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Peroxisome proliferator-activated receptor β/δ activation inhibits hypertrophy in neonatal rat cardiomyocytes

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Abstract

Objective: Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism. However, the role of PPARβ/δ activators in cardiac hypertrophy is not yet known.

Methods and Results: In cultured neonatal rat cardiomyocytes, the selective PPARβ/δ activator L-165041 (10 μmol/L) inhibited phenylephrine (PE)-induced protein synthesis ([^{3}H]leucine uptake), induction of the fetal-type gene atrial natriuretic factor (ANF) and cardiac myocyte size. Induction of cardiac hypertrophy by PE stimulation also led to a reduction in the transcript levels of both muscle-type carnitine palmitoyltransferase (50%, $P<0.05$) and pyruvatedehydrogenase kinase 4 (30%, $P<0.05$), and these changes were reversed in the presence of the PPARβ/δ agonist L-165041. Stimulation of neonatal rat cardiomyocytes with PE and embryonic rat heart-derived H9c2 cells with lipopolysaccharide (LPS) enhanced the expression of the nuclear factor (NF)-κB-target gene monocyte chemoattractant protein 1 (MCP-1). The induction of MCP-1 was reduced in the presence of L-165041, suggesting that this compound prevented NF-κB activation. Electrophoretic mobility shift assay (EMSA) revealed that L-165041 significantly decreased LPS-stimulated NF-κB binding activity in H9c2 myotubes. Finally, coimmunoprecipitation studies showed that L-165041 strongly enhanced the physical interaction between PPARβ/δ and the p65 subunit of NF-κB, suggesting that increased association between these two proteins is the mechanism responsible for antagonizing NF-κB activation by PPARβ/δ activators.

Conclusion: These results suggest that PPARβ/δ activation inhibits PE-induced cardiac hypertrophy and LPS-induced NF-κB activation.

Keywords: Cardiac hypertrophy; NF-κB; L-165041; p65

This article is referred to in the Editorial by N.N. Petrashevskaya and A. Schwarz (pages 770–771) in this issue.

Cardiac hypertrophy is a response of the heart to a wide range of extrinsic stimuli, such as arterial hypertension, valvular heart disease, myocardial infarction and hypertrophic cardiomyopathy. Although this process is initially compensatory for an increase workload, its prolongation frequently results in congestive heart failure, arrhythmia and sudden death [1,2]. Among the signal transduction pathways involved in the hypertrophic growth of the myocardium, the nuclear factor (NF)-κB signaling pathway plays a pivotal role, since it has been shown that NF-κB inhibition blocks or attenuates the hypertrophic response of cultured cardiac myocytes [3–6]. In addition, cardiac hypertrophy is associated with an increase in glucose utilization and a decrease in fatty acid oxidation, which is

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characteristic of fetal heart [7,8]. Defects in mitochondrial fatty acid oxidation enzymes cause childhood hypertrophic cardiomyopathy [9], and perturbation of fatty acid oxidation in animal models causes cardiac hypertrophy [10,11], demonstrating that substrate utilization is important in the pathogenesis of hypertrophy. Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the expression of genes involved in fatty acid uptake and oxidation, lipid metabolism and inflammation [12]. The PPAR subfamily consists of three subtypes, PPARα (NR1C1 according to the unified nomenclature system for the nuclear receptor superfamily), PPARβ (also known as PPARδ) (NR1C2) and PPARγ (NR1C3) [13]. PPARα is expressed primarily in tissues that have a high level of fatty acid catabolism such as liver, brown fat, kidney, heart and skeletal muscle [14]. PPARβ/δ is ubiquitously expressed, and PPARγ has a restricted pattern of expression, mainly in white and brown adipose tissues, whereas other tissues such as skeletal muscle and heart contain limited amounts. In order to be transcriptionally active, PPARs need to heterodimerize with the 9-*cis* retinoic acid receptor (RXR) (NR2B). PPAR-RXR heterodimers binds to DNA specific sequences called peroxisome proliferator-response elements (PPREs), consisting of an imperfect direct repeat of the consensus binding site for nuclear hormone receptors (AGGTCA) separated by one nucleotide (DR-1). These sequences have been characterized within the promoter regions of PPAR target genes. However, the regulation of gene transcription by PPARs extends beyond their ability to transactivate specific target genes. PPARs are also able of regulating gene expression independently of binding to DNA through a mechanism termed receptor-dependent *trans*repression [15]. One of these mechanisms involves a physical interaction of PPARα with NF-κB, leading to suppression activity of the latter [16].

It has been demonstrated that, of the three PPAR subtypes, activation of both PPARα [17,18] and PPARγ [19,20] results in inhibition of cardiac hypertrophy. However, the role of PPARβ/δ in the development of this process is unknown. The recent availability of specific synthetic ligands for PPARβ/δ, such as L-165041, now makes possible to study the role of this nuclear receptor in cardiac cells. Thus, recently, Gilde et al. [21], using neonatal rat cardiomyocytes as well as the embryonic rat heart-derived H9c2 cells, clearly demonstrated that PPARβ/δ is the predominant PPAR subtype in cardiac cells and plays a prominent role in the regulation of cardiac lipid metabolism, suggesting that PPARβ/δ, similarly to PPARα and γ, may play an important role in cardiac disease.

In this study, we examined the role of PPARβ/δ activation in phenylephrine (PE)-induced hypertrophy in neonatal rat cardiac myocytes and in lipopolysaccharide (LPS)-estimulated H9c2 myotubes. We found that activation of PPARβ/δ inhibits PE-induced hypertrophy and LPS-induced NF-κB activation.

1. Methods

1.1. Materials

L-165041 was synthesized according to Berger et al. [22]. [γ-^{32}P]dATP (3000 Ci/mmol) and [^{3}H]leucine (50 Ci/mmol) were purchased from Amersham Pharmacia Biotech KK. Anti-atrial natriuretic factor (ANF) polyclonal antiserum was from Peninsula Laboratories and Alexa flouro 488 goat anti-rabbit and 568 goat anti-mouse antibodies were from Molecular Probes. All other chemicals were purchased from Sigma.

1.2. Cell culture

Neonatal rat ventricular myocytes from 1- to 2-day-old Sprague–Dawley rats were prepared and cultured overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously [23]. The media was changed to serum-free DMEM supplemented with transferrin (10 μg/mL), insulin (1 μg/mL) and bromodeoxyuridine (0.1 mmol/L) 24 h before treatments. In this study, PE was used to stimulate neonatal rat cardiomyocytes. Animal handling and disposal were performed in accordance with NIH guidelines.

The embryonic rat-heart derived H9c2 cells (ATCC) were maintained in growth medium composed of DMEM supplemented with 10% fetal bovine serum. H9c2 cells were plated at a density of 5000 cells/cm^2 and allowed to proliferate in growth medium. Medium was changed every 3 days. To induce differentiation of H9c2 myoblasts into myotubes, growth medium was replaced with differentiation medium (DMEM containing 2% horse serum) when cells had reached near confluence. For mRNA analysis H9c2 cells were treated with 10 μmol/L L-165041 and LPS (10 ng/mL) for 24 h.

1.3. Incorporation of [^{3}H]leucine

To examine the effect of PE on protein synthesis, the incorporation of [^{3}H]leucine was measured essentially by the method of Thaik et al. [24]. Cultured neonatal rat ventricular myocytes were treated with PE in the presence or in the absence of L-165041 and coincubated with [^{3}H]leucine (1 μCi/mL) for 24 h. The cells were washed with PBS and then treated with 10% trichloroacetic acid at 4 °C for 30 min to precipitate the proteins. The precipitates were then dissolved in NaOH (0.25 N). Aliquots were counted with scintillation counter.

1.4. Immunocytochemistry

Neonatal rat ventricular myocytes were fixed in ice-cold 100% methanol for 10 min. Anti-α-actinin antibody and anti-ANF polyclonal antiserum were added at dilutions 1:400 and 1:150, respectively, in PBS containing 1% BSA and incubated for 1 h at room temperature. Secondary antibodies,

Alexa flouro 488 goat anti-rabbit and Alexa flouro 568 goat anti-mouse, were used at a dilution of 1:300 in PBS containing 5% rat serum and incubated for 30 min at room temperature. Immunofluorescence was visualized using a confocal laser fluorescence microscope Olympus Fluoview FV500. Photographic images were taken from five random fields.

1.5. RNA preparation and analysis

Relative levels of specific mRNAs were assessed by the reverse transcription–polymerase chain reaction (RT–PCR) as previously described [25]. The sequences of the sense and antisense primers used for amplification were: ANF, 5′-TCCTCTTCCTGGCCTTTTGGC-3′ and 5′-

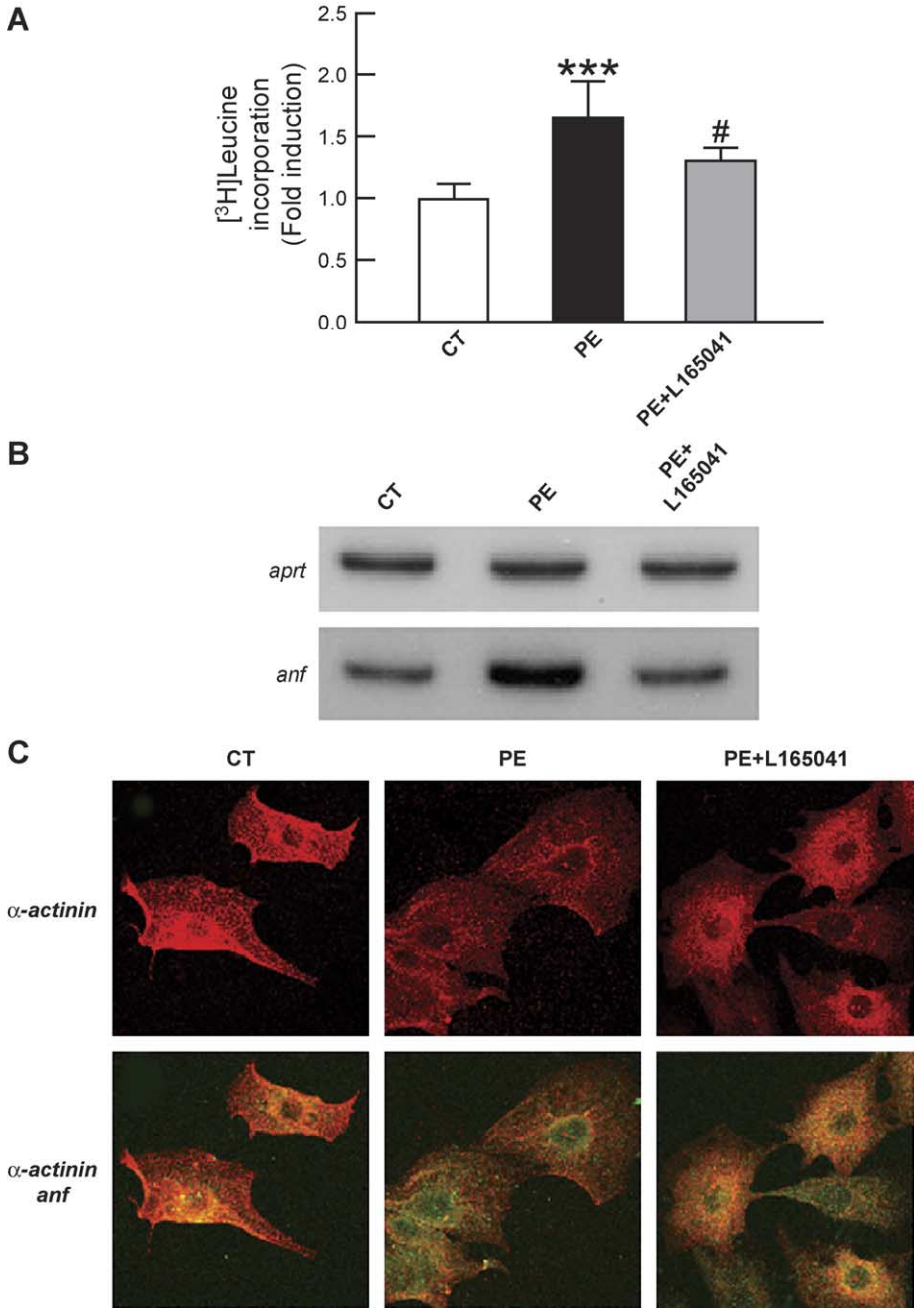

Fig. 1. The PPARβ/δ activator L-165041 inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes. Cardiac myocytes were stimulated with 100 μmol/L PE in the presence or absence of 10 μmol/L L-165041 that was added 30 min before experiments. (A) [^{3}H]leucine incorporation was determined by coincubating cardiac myocytes with 1.0 μCi/mL [^{3}H]leucine for 24 h. Data are expressed as mean±S.D. (n=6) of the treated-to-control ratio. (B) Analysis of the mRNA levels of ANF in PE-stimulated cardiomyocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram is shown. (C) Effects of PE with and without L-165041 on cardiac myocyte ANF protein expression and cardiac myocyte size. Double immunofluorescent microscopy was performed using specific antibodies to α-actinin (upper panel, red color) and ANF (lower panel, green color). Experiments were performed three times with similar results. ***P<0.001 vs. control. $^{\#}P$<0.05 vs. PE-stimulated cardiac myocytes.

AGACGGGTTGCTTCCCCAGTC-3′; gp91, 5′-CACCTGCAGCCTGCCTGAATT-3′ and 5′-ATGGTGTGAATGGCGGTGTGA-3′; inducible nitric oxide synthase (iNOS), 5′-GCATGGACCAGTATAAGGCAAGCA-3′ and 5′-GCTTCTGGTCGATGTCATGAGCAA-3′; malonyl-CoA decarboxylase (MCD), 5′-TACGGTGAGAAGCACCGAGGC-3′ and 5′-GGGGCCTGTCTCCTCCAGGTA-3′; monocyte chemoattractant protein 1 (MCP-1), 5′-GGGCCTGTTGTTCACAGTTGC-3′ and 5′-GGGACACCTGCTGCTGGTGAT-3′; muscle-type carnitine palmitoyltransferase (M-CPT-I), 5′-TTCACTGTGACCCCAGACGGG-3′ and5′-AATGGACCAGCCCCATGGAGA;pyruvatedehydrogenase kinase 4 (PDK-4), 5′-GAACACCCCTTCCGTCCAGCT-3′ and 5′-TGTGCCATCGTAGGGACCACA-3′; PPARγ coactivator-1 (PGC-1), 5′-AGAAAGGGCCCGAGCAATCTG-3′ and 5′-AGATGTGCCCCTGCCAGTCAC-3′; p22, 5′-CCCCGGGGAAAGAGGAAAAAG-3′ and 5′-GGATGGCTGCCAGCAGGTAGA-3′; and APRT (adenosyl phosphoribosyl transferase), 5′-GCCTCTTGGCCAGTCACCTGA-3′ and 5′-CCAGGCTCACACACTCCACCA-3′. Amplification of each gene yielded a single band of the expected size (ANF: 234 bp, gp91: 200 bp, iNOS: 198 bp, MCD: 231 bp, MCP-1: 157 bp, M-CPT-I: 222 bp, PDK-4: 168 bp, PGC-1: 234 bp, p22: 215 bp and APRT: 329 bp). The results for the expression of specific mRNAs are always presented relative to the expression of the control gene (*aprt*).

1.6. Immunoblotting

Cell lysates and nuclear extracts from H9c2 cells were obtained as previously described [25]. Proteins (50 μg) were separated by SDS–PAGE on 10% separation gels and transferred to Immobilon polyvinylidene diflouride mem-

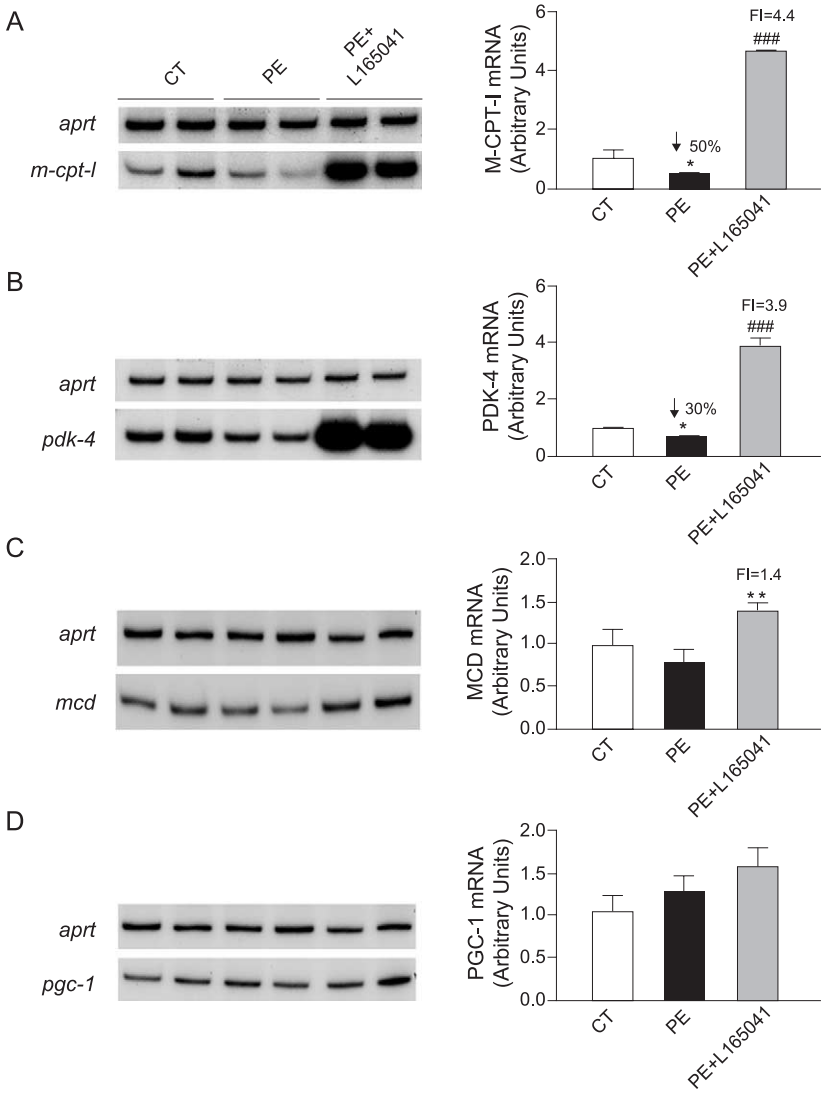

Fig. 2. L-165041 prevents downregulation of the expression of several genes involved in fatty acid lipid metabolism in PE-stimulated neonatal rat cardiomyocytes. Analysis of the mRNA levels of M-CPT-I (A), PDK-4 (B), MCD (C) and PGC-1 (C) in PE-stimulated cardiac myocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean±S.D. of five different experiments. *$P<0.05$ and **$P<0.01$ vs. control. $^{###}P<0.001$ vs. PE-stimulated cardiac myocytes.

branes (Millipore, Bedford, MA). Western blot analysis was performed using antibodies against IκBα, IκBβ, p65 and PPARβ/δ (Santa Cruz Biotechnology) and β-tubulin (Sigma). Detection was achieved using the EZ-ECL chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel). Size of detected proteins was estimated using protein molecular-mass standards (Life Technologies).

1.7. Electrophoretic mobility shift assay (EMSA)

H9c2 cells were pretreated with 10 μmol/L L-165041 for 24 h before stimulation with LPS (10 ng/ml) for 1 h. Isolation of nuclear extracts and EMSA were performed as previously described [25].

1.8. Coimmunoprecipitation

Cell nuclear extracts were brought to a final volume of 0.5 mL with buffer containing 10 mM PBS, 50 mM KCl, 0.05 mM EDTA, 2.5 mM $MgCl_2$, 8.5% glycerol, 1 mM dithiothreitol, 0.1% Triton X-100, BSA 2% and 1 mg/ml nonfat milk for 6 h at 4 °C and incubated with 4 μg of anti-p65. Immunocomplex were captured by incubating the samples with protein A-agarose suspension overnight at 4 °C on a rocker platform. Agarose beads were collected by centrifugation and washed three times with PBS containing protease inhibitors. After microcentrifugation, the pellet was washed with 60 μl of SDS–PAGE sample buffer and boiled for 5 min at 100 °C. An aliquot of the supernatant was subjected to electrophoresis on 10% SDS–PAGE and immunoblotted with an antibody against PPARβ/δ.

1.9. Statistical analyses

Results were obtained from at least four independent experiments and presented as mean±S.D. Comparisons between groups were performed with one-way ANOVA using the computer program GraphPad Instat (GraphPad Softwware, San Diego, CA). When significant variations were found, the Tukey–Kramer multiple comparisons test was performed. Differences were considered significant at $P<0.05$.

2. Results

2.1. PPARβ/δ activation by L-165041 inhibits PE-induced cardiac hypertrophy in neonatal rat cardiomyocytes

Cardiac hypertrophy leads to significant increases in protein content (e.g., [^{3}H]leucine uptake), induction of fetal-type genes (e.g., ANF) and cardiac myocyte size [26]. Therefore, we first examined the effects on these parameters of the specific PPARβ/δ agonist L-165041, which was previously shown to be selective for this PPAR-subtype at 10 μmol/L [27]. The cells were pretreated with either vehicle or L-165041 for 30 min and subsequently stimulated with 100 μmol/L PE for 24 h. As shown in Fig. 1A, [^{3}H]leucine incorporation was significantly increased by PE (1.6-fold, $P<0.001$) and this was inhibited by L-165041 (−20%, $P<0.05$). PE-induced cardiomyocyte hypertrophy also led to approximately twofold induction in the mRNA levels of the fetal cardiac gene ANF (Fig. 1B). In contrast, in the presence of L-165041 PE-induced ANF expression was abolished. Immunostaining of cardiac myocytes for the sarcomere-associated protein α-actinin and ANF clearly shown an increase in cardiac myocyte size and ANF protein expression following PE stimulation (Fig. 1C). These changes were blocked by the presence of the PPARβ/δ agonist L-165041.

2.2. Treatment with the PPARβ/δ agonist L-165041 prevents the reduction in the expression of genes involved in lipid metabolism caused by PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes

An important molecular adaptation in cardiac hypertrophy is the increase in glucose utilization and decrease in fatty acid oxidation associated to a downregulation of the expression of the mRNA levels of genes involved in fatty acid metabolism [8]. Interestingly, PPARβ/δ activates the expression of several PPAR target genes involved in fatty acid utilization in cardiac myocytes [21], including M-CPT-I, which determines the flux of mitochondrial β-oxidation [28], and PDK-4, which suppresses glucose

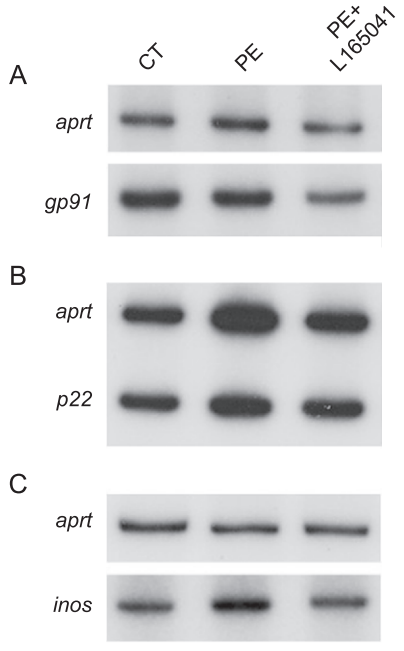

Fig. 3. Effects of L-165041 of the expression of NADPH oxidase subunits and iNOS. Analysis of the mRNA levels of gp91 (A), p22 (B) and iNOS (C) in PE-stimulated cardiac myocytes in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram is shown. Experiments were performed three times with similar results.

oxidation by its inhibitory effect on the pyruvate dehydrogenase complex leading to an increase in fatty acid utilization [29]. Taking into account these data, we next assessed the effects of PE-induced cardiomyocyte hypertrophy on the mRNA levels of these genes in the presence or in the absence of L-165041. Induction of cardiomyocyte hypertrophy by PE led to a reduction in the transcript levels of both M-CPT-I (50%, $P<0.05$) and PDK-4 (30%, $P<0.05$). In contrast, in the presence of the PPARβ/δ agonist, PE did not reduce the levels of these genes and even a robust induction (about fourfold, $P<0.001$) was observed compared to control values (Fig. 2A and B). PE treatment did not affect the mRNA levels of MCD and PGC-1, two genes involved in lipid metabolism, whereas coincubation with L-165041 caused a significant increase in MCD expression (Fig. 2C and D).

2.3. Treatment with L-165041 inhibits the upregulation of MCP-1 caused by PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes

PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes is mediated through NF-κB activation via the generation of reactive oxygen species (ROS) [4]. NADPH oxidase is one of the systems generating ROS whose expression is increased in cardiac hypertrophy [30]. We therefore evaluated whether PPARβ/δ activation by L-165041 affected the expression of the NADPH oxidase subunits gp91 and p22 (Fig. 3A and B). No changes were observed in the mRNA levels of these genes in neonatal rat cardiomyocytes, making unlikely that reduced NADPH oxidase expression may account for the antihypertrophic effect of L-165041. Similarly, L-165041 treatment did not

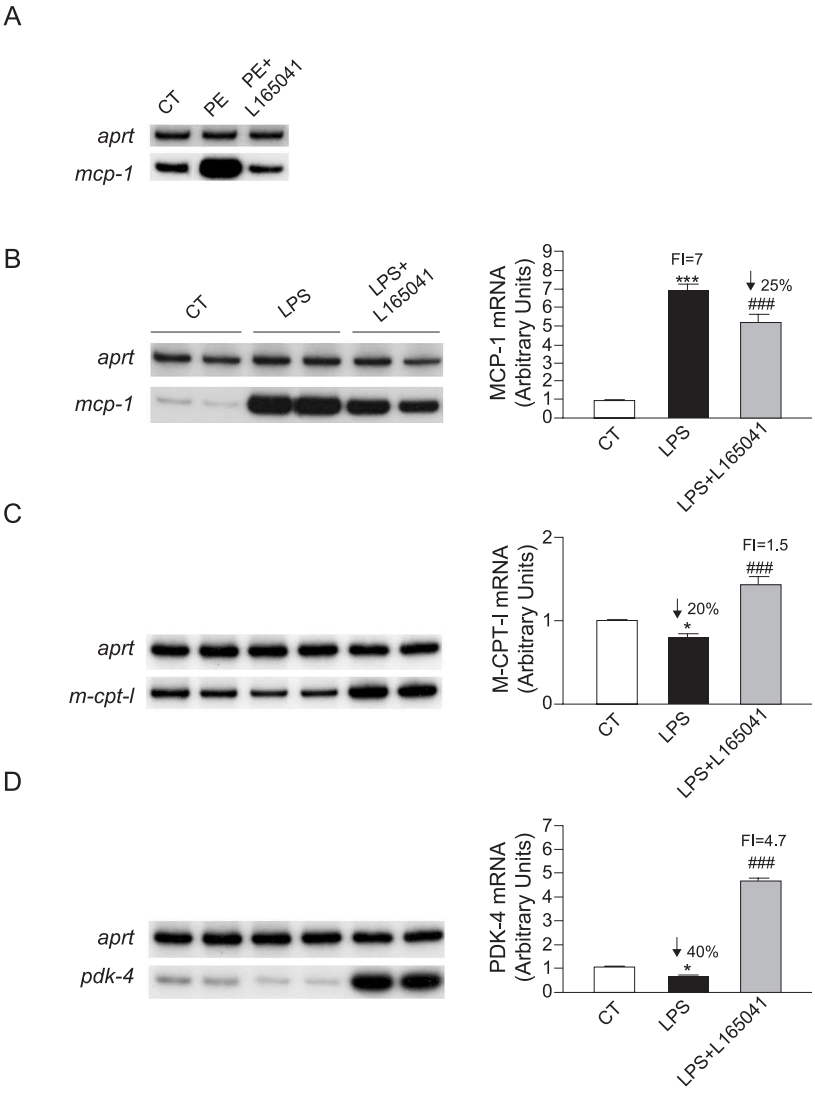

Fig. 4. L-165041 inhibits the upregulation of MCP-1 caused by PE and LPS stimulation in myocytes. Analysis of the mRNA levels of MCP-1 in PE-stimulated in neonatal rat cardiomyocytes (A) in the presence or absence of 10 μmol/L L-165041. Analysis of the mRNA levels of MCP-1 (B), M-CPT-I (C) and PDK-4 (D) in LPS-stimulated H9c2 cells in the presence or absence of 10 μmol/L L-165041. A representative autoradiogram and the quantification normalized to the APRT mRNA levels are shown. Data are expressed as mean±S.D. of six different experiments. *$P<0.05$ and ***$P<0.001$ vs. control. $^{###}P<0.001$ vs. either PE- or LPS-stimulated cells.

affect the expression of iNOS, which has been previously involved in maladaptive consequences of cardiac hypertrophy [31] (Fig. 3C). Next, we determined the effects of L-165041 on the expression of MCP-1, a gene under the transcriptional control of NF-κB [32], in neonatal rat cardiomyocytes. Stimulation of cardiac myocytes with PE enhanced twofold the expression of this gene and this was abolished by L-165041 (Fig. 4A). This data suggests that prevention of NF-κB activation may be involved in the antihypertrophic effect attained by PPARβ/δ activation. Because of limited amount of mRNA and proteins obtained from neonatal rat cardiomyocytes, we continue our studies in the embryonic rat-heart derived H9c2 cells to confirm the involvement of NF-κB in the changes observed after L-165041 treatment. Gilde et al. [21] recently reported that H9c2 cells abundantly express the PPARβ/δ subtype, whereas PPARα and γ were undetectable. This fact converts H9c2 cells in a proper tool to investigate the role of PPARβ/δ activation without the potential interference of the other PPAR subtypes. In order to activate NF-κB, H9c2 cells were stimulated for 24 h with LPS, which has been reported to activate NF-κB in cardiomyocytes [33]. As expected, a robust induction (sevenfold, $P<0.001$) was observed in the mRNA levels of the NF-κB target gene MCP-1 (Fig. 4B), that was significantly reduced (−25%, $P<0.001$) in the presence of L-165041. The stimulation of H9c2 cells with LPS also caused a similar pattern of changes in the expression of genes involved in fatty acid metabolism to those observed in PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes. Thus, a 20% and a 40% reduction were observed in the mRNA levels of M-CPT-I and PDK-4, respectively, and these changes were prevented in the presence of L-165041 (Fig. 4C and D).

2.4. Treatment with the PPARβ/δ Activator L-165041 reduces LPS-induced NF-κB activation

Since activation of NF-κB is required for hypertrophic growth of cardiomyocytes [3–6] and MCP-1 transcription

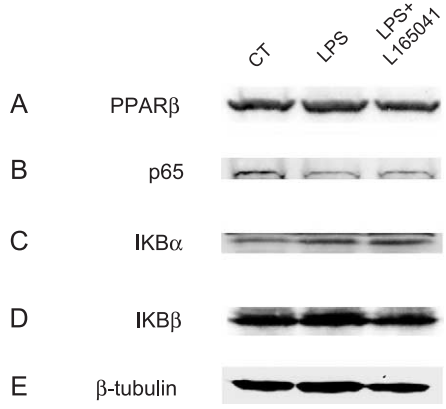

Fig. 6. Treatment with L-165041 does not affect the protein levels of IκBα. Protein extracts from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041 were assayed for Western blot analysis with PPARβ/δ (A), p65 (B), IκBα (C) IκBβ (D) and β-tubulin (E) antibodies.

is regulated by this transcription factor, we performed EMSAs to investigate whether the PPARβ/δ activator L-165041 inhibited LPS-induced NF-κB activation in H9c2 cells. EMSA studies shown that the NF-κB probe formed three complexes with cardiac nuclear proteins (complexes I to III, Fig. 5). Specificity of the three DNA-binding complexes was assessed in competition experiments by adding an excess of unlabeled NF-κB oligonucleotide to incubation mixtures (Fig. 5A). NF-κB binding activity, mainly of specific complex II, increased in cells stimulated with LPS for 1 h (Fig. 5B). In contrast, in the presence of L-165041 the LPS-induced increase in NF-κB binding activity was abolished. Characterization of NF-κB was performed by incubating nuclear extracts with an antibody directed against the p65 subunit of NF-κB. Addition of this antibody to incubation mixtures resulted in complete supershift of complex II, thus showing that this complex contained p65. No changes were observed in the DNA binding of nuclear proteins to an Oct-1 probe, indicating that the increase observed for the NF-κB probe was specific (data not

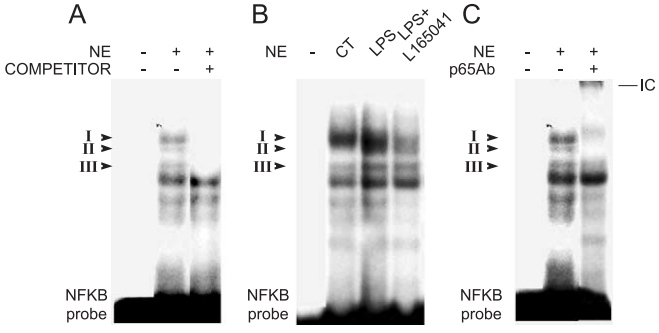

Fig. 5. Treatment with the PPARβ/δ activator L-165041 reduces LPS-induced NF-κB activation in H9c2 myotubes. (A) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and crude nuclear protein extract (NE) shows three specific complexes (I to III), based on competition with a molar excess of unlabeled probe. (B) Autoradiograph of EMSA performed with a ^{32}P-labeled NF-κB nucleotide and NE from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041. (C) Supershift analysis performed by incubating NE with an antibody directed against the p65 subunit of NF-kB. Supershifted immune complex (IC) is denoted.

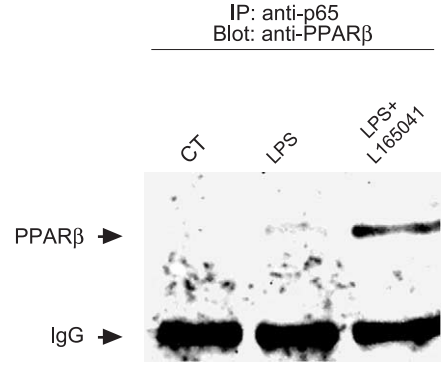

Fig. 7. L-165041 enhances PPARβ/δ association with the p65 subunit of NF-κB. Nuclear extracts from H9c2 myotubes stimulated with LPS for 1 h in the presence or the absence of 10 μmol/L L-165041 were subjected to immunoprecipitation using anti-p65 antibody coupled to protein-A agarose beads. Immunoprecipitates were subjected to SDS–PAGE and immunoblotted with an anti-PPARβ/δ antibody. The blot data are representative of three separate experiments.

shown). Overall, these data demonstrate that PPARβ/δ activation by L-165041 inhibits LPS-induced NF-κB activation.

2.5. Treatment with the PPARβ/δ activator enhances its interaction with the p65 subunit of NF-kB

Finally, we sought to determine the molecular mechanism by which the PPARβ/δ activator L-165041 inhibits LPS-induced NF-κB activation. Since it has been proposed that PPARβ/δ and PPARα share similar biological roles [21], we studied whether PPARβ/δ inhibited NF-κB signaling through mechanisms similar to those reported for PPARα. Activation of PPARα may result in inhibition of NF-κB signaling through different mechanisms. First, PPARα activators have been reported to induce the expression of IκBα, which forms a cytoplasmic inactive complex with the p65–p50 heterodimeric complex [34,35]. We did not observe significant changes in the protein expression of PPARβ/δ, the p65 subunit of NF-κB, IκBα or IκBβ after L-165041 treatment (Fig. 6), suggesting that PPARβ/δ activation did not act through this mechanism. In addition, PPARα activators may act through DNA-binding independent mechanisms that may involve a physical interaction with NF-κB. This association prevents NF-κB from binding to its response element and thereby inhibits its ability to induce gene transcription [16]. In order to evaluate whether PPARβ/δ activation acts through a similar mechanism, we performed coimmunoprecipitation studies with isolated nuclear extracts using antibodies against the p65 subunit of NF-κB and PPARβ/δ. Data shown in Fig. 7 demonstrate that addition of the PPARβ/δ agonist L-165041 strongly enhanced the physical interaction between p65 and PPARβ/δ, suggesting that increased association between these two proteins is the mechanism through which PPARβ/δ activation prevents NF-κB activation.

3. Discussion

In the present study, we demonstrate that activation of PPARβ/δ by the specific ligand L-165041 inhibits PE-induced cardiomyocyte hypertrophy in neonatal rat cardiomyocytes. Treatment with L-165041 also inhibited PE-induced expression of the NF-κB-target gene MCP-1, suggesting that the antihypetrophic effect of this compound involves downregulation of NF-κB signaling pathway. Further, it is shown that L-165041 may inhibit LPS-induced NF-κB activation through enhanced physical interaction of PPARβ/δ with the p65 subunit of NF-κB.

Several studies have reported that both PPARα and PPARγ activators inhibit cardiac hypertrophy [36–39]. In contrast, the biologic role of PPARβ/δ activation in cardiac hypertrophy was unknown. The availability of selective PPARβ/δ ligands, such as L-165041, opened the possibility of studying the role of this PPAR subtype in cardiac cells. Thus, previous data of a recent study [21] pointed to an important function of PPARβ/δ in the heart. The authors demonstrated that both PPARα and PPARβ/δ were expressed in comparable levels in heart, whereas PPARγ was barely detectable. Further, PPARβ/δ was fatty acid inducible and activated the expression of PPARα target genes involved in fatty acid utilization in cardiac myocytes. The authors of this study suggested that PPARα and PPARβ/δ shared similar functions in cardiac cells regarding cardiac fatty acid metabolism. In agreement with this idea, Muoio et al. [40] shown that fatty acid oxidation in skeletal muscle of PPARα$^{-/-}$ mice was not impaired, probably because of PPARβ/δ compensated for the lack of PPARα in these mice. In the present study, we define a new role for PPARβ/δ activation, inhibition of cardiomyocyte hypertrophy. Therefore, given the abundant expression of both PPARα and PPARβ/δ in heart and the fact that PPARα activation also inhibits cardiac hypertrophy [41,42], these PPAR subtypes may also share similar roles in the development of cardiac hypertrophy.

It is still a matter of controversy whether changes in intracellular substrate and metabolite levels in cardiomyocytes are the consequence or the reason for cardiac hypertrophy. However, several factors support a role for cardiac metabolism in the development of cardiac hypertrophy. Thus, an increase in the activities of several glycolytic enzymes has been reported prior to cardiac hypertrophy [7]. Moreover, the fact that PPARα gene influences human left ventricular growth in response to exercise and hypertension, indicates that maladaptative cardiac substrate utilization can play a causative role in the pathogenesis of left ventricular hypertrophy [43]. In the present work, stimulation of rat neonatal cardiomyocytes with PE, which leads to NF-κB activation [4], caused cardiomyocyte hypertrophy that was accompanied by a fall in the expression of genes involved in fatty acid metabolism, such as M-CPT-I and PDK-4. This effect was abolished by the addition of the PPARβ/δ activator

L-165041, which strongly induced the expression of these genes. Further studies are necessary to clearly establish whether pharmacological modulation of cardiac fatty acid metabolism with either PPARα or PPARβ/δ activators is enough to alleviate or inhibit cardiac hypertrophy. However, it is worth noting that treatment of H9c2 cells with LPS for 24 h caused a similar pattern of changes in the expression of M-CPT-I and PDK-4 to those observed in PE-induced cardiomyocyte hypertrophy. Since both PE-induced cardiomyocyte hypertrophy and LPS lead to NF-κB activation, these data point to the involvement of this transcription factor in the downregulation of genes involved in fatty acid metabolism. Activation of PPARβ/δ would inhibit NF-κB signaling pathway avoiding both cardiomyocyte hypertrophy and downregulation of genes involved in fatty acid metabolism. Furthermore, and although, it is not the objective of this study, apoptosis is considered an important factor in the progression from cardiac hypertrophy to heart failure. Activation of NF-κB is involved in direct regulation of both anti- and proapoptotic effects [44] and the latter maybe stimulated by LPS.

Interestingly, L-165041 reduced the induction of the NF-κB target gene MCP-1 in cardiac cells stimulated by either PE or LPS, suggesting that PPARβ/δ may antagonize NF-κB activation. Enhanced myocardial MCP-1 has been described in the hypertrophied and failing heart [45] and may lead to the infiltration and activation of inflammatory cells, such as monocytes/macrophages and lymphocytes. In addition, it has been reported that activation of MCP-1 expression contributes to left ventricular remodeling and failure after myocardial infarction [46]. Therefore, PPARβ/δ activation may become a therapeutic option to reduce the expression of MCP-1 in heart. It is important to note that the inhibitory effect of L-165041 on LPS-induced MCP-1 expression was of lower intensity that the observed for PE. This probably reflects the higher induction achieved by LPS stimulation (sevenfold induction) compared to PE (twofold induction) and/or differences in the two cell system used. The use of H9c2 myotubes, which only express PPARβ/δ, offers the advantage of avoiding the interference of other PPAR subtypes and, therefore, permits to adscribe the changes observed to this transcription factor. However, LPS-treatment of H9c2 myotubes was performed to achieve NF-κB activation, but not cardiac hypertrophy, and consequently the findings observed in these cells should be limited to the activation of NF-κB by LPS.

PPARα activators may inhibit NF-κB signaling through different mechanisms [16,47,48]. One of these mechanisms involves physical interaction of PPARα and the p65 subunit of NF-κB [16]. Here, we demonstrate that PPARβ/δ activation by L-165041 enhances the protein-protein association between PPARβ/δ and p65, indicating that this mechanism may interfere NF-κB transactivation capacity. Therefore, PPARα and PPARβ/δ may also share similar mechanisms of action inhibiting NF-κB signaling. Further studies are necessary to investigate whether PPARβ/δ activation may inhibit the NF-κB signaling pathway through additional mechanisms or affects the activity of other transcription factors involved in cardiac hypertrophy, such as nuclear factor of activated T lymphocyte (NFAT).

In summary, in the present study, we show that PPARβ/δ activation inhibits PE-induced cardiomyocyte hypertrophy in neonatal rat ventricular cardiomyocytes. PPARβ/δ activation also inhibits LPS-induced NF-κB activation through a mechanism that may involve enhanced protein–protein interaction between this PPAR subtype and the p65 subunit of NF-κB. These data indicate that inhibition of the NF-κB signaling pathway may be the underlying mechanism responsible for the inhibition of cardiomyocyte growth.

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