(\text{mmol/L}\) Na\(_2\)VO\(_4\), 1 \(\text{mmol/L}\) dithiothreitol, and 200 \(\text{mmol/L}\) phenylmethylsulfonyl fluoride, and then sonicated briefly. After being boiled for 2 minutes, the samples were centrifuged at 15 000 rpm for 5 minutes and then analyzed by SDS-PAGE. The gels were transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) by blotting at 100 V for 2 hours. After incubation for 1 hour in blocking buffer (5% skim milk powder in TBS-T [20 mmol/L Tris, 55 mmol/L NaCl, 0.1% Tween 20]), the membranes were incubated with primary antibody overnight at 4°C. After washing with TBS-T, the membranes were incubated with secondary antibody at room temperature for 1 hour. The membranes were then washed with TBS-T, and signals were visualized by chemiluminescence (Amersham) and exposed to x-ray films (Fujifilms).

**Northern Blot Analysis**

BAECs grown on 10-cm collagen-coated dishes were treated for test compounds or vehicle in DMEM with 2% FBS for the indicated periods. Total RNA extracted by the acid guanidinium thiocyanate-phenol-chloroform method was electrophoresed in 1% agarose-5% formaldehyde gels. The gels were transferred to nylon membranes (Hybond N+, Amersham), and the membranes were hybridized at 68°C with \(^{32}\text{P}\)-labeled bovine eNOS cDNA probe or GAPDH cDNA probe using Perfect hybridization buffer (Toyobo). After the hybridization, the filters were washed two times in 2\(\times\)SSC and 0.1% SDS at 60°C for 10 minutes, followed by washing in 0.1\(\times\)SSC and 0.1% SDS at 60°C for 30 minutes. The membranes were then dried and exposed to x-ray films.

**Transcript Transfection and Luciferase Reporter Assay**

To examine the effect of test compounds on eNOS promoter activity, BAECs were transiently transfected with an eNOS reporter construct. The eNOS reporter consists of 5’-flanking region (~1600 to +26) of the human eNOS gene and firefly luciferase. BAECs on 24-well collagen-coated dishes were transfected with the reporter plasmids (1 \(\mu\)g) together with seapansy luciferase control plasmid (0.05 \(\mu\)g; Toky Beanet) using SuperFect Transfection Reagent (QIAGEN). Two hours after the transfection, the cells were treated for 24 hours with test compounds or vehicle. The cell lysates were assayed for each luciferase activity in Lumat LB9501 luminometer (Berthold System).

**eNOS mRNA Stability Assay**

Subconfluent BAECs on 10-cm collagen coated dishes were treated for the indicated periods with fenofibrate or vehicle in the presence of 2.5 \(\mu\)g/mL actinomycin D in DMEM with 2% FBS. Northern blot analyses for eNOS mRNA and GAPDH mRNA were performed, as described.

**Statistics**

All values represent mean\(\pm\)SD. The data were analyzed by ANOVA, and the Bonferroni method was used to estimate the level of significance of differences between means. \(P<0.05\) was considered statistically significant.

**Results**

**Effects of Fenofibrate on eNOS Activity**

First, we investigated whether fenofibrate alters eNOS enzymatic activity in lysates of BAECs. As shown in Figure 1A, fenofibrate treatment for 48 hours increased eNOS activity in a concentration-dependent manner: At concentrations of 10 \(\mu\)mol/L or more, fenofibrate treatment caused a significant increase in eNOS activity. There was no increase in eNOS activity by fenofibrate treatment within 1 hour, and the fenofibrate-stimulated induction of eNOS activity was observed 24 hours after stimulation (Figure 1B).

**Effects of Fenofibrate on eNOS Protein Expression**

Treatment of BAECs with fenofibrate for 48 hours demonstrated a concentration-dependent increase in eNOS protein levels as measured by Western blot analysis (Figure 2A and 2B). Densitometric analysis indicated that there was a significant increase in eNOS to \(\beta\)-actin ratios after fenofibrate treatment at concentrations of 10 and 50 \(\mu\)mol/L. The significant increase in eNOS protein levels was observed 12 hours after treatment and lasted for 48 hours (Figure 2C and 2D).

As shown in Figure 3, treatment of BAECs with 50 \(\mu\)mol/L bezafibrate as well as 50 \(\mu\)mol/L WY14643 for 48 hours also caused a significant increase in eNOS protein levels, whereas 50 \(\mu\)mol/L rosiglitazone elicited no change. The simultaneous treatment of BAECs with RU486 (10 \(\mu\)mol/L) resulted in the inhibition of fenofibrate (50 \(\mu\)mol/L)-induced increase in eNOS protein levels (Figure 4).

**Effects of Fenofibrate on eNOS mRNA Expression**

Northern blot analysis demonstrated that treatment with fenofibrate for 24 hours resulted in a concentration-dependent increase in eNOS mRNA levels (Figure 5A). Densitometric scan revealed that eNOS mRNA relative to GAPDH mRNA...
significantly increased at concentrations of 5 μmol/L or more: it reached 1.9-fold by the treatment with 50 μmol/L fenofibrate (Figure 5B). Significant increase in eNOS mRNA levels was observed after 6 hours, and subsequently there was a gradual decrease of eNOS mRNA levels (Figure 5C and 5D).

**Effects of Fenofibrate on eNOS Promoter Activity and eNOS mRNA Stability**

To investigate whether or not fenofibrate increases eNOS mRNA levels at transcription levels, we performed transient transfection assay using eNOS promoter fragment (Figure 6A). PMA, used as a positive control, increased eNOS promoter activity by 4.0-fold compared with that in unstimulated cells. Fenofibrate failed to increase eNOS promoter activity.

Next, we studied the effects of fenofibrate on eNOS mRNA stability after stopping the transcription with actinomycin D. The half-life of eNOS mRNA relative to GAPDH mRNA levels was about 24 hours in control, and it increased to about 110 hours by treatment with 50 μmol/L fenofibrate (Figure 6B).

**Discussion**

The PPARα activators fibrates are shown to slow atherosclerosis progression and reduce the number of events of coronary heart diseases.3–5 Such effects may be attributable at least in part to their effects on lipoprotein abnormalities, which are mainly exerted by PPARα expressed in the liver. Several studies9,10,17,18 have demonstrated that PPARα is also expressed in vascular endothelial cells. Recently, direct roles of fibrates in vascular endothelial cells have become evident, clarifying the inhibitory effects of the PPARα activators on the expression of VCAM-1, IL-6, and endothelin-1.8–10 IL-6 is known to control macrophage and T cell activation as well
as vascular smooth muscle cell proliferation,\textsuperscript{19,20} and has been detected in human and rabbit atherosclerotic regions.\textsuperscript{21,22} VCAM-1 plays an important role in mediating mononuclear leukocyte-selective adhesion to vascular endothelium, which may be involved in the initial steps of atherosclerotic process.\textsuperscript{23} Endothelin-1 is a potent vasoconstrictor peptide and inducer of vascular smooth muscle cell proliferation.\textsuperscript{24,25} Thus, all these molecules are known to be involved in the atherosclerotic process, and suppression of their expression may also be attributable partly to the antiatherogenic effects of PPAR\textsubscript{\alpha} activators.

Endothelium-derived NO is a potent chemical mediator with antiatherogenic properties, such as stimulation of vasorelaxation and repression of endothelial leukocyte adhesion molecules, platelet aggregation, and smooth muscle cell proliferation.\textsuperscript{16,26,27} To date, it is unknown whether PPAR\textsubscript{\alpha} activators can directly modulate eNOS activity and its expression, which mediates endothelial NO production. Our study clearly demonstrates that fenofibrate upregulated eNOS protein levels in cultured vascular endothelial cells. This effect of fenofibrate was observed at concentrations at 10\,\mu M or more, which are near to plasma concentrations of its active metabolite fenofibric acid found in humans administered.\textsuperscript{28} In addition, such concentrations corresponded closely to the range of activation of PPAR\textsubscript{\alpha} by fenofibrate, although this compound can also activate PPAR\textsubscript{\gamma} with approximately 10-fold lower selectivity than PPAR\textsubscript{\alpha}.\textsuperscript{29,30} WY14643, a selective PPAR\textsubscript{\alpha} activator,\textsuperscript{30} and bezafibrate, a hypolipidemic drug that potentially activates three types of PPAR (PPAR\textsubscript{\alpha}, \textsubscript{\gamma}, and \textsubscript{\delta}),\textsuperscript{30,31} also increased eNOS protein expression. By contrast, rosiglitazone, a specific ligand for PPAR\textsubscript{\gamma},\textsuperscript{32} failed to increase eNOS protein levels. In addition, RU486, which has been shown to have a potential to antagonize the inhibitory effect of PPAR\textsubscript{\alpha} on IL-6 production probably via interference with nuclear translocation of PPAR\textsubscript{\alpha},\textsuperscript{33} inhibited the fenofibrate-induced upregulation of eNOS protein levels in cultured vascular endothelial cells.
eNOS expression. The sum total of these observations suggests that fenofibrate-induced upregulation of eNOS protein expression may be mediated through PPARα.

We demonstrated that eNOS mRNA levels increased after the treatment of vascular endothelial cells with fenofibrate, which is parallel with the increase in protein levels and enzymatic activity of eNOS. Thus, the effect of fenofibrate is primarily caused by the elevation of steady state levels of eNOS mRNA. It is known that eNOS activity is regulated by genomic mechanisms as well as nongenomic mechanisms. The latter mechanisms imply activation by phosphorylation of eNOS by the protein kinase Akt/protein kinase B (PKB).34,35 In our experiments, eNOS activity could not increase so rapidly (≤1 hour) after the fenofibrate treatment, indicating that PPARα does not exert posttranslational modification of eNOS activity. This seems to be in contrast with cases of other nuclear receptors such as estrogen receptor36 and glucocorticoid receptor,37 both of which are capable of stimulating phosphatidylinositol 3-kinase (PI3-K) and Akt/PKB. Simoncini et al36 have shown that WY14643 failed to activate PI3-K activity in PPARα immunoprecipitates. However, it is not possible from our results to determine the effect of fenofibrate on phosphorylation of eNOS. Further studies will be needed to clarify this issue.

Analysis of the human eNOS promoter sequence (−1600 to +22 nucleotides) did not reveal the presence of any discernible PPAR response elements,38 suggesting that PPARα may not directly interact with eNOS promoter region.

Figure 5. Fenofibrate increases eNOS mRNA levels in BAECs, as demonstrated by Northern blot analyses. A and B, BAECs were treated for 24 hours with increasing concentrations of fenofibrate. C and D, BAECs were treated for the indicated periods with 50 μmol/L fenofibrate. Representative blots are illustrated in A and C. The densitometric readings as percent control of eNOS mRNA relative to GAPDH mRNA levels are shown in B and D. Data are mean±SD in three experiments. cont indicates control; Feno, fenofibrate. *P<0.0001 vs control, by ANOVA.

Figure 6. Fenofibrate fails to increase eNOS promoter activity (A). It increases half-life of eNOS mRNA (B). A, Two hours after transfection with −1600 to +26 of human eNOS gene-based luciferase reporter construct, BAECs were treated for 24 hours with 50 μmol/L fenofibrate or 100 nM PMA. Luciferase activity was adjusted for control reporter activity. Data are mean±SD in triplicated assays typical of three separate experiments. B, BAECs were treated with or without 50 μmol/L fenofibrate up to 15 hours in the presence of actinomycin D (2.5 μg/mL). Northern blot analyses were performed. The densitometric readings as percent of initial eNOS mRNA relative to GAPDH mRNA levels are shown. Data are mean±SD in three experiments. cont indicates control; Feno, fenofibrate. *P<0.001, **P<0.0005 vs control−, by ANOVA.
In fact, transient transfection experiments of the eNOS promoter construct showed that fenofibrate could not elevate its promoter activity. mRNA stability assays using the transcriptional inhibitor actinomycin D revealed that fenofibrate increased the half-life of eNOS mRNA, suggesting that fenofibrate performs the novel action of stabilizing mRNA for eNOS. The precise mechanism of fenofibrate-mediated mRNA stabilization is unknown. In this relation, it has been postulated that estrogens stabilize mammalian mRNAs, requiring specific sequences of the mRNA and the presence of estrogen receptor. Similar mechanisms may operate in the stabilizing effect of PPARs on eNOS mRNA.

The PPARγ activators glitazones are also shown to improve vascular and endothelial function. In our experiments, rosiglitazone failed to upregulate eNOS protein levels. Recently, PPARγ ligands have been shown to increase NO release from vascular endothelial cells without altering eNOS mRNA levels, probably through a transcriptional mechanism unrelated to eNOS expression. Together with these results, our findings show that PPARs and PPARγ may increase NO production in endothelium by different mechanisms.

Tabernero et al have shown that fenofibrate improves NO-mediated vasodilatation in the aorta and in the mesenteric vascular bed, which cannot be explained by an increase in eNOS expression. In addition, Deplanque et al have demonstrated that fenofibrate reduces susceptibility to stroke in apolipoprotein E-deficient mice and decreases infarct size volume in mice. This neuroprotective effect is partly associated with an improvement in middle cerebral artery sensitivity to endothelium-dependent relaxation, which is unrelated to an increase in eNOS expression. Thus, the effects of fenofibrate on eNOS expression in these mouse models are not the same as those observed in our study. These in vivo experiments may not always reflect the direct effect of fenofibrate on endothelial cells.

Diabetes Atherosclerosis Intervention Study (DAIS) as well as Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT) suggest that treatment with fenofibrate or Bezafibrate Coronary Atherosclerosis Intervention Trial (BECAIT). J Am Coll Cardiol. 1998;32:1648–1656. The expression of eNOS and endothelin-1 is known to be coordinately regulated in diverse pharmacological and experimental conditions. Since PPARγ agonists have the potential to inhibit endothelin-1 expression, our results may give another example of the coordinated regulation between eNOS and endothelin-1 systems. These effects might explain the vasodilator function of PPARγ agonists.

Acknowledgments
We thank Dr Yasuko Kureishi (Mie University) for providing us bovine eNOS cDNA probe and Dr Masafumi Nakayama (Kumamoto University) for providing human eNOS reporter construct. This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (to S. Kasayama).

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Arterioscler Thromb Vasc Biol. published online January 29, 2004;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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