Hypoxia Inhibits the Peroxisome Proliferator-activated Receptor α/Retinoid X Receptor Gene Regulatory Pathway in Cardiac Myocytes

A MECHANISM FOR O2-DEPENDENT MODULATION OF MITOCHONDRIAL FATTY ACID OXIDATION*

Received for publication, January 11, 2001, and in revised form, March 15, 2001
Published, JBC Papers in Press, May 22, 2001, DOI 10.1074/jbc.M100277200

Janice M. Huss‡§, Fiona H. Levy‖, and Daniel P. Kelly‡¶†‡‡
From the Center for Cardiovascular Research, Departments of Medicine, Pediatrics, and ‡‡Molecular Biology & Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Hypoxia triggers a cascade of cellular energy metabolic responses including a decrease in mitochondrial oxidative flux. To characterize gene regulatory mechanisms by which mitochondrial fatty acid oxidative capacity is diminished in response to hypoxia, cardiac myocytes in culture were exposed to long-chain fatty acids (LCFA) under normoxic or hypoxic conditions. Hypoxia prevented the known LCFA-induced accumulation of mRNA encoding muscle carnitine palmitoyltransferase I (M-CPT I), an enzyme that catalyzes the rate-limiting step in mitochondrial fatty acid oxidation (FAO). Under hypoxic conditions, myocytes exhibited significant accumulation of intracellular neutral lipid consistent with reduced CPT I activity and diminished FAO capacity. Transient transfection experiments demonstrated that the hypoxia-mediated blunting of M-CPT I gene expression occurs at the transcriptional level, is localized to an LCFA/peroxisome proliferator-activated receptor-α (PPARα/rexinoid X receptor (RXR)) response element within the M-CPT I gene promoter, and is PPARα-dependent. DNA-protein binding studies demonstrated that exposure to hypoxia reduces PPARα/RXR binding activity. Immunoblotting studies demonstrated that whereas hypoxia had no effect on nuclear levels of PPARα protein, nuclear and cellular RXRα levels were reduced. Hypoxia also diminished the 9-cis-retinoic acid-mediated activation of a reporter containing an RXR homodimer response element. These results demonstrate that hypoxia deactivates PPARα by reducing the availability of its obligate partner RXR.

Energy metabolic responses have evolved to preserve preservation of cellular function in hypoxic environments. One critical adaptive response involves the suppression of overall cellular energy consumption and improved efficiency of oxygen utilization during ATP production. Under hypoxic conditions, decreased oxygen consumption is achieved in part by increasing cellular glycolytic capacity while down-regulating mitochondrial fatty acid oxidative capacity, such as heart and brown adipose (15, 16). Tight post-translational control of M-CPT I activity is effected by malonyl-CoA, an intermediate in fatty acid synthesis, via a well-characterized mechanism of allosteric inhibition (14). Recently, we and others (17, 18) have shown that M-CPT I expression is also regulated at the gene transcriptional level by LCFA via the lipid-activated nuclear receptor, peroxisome proliferator-activated receptor α (PPARα). Specifically, transcriptional activation is mediated by LCFA via a heterodimeric complex composed of muscle carnitine palmitoyltransferase I, PPARα, peroxisome proliferator-activated receptor α; RXR, retinoid X receptor; LCFA, long-chain fatty acid; HIF-1α, hypoxia inducible factor-1α (HIF-1α) (4–6). Hypoxia also triggers additional transcriptional control mechanisms involving the Sp factor family (7) as well as regulatory effects at the post-transcriptional level (8).

Despite recent progress in characterizing the regulation of cellular glucose utilization in response to hypoxia, little is known about the mechanisms involved in hypoxia-mediated suppression of mitochondrial flux. This response is particularly relevant for tissues such as the mammalian heart which relies largely on mitochondrial FAO for energy (9) yet exhibits a dynamic plasticity related to energy substrate preferences. For example, during the transition from the fetal to postnatal period, the predominant myocardial energy source switches from glycolysis to FAO (9, 10). The adult human heart is also capable of adaptive metabolic responses. Under conditions of physiologic hypoxia (e.g. high altitude environments), myocardial energy substrate preference shifts from fatty acids to glucose (11). Studies performed in animals as well as in cell culture have shown a marked decrease in the rate of long-chain fatty acid (LCFA) β-oxidation in cardiac myocytes following exposure to a hypoxic environment (12, 13). The molecular mechanisms responsible for this hypoxia-mediated decrease in cardiac FAO are unknown.

The activity of the enzyme, carnitine palmitoyltransferase I (CPT I), is an important determinant of mitochondrial FAO cycle flux. CPT I catalyzes the rate-limiting step in the mitochondrial import of LCFA into mitochondria to the β-oxidation cycle (14). The muscle CPT I isoform, M-CPT I (or CPT Iβ), is expressed abundantly in tissues with high mitochondrial FAO capacity, such as heart and brown adipose (15, 16). Tight post-translational control of M-CPT I activity is effected by malonyl-CoA, an intermediate in fatty acid synthesis, via a well-characterized mechanism of allosteric inhibition (14). Recently, we and others (17, 18) have shown that M-CPT I expression is also regulated at the gene transcriptional level by LCFA via the lipid-activated nuclear receptor, peroxisome proliferator-activated receptor α (PPARα). Specifically, transcriptional activation is mediated by LCFA via a heterodimeric complex composed of muscle carnitine palmitoyltransferase I, PPARα, peroxisome proliferator-activated receptor α; RXR, retinoid X receptor; LCFA, long-chain fatty acid; HIF-1α, hypoxia inducible factor-1α (HIF-1α) (4–6). Hypoxia also triggers additional transcriptional control mechanisms involving the Sp factor family (7) as well as regulatory effects at the post-transcriptional level (8).
posed of PPARα and the retinoid X receptor (RXR), bound to a fatty acid-responsive element (FARE-1) within the M-CPT I gene promoter. This regulatory mechanism likely serves to modulate mitochondrial FAO enzyme levels and substrate flow into the β-oxidation spiral in accordance with myocardial fatty acid availability during development and in diverse dietary and physiologic conditions (19–21).

We hypothesized that hypoxia leads to decreased capacity for mitochondrial FAO through its effect on the same gene regulatory pathways involved in LCFA-mediated control of M-CPT I expression. Accordingly, we characterized the effects of hypoxia on the regulation of M-CPT I gene expression by LCFAWs. We demonstrate that hypoxia antagonizes the LCFA-mediated, PPARα-dependent transcriptional activation of the M-CPT I gene via an HIF-1α-independent pathway. Our results indicate that hypoxia elicits a decrease in the nuclear levels of RXR which, in turn, reduces the formation of functional PPARα/RXR heterodimers. Accordingly, alteration of nuclear receptor signaling represents a novel mechanism by which hypoxia regulates gene expression.

EXPERIMENTAL PROCEDURES

Primary Rat Neonatal Cardiac Myocyte Cell Culture—Ventricular cardiac myocytes were prepared from 1-day-old Harlan Sprague-Dawley rats as described (22). After 24 h, serum was withdrawn from the media. A hypoxic culture environment was created by the infusion of a pre-analyzed gas mixture of 95% N2, 5% CO2 into an airtight modular chamber (Billups-Rothenberg, Inc.) as described (23). Intracellular neutral lipid was detected by oil red O staining (24). The viability of the cells under various treatment was assessed by measuring dehydrogenase metabolism of tetrazolium to formazan in live cells using the Cell Proliferation Kit II (Roche Molecular Biochemicals). Animal protocols were approved by the Washington University Animal Care Committee.

Northern Blot Analyses—Total cellular RNA isolation and blotting was performed as described (17). Blots were hybridized with a radiolabeled probe derived from a rat M-CPT I cDNA clone (17). Band intensities were quantified by phosphorimaging using a Bio-Rad GS 525 Molecular Imaging System.

Reporter Plasmids—The M-CPT I promoter-luciferase plasmids and the heterologous TK promoter-luciferase constructs have been described (17, 19). The (RXRE)2TK.Luc was constructed by ligation of the (RXRE)2 consensus sequence (5′-AGTTCAGTGTCAGTGTCAGT-3′) into the BamHI site upstream of the thymidine kinase promoter of the pGL2-TK.Luc reporter plasmid. The sequence is based on the RXR response element contained in the rat cardiac retinol-binding protein (CRBP) II gene promoter (25). The HIF-1α-responsive reporter, PGHKRE.TK.Luc, containing the HIF-1α response element from the phosphoglycerate kinase gene, was generously provided by C. Simone (University of Pennsylvania).

Mammalian Cell Transient Transfections—Transient transfections were performed by the calcium phosphate coprecipitation method as described (22). Reporter plasmids were cotransfected with SV40-βGal, containing a β-galactosidase gene downstream of the simian virus promoter, to control for transfection efficiency and cell survival. For cotransfection experiments, the mammalian expression vector, pCDM-P-PPARα, containing mouse PPARα cDNA (26), pCDM-RXRα, containing the human RXRα cDNA (27), or pCDM (−), the vector backbone alone, was used. Ligands or vehicle were added just before cells were exposed to hypoxia. Oleate complexed to bovine serum albumin at a molar ratio of 2:1 was added to cell media to a final concentration of 250 μM oleate.

Northern blot analysis performed with total RNA isolated from primary neonatal cardiac ventricular myocytes incubated in the presence or absence of 250 μM oleate under normoxic and hypoxic conditions. As shown previously under normoxic conditions, oleate induced M-CPT I mRNA levels over 5-fold (17). In contrast, whereas hypoxia did not alter basal steady-state levels of M-CPT I mRNA, it abolished the oleate-mediated induction of M-CPT I gene expression (Fig. 1).

The effects of hypoxia on basal and fatty acid-induced M-CPT I gene expression were characterized using Northern blot analysis performed with total RNA isolated from rat neonatal cardiac myocytes cultured under normoxic or hypoxic (48 h) conditions and EMSA reactions were performed as described (29). The adenosinergic construct, ad-PPARα, was constructed by subcloning a Gal4/PPARα/2xGal4 construct containing the PPARα cDNA encoding amino acids 1–468 plus an N-terminal FLAG epitope into pAD Track-CMV vector. Recombination and propagation of adenovirus expressing PPARα was performed as described (28). The M-CPT I FARE-1 (17), HIF-RE (4), and GATA-RE (Santa Cruz Biotechnology, Inc.) probes were end-labeled with [γ-32P]dATP. Antibody recognition experiments were performed with a monoclonal antibody directed against the FLAG epitope (M5 antibody, Sigma) or with a polyclonal anti-PPARα antibody (N-19, Santa Cruz Biotechnology).

Immunoblotting Studies—Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (7.5%) and electroblotted onto Polyvinylidene difluoride (Schleicher & Schuell). Immunodetection of overexpressed FLAG-PPARα was performed with the same anti-FLAG antibody used in the antibody supershift reactions. The anti-PPARα antibody (FP2) is directed toward the hinge region of PPARα and has been described previously (29). The anti-RXRα antibody (D-20, Santa Cruz Biotechnology) is directed toward the N terminus of the protein. A universal anti-actin antibody (Sigma) directed against the conserved C terminus of actin was used as a control. Proteins were detected using ECL reagents (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays (EMSA)—Preparation of crude nuclear protein extracts from rat neonatal cardiomyocytes cultured under normoxic or hypoxic conditions and EMSA reactions were performed as described (22). The adenosinergic construct, ad-PPARα, was constructed by subcloning a SalI/XbaI fragment from a Gal4-PPARα/Gal4 construct containing the PPARα cDNA encoding amino acids 1–468 plus an N-terminal FLAG epitope into pAD Track-CMV vector. Recombination and propagation of adenovirus expressing PPARα was performed as described (28). The M-CPT I FARE-1 (17), HIF-RE (4), and GATA-RE (Santa Cruz Biotechnology, Inc.) probes were end-labeled with [γ-32P]dATP. Antibody recognition experiments were performed with a monoclonal antibody directed against the FLAG epitope (M5 antibody, Sigma) or with a polyclonal anti-PPARα antibody (N-19, Santa Cruz Biotechnology).

RESULTS

Hypoxia Blocks the LCFA-mediated Activation of M-CPT I Gene Expression—The effects of hypoxia on basal and fatty acid-induced M-CPT I gene expression were characterized using Northern blot analysis performed with total RNA isolated from rat neonatal cardiac myocytes cultured under normoxic or hypoxic conditions and EMSA reactions were performed as described (29). The adenosinergic construct, ad-PPARα, was constructed by subcloning a Gal4/PPARα/2xGal4 construct containing the PPARα cDNA encoding amino acids 1–468 plus an N-terminal FLAG epitope into pAD Track-CMV vector. Recombination and propagation of adenovirus expressing PPARα was performed as described (28). The M-CPT I FARE-1 (17), HIF-RE (4), and GATA-RE (Santa Cruz Biotechnology, Inc.) probes were end-labeled with [γ-32P]dATP. Antibody recognition experiments were performed with a monoclonal antibody directed against the FLAG epitope (M5 antibody, Sigma) or with a polyclonal anti-PPARα antibody (N-19, Santa Cruz Biotechnology).

Immunoblotting Studies—Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (7.5%) and electroblotted onto Polyvinylidene difluoride (Schleicher & Schuell). Immunodetection of overexpressed FLAG-PPARα was performed with the same anti-FLAG antibody used in the antibody supershift reactions. The anti-PPARα antibody (FP2) is directed toward the hinge region of PPARα and has been described previously (29). The anti-RXRα antibody (D-20, Santa Cruz Biotechnology) is directed toward the N terminus of the protein. A universal anti-actin antibody (Sigma) directed against the conserved C terminus of actin was used as a control. Proteins were detected using ECL reagents (Amersham Pharmacia Biotech).

Electrophoretic Mobility Shift Assays (EMSA)—Preparation of crude nuclear protein extracts from rat neonatal cardiomyocytes cultured under normoxic or hypoxic conditions and EMSA reactions were performed as described (22). The adenosinergic construct, ad-PPARα, was constructed by subcloning a Gal4/PPARα/2xGal4 construct containing the PPARα cDNA encoding amino acids 1–468 plus an N-terminal FLAG epitope into pAD Track-CMV vector. Recombination and propagation of adenovirus expressing PPARα was performed as described (28). The M-CPT I FARE-1 (17), HIF-RE (4), and GATA-RE (Santa Cruz Biotechnology, Inc.) probes were end-labeled with [γ-32P]dATP. Antibody recognition experiments were performed with a monoclonal antibody directed against the FLAG epitope (M5 antibody, Sigma) or with a polyclonal anti-PPARα antibody (N-19, Santa Cruz Biotechnology).

Immunoblotting Studies—Protein extracts were resolved by SDS-polyacrylamide gel electrophoresis (7.5%) and electroblotted onto Polyvinylidene difluoride (Schleicher & Schuell). Immunodetection of overexpressed FLAG-PPARα was performed with the same anti-FLAG antibody used in the antibody supershift reactions. The anti-PPARα antibody (FP2) is directed toward the hinge region of PPARα and has been described previously (29). The anti-RXRα antibody (D-20, Santa Cruz Biotechnology) is directed toward the N terminus of the protein. A universal anti-actin antibody (Sigma) directed against the conserved C terminus of actin was used as a control. Proteins were detected using ECL reagents (Amersham Pharmacia Biotech).

Statistical Analysis—Data are presented as mean ± S.E. Differences between mean mRNA levels were compared using an unpaired Student’s t test, and transfection data were analyzed by either a one-factor analysis of variance or an unpaired Student’s t test. A p value < 0.05 was considered significant.

Hypoxia Antagonizes PPARα/RXR Signaling
cardiac myocytes exposed to 250 μM oleate complexed to albumin and cultured under normoxic or hypoxic conditions. Oil red O staining of the cardiac myocytes demonstrated a marked accumulation of intracellular neutral lipid within the hypoxic myocytes compared with control cells (Fig. 2). This hypoxia-induced lipid accumulation occurred in the absence of exogenous fatty acid but was more pronounced following exposure to oleate (Fig. 2). These data, consistent with the hypoxia-mediated reduction in M-CPT I gene expression, indicate that reduced oxygen availability leads to a diminution in the capacity for myocyte fatty acid catabolism.

**Hypoxia Inhibits LCFA-mediated Transcriptional Activation of the M-CPT I Gene**—To determine if the antagonistic effects of hypoxia on LCFA-induced M-CPT I gene expression occur at the transcriptional level, we performed promoter activity assays in cardiac myocytes. Myocytes were transiently transfected with a reporter plasmid containing the human M-CPT I gene 5′-flanking region from −1025 to −12 bp fused to a luciferase gene reporter (MCPT.Luc.1025) (17). As shown schematically in Fig. 3A, MCPT.Luc.1025 contains the fatty acid response element-1 (FARE-1), a previously described PPARα-responsive element (17), upstream of two untranslated exons, 1A and 1B (15, 30). Following transfection, the myocytes were incubated in the presence of oleate or vehicle for 48 h under normoxic or hypoxic conditions. Based on the tetrazolium conversion assay (see “Experimental Procedures”), cell survival was not significantly reduced in any of the hypoxic conditions tested (data not shown). As expected, under normoxic conditions, oleate induced the transcriptional activity of MCPT.Luc.1025 over 8-fold (Fig. 3A). This oleate-mediated induction of MCPT.Luc.1025 activity was abolished in hypoxic conditions. In the absence of oleate, exposure to a hypoxic environment had no effect on the transcriptional activity of MCPT.Luc.1025 (Fig. 3A).

To verify the effectiveness of our hypoxic system, parallel transfection experiments were performed using a thymidine kinase promoter-reporter construct containing three copies of the HIF-1α response element (HRE) derived from the phosphoglycerate kinase gene (PGKHER.TKLuc) (5). PGKHER.TKLuc was markedly induced following a 48-h incubation within the hypoxia chamber confirming that a hypoxic cellular environment had been established (Fig. 3A).

**The Effect of Hypoxia on M-CPT I Gene Transcription Maps to FARE-1, a PPARα Response Element**—Previous studies have mapped the LCFA-responsive region of the M-CPT I gene to −775 to −763 bp of the 5′-flanking region (17). To determine the importance of FARE-1 in the hypoxia response, we repeated cardiac myocyte transfections using MCPT.Luc.1025 and two additional M-CPT I promoter constructs as follows: MCPT.Luc.781, which contains the −781 to −12-bp region of the M-CPT I gene (17) including an intact FARE-1 element, and a mutated construct, MCPT.Luc.781m1, which is identical to MCPT.Luc.781 except that it bears a G to C substitution in the upstream half-site of FARE-1 rendering it unable to bind PPARα (17). Compared with MCPT.1025.Luc, a similar hypoxia-mediated repression of LCFA-induced transcription was observed with MCPT.Luc.781, suggesting that the −781 to −1025 region was not essential for the inhibition (Fig. 3B). However, in the absence of an intact FARE-1 (MCPT.Luc.781 m1) the hypoxia-mediated transcriptional repression was absent, indicating that a functional PPARα response element is necessary for the hypoxia response.

**PPARα Is Necessary and Sufficient for the Effect of Hypoxia on Fatty Acid-mediated Activation of M-CPT I Gene Transcription**—The results shown in Fig. 3 suggested that the PPARα regulatory pathway is attenuated following exposure to hypoxia. However, the basal activity of the mutated FARE-1 construct, MCPT.Luc.781m1, is significantly lower than that of the corresponding wild-type construct (Fig. 3B) precluding a definitive conclusion regarding the necessity of PPARα for the transcriptional repression by hypoxia. Accordingly, experiments were performed in the hepatoma G2 (HepG2) cell line, which provides a relative PPARα-deficient background (17). HepG2 cells were cotransfected with either a PPARα expression vector (CDM.PPARα) or the vector backbone (CDM−) and MCPT.Luc.781. For these studies, the known PPARα activator ETYA was used to exclude potential PPAR-independent regulatory effects mediated by oleate. MCPT.781.Luc activity was not significantly affected by ETYA treatment or by hypoxic conditions in the absence of PPARα expression vector. However, upon cotransfection with CDM.PPARα, MCPT.781.Luc exhibited ETYA responsiveness, which was markedly diminished under hypoxic conditions (Fig. 4A). As expected, similar results were obtained using oleate as the PPARα activator.
Hypoxia Antagonizes PPARα/RXR Signaling

Fig. 3. Hypoxia abolishes the fatty acid-mediated activation of the M-CPT I promoter. A, left panel, cardiac myocytes were transfected with MCPT.Luc.1025 (shown schematically at the top). Cells were exposed to vehicle (solid bars) or 250 μM oleate (hatched bars) for 48 h under normoxic or hypoxic conditions. Right panel, cardiac myocytes were transfected with the HIF-1α-responsive plasmid PGKHRE.TK.Luc. Cells were exposed to normoxic or hypoxic conditions as described for the left panel. The bars in both panels represent mean luciferase activity in relative luciferase units (RLU) normalized to the activity of the normoxic control condition (=1.0). The values were corrected for transfection efficiency using the activity of cotransfected pSV40 β-galactosidase plasmid. The data represent the mean of at least three independent experiments performed in triplicate. The * denotes a significant difference (p < 0.05) between values representing oleate treatment and corresponding vehicle control. B, cardiac myocytes were transfected with the M-CPT I promoter luciferase constructs shown on the left. The numeric plasmid designations indicate the 5′ end of each construct. MCPT.Luc.781m1 contains a single bp mutation within FARE-1 rendering this element unresponsive to PPARα. Cells were treated as described in A. The bars represent fold induction (oleate-treated activity/normoxic, vehicle-treated activity). The data represent the mean of at least 2 independent experiments performed in triplicate. The * denotes a statistically significant difference (p < 0.05) between the fold induction of a construct in hypoxic conditions compared with its induction in normoxic conditions.

(data not shown). These data demonstrate that PPARα is a target for the hypoxia-induced block in fatty acid-mediated activation of M-CPT I gene expression. Additionally, we conclude that this effect is not PPARα ligand-specific, suggesting that hypoxia does not mediate its effects via altered metabolism of oleate or other potential fatty acid PPARα ligands.

To determine whether a PPARα-responsive element is sufficient to confer the inhibitory effect of hypoxia, transfection experiments were repeated in HepG2 cells with two heterologous promoter-reporter plasmids, MCPT(FARE)2TK.LUC, containing 2 copies of FARE-1 and (ACO)3TK.LUC, containing 3 copies of a PPAR-RE derived from the peroxisomal acyl-CoA oxidase (ACO) gene (Fig. 4B). Both reporters contain a minimal viral thymidine kinase promoter. The MCPT(FARE)2TK.LUC and (ACO)3TK.LUC constructs, when cotransfected with CDM.PPARα, displayed 10- and 17-fold activation by ETYA, respectively, under normoxic conditions. In contrast, the ligand-induced activities of the reporter constructs were significantly blunted by hypoxia (Fig. 4B). The activity of the TK promoter lacking a PPARα response element was not repressed by hypoxia, rather it was modestly increased (data not shown). These results demonstrate that a PPARα response element is sufficient to confer the hypoxia-mediated inhibitory response and that this effect is not unique to the M-CPT I gene PPARα, rather, it likely reflects a global effect on PPARα signaling.

Hypoxia Interferes with PPARα-mediated Control of M-CPT I Gene Transcription via a HIF-1α-independent Mechanism—The best characterized mechanism whereby hypoxia regulates gene expression involves the transcription factor HIF-1α. HIF-1α mediates hypoxia-induced transcriptional regulation of a wide variety of genes encoding proteins necessary for cellular and physiologic adaptation to hypoxia (4, 6, 7, 31). To investigate whether HIF-1α is involved in the observed effects of hypoxia on PPARα function, we explored the effects of CoCl2, a hypoxia-mimicking compound known to activate HIF-1α and to induce HIF-1α-dependent changes in target gene expression (32). For these experiments, transient transfection assays were repeated in HepG2 cells using the MCPT.Luc.781 reporter plasmid. In contrast to the effects with hypoxia, treatment with 75 μM CoCl2 did not blunt MCPT.Luc.781 activation by oleate (Fig. 5). In parallel experiments, the HIF-1α-responsive construct PGKHRE.TK.Luc displayed a robust induction following exposure to CoCl2 (Fig. 5). These results suggest that an HIF-1α-independent pathway is involved in the M-CPT I gene transcriptional response to hypoxia.

Hypoxia Decreases PPARα/RXR DNA Binding Activity—Results from the PPARα overexpression studies shown above indicated that hypoxia alters PPARα function at the post-translational level. To determine whether hypoxia influences the interaction of PPARα with cognate DNA-binding sites, the FARE-1 element was used in EMSA to assess PPARα DNA binding activity. To increase the sensitivity of the analysis, EMSA was performed with nuclear protein extracts prepared from cardiac myocytes infected with a FLAG epitope-tagged PPARα-expressing adenoviral vector (Ad-PPARα) and cultured under normoxic or hypoxic conditions for 48 h. The FARE-1
oligonucleotide probe formed one major DNA-protein complex of low mobility and two additional fainter complexes, exhibiting high and intermediate mobilities, respectively (Fig. 6, lane 2). All of the complexes represent specific FARE-1-protein interactions, based on competition studies performed with a molar excess of unlabeled FARE-1 or a mutated FARE-1 (data not shown). We have shown previously (17) that the low mobility complex corresponds to PPARα/RXR heterodimer, the functional form of PPARα. Antibody recognition experiments using anti-FLAG epitope or anti-PPARα antibodies (Fig. 6, lanes 3–5) confirmed that this complex contained PPARα. The faint complex of intermediate mobility was also recognized by the anti-FLAG antibody and could represent a proteolytic product of PPARα. A comparison of FARE-1 binding activity between normoxic and hypoxic extracts revealed that formation of the PPARα/RXR complex was significantly reduced in response to hypoxia (Fig. 6, lanes 6–9), indicating that hypoxia decreases DNA binding activity of the PPARα/RXR heterodimer. As controls, EMSA experiments were also performed with probes containing either the recognition sequence for the PPARα/RXR and is only observed in PPARα-overexpressing cells (data not shown), whereas the rapid mobility complex is observed in extracts from uninfected cells. Antibody recognition reactions (lanes 3–5) were performed using extracts from normoxic, vehicle-treated cells. Extracts were incubated with PPARα antibody (PPARα), FLAG antibody (FLAG), or with pre-immune serum (PI). The supershifted complex is indicated by the asterisk. In lanes 6–9, reactions were performed with extracts from Ad-PPARα-infected cardiac myocytes cultured for 48 h under normoxic (N) or hypoxic (H) conditions (two independent samples shown). The right panel depicts the results of EMSA performed with probes containing the HIF-1 (HIF-RE) or GATA (GATA-RE) binding sites as controls.

Fig. 4. PPARα is required and is sufficient for the hypoxia-induced deactivation of M-CPT 1 gene transcription. A, MCPT.Luc.781 was cotransfected into HepG2 cells with or without co-transfected PPARα expression vector (CDM.PPARα) as denoted at the bottom and subsequently incubated in the absence or presence of the PPARα activator ETYA (10 μM). The bars (black, normoxic, and gray, hypoxic) represent mean relative luciferase units (RLU) normalized (=1.0) to the activity of the reporter cotransfected with the expression vector backbone, pCDM(−), under normoxic conditions. Values obtained for samples in hypoxic versus normoxic conditions in the presence of ligand (denoted by brackets) were analyzed. * denotes a significant difference (p < 0.05), and NS denotes no significant difference. The data represent the mean of at least 3 independent experiments performed in triplicate. B, two PPAR-RE heterologous promoter constructs, MCPT(FARE)/TKLuc and (ACO)3TKLuc, were independently cotransfected with CDM.PPARα into HepG2 cells and subsequently treated with ETYA (10 μM) under normoxic (black bars) or hypoxic (gray bars) conditions. The bars represent mean ETYA-induced fold activation in the presence of CDM.PPARα relative to the corresponding CDM(−) untreated control. Activities were normalized to the activity of their respective TKLuc controls due to a modest activation of the TK promoter in response to hypoxia. The * indicates a significant difference (p < 0.05) between hypoxic and normoxic conditions for a given reporter. These data represent the mean of at least 3 independent experiments performed in triplicate.

Fig. 5. Exposure to cobalt chloride (CoCl2) does not mimic the effects of hypoxia on M-CPT 1 gene promoter function. MCPT.Luc.781 was cotransfected into CDM.PPARα into HepG2 cells and subsequently cultured in the presence or absence of oleate (250 μM). 75 μM CoCl2 or vehicle was added to the media after oleate treatment as indicated. The activity of the HIF-1α-responsive reporter, PGKHRE.TKLuc, was analyzed in parallel experiments to serve as a positive control for the effects of CoCl2. The bars represent mean relative luciferase units (RLU) normalized to the activity in untreated controls (=1.0). The * denotes a significant difference (p < 0.05) between treated and untreated controls. These data represent the mean of at least 2 independent experiments performed in triplicate.
factor. As expected, formation of the HIF-1-DNA complex was induced in extracts prepared from hypoxic cells (Fig. 6, right panel). In contrast, the intensity of the GATA-RE-protein complex was not significantly different between the hypoxic and control samples (Fig. 6, right panel).

**Hypoxia Reduces Nuclear Levels of RXRα but Not PPARα in Cardiac Myocytes**—The results shown above indicate that hypoxia attenuates PPARα signaling by reducing its DNA binding activity. Therefore, nuclear levels of PPARα protein were measured to determine whether the altered binding activity of PPARα/RXR was due to diminished availability of PPARα. For these experiments, Western blot analyses were performed using anti-PPARα and anti-FLAG epitope antibodies with nuclear- and cytosol-enriched protein extracts prepared from Ad-PPARα-infected cardiac myocytes exposed to normoxic or hypoxic (48 h) conditions. As expected, the majority of PPARα was detected in the nucleus under normoxic conditions although a small amount was detected in the cytosol using the anti-FLAG antibody (Fig. 7A). Surprisingly, hypoxic conditions did not alter the nuclear levels of PPARα (Fig. 7A).

Immunoblotting studies were next performed to evaluate the effect of hypoxia on the levels of the obligate PPARα partner, RXR. Nuclear levels of endogenous RXRα were significantly lower in hypoxic myocytes compared with normoxic control cells (Fig. 7B). RXRα was not detected in the cytosolic fraction of normoxic or hypoxic cells suggesting that the observed reduction in nuclear levels of RXRα was not due to redistribution of RXRα from the nucleus to cytosol (data not shown). Consistent with this latter finding, levels of RXRα in total cell protein extracts were also significantly reduced (Fig. 7B). Recent studies have shown that ligand binding leads to increased proteasomal-mediated degradation of RXR (33, 34). As expected, exposure of the normoxic myocytes to the RXR ligand, 9-cis-RA, resulted in reduced levels of RXRα (Fig. 7B). The combination of hypoxia and exposure to RXR ligand resulted in a greater reduction in RXRα levels compared with either condition alone.

The results shown above suggested that hypoxia inhibits PPARα-dependent activation by decreasing nuclear levels of RXRα, thereby limiting formation of the functional PPARα/RXRα complex. To test this hypothesis further, experiments were performed to determine whether the observed effects of hypoxia on RXRα nuclear protein levels correlated temporally with the inhibition of PPARα signaling. PPARα-mediated induction of MCPT.Luc.781 and RXRα protein levels were determined at identical time points (12, 24, and 48 h) in cardiac myocytes following the onset of hypoxia. RXRα protein levels began to decrease between 12 h and 24 h of hypoxic exposure and remained low at 48 h compared to levels in normoxic conditions. In contrast, a significant reduction in PPARα-mediated activation of MCPT.Luc.781 did not occur until the period between 24 and 48 h of hypoxia exposure (Fig. 7C), whereas induction of the HIF-1-responsive PGKHRRE,TKLuc occurred within 12 h (data not shown). As expected, PPARα-dependent activation by ETYA was observed at all time points in normoxic conditions (Fig. 7C). These results demonstrate that the hypoxia-mediated alteration in RXRα protein levels precedes the onset of functional inhibition of PPARα-dependent transactivation. Collectively, these data indicate that hypoxia leads to reduced nuclear levels of RXRα, accounting, at least in part, for the observed reduction in PPARα/RXR binding and activity.

**Hypoxia Inhibits RXRα-mediated Transcriptional Activation**—RXR serves as the obligate heterodimeric partner for a number of nuclear receptors in addition to PPARα and can mediate 9-cis-RA effects additionally as a homodimer. Given that hypoxia results in a reduction in the nuclear levels of RXRα, it would be predicted that signaling through other RXR-dependent pathways would be similarly affected. To investigate this possibility, the hypoxia transfection experiments were repeated using an RXR-responsive heterologous reporter construct containing 2 copies of a well characterized RXR-RE

![Fig. 7](http://www.jbc.org/)
derived from the cellular retinol-binding protein II (CRBP II) gene promoter. The CRBP II RXR-RE has been shown to confer transcriptional activation by 9-cis-RA through interaction with RXR homodimers (25). When cotransfected with CDM.RXRα into HepG2 cells, the (RXRE)2TK.Luc was induced 54-fold by 9-cis-RA under normoxic conditions (Fig. 8). In contrast, the 9-cis-RA-mediated induction of (RXRE)2TK.Luc was reduced by ~50% in hypoxic conditions. Hypoxia had no significant effect on the basal transcriptional activity of (RXRE)2TK.Luc in the absence of 9-cis-RA (Fig. 8). These data indicate that as predicted by the observed hypoxia-induced diminution in nuclear RXR levels, hypoxia alters the activity of a PPARα-independent RXR gene target.

DISCUSSION

Preservation of cellular function in a hypoxic environment requires metabolic adaptation to improve oxygen utilization efficiency during energy production. While the oxidation of fatty acids provides more ATP per mol of substrate compared with that of glucose, it does so at the expense of increased oxygen consumption. Therefore, the hypoxia-mediated switch from mitochondrial β-oxidation to glycolysis in heart is thought to serve as a protective cellular response. Although previous studies have identified mechanisms whereby glycolytic capacity is increased under hypoxic conditions, little is known regarding hypoxia-driven decreases in mitochondrial flux. In this report, we identify one such hypoxia-mediated regulatory response, involving attenuation of the PPAR response element, involving attenuation of the PPARα/RXR heterodimeric complex. In addition, we found that the hypoxia-mediated induction of M-CPT I expression is dependent on the hypoxia response element (RXRE)2TK.Luc in the absence of 9-cis-RA (Fig. 8). These results indicate that the observed reduction in PPARα-mediated activation of M-CPT I gene expression in the hypoxic cardiac myocyte occurs, at least in part, through decreased availability of RXR resulting in reduced formation of the heterodimeric functional transcriptional regulatory complex. In addition, we found that the 9-cis-RA-induced activity of a PPARα-independent RXR response element was abrogated by hypoxia. These findings suggest a mechanism whereby O2 levels, directly or indirectly, modulate the activity of the retinoid signaling pathway. This mechanism could be important not only for physiologic regulation of postnatal cardiac function but also during early cardiac development in which retinoid signaling likely plays a critical role in morphogenesis and cellular differentiation.

Several mechanisms could be responsible for the reduction of nuclear RXRα protein levels following exposure to hypoxic conditions. Hypoxia could lead to redistribution of RXRα from the nucleus. However, we found that RXRα levels are reduced in both nuclear and whole cell fractions. Alternatively, hypoxia could cause reduced RXRα protein synthesis or increased degradation. Interestingly, recent studies have demonstrated that hypoxia induces RXRα degradation via the ubiquitin-proteasome pathway (33, 34). We show here that the effects of hypoxia and 9-cis-RA are additive in reducing steady-state levels of RXRα indicating that the hypoxia effect is active even when RXR is engaged by ligand. Although we did not observe a direct effect of hypoxia on PPARα levels in cardiac myocytes, we cannot exclude the possibility that additional post-translational modifications of PPARα directly inhibit its transactivating properties.

Our results suggest that the upstream events involved in the hypoxia-mediated inhibition of M-CPT I gene expression are independent of the well-characterized HIF-1α pathway (39). Other transcription factors, such as Sp1, AP-1, HNF-4, and NFκB, have also been implicated in the gene regulatory response to hypoxia (7, 40, 41). Herein, we identify a potential novel transcriptional regulatory pathway involved in the hypoxia response, alteration of nuclear receptor signaling. The
results of our time course experiments indicate that in contrast to the rapid activation of the HIF-1 pathway, the levels of RXRa fall between 12 and 24 h after initiation of hypoxic exposure leading to a reduction in PPARa activity. This observation together with the results of the cobalt chloride experiments suggest that the effects of hypoxia on the activity of PPARs/RXR are mediated independent of the HIF-1 pathway. Given that the effects of hypoxia on RXR/PPAR activity is delayed, it is tempting to speculate that a secondary signaling pathway or the level of a regulatory cellular metabolite confers the downstream effect on transcriptional control.

In summary, we have shown that the regulated expression of the gene encoding M-CPT I, a critical enzyme in the cardiac mitochondrial FAO pathway, is down-regulated following exposure leading to a reduction in PPARa activity. This observation together with the results of the cobalt chloride experiments suggest that the effects of hypoxia on the activity of PPARs/RXR are mediated independent of the HIF-1 pathway. Given that the effects of hypoxia on RXR/PPAR activity is delayed, it is tempting to speculate that a secondary signaling pathway or the level of a regulatory cellular metabolite confers the downstream effect on transcriptional control.

Acknowledgments—We thank Philip Barger for providing the Ad-PPARa vector and Mary Wingate for assistance with preparation of the manuscript.

REFERENCES
1. Fahey, J. T., and Lister, G. (1989) Pediatr. Res. 26, 180–187
2. Hochachka, P. W., Buck, L. T., Doll, C. J., and Land, S. C. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9493–9498
3. Poizat, C., Keriel, C., Garnier, A., Dubois, F., Cand, F., and Cuchet, P. (1993) Arch. Int. Physiol. Biochim. Biophys. 101, 347–356
4. Semenza, G. L., Roth, P. H., Fang, H. M., and Wang, G. L. (1994) J. Biol. Chem. 269, 23757–23763
5. Firth, J. D., Ebert, B. L., Pugh, C. W., and Ratcliffe, P. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 6496–6500
6. Semenza, G. L., Jiang, B.-H., Leung, S. W., Passantino, R., Concordet, J.-P., Maire, P., and Giallongo, A. (1996) J. Biol. Chem. 271, 32529–32537
7. Discher, D. J., Bishopric, N. H., Wu, X., Peterson, C. A., and Webster, K. A. (1998) J. Biol. Chem. 273, 26887–26893
8. Levy, A. P., Levy, N. S., and Goldberg, M. A. (1996) J. Biol. Chem. 271, 25492–25497
9. Bing, R. J. (1955) Harvey Lect. 50, 27–70
10. Warshaw, J. B. (1972) Dev. Biol. 28, 537–544
11. Holden, J. E., Stone, C. K., Clark, C. M., Brown, W. D., Nickles, R. J., Stanley, C., and Hochachka, P. W. (1995) J. Appl. Physiol. 79, 222–228
12. Abdel-aleem, S., St. Louis, J., Hendrickson, S. C., El-Shewy, H. M., El-Dawy, K., Taylor, D. A., and Lowe, J. E. (1998) Mol. Cell. Biol. 18, 95–103
13. Rumsey, W. L., Abbott, B., Bertelsen, D., Mallamaci, M., Hagan, K., Nelson, D., and Erecinska, M. (1999) Am. J. Physiol. 276, H171–H80
14. McGarry, J. D., Wooley, K. F., Kawajima, M., and Foster, D. W. (1989) Diabetes Metab. Rev. 5, 271–284
15. Yamazaki, N., Shinozohra, Y., Shima, A., Yamanaka, Y., and Terada, H. (1996) Biochim. Biophys. Acta 1307, 157–161
16. Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W., and McGarry, J. D. (1996) J. Biol. Chem. 271, 6972–6977
17. Brandt, J., Djouadi, F., and Kelly, D. P. (1998) J. Biol. Chem. 273, 23786–23792
18. Yu, G. S., Liu, Y. C., and Gilmour, T. (1998) J. Biol. Chem. 273, 32901–32909
19. Djouadi, F., Weinheimer, C. J., Saffitz, J. E., Pitchford, C., Bastin, J., Gonzalez, F. J., and Kelly, D. P. (1996) J. Clin. Invest. 102, 1083–1091
20. Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7473–7478
21. Crescenti, S., Wright, L. D., Spratt, J. A., Briggs, F. N., and Kelly, D. P. (1996) Am. J. Physiol. 270, C1413–C1429
22. Dische, D. L., Ruder, T. A., Crescenti, S., Leone, T. C., Barger, P. M., Vega, R., Wood, A. P., and Kelly, D. P. (1996) Mol. Cell. Biol. 16, 4043–4051
23. Levy, F. H., and Kelly, D. P. (1997) Am. J. Physiol. 272, C457–C465
24. Barger, P. M., Brandt, J. M., Leone, T. C., Weinheimer, C. J., and Kelly, D. P. (2000) J. Clin. Invest. 105, 1723–1730
25. Mangelendorf, D. J., Umesono, K., Kii, Shiman, A., Borgmeyer, U., Ong, E. S., and Evans, R. M. (1991) Cell 66, 555–561
26. Gullick, T., Crescenti, S., Cair, T., Moore, D. D., and Kelly, D. P. (1996) Proc. Natl. Acad. Sci. U. S. A. 91, 11012–11016
27. Carter, M. E., Gullick, T., Moore, D. D., and Kelly, D. P. (1994) Mol. Cell. Biol. 14, 4360–4372
28. He, T. C., Zhou, S., da Costa, L. T., Yu, J., Klinzr, K. W., and Vogelstein, B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2509–2514
29. Gebel, T., Arand, M., and Oesch, F. (1992) FEBS Lett. 309, 37–40
30. Yamazaki, N., Yamanaka, Y., Hasimoto, Y., Shinozohra, Y., Shima, A., and Terada, H. (1997) FEBS Lett. 409, 401–406
31. Wang, G. L., and Semenza, G. L. (1995) J. Biol. Chem. 270, 1230–1237
32. Goldberg, M. A., Dunning, S. F., and Bunn, H. F. (1988) Science 242, 1412–1415
33. Nomura, Y., Nagaya, T., Hayashi, Y., Kame, F., and See, H. (1999) Biochem. Biophys. Res. Commun. 260, 729–733
34. Kopf, E., Plassat, J.-L., Vivat, V., deThe, H., Chambon, P., and Rochette-Egly, C. (2000) J. Biol. Chem. 275, 33280–33288
35. Saitummar, P., Dong, Z., Weinberg, J. M., and Venkatakalam, M. A. (1998) Oncogene 17, 3341–3349
36. Yamada, K. A., McIlwatt, J., Yan, G. X., Donahue, K., Peirick, J., Kleber, A. G., and Cor, P. B. (1994) J. Biol. Chem. 269, 74579–74587
37. Wu, J., and Cor, P. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1083–1091
38. Wu, J., and Cor, P. B. (1998) J. Biol. Chem. 273, 23793–23799
39. Semenza, G. L. (1999) Annu. Rev. Cell Dev. Biol. 15, 551–578
40. Webster, K. A., Discher, D. J., and Bishopric, N. H. (1996) J. Mol. Cell. Cardiol. 27, 453–458
41. Galson, D. L., Tsuiyi, T., Tendler, D. S., Huang, L. E., Ren, Y., Ogura, T., and Bunn, H. F. (1995) Mol. Cell. Biol. 15, 2135–2144
Hypoxia Inhibits the Peroxisome Proliferator-activated Receptor α/Retinoid X Receptor Gene Regulatory Pathway in Cardiac Myocytes: A MECHANISM FOR O2-DEPENDENT MODULATION OF MITOCHONDRIAL FATTY ACID OXIDATION

Janice M. Huss, Fiona H. Levy and Daniel P. Kelly

J. Biol. Chem. 2001, 276:27605-27612.
doi: 10.1074/jbc.M100277200 originally published online May 22, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100277200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 41 references, 19 of which can be accessed free at http://www.jbc.org/content/276/29/27605.full.html#ref-list-1