Endothelin-1–Induced Cardiac Hypertrophy Is Inhibited by Activation of Peroxisome Proliferator–Activated Receptor-α Partly Via Blockade of c-Jun NH₂-Terminal Kinase Pathway

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Background—Peroxisome proliferator-activated receptor-α (PPAR-α) is a lipid-activated nuclear receptor that negatively regulates the vascular inflammatory gene response by interacting with transcription factors, nuclear factor-κB, and AP-1. However, the roles of PPAR-α activators in endothelin (ET)-1–induced cardiac hypertrophy are not yet known.

Methods and Results—First, in cultured neonatal rat cardiomyocytes, a PPAR-α activator, fenofibrate (10 μmol/L), and PPAR-α overexpression markedly inhibited the ET-1–induced increase in protein synthesis. Second, fenofibrate markedly inhibited ET-1–induced increase in c-Jun gene expression and phosphorylation of c-Jun and JNK. These results suggest that this PPAR-α activator interferes with the formation and activation of AP-1 protein induced by ET-1 in cardiomyocytes. Third, fenofibrate significantly inhibited the increase of ET-1 mRNA level by ET-1, which was also confirmed by luciferase assay. Electrophoretic mobility shift assay revealed that fenofibrate significantly decreased the ET-1–stimulated or phorbol 12-myristate 13-acetate–stimulated AP-1 DNA binding activity, and the nuclear extract probe complex was supershifted by anti-c-Jun antibody. Fourth, 24 hours after aortic banding (AB) operation, fenofibrate treatment significantly inhibited left ventricle hypertrophy and hypertrophy-related gene expression pattern (ET-1, brain natriuretic peptide, and β-myosin heavy chain mRNA) in AB rats.

Conclusions—These results suggest that PPAR-α activation interferes with the signaling pathway of ET-1–induced cardiac hypertrophy through negative regulation of AP-1 binding activity, partly via inhibition of the JNK pathway in cultured cardiomyocytes. We also revealed that fenofibrate treatment inhibited left ventricle hypertrophy and phenotypic changes in cardiac gene expression in AB rats in vivo. (Circulation. 2004;109:904-910.)

Key Words: endothelin || hypertrophy || signal transduction

Peroxisome proliferator-activated receptor-α (PPAR-α) is abundant in tissues with high oxidative energy demands that depend on mitochondrial fatty acid oxidation as a primary energy source, such as the heart and liver.1 Fibric acid derivatives including fenofibrate are thought to act as specific activators of PPAR-α.2 PPAR-α together with PPAR-δ and PPAR-γ form a subgroup within the nuclear receptor superfamily.3 PPARs regulate gene expression by binding with retinoid X receptors as a heterodimeric partner to peroxisome proliferator response elements.4 Recently, Yamamoto et al5 demonstrated that PPAR-γ activators inhibit mechanical strain–induced hypertrophy in cardiomyocytes. However, it is unclear how PPAR-α activation contributes to the development of cardiac hypertrophy.

Endothelin (ET)-1, a potent vasoconstrictor peptide from vascular endothelial cells,6 is also synthesized and secreted by cardiomyocytes6 and induces hypertrophy of cardiomyocytes6,7 through activating phospholipase C, protein kinase C, extracellular signal-regulated kinase (ERK) 1 and ERK2, and upregulation of c-Fos and c-Jun. ET-1 is moderately effective in activating JNK8 and p38.9 Upregulation of preproET-1 mRNA expression is induced by several stimuli that activate protein kinase C, such as angiotensin II, ET-1 itself, phorbol ester, and stretch.10 The ET-1 gene has AP-1 binding sites in the promoter region, and these factors are known to upregulate AP-1, suggesting that the ET-1 gene is partly induced through AP-1 binding.6 We have reported that the production of ET-1 is markedly increased both in the hypertrophied heart and the failing heart11–14 and that chronic treatment with ET type A receptor antagonists significantly inhibited the development of cardiac hypertrophy and heart failure.11,13,14 These data suggest that ET-1 plays an important role in the
development of cardiac hypertrophy and heart failure, both in vitro and in vivo. However, the roles of PPAR-α activators in ET-1–induced cardiac hypertrophy are not yet known. The present study was designed to examine this question.

Methods

Primary Culture of Rat Cardiomyocytes
As described previously,13,14 ventricular cardiomyocytes were isolated from 2–3-day-old Sprague-Dawley rats, cultured in DMEM/Ham F12 medium supplemented with 0.1% fatty acid–free BSA (Sigma) for 2 days, and then used for additional experiments.

RNA Extraction and Reverse Transcription–Polymerase Chain Reaction
Total RNA from cardiomyocytes or the left ventricle (LV) was isolated as described previously.15,16 The mRNA level was analyzed by reverse transcription–polymerase chain reaction (RT-PCR). RT-PCR for ET-1 and brain natriuretic peptide (BNP) was performed according to our previous report.14 The expression of GAPDH and β-actin was also determined as an internal control. The sequences of the specific primers were as follows: c-Fos (sense), 5′-CCCTCTTCACTACCCATCCCG-3′; c-Fos (antisense), 5′-GCAGCCAATCTATTCCTTTCCC-3′; c-Jun (sense), 5′-AACTCGGACCTTCTAGGC-3′; c-Jun (antisense), 5′-TCTGAGGTGGCTGACCC-3′. Distinction between α- and β-myosin heavy chain (MHC) and α-MHC was determined by our previous method.14

In Vitro Experiments

Cardiac Myocyte Surface Area
Cell images captured by CCD Camera (Olympus) were traced and analyzed with NIH image 1.56. The area was then doubled to account for the surface portion in contact with the dish. All cells (102 cells in total) from randomly selected fields in 2 or 3 dishes were examined for each condition.

Cloning and Expression of PPAR-α
Rat PPAR-α was cloned from rat cardiomyocyte cDNA using the following primers: PPAR-α (sense), 5′-CGGGATCCATGGTGGAACCTTCT-3′; PPAR-α (antisense), 5′-GGATCCGATACATGTCTCTGTA-3′. After sequence analysis, the PCR product was cloned into the mammalian expression vector phrGFP (STRATAGENE, North Torrey). Transfected cardiomyocytes were cultured for ~24 to 26 hours and treated with fenofibrate for 1 hour. Then ET-1 (100 nmol/L) or phospholipid 12-myristate 13-acetate (PMA) (SIGMA-ALDRICH, USA) (100 nmol/L) was added for 3 hours. Cells were harvested, and luciferase activity was measured as described previously.13

Nuclear Extracts and EMSA
Nuclear extracts of cardiomyocytes were prepared as described previously.15 Cardiomyocytes were stimulated for 30 minutes with PMA (100 nmol/L) or ET-1 (100 nmol/L) with or without fenofibrate pretreatment (1 hour) before nuclear extracts preparation. Double-stranded oligonucleotide probes containing the AP-1 consensus sequence 5′-d(CGGGATCCATGGTGGAACCTTCT)-3′ (Promega) or mutant AP-1 sequence 5′-d(CGCGGATCCATGGTGGAACCTTCT)-3′ (mAP-1) were end-labeled with [γ-P]ATP (3000 Ci/mmol; NEN Life Science Products Inc) according to standard protocols. Protein-DNA complex was separated from free DNA probe by electrophoresis in a nondenaturing 4% polyacrylamide gel in 0.5 Tris-Borate-EDTA at 4°C and analyzed by BAS 5000 (Fuji Film Ltd). Nonlabeled double-stranded oligonucleotides corresponding to AP-1 binding site were used as specific competitor DNAs. For supershift experiments, 200 ng of goat polyclonal c-Jun/AP-1 (N)-G affinity–purified IgG (Santa Cruz Biotechnology) was used.

In Vivo Experiments

Rats With Cardiac Hypertrophy Attributable to Aortic Banding
Rats were orally administered fenofibrate (80 mg/kg per d) (Kaken Seiyaku Ltd) or vehicle (3% arabic gum) (Wako Ltd) for 1 week before surgery.17 The operation of aortic banding was carried out according to the method described by Jouannot and Hatt18 and Yorikane et al12 with minor modification. This animal study was approved by the University of Tsukuba and conformed to the Position of the American Heart Association on Research Animal Use.

Hemodynamic Measurement, Tissue Sampling, and mRNA Expression
Twenty-four hours after surgery, the hemodynamic parameter of each rat and the ratio of LV wet weight to body weight (BW) (LV mass index for BW) were measured according to our previous reports.13,14 The expression of preproET-1 and BNP mRNA and the transition of α-MHC to β-MHC mRNA, the alterations of which are regarded as molecular markers of cardiac hypertrophy, were investigated according to our previous report.14 The expression of 18 S ribosomal RNA was determined as an internal control.

Data Analysis
Data were expressed as mean±SEM. One-way ANOVA followed by a post hoc test was used for statistical comparison among the various treatment groups. Differences were considered significant at P<0.05.

Results

In Vitro Experiments

Inhibitory Effect of Fenofibrate and PPAR-α Overexpression on Cardiomyocytes Hypertrophy-Induced by ET-1
As shown in Figure 1A, ET-1 significantly increased enlargement of cardiomyocytes (1.8-fold versus control, n=100 cells, P<0.0001). A PPAR-α activator, fenofibrate (10 μmol/L), significantly inhibited the effect (−64%,
n=100 cells, P<0.0005). Fenofibrate (10 μmol/L) by itself did not affect the myocyte surface area (n=100 cells). Leucine uptake was significantly increased by ET-1 (1.8±4.0-fold versus control, n=4, Figure 1B), and this was inhibited by fenofibrate in a concentration-dependent manner (5 and 10 μmol/L: −20% at 5 μmol/L, n=10; −28% at 10 μmol/L, n=10). Fenofibrate by itself did not affect basal levels of leucine incorporation (n=10, Figure 1B). Overexpression of PPAR-α also inhibited ET-1-induced leucine uptake in cardiomyocytes (Figure 1C).

Effect of Fenofibrate on c-Fos and c-Jun mRNA Expression Induced by ET-1

We investigated the effect of fenofibrate on ET-1-stimulated expression of immediate early genes such as c-Fos and c-Jun, which are known to be the AP-1 components. ET-1 increased the levels of c-Fos and c-Jun mRNA (Figures 2A and 2B). The increase in c-Fos mRNA level was inhibited by MEK1/2 inhibitor, PD98059 (20 μmol/L), but not fenofibrate (10 μmol/L). In contrast, the increase in c-Jun mRNA level was inhibited by fenofibrate but not PD98059 (Figure 2B). More-
over, c-Jun protein level was increased after 15, 30, and 60 minutes of ET-1 stimulation (Figure 3A), which was also inhibited by fenofibrate (Figure 3A).

**Effect of Fenofibrate on ET-1–Induced Phosphorylation of c-Jun and JNK**

To additionally elucidate the counter effect of fenofibrate on AP-1, we determined whether fenofibrate affected the JNK/c-Jun pathway. The level of phosphorylation on Ser73 of c-Jun was increased after 15, 30, and 60 minutes of ET-1 stimulation, which was markedly inhibited by fenofibrate (Figures 3B and 3C). Fenofibrate did not affect phosphorylation of c-Jun without ET-1 (Figures 3B and 3C). As shown in Figure 4A, ET-1 (100 nmol/L) markedly induced the phosphorylation of JNKs (JNK1/2 at 54 KDa/46 KDa), which was significantly inhibited by fenofibrate. Neither application of ET-1 nor fenofibrate treatment affected the expression level of JNKs in cardiomyocytes.

**Effects of Fenofibrate on ET-1–Induced ERK1/2 Phosphorylation**

As described in previous reports, ERK activation, a sensitive and quantitative marker for hypertrophic responses to external stimuli, is strongly activated by ET-1 in cardiomyocytes.10 Also in this study, ET-1 (100 nmol/L) induced the phosphorylation of ERKs in cardiomyocytes. This was not inhibited by fenofibrate (10 μmol/L) (Figure 4B). Neither application of ET-1 nor fenofibrate treatment affected the expression level of ERKs in cardiomyocytes.

**Effect of Fenofibrate on PreproET-1 mRNA Expression and ET-1 Gene Promoter Activity Induced by ET-1**

As shown in Table 1, ET-1 (100 nmol/L) by itself increased ET-1 mRNA level in cardiomyocytes (308.6±17.9% versus control, \( P < 0.05 \)), which was inhibited by fenofibrate (1, 5, and 10 μmol/L) in a dose-dependent manner (165.3±14.5%, 106.0±1.0%, and 83.3±9.9% versus ET-1, \( P < 0.05 \)).

**TABLE 1. Effect of Fenofibrate on ET-1 mRNA Expression in Cardiomyocytes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ET-1 mRNA Expression, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>ET-1</td>
<td>308.6±17.9*</td>
</tr>
<tr>
<td>ET-1+fenofibrate (1 μmol/L)</td>
<td>165.3±14.5†</td>
</tr>
<tr>
<td>ET-1+fenofibrate (5 μmol/L)</td>
<td>106.0±1.0†</td>
</tr>
<tr>
<td>ET-1+fenofibrate (10 μmol/L)</td>
<td>83.3±9.9†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. Each result shown is representative of 3 independent experiments performed in triplicate. *\( P < 0.05 \) compared with control; †\( P < 0.05 \) compared with ET-1.
Inhibitory Effects of Fenofibrate on ET-1–Induced or PMA-Induced AP-1 DNA Binding Activity

To investigate whether fenofibrate inhibits AP-1 DNA binding activity, we performed EMSA using radiolabeled oligonucleotides corresponding to AP-1 site. As shown in Figure 5A, ET-1 produced an almost 4-fold increase in AP-1 DNA binding activity. This was significantly inhibited by fenofibrate. The shifted complexes were specific for c-Jun because they were supershifted in the presence of antibody to the c-Jun and disappeared with excess unlabeled oligonucleotide. The activity of AP-1 DNA binding using mutant AP-1 oligonucleotide was hardly detected in ET-1–stimulated cardiomyocytes. The PMA (100 nmol/L)–induced AP-1 DNA binding activity was also inhibited by fenofibrate (Figure 5B).

In Vivo Experiments

Effect of Fenofibrate Treatment on Ventricular Hypertrophy Attributable to Pressure Overload in Rats With Aortic Banding

At 24 hours after operation, systolic arterial pressure was significantly elevated in the aortic banding (AB) groups (145±14 versus 114±7 mm Hg, AB+vehicle versus sham+vehicle). Administration of fenofibrate did not affect the elevation of arterial pressure in AB rats compared with vehicle administration (155±8 mm Hg). BWs of 3 groups (sham+vehicle, AB+vehicle, and AB+fenofibrate) were not significantly different (338±2, 332±7, and 345±3 g). LV weight and LV mass index were significantly higher in the AB+vehicle group than in the sham+vehicle group. These were significantly lower in fenofibrate-treated AB rats than in vehicle-

**TABLE 2. Effect of Fenofibrate on Promoter Activity of Rat ET-1 Gene in Cardiomyocytes**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mock</th>
<th>pET-1</th>
<th>pmET-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.0±0.4</td>
<td>0.3±0.1</td>
<td>0.3±0.1</td>
</tr>
<tr>
<td>ET-1</td>
<td>†</td>
<td>9.4±0.6*</td>
<td>3.6±0.3†</td>
</tr>
<tr>
<td>ET-1+fenofibrate</td>
<td>†</td>
<td>4.7±0.4†</td>
<td>3.8±0.8†</td>
</tr>
<tr>
<td>PMA</td>
<td>†</td>
<td>10.8±2.5*</td>
<td>2.1±0.6†</td>
</tr>
</tbody>
</table>

Plasmids used for transfection were as follows: mock, basic Luc-vector; pET-1, pET-1Luc vector; and pmET-1, pmET-1Luc vector. Data for promoter activity were expressed as light units. Values are mean±SE. Each results shown is representative of 3 independent experiments performed in triplicate.

*P<0.05 compared with pET-1; †P<0.05 compared with pET-1 ET-1 (+); ‡P<0.05 compared with pET-1 PM (+).
treated AB rats (Figure 6A). Hypertrophy-related gene expression pattern was also elucidated. The level of preproET-1 and BNP mRNA expression and the ratio of the expression of β-MHC mRNA to α-MHC mRNA in the LV were higher in the AB+vehicle group than in sham-operated rats, and fenofibrate treatment decreased these parameters for alterations in AB rats (Figure 6B).

Discussion

In this study, we demonstrated that activation of PPAR-α interrupted the earliest ET-1–induced events, ie, JNK activation, c-Jun phosphorylation, and c-Jun induction in cardiomyocytes. Because JNKs regulate the AP-1 DNA binding activity through phosphorylation of the 2 serine residues in the NH₂-terminal region of c-Jun, 1 of the AP-1 components, fenofibrate, would inhibit AP-1 activity partly via JNK pathway inhibition. Activation of ERK has been implicated in features of the hypertrophic response in an in vitro model. In our study, ERKs were markedly activated by ET-1 in cardiomyocytes, but this was not affected by fenofibrate. Therefore, the inhibitory effect of fenofibrate on ET-1–related hypertrophic responses might be mediated through interfering with the JNK pathway rather than the ERK pathway.

We demonstrated that fenofibrate inhibited ET-1 promoter activity, preproET-1 mRNA expression, and hypertrophy in ET-1–stimulated cardiomyocytes. To gain additional insight into the mechanism of the PPAR-α–mediated regulation of ET-1 gene expression, we focused on the effect of fenofibrate on AP-1 transcription activity, because the AP-1 site in the promoter region of the ET-1 gene is considered to be essential in the ET-1–induced transcription of ET-1. Indeed, by using p(AP-1)7-Luc vector, we confirmed that the AP-1–dependent reporter gene expression induced by ET-1 or PMA in cardiomyocytes was significantly inhibited by fenofibrate (data not shown). It has been reported that transactivation studies using c-Fos and c-Jun expression plasmids confirmed strong activation of the ET-1 promoter and that the markedly increased ET-1 promoter activity by c-Fos and c-Jun was inhibited by cotransfection of the PPAR-α expression plasmid. PPARs have been suggested to interfere negatively with the AP-1, Stat, and nuclear factor–κB signaling pathways via competition for essential cofactors. Therefore, it is considered that activated PPAR-α binds to AP-1 or its cofactor and that AP-1 is prevented from binding to the cis-elements of the promoter region, which results in impairment of ET-1 gene induction. Thus, one possible mechanism of the inhibitory action of PPAR-α activation on ET-1–induced cardiac hypertrophy seems to be through interference with AP-1 binding to its specific site in the ET-1 gene promoter region. This notion is supported by the fact that the in vitro complex formation of the AP-1 site with nuclear protein from cardiomyocytes stimulated by either ET-1 or PMA was significantly inhibited by fenofibrate.

In this study, we revealed that fenofibrate inhibited preproET-1 mRNA expression and molecular markers of hypertrophy in the LV of AB rats of pressure overload in vivo. An increase in ET-1 production has also been shown in hypertrophied hearts in various models of pressure overload. These data suggest that the ET-1 signal is involved in the development of cardiac hypertrophy by pressure overload. Therefore, a PPAR-α activator may inhibit cardiac hypertrophy through suppression of the ET-1 signal in vivo as well as in vitro.

In conclusion, we demonstrated that PPAR-α activation inhibits ET-1–induced cardiac hypertrophy by negative interference with AP-1, partly via JNK pathway inhibition in cardiomyocytes. Furthermore, we revealed that fenofibrate treatment inhibited LV hypertrophy and alterations in the expression of cardiac genes in the LV of AB rats in vivo. Therefore, it is possible that treatment with PPAR-α activators leads to improvement in pathological myocyte hypertrophy.

Acknowledgments

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (11357019, 11557047, 12470147, 12680012, 00005167, 0.05 vs AB+vehicle, *P<0.05 vs sham+vehicle, †P<0.05 vs AB+vehicle.

Figure 6. Inhibitory effects of fenofibrate treatment on ventricular hypertrophy attributable to pressure overload in rats with AB. A, Weight data 24 hours after operation. Values are mean±SEM. *P<0.05 vs sham+vehicle, †P<0.05 vs AB+vehicle. B, Effects of fenofibrate on expression levels of preproET-1 (a) and BNP (b) and on the ratio of β-MHC to α-MHC mRNA expression levels (c) in the LV of rats with AB. Values are mean±SEM. *P<0.01 vs sham+vehicle, †P<0.05 vs AB+vehicle.
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References


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