Published in final edited form as:
Arch Biochem Biophys. 2012 February 1; 518(1): 71–78. doi:10.1016/j.abb.2011.11.024.

PPARα activation inhibits endothelin-1-induced cardiomyocyte hypertrophy by prevention of NFATc4 binding to GATA-4

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Abstract

Peroxisome proliferator-activated receptor alpha (PPARα) has been implicated in the pathogenesis of cardiac hypertrophy, although its mechanism of action remains largely unknown. To determine the effect of PPARα activation on endothelin-1 (ET-1)-induced cardiomyocyte hypertrophy and explore its molecular mechanisms, we evaluated the interaction of PPARα with nuclear factor of activated T-cells c4 (NFATc4) in nuclei of cardiomyocytes from neonatal rats in primary culture. In ET-1-stimulated cardiomyocytes, data from electrophoretic mobility-shift assays (EMSA) and co-immunoprecipitation (co-IP) revealed that fenofibrate (Fen), a PPARα activator, in a concentration-dependent manner, enhanced the association of NFATc4 with PPARα and decreased its interaction with GATA-4, in promoter complexes involved in activation of the rat brain natriuretic peptide (rBNP) gene. Effects of PPARα overexpression were similar to those of its activation by Fen. PPARα depletion by small interfering RNA abolished inhibitory effects of Fen on NFATc4 binding to GATA-4 and the rBNP DNA. Quantitative RT-PCR and confocal microscopy confirmed inhibitory effects of PPARα activation on elevation of rBNP mRNA levels and ET-1-induced cardiomyocyte hypertrophy. Our results suggest that activated PPARα can compete with GATA-4 binding to NFATc4, thereby decreasing transactivation of NFATc4, and interfering with ET-1 induced cardiomyocyte hypertrophy.

Keywords

NFATc4; PPARα; Cardiac hypertrophy; GATA-4; Fenofibrate

Introduction

Cardiac hypertrophy is thought to be an adaptive response of the heart to preserve pump function under adverse conditions, and prolonged hypertrophy is a major predictor of arrhythmias and sudden death or heart failure [1–4]. Evidence is increasing that endothelin-1 (ET-1)[1], a 21-amino acid peptide, contributes to the adaptive process by inducing

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transcription of several genes, including atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), and cardiac α- and β-myosin heavy chain [5–10]. Several nuclear factor of activated T-cells (NFAT)-related signaling systems, e.g., calcineurin/NFAT [11], PI3K/Akt/GSK3, NFATc4 [12], are believed to participate in ET-1-induced cardiomyocyte hypertrophy. The calcineurin/NFATc4 pathway has been of special interest for its role in cardiac hypertrophy [13], and reversal of ET-1-induced cardiac hypertrophy by expression of a dominant-negative NFATc4 protein supported an important role of NFATc4 [11].

Peroxisome proliferator-activated receptors (PPARs) are family of nuclear receptor transcription factors that bind specific DNA sequences known as PPAR response elements (PPREs) [14–16]. Among the three PPAR isoforms [17,18], PPARα and PPARγ share 60–80% identity of amino acid sequences in their ligand- and DNA-binding domains [19], and both are present in cardiomyocytes. Evidence suggests that PPARα activation inhibited cardiomyocyte hypertrophy via different pathways; a PPARα activator, fenofibrate (Fen) was reported to suppress ET-1-induced cardiac hypertrophy by down-regulation of AP-1-binding capability and inhibition of p38 signaling [20,21]. Atorvastatin inhibited cardiac hypertrophy through inhibition of negative cross-talk between PPARα and nuclear factor-kappaB (NF-κB) [22], but the molecular mechanism of inhibition of cardiomyocyte hypertrophy by activated PPARα is not clear. In T lymphocytes, PPARγ associated with NFAT to form a complex that inhibited transcription of IL-2 and IL-4 [23,24]. In cardiomyocytes, association of PPARγ with NFATc4 partially inhibited hypertrophy induced by ET-1 [25].

Based on the interaction between PPARγ and NFATc4 in cardiomyocytes, we hypothesized that such a link may also exist between PPARα and NFATc4. To clarify this hypothesis, we employed co-immunoprecipitation (co-IP) and electrophoretic mobility-shift assays (EMSA) to investigate the association of PPARα and NFATc4 in cardiomyocyte nuclei, and in particular, its effects on NFATc4 binding to the rat BNP (rBNP) promoter. We also evaluated the effect of this interaction on the association of NFATc4 with its cofactor GATA-4, and whether it will interfere with ET-1-induced cardiomyocyte hypertrophy.

Materials and methods

Chemicals and antibodies

Fenofibrate, endothelin-1, DMSO, and 5-bromodeoxyuridine were purchased from Sigma–Aldrich, mouse monoclonal antibodies against PPARα (ab2779) from Abcam, rabbit polyclonal antibodies against NFATc4 (sc-13036) or GATA-4 (sc-9053), and normal (rabbit and mouse) IgG from Santa Cruz, mouse monoclonal antibodies against α-tubulin from Sigma–Aldrich, and HRP-conjugated secondary antibodies (goat anti-rabbit and goat anti-mouse) from Promega.

Primary culture and studies of rat cardiomyocytes

Primary cultures of neonatal rat cardiomyocytes were obtained from the hearts of 1- to 3-day-old Sprague-Dawley (SD) rats using the optimized repetitive trypsinization method established in our laboratory [26]. Briefly, after decontaminated with 75% ethanol, the hearts were removed from rats to a Luminer flow hood immediately. The ventricles were excised and chopped into small pieces, then digested with repetitive trypsinization (0.08% trypsin solution). After differential adhesion, cardiomyocytes were seeded in DMEM

[1] *Abbreviations used*: ET-1, endothelin-1; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; NFATc4, nuclear factor of activated T-cells c4; PPARα, peroxisome proliferator-activated receptor alpha; PPREs, PPAR response elements; Fen, fenofibrate; NF-κB, nuclear factor-kappaB; EMSA, electrophoretic mobility-shift assays; co-IP, co-immunoprecipitation; AngII, angiotenin II.

supplied with 10% (vol/vol) fetal bovine serum and 0.1 mM 5-bromodeoxyuridine. All experimental procedures conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and we confirmed that Institutional Ethics Review Board of Sun Yat-sen University approved this study (Approved No. 20080401003). Cardiomyocytes in 6-well plates (2×10^5 cells/ cm^2) were transfected with 4 μg PPARα-EGFP plasmid (kindly provided by Dr. Ruifang Li-Department of Pharmacology, Henan University of Science and Technology, PR China) using 10 μl Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Three 25-nucleotide duplex siRNAs for PPARα (siR-NA169, siRNA168, siRNA167) and non-target siRNA were purchased from Invitrogen. Rat cardiomyocytes were transfected with 100 pmol PPARα-specific or non-target siRNA using 5 μl Lipofectamine 2000 (Invitrogen) and harvested at indicated time thereafter.

Immunoprecipitation and Western blotting

Nuclear extracts were prepared using the CelLytic™ NuCLEAR™ Extraction Kit (Sigma–Aldrich), according to the manufacturer's instructions. Protein concentrations were measured using a BCA Protein Assay Kit (Pierce). Nuclear proteins (200 μg) were immunoprecipitated with anti-PPARα or anti-GATA-4 antibodies or normal (rabbit and mouse) IgG (control). Immunoprecipitated proteins bound to protein G-agarose beads (Pierce) were separated by SDS–PAGE in 8% gels and transferred to PVDF membranes (Millipore), which were blocked with 5% nonfat milk (Bio-Rad), reacted with antibodies against NFATc4, and then with appropriate horse-radish peroxidase-conjugated secondary antibodies. Blots were developed using enhanced chemiluminescence (Pierce) and exposed to X-ray films.

Electrophoretic mobility shift assay

Assays were conducted according to instructions of "The LightShift™ Chemiluminescent EMSA kit" (Pierce). Briefly, nuclear extracts were prepared using the Nuclear Extract Kit (Active Motif), according to the manufacturer's instructions. Sequence of oligonucleotide used for EMSA was 5′-AGGGTGGGAAAACTGGGGGTTC-3′ (3′-biotinylated by Shanghai Sangon) from the region (−330/−351) of rat BNP promoter containing a putative binding site (underlined) for nuclear factor NFATc4 (GGAAAAT) (11). Mutant sequence (without biotinylation) was 5′-AGGGTGGTAGCACTGGGG GTTC-3′. Each 20-μl binding reaction was incubated for 20 min at room temperature with 1 μg of poly (dI.dC), 0.05% Nonidet P-40, 5 mM $MgCl_2$, 2.5% glycerol, 1 pmol of biotin-end-labeled probe DNA, and 4 μg nuclear protein (determined by Bradford assay). For supershift assays, NFATc4 antibodies (2 μg) were incubated (4 °C, 1 h) in binding mixture (20 μl) before addition of the biotinylated probe. Bound complexes and free probe were separated by electrophoresis in6%non-denaturingpolyacrylamidegel at100 Vfor1 h, transferred to positively charged nylon membrane (Biodyne PLUS, supplied by PALL), immediately cross-linked to membrane with UV (254 nm), and detected using Stabilized Streptavidin–Horseradish Peroxidase Conjugate (Pierce), according to the manufacturer's instructions.

RNA isolation and quantitative RT-PCR

Total cell RNA, isolated using TRIzol reagent (Invitrogen), and reverse transcribed, was the template for quantitative PCR using an iCycler iQ system (Bio-Rad) with sequence-specific primer pairs and intercalated SYBR Green (Takara) as fluorescent probe. Results were evaluated using iCycler iQ real-time detection system software (Bio-Rad). Primers were: BNP, 5′-TTTGGGCAGAAGATAGACCG-3′ (forward) and 5′-AGAAGAGCCGCAGGCAGAG-3′ (reverse); GAPDH, 5′-AGGAGTAAGAAACCCTGGAC-3′ (forward) and 5′-CTGGGATGGA ATTGTGAG-3′ (reverse).

Microscopic evaluation

Rhodamine phalloidin (Invitrogen), prepared in methanol according to the manufacturer's specifications and stored at −20 °C, was used to visualize actin fibers by fluorescence microscopy as previously described [27,28]. Cardiomyocytes grown on coverslips were fixed with 4% paraformaldehyde in PBS (15 min, room temperature) and incubated with 0.1% rhodamine–phalloidin plus 0.1% saponin in PBS for 1 h at room temperature. Cells were washed with PBS, mounted in "Prolong Gold Anti-fade Reagent" with DAPI (Invitrogen), and inspected using a confocal microscope (Zeiss 710). The cell surface area of at least 60 cardiomyocytes from 10 randomly selected fields in three independent experiments was measured by a Leica QWin-Plus software (Leica Microsystem, Germany).

Statistical analysis

Data are presented as means ± S.E.M. Multiple group comparisons test was performed with one-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc test with Graphpad Prism Software (Version 5.03). Criterion for statistical significance was $P < 0.05$.

Results

Activated PPARα co-IP of NFATc4 from nuclei of cardiomyocytes

To explore a potential interaction of PPARα with NFATc4 in cardiomyocyte nuclei, proteins immunoprecipitated with anti-PPARα antibodies from nuclear extracts were analyzed by immunoblotting with antibodies against NFATc4 and PPARα, each of which reacted with a single protein band consistent with the molecular size of PPARα (50 kDa) or dephosphorylated NFATc4 (140 kDa). Neither protein was immunoprecipitated with non-immune IgG (Fig. 1A). Incubation of cells for 1 h with 10 μM Fen significantly enhanced IP of NFATc4 with PPARα. The effect of Fen was more significant after incubation of cells for 3 h with 100 nM ET-1, which itself did not alter co-IP of NFATc4 with PPARα (Fig. 1B). Results were similar in cells overexpressing PPARα-EGFP (80 kDa) for 24 h (Fig. 1C).

Increased NFATc4 co-IP with PPARα accompanied by decreased NFATc4–GATA-4 interaction in cardiomyocyte nuclei

Consistent with the report of Kakita et al. [29], our data showed that co-IP of NFATc4 with antibodies against GATA-4 from cardiomyocyte nuclei was significantly ($P < 0.01$) enhanced by incubation cells with 100 nM ET-1, reaching a peak between 1 and 6 h (Fig. 2A). Activation of PPARα by treatment of cardiomyocytes with 10 μM Fen for 1 h, or overexpression of PPARα for 24 h decreased the enhanced interaction of NFATc4 and GATA-4 induced by ET-1. Without ET-1 stimulation, however, neither activation nor overexpression of PPARα affected the association of NFATc4 with GATA-4 (Fig. 2B and C).

Effects of PPARα activation or overexpression on NFATc4 binding to rBNP promoter in cardiomyocytes stimulated by ET-1

Binding of NFATc4 to the target BNP DNA sequence regulates BNP transcription [13]. To determine whether the PPARα/NFATc4 interaction affected this function, we used EMSA to assess DNA binding by NFATc4. In Fig. 3A, binding of NFATc4 to the −330/−351bp region of rat BNP DNA was increased after 100 nM ET-1 treatments, reaching a peak between 1 and 6 h. Without nuclear extract, no retarded band was seen (Fig. 3A, lane1). Incubation of cells 1 h with Fen or overexpression of PPARα (24 h) alone had relatively little effect on NFATc4 binding to DNA. In cells treated with ET-1, however, either Fen or overexpression of PPARα clearly decreased DNA-binding by NFATc4 (Fig. 3B). In competition assays with a 400-fold molar excess of unlabeled (cold) normal (N) and mutant

(M) probes, the former completely abolished the shifted band, whereas the latter was without effect (Fig. 3C). The shifted band was markedly diminished by anti-NFATc4 antibodies, consistent with the specificity of this DNA binding (Fig. 3C).

Effects of Fen concentration on interaction of NFATc4 with GATA-4 and rBNP promoter

As shown in Fig. 4, Fen, in a concentration-dependent manner, enhanced co-IP of NFATc4 with antibodies against PPARα. Inhibition by Fen of co-IP of NFATc4 with GATA-4 and DNA binding to the rBNP promoter, was similarly concentration-dependent, suggesting that in ET-1-induced cardiomyocyte hypertrophy, PPARα and GATA-4 competition for interaction with NFATc4 might result in Fen inhibition of NFATc4 binding to DNA.

Effects of activation or overexpression of PPARα on ET-1-induced elevation of BNP mRNA levels and cardiomyocyte surface area

To evaluate effects of PPARα and NFATc4 interaction on BNP transcription, BNP mRNA was quantified by real-time RT-PCR, showing a significant increase after incubation of cardiomyocytes with 100 nM ET-1, peaking between 12 and 36 h (Fig. 5A). After treatment with Fen (1 h) or overexpression of PPARα (24 h), elevation in BNP mRNA levels induced by ET-1 was significantly suppressed (Fig. 5B), and the increase in cardiomyocyte size seen after 48 h of ET-1 exposure was also much less after activation or overexpression of PPARα. (Fig. 5C and D)

Effects of PPARα depletion on Fen inhibition of ET-1-induced BNP mRNA elevation and cardiomyocyte surface area

To perturb the PPARα interaction with NFATc4, three different duplex siRNAs (167–169) were used to deplete PPARα. Cell lysates were prepared 72 h after siRNA transfection for Western blot analyses, which suggested that siRNA168 was the most effective, with no significant effect of the non-target siRNA (Fig. 6A). We used siRNA168, therefore, in experiments like that shown in Fig. 6, where PPARα depletion blocked the inhibitory effects of Fen on interaction of NFATc4 with GATA-4 (Fig. 6B) and rBNP promoter (Fig. 6C). The decrease in levels of BNP mRNA (Fig. 6D) and cell size caused by Fen in ET-1-stimulated cardiomyocytes were attenuated (Fig. 6E and F) by siRNA-induced depletion of PPARα. These results suggested that interaction of activated PPARα and NFATc4 could diminish binding of NFATc4 to DNA by blocking its association with GATA-4 in ET-1-stimulated cardiomyocytes.

Discussion

PPARs are known widely for their roles in lipid metabolism and inflammation. Agonist ligands for PPARα and PPARγ have been used clinically for the management of dyslipidemia and control of glycemia in patients with type 2 diabetes [30,31]. More recently, attention to pleiotropic effects of these agents has grown with evidence that PPARγ is a negative regulator of cardiomyocyte hypertrophy through its interaction with NFATc4, an important transcription factor that is both necessary and sufficient for development of cardiomyocyte hypertrophy [13,25,32–34]. Proof of PPARα participation in the pathophysiology of hypertensive heart diseases [35–38], suggested that PPARα ligands could be useful beyond their hypolipidaemic effects, in the management of disorders associated with hypertrophy and myocardial remodeling.

Multiple signaling systems function as downstream effectors of ET-1 [39–42], including the calcineurin/NFAT pathway, which is important for cardiomyocyte hypertrophy [13]. In a current model for the calcineurin/NFATc4 pathway in cardiomyocyte hypertrophy, NFATc4 is usually hyperphosphorylated and sequestered in the cytoplasm. Actions of ET-1,

angiotenin II (AngII), and possibly other hypertrophic stimuli lead to elevation of intracellular Ca^{2+} and activation of cytoplasmic calcineurin. Activated calcineurin dephosphorylates NFATc4, resulting in its translocation to the nucleus, where it interacts with molecules such as GATA-4 to bind to a target sequence in the promoter of hypertrophic genes and trigger gene transcription [13,43].

Here, we identified by co-IP a interaction of PPARα and NFATc4 in nuclei of cardiomyocytes. This interaction was enhanced by Fen, an activator of PPARα, or by overexpression of PPARα-EGFP. Association of NFATc4 with PPARα was significantly increased by Fen treatment of ET-1-stimulated cardiomyocytes, suggesting a novel mechanism of action of PPARα in cardiomyocyte hypertrophy induced by ET-1 through the NFATc4 pathway. To understand better how PPARα regulated NFATc4 function, we investigated two important actions of NFATc4 on binding to its target gene and its interaction with cofactors.

It had been reported that the binding of NF-AT3 (analogous to NFATc4) at a position −927 nucleotides upstream of the human BNP gene, which is a marker of cardiac hypertrophy and heart failure, was involved in the activation of the promoter [13,44]. Promoter analysis based on transcription factor-binding sites, revealed a putative binding site for NFATc4 in region −330 to −351bp of the rat BNP promoter; EMSA confirmed this. Although a supershifted band was not observed with anti-NFATc4 antibodies, the shifted band was markedly diminished by the antibodies. One possible reason for the lack of a supershifted band was shielding the DNA binding site after NFATc4 binding with the antibody. These data are consistent with the findings of Zhu et al. [45]. We showed that binding of NFATc4 to this site was increased in a time-dependent manner by ET-1 treatment. The enhanced binding was clearly decreased by activation or overexpression of PPARα, suggesting that interaction of NFATc4 with PPARα interfered with its binding to the BNP promoter.

GATA-4, a zinc-finger transcription factor was an important role in cardiac hypertrophy [46–49]. GATA-4 also acted synergistically with NF-AT3 to activate the BNP promoter in cardio myocytes [13]. Consistent with the report of Kakita et al. that ET-1 translocated NFATc into nuclei and enhanced its interaction with GATA-4 [24], the association of NFATc4 and GATA-4 was significantly increased in nuclei of cardiomyocytes after ET-1 stimulation. The interaction between NFATc4 and GATA-4 was markedly decreased by PPARα activation or overexpression, indicating the interaction of NFATc4 with PPARα decreased its association with GATA-4. Our data also showed that Fen or overexpression of PPARα significantly attenuated increases in BNP mRNA and cardiomyocyte size induced by ET-1, consistent with a significant contribution of PPARα to ET-1-induced BNP transcription and cardiomyocyte hypertrophy.

In addition, using siRNA to deplete cells of PPARα (without affecting PPARβ/δ or PPARγ), we showed that PPARα siRNA failed to block the enhanced interaction of NFATc4 with BNP promoter or GATA-4, resulting from Fen and ET-1 co-stimulation. Quantitative RT-PCR and confocal microscopy similarly confirmed the effects of PPARα siRNA on elevation of BNP mRNA content and cardiomyocyte hypertrophy.

Although the mechanism by which NFATc4 binding to PPARα decreased its binding to GATA-4 remains to be determined, it is possible that the two molecules compete for the same binding site. Molkentin et al. [13] reported that NF-AT3 interacted with GATA-4 through its Rel-homology domain, which also mediated DNA binding. In our study, Fen activation of PPARα apparently enhanced its interaction with NFATc4 while, in a concentration-dependent manner, decreasing NFATc4 interactions with GATA-4 and the

BNP promoter (Fig. 4), consistent with the notion that PPARα and GATA-4 competed for binding to the Rel-homology domain of NFATc4.

Overall, our data fit a model (Fig. 7) in which PPARα can participate in transcription complexes with NFATc4 that interfere with an NFATc4–GATA-4 interaction in cardiomyocytes, thereby decreasing its transactivation potential and preventing induction of cardiomyocyte hypertrophy by ET-1. These findings provide novel mechanistic insight into a role for PPARα in cardiac hypertrophy, and suggest that interference with interactions of nuclear transcription factors could be a useful therapeutic approach to prevent cardiac hypertrophy.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 81072641); NSFC–CIHR China–Canada Joint Health Research Initiative Proposal (No. 30811120434); the National Science and Technology Major Project of China “Key New Drug Creation and Manufacturing Program” (No. 2009ZX09102-152, 2011ZX09401-307); Major Project of Guangdong Province (2008A030201013) Major Project of Guangz-hou City (2008Z1-E571), and Intramural Research Program, National Institutes of Health, NHLBI (J.M.). We thank Dr. Martha Vaughan (Cardiovascular and Pulmonary Branch, NHLBI, National Institutes of Health) for valuable discussions and manuscript review. We extend our gratitude to all members of the Department of Pharmacology and Toxicology for technical assistance.

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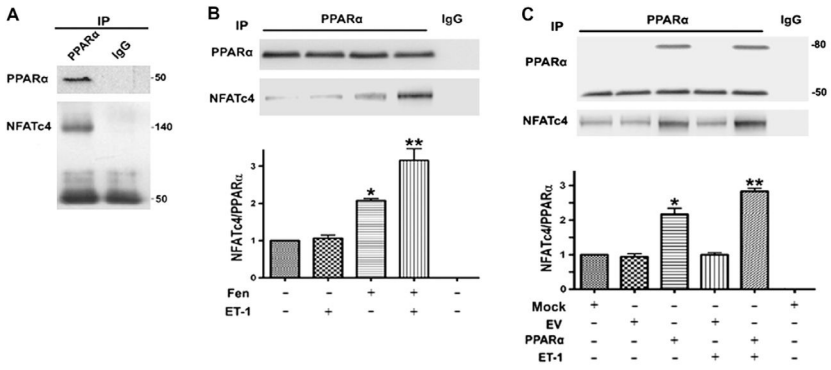

Fig. 1.

Activation or overexpression of PPARα enhanced the association of NFATc4 with PPARα in the nuclei of cardiomyocytes. (A) Proteins precipitated from nuclei of rat cardiomyocytes with antibodies against PPARα or control mouse IgG were separated in 8% gels before Western blotting with antibodies against PPARα or NFATc4. Data were similar in two other experiments. (B) Cardiomyocytes were incubated without or with 10 μM fenofibrate (Fen) 1 h and/or 100 nM ET-1 3 h, before IP of nuclear proteins with antibodies against PPARα. Proteins separated by PAGE were quantified by densitometry of Western blots. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.01$ vs. no additions, $^{**}P < 0.01$ vs. Fen alone. (C) For overexpression of PPARα-EGFP (80 kDa), cardiomyocytes were incubated 24 h with vehicle (Mock), empty vector (EV), or PPARα-EGFP (PPARα), followed by 3 h without or with 100 nM ET-1 as indicated. Proteins precipitated from nuclei with antibodies against PPARα were separated and analyzed as in Fig. 1B. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. Mock, $^{**}P < 0.01$ vs. PPARα.

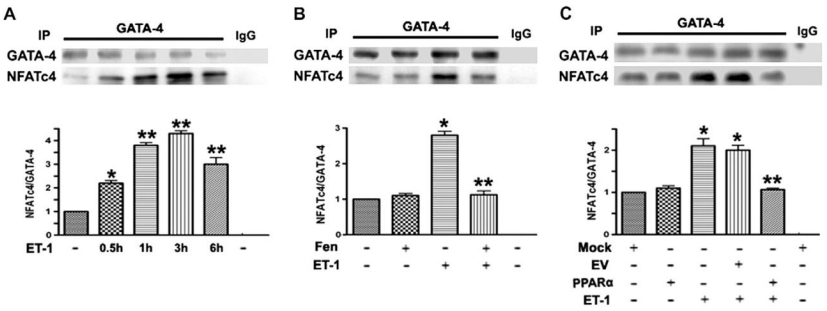

Fig. 2.

Activation or overexpression of PPARα inhibited the interaction between NFATc4 and GATA-4. (A) Cardiomyocytes were incubated without or with 100 nM ET-1 for indicated times, before IP of nuclear proteins with GATA-4 antibodies or control rabbit IgG. Proteins were separated in 8% gels before Western blotting with antibodies against NFATc4 and GATA-4. Data are means ± S.E.M. of values from three independent experiments. $^{*}P <$ 0.01, $^{**}P < 0.001$ vs. no additions. (B) Cardiomyocytes were incubated without or with 10 μM Fen (1 h) and/or 100 nM ET-1 (3 h), before IP of nuclear proteins with antibodies against GATA-4. Proteins separated by PAGE were quantified by densitometry of Western blots. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1. (C) In studies involving PPARα overexpression, cardiomyocytes were incubated for 24 h with vehicle, empty vector, or PPARα-EGFP, followed by 3 h without or with 100 nM ET-1 as indicated. Proteins precipitated from nuclei with antibodies against GATA-4 were separated and analyzed as in Fig. 2B. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. Mock, $^{**}P < 0.001$ vs. ET-1 + EV.

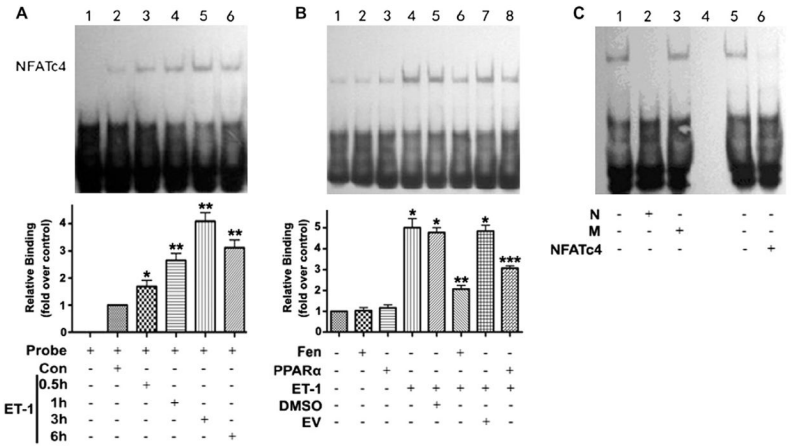

Fig. 3.

The binding activity of NFATc4 to rBNP promoter (−330/−351bp) was decreased by PPARα activation or overexpression in the nuclei of cardiomyocytes. (A) Cardiomyocytes were incubated without or with 100 nM ET-1 for 0.5, 1, 3, or 6 h as indicated before EMSA. Con (control) means without ET-1. The amounts of NFATc4–DNA complexes were quantified by densitometry. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. no additions. (B) EMSA was used to assess binding of NFATc4 with probe in cardiomyocytes treated as described in Fig. 1. The amounts of NFATc4–DNA complexes were quantified by densitometry. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1 + DMSO, $^{***}P < 0.001$ vs. ET-1 + EV. (C) Effects of unlabeled (cold) probes or NFATc4 antibodies on EMSA. Cold normal (N) or mutant (M) probe was present at 400-fold the concentration of labeled probe, where indicated. Supershift assay were performed by preincubating the nuclear extracts with anti-NFATc4 antibodies (NFATc4). Data were similar in two other experiments.

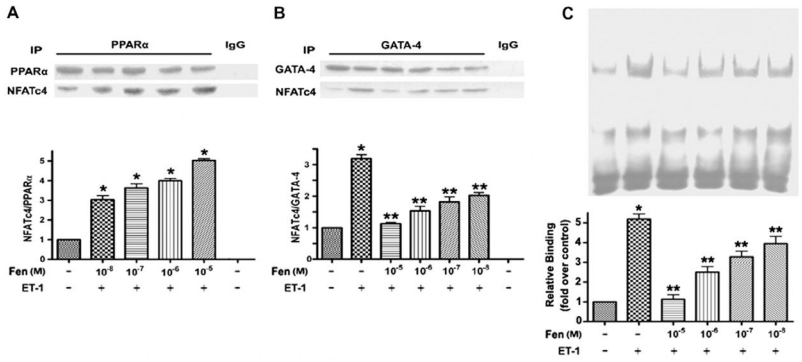

Fig. 4.

Fen inhibited the co-IP of NFATc4 with GATA-4 and DNA binding to the rBNP promoter in a concentration-dependent manner. Cells were incubated for 1 h with the indicated concentration of Fen, followed by 3 h with 100 nM ET-1 before IP with anti-PPARα antibodies or control IgG, for Western blotting of proteins with antibodies against NFATc4 or PPARα and densitometric quantitation. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions. (B) Cardiomyocytes were incubated with Fen and/or ET-1 as in Fig. 4A, before IP with antibodies against GATA-4 or control IgG, for Western blotting of proteins with antibodies against NFATc4 or GATA-4, and densitometric quantitation. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1. (C) EMSA contained nuclear extracts from cardiomyocytes incubated with Fen and/or ET-1. The amounts of NFATc4-DNA complexes were quantified by densitometry. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1.

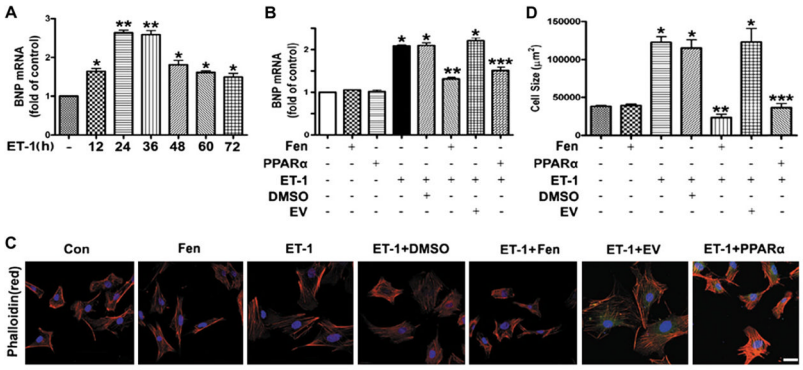

Fig. 5.

Activation or overexpression of PPARα attenuated ET-1 induced BNP mRNA upregulation and cardiomyocyte hypertrophy. (A) Levels of BNP mRNA in rat cardiomyocytes treated with 100 nM ET-1 for indicated times were assessed by quantitative RT-PCR. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ vs. no additions. (B) Total RNA was extracted from cardiomyocytes for BNP mRNA detection after 24 h of indicated treatment. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1 + DMSO, $^{***}P < 0.001$ vs. ET-1 + EV. (C) and (D) Cardiomyocytes after 48 h of indicated treatment were stained with rhodamine–phalloidin (C), followed by cell surface area quantitation (D). Scale bar, 20 μm. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1 + DMSO, $^{***}P < 0.001$ vs. ET-1 + EV.

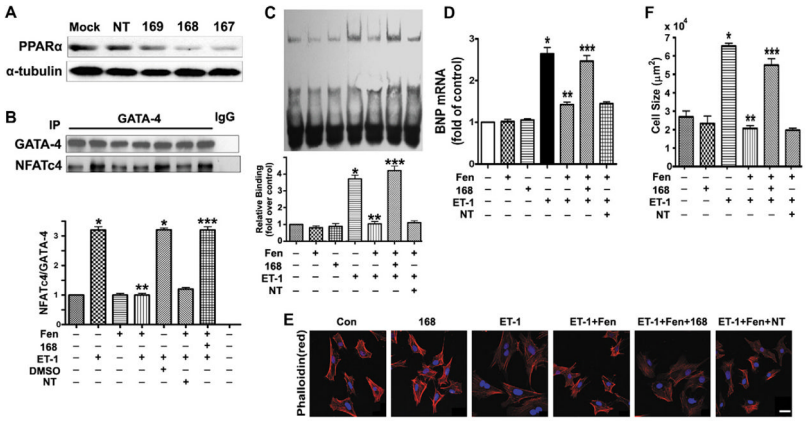

Fig. 6.

PPARα depletion blocked the inhibitory effects of fenofibrate on ET-1 induced BNP mRNA upregulation and cardiomyocyte hypertrophy. (A) Lysates of cardiomyocytes prepared after incubation for 72 h with vehicle alone (Mock), or with non-target siRNA (NT), or one of three (167, 168, 169) PPARα-directed siRNAs were analysed by Western blotting with α-tubulin as loading control. (B) IP with anti-GATA-4 antibodies or control IgG of the nuclear extracts of cardiomyocytes 72 h after siRNA transfection followed by indicated treatments were immunoblotted with antibodies against NFATc4 and GATA-4, and followed by densitometric quantitation. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1, $^{***}P < 0.001$ vs. ET-1 + Fen. (C) EMSA was performed with nuclear extracts of cardiomyocytes that had been treated as indicated to assess differences in association of NFATc4 with BNP promoter. The amounts of NFATc4–DNA complexes were quantified by densitometry. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1, $^{***}P < 0.001$ vs. ET-1 + Fen. (D) Total RNA from cardiomyocytes was template for quantitation of BNP mRNA 72 h after siRNA transfection followed by indicated treatments. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1, $^{***}P < 0.001$ vs. ET-1 + Fen. (E) and (F) Cardiomyocytes 72 h after siRNA transfection following indicated treatments were stained with rhodamine–phalloidin (E), followed by surface area quantitation (F). Scale bar, 20 μm. Data are means ± S.E.M. of values from three independent experiments. $^{*}P < 0.001$ vs. no additions, $^{**}P < 0.001$ vs. ET-1, $^{***}P < 0.001$ vs. ET-1 + Fen.

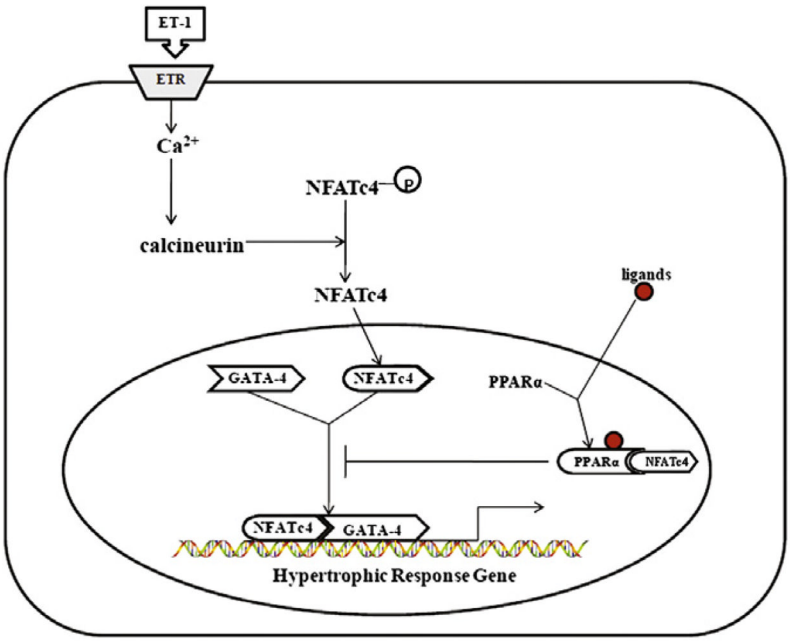

Fig. 7.

A model for the function of PPARα activation on cardiomyocyte hypertrophy induced by ET-1. Activated PPARα associates with activated NFATc4 induced by ET-1 in the nucleus to prevent the interaction of NFATc4 with GATA-4, and further reduce the binding of NFATc4 to the promoter of hypertrophic response genes, and thereby inhibit cardiomyocyte hypertrophy.