

Peroxisome Proliferator Activated Receptor (PPAR) α Agonists Inhibit Hypertrophy of Neonatal Rat Cardiac Myocytes

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The peroxisome proliferator activated receptors (PPARs) appear to have beneficial effects in the cardiovascular system. PPAR γ has been shown previously to exert an inhibitory effect on cardiac myocyte hypertrophy *in vivo* and *in vitro*. Using endothelin to activate the hypertrophic program in neonatal rat cardiac myocytes, we demonstrate that PPAR α ligands (fenofibrate and WY14,643) suppress hypertrophy-dependent increases in protein synthesis, cell surface area, and sarcomeric organization *in vitro*. This was accompanied by a decrease in brain natriuretic peptide gene expression, a marker of transcriptional activation in hypertrophy. These effects were equivalent to or greater than those seen with the PPAR γ agonist rosiglitazone. Fenofibrate and rosiglitazone

suppressed endothelin stimulation of human brain natriuretic peptide gene promoter activity, and this effect was amplified by cotransfection of PPAR α and PPAR γ expression vectors, respectively. The fenofibrate-dependent suppression of endothelin's stimulatory activity was dependent upon promoter sequence positioned between –904 and –40 relative to the transcription start site and did not appear to involve a number of positive and negative regulatory elements that are known to govern transcription of this gene. These findings suggest that PPAR α ligands could prove to be useful in the management of disorders associated with hypertrophy and remodeling of the myocardium. (*Endocrinology* 144: 4187–4194, 2003)

THE PEROXISOME PROLIFERATOR activated receptors (PPARs) are members of the extended nuclear receptor family of transcription factors (1). They play an important role in the regulation of lipid synthesis and degradation by virtue of their ability to control key transport proteins and enzymes involved in fatty acid and triglyceride metabolism. PPARs can be subdivided into three independent isoforms, PPAR α , β/δ , and γ (2, 3). PPAR γ is expressed in vascular smooth muscle cells, immune cells, and white adipose tissue, in which it is thought to contribute to adipocyte differentiation (2, 3). Putative endogenous ligands for PPAR γ include 15-deoxy- Δ 12,14-prostaglandin J₂ (4) and the pathophysiological oxidized metabolites of linoleic acid, 9-hydroxy and 13-hydroxy octadecadienoic acid, which are components of oxidized low-density lipoprotein (5). Thiazolidinediones, synthetic ligands that bind to and activate the PPAR γ receptor, have been shown to function as insulin sensitizers controlling both the hyperglycemia and dyslipidemia associated with type 2 diabetes mellitus. Interestingly, the thiazolidinediones have also been shown to have a number of salutary properties in the cardiovascular system, which are largely independent of their ability to lower blood glucose. They reduce blood pressure in hypertensive animal models (6–8) and humans (9). They suppress smooth muscle cell proliferation and migration *in vitro*, and they inhibit luminal narrowing in the experimentally damaged arterial wall *in*

vivo (10, 11). PPAR γ is expressed in most cell types involved in the generation of atheromata in the vascular wall and may play an inhibitory role in controlling the lipid deposition and vascular inflammation that typify the atherosclerotic lesion (12, 13), although this remains controversial. An independent line of studies suggests that PPAR γ may promote formation of foam cells and exacerbate the atherosclerotic process (5, 14). PPAR γ ligands have also been shown to limit infarct size after experimental coronary occlusion (15) and to suppress the development of cardiac myocyte hypertrophy both *in vitro* (16, 17) and in the *in vivo* (17) setting.

PPAR α is expressed in liver, intestine, renal cortex, and heart. Polyunsaturated fatty acids, leukotriene B₄, and products of the lipoxygenase pathway are putative endogenous ligands for PPAR α (3). The fibrates, pharmacological agents that ligand and activate the PPAR α receptor, are used clinically for control of a variety of dyslipidemias. In the heart, liganded PPAR α is thought to play an important role in regulating fuel homeostasis. It induces the expression of genes encoding proteins involved in fatty acid uptake (*e.g.* fatty acid transport protein, fatty acid translocase/CD36), mitochondrial import (mitochondrial carnitine pantoic transferase-1), and mitochondrial (*e.g.* medium chain acyl-coenzyme A dehydrogenase) and peroxisomal (acyl-coenzyme A oxidase) fatty acid β -oxidation (18–21). Fatty acids represent the primary energy source of the adult cardiac myocyte under normal physiological conditions. Noteworthy, acquisition of the hypertrophic phenotype by the cardiac myocyte leads to a shift away from fatty acids toward glucose as the primary energy source (21). This is accompanied by a reduction in PPAR α expression and activity (22, 23), the latter by virtue of a posttranslational modification (22), implying

Abbreviations: ANP, Atrial natriuretic peptide; BNP, brain natriuretic peptide; ET, endothelin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; h, human; NRSE, neuron restricted silencer element; PPAR, peroxisome proliferator activated receptors; RXR, retinoid X receptor; SSRE, shear stress response element; TR, thyroid hormone receptor; TRE, thyroid receptor binding element.

that PPAR α levels may dictate the type and pace of nutrient consumption in the heart.

Neonatal rat cardiac myocytes respond to hypertrophic agonists [*e.g.* endothelin (ET), α -adrenergic agonists, angiotensin, or mechanical strain] with an increase in protein synthesis, cell size, and sarcomeric organization, and a shift in the gene expression profile toward a more primitive fetal program (24). Both PPAR α and γ are expressed in neonatal rat ventricular myocytes (25). Brain natriuretic peptide (BNP) is a cardiac hormone that is expressed at low levels in the normal adult ventricle. BNP expression is activated by virtually any stimulus that elicits hypertrophy of the ventricular myocyte either *in vivo* or *in vitro*. In states of advanced hypertrophy and cardiac failure, plasma BNP levels are dramatically increased. In fact, plasma BNP levels have come to represent one of the most reliable markers of cardiac hypertrophy and heart failure. They are presently being used in the detection and differential diagnosis of early heart failure (26) and as a guide in the management of more advanced disease (27).

In the present study, we have investigated the role of PPAR ligands in controlling both hypertrophy and BNP gene expression in the neonatal rat ventricular myocyte model. We show that the PPAR α ligands fenofibrate and WY14,643 are as effective as the PPAR γ ligand rosiglitazone in blocking the increase in protein synthesis, cell size, sarcomeric organization, and BNP gene expression and promoter activity that accompanies hypertrophy in these cells.

Materials and Methods

Materials

[α -³²P]Deoxycytidine triphosphate and [³H]leucine were purchased from NEN Life Science Products (Boston, MA). Endothelin-1 was obtained from Phoenix Pharmaceuticals, Inc. (Belmont, CA). Fenofibrate was purchased from Sigma (St. Louis, MO). WY14,643 was purchased from Biomol Research Laboratory (Plymouth Meeting, PA). Rosiglitazone was the generous gift of T. Scanlan (University of California San Francisco). GW9662 was purchased from Sigma RBL. All oligonucleotides were synthesized by Invitrogen, Inc. (San Diego, CA). Other reagents were obtained through standard commercial suppliers.

Cell culture

Ventricular myocytes were prepared from 1-d-old neonatal rat hearts by alternate cycles of 0.05% trypsin digestion and mechanical disruption as described previously (28). Cells were cultured in DMEM containing 10% enriched calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin for 24 h. Cells were changed to serum-free medium containing 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin before initiation of each experiment.

Protein synthesis

Protein synthesis was assessed by measuring [³H]leucine incorporation into acid-insoluble material in cultured ventricular myocytes. Cells were cultured in 24-well plates for 24 h, then changed to serum-free medium and subjected to treatment for 48 h. Cells were pulsed with [³H]leucine in leucine-free medium for the final 4 h of the treatment period, washed three times with PBS, and extracted with 10% trichloroacetic acid at 4 C for 30 min. Cell residues were rinsed in 95% ethanol, solubilized in 0.25 N NaOH for 2 h, and neutralized with 2.5 mM HCl plus 1 mM Tris-HCl (pH 7.5). Radioactivity was measured in a liquid scintillation counter.

RNA isolation and Northern blot analysis

Total RNA was isolated from cultured ventricular myocytes using RNAzol (LPS Industries Inc., Moonachie, NJ). Fifteen micrograms of RNA were size-fractionated on a 1.2% agarose gel containing 2% formaldehyde, transferred to a nitrocellulose membrane, and hybridized simultaneously with ³²P-labeled 640-bp rat BNP cDNA and ³²P-labeled 1.3-kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. The membrane was washed and exposed to x-ray film. Autoradiographic signals were scanned and quantified by the National Institutes of Health Image program. Normalized data are presented as the ratio of BNP to GAPDH mRNA levels in individual samples.

Plasmid construction and site-directed mutagenesis

The construction of -1595, -904, -198, and -40 human (h) BNP luciferase have been described previously (29). Site-directed mutagenesis was carried out with the QuikChange kit (Stratagene, La Jolla, CA) using conditions recommended by the manufacturer. The sequence of each mutagenic primer (sense strand) was as follows (mutagenized bases are identified by lowercase letters): thyroid receptor binding element (TRE), 5'-CGATCTCCTtAtCaGcGATCCGACCGCCTCG-3'; neuron restricted silencer element (NRSE), 5'-GGGTGATCgtCgCtActGACAtGGGCCAGGG-3'; GATA (upstream), 5'-CCGGAAATGTGGCT-CgcAAATCAGAGACTGAGAC-3'; GATA (downstream), GACACT-CAGCTCCAGGcgAAAAGGCCACGGTG-3'; MCAT, 5'-GCGGAGGG-GCTCtTCGCGGGCCCTGATCTC-3'; reverse MCAT, 5'-GATCTCA-GAGGCCCGGgAgGTGGCTGATAAATCAG-3'; double shear stress response element (SSRE) (upstream), 5'-GTGAGAGCATAGGGAAA-tacagtGGAtacagtTTGTCCTTGCTCCACG-3'; SSRE (downstream), 5'-GGTCGGCTCTGCCctacagtCACCTCCACGTCG-3'.

Transfection and luciferase assay

Freshly prepared ventricular myocytes were transiently transfected with the indicated reporters by electroporation (GenePulser; Bio-Rad Laboratories, Hercules, CA) at 280 mV and 250 μ F. DNA content in individual cultures was normalized with pUC18. Cells were plated and cultured in DMEM H-21 containing 10% enriched calf serum for 24 h, then changed to serum-free medium overnight before treatment. Cells were lysed in 200 μ l of cell culture lysis reagent (Promega, Madison, WI). Cell lysates were assayed for luciferase activity using a commercially available kit (Promega). Luciferase levels were normalized for concentrations of soluble protein in the extracts. To ensure reproducibility, experiments were repeated with three to five independent cell preparations.

Immunostaining

Ventricular myocytes were cultured in four-chamber slides for 24 h, then changed to serum-free medium and subjected to treatments indicated for 48 h. Cells were then washed with PBS and incubated with 3.7% paraformaldehyde at room temperature for 20 min followed by PBS containing 0.2% Triton X-100 for 2 min. Slides were blocked with PBS containing 0.2% BSA and 0.1 μ g/ml normal horse IgG and incubated with mouse antirat sarcomeric α -actinin antibody (EA-53 from Sigma) at 4 C overnight. Cells were washed three times with PBS and incubated with Texas Red-conjugated horse antimouse secondary antibody (Vector Laboratories, Burlingame, CA) at room temperature for 30 min. After three consecutive washes, slides were mounted with VECTASHIELD mounting medium and viewed using fluorescence microscopy. Linear dimensions and surface area of individual cells were calculated using semiautomatic computer-assisted planimetry from two-dimensional images.

Statistics

Data were analyzed using one-way ANOVA and the Newman-Keuls test to assess significance.

Results

Each of the PPAR agonists diminished ET-dependent protein synthesis in these cultures (Fig. 1). At the highest dose

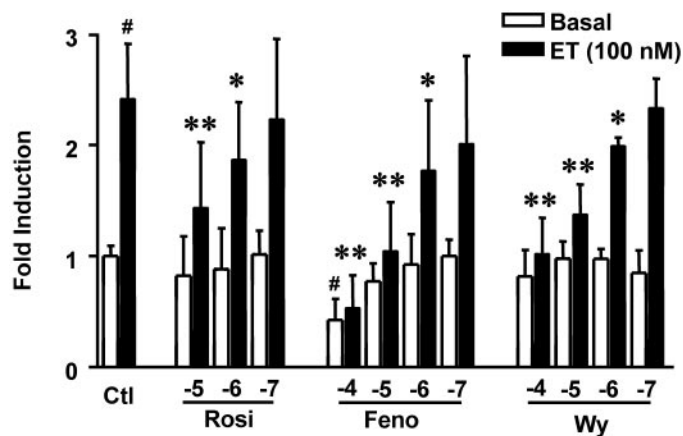


FIG. 1. PPAR agonists suppress ET-dependent protein synthesis in cultured neonatal rat ventricular myocytes. After 24 h of culture, cells were treated with increasing concentrations of fenofibrate, WY14,643, or rosiglitazone in the presence or absence of 100 nM ET-1 for 48 h. Cells were pulsed with [3 H]leucine for 4 h, and incorporation of radioactivity into acid insoluble protein was measured as described in *Materials and Methods*. Pooled data from four independent experiments are presented as means \pm SD. **, $P < 0.01$; and *, $P < 0.05$ vs. ET control. #, $P < 0.01$ vs. basal control.

of fenofibrate and WY14,643 employed (10^{-4} M), the ET effect was reduced to close to basal levels. Only in the case of 10^{-4} M fenofibrate was there an appreciable reduction in basal protein synthetic activity.

The reduction in protein synthesis was accompanied by a reduction in overall cell surface area (Fig. 2A). Cells treated with fenofibrate or rosiglitazone under basal (*i.e.* unstimulated) conditions were not affected appreciably; however, in those cultures stimulated with ET (10^{-7} M), there was a significant reduction in cellular dimensions and surface area (Fig. 2B). This was associated with an overall reduction in the level of sarcomeric organization, which, as seen in Fig. 2A, typically increases in these cells following ET treatment. Collectively, these data and those presented in Fig. 1 suggest that the PPAR α ligands antagonize the hypertrophic properties of ET in this model system.

As expected, ET also effected a significant increase (~ 4.5 -fold) in BNP gene expression in these cells, implying activation of the hypertrophic gene expression program (Fig. 3). Rosiglitazone, fenofibrate, and WY14,643 each effected a reduction in ET-stimulated BNP transcript levels, albeit not to the levels seen under unstimulated conditions. Maximal inhibition was in the range of 70–80%. In no instance was basal expression of the BNP gene transcript affected by these agonists.

The PPAR agonists also blocked the ET-dependent stimulation of BNP promoter activity. Treatment of cells transiently transfected with a -1595 hBNP luciferase reporter with rosiglitazone (10^{-5} M) resulted in approximately 35% reduction in promoter activity whereas treatment with either fenofibrate (10^{-5} M) or WY14,643 (10^{-5} M) resulted in approximately 65% inhibition of the ET stimulation (Fig. 4). As noted with the RNA studies above, in no instance did these agents have an effect on basal hBNP promoter activity. The inhibition by rosiglitazone or WY14,643 was observed across a concentration range of 10^{-9} to 10^{-7} M ET (data not shown).

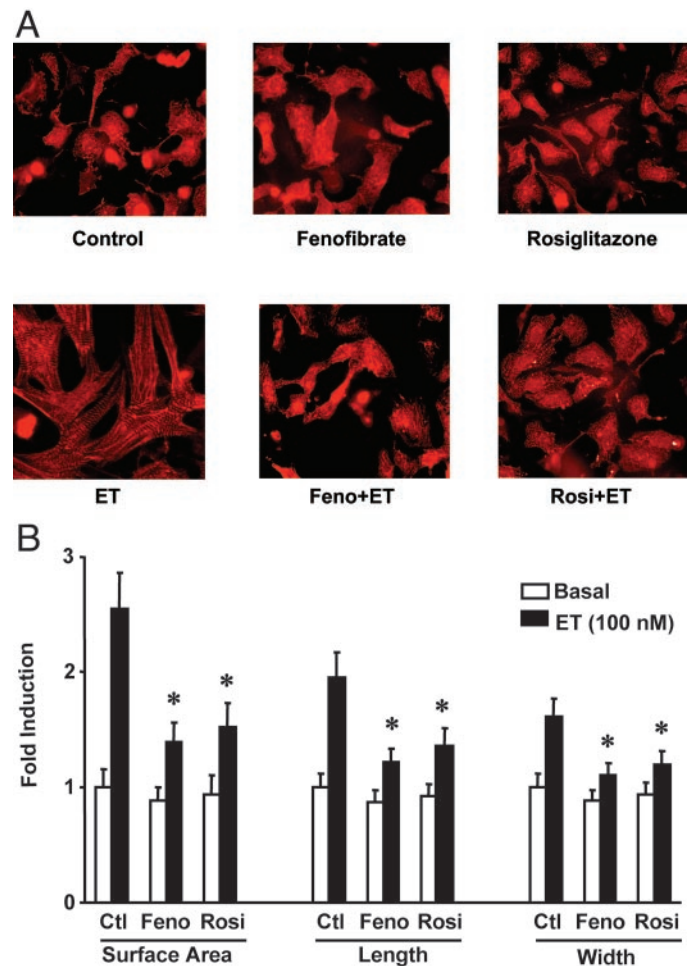


FIG. 2. PPAR agonists inhibit ET-dependent increases in cell size and sarcomeric organization in cultured ventricular myocytes. After 24 h of culture, cells were treated with fenofibrate (10^{-5} M), WY14,643 (10^{-5} M), or rosiglitazone (10^{-5} M) in the presence or absence of 100 nM ET-1 for 48 h. A, Cells were fixed with 3.7% paraformaldehyde, stained with monoclonal antibody directed against sarcomeric α -actinin, and processed for fluorescence microscopy as described in *Materials and Methods*. B, Myocyte length, width (each at maximal dimension), and surface area were measured using semiautomatic, computer-assisted planimetry as described in *Materials and Methods*. Thirty cells from each group were randomly selected for measurement of surface area in each experiment. Pooled data from three independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control.

The magnitude of the inhibition was amplified by cotransfection of the relevant PPAR into these cells. Cotransfection of PPAR γ alone resulted in approximately 40% inhibition of ET-stimulated hBNP promoter activity (Fig. 5), and this inhibition was increased further by inclusion of rosiglitazone ($\sim 60\%$ inhibition). Cotransfection of PPAR α resulted in near 50% inhibition of ET-stimulated promoter activity, and this was increased to approximately 70% in the presence of the PPAR α ligand WY14,643.

Pretreatment of cells with GW9662, a PPAR γ antagonist, significantly increased basal BNP promoter activity (~ 2.7 -fold) and amplified the stimulation by ET (~ 1.8 -fold). The inhibitory effect of rosiglitazone was abolished in the presence of GW9662; however, the effects of fenofibrate and

FIG. 3. Effect of PPAR agonists on ET-induced BNP mRNA levels in neonatal rat ventricular myocytes. After 24 h of culture, cells were treated with increasing concentrations of fenofibrate, WY14,643, or rosiglitazone in the presence or absence of 100 nM ET-1 for 24 h. Cells were collected for isolation of total RNA. Fifteen micrograms of RNA were subjected to blot hybridization analysis. Blots were hybridized simultaneously with radiolabeled BNP and GAPDH probes. Autoradiographs were quantified by NIH Image and normalized for GAPDH. Pooled data from four independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control.

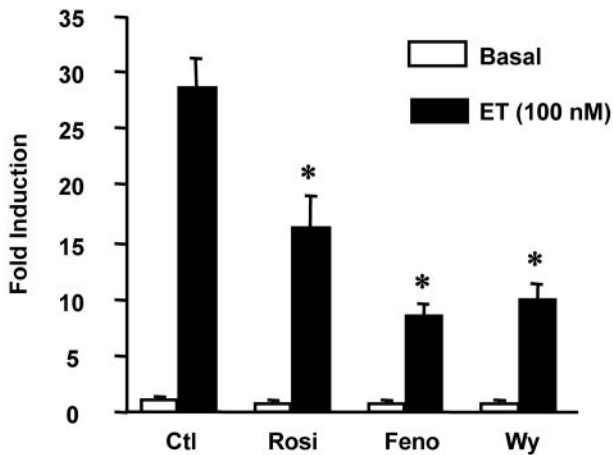
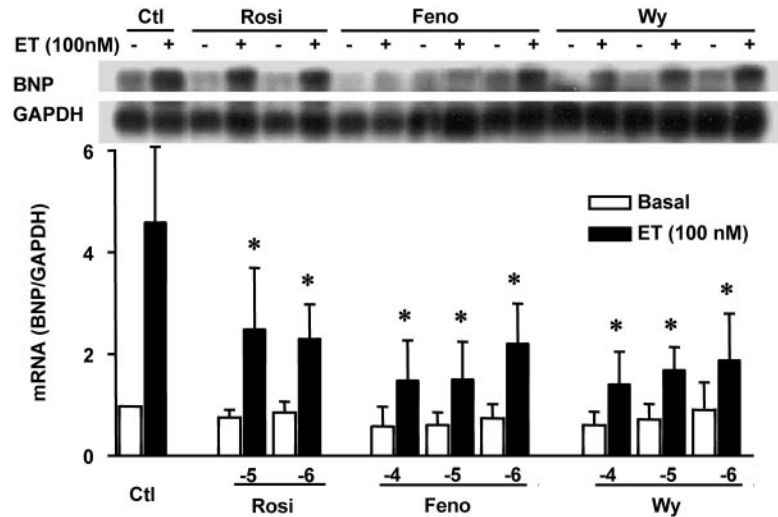


FIG. 4. Effect of PPAR agonists on ET-dependent BNP promoter activity in neonatal rat ventricular myocytes. Cells were transfected with 2 μ g of -1595 hBNP-luciferase. After 24 h of culture, cells were treated with 10^{-5} M fenofibrate, WY14,643, or rosiglitazone in the presence or absence of 100 nM ET. After 48 h, cells were collected and lysates were prepared for luciferase assay. Pooled data from four independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control.

WY14,643 were unaffected by the antagonist (Fig. 6), indicating that the effects of the latter are likely mediated through the PPAR α rather than the PPAR γ receptor.

Next, we attempted to localize the site of PPAR α activity on the hBNP gene promoter using a series of 5' deletion constructs available in our laboratory. As shown in Fig. 7, ET effected a robust increase in activity of the reporter incorporating the largest segment of hBNP gene 5' flanking sequence (-1595 hBNP luciferase). Fenofibrate reduced this increment to close to basal levels. The fenofibrate inhibition was maintained as the deletion was pushed to -904 and then on to -198, although the latter demonstrated a significant increment in basal activity, resulting in a reduction in the magnitude of the ET induction and diminished efficacy of fenofibrate in antagonizing this induction. Deletion of the promoter to -40 diminished basal activity of the reporter dramatically. Although a modest 2-fold increment in promoter activity was seen after ET stimulation, fenofibrate no

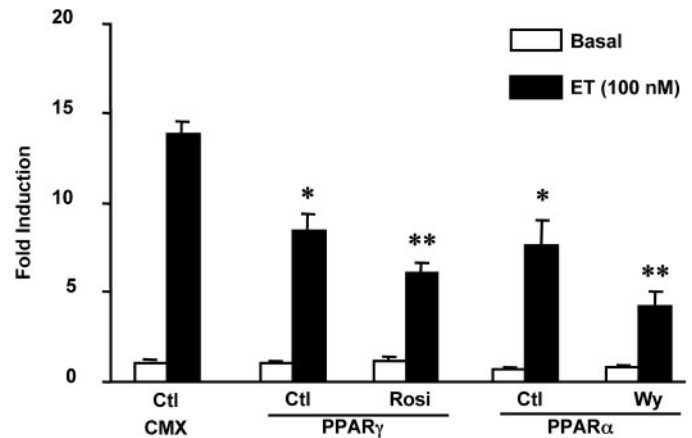


FIG. 5. Overexpression of PPAR α or PPAR γ enhances PPAR agonist-mediated inhibition of ET-dependent hBNP promoter activity in neonatal rat ventricular myocytes. Two micrograms of -1595 hBNP-luciferase were cotransfected into ventricular myocytes with 2 μ g of expression vector encoding PPAR α or PPAR γ . After 24 h of culture, cells were treated with 10^{-5} M fenofibrate or WY14,643 for an additional 48 h in the presence or absence of 100 nM ET. Cells were collected and lysates were prepared for luciferase assay. Pooled data from three independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control; **, $P < 0.01$ vs. unliganded PPAR α or PPAR γ -transfected group.

longer proved capable of reversing this effect (control, 681 \pm 67; ET, 1385 \pm 102; ET plus fenofibrate, 1231 \pm 168 light units/ μ g soluble protein; data presented as means \pm SD).

Finally, we examined the potential role of a number of basal transcriptional regulatory elements in the hBNP promoter as mediators the PPAR α /fenofibrate effect. A schematic diagram depicting these elements is presented in Fig. 8. GATA regulatory elements have been shown previously to play an important role in controlling both basal as well as regulated transcription of the rat BNP gene. Paired GATA elements have been identified in the hBNP gene promoter (located between -85 and -82 and -32 and -29; Refs. 29 and 30) and appear to play an important role in the maintenance of basal gene expression (Fig. 9). Similarly, we have identified two regions with sequence identity to the MCAT regulatory element in the proximal hBNP gene promoter.

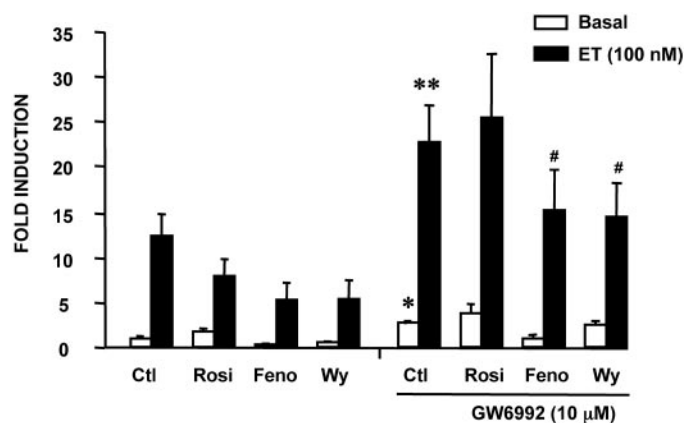


FIG. 6. Effect of PPAR γ antagonist on ET-dependent BNP gene promoter activity. Ventricular myocytes were transfected with 2 μ g of -1595 hBNP-luciferase. Twenty four hours later, cells were pretreated with 10^{-5} M GW9662 for 1 h, then treated with 10^{-5} M fenofibrate, WY14,643, or rosiglitazone in the presence or absence of 100 nM ET for 48 h. Cells were collected and lysates prepared for luciferase assay. Pooled data from five independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. basal control; **, $P < 0.01$ vs. ET in the absence of GW9662; #, $P < 0.05$ vs. GW9662 plus ET.

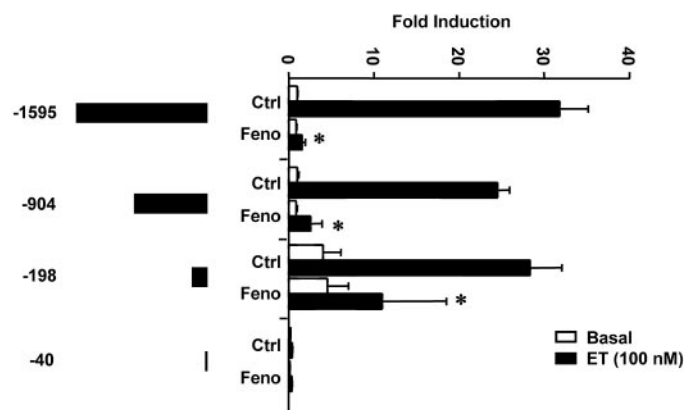


FIG. 7. Deletion analysis of hBNP gene promoter. Ventricular myocytes were transfected with 2 μ g of -1595 hBNP-luciferase, -904 hBNP-luciferase, -198 hBNP-luciferase, or -40 hBNP-luciferase. After 24 h of culture, cells were treated with 10^{-5} M fenofibrate or 10^{-5} M WY14,643 for an additional 48 h in the presence of 100 nM ET. Cells were collected and lysates were generated for luciferase assay. Pooled data from three independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control.

MCAT encodes a regulatory element that binds the transcription enhancing factor in other systems (29, 31). One of these is positioned on the sense strand in the forward orientation (positions -124 to -120) and one is on the antisense strand in the reverse orientation (positions -95 to -91). Selective mutation of the paired GATA elements, the forward MCAT site or the reverse MCAT site, resulted in significant reductions in basal hBNP promoter activity (Fig. 9), but, in each case, the ET induction was at least partially preserved, as was the fenofibrate-dependent suppression of this induction. A NRSE has been implicated as playing a major role in the regulation of basal and agonist-stimulated (ET or fibronectin) rat atrial natriuretic peptide (ANP) (32) and rat BNP (33) gene transcription. We have identified a homologous NRSE in the human gene between positions

-543 and -540 . Mutation of this site resulted in an increase in basal promoter activity, reflecting loss of the silencer function; however, once again, both the ET induction and the fenofibrate suppression of this induction were preserved (Fig. 9).

We have recently identified a thyroid hormone receptor (TR) binding element half-site positioned at -1000 to -995 in the hBNP gene (34). This element binds to the TR, either as a homodimer or a heterodimer with the retinoid X receptor (RXR), and signals virtually the entire thyroid hormone-dependent induction of this promoter. Based on the known homology between the PPAR and TR regulatory elements (1), we asked whether PPAR α might traffic its inhibitory activity through the hBNP TRE. This element binds to PPAR α -RXR heterodimers in conventional EMSAs *in vitro* (data not shown); however, mutation of the element does not eliminate the fenofibrate-dependent inhibition (Fig. 9). We have also identified a series of three nuclear factor- κ B binding, SSRE-like structures in the hBNP promoter that appear to confer sensitivity to mechanical strain (35). Although mutation of these three elements in combination results in a modest reduction in the ET stimulation of promoter activity (36), the fenofibrate inhibition is preserved. Collectively, these data indicate that none of the known elements responsible for governing basal or stimulated expression of the hBNP promoter are involved in mediating the PPAR α -dependent inhibition.

Discussion

The studies presented here demonstrate that ligands for PPAR α inhibit ET-dependent hypertrophy in this neonatal rat cardiac myocyte model, based on changes in protein synthesis, cell size, and morphological characteristics. This is accompanied by a reduction in the expression of the BNP gene, a well-defined marker of hypertrophy, and hBNP gene promoter activity. The latter activity appears to depend on promoter sequences between -904 and -40 and, more critically, between -198 and -40 relative to the start site of transcription. In direct comparisons, the PPAR α ligands appear to be as effective as the PPAR γ ligand rosiglitazone in promoting these antihypertrophic effects.

Since their initial discovery, PPARs have attracted considerable attention with regard to their unique properties in controlling fuel metabolism. In fact, agonists for both PPAR α and PPAR γ are currently used clinically for management of dyslipidemias (α and γ) and establishing glycemic control in patients with type 2 diabetes mellitus (8, 9). More recently, attention has turned to other effects of these agents. In the immune system, PPAR γ agonists display antiinflammatory properties that may reflect the ability of these drugs to interfere with the activity of a variety of cytokines and lymphokines that are activated as part of the inflammatory process (12). In the cardiovascular system, evidence has been presented supporting a salutary role for PPAR γ agonists in slowing the progression of atherosclerosis, an effect that may be based in part on its antiinflammatory properties (12). They also reduce blood pressure in hypertensive rodent models (6–8), inhibit smooth muscle cell proliferation and migration in cell culture (7, 8) and in *in vivo* models of arterial injury

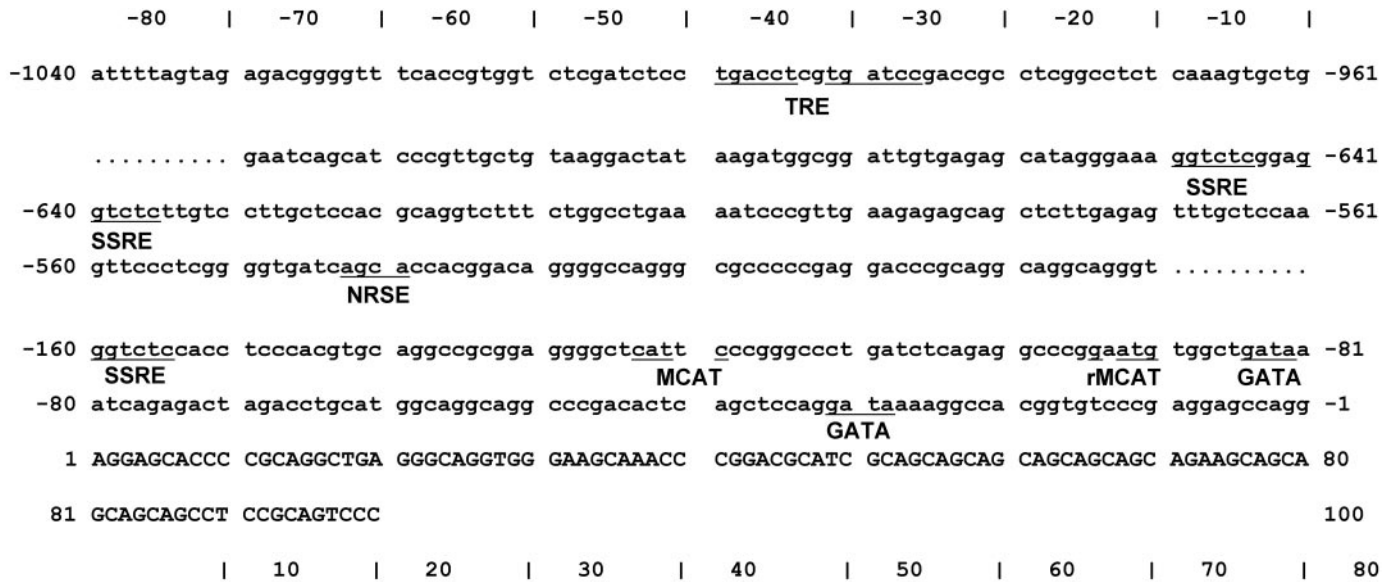
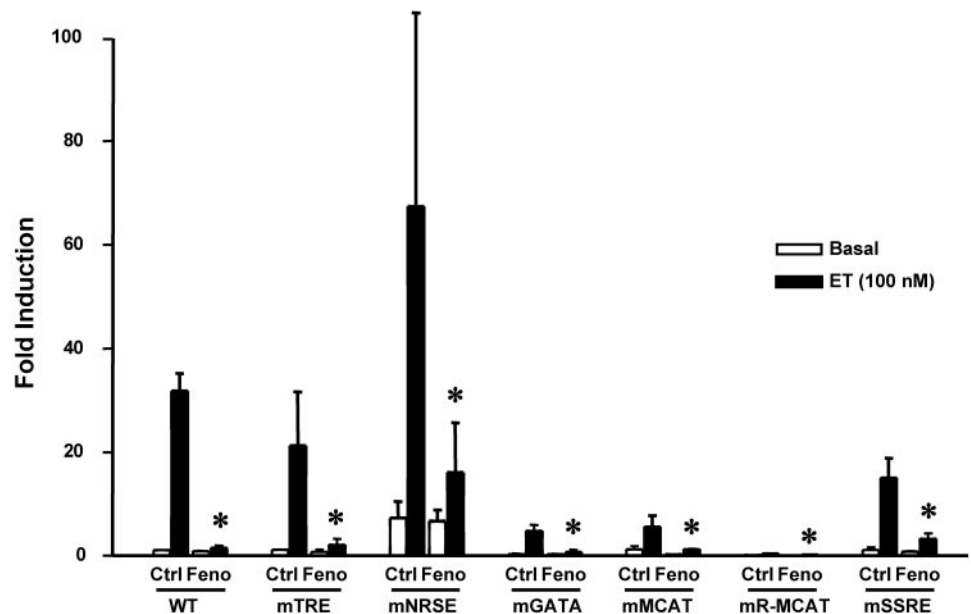


FIG. 8. Schematic diagram depicting putative regulatory elements in the 5' flanking sequence of the hBNP gene. The sequence of each regulatory element is *underlined*. Two GATA sites, two MCAT sites, a NRSE, a TRE, and three SSREs were identified in the hBNP gene promoter.

FIG. 9. Effect of regulatory element mutations on PPAR α -dependent reduction in ET stimulation of hBNP promoter activity. Ventricular myocytes were transfected with 2 μ g of double GATA (mGATA), MCAT (m-MCAT), reverse MCAT (mR-MCAT), NRSE (mNRSE), TRE (mTRE), or triple SSRE (mSSRE) mutant. After 24 h of culture, cells were treated with 10^{-5} M fenofibrate for additional 48 h in the presence or absence of 100 nM ET. Cells were collected and lysates were prepared for luciferase assay. Pooled data from four independent experiments are presented as means \pm SD. *, $P < 0.01$ vs. ET control.



(10), and suppress the growth and activation of cardiac myocytes under conditions leading to myocyte hypertrophy *in vitro* (16, 17) or cardiac hypertrophy *in vivo* (17).

The story with PPAR α is also suggestive of potential benefit in the cardiovascular system. PPAR α is present in the heart (3) and appears to play an important role in the maintenance of myocardial homeostasis, through regulation of target genes involved in the uptake and β -oxidation of fatty acids in cardiac myocytes (see above). Under normal conditions, fatty acids represent a major energy source for working myocytes; however, under conditions associated with myocardial hypertrophy, the heart shifts away from oxidation of fatty acids and toward glucose utilization to supply its energy needs (21). Interestingly, this is accompanied by a

reduction in PPAR α gene expression and activity in the heart (22, 23), suggesting a potential mechanism for the observed shift in energy metabolism and, possibly, for the development of hypertrophy itself. In fact, a recent genetic study identified a link between a common polymorphism in the PPAR α gene and a propensity for the development of physiological and pathological ventricular hypertrophy in humans (37). Our own studies provide some support for this latter hypothesis in that the PPAR α agonists fenofibrate and WY14,643 clearly demonstrated antihypertrophic activity in our cultured myocytes, and this effect was as robust as that seen with the PPAR γ agonist rosiglitazone. It remains unclear whether this antihypertrophic activity reflects a direct action of the liganded PPARs on hypertrophy-sensitive tar-

get genes *vs.* an indirect action of PPARs resulting from the metabolic effects of these agonists on fatty acid and glucose consumption by the myocyte *vs.* a PPAR-independent effect of the ligands (38–40). The latter possibility seems less likely in view of the amplified inhibition seen after cotransfection of the PPAR expression vectors (Fig. 5) and the observed reversal of the rosiglitazone inhibition with GW9662. PPARs have been shown to directly interfere with the activity of activator protein-1, nuclear factor- κ B, and CCAAT/enhancer binding protein- α in other systems (41, 42), findings that favor the direct action hypothesis alluded to above.

Of note, a recent study by Finck *et al.* (43) reported that forced expression of PPAR α in the myocardium resulted in a shift in energy utilization from glucose to fatty acids and a cardiac phenotype that was similar to that seen in diabetes mellitus, implying that chronic PPAR α activation may be detrimental to the myocyte. This finding diverges from those in the current study and may reflect differences in the levels of PPAR activity seen in the *in vivo* paradigm *vs.* our *in vitro* studies, a differential effect of high levels of PPAR α expression in the myocardium during development, or the consequence of chronically high levels of fatty acid oxidation in the PPAR α transgene expressing hearts.

The nature of the antihypertrophic effects of the PPAR ligands parallels those previously described for the liganded vitamin D receptor (44), raising the possibility of a shared mechanism between these two nuclear receptors and contrasting with the prohypertrophic activity of the TR, a third member of this extended nuclear receptor family (34). Like the PPAR agonists, 1,25 dihydroxyvitamin D inhibits protein synthesis, increments in cell size and sarcomeric organization, and BNP, as well as ANP, gene expression in cultured cardiac myocytes (44). Optimal inhibitory activity for the liganded vitamin D receptor requires the ability to pair with its heterodimeric partner RXR (45) and preservation of the activation domain that allows it to associate with coregulatory proteins that signal activation of gene transcription in other systems (46). It remains unknown whether the same structural requirements apply to the PPARs with regard to their antihypertrophic activity.

Interestingly, the PPAR γ antagonist GW9662 increased both basal and ET-stimulated BNP gene promoter activity. This raises the intriguing possibility that endogenous PPAR γ ligands produced in these myocytes may exert a tonic suppression of BNP gene promoter activity. Furthermore, the fact that GW9662 reversed the rosiglitazone-dependent inhibition but not that associated with fenofibrate or WY14,643 indicates that the latter are not operating through the PPAR γ receptor but, presumably, through PPAR α .

As with the liganded vitamin D receptor, the liganded PPARs have yet to be linked to a specific regulatory element on the BNP gene promoter. The current study suggests the presence of an element or elements between –904 and –198 and again between –198 and –40 (90 *vs.* 70 *vs.* 0% inhibition of ET-stimulated hBNP promoter activity by fenofibrate at –904, –198, and –40, respectively). We have identified a number of core elements in the proximal promoter that appear to confer either stimulatory (GATA and MCAT) or inhibitory (NRSE) control on the basal transcription of this gene. The PPAR-dependent inhibition does not appear to

directly involve any of these proximal elements. It does not involve the SSREs shown previously to confer strain sensitivity (35) and at least a component of the response to ET (36) upon this promoter, nor does it involve the TRE that traffics the stimulatory activity of the liganded TR (34). The NRSE, which in the rat BNP promoter has been shown to traffic fibronectin-dependent stimulation of promoter activity (32) and in the rat ANP 3' flanking sequence appears to signal ET-dependent induction of the gene (33), does convey inhibitory activity upon the proximal promoter, but mutation of the site does not abrogate the PPAR α effect. Thus, it appears that further attention will need to be devoted to more novel sites if the locus of PPAR's regulatory activity is to be defined with precision.

In summary, like PPAR γ ligands reported previously (16, 17), PPAR α ligands inhibit agonist-induced hypertrophy of neonatal rat cardiac myocytes in culture. Thus, in addition to their beneficial lipid-lowering properties, these agents may provide a salutary effect directly at the level of the myocardium. Such activity could prove useful in the management of patients with different types and levels of cardiac dysfunction, particularly those with coexistent dyslipidemia or diabetes mellitus.

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