Inflammatory Pathways Are Activated during Cardiomyocyte Hypertrophy and Attenuated by Peroxisome Proliferator-activated Receptors PPAR\(\alpha\) and PPAR\(\delta\)*

Pascal J. H. Smeets, Birgit E. J. Teunissen, Anna Planavila, Heleen de Vogel-van den Bosch, Peter H. M. Willemsen, Ger J. van der Vusse, and Marc van Bilsen

From the Department of Physiology, Cardiovascular Research Institute Maastricht, Maastricht University, 6200 MD Maastricht, the Netherlands

Evidence is accumulating that chronic inflammation plays an important role in cardiac disease. This notion is based, among others, on the observation that heart failure patients show elevated plasma levels of pro-inflammatory cytokines, such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\))\(^3\). In addition, cytokines have been implicated in the development of cardiac hypertrophy (2, 3), most likely via downstream activation of pro-inflammatory transcription factors such as nuclear factor-\(\kappa\)B (NF-\(\kappa\)B) (4). NF-\(\kappa\)B is composed of different homo- and heterodimers of which p50/p65 is transcriptionally the most active dimer in the heart (5). In its inactive state, NF-\(\kappa\)B resides within the cytoplasm through its interaction with inhibitor-\(\kappa\)B\(\alpha\) (I\(\kappa\)B\(\alpha\)). Upon phosphorylation of I\(\kappa\)B\(\alpha\) by inhibitor-\(\kappa\)B kinase-\(\beta\) (IKK\(\beta\)), NF-\(\kappa\)B dissociates from I\(\kappa\)B\(\alpha\) allowing its translocation to the nucleus where it binds to NF-\(\kappa\)B response \(\text{cis}\)-elements in target genes. It is proposed that inhibition of pro-inflammatory pathways, in general, and NF-\(\kappa\)B, in particular, could be beneficial for the diseased heart (6, 7).

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors acting as modulators of lipid metabolism as well as inflammation (8, 9). The anti-inflammatory effect of PPARs appears to be related to interference with NF-\(\kappa\)B signaling (6, 10). Indeed, treatment of animals with PPAR ligands has been reported to mitigate inflammation following cardiac ischemia and reperfusion (11–14). Two of the three PPAR isoforms, i.e. PPAR\(\alpha\) (NR1C1) and PPAR\(\delta\) (NR1C2), are abundantly expressed in the cardiac muscle cell (15). Although the majority of studies focused on the anti-inflammatory properties of PPAR\(\alpha\) and PPAR\(\gamma\) (16–19), several studies indicate that PPAR\(\delta\) is likely to possess similar features (10, 20, 21). Whether the putative anti-inflammatory properties of PPAR\(\alpha\) and PPAR\(\delta\) affect cardiomyocyte hypertrophy still remains to be established.

We hypothesized that growth-promoting stimuli elicit an inflammatory response in cardiomyocytes and that pro-hypertrophic and inflammatory signaling pathways converge on NF-\(\kappa\)B. Secondly, it was investigated whether PPAR\(\alpha\) and PPAR\(\delta\) are able to inhibit NF-\(\kappa\)B activation in the cardiomyocyte and whether this provides a molecular mechanism to mit-

\* This work was supported by a grant from the Netherlands Organization for Scientific Research (NWO 912-04-017, to M. v. B.) and EU FP6 Grant LSHM-CT-2005-018833/EUGeneHeart. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked \“advertisement\” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\[3\] The abbreviations used are: TNF, tumor necrosis factor; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility shift assay; PPAR, peroxisome proliferator-activated receptor; PE, phenylephrine; qPCR, quantitative PCR; TGF, transforming growth factor; NCM, neonatal cardiomyocytes; ANF, atrial natriuretic factor.
igate cardiac hypertrophy. Therefore, rat neonatal cardiomyocytes (NCM) were stimulated with several growth-promoting neuroendocrine factors or with pro-inflammatory agents for up to 48 h, and time-dependent changes in the expression of established markers of hypertrophy and inflammation were assessed by quantitative PCR (qPCR). Activation of NF-κB was monitored by electrophoretic mobility shift assay (EMSA). Transient transfection studies with expression vectors and promoter/reporter constructs were performed to investigate the functional interaction of the NF-κB subunit p65 with PPARs. In addition, the ability of PPARs to attenuate cardiomyocyte hypertrophy was explored using pharmacological (isoform-specific PPAR ligands) and molecular tools (adenoviral overexpression of PPARα and PPARδ).

The collective findings reveal a close relationship between hypertrophic and inflammatory signaling pathways in the cardiomyocyte. We demonstrate for the first time that both PPARs and PPARδ are able to inhibit NF-κB activation in vitro and, in this way, may mitigate hypertrophic remodeling of the cardiac muscle cell.

EXPERIMENTAL PROCEDURES

Chemicals—TNF-α and transforming growth factor-β (TGF-β) were purchased from PeproTech (Rocky Hill, NJ) and angiotensin II (AngII) from Bachem (Bubendorf, Switzerland). Isoform-specific ligands for PPARα (Wy-14,643) and PPARδ (GW501516) were obtained from Biomol (Plymouth Meeting, PA) and Calbiochem (EMD Biosciences, Darmstadt, Germany), respectively. All other chemicals were purchased from Sigma. The PPAR ligands were dissolved in DMSO; TGF-β and AngII were obtained from Biomol (Plymouth Meeting, PA) and Calbiochem (EMD Biosciences, Darmstadt, Germany), respectively. The PPAR ligands were added to NCM at 0.25 mM and 0.25 milliunits/ml insulin, and 1% bovine serum albumin. Ara C was present during the entire cell culture.

NCM were stimulated with either hypertrophic growth factors, i.e. the α1-adrenergic agonist PE (10 μM), endothelin-1 (ET-1, 1 nM), AngII (10 μM), TGF-β (10 ng/ml), or with the inflammatory agents lipopolysaccharide (LPS, 100 ng/ml) and TNF-α (50 ng/ml), and cells were harvested for RNA or protein isolation, 2, 12, 24, and 48 h after exposure. Appropriate vehicles served as controls. At the concentrations used, none of the agents induced significant cell loss.

RNA Isolation and qPCR—RNA isolation, cDNA synthesis, and qPCR were performed as previously described (23). 100 ng of RNA were used for cDNA synthesis. Primers used are given in supplemental Table S1. Results were normalized to the housekeeping gene cyclophilin A. Relative changes in expression were calculated using GeneX software (Bio-Rad).

Plasmids—For transient transfection studies the following promoter/reporter constructs were used; mCPT-I-luc (15), mutated mCPT-I-luc construct (23), (−638 bp) ANF promoter-luc, and p19TK-luc containing three NF-κB consensus sites (kind gift from Dr. Van der Saag, Hubrecht Lab, Utrecht, the Netherlands). Expression vectors used were: pSG5 containing cDNA of mouse PPARα or PPARδ (kindly provided by Dr. Staeli, Institut Pasteur, Lille, France); pCMV4 containing cDNA of human p65 (kind gift from Dr. Van der Saag) and pON249 containing CMV-β-galactosidase. As a positive control for the transfection assay, we used pSVOAL-luc in which luciferase is driven by the RSV promoter.

Transient Transfection—NCM were transfected 24 h after cell isolation using the transfection reagent FuGENE 6 (Roche Applied Sciences, Indianapolis IN) with 0.5 μg of promoter/reporter vector (15). The CMV-β-galactosidase containing vector pON249 (0.25 μg) was co-transfected to check for transfection efficiency. In a subseries of experiments 0.25 μg of expression vectors containing the cDNA of PPARα, PPARδ, or p65 were co-transfected. The total amount of plasmid DNA per well (1.25 μg) was kept constant by adding empty pSG5 vector (Promega, Madison, WI). After 16 h of transfection, experimental medium was added to the cells followed by exposure to PE (10 μM) in the absence or presence of Wy-14,643 (10 μM, EC50 = 0.63 μM) or GW501516 (1 μM, EC50 = 0.024 μM). The affinity and selectivity of these ligands for their PPAR isoforms have been described previously (15). Cells were harvested 24 h (m-CPT-I and NF-κB reporter/promoter) or 48 h (ANF reporter/promoter) after start of treatment and immediately processed for the determination of reporter activity. Luciferase activity was determined using a commercial firefly luciferase assay according to the supplier’s instructions (Steady Glo, Promega) in white 96-well plates (Nalge Nunc International, Maperville, IL) on a FluorS imager (Bio-Rad) for measuring luminescence. β-Galactosidase activity was assessed spectrophotometrically (Titertek Multiskan Plus MKII, Thermo LabSystems, Helsinki, Finland) as described previously (24).

Preparation of Nuclear Extracts—NCM were exposed to TNF-α or PE for the times indicated. Cells were harvested by scraping in ice-cold PBS supplemented with phosphatase inhibitors (125 mM NaF, 250 mM β-glycerophosphate, and 25 mM NaVO3), followed by centrifugation (3000 × g, 5 min). The cell pellet was resuspended in 400 μl of ice-cold low-salt buffer (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin). Cells were allowed to swell at 4 °C for 10 min.
and then vortexed for 10 s. Subsequently, the supernatant fraction was discarded after a short spin. Pellets were resuspended in 30 μl of ice-cold high-salt buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin) and incubated at 4 °C for 30 min. Nuclear debris was removed by centrifugation at 1000 x g at 4 °C for 10 min, and the supernatant (containing DNA-binding proteins) was stored at −80 °C. Protein concentration of nuclear extracts was determined with the Bradford method (25).

**EMSA**—A double-stranded oligonucleotide containing the NF-κB consensus binding site (5'-AGTTGAGGACCTTGCCAGGC-3') was end-labeled at 37 °C in the following reaction mix: 2 μl of ds oligonucleotide (1.75 pmol/μl), 2 μl of 5X kinase buffer, 1 μl of T₄ polynucleotide kinase (10 units/μl), and 2.5 μl of [γ-³²P]dATP (3000 Ci/mmol at 10 mCi/ml). After 2 h, the reaction was stopped by adding 90 μl of TE buffer. To separate the labeled probe from unbound ATP, the reaction mixture was eluted on ProbeQuant G-50 Micro columns (Amersham Biosciences). Nuclear extracts (4 μg of protein) were incubated for 10 min at 4 °C in binding buffer containing 10 mM Tris-HCl, pH 8.0, 25 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA (pH 8.0), 5% glycerol, 5 mg/ml bovine serum albumin, and 50 μg/ml poly(dI-dC) Next, ³²P-labeled oligonucleotide probe (~60,000 cpm) was added, and the reaction was incubated for 15 min at 4 °C. Where indicated, p65 antibody (Santa Cruz Biotechnology) or specific competitor oligonucleotide were added 10 min prior to incubation with labeled probe at 4 °C. Protein-DNA complexes were separated by electrophoresis at 4 °C on a 5% acrylamide gel. After drying of the gels, autoradiography was performed using Hyperfilm (Amersham Biosciences) and by scanning on a Phosphorimager (Bio-Rad).

**Adenoviral Infection**—Recombinant adenoviruses expressing human PPARα (AdPPARα), human PPARδ (AdPPARδ), and control virus (AdCMV) were kindly provided by Dr. Staels (Institut Pasteur, Lille, France). Adenoviruses were multiplied and control virus (AdCMV) were kindly provided by Dr. Staels (Institut Pasteur, Lille, France). Adenoviruses were multiplied at 4 °C. Protein-DNA complex was separated by electrophoresis at 4 °C on a 5% acrylamide gel. After drying of the gels, autoradiography was performed using Hyperfilm (Amersham Biosciences) and by scanning on a Phosphorimager (Bio-Rad).

**Assessment of Cell Size**—NCM were grown on laminin (10 μg/ml)-coated glass coverslips and fixated with methanol for 3 min at −20 °C. After permeabilization with 0.1% Triton X-100 for 5 min and blocking with 2% bovine serum albumin for 30 min, cells were incubated overnight with an antibody against α-actinin (Sigma) dissolved in PBS containing 10% normal goat serum (Dako, Glostrup, Denmark). Next, cells were washed with PBS and incubated with Texas Red-conjugated secondary anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), dissolved in PBS containing 10% normal goat serum. Cells were washed again, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and analyzed using a fluorescence microscope. Cardiomyocyte surface area (μm²) was determined from randomly selected fields from three independent cell cultures using Quantimet 570 software (Leica, the Netherlands).

![Figure 1. Effect of hypertrophic and inflammatory stimuli on mRNA levels of the inflammatory marker COX-2 (A) and the hypertrophy marker ANF (B).](image)

**Statistics**—Each experiment was conducted in triplicate in at least three independent cardiomyocyte isolations. Results are presented as mean ± S.E. Data were analyzed by one-way analysis of variance, followed by Bonferroni Post-Hoc when appropriate using SPSS 12 software (SPSS Inc.). A p value of less than 0.05 was considered to be statistically significant.

**RESULTS**

**Cardiomyocyte Hypertrophy and Inflammation**—To investigate the relationship between cardiac hypertrophy and inflammation, NCM were incubated with either neuroendocrine growth factors (AngII, ET-1, PE, or TGF-β) or pro-inflammatory agents (LPS or TNF-α). The early (2 h) and late (24 h) effects of these distinct stimuli on the expression of COX-2 and ANF, markers of inflammation and hypertrophy, respectively, were determined. Already 2 h after administration of pro-hypertrophic as well as inflammatory agents, COX-2 mRNA levels were increased severalfold (Fig. 1A), whereas the mRNA level of ANF was not yet affected to a significant extent (Fig. 1B). After 24 h, however, COX-2 expression had returned to near basal levels, whereas expression of ANF was markedly up-regulated at this later time point.

Given the temporal differences in response of COX-2 or ANF to the pro-hypertrophic and pro-inflammatory stimuli, the time course of changes in the expression of an extended set of...
markers of hypertrophy (the natriuretic proteins ANF and BNP, the sarcomeric protein α-skeletal actin, α-SKA) and inflammation (COX-2, TNF-α) was examined in more detail using PE and TNF-α as hypertrophic and inflammatory stimulus, respectively. Following PE or TNF-α stimulation the expression of ANF showed a gradual increase as a function of time, reaching statistical significance at 12 h (Fig. 2A). After 2 h of stimulation with either PE or TNF-α BNP mRNA was already up-regulated (Fig. 2B). Only in PE-treated cells, BNP expression increased further thereafter. The expression of α-SKA was found to increase slowly during PE and TNF-α stimulation (Fig. 2C). In contrast, the expression of the two inflammatory genes COX-2 and TNF-α (Fig. 2D and E) rapidly increased within the first 2–12 h and declined thereafter in both PE- and TNF-α-treated cardiomyocytes. Collectively, these findings point to a functional relationship between hypertrophy and inflammation in that established hypertrophic stimuli give rise to the expression of inflammatory genes and, vice versa, that inflammatory stimuli induce expression of established hypertrophic genes.

NF-κB Activation during Hypertrophy and Inflammation—To explore whether the early expression of pro-inflammatory genes following application of the α1-adrenergic agonist PE and the cytokine TNF-α was associated with activation of the NF-κB pathway, NF-κB DNA binding activity was monitored by EMSA (Fig. 3). The NF-κB oligonucleotide incubated with
nuclear extracts of NCM gave rise to 3 distinct bands. The p65 antibody resulted in a supershift of only one band, indicating that this band contains the p65 subunit of NF-κB (Fig. 3A).

Exposure of NCM to TNF-α evoked a robust increase in NF-κB DNA binding, reaching maximal intensity after 30 min (Fig. 3B). PE stimulation of NCM also resulted in an early increase in NF-κB DNA binding, albeit that peak levels were lower. At later time points (2–48 h), NF-κB DNA binding activity in PE-stimulated cells returned to control levels, whereas binding activity remained elevated in TNF-α stimulated NCM (Fig. 3C).

Interaction of PPAR and NF-κB—Results above demonstrate that hypertrophic stimuli cause NF-κB activation and increased expression of pro-inflammatory genes. Previous studies provide evidence that cross-interaction exists between PPARs and pro-inflammatory signaling pathways (16, 21). Therefore, it was examined whether in the cardiomyocyte context PPARα and PPARδ inhibit the activity of NF-κB. Therefore, NCM were co-transfected with a p65 expression vector along with PPARα and PPARδ expression vectors. Where indicated, cells were treated with 10 μM Wy-14,643 or 1 μM GW501516 for 24 h. Changes in NF-κB reporter activity are expressed relative to vehicle-treated, control cells. Data are presented as mean ± S.E. * indicates statistically significant (p < 0.05) compared with corresponding control cells. # indicates statistically significant (p < 0.05) compared with NF-κB transcriptional activity by p65 alone.

FIGURE 3. Effect of PE and TNF-α on NF-κB activation as measured by EMSA. Incubation of 32P-labeled NF-κB oligonucleotide with nuclear extract (+NE) of NCM showed several complexes. The specificity of the signal was checked by adding a 100-fold excess of unlabeled NF-κB probe. Incubation with an antibody against p65 resulted in a supershift of the upper band (open arrow) (panel A). Nuclear extracts were prepared from untreated and PE- and TNF-α treated NCM at the indicated time points to monitor acute effects (panel B) and more long-term effects (panel C). The specific p65 signal is indicated by the black arrow.

DNA binding, reaching maximal intensity after 30 min (Fig. 3B). PE stimulation of NCM also resulted in an early increase in NF-κB DNA binding, albeit that peak levels were lower. At later time points (2–48 h), NF-κB DNA binding activity in PE-stimulated cells returned to control levels, whereas binding activity remained elevated in TNF-α stimulated NCM (Fig. 3C).

Interaction of PPAR and NF-κB—Results above demonstrate that hypertrophic stimuli cause NF-κB activation and increased expression of pro-inflammatory genes. Previous studies provided evidence that cross-interaction exists between PPARs and pro-inflammatory signaling pathways (16, 21). Therefore, it was examined whether in the cardiomyocyte context PPARα and PPARδ inhibit the activity of NF-κB. Therefore, NCM were co-transfected with a p65 expression vector along with the NF-κB reporter construct. Where indicated, cells were treated with 10 μM Wy-14,643 or 1 μM GW501516 for 24 h. Changes in NF-κB reporter activity are expressed relative to vehicle-treated, control cells. Data are presented as mean ± S.E. * indicates statistically significant (p < 0.05) compared with corresponding control cells. # indicates statistically significant (p < 0.05) compared with NF-κB transcriptional activity by p65 alone.

FIGURE 4. Effect of PPARα and PPARδ on p65-induced transcriptional activity of NF-κB. NCM were transfected with a 3× NF-κB reporter/luciferase construct and co-transfected with a control vector (open bars) or a p65 expression vector (black bars) along with PPARα (panel A) and PPARδ (panel B) expression vectors. Where indicated, cells were treated with 10 μM Wy-14,643 or 1 μM GW501516 for 24 h. Changes in NF-κB reporter activity are expressed relative to vehicle-treated, control cells. Data are presented as mean ± S.E. * indicates statistically significant (p < 0.05) compared with corresponding control cells. # indicates statistically significant (p < 0.05) compared with NF-κB transcriptional activity by p65 alone.
cognate ligands co-transfection with PPARα and PPARδ significantly blunted NF-κB activity by 40–50% (Fig. 4, A and B).

Next, it was tested whether p65 interferes with PPAR-mediated transactivation of the PPAR-responsive mCPT-1 promoter/reporter construct. Addition of Wy-14,643 and GW501516 resulted in a 3- and 4-fold increase of the transcriptional activity of the mCPT-1 promoter, respectively (Fig. 5, A and B). Interestingly, following co-transfection with p65, Wy-14,643, and GW501516-mediated mCPT-1 promoter activation was almost completely abrogated. The experiments were repeated with a mCPT-1 promoter in which the PPAR response element (PPRE) binding site was mutated. None of the above conditions affected the transcriptional activity of the mutated promoter, thereby verifying that the effects were dependent on a functional PPRE (Fig. 5, A and B).

**PPAR Overexpression and Hypertrophy**—Subsequently, it was investigated whether PPARα or PPARδ are able to attenuate hypertrophy. NCM were infected with either AdPPARα or AdPPARδ, or with AdCMV (control) prior to stimulation with PE. The functionality of the PPAR viruses was confirmed by showing that the expression of the PPAR-responsive gene PDK4 increased substantially following infection (data not shown). Treatment of AdCMV-infected, control NCM with PE for 24 h significantly increased sarcomere organization (Fig. 6A) and cardiomyocyte size (Fig. 6B), indicative of cellular hypertrophy. AdPPARα or AdPPARδ-infected cells are not shown since their appearance did not change. B, cardiomyocyte cross-sectional area. Cell size was quantified using Quantimet 570 software. Data are presented as mean ± S.E. * indicates statistically significant (p < 0.05) compared with AdCMV-infected, vehicle-treated, control cells (open bars). # indicates statistically significant (p < 0.05) compared with AdCMV-infected, PE-stimulated cells.
did not affect the PE-induced cell morphology (data not shown).

Stimulation of NCM with TNF-α did not give rise to a significant increase in NCM size. In line with previous studies (10, 26), infection with AdPPARα or AdPPARδ blunted the TNF-α induced expression of inflammatory markers like COX-2 (data not shown).

Secondly, in AdPPARα or AdPPARδ infected NCM the PE-induced ANF mRNA expression was lowered by almost 50% (Fig. 7, A and B) and induction of BNP was almost completely blunted (Fig. 7, C and D). Treatment of AdPPARα- or AdPPARδ-infected NCM with Wy-14,643 or GW501516 did not result in an additional effect.

Finally, transient transfection assays were performed using an ANF promoter/reporter construct as readout for the hypertrophic gene program. PE stimulation led to an almost 15-fold increase in ANF promoter activity (Fig. 8). The PE-induced ANF promoter activity was not affected when cardiomyocytes were exposed to either Wy-14,643 or GW501516. When the cells were co-transfected with PPARα or PPARδ expression vectors, ANF promoter activity was further enhanced. However, in combination with their corresponding ligands over-expression of each PPAR isoform profoundly inhibited PE-induced ANF promoter activity. The collective findings indicate that PPAR overexpression mitigated cardiomyocyte hypertrophy.

DISCUSSION

In the present study it is demonstrated that, similar to *bona fide* pro-inflammatory stimuli, a variety of growth-promoting factors elicit a marked inflammatory response, characterized by induction of TNF-α and COX-2 expression by NCM. The pro-hypertrophic and inflammatory signaling pathways in the cardiomyocyte appear to converge on NF-κB. These observations...
Mutually Related—The present study demonstrates that PPARs and inflammatory signaling pathways are apparently linked. Accordingly, the ability of PPARα and PPARδ to attenuate inflammation and hypertrophy, was investigated. Both PPAR isoforms inhibited NF-κB activity and their overexpression exerted anti-inflammatory effects, as evidenced from the downregulation of ANF and BNP mRNA expression and the blunted increase in cardiomyocyte size.

Cardiac Hypertrophic and Inflammatory Pathways Are Mutually Related—The present study demonstrates that hypertrophic as well as inflammatory stimuli exert a direct effect on both cardiac hypertrophy and inflammatory pathways. It is of interest to note that the inflammatory response seems to precede the hypertrophy response. The induction of inflammatory genes was found to be relatively fast (within 2 h after stimulation) and transient. More detailed analysis revealed that maximal induction was reached within 30–60 min (data not shown). In contrast, the increase in expression of hypertrophic marker genes was more gradual in nature (peaking at 24–48 h). These time-related changes in gene expression are suggestive of a causal relationship between activation of inflammatory pathways and the subsequent hypertrophic response and, to the best of our knowledge, have not been reported before. Furthermore, as expected the pro-inflammatory factors TNF-α and LPS not only elicited a prominent inflammatory response (27), but also gave rise to a hypertrophic response. The latter finding is in line with earlier observations that cytokines, e.g. IL-1β or TNF-α, are able to induce hypertrophic growth of NCM and can activate signaling pathways involved in cardiac hypertrophy (2, 3, 28). Additional support is provided by in vitro studies, indicating that mice with cardiomyocyte-specific overexpression of IL-1α and TNF-α develop left ventricular hypertrophy and dilated cardiomyopathy, respectively (29, 30). It should be noted, however, that in the present study the degree of cellular hypertrophy induced by TNF-α is less prominent than that by PE, as reflected by the absence of a statistically significant increase in cell size and a less prominent induction of the hypertrophic marker genes ANF, BNP, and α-SKA. The latter findings indicate that, in addition to inflammatory signaling, other pathways most likely contribute to the hypertrophic growth response elicited by α-adrenergic stimulation.

NF-κB in Cardiac Inflammation and Hypertrophy—The intricate relationship between hypertrophic and inflammatory pathways suggests the presence of a common factor in the two seemingly unrelated processes. Previous studies have shown that α1-adrenergic stimulation activates NF-κB in vitro (4) and that NF-κB activation is required for the induction of cardiac hypertrophy in vivo (31). Furthermore, treatment of aortic-banded and spontaneously hypertensive rats with a specific NF-κB inhibitor mitigated the cardiac hypertrophic response (32, 33), and cardiac-specific ablation of NF-κB was found to attenuate cardiac hypertrophy in TNF-α-overexpressing mice (34). Accordingly, NF-κB might serve as the common factor, functionally linking the hypertrophy and inflammatory pathways in the cardiac muscle cell. In support of this contention, we observed that DNA binding activity of NF-κB is significantly increased after stimulation of the cardiomyocytes by both an inflammatory (TNF-α) and hypertrophic stimulus (PE). Our study also shows that PE increased the expression of NF-κB-regulated genes, implying that NF-κB is an important factor in integrating the inflammatory and hypertrophic signaling pathways.

Mutual Interaction of NF-κB and PPARs—To date PPARs are under extensive investigation as targets to inhibit inflammation (8, 35). The present study shows that also in the cardiac context PPARα and PPARδ are able to attenuate the inflammatory response, because in transient transfection studies on cardiac muscle cells the combination of overexpression of PPARα or PPARδ and treatment with their respective synthetic ligands inhibited p65-induced activation of a NF-κB-responsive promoter reporter. In vascular smooth muscle cells a comparable inhibitory effect of PPARα was demonstrated, following p65-induced transcription of the IL-6 promoter (16). Recently, Ding et al. (10) have shown anti-inflammatory properties of PPARδ in LPS-treated NCM. Several mechanisms have been proposed to explain the inhibitory effect of PPARs on NF-κB (reviewed in...
Ref. 36). At present, protein–protein interaction, as revealed by co-immunoprecipitation assays (21), is considered the predominant mechanism for the transrepression property of PPARs. By inference, this implies that NF-κB also modulates PPAR-mediated events. Consistent with this notion, p65 overexpression in cardiomyocytes inhibited mCPT-1 promoter activity, an effect shown to be dependent on an intact PPAR response element. Moreover, Planavila et al. (37) recently showed that exposure of H9c2 myotubes to the pro-inflammatory agent LPS caused a reduction in the expression of PDK4, a PPAR-regulated gene, as well.

The collective observations point to a pivotal role of PPARs in limiting the inflammatory response. This property could be of paramount importance in pathophysiological conditions, such as cardiac hypertrophy or ischemia/reperfusion, when NF-κB gets activated.

**PPARs and Hypertrophy**—The current observations indicate that PPARs, through their anti-inflammatory actions, exert anti-hypertrophic effects on the cardiac muscle in vitro. Adenoviral overexpression of PPARα or PPARδ blunted the increase in ANF and BNP mRNA levels and inhibited cell size enlargement of PE-stimulated NCM both in the absence and presence of ligands. Likewise, PE-mediated induction of the −638 bp ANF promoter/reporter construct was substantially attenuated when PPARα and PPARδ were co-transfected. However, the response of the ANF promoter appeared to be ligand-dependent, showing an increase or decrease in the absence or presence of PPAR ligands, respectively. In this respect, the −638 bp ANF promoter and the endogenous ANF gene appeared to react differently. It is feasible that regulatory sequences outside the −638/+62 ANF promoter region are responsible for the differences observed.

The finding that PPARα and PPARδ inhibited the induction of the fetal gene program is in line with previous studies showing that the PPARα agonist fenofibrate attenuated ET-1-induced protein synthesis and enlargement of NCM (38, 39). Moreover, recently we showed that the hearts of mice deficient in PPARα developed a more pronounced hypertrophy after pressure overload, a response that was associated with enhanced expression of inflammatory genes (40).

Collectively, these earlier and present observations indicate that the α- and δ-isof orm of PPAR suppress inflammation, a process closely linked to the induction of cardiac hypertrophy. In this respect, it is of note that Jucker et al. (41) reported that PPARδ activation in hearts with regional infarction significantly reduced right ventricular hypertrophy in vivo.

In summary, the present findings demonstrate that in the cardiac muscle cell inflammatory and hypertrophic stimuli converge on NF-κB. In addition, the transcription factors PPAR and NF-κB are functionally intertwined in their ability to regulate hypertrophic and inflammatory processes in the cardiac muscle cell. The close relationship between hypertrophy and inflammation is also from a clinical point of view of great importance, because chronic low-grade inflammation is thought to play a significant role in cardiac hypertrophy and failure (42). PPARs are able to inhibit myocardial inflammation and hypertrophy via inhibition of NF-κB. It is therefore tempting to speculate that PPARs serve as therapeutic targets to inhibit inflammatory signaling and, consequently, prevent hypertrophic growth of the heart.

**REFERENCES**

1. Levine, B., Kalman, J., Mayer, L., Fillit, H. M., and Packer, M. (1990) *N. Engl. J. Med.* 323, 236–241
2. Thaik, C. M., Calderone, A., Takahashi, N., and Colucci, W. S. (1995) *J. Clin. Invest.* 96, 1093–1099
3. Yokoyama, T., Nakano, M., Bednarczyk, J. L., McIntyre, B. W., Entman, M., and Mann, D. L. (1997) *Circulation* 95, 1247–1252
4. Purcell, N. H., Tang, G., Yu, C., Mercurio, F., DiDonato, J. A., and Lin, A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 6668–6673
5. Hall, G., Hasday, J. D., and Rogers, T. B. (2006) *J. Mol. Cell Cardiol.* 41, 580–591
6. Ogata, T., Miyautchi, T., Sakai, S., Takeshita, A., Irukayama- Tomobe, Y., and Yamaguchi, I. (2004) *J. Am Coll. Cardiol.* 43, 1481–1488
7. Kawano, S., Kubota, T., Monden, Y., Kawamura, N., Tsutsui, H., Takehisa, Y., and Sunagawa, K. (2005) *Cardiovasc. Res.* 67, 689–698
8. Chinetti, G., Fruchart, J. C., and Staels, B. (2000) *Inflamm. Res.* 49, 497–505
9. van Bilsen, M., van der Vusse, G. J., Gilde, A. J., Lindhout, M., and van der Lee, K. A. (2002) *Mol. Cell Biochem.* 239, 131–138
10. Ding, G., Cheng, L., Qin, Q., Fronton, S., and Yang, Q. (2006) *J. Mol. Cell Cardiol.* 40, 821–828
11. Wayman, N. S., Ellis, B. L., and Thiemermann, C. (2002) *Med. Sci. Monit.* 8, BR243–247
12. Wayman, N. S., Hattori, Y., McDonald, M. C., Mota-Filipe, H., Cuzzocrea, S., Pisano, B., Chatterjee, P. K., and Thiemermann, C. (2002) *Faseb J.* 16, 1027–1040
13. Yeh, C. H., Chen, T. P., Lee, C. H., Wu, Y. C., Lin, Y. M., and Lin, P. J. (2006) *Shock* 26, 262–270
14. Liu, H. R., Tao, L., Gao, E., Lopez, B. L., Christopher, T. A., Willette, R. N., Ohlstein, E. H., Yue, T. L., and Ma, X. L. (2004) *Cardiovasc. Res.* 62, 135–144
15. Gilde, A. J., van der Lee, K. A., Willemse, P. H., Chinetti, G., van der Leij, F. R., van der Vusse, G. J., Staels, B., and van Bilsen, M. (2003) *Circ. Res.* 92, 518–524
16. Delerive, P., De Bosscher, K., Besnard, S., Vanden Bergh, W., Peters, J. M., Gonzalez, F. J., Fruchart, J. C., Tegdju, A., Haegeman, G., and Staels, B. (1999) *J. Biol. Chem.* 274, 32048–32054
17. Delerive, P., Gervois, P., Fruchart, J. C., and Staels, B. (2000) *J. Biol. Chem.* 275, 36703–36707
18. Cuzzocrea, S., Pisano, B., Dugo, L., Iarano, A., Maffia, P., Patel, N. S., Di Paola, R., Ialenti, A., Genovese, T., Chatterjee, P. K., Di Rosa, M., Caputi, A. P., and Thiemermann, C. (2004) *Eur. J. Pharmacol.* 483, 79–93
19. Lee, K. S., Park, S. J., Hwang, P. H., Yi, H. K., Song, C. H., Chai, O. H., Kim, J. S., Lee, M. K., and Lee, Y. C. (2005) *Faseb J.* 19, 1033–1035
20. Lee, C. H., Chawla, A., Urbiztondo, N., Diao, D., Boisvert, W. A., and Evans, R. M. (2003) *Science* 302, 453–457
21. Planavila, A., Rodriguez-Calvo, R., Jove, M., Michalik, L., Wahl, W., Laguna, J. C., and Vazquez-Carrera, M. (2005) *Cardiovasc. Res.* 65, 832–841
22. de Vries, J. E., Vork, M. M., Roemen, T. H., de Jong, Y. F., Cleutjens, J. P., van der Vusse, G. J., and van Bilsen, M. (1997) *J. Lipid Res.* 38, 1384–1394
23. Teunissen, B. E., Smeets, P. J., Willemse, P. H., De Windt, L. J., Van der Vusse, G. J., and Van Bilsen, M. (2007) *Cardiovasc. Res.* 75, 519–529
24. Shubietta, H. E., Martinson, E. A., Van Bilsen, M., Chien, K. R., and Brown, J. H. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 1305–1309
25. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248–254
26. Takano, H., Nagai, T., Asakawa, M., Toyozaki, T., Oka, T., Komuro, I., Saito, T., and Masuda, Y. (2000) *Circ. Res.* 87, 596–602
27. Peng, T., Lu, X., and Feng, Q. (2005) *Faseb J.* 19, 293–295
28. Clerk, A., Harrison, J. G., Long, C. S., and Sugden, P. H. (1999) *J. Mol. Cell Cardiol.* 31, 2087–2099
29. Nishikawa, K., Yoshida, M., Kusuahara, M., Ishigami, N., Isoda, K., Miyazaki, K., and Ohsumi, F. (2006) *Am. J. Physiol. Heart Circ. Physiol.* 291, H176–H183

**PPARs and Inflammation during Hypertrophy**

Inflammation is also from a clinical point of view of great importance, because chronic low-grade inflammation is thought to play a significant role in cardiac hypertrophy and failure. PPARs are able to inhibit myocardial inflammation and hypertrophy via inhibition of NF-κB. It is therefore tempting to speculate that PPARs serve as therapeutic targets to inhibit inflammatory signaling and, consequently, prevent hypertrophic growth of the heart.
30. Kubota, T., McTiernan, C. F., Frye, C. S., Slawson, S. E., Lemster, B. H., Koretsky, A. P., Demetris, A. J., and Feldman, A. M. (1997) Circ. Res. 81, 627–635
31. Li, Y., Ha, T., Gao, X., Kelley, J., Williams, D. L., Browder, I. W., Kao, R. L., and Li, C. (2004) Am. J. Physiol. Heart Circ. Physiol. 287, H1712–H1720
32. Ha, T., Li, Y., Gao, X., McMullen, J. R., Shioi, T., Izumo, S., Kelley, J. L., Zhao, A., Haddad, G. E., Williams, D. L., Browder, I. W., Kao, R. L., and Li, C. (2005) Free Radic. Biol. Med. 39, 1570–1580
33. Gupta, S., Young, D., and Sen, S. (2005) Am. J. Physiol. Heart Circ. Physiol. 289, H20–H29
34. Higuchi, Y., Chan, T. O., Brown, M. A., Zhang, J., DeGeorge, B. R., Jr., Funakoshi, H., Gibson, G., McTiernan, C. F., Kubota, T., Jones, W. K., and Feldman, A. M. (2006) Am. J. Physiol. Heart Circ. Physiol. 290, H590–H598
35. Blanquart, C., Barbier, O., Fruchart, J. C., Staels, B., and Glineur, C. (2003) J. Steroid Biochem. Mol. Biol. 85, 267–273
36. Smeets, P. J., Planavila, A., Van der Vusse, G. J., and Van Bilsen, M. (2007) Acta Physiol. 191, 171–188
37. Planavila, A., Laguna, J. C., and Vazquez-Carrera, M. (2005) J. Biol. Chem. 280, 17464–17471
38. Irukayama-Tomobe, Y., Miyauchi, T., Sakai, S., Kasuya, Y., Ogata, T., Takanashi, M., Iemitsu, M., Sudo, T., Goto, K., and Yamaguchi, I. (2004) Circulation 109, 904–910
39. Liang, F., Wang, F., Zhang, S., and Gardner, D. G. (2003) Endocrinology 144, 4187–4194
40. Smeets, P. J., Teunissen, B. E., Willemsen, P. H., van Nieuwenhoven, F. A., Brouns, A. E., Janssen, B. J., Cleutjens, J. P., Staels, B., van der Vusse, G. J., and van Bilsen, M. (2008) Cardiovasc. Res. 78, 79–89
41. Jucker, B. M., Doe, C. P., Schnackenberg, C. G., Olzinski, A. R., Maniscalco, K., Williams, C., Hu, T. C., Lenhard, S. C., Costell, M., Bernard, R., Sarov-Blat, L., Steplewski, K., and Willette, R. N. (2007) J. Cardiovasc. Pharmacol. 50, 25–34
42. Yndestad, A., Damas, K. O., Ole, E., Ueland, T., Gullestad, L., and Aukrust, P. (2006) Heart Fail Rev. 11, 83–92