

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

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ABSTRACT

Dehydroepiandrosterone (DHEA), a C19 human adrenal steroid, activates peroxisome proliferator-activated receptor α (PPAR α) in vivo but does not ligand-activate PPAR α in transient transfection experiments. We demonstrate that DHEA regulates PPAR α action by altering both the levels and phosphorylation status of the receptor. Human hepatoma cells (HepG2) were transiently transfected with the expression plasmid encoding PPAR α and a plasmid containing two copies of fatty acyl coenzyme 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 PPAR α mRNA and protein levels, as well as PP2A message in primary rat hepatocytes. Western blot analysis showed that the serines 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) is responsible for DHEA action, and protein phosphatase 1 might be involved in nafenopin induction. Mutation of serines at position 6, 12, and 21 to an uncharged alanine residue significantly increased transcriptional activity, whereas mutation to negative charged aspartate residues (mimicking receptor phosphorylation) decreased transcriptional activity. DHEA action involves induction of PPAR α mRNA and protein levels as well as increased PPAR α transcriptional activity through decreasing receptor phosphorylation at serines in the AF1 region.

Peroxisome proliferator-activated receptor α (PPAR α , NR1C1) is a member of the nuclear receptor superfamily and functions as a ligand-activated transcription factor (Isseman et al., 1990; Sher et al., 1993). PPAR α 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 PPAR α mRNA levels have been re-

ported to be lower in humans than in rodents (Palmer et al., 1998). In vivo, PPAR α 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

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ABBREVIATIONS: PPAR α , peroxisome proliferator-activated receptor α ; PP, peroxisome proliferator; DHEA, dehydroepiandrosterone; MAPK, mitogen-activated protein kinase; FACO, fatty acyl coenzyme oxidase; Wy-14643, [[4-chloro-6-[(2,3-dimethylamino)-2-pyrimidinyl]thio] acetic acid; PPRE, peroxisome proliferator-activated receptor responsive element; RT, reverse transcription; DMSO, dimethyl sulfoxide; PP2A, protein phosphatase 2A; RT, reverse transcription; PCR, polymerase chain reaction.

of the receptor or through perturbation of lipid metabolism/transport, leading to release of PPAR α 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 PPAR α receptor by DHEA has never been demonstrated in assays using immortalized cell lines, although it is well established that DHEA affects PPAR α 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 rat hepatocytes, DHEA, nafenopin, and Wy-14643 induce protein and mRNA levels of PPAR α and, thereby, PPAR α -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 PPAR α and decreases phosphorylation status of the PPAR α receptor in hepatocytes, leading to increased transcriptional activity. This indirect mechanism of activation of PPAR α 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 PPAR α , PPAR α (phospho S12) antibody, and PPAR α (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 transferase 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 PPAR α (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 FACO peroxisome proliferator-activated receptor responsive element (PPRE) inserted upstream of the minimal promoter construct GSTmin. The expression plasmid for β -galactosidase (pCMV β) was purchased from Clontech (Mountain View, CA). All plasmids were transformed into DH5 α *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 or nafenopin daily for 4 days. The animals were anesthetized with carbon dioxide and killed by cervical dislocation. These procedures were approved by the University of Louisville Institutional Animal Care and Use Committee. The livers were flash-frozen in liquid nitrogen and stored at -80°C until mRNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Primary Hepatocyte Culture. Male Sprague-Dawley rats (180–200 g; Hsd:SD, Harlan) were used for liver perfusion as described by

the method of Bayliss and Skett (1996). Cell survival was determined by trypan blue exclusion (Berry et al., 1991), and hepatocytes having viability greater than 80% were used and were plated at a density of 4×10^6 cells/dish onto 60-mm plastic dishes precoated with collagen in Williams E/Ham's F12 1:1 mixed medium as described by Ferrini et al. (1998) and Isom and Georgoff (1984). After allowing the cells to attach for 4 h, the medium was changed and renewed every 24 h thereafter in the absence of serum. The cells were treated with various concentrations of the following inducers: nafenopin, DHEA, and Wy-14643. Okadaic acid and tautomycin dissolved in DMSO were used as phosphatase inhibitors.

Peroxisomal β -Oxidation of Fatty Acid Activity (FACO). Cultured rat hepatocytes were collected to measure NADH formation using 0.01 μM palmitoyl-CoA (Sigma) as a substrate as described by Lazarow (1981). Subcellular fractions (5000g supernatant) were diluted in 0.25 M sucrose, and the enzymatic reaction was carried out in a mixture containing the following: 0.2 mM NAD $^{+}$, 1 mM DTT, 0.1 mM CoA, 0.01 mM FAD, and 1 mM potassium cyanide. Protein concentration was measured using the Bradford assay (Bradford, 1976).

Quantitative Real-Time PCR of mRNA. Total RNA from rat liver or primary hepatocytes was extracted using TRIzol reagent following the manufacturer protocol (Invitrogen, Carlsbad, CA). The genomic DNA was digested with DNase I (Invitrogen), and the RNA was cleaned using an RNeasy mini kit (QIAGEN). Reverse transcription (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 μM dNTP mix, and 200 U of Moloney murine leukemia virus reverse transcriptase at 42°C for 1 h (Promega, Madison, WI). The cDNA product was treated to remove 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 kit (Pierce Chemical, Rockford, IL) according to the manufacturer protocol. Protein (20 μg) was loaded to the gel and run using the method described by Laemmli (1970). Proteins were transferred to polyvinylidene difluoride membranes (Towbin et al., 1979) and rinsed with either anti-rat PPAR α , anti-rat PPAR α (phospho-ser-12), or anti-rat PPAR α (phospho-ser-21) as a primary antibody (Abcam). Enhanced chemiluminescence (ECL detection system; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to detect the target protein of interest. The Western blots were scanned with fluorescence scanning using a Storm 860 PhosphorImager, and the immune complexes were quantified with ImageQuant software (GE Healthcare).

Mutation of Serines 6, 12, and 21 of Murine PPAR α . Site-directed mutagenesis of PPAR α 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 (TCCser) to (GCCala); for the S21A mutant, codon 21 (AGTser) to (GCTala); for the double mutant S12A-S21A, codon 21 of the S12A

TABLE 1
Sequence of primers used for quantitative RT-PCR

Gene	Base Pair Position	Sequence
CYP4A1	306aFL	5'-GAACCAAGTGCCTTTCTCGATGGT[FAM]C-3'
CYP4A1	347R	5'-GCCCGAGAATCACCTTCATGT-3'
Ppp2ca	552FL	5'-CGTGACATCTCCGAGGGAATCA[FAM]G-3'
Ppp2ca	562FL/65	5'-CCAAGGCAGTGAGAGAAGGT-3'
rPPAR	Sense	5'-GTAGGCCCAAGAGAATCCACGAAGCC[FAM]AC
rPPAR	Antisense	5'-AGCCATGCACAAGGTCTCCA-3'

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 (GCCala) 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 1.5×10^5 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 \pm 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.

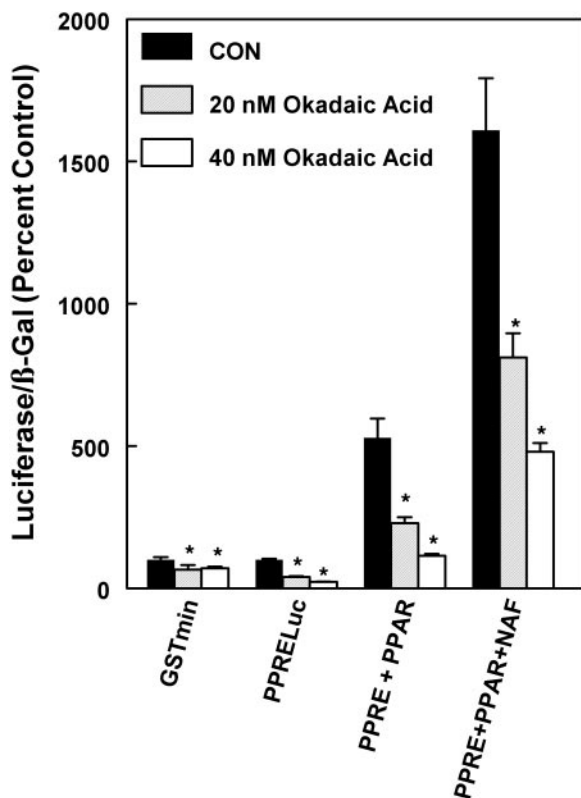


Fig. 1. Effect of okadaic acid on activation of a PPAR α -dependent FACO luciferase reporter by nafenopin in HepG2 cells. HepG2 cells were treated with various concentrations of okadaic acid in the presence of 50 μ M nafenopin and/or expression constructs for PPAR α . The luciferase and β -galactosidase were measured as described under *Materials and Methods*. The values shown are expressed as the average and standard deviation of three separate transient transfection experiments performed in triplicate. Statistical significance: *, $p < 0.05$

Results

Okadaic Acid Alters Nafenopin-Dependent Induction of PPRELuc Reporter Expression in HepG2 Cells.

In our experiments, the role of PPAR α phosphorylation was investigated by treatment with different peroxisome proliferators and using protein phosphatase inhibitors. HepG2 cells were transfected with PPAR α expression vector in the presence of PPRELuc reporter and treated with various concentrations of okadaic acid (20–40 nM) or 50 μ M 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 PPAR α expression plasmid. PPAR α 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 PPAR α 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 PPAR α -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 5000g and FACO activity was measured. Concentrations between 10 and 25 μ M 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 μ M nafenopin

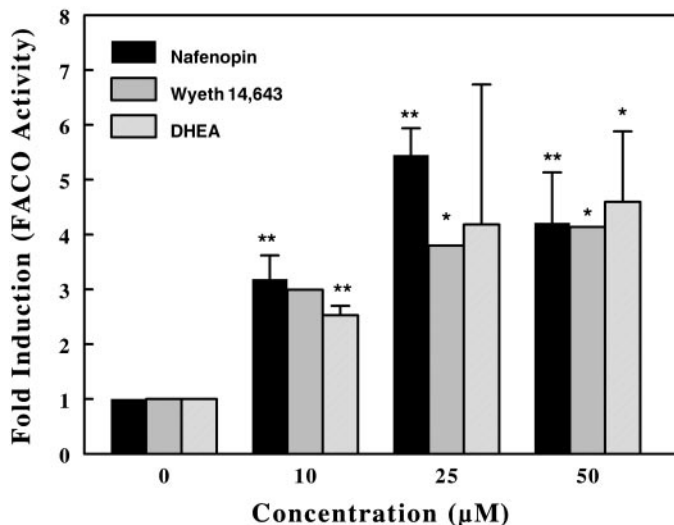


Fig. 2. Nafenopin, Wy-14643, and DHEA induce FACO activity in primary rat hepatocytes. Primary cultures of rat hepatocytes were treated with various concentrations of inducers for 24 h. As described under *Materials and Methods*, a 5000g supernatant was collected, and FACO activity was determined using palmitoyl-CoA and NAD $^{+}$ as substrates. The normalized values shown are expressed as the average and standard deviation of three separate assays performed in triplicate. Significantly different from untreated control. *, $p < 0.05$; **, $p < 0.01$.

(5.5-fold induction), 50 μ M Wy-14643 (4-fold induction), and 50 μ M DHEA (4-fold induction). These results demonstrate that primary rat hepatocytes display PPAR α 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 μ M 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 PPAR α mRNA and Protein Expression in Vivo and in Primary Cultures of Rat Hepatocytes. To verify that these agents altered the levels of PPAR α mRNA in vivo, we performed experiments with male Sprague-Dawley rats to demonstrate whether DHEA or nafenopin caused changes in the levels of hepatic PPAR α 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 levels of PPAR α mRNA using quantitative RT-PCR. As seen in Table 2, DHEA treatment caused a 5.9-fold increase in relative PPAR α mRNA levels, whereas nafenopin caused a 30% decrease. Treatment with either DHEA or nafenopin resulted in significant induction of P450A1, demonstrating that both compounds induced expression of this PPAR α target gene. These results document that DHEA does alter the levels of PPAR α message in vivo, increasing the mRNA levels

by approximately 6-fold; however, Nafenopin did not seem to increase PPAR α message levels.

To investigate whether peroxisome proliferators induce the levels of PPAR α 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 μ M inducer, whereas the cells treated with 25 to 50 μ M showed lower PPAR α protein levels than those given 10 μ M (Fig. 4A). Nafenopin was as potent as DHEA causing a 1.5-fold increase in PPAR α levels at 50 μ M concentration. Treatment with 50 μ M Wy-14643 caused the most pronounced induction of PPAR α of 1.7-fold. The levels of PPAR α 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 PPAR α . Because okadaic acid, a protein phosphatase inhibitor, decreased gene expression, we sought to establish whether DHEA may decrease levels of phosphorylation of PPAR α . 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. PPAR α phosphorylation status was analyzed by immunoblotting using specific antibodies against monoclonal PPAR α phosphoserine 12 and phosphoserine 21. Total PPAR α levels were quantitated with a polyclonal antibody against PPAR α . 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

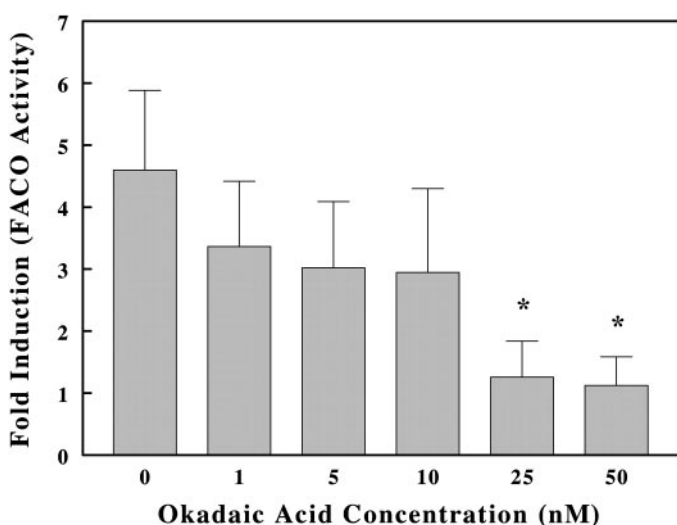


Fig. 3. Effect of okadaic acid on induction of FACO activity in cultured rat hepatocytes treated with 50 μ M DHEA. Hepatocytes were treated with various concentrations of okadaic acid and 50 μ M DHEA for 24 h. FACO activity was determined as described under *Materials and Methods*. The values shown are expressed as the average and standard deviation of 3 separate assays performed in triplicate. *, $p < 0.05$.

TABLE 2

Relative PPAR α mRNA levels in livers of male Sprague-Dawley Rats treated with either DHEA or Nafenopin

Male Sprague-Dawley rats were maintained on AIN-76A diet and injected i.p. daily for 4 days with corn oil or corn oil containing either DHEA or nafenopin to attain a 100 mg/kg dosage. The rats were euthanized with CO₂, and livers were removed to prepare mRNA as described under *Materials and Methods*. The statistical values are relative to control.

Treatment Condition	Relative mRNA Expression	P Value
Control	100 \pm 1	
DHEA	594 \pm 73	.005
Nafenopin	69 \pm 8	.05

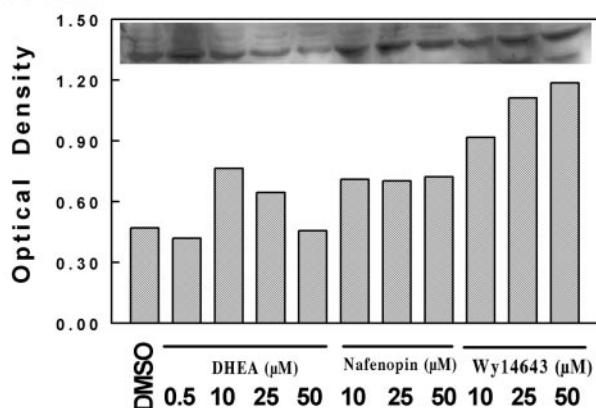
by pretreating cells with okadaic acid. In the presence of either DHEA or nafenopin, treatment with okadaic acid caused the level of the phosphoreceptor to be slightly higher than that seen in untreated cells (130–110%). Treatment with Wy-14643 caused a lower level of phosphorylation, but combined treatment 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 PPAR α .

Mutation of Phosphorylation Sites in PPAR α Receptor Modulates Nafenopin Induction in HepG2 Cells. To test the functionality of the PPAR α phosphorylation sites in the AF1 region, mutated PPAR α 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 PPARE-Luc and pCMV β and were subsequently treated with 50 μ M nafenopin or cotreated with nafenopin plus increasing concentrations of okadaic acid for 24 h.

Transfecting the cells with the wild-type receptor resulted in an 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

A Protein



B mRNA

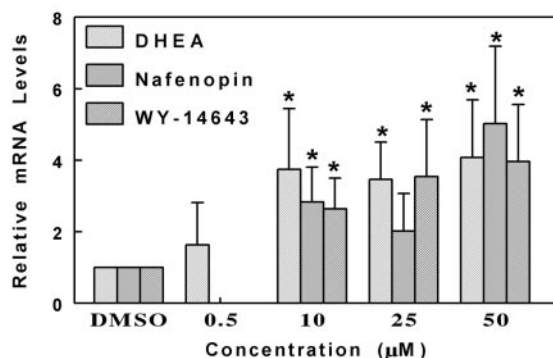


Fig. 4. Effect of DHEA, nafenopin, and Wy-14643 on PPAR α protein and mRNA expression in primary cultures of rat hepatocytes. A, cells were treated with different concentrations of DHEA, nafenopin and Wy-14643 for 48 h. Cellular protein (20 μ g) were analyzed by SDS-PAGE and immunoblotting was performed using anti-PPAR α 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. PPAR α 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.

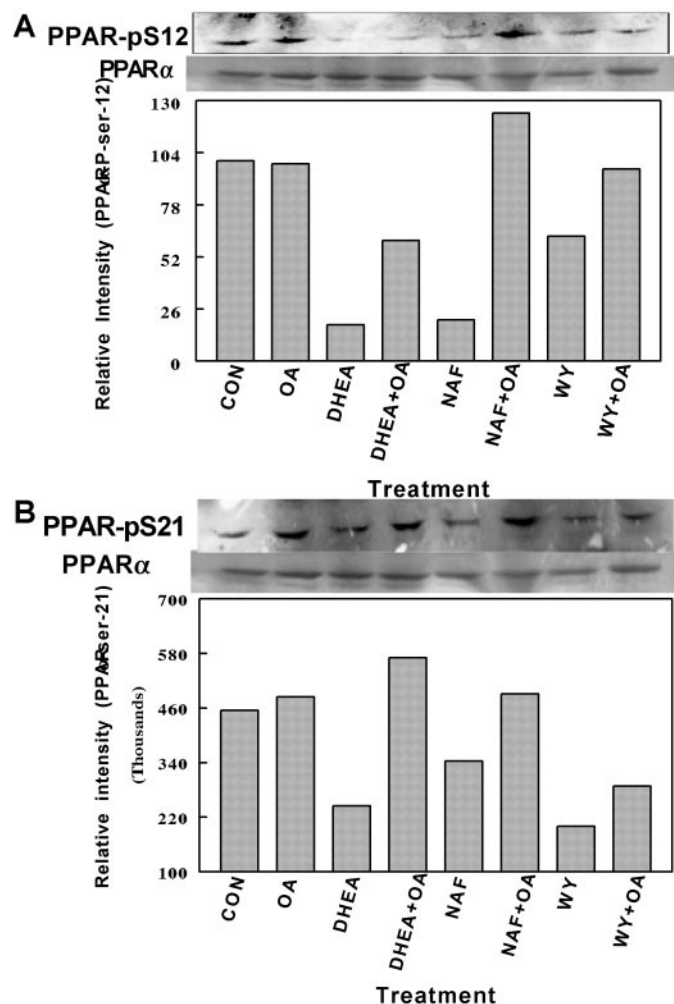


Fig. 5. Phosphorylation status of serine-12 or serine-21 of PPAR α after treatment with peroxisome proliferators. Primary hepatocytes were pretreated with okadaic acid (OA) for 1 h and subsequently treated with 50 μ M peroxisome proliferators for 4 h. Cells were collected and the amount of phosphoprotein was determined by immunoblot analysis using an anti-PPAR α -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 PPAR α . B, relative levels of phosphoserine-21 PPAR α .

PPAR α s, 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

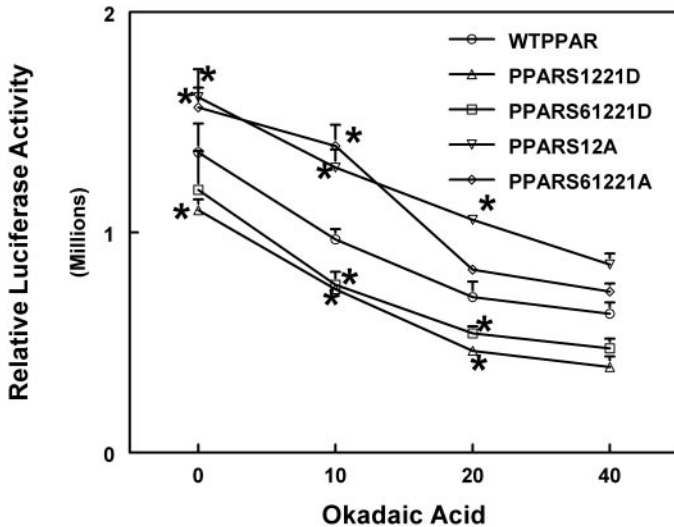


Fig. 6. Effect of mutation of serine residues in the AF-1 region of PPAR α on transactivation activity. Two alanine [S12A and S6,12,21A] or aspartic acid [S12,21D and S6,12,21D] mutant PPAR α expression vectors were prepared as described under *Materials and Methods*. HepG2 cells were treated with varying concentrations of okadaic acid in the presence of 50 μ M nafenopin and/or expression constructs for the mutant, and wild-type PPAR α and luciferase and β -galactosidase assays were measured. The values shown are expressed as the average and standard deviation of three separate transient transfection experiments performed in triplicate. *, significantly different from wild type, $p < 0.05$.

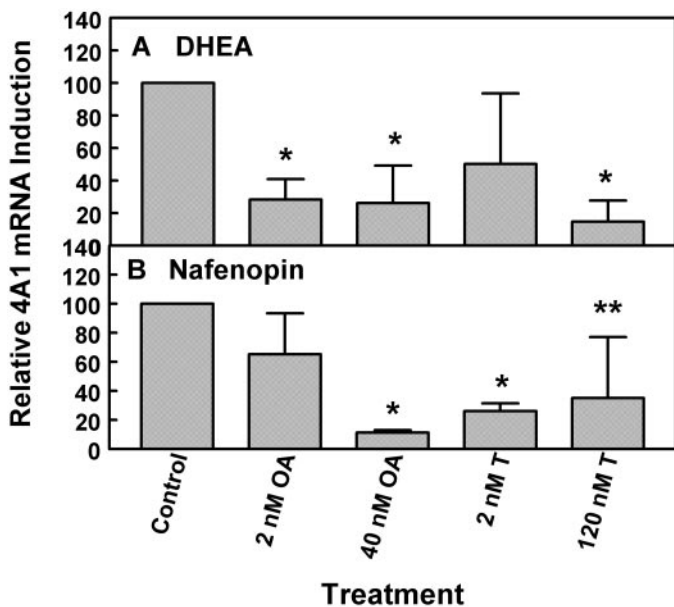


Fig. 7. CYP4A1 mRNA induction with DHEA or nafenopin in primary rat hepatocytes after treatment with different phosphatase inhibitors. Cells were pretreated with okadaic acid (OA; 2 or 40 nM) or tautomycin (T; 2 or 120 nM) for 1 h and then treated with either 10 μ M DHEA (A) or 50 μ M nafenopin (B) for 48 h. CYP4A1 mRNA expression was measured as described under *Materials and Methods*. Ribosomal 18S RNA levels were used as normalization standards. The values shown are expressed as the average and standard deviation of three separate assays performed in triplicate. All values are relative values to samples treated with peroxisome proliferators only. *, significantly different from control, $p < 0.05$.

in HepG2 cells using the reporter PPRELUC and pCMV β as a control (data not shown). The maximal level of luciferase expression was greatest with the wild-type PPAR α 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 PPAR α 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 μ M DHEA or 50 μ M 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

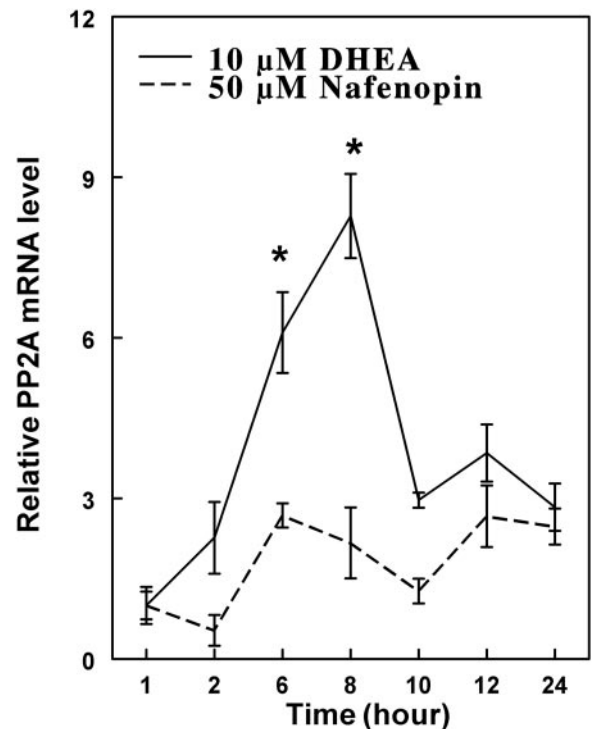


Fig. 8. Time-dependent changes in PP2A mRNA levels after treatment with DHEA or nafenopin. Primary rat hepatocytes were treated with 10 μ M DHEA or nafenopin for various time periods between 0 and 24 h. PP2A mRNA expression was quantified using quantitative RT-PCR, as described under *Materials and Methods*. Ribosomal 18S RNA levels were used as normalization standards. The values shown are expressed as the average and standard deviation of three separate assays performed in triplicate. *, $p < 0.05$.

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 μ M 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 μ M) 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). PPAR α 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 PPAR α is phosphorylated in response to insulin on serine 12 and 21, by action of MAPK pathways leading to increased AF-1 activity of PPAR α .

Some peroxisome proliferators, like DHEA, apparently act via PPAR α receptor, although not as a direct ligand of the receptor. Our results demonstrate that DHEA treatment elevates PPAR α levels in the cell and increases the expression of other proteins involved in regulation of PPAR α activation. Besides direct induction of PPAR α 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 PPAR α action. Okadaic acid also antagonized DHEA induction in primary rat hepatocytes, suggesting that the action of DHEA in inducing PP2A leads to dephosphorylation of PPAR α , and the PP2A inhibitor okadaic acid blocks dephosphorylation.

To test the hypothesis that peroxisome proliferators alter phosphorylation status of the receptor, the levels of phospho-ser-12- and phospho-ser-21-PPAR α were determined by Western blot analysis with specific anti-phospho-ser-PPAR α 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 PPAR α : ligand activation, induction of PPAR α levels, and dephosphorylation of PPAR α .

Our results correlate with the work of Passilly et al. (1999), who measured acyl-CoA oxidase activity as a marker gene for PPAR α 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 PPAR γ action; e.g., pread-

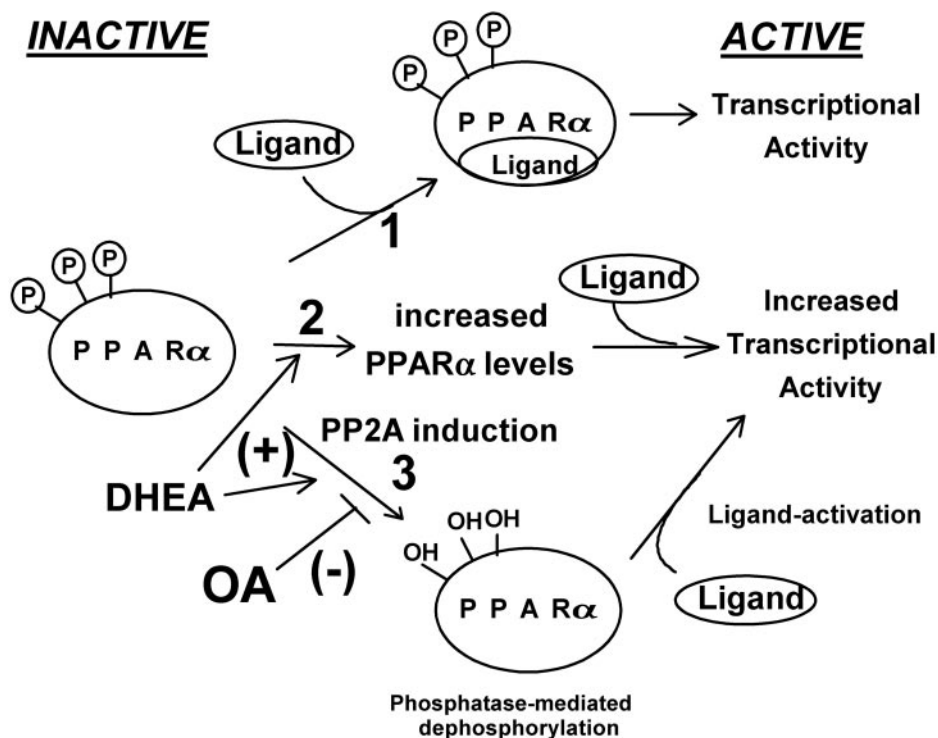


Fig. 9. Schematic of three possible pathways for activation of PPAR α by peroxisome proliferators. 1) Ligand-induced receptor activation, 2) increased expression of receptor, and 3) dephosphorylation to yield more active transcription factor.

pocytes and adipocytes display similar decreases in PPAR γ action, when PPAR γ phosphorylation is stimulated by insulin acting through MAPK. The phosphorylation of PPAR γ leads to a decrease in PPAR γ -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 PPAR γ 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 PPAR α activity. Serine phosphorylation in the DNA binding (C-domain) and hinge (D-domain) were shown to contribute to decreased interaction of PPAR α and retinoid X receptor α and therefore DNA binding.

Mutation of PPAR α -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 PPAR α -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 PPAR α -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 IC₅₀ 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 reg-

ulate PPAR α on at least two levels (Fig. 9); they increase PPAR α mRNA and protein expression, and they also may change 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 PPAR α in rodents, a third mechanism of receptor regulation.

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