Modulation of Receptor Phosphorylation Contributes to Activation of Peroxisome Proliferator Activated Receptor α by Dehydroepiandrosterone and Other Peroxisome Proliferators

Viola Tamasi, Kristy K. Michael Miller, Sharon L. Ripp, Ermin Vila, Thomas E. Geoghanen, and Russell A. Prough

Department of Biochemistry and Molecular Biology, University of Louisville School of Medicine, Louisville, Kentucky (V.T., E.V., T.E.G., R.A.P.); Department of Chemistry, University of Evansville, Evansville, Indiana (K.K.M.); and Pharmacokinetics, Dynamics & Metabolism, Pfizer Global Research & Development, Groton, Connecticut (S.L.R.)

ABSTRACT

Dehydroepiandrosterone (DHEA), a C19 human adrenal steroid, activates peroxisome proliferator-activated receptor α (PPARα) in vivo but does not ligand-activate PPARα in transient transfection experiments. We demonstrate that DHEA regulates PPARα action by altering both the levels and phosphorylation status of the receptor. Human hepatoma cells (HepG2) were transiently transfected with the expression plasmid encoding PPARα and a plasmid containing two copies of fatty acyl co-enzyme oxidase (FACO) peroxisome proliferator activated receptor responsive element consensus oligonucleotide in a luciferase reporter gene. Nafenopin treatment increased reporter gene activity in this system, whereas DHEA treatment did not. Okadaic acid significantly decreased nafenopin-induced reporter activity in a concentration-dependent manner. Okadaic acid treatment of primary rat hepatocytes decreased both DHEA- and nafenopin-induced FACO activity in primary rat hepatocytes. DHEA induced both PPARα mRNA and protein levels, as well as PP2A message in primary rat hepatocytes. Western blot analysis showed that the series at positions 12 and 21 were rapidly dephosphorylated upon treatment with DHEA and nafenopin. Results using specific protein phosphatase inhibitors suggested that protein phosphatase 2A (PP2A) might be involved in nafenopin induction. Mutation of series at position 6, 12, and 21 to an uncharged alanine residue significantly increased transcriptional activity, whereas mutation to negative charge aspartate residues (mimicking receptor phosphorylation) decreased transcriptional activity. DHEA action involves induction of PPARα mRNA and protein levels as well as increased PPARα transcriptional activity through decreasing receptor phosphorylation at serines in the AF1 region.

Peroxisome proliferator-activated receptor α (PPARα, NR1C1) is a member of the nuclear receptor superfamily and functions as a ligand-activated transcription factor (Issam et al., 1990; Sher et al., 1993). PPARα is expressed in significant levels in human liver, heart, kidney, skeletal muscle, intestine, and pancreas, and it is also detectable in lung, placenta, and adipose tissue (Auboeuf et al., 1997; Mukherjee et al., 1997). Hepatic PPARα mRNA levels have been reported to be lower in humans than in rodents (Palmer et al., 1998). In vivo, PPARα can be activated with peroxisome proliferators (PP), such as fibrate drugs (e.g., Wy-14643, clofibrate), nonfibrate drugs (nafenopin), fatty acids, chlorinated compounds, nonsteroidal anti-inflammatory drugs, and endogenous steroids, such as dehydroepiandrosterone (DHEA) (Escher and Whali, 2000). Most PP serve as ligands for the receptor, and their binding results in ligand-dependent activation of a distinct set of genes involved in lipid metabolism and homeostasis, peroxisome proliferation and cell growth (Rao et al., 1992; Tirona and Kim, 2004). However, ligand-independent processes can also activate these receptors, such as elevated levels of retinoid X receptor α. Shipley and Waxman (2003) have concluded that PP act through mechanisms leading to either direct ligand-activation
of the receptor or through perturbation of lipid metabolism/transport, leading to release of PPARα ligands. In addition, several PP have been suggested to also activate mitogen-activated protein kinase (MAPK) pathways, providing three distinct mechanisms of receptor activation (Gardner et al., 2003).

Ligand-activation of the PPARα receptor by DHEA has never been demonstrated in assays using immortalized cell lines, although it is well established that DHEA affects PPARα target genes in vivo, such as cytochrome P450 genes (CYP4A1, CYP2C11) and fatty acyl coenzyme oxidase (FACO) (Wu et al., 1989; Prough et al., 1994, Peters et al., 1996; Ripp et al., 2003).

In this study, we provide evidence that in primary hepatocytes, DHEA, nafenopin, and Wy-14643 induce protein and mRNA levels of PPARα and, thereby, PPARα-regulated ω-oxidation of fatty acids. In transiently transfected HepG2 cells and cultured rat hepatocytes, the phosphatase inhibitor okadaic acid also alters regulation by these inducers. In our studies, we sought to test the hypothesis that DHEA increases the levels of PPARα and decreases phosphorylation status of the PPARα receptor in hepatocytes, leading to increased transcriptional activity. This indirect mechanism of activation of PPARα via increases in protein content and decreases in phosphorylation status of the receptor may account for the action of DHEA in regulating this important transcription factor, instead of through direct ligand activation.

Materials and Methods

Chemicals. DHEA was purchased from Steraloids (Newport, RI), and nafenopin was a gift from Novartis (Ardsley, NY). Wy-14643, okadaic acid, and the other biochemicals used were purchased from the Sigma Chemical Co. (St. Louis, MO). Tautomycin was purchased from Calbiochem (La Jolla, CA). Antibodies against rat PPARα, PPARα (phospho S12) antibody, and PPARα (phospho S21) antibody were purchased from Abcam, Inc. (Cambridge, MA).

Plasmids. The luciferase reporter plasmid GSTmin was constructed by isolating 164 base pairs of the minimal promoter from the 5′-flanking region of the rat glutathione transerase A2 gene (Falkner et al., 2001) and inserting it into the HindIII/KpnI sites of pGL3-basic (Invitrogen, Carlsbad, CA). The expression plasmids for the murine PPARα (Boie et al., 1993) were provided by Thomas Rushmore (Merck Research Laboratory, West Point, PA). The luciferase reporter plasmid PPRELuc contained two copies of the peroxisome proliferator responsive element from rat PPARc peroxisome proliferator-activated receptor responsive element (PPRE) inserted upstream of the minimal promoter construct GSTmin. The expression plasmid for β-galactosidase (pCMVβ) was purchased from Clontech (Mountain View, CA). All plasmids were transformed into DH5α Escherichia coli bacteria, isolated, and prepared for use in transient transfections using QIAGEN plasmid prep kits (QIAGEN, Valencia, CA).

Treatment of Rats. Male Sprague-Dawley rats (180–200 g; Hsd:SD, Harlan, Indianapolis, IN) were fed a diet of AIN76A diet (Purina, St. Louis, MO) and treated with either 80 mg/kg body weight DHEA and 200 μg of random hexamer, 20 U of RNasin, 0.5 mM dNTP mix, and 200 μl of Moloney murine leukemia virus reverse transcriptase at 42°C for 1 h (Promega, Madison, WI). The cDNA was performed using reverse RNA and the oligo DNA mixture with RNase H digestion and QIAquick PCR purification kit (Promega). The Taqman reaction was carried out with a DNA sequence detection analyzer (ABI7900). Applied Biosystems, Foster City, CA) in a total volume of 25 μl using 2.2 pmol of reverse and forward primers. The primers used are shown in Table 1.

Primers for 18S rRNA (5-carboxyfluorescein-labeled and JOE-labeled) were purchased from Invitrogen.

Immunoblot Analysis. Hepatocytes were harvested in M-Per reagent (Sigma) supplemented with phosphatase inhibitor cocktail I-II (Sigma) and with Halt protease inhibitor (Pierce Chemical, Rockford, IL) according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). The genomic DNA was digested with DNase I (Invitrogen), and the RNA was cleaned using an RNaseasy mini kit (QIAGEN). Reverse transcript (RT) reactions were carried out in a final volume of 20 μl with 1 μg of rat total RNA, 0.45 μg of random hexamer, 20 U of RNasin, 0.5 mM dNTP mix, and 200 μl of Moloney murine leukemia virus reverse transcriptase at 42°C for 1 h (Promega, Madison, WI). The cDNA product was treated with enzymes and the elution DNA mixture with RNase H digestion and QIAquick PCR purification kit (Promega). The Taqman reaction was carried out with a DNA sequence detection analyzer (ABI7900). Applied Biosystems, Foster City, CA) in a total volume of 25 μl using 2.2 pmol of reverse and forward primers. The primers used are shown in Table 1. Primers for 18S rRNA (5-carboxyfluorescein-labeled and JOE-labeled) were purchased from Invitrogen.

Mutation of Serines 6, 12, and 21 of Murine PPARα. Site-directed mutagenesis of PPARα expression vector was performed with the Gene Tailor site-directed mutagenesis kit purchased from Invitrogen. Oligonucleotides for mutagenesis were purchased from Integrated DNA Technologies (Coralville, IA). The following nucleotide sequence changes were performed: for the S12A mutant, codon 12 (TCC toer) to (GCA); for the S21A mutant, codon 21 (AGT toer) to (GCT); for the double mutant S12A-S21A, codon 21 of the S12A

<table>
<thead>
<tr>
<th>Gene</th>
<th>Base Pair Position</th>
<th>Sequence</th>
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<tr>
<td>CYP4A1</td>
<td>306aFL</td>
<td>5′-GAACGAGCCTGTCTGCTGCTGGTCTGCTGC[Can]-3′</td>
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<tr>
<td>CYP4A1</td>
<td>347R</td>
<td>5′-CCCCGAATTCACACTTTGTTG[Can]-3′</td>
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<tr>
<td>Fpp2α</td>
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<td>5′-CGTCATCACTTCCAAGAAGGAACC[Can]-3′</td>
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<td>562FLR</td>
<td>5′-CAAAGGAGGAGGAGGAGGAGGAGG[Can]-3′</td>
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</tr>
<tr>
<td>rPPAR</td>
<td>Antisense</td>
<td>5′-GCCAGCGCGCGCGCAGAGCGGAGGAGG[Can]-3′</td>
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TABLE 1

Sequence of primers used for quantitative RT-PCR

DHEA Induces PPARα Expression and Phosphorylation

969

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mutant (AGTser) to (GCTala); for the triple mutant S6A-S12A-S21A, codon 6 of the S12A-S21A double mutant (AGCser) to (GCCala); for the S12D mutant, codon 12 of the S12A-S21A double mutant (GC gala) to (GACasp); for the S21D mutant, codon 21 of the S12A-S21A double mutant (GCTala) to (GATasp); for the S12D-S21D double mutant, codon 21 of the S12D (GCTala) to (GACasp). Note that the two individual aspartic acid mutants S12D and S21D each had alanine at the other phosphorylation site (S12D is also S21A; S21D is also S12A). The triple serine mutants (S6A-S12A-S21A and S6D- S12D-S21D) were also prepared using this mutation cloning strategy. In each case, the constructs were sequenced in the University of Louisville’s Center for Genetics and Molecular Medicine core facility, to assure that the appropriate nucleotide changes had been made.

HepG2 Cell Culture and Transient Transfection. HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and penicillin/streptomycin/Fungizone. Cells were plated in 12-well plates at a cell density of $1.5 \times 10^5$ per well. The following day, cells were transiently transfected with various plasmids using LipofectAMINE (Invitrogen, Carlsbad, CA). After transfection, cells were treated at 24 h with several peroxisome proliferators (DHEA, nafenopin, Wy-14643). β-Galactosidase and luciferase activities were determined as described by Falkner et al. (2001). Data are expressed as luciferase activity relative to β-galactosidase activity to correct for transfection efficiency. All transient transfection experiments were performed in triplicate and experiments were performed at least in duplicate.

Statistical Analysis. Experiments were conducted in triplicate or quadruplicate and means ± S.D. were determined. Statistical comparisons among treatment groups were determined using a two-tailed t test, with $p < 0.05$ as the criterion for significance.

Results

Okadaic Acid Alters Nafenopin-Dependent Induction of PPRELuc Reporter Expression in HepG2 Cells. In our experiments, the role of PPARα phosphorylation was investigated by treatment with different peroxisome proliferators and using protein phosphatase inhibitors. HepG2 cells were transfected with PPARα expression vector in the presence of PPRELuc reporter and treated with various concentrations of okadaic acid (20–40 nM) or 50 μM nafenopin plus okadaic acid for 24 h (Fig. 1). Unexpectedly, okadaic acid itself significantly inhibited the activity of the PPRELuc reporter gene in the absence of cotransfection with the PPARα expression plasmid. PPARα was able to positively regulate the PPRE responsive element, an effect that was reversed with increasing concentration of okadaic acid. In the presence of nafenopin, the activation of the luciferase reporter gene was over 3-fold higher than what was observed in untreated cells. Okadaic acid cotreatment caused a concentration-dependent reversal of nafenopin-induced activation of gene expression under all conditions (Fig. 1).

Nafenopin, Wy-14643, and DHEA Induce FACO Activity in Primary Rat Hepatocytes. Based on our previous findings that DHEA does not serve as a direct activator of PPARα to induce PPRE-containing genes in cultured cell lines, but does induce FACO and CYP4A1 mRNA and protein in vivo and in primary cultures of rat hepatocytes, we used primary hepatocytes to evaluate the mechanism of PPARα-dependent induction. Hepatocytes were treated with varying concentrations of DHEA and two other peroxisome proliferators for 48 h. Cell supernatant fractions were obtained by sedimentation at 5000 g and FACO activity was measured. Concentrations between 10 and 25 μM of either DHEA, nafenopin, or Wy-14643 caused a 2- to 3.5-fold induction of FACO activity (Fig. 2). Maximal inductive effect was observed at the following concentrations: 25 μM nafenopin.
(5.5-fold induction), 50 μM Wy-14643 (4-fold induction), and 50 μM DHEA (4-fold induction). These results demonstrate that primary rat hepatocytes display PPARα activation by DHEA to the same level as other PP.

Effect of Okadaic Acid on Induction of FACO Activity in Primary Cultures of Rat Hepatocytes. To test whether okadaic acid reverses the effect of peroxisome proliferators in primary hepatocytes, cells were pretreated with various concentrations of okadaic acid for 1 h and then treated with 50 μM DHEA for 24 h. Cell supernatant fractions were collected, and FACO activity was measured from cell supernatants. FACO activity was induced approximately 5-fold in DHEA-treated cells relative to control cells. Pretreating cells for 1 h with various concentrations of okadaic acid antagonized the inductive effect of DHEA in a concentration-dependent manner. The suppression by 25 nM okadaic acid reached the level of activity seen in untreated cells (Fig. 3). Similar results were observed with nafenopin (results not shown).

Effect of DHEA, Nafenopin, and Wy-14643 on PPARα mRNA and Protein Expression in Vivo and in Primary Cultures of Rat Hepatocytes. To verify that these agents altered the levels of PPARα mRNA in vivo, we performed experiments with male Sprague-Dawley rats to demonstrate whether DHEA or nafenopin caused changes in the levels of hepatic PPARα mRNA. The rats were maintained on AIN76A diet and administered i.p. either DHEA or nafenopin in corn oil at a dose of 100 mg/kg body weight daily for 4 days. The mRNA was isolated from liver and analyzed for relative expression specific antibodies against monoclonal PPARα antibody, using quantitative RT-PCR. As seen in Table 2, DHEA treatment caused a 5.9-fold increase in relative PPARα mRNA levels, whereas nafenopin caused a 30% decrease. Treatment with either DHEA or nafenopin resulted in significant induction of P4504A1, demonstrating that both compounds induced expression of this PPARα target gene. These results document that DHEA does alter the levels of PPARα message in vivo, increasing the mRNA levels by approximately 6-fold; however, Nafenopin did not seem to increase PPARα message levels.

To investigate whether peroxisome proliferators induce the levels of PPARα protein, primary cultures of rat hepatocytes were treated with varying concentrations of DHEA, nafenopin, or WY14643 and receptor mRNA and protein expression was measured. At the protein level, maximal induction by DHEA of 1.5-fold was observed using 10 μM inducer, whereas the cells treated with 25 to 50 μM showed lower PPARα protein levels than those given 10 μM (Fig. 4A). Nafenopin was as potent as DHEA causing a 1.5-fold increase in PPARα levels at 50 μM concentration. Treatment with 50 μM Wy-14643 caused the most pronounced induction of PPARα of 1.7-fold. The levels of PPARα mRNA in primary hepatocytes were also measured after treatment with DHEA, nafenopin, or Wy-14643; all three displayed similar concentration-dependent induction (Fig. 4B) and resulted in a 4- to 5-fold increase in mRNA in the primary hepatocytes cultures.

Peroxisome Proliferators Dephosphorylate Serine 12 and 21 of PPARα. Because okadaic acid, a protein phosphatase inhibitor, decreased gene expression, we sought to establish whether DHEA may decrease levels of phosphorylation of PPARα. Primary rat hepatocytes were pretreated with either DMSO or okadaic acid in DMSO for 1 h and then treated with various peroxisome proliferators for 4 h. PPARα phosphorylation status was analyzed by immunoblotting using specific antibodies against monoclonal PPARα phosphoserine 12 and phosphoserine 21. Total PPARα levels were quantitated with a polyclonal antibody against PPARα. It is noteworthy that okadaic acid treatment alone did not cause increased phosphorylation of ser-12, suggesting that the receptor may be maximally phosphorylated under basal conditions (Fig. 5A). A striking decrease in phosphorylation status of serine-12 of around 80% was observed when the cells were treated with DHEA. This effect was partially reversed by okadaic acid pretreatment. Nafenopin also caused a 75% reduction in phosphorylation of the receptor compared with the receptor in untreated cells. In the presence of okadaic acid, a slightly elevated level of phosphorylation was observed (130%). Wy-14643 was less effective than DHEA or nafenopin that it only decreased phosphorylation levels by 40%. Addition of okadaic acid with nafenopin or Wy-14643 only increased phosphorylation to the initial level. This experiment was repeated twice with similar results.

Another serine in the AF-1 region proposed to be phosphorylated by action of insulin is serine 21. Upon treatment with DHEA and Wy-14643, the receptor becomes dephosphorylated by approximately 50% or approximately 30% with nafenopin (Fig. 5B). In addition, the levels of dephosphorylation caused by peroxisome proliferators could be prevented.

**Table 2**

<table>
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<tr>
<th>Treatment Condition</th>
<th>Relative mRNA Expression</th>
<th>P Value</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 1</td>
<td></td>
</tr>
<tr>
<td>DHEA</td>
<td>594 ± 73</td>
<td>.005</td>
</tr>
<tr>
<td>Nafenopin</td>
<td>69 ± 8</td>
<td>.05</td>
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**Fig. 3.** Effect of okadaic acid on induction of FACO activity in cultured rat hepatocytes treated with 50 μM DHEA. Hepatocytes were treated with various concentrations of okadaic acid and 50 μM DHEA for 24 h. FACO activity was determined as described under Materials and Methods. The values shown are expressed as the average and standard deviation of 3 separate assays performed in triplicate. *p < 0.05.
by pretreating cells with okadaic acid. In the presence of either DHEA or nafenopin, treatment with okadaic acid caused the level of the phosphoreceptor to be slightly higher than that seen in untreated cells (130–110%). Treatment with Wy-14643 caused a lower level of phosphorylation, but combined treatment with Wy-14643 and okadaic acid did not cause the receptor phosphorylation levels to return toward control levels. Treatment with okadaic acid alone had no effect on the basal level of serine-21 phosphorylation. This experiment was repeated twice with similar results. These results demonstrate that addition of DHEA to cultured rat hepatocytes results in rapid dephosphorylation of serine 12 and serine 21 of PPARα.

**Mutation of Phosphorylation Sites in PPARα Receptor Modulates Nafenopin Induction in HepG2 Cells.** To test the functionality of the PPARα phosphorylation sites in the AF1 region, mutated PPARα expression vectors were designed. Serine phosphorylation sites at residues 6, 12, and 21 were changed to the apolar alanine residues or the negatively charged aspartic acid residue. HepG2 cells were transfected with the expression plasmid containing the receptor mutated at positions 6, 12, and 21 in the presence of PPRE-Luc and pCMVβ and were subsequently treated with 50 μM nafenopin or cotreated with nafenopin plus increasing concentrations of okadaic acid for 24 h. 

Transfecting the cells with the wild-type receptor resulted in a 3.5-fold induction after nafenopin treatment (data not shown). In the presence of okadaic acid, PPAR-dependent gene expression was less active than after treatment with peroxisome proliferators alone. Mutation from serine to alanine in the position of 6, 12, or 21 significantly increased the transcriptional activity of the receptor (Fig. 6). Mutating all three serine sites (6, 12, and 21) or mutating ser12 to alanine only in the AF-1 region causes a 30 to 40% increase in gene expression. When aspartic acid mutations were introduced at serine 21 or serines 6, 12, and 21, the cells show much less activity (20–30% decrease) after nafenopin induction (Fig. 7). Okadaic acid cotreatment was able to decrease this induction, in a concentration-dependent manner for all mutant

![Fig. 4. Effect of DHEA, nafenopin, and Wy-14643 on PPARα protein and mRNA expression in primary cultures of rat hepatocytes. A, cells were treated with different concentrations of DHEA, nafenopin and Wy-14643 for 48 h. Cellular protein (20 μg) were analyzed by SDS-PAGE and immunoblotting was performed using anti-PPARα antibody. Images of a representative immunoblot and bar graph of the quantification of scanned blots. B, cells were treated with different concentrations of DHEA, nafenopin, and Wy-14643 (WY) for 24 h. PPARα mRNA was measured by quantitative RT-PCR as described under Materials and Methods. Ribosomal 18S RNA levels were used as normalization standards. The values shown are expressed as the average and S.D. of six separate assays performed in triplicate.

![Fig. 5. Phosphorylation status of serine-12 or serine-21 of PPARα after treatment with peroxisome proliferators. Primary hepatocytes were pretreated with okadaic acid (OA) for 1 h and subsequently treated with 50 μM peroxisome proliferators for 4 h. Cells were collected and the amount of phosphoprotein was determined by immunoblot analysis using an anti-PPARα-phospho-ser-12 antibody. The Images of representative experiments are shown with quantitation of scanned blots. Experiments were performed in duplicate with nearly identical data. A, relative levels of phosphoserine-12 PPARα. B, relative levels of phosphoserine-21 PPARα.]
PPARs, suggesting an additional role for other residues in regulation by phosphorylation.

We evaluated the concentration-dependence of activation of the wild-type, S6,12,21A, and S6,12,21D forms of the receptor by nafenopin, Wyeth 14,643, and ciprofibrate in HepG2 cells using the reporter PPRELUC and pCMVβ as a control (data not shown). The maximal level of luciferase expression was greatest with the wild-type PPARα and lowest with the triple and quadruple serine-to-aspartate mutants. However, the concentration to achieve half-maximal activation of the respective receptors was unchanged among the receptor mutants tested (data not shown). These results clearly demonstrate that the mutants that resemble phosphorylated receptor display decreased levels of gene expression.

**Protein Phosphatase Inhibitors Modulate CYP4A1 mRNA Induction after Treatment with Peroxisome Proliferators.** Based on the hypothesis that peroxisome proliferators can induce PPARα target genes such as CYP4A1 by dephosphorylation of the PPAR receptor, primary rat hepatocytes were treated with specific phosphatase inhibitors such as okadaic acid (IC$_{50}$ for PP1 is 20–100 nM for PP2A 0.1–1 nM), or tautomycin (IC$_{50}$ for PP1 is 0.2 nM for PP2A 1–10 nM; Chatfield and Eastman, 2004), followed by treatment with either 10 μM DHEA or 50 μM nafenopin for 48 h (Fig. 7). Okadaic acid at low concentrations (2 nM) caused significant decreases in CYP4A1 induction in cultured rat hepatocytes; the level of inhibition was the same as that seen at 40 nM. However, low concentrations of okadaic acid (2 nM) had little or no effect on nafenopin induction, but higher concentrations, such as 40 nM, almost completely prevented the increase in CYP4A1 mRNA synthesis. Tautomycin (2 nM) was not as effective as okadaic acid in suppressing induction of CYP4A1 message with DHEA but was effective...
at both concentrations (2 and 120 nM being maximal) in suppressing induction of CYP4A1 mRNA by nafenopin. These data suggest that PP2A is more involved in the DHEA induction of CYP4A1 message than PP1 (Fig. 7). The opposite was observed for nafenopin.

**DHEA, but Not Nafenopin, Causes Changes in PP2A mRNA Levels.** Primary rat hepatocytes were treated with 10 μM DHEA for 0 to 24 h, and PP2A mRNA was measured. PP2A mRNA increased with time, reaching a 9-fold increase after 8 h (Fig. 8). Thereafter, there was a decline in mRNA levels, possibly because of the low concentration of DHEA used in this experiment. After 8 h, there was a rapid decline in mRNA specific for PP2A, suggesting that this mRNA has a short half-life. These results suggest that DHEA induces PP2A mRNA levels, accounting for the increase in dephosphorylation of serine 12 and serine 21 (Figs. 5, A and B), supporting our results with different phosphatase inhibitors (Fig. 7). Nafenopin treatment (50 μM) did not cause significant changes in PP2A mRNA.

**Discussion**

Phosphorylation or dephosphorylation of transcription factors often alters functional activity of that factor. Phosphorylation status of a transcription factor is regulated by action of various kinases and phosphatases, which act in a time-dependent manner (Hunter, 1995). Phosphorylation of nuclear receptors is a major determinant of their transcriptional activity as shown for many nuclear proteins (Sun et al., 1994; Reifel-Miller et al., 1994; Blanquart et al., 2003; Tan et al., 2005). PPARα is also a phosphoprotein, as was shown by Shalev et al. (1996), and its phosphorylation status could be modified by insulin (Juge-Aubry et al., 1999). Indeed, these authors have shown that PPARα is phosphorylated in response to insulin on serine 12 and 21, by action of MAPK pathways leading to increased AF-1 activity of PPARα.

Some peroxisome proliferators, like DHEA, apparently act via PPARα receptor, although not as a direct ligand of the receptor. Our results demonstrate that DHEA treatment elevates PPARα levels in the cell and increases the expression of other proteins involved in regulation of PPARα activation. Besides direct induction of PPARα expression, a second pathway of DHEA action is changing the phosphorylation status of the receptor. In our experiments, okadaic acid, a protein phosphatase inhibitor, reduced PPRE-dependent luciferase activity in nafenopin-treated HepG2 cells, supporting an important role of phosphatases in PPARα action. Okadaic acid also antagonized DHEA induction in primary rat hepatocytes, suggesting that the action of DHEA in inducing PP2A leads to dephosphorylation of PPARα, and the PP2A inhibitor okadaic acid blocks dephosphorylation.

To test the hypothesis that peroxisome proliferators alter phosphorylation status of the receptor, the levels of phosphoser-12- and phospo-ser-21-PPARα were determined by Western blot analysis with specific anti-phospho-ser-PPARα antibodies. Both ser-12 and ser-21 are dephosphorylated after treatment with DHEA, and this effect was reversed by okadaic acid. This demonstrated that phosphorylation sites in the AF-1 region of the receptor are important in the regulation with DHEA and perhaps other peroxisome proliferators (Fig. 5, A and B); phosphorylation decreased gene activation, whereas dephosphorylation increased gene activation, as shown in Fig. 9. There seem to be three pathways for activation of PPARα: ligand activation, induction of PPARα levels, and dephosphorylation of PPARα.

Our results correlate with the work of Passilly et al. (1999), who measured acyl-CoA oxidase activity as a marker gene for PPARα in Fao cells. In their experiments, okadaic acid and vanadate treatment led to decreased acyl-CoA oxidase activity. This negative regulation phenomenon due to phosphorylation status is also observed in PPARγ action; e.g., preadi...
DHEA Induces PPARα Expression and Phosphorylation

DHEA induces PPARα on at least two levels (Fig. 9): they increase PPARα mRNA and protein expression, and they also may alter the phosphorylation status of the receptor. Both of these mechanisms result in increases in transcriptional activity of the receptor. However, no evidence exists that DHEA functions as a ligand activator for PPARα in rodents, a third mechanism of receptor regulation.

Acknowledgments

We thank Mary Pendleton for technical assistance in the transient transfection assays and Tom Rushmore and K. Cameron Falkner for expression plasmids.

References


...ocytes and adipocytes display similar decreases in PPARγ action, when PPARγ phosphorylation is stimulated by insulin acting through MAPK. The phosphorylation of PPARγ leads to a decrease in PPARγ-mediated preadipocyte differentiation into adipocytes (Zhang et al., 1996; Latruffe et al., 2000). Other phosphorylation pathways stimulated by epidermal growth factor and platelet-derived growth factor acting through MAPK have a negative effect on the PPARγ action (Hu et al., 1996; Adams et al., 1997; Camp and Tafuri, 1997). Additional studies have demonstrated that this negative effect of MAPK was due to the inhibition of ligand binding resulting from an alteration of the three-dimensional structure of the receptor (Shao et al., 1998). Gray et al. (2005) have also demonstrated that protein kinase C is stimulated by the peroxisome proliferators Wy-14,643. Subsequent studies using mutated receptors or overexpressing forms of PKC affected both basal and Wy-14,643-induced PPARα activity. Serine phosphorylation in the DNA binding (C-domain) and hinge (D-domain) were shown to contribute to decreased interaction of PPARα and retinoid X receptor α and therefore DNA binding.

Mutation of PPARα-Ser 6, 12, and 21 sites to alanine, a neutral change in charge to a residue that cannot be phosphorylated, causes an increase in the activity of the receptor after treatment with nafenopin in HepG2 cells, but mutation of serines in the AF-1 region to negatively charged aspartic acid residues, which may mimic the phosphorylated form of the receptor, significantly reduces the transactivation of the gene. It is noteworthy that okadaic acid treatment was still able to reduce the activity after mutating serine residues to alanine, suggesting that other phosphorylation sites may alter activation of PPARα-dependent gene expression. Aspartate mutants (except the Ser12 mutant) were not as sensitive to a phosphatase inhibitor, which demonstrates the importance of the phosphorylation status of these serine sites after nafenopin induction.

Swales and Negishi (2004) reported that transcriptional activity of constitutive androstane receptor is also sensitive to okadaic acid and that this effect could be explained by the action of a specific phosphatase (PP2A), which binds to the receptor after activation and mediates receptor translocation to the nucleus. To investigate the role of phosphatases in PPARα-dependent regulation of rat hepatocytes, changes in CYP4A1 expression were measured after treatment with peroxisome proliferators and specific phosphatase inhibitors. Higher concentrations of okadaic acid (20–40 nM) are not specific for a certain phosphatase type, but in low concentrations (2 nM), okadaic acid inhibits PP2A, but not members of the PP1 family at this concentration (Chatfield and Eastman, 2004). Tautomycin has an opposite effect; it displays a lower IC_{50} value for PP1, relative to PP2A. Induction of CYP4A1 message by nafenopin was diminished in the presence of 2 nM tautomycin, but not with 2 nM okadaic acid, suggesting a role for PP1 in activation of gene expression by nafenopin. Induction of CYP4A1 by DHEA was sensitive to low concentrations of okadaic acid, but not for 2 nM tautomycin. This suggests that DHEA induction of CYP4A1 requires PP2A for the receptor dephosphorylation mechanism. Monitoring PP2A mRNA level after DHEA treatment provides further evidence that the sterol induces PPA2 to cause dephosphorylation of the receptor.

In conclusion, peroxisome proliferators such as DHEA regulate PPARα on at least two levels (Fig. 9); they increase PPARα mRNA and protein expression, and they may also alter the phosphorylation status of the receptor. Both of these mechanisms result in increases in transcriptional activity of the receptor. However, no evidence exists that DHEA functions as a ligand activator for PPARα in rodents, a third mechanism of receptor regulation.
receptor (PPAR) isoforms PPAR\(_2\) versus PPAR\(_1\) and activation with retinoid X receptor agonists and antagonists. *J Biol Chem* **272**:8071–8076.


Address correspondence to: Dr. R. A. Prough, Department of Biochemistry and Molecular Biology, U. Louisville School of Medicine, Louisville, KY 40292. E-mail: russ.prough@louisville.edu