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CELL AND MOLECULAR PHYSIOLOGY

Inactivation of peroxisome proliferator-activated receptor isoforms α, β/δ, and γ mediate distinct facets of hypertrophic transformation of adult cardiac myocytes

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Abstract Inactivation of peroxisome proliferator-activated receptor (PPARs) isoforms α, β/δ, and γ mediate distinct facets of hypertrophic transformation of adult cardiac myocytes. PPARs are ligand-activated transcription factors that modulate the transcriptional regulation of fatty acid metabolism and the hypertrophic response in neonatal cardiac myocytes. The purpose of this study was to determine the role of PPAR isoforms in the morphologic and metabolic phenotype transformation of adult cardiac myocytes in culture, which, in medium containing 20% fetal calf serum, undergo hypertrophy-like cell growth associated with downregulation of regulatory proteins of fatty acid metabolism. Expression and DNA-binding activity of PPARα, PPARβ/δ, and PPARγ rapidly decreased after cell isolation and remained persistently reduced during the 14-day culture period. Cells progressively increased in size and developed both re-expression of atrial natriuretic factor and downregulation of regulatory proteins of fatty acid metabolism. Supplementation of the medium with fatty acid (oleate 0.25 mM/palmitate 0.25 mM) prevented inactivation of PPARs and downregulation of metabolic genes. Furthermore, cell size and markers of hypertrophy were markedly reduced. Selective activation of either PPARα or PPARβ/δ completely restored expression of regulatory genes of fatty acid metabolism but did not influence cardiac myocyte size and markers of hypertrophy. Conversely, activation of PPARγ prevented cardiomyocyte hypertrophy but had no effect on fatty acid metabolism. The results indicate that PPAR activity markedly influences hypertrophic transformation of adult rat cardiac myocytes. Inactivation of PPARα and PPARβ/δ accounts for downregulation of the fatty acid oxidation pathway, whereas inactivation of PPARγ enables development of hypertrophy.

Keywords Metabolism · Genes · Hypertrophy · Phenotype · Cardiomyocyte

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Introduction

Phenotypic modification of cardiac myocytes plays a critical role during the development of myocardial hypertrophy and progression to heart failure [25]. A number of observations suggest that morphological and functional changes of cardiac myocytes are associated with modification of transcriptional regulation of substrate metabolism including altered expression of glucose transporter isoforms [32, 36] and downregulation of genes encoding regulatory proteins of fatty acid oxidation [26, 27, 39]. To date, very little is known on the mechanisms linking alterations of the morphologic and metabolic phenotype.

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptor transcription factors that are activated by fatty acid derivatives and, therefore, may modify the cellular phenotype in response to alteration of cytoplasmic fatty acid concentration [16, 44]. PPARs bind to the DNA consensus response element as obligate heterodimers with retinoid X receptor (RXR) [16]. All three presently known isoforms, PPARα, PPARβ/δ, and PPARγ, are expressed in cardiac myocytes [14, 16]. PPARα and PPARβ/δ play a central role in the transcriptional regulation of fatty acid oxidation [14]. In fact, PPARα-deficient mice [46] and mice with cardiac-myocyte-restricted deletion of PPARβ/δ in the heart [5] exhibit marked reduction of fatty acid oxidation enzymes in the myocardium and a corresponding

decrease of fatty acid oxidation. Observations in animal models have implicated inactivation of PPARα signaling in the downregulation of fatty acid oxidation enzymes during overload-induced myocardial hypertrophy and heart failure [2, 19, 48]. In adipocytes, PPARγ is involved in the expression of enzymes of synthesis of fatty acids and triglycerides [16], but its role in cardiac myocytes is still poorly defined.

Studies in neonatal rat cardiac myocytes suggest that PPAR signaling not only influences regulation of fatty acid metabolism but also may modify the hypertrophic response. Selective activation of either PPARα [17, 21], PPARβ/δ [30], or PPARγ [47] reduced protein synthesis in neonatal cardiac myocytes exposed to phenylephrine [30, 47], angiotensin II [47], endothelin-1 [17, 21], or mechanical strain [47]. Cell size and expression of atrial natriuretic factor was reduced concomitantly [1, 21, 47]. These observations are compatible with the hypothesis that inactivation of PPARα, and possibly of other PPAR isoforms, may not only mediate downregulation of the fatty acid oxidation pathway, but also contribute to cardiac myocyte hypertrophy and dedifferentiation during maladaptive remodeling. However, the role of individual PPAR isoforms in the modification of the morphologic and metabolic phenotype has not been studied in adult cardiac myocytes.

In the present study, we investigated the role of PPAR expression and activity in the phenotypic modification of adult rat cardiac myocytes (ARC) in long-term culture. ARC cultured in medium containing 20% fetal calf serum (FCS) undergo phenotypic transformation that exhibits similarities to that observed during the development of maladaptive hypertrophy and heart failure in vivo [41]. The changes include cellular spreading, re-expression of "immature" isoforms of contractile proteins, and re-expression of so-called fetal genes [9, 12, 22]. We have previously observed that the phenotypic modification of ARC is associated with the return to a fetal-like expression pattern of metabolic genes. There occurs a shift from glucose transporter-4 (GLUT4) to glucose transporter-1 (GLUT1) [23, 35], and downregulation of messenger RNA (mRNA) expression of a number of regulatory genes of fatty acid oxidation [37]. We hypothesized that hypertrophy-like phenotype transformation and downregulation of the fatty acid oxidation pathway is associated with inactivation of PPAR signaling.

Materials and methods

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publications no. 85–23, revised 1996) and was approved by the local animal protection authorities.

Cell isolation and culture

ARC were isolated from male oncins France strain A (OFA) rats (Charles River, France) weighing 110–120 g by retrograde perfusion of the hearts with collagenase (Worthington Biochemical) as described previously [13, 23]. After preplating in uncoated dishes (2 h; 37°C) to remove non-myocyte cells, cardiac myocytes were plated onto dishes coated with 0.1% gelatin. The culture medium was M199 supplemented with 20% (vol/vol) FCS (Invitrogen), 1% penicillin/streptomycin, and 20 mmol/l creatine throughout the culture period. To avoid proliferation of residual non-myocyte cells, 10 μmol/l 1-β-D-arabinofuranosylcytosine was added to the culture medium. More than 90% of the cells were myocytes upon visual inspection at light microscopy after each culture interval with increasing purity over time. The standard culture medium contained glucose (5.5 mmol/l). In selected experiments, palmitate and oleate at equimolar concentration (0.25 mmol/l each, unless otherwise stated) were added to the culture medium throughout the entire culture period. Fatty acids were prebound to serum albumin (0.2 mmol/l) as described previously [8]. Unlike palmitate alone, palmitate plus oleate does not elicit apoptosis in ARC [11]. PPAR isoforms were selectively activated with the PPARα ligand WY-14643 (100 nmol/l; Biomol), the PPARβ/δ ligand L 165–041 (1 μmol/l; Fluka), or the PPARγ ligand ciglitazone (10 nmol/l; Biomol). PPAR agonists were dissolved in dimethyl sulphoxide (DMSO), which was also added (0.1% vol/vol) to the control cells.

Immunofluorescence and cell surface area determination

ARC were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.3% Triton ×−100 in PBS. Primary antibody against sarcomeric α-actinin (Sigma) was recognized by an anti-mouse IgG-FITC-conjugated antibody (Sigma). Counterstaining for F-actin was obtained with rhodamine–phalloidin (Molecular Probes, Eugene) and DNA staining with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Slides were mounted with VectaShield (Vector Laboratories) and examined using a Carl Zeiss Axiophot microscope equipped with an Axiocam color charge-coupled device (CCD) camera (Carl Zeiss). Images were acquired with the AxioVision software (Carl Zeiss) and processed with Photoshop 3.0.5 (Adobe Systems).

Relative cell surface area was calculated from digitized cardiac myocyte images taken from random fields of view using NIH Image software (version 1.62). At least 100 cells

were measured for each condition by an observer blinded to the culture conditions.

Protein extraction and western blot

Cells were homogenized in lysis buffer containing Tris–HCl 10 mmol/l, NaCl 150 mmol/l, and ethylenediaminetetraacetic acid (EDTA) 1 mmol/l, Igepal 1%, sodium deoxycholate 0.5%, sodium dodecyl sulfate (SDS) 0.1%, and protease inhibitor cocktail (Roche). Proteins (100 μg) were loaded onto 8.5% SDS polyacrylamide gel electrophoresis (SDS–PAGE) gels. Ponceau's red staining was performed to verify equal loading. Western blot analysis was performed using polyclonal antibodies against PPARα and PPARγ (Santa Cruz Biotechnology), PPAR β/δ (Cayman chemicals), fatty acid translocase (FAT/CD36; Santa Cruz Biotechnology), medium-chain acyl-CoA dehydrogenase (MCAD; Cayman Chemicals), and muscle-type carnitine palmitoyltransferase-I (mCPT-I; a gift from Dr. Gebre Woldegiorgis). Antibodies were diluted in Tris-buffered saline containing 0.1% Tween 20. All antibodies identified the proper protein band. Specific signals were detected by chemiluminescence (ECL, Amersham) and quantified by laser densitometry (Imagequant 3.3, Molecular Dynamics).

DNA-binding activity of PPAR

DNA-binding activities of PPARα and PPARγ were measured in nuclear extracts prepared as described previously [23] using an enzyme-linked immunosorbent assay (ELISA) assay (Active Motif). Briefly, active PPARs in nuclear extracts bind specifically to consensus oligonucleotides coated on a well plate and are detected using an antibody directed against PPAR. Addition of a secondary antibody conjugated to horseradish peroxidase provides a sensitive colorimetric readout that is quantified by spectrophotometry.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was purified using Trizol reagent. After DNAse (Invitrogen) treatment, RNA was analyzed by reverse transcription (Superscript II, Invitrogen) followed by real-time quantitative polymerase chain reaction (PCR; TaqMan, BioRad). The rat nucleotide sequences of probes, as well as of forward and reverse primers, were designed from unconserved sequences of the genes, allowing isoform specificity (Table 1). The transcripts of the constitutive housekeeping gene product cyclophilin were quantitatively measured in each sample, and PCR data were reported as the number of transcripts per cyclophilin transcript. Cycle number for cyclophilin was virtually identical in ARC cultured for 14 days (27.5±2.0 cycles/μgRNA) and in freshly isolated ARC (27.5±1.8 cycles/μg RNA), indirectly suggesting expression stability over time. Furthermore, identical results to normalization by cyclophilin were obtained using 18S ribosomal RNA (rRNA) as housekeeping gene.

Palmitate oxidation

Palmitate oxidation was estimated based on the release of $^{14}CO_2$ from [1-^{14}C]-palmitate (Amersham). For measurement of palmitate oxidation, cells were incubated for 60 min in sealed flasks containing a suspended filter paper soaked with an organic base (NCS-II, Amersham) and medium containing palmitate (0.05 mmol/l), oleate (0.05 mmol/l), and 1 μCi/ml [1-^{14}C]-palmitate. All fatty acids were complexed to bovine serum albumin (0.2 mmol/l). The reaction was stopped by addition of 2N perchloric acid. $^{14}CO_2$ produced by [1-^{14}C]-palmitate metabolism was collected overnight on the filter paper and quantified by scintillation counting.

Table 1 Primers of RT-PCR assays. F: forward primer, R: reverse primer, P: probe

Genes		Primers and probe sequences
Cyclophilin	F	5′-CTG ATG GCG AGC CCT TG-3′
	R	5′-TCT GCT GTC TTT GGA ACT TTG TC-3′
	P	5′-CGC GTC TGC TTC GAG CTG TTT GCA-3′
ANF	F	5′-AGT GCG GTG TCC AAC ACA G-3′
	R	5′-CTT CAT CGG TCT GCT CGC T-3′
	P	5′-TCT GAT GGA TTT CAA GAA CCT GCT AGA CCA-3′
BNP	F	5′-AAG TCC TAG CCA GTC TCC A-3′
	R	5GTC TCT GAG CCA TTT CCT C-3′
	P	5′-GCA GAA GCT GCT GGA GCT GAT AA-3′
PPARα	F	5′-ACT ACG GAG TTC ACG CAT GTG-3′
	R	5′-TTG TCG TAC ACC AGC TTC AGC-3′
	P	5′-AGG CTG TAA GGG CTT CTT TCG GCG-3′
PPARβ/δ	F	5′-TGG ATA CGC TTC ACA GTC C-3′
	R	5′-GCA GGC TCT AGA ATT CCA TC-3′
	P	5′-ACC TGC GGC AGC TGG TCA CTG A-3′
PPARγ	F	5′-CCA TTC TGG CCC ACC AAC-3′
	R	5′-AAT GCG AGT GGT CTT CCA TCA-3′
	P	5′-TCG GAA TCA GCT CTG TGG ACC TCT CC-3′
FAT/CD36	F	5′-TCC AGC CAA TGC CTT TGC-3′
	R	5′-TGG AGA TTA CTT TTC AGT GCA GAA-3′
	P	5′-TCA CCC CTC CAG AAT CCA GAC AAC CA-3′
MCAD	F	5′-TGG CAT ATG GGT GTA CAG GG-3′
	R	5′-CCA AAT ACT TCT TCT TCT GTT GAT CA-3′
	P	5′-AGG CAT TTG CCC CAA AGA ATT TGC TTC-3′
mCPT-I	F	5′-ATC ATG TAT CGC CGC AAA CT-3′
	R	5′-ATC TGG TAG GAG CAC ATGG GT-3′
	P	5′-TCA AGC CGG TAA TGG CAC TGG G-3′

[^{3}H]-Phenylalanine incorporation

To obtain an index for the rate of de novo protein synthesis, ARC were incubated after selected culture intervals for 2 h at 37°C in serum-free M199 containing 2 μCi/ml of L-[2,3,4,5-^{3}H]phenylalanine (Amersham) with or without PPAR agonists. At the end of the incubation period, the culture medium was removed, and cardiac myocytes were rinsed three times with ice-cold PBS. DNA and protein were precipitated by trichloroacetic acid and transferred into 0.3-M sodium hydroxide. [^{3}H]-Phenylalanine incorporation and DNA content were determined as reported previously [34]. [^{3}H]-phenylalanine incorporation was normalized to nanograms of DNA per sample. Results are expressed as percentage of the mean value measured in freshly isolated ARC cultured for measurement of [^{3}H]-phenylalanine incorporation in standard medium.

Statistics

Results are obtained from at least three independent experiments and expressed as mean±SEM. Mean group values were compared by analysis of variance (ANOVA; Prism 4), applying Bonferroni's adjustment for multiple comparisons. P values<0.05 were considered significant.

Results

Fatty acid supplementation of culture medium prevents ARC hypertrophy and downregulation of mRNA expression of regulatory proteins of fatty acid metabolism

ARC cultured in medium containing 20% FCS underwent morphological remodeling as described previously [12, 23]. After 3–4 days of culture, cells resumed spontaneous beating

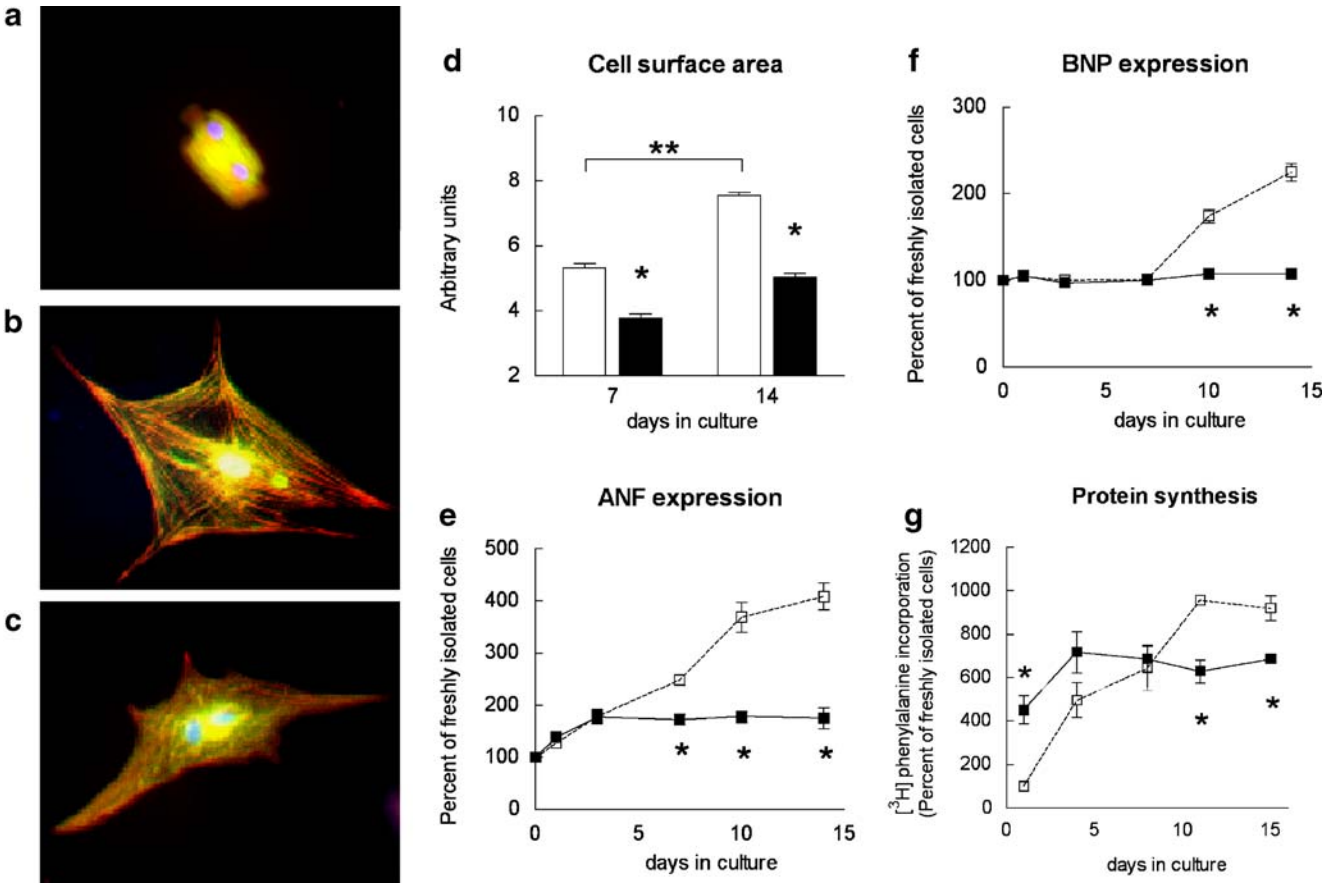

Fig. 1 Effect of supplementation of the culture medium with fatty acid (oleate 0.25 mmol/l/palmitate 0.25 mmol/l) on cardiac myocyte size and ANF expression. **a** Freshly isolated cardiac myocyte. After 14 days of culture in medium containing 20% FCS, cardiac myocytes had increased in size and assumed a polymorphic shape (**b** and **c**). Cells cultured in fatty-acid-free medium were larger (**b**) than in medium containing oleate/palmitate (**c**). *Green* α-Actinin, *blue* nucleus, and *red* phalloidine. **d** Relative difference of cell size estimated by planimetry on days 7 and 14 (control, *open bars*; fatty acid, *filled bars*; at least 100 cells in three to six different cultures were measured for each condition). **e** and **f** Change in ANF and BNP mRNA expression relative to freshly isolated cardiac myocytes (n=6 for each condition). ANF (**e**) and BNP (**f**) expression were markedly lower in cells cultured during 14 days with fatty acids (*filled squares*) compared with those cultured without fatty acids (*open squares*). **g** De novo protein synthesis, estimated from [^{3}H]-phenylalanine incorporation (n, at least four independent experiments), increased throughout the culture period (*open squares*) and was attenuated by fatty acids (*filled squares*). Values are means+SEM. *P<0.05 compared with "without fatty acid." **P<0.05 between indicated groups

and continuously increased in size throughout the 14-day culture period (Fig. 1a–d). The rate of de novo protein synthesis, estimated from [^{3}H]-phenylalanine incorporation, also continuously increased (Fig. 1g). Moreover, mRNA expression of atrial natriuretic factor (ANF) increased progressively and averaged, after 14 days, 409±25% of the value measured in freshly isolated cardiac myocytes (Fig. 1e). Similarly, brain natriuretic peptide (BNP) mRNA increased to 225±9% ($P<0.05$; Fig. 1f). Supplementation of the culture medium with 0.5 mmol/l fatty acids (palmitate 0.25 mmol/l plus oleate 0.25 mmol/l) during the entire culture period reduced cell spreading with a decrease of cell surface area by 29 and 33% after 7 and 14 days, respectively, compared with cells cultured in standard medium (Fig. 1b–d). The rate of de novo protein synthesis leveled off after 5 days in the presence of fatty acids and remained constant thereafter (Fig. 1g). Fatty acids markedly attenuated the increase of ANF mRNA (Fig. 1e) and prevented the increase of BNP mRNA, which averaged, after 14 days, 107±14% of the values measured in freshly isolated cardiac myocytes (Fig. 1f). No appreciable effect of fatty acids on contractile function was observed.

We monitored the expression of three PPAR target genes that are involved in the regulation of fatty acid metabolism: fatty acid translocase (FAT/CD36), medium-chain acyl-CoA dehydrogenase (MCAD), and muscle-type carnitine palmitoyltransferase-I (mCPT-I). In standard medium, mRNA expression of MCAD, FAT/CD36, and mCPT-I decreased rapidly after cell isolation to 50 to 70% of values measured in freshly isolated cardiac myocytes and continued to decrease thereafter to 15 to 35% after 14 days (Fig. 2a–c). Interestingly, there was no concomitant decrease of protein expression of

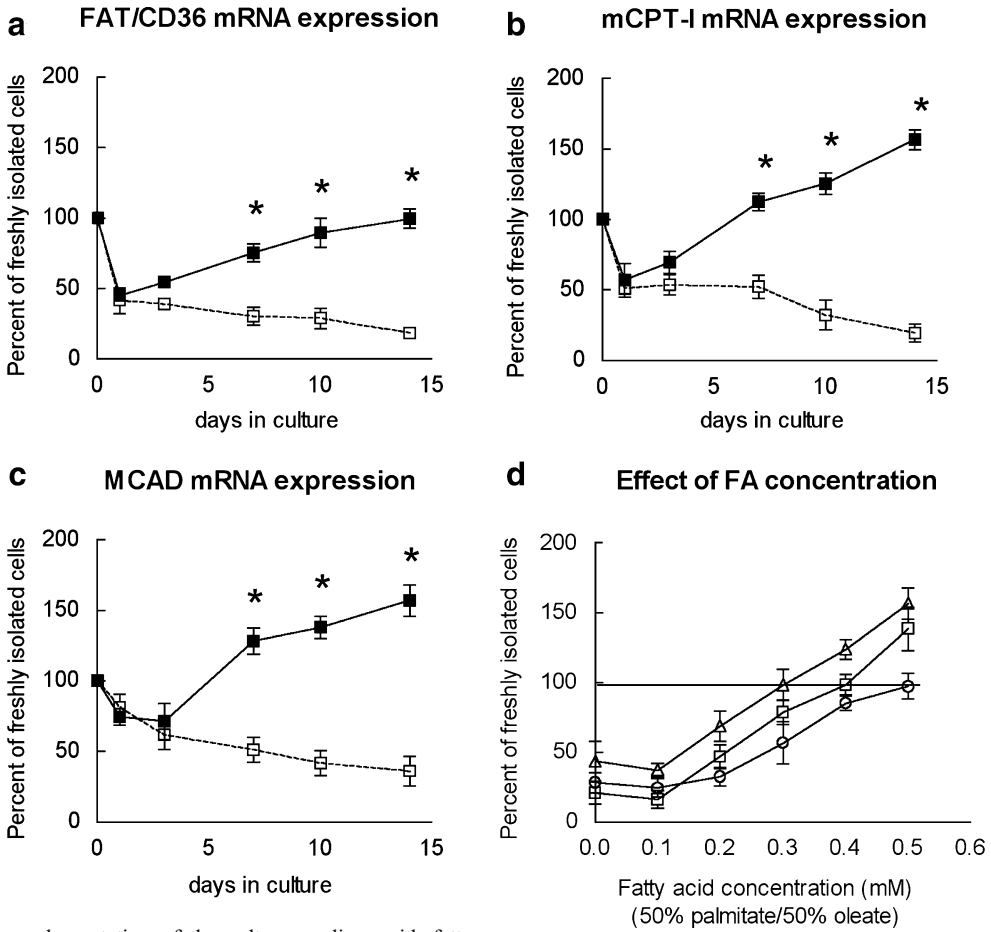

Fig. 2 Effect of supplementation of the culture medium with fatty acids on mRNA expression of regulatory proteins of fatty acid metabolism. mRNA expression of FAT/CD36 (**a**), mCPT-I (**b**), and MCAD (**c**) decreased rapidly after isolation and remained low in cardiac myocytes cultured without fatty acid (*open squares*). The reduction compared with freshly isolated cardiac myocytes was significant for each culture period ($P<0.05$). In the presence of 0.5 mmol/l fatty acids (oleate 0.25 mmol/l plus palmitate 0.25 mmol/l; *filled squares*), mRNA expression of all three proteins steadily increased to supranormal values (*n*, at least five determinations for each data point). **d** Effect of increasing fatty acid (*FA*) concentration on mRNA expression of FAT/CD36 (*circles*), mCPT-I (*squares*), and MCAD (*triangles*). Stimulation of mRNA expression was observed at all examined total fatty acid concentrations>0.1 mmol/l. Each fatty acid concentration consisted of equimolar proportions of oleate and palmitate. Values are means±SEM. *$P<0.05$ compared with "without fatty acid"

MCAD, FAT/CD36, and mCPT-I after 14 days (Fig. 3a). Likewise, oxidation of palmitate was only slightly reduced by 21% compared with freshly isolated ARC (Fig. 3b).

Supplementation of the medium with fatty acid did not prevent the initial drop of mRNA expression of MCAD, FAT/CD36, and mCPT-I (Fig. 2a–c). However, mRNA expression increased steadily thereafter. Stimulation of mRNA expression was observed at total fatty acid concentrations higher than 0.1 mmol/l (Fig. 2d). In the presence of a total fatty acid concentration of 0.5 mmol/l, values measured after 14 days were equal (FAT/CD36) or higher (mCPT-I, MCAD) than those measured in freshly isolated cardiac myocytes (Fig. 2a–c). Concomitantly, protein expression of MCAD, FAT/CD36, and mCPT-I was increased roughly by 50% compared with values measured in ARC cultured without fatty acid (Fig. 3a), and oxidation of palmitate was approximately doubled (Fig. 3b).

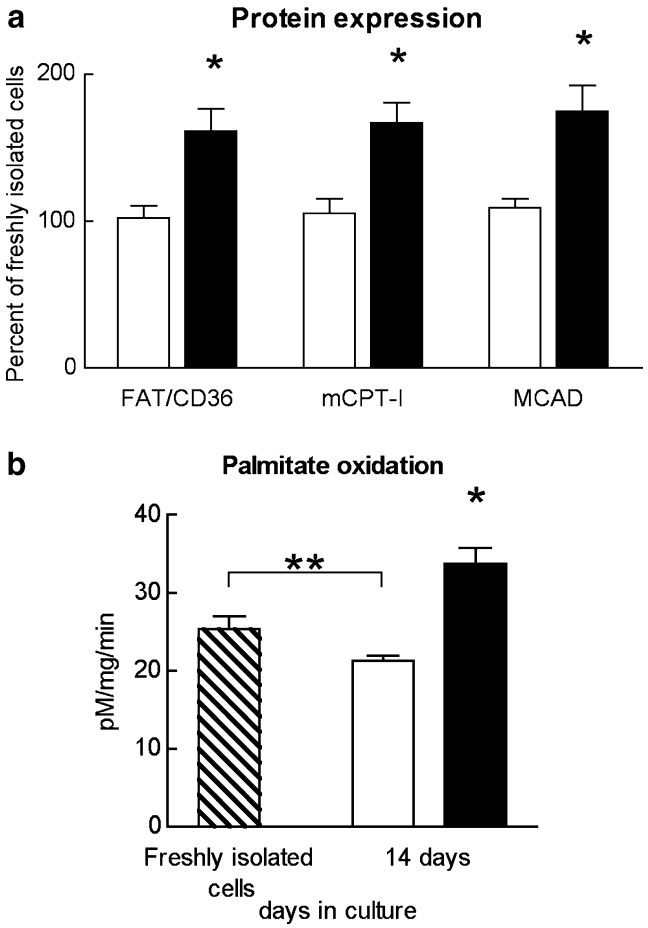

Fig. 3 Effect of supplementation of the culture medium with fatty acids (oleate 0.25 mmol/l plus palmitate 0.25 mmol/l) on protein expression of regulatory proteins of fatty acid oxidation (**a**) and palmitate oxidation (**b**). **a** On day 14, protein expression of FAT/CD36, mCPT-I, and MCAD was approximately 50% higher in ARC cultured with fatty acids (*filled bars*) compared to ARC cultured without fatty acids (*open bars*; *n*, at least six determinations for each data point). **b** Palmitate oxidation in cardiac myocytes immediately after isolation (freshly isolated ARC, $n=6$) and in cells cultured for 14 days without (*open bar*, $n=5$) and with fatty acids (*filled bar*, $n=4$). Palmitate oxidation was higher in the presence of fatty acids. Values are means±SEM. *$P<0.05$ compared with "without fatty acid". **$P<0.05$ between indicated groups

Reduction of transcriptional activity of PPARα, PPARβ/δ, and PPARγ in ARC is restored by fatty acid supplementation

We determined whether the expression and/or DNA-binding activity of PPARs are altered in ARC undergoing phenotypic changes. In ARC cultured in standard medium, mRNA expression levels of PPARα, PPARβ/δ, and PPARγ were decreased to less than 50% of the values measured in freshly isolated ARC throughout the entire 14-day culture interval (Fig. 4a). However, protein expression decreased only transiently (day 1) and was restored by day 3 to values comparable to those measured in freshly isolated ARC, without further change up to day 14 (Fig. 4b). DNA-binding activity of PPARα and PPARγ (binding activity of PPARβ/δ was not measured) decreased to approximately 50% of values measured in freshly isolated ARC and, in contrast to protein expression, did not appreciably recover thereafter (Fig. 4c and d). Thus, cardiac myocyte remodeling seems to be associated with reduced DNA binding, rather than reduced protein expression of PPAR isoforms.

Supplementation of the culture medium with fatty acids largely restored DNA binding of PPARα and PPARγ by day 7 (Fig. 4c and d). mRNA expression of all PPAR isoforms was also restored by fatty acid, showing a similar time course (only data for day 14 displayed; Fig. 4a). This presumably reflects the known presence of PPAR response elements (PPRE) in the promoter region of PPAR genes [30].

Differential effects of PPAR isoforms on metabolic and morphologic phenotype of ARC

To determine the role of individual PPAR isoforms, ARC were treated with specific agonists of either PPARα (WY-14643), PPARβ/δ (L 165-041), or PPARγ (ciglitazone). As each PPAR isoform transactivates its own expression, specificity of compounds was validated by measurement of the effect on PPAR mRNA expression in standard medium. At the selected concentration, each agonist selectively restored mRNA expression of the corresponding PPAR isoform to values comparable to those measured in freshly isolated cells (Fig. 5a). DNA-binding activity was measured for PPARα and PPARγ. WY-14643 and ciglitazone selectively increased DNA-binding activity of PPARα and PPARγ, respectively (Fig. 5b). Thus, the selected agonists selectively activated PPAR isoforms in ARC.

The effect of selective activation of each PPAR isoform on the expression of regulatory genes of fatty acid metabolism

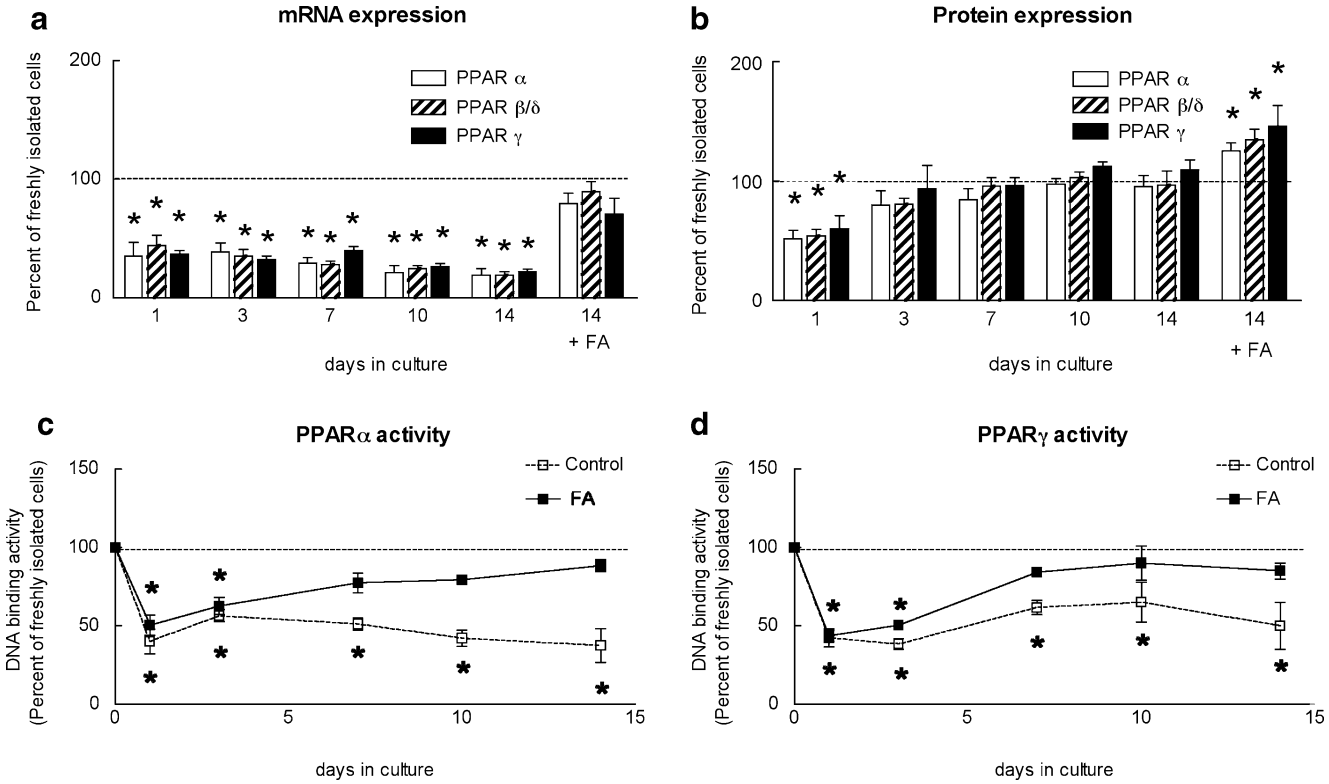

Fig. 4 Changes of expression and DNA-binding activity of PPAR isoforms. In cardiac myocytes cultured in standard medium. mRNA expression by RT-PCR of PPARα, PPARβ/δ, and PPARγ (**a**) was reduced by at least 50% during the entire culture period. In the presence of fatty acids, mRNA expression was not reduced after 14 days. Protein expression (**b**) dropped only transiently, immediately after isolation, and completely recovered thereafter. DNA-binding activity (measured by ELISA) of PPARα (**c**) and PPARγ (**d**) decreased immediately after isolation and remained low in standard medium (*open squares*). Fatty acid supplementation largely restored DNA-binding activity (*filled squares*). Values are means±SEM of at least three determinations for each data point. *$P<0.05$ compared with freshly isolated ARC

and hypertrophic markers is shown in Figs. 6 and 7. Activation of PPARα or PPARβ/δ by WY-14643 or L 165–041, respectively, restored mRNA expression of FAT/CD36, mCPT-I, and MCAD to values comparable to those measured in freshly isolated cardiac myocytes (Fig. 6a–c). Conversely, activation of PPARγ by ciglitazone had no effect on the expression of genes of fatty acid metabolism (Fig. 6a–c).

Cell surface area of ARC cultured for 14 days in standard medium was significantly reduced by ciglitazone by 37% compared with the control (Fig. 7a). Ciglitazone also decreased the rate of de novo protein synthesis by 22% (Fig. 7c). Furthermore, ciglitazone reduced expression of ANF by 53% (Fig. 7b) and of BNP by 31% ($P<0.05$). In contrast, WY-14643 and L 165–041 did not alter cell surface area, protein synthesis, expression of ANF, or expression of BNP (Fig. 7a–c).

Discussion

Ventricular myocytes of adult rat hearts cultured in medium containing 20% FCS undergo a progressive remodeling process. Phenotypic changes include an increase in cell size, acceleration of de novo protein synthesis and re-expression of so-called "fetal genes," resembling cardiac myocyte hypertrophy in vivo [9, 12, 23, 41]. ARC, therefore, offer a homogenous system for delineating the effects of individual stimuli on the hypertrophic response in terms of gene expression and cell morphology. The present study implicates all three PPAR isoforms expressed in the myocardium in an isoform-specific manner as modulators of the morphologic and metabolic phenotype of adult cardiac myocytes. The results demonstrate in ARC in long-term culture that: (1) the progressive hypertrophic response and the downregulation of genes controlling fatty acid oxidation are associated with inactivation of PPARα, PPARβ/δ, and PPARγ at unaltered protein levels; (2) PPAR-inactivation is reversed, and both metabolic and hypertrophic changes are attenuated, by supplementation of the culture medium with fatty acid; and (3) in fatty-acid-free medium, selective activation of PPARα or PPARβ/δ, but not of PPARγ, restores expression of regulatory proteins of fatty acid metabolism, without a change of markers of hypertrophy. Conversely, activation of PPARγ attenuates

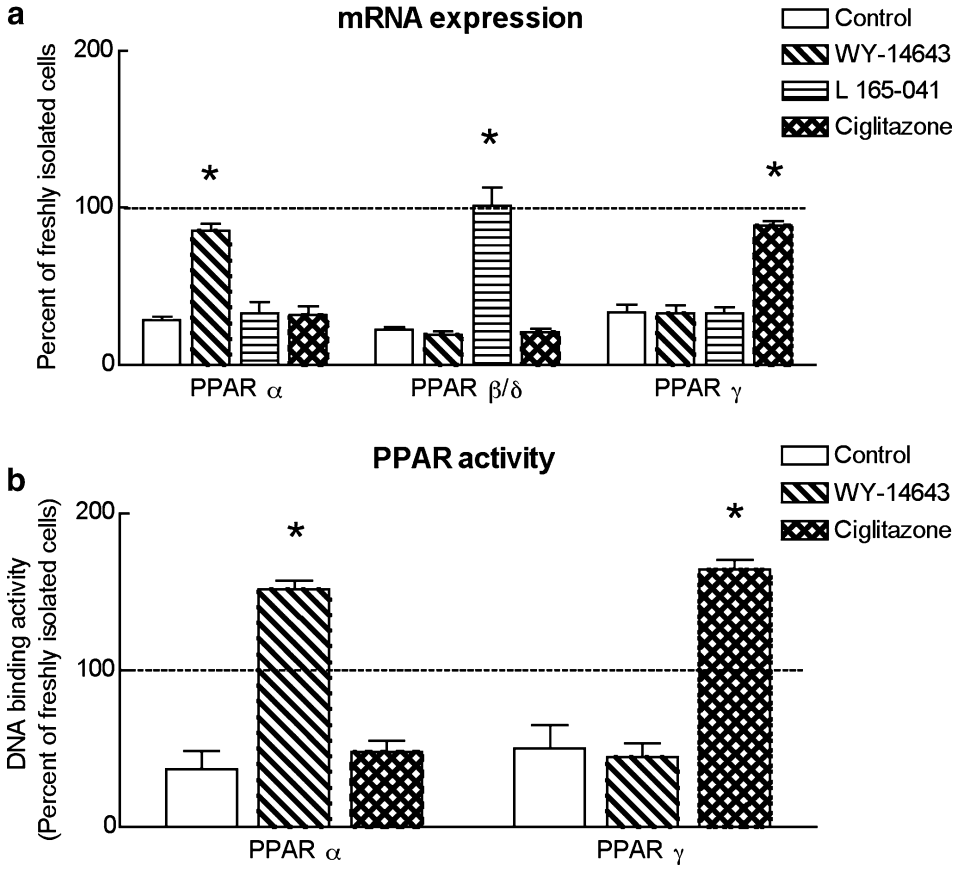

Fig. 5 Effect of specific activation of PPAR isoforms on mRNA expression (**a**) and DNA-binding activity (**b**) after 14 days of culture. **a** Activation of PPARα by WY-14643 (100 nmol/l), of PPARβ/δ by L 165–041 (1 μmol/l), and of PPARγ by ciglitazone (10 nmol/l) restored mRNA expression of the corresponding PPAR isoform. **b** DNA binding of PPARα was increased by WY-14643 and that of PPARγ by ciglitazone. Values are means±SEM of at least five determinations. *$P<0.05$ compared with untreated cells (*control*)

markers of hypertrophy, without influencing expression of genes of fatty acid metabolism.

Similar to previous observations by others in neonatal cardiac myocytes, we have found that all three isoforms, PPARα, PPARβ/δ, and PPARγ, are expressed in adult rat cardiac myocytes. mRNA expression of each isoform rapidly decreased in ARC cultured in standard medium and remained low throughout the 14-day culture period. Conversely, protein

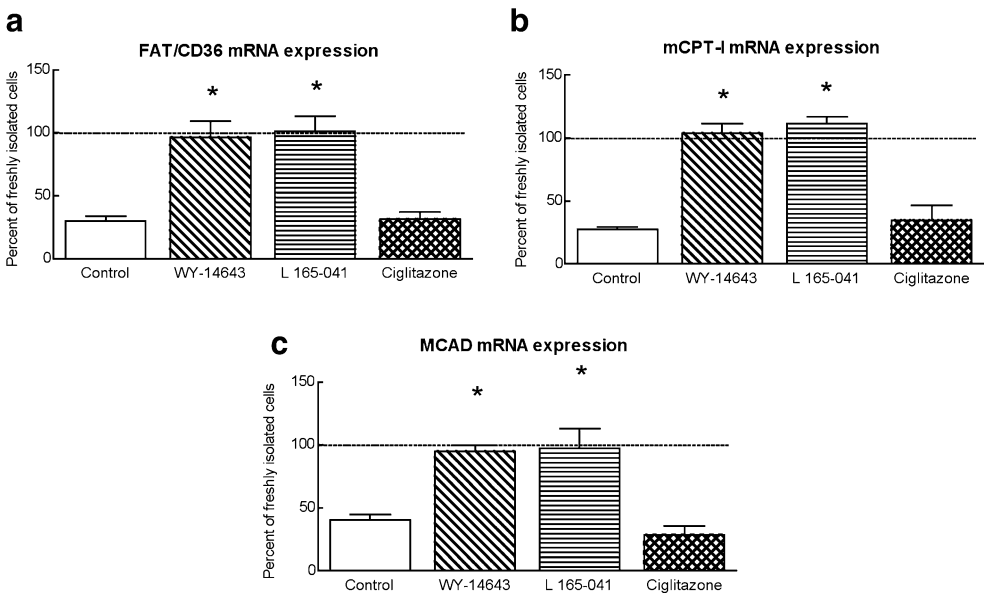

Fig. 6 Effect of activation of PPARα, PPARβ/δ, and PPARγ by WY-14643, L-165–041, and ciglitazone, respectively, on mRNA expression of FAT/CD36 (**a**), mCPT-I (**b**), and MCAD (**c**). Specific ligands of PPARα and PPARβ/δ (WY-14643, L 165–041) restored expression of each regulatory protein of fatty acid oxidation. Activation of PPARγ by ciglitazone had no effect. Values are means±SEM of at least four determinations. *$P<0.05$ compared with untreated cells (*control*)

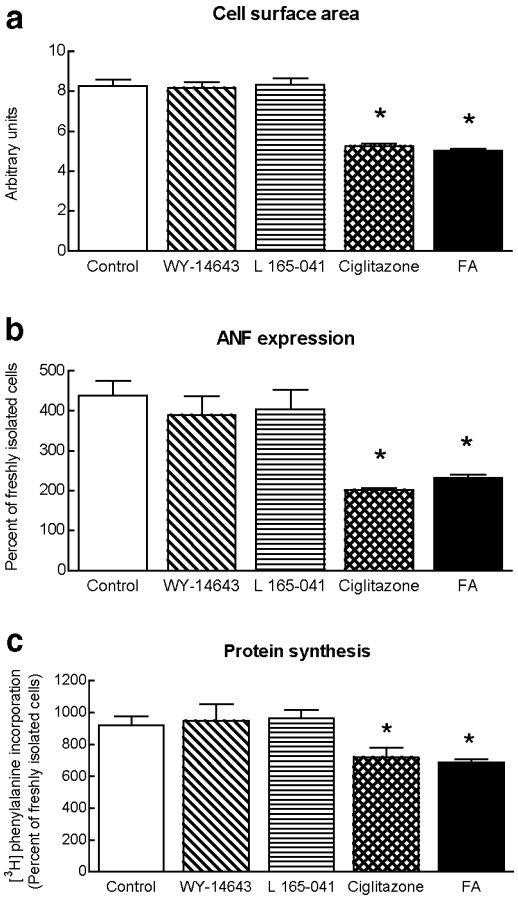

Fig. 7 Effect of activation of PPAR isoforms by specific ligands on cell surface (**a**), ANF mRNA expression (**b**), and de novo protein synthesis (**c**). Activation of PPARα by WY-14643 and PPARβ/δ by L 165–041 had no effect. Activation of PPARγ by ciglitazone decreased cell surface, ANF expression, and de novo protein synthesis to a comparable extent as fatty acid. Values are means±SEM of at least four determinations. *$P<0.05$ compared with untreated cells (*control*)

expression decreased only transiently and returned to levels measured immediately after isolation within 3 days, suggesting posttranscriptional compensation by as yet unknown mechanisms. However, DNA-binding activity of PPARα and PPARγ was reduced by roughly 50% during the entire culture period, compared with freshly isolated cells (binding activity of PPARβ/δ not measured). mRNA expression and DNA-binding activity exhibited a similar time-course most likely reflecting the fact that PPARs transactivate their own expression [3]. This is further supported by the observation that specific activation of PPARα and PPARγ by WY-14643 and ciglitazone, respectively, restored both DNA-binding activity and mRNA expression of the corresponding isoform. Although not measured, we anticipate that PPARβ/δ was also inactivated because mRNA expression was reduced and could be reactivated by L 165–041. Collectively, the results provide circumstantial evidence that transcriptional activity of all three PPAR isoforms is reduced in ARC undergoing phenotype transformation, despite unaltered protein expression.

There is evidence that PPAR signaling is altered during the development of maladaptive myocardial hypertrophy in vivo. Reduction of both myocardial expression and activity of PPARα has been observed in rodents with mechanical overload induced by constriction of either the aorta [2, 48] or the pulmonary artery [38]. Mechanisms that may contribute to the inactivation of PPARα during cardiac hypertrophy include phosphorylation of PPARα by extracellular signal-regulated MAP kinase [2], increased expression of the competing repressor chicken ovalbumin upstream promoter-transcription factor (COUP-TF) [38], or reduced availability of the co-activator PGC-1α [2, 20].

mRNA expression of FAT/CD36, CPT-I, and MCAD, which are target genes of PPARα and PPARβ/δ, decreased in ARC concomitantly with the reduction of DNA-binding activity (and mRNA expression) of PPARs. An interesting finding was that both protein expression and palmitate oxidation were not reduced concomitantly but, similar to PPARs protein expression, was maintained at levels comparable to those measured in freshly isolated cardiac myocytes. A dissociation between mRNA and protein expression of genes of fatty acid oxidation has also been observed in hypertrophic myocardium of spontaneously hypertensive rats [39] and of mice with targeted overexpression of angiotensinogen in the myocardium [27]. In these rodent models of progressive hypertrophy, in vivo protein expression and enzyme activity of MCAD was maintained at control level during compensated hypertrophy, despite marked reduction of mRNA, but dropped markedly after the onset of heart failure [27, 39]. Taken together, these observations suggest the involvement of posttranscriptional mechanisms in the control of levels of regulatory proteins of fatty acid metabolism during cardiac myocyte remodeling. However, the underlying molecular events are presently not known.

Inclusion of fatty acids (oleate 0.25 mmol/l plus palmitate 0.25 mmol/l) in the culture medium restored mRNA expression of PPARα, PPARβ/δ, and PPARγ, and DNA-binding activity. Both mRNA and protein expression of FAT/CD36, mCPT-I, and MCAD increased to levels exceeding those measured in freshly isolated ARC. Concomitantly, fatty acid oxidation rate was higher than in freshly isolated cells. Analogous findings have been reported for neonatal rat cardiac myocytes [14, 45]. However, in contrast to observations in neonatal cardiac myocytes cultured for 48 h [45], exposure to fatty acid not only increased the expression of the fatty acid oxidation pathway, but also attenuated cardiac myocyte growth, protein synthesis, and expression of hypertrophy marker genes. After 14 days of culture, adult rat cardiac myocytes were smaller by 33%, and both ANF and BNP mRNAs were reduced by more than 50%. The results are compatible with the hypothesis that inactivation of PPAR signaling contributes to morphologic and metabolic remodeling of cardiac myocytes.

The contribution of individual PPAR isoforms to phenotype modification of ARC has been determined in experiments using specific agonists. The results indicate that PPAR isoforms have distinct effects. Consistent with previous studies in neonatal cardiac myocytes in short-term culture [14], activation of PPARα or PPARβ/δ by WY-14643 and L 165-041, respectively, restored expression of regulatory genes of fatty acid metabolism. Conversely, activation of PPARγ by ciglitazone, as previously observed in neonatal cardiac myocytes [14], had no effect on metabolic genes.

In the present study, activation of PPARα and PPARβ/δ had no effect on cardiac myocyte size, protein synthesis, and the expression of ANF and BNP. This is in apparent contrast to previous observations in neonatal cardiac myocytes. In fact, activation of PPARα reduced the hypertrophic response in neonatal rat cardiac myocytes exposed to endothelin-1 [17, 21]. A similar effect has been observed after activation of PPARβ/δ by L 165–041 in phenylephrine-stimulated neonatal rat cardiac myocytes [30]. The reason for the absence of an antihypertrophic effect of activation of PPARα and PPARβ/δ in ARC is not apparent from the available data. However, at least three major differences in the experimental conditions should be considered: First, ARC and neonatal cardiac myocytes exhibit differences in the signaling pathways involved in hypertrophy [42]. Second, in neonatal cardiac myocytes, antihypertrophic effects of PPAR activation have been studied during stimulation by endothelin-1 or phenylephrine [17, 21, 30]. Third, studies in neonatal cardiac myocytes were limited to 1 to 3 days [30, 43, 47], whereas in the present study ARC were followed for 14 days.

In contrast to activation of PPARα and PPARβ/δ, activation of PPARγ by ciglitazone markedly reduced cardiac myocyte growth and protein synthesis in ARC, and decreased mRNA expression of ANF and BNP. Antihypertrophic effects of PPARγ signaling have also been observed in neonatal cardiac myocytes stimulated by angiotensin II [1, 47], phenylephrine [47], or mechanical strain [47]. Preliminary observations from our laboratory indicate that reactivation of PPARγ also attenuates angiotensin-II-mediated hypertrophy of ARC [28]. Interestingly, mice with targeted knockout of PPARγ in the myocardium develop ventricular hypertrophy even without exogenous hypertrophic stimulus, suggesting that PPARγ may suppress the hypertrophic gene program even in unstressed conditions [10]. Moreover, the hypertrophic response to pressure overload is enhanced in mice with cardiac-myocyte-restricted PPARγ deletion [10]. Conversely, PPARγ agonists inhibit ventricular hypertrophy induced by aortic constriction in wild-type mice [1, 40].

Whereas upregulation of genes of fatty acid metabolism most likely is mediated by direct interaction of the PPAR/RXR heterodimer with the promoter region [4, 6], the molecular mechanisms of attenuation of the hypertrophic response is less clear. DNA-binding-independent inactivation of other transcription factors (transrepression) may be involved in the antihypertrophic effects [7, 18, 29]. Studies in neonatal cardiac myocytes exposed to hypertrophic stimuli have demonstrated that activation of PPARγ [43, 47], but also of PPARα [43] and PPARβ/δ [30], inactivate NF-κB that is essential for hypertrophy in this model [15, 31]. Observations in macrophages raise the possibility that PPARγ may antagonize activity of other transcription factors potentially involved in hypertrophy, including activator protein-1 (AP-1) and STAT [33].

Collectively, this study demonstrates that PPAR activity considerably modulates phenotypic transformation of adult rat cardiac myocytes in culture. PPARα and PPARβ/δ on one hand, and PPARγ on the other hand, differ not only in their effects on the expression of regulatory proteins of fatty acid metabolism, but also in their modification of the hypertrophic response. This observation may be relevant for the development of therapeutic strategies designed to modify myocardial remodeling because restoration of fatty acid metabolism and attenuation of the hypertrophic response may differentially affect the remodeling process [24, 48].

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