Regulation of Peroxisome Proliferator-Activated Receptor α by Protein Kinase C†

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ABSTRACT: Peroxisome proliferator-activated receptor α (PPARα) is a nuclear receptor activated by fatty acids, hypolipidemic drugs, and peroxisome proliferators (PPs). Like other nuclear receptors, PPARα is a phosphoprotein whose activity is affected by a variety of growth factor signaling cascades. In this study, the effects of protein kinase C (PKC) on PPARα activity were explored. In vivo phosphorylation studies in COS-1 cells transfected with murine PPARα showed that the level of phosphorylated PPARα is increased by treatment with the PP Wy-14,643 as well as the PKC activator phorbol myristol acetate (PMA). In addition, inhibitors of PKC decreased Wy-14,643-induced PPARα activity in a variety of reporter assays. Overexpressing PKCα, -*β*, -*δ*, and -ζ affected both basal and Wy-14,643-induced PPARα activity. Four consensus PKC phosphorylation sites are contained within the DNA binding (C-domain) and hinge (D-domain) regions of rat PPARα (S110, T129, S142, and S179), and their contribution to receptor function was examined. Mutation of T129 or S179 to alanine prevented heterodimerization of PPARα with RXRα, lowered the level of phosphorylation by PKCα and PKC*δ* in vitro, and lowered the level of phosphorylation of transfected PPARα in transfected cells. In addition, the T129A mutation prevented PPARα from binding DNA in an electromobility shift assay. Together, these studies demonstrate a direct role for PKC in the regulation of PPARα, and suggest several PKCs can regulate PPARα activity through multiple phosphorylation sites.

Peroxisome proliferators (PPs)[1] are xenobiotic ligands of the peroxisome proliferator-activated receptor α (PPARα) and mediate most of their effects through this protein (*1*). PPARα is a nuclear receptor (NR1C1) which, following ligand binding, heterodimerizes with retinoid X receptor (RXR), binds to peroxisome proliferator response elements (PPREs), and regulates transcription (*2*−*4*). Transcriptional targets of PPARα include acyl-CoA oxidase (ACO), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase (HD), and other fatty acid metabolism genes (*5*, *6*). DNA response elements in target genes consist of direct repeat 1 (DR1) elements similar to several other nuclear receptor binding sites (*7*, *8*).

PPARα plays a role in the maintenance of fatty acid and cholesterol homeostasis (*9*, *10*). In the absence of PPARα (PPARα$^{-/-}$), mice have abnormal triglyceride and cholesterol metabolism and accumulate lipid droplets in their liver cells (*11*−*13*). PPAR$^{-/-}$ mice have higher levels of glycolysis and glucose oxidation, particularly in cardiac tissue, which normally relies heavily on fatty acids as an energy source (*14*). PPs are hepatocarcinogenic in rats, mice, and hamsters, but not in guinea pigs (*15*−*17*). The mechanism of carcinogenesis is not clear, but PP treatment is followed by an increase in cell proliferation and immediate early genes in liver (reviewed in ref *18*). The PP-induced hepatocarcinogenesis seen in rodents requires PPARα, as PPARα$^{-/-}$ mice are resistant to tumors normally caused by these xenobiotics (*11*).

In addition to ligand binding, phosphorylation is another mechanism by which nuclear receptor activity is modulated. PPARα is a phosphoprotein, and phosphorylation regulates function of this nuclear receptor (*19*−*24*). Physiological signals, including insulin, induce phosphorylation of PPARα and enhance its transcriptional response (*19*). This regulation is MAPK-dependent and involves serine residues 12 and 21 (*20*). Statin treatment, however, decreases the level of phosphorylation of PPARα (*21*). Activation of p38 MAPK activates PPARα-driven reporter activity in CV-1 cells, while lowering it in cardiac cells (*22*). Inhibition of PI3K using wortmannin and LY294002 dramatically enhances PPARα activity in a cell culture system (*23*). Finally, PKA enhances basal and Wy-14,643-induced reporter activity in transfected cells (*24*). These studies provide strong evidence for the regulation of PPARα by phosphorylation.

In addition to the signaling cascades described above, the protein kinase C (PKC) pathway also influences PPARα activity. Treatment of mice with 1-(5-isoquinolinesulfonyl)-2-methylpiperizine dihydrochloride (H-7) inhibited Wy-14,643-mediated induction of fos and jun mRNA (*25*). PPARα activity is also induced in human keratinocyte cultures by treatment with calcium, presumably through PKC (*26*, *27*). Treatment of primary rat hepatocytes with H-7

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[1] Abbreviations: GFP, green fluorescent protein; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PMA, phorbol myristol acetate; PPARα, peroxisome proliferator-activated receptor α; PPs, peroxisome proliferators; RXRα, retinoid X receptor α.

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blocked induction of fatty acid oxidizing enzymes by clofibrate (*28*). Recent studies have shown human PPARα purified protein is phosphorylated in vitro by recombinant PKCα and -βII at serines 179 and 230 (*29*). PPARα S179A/S230A protein displays an impaired ligand-induced transactivation activity. The mechanistic reason for this decreased activity was not examined.

The purpose of this work was to verify the cross-talk between PPARα and PKC and to investigate the mechanism by which the PKC pathway affects PPARα signaling. Cell culture studies show that activation of the PKC pathway increased endogenous and Wy-14,643-induced PPARα activity. Likewise, inactivation of PKC inhibits Wy-14,643-mediated induction of various reporters. In vitro phosphorylation studies show that PPARα is phosphorylated directly by PKC, which in vivo studies confirm. Finally, mutations at potential phosphorylation sites within the C- and D-domains of PPARα block its phosphorylation, alter heterodimerization, and lower transactivation activity. These results confirm the direct role of PKC in modulating PPARα activity in vivo and provide insight into the molecular mechanisms that are involved.

EXPERIMENTAL PROCEDURES

Materials. Hybond-C extra nitrocellulose, [^{35}S]methionine, [^{32}P]adenosine triphosphate, and an ECL chemiluminescent detection system were purchased from Amersham (Arlington Heights, IL). [^{32}P]Orthophosphate was purchased from Perkin-Elmer (Boston, MA). Wy-14,643 {[4-chloro-6-(2,3-xylindino)-2-pyrimidinylthio]acetic acid, CAS registry no. 50892-23-4, >98% pure} was purchased from Chemsyn Science Laboratories (Lenexa, KS). Go6976, HBDDE, Rottlerin, Staurosporine, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12-myristate 13-acetate (4α-PMA), and A23187 were purchased from Biomol (Plymouth Meeting, PA). Dexamethasone, α-MEM, D-MEM, dimethyl sulfoxide (DMSO), disodium ethylenediaminetetraacetic acid (EDTA), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), myelin basic protein, and Protein G−Sepharose were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from HyClone Laboratories (Logan, UT). Restriction endonucleases, purified protein kinase C (a mixture of multiple isozymes), in vitro coupled transcription/translation kits, and all cofactors and nucleotides necessary for PCR were obtained from Promega (Madison, WI). Plasmid purification kits were purchased from Qiagen (Chatsworth, CA). Primers for PCR were purchased from Operon (Gaithersburg, MD). Site-directed mutagenesis kits were purchased from Stratagene (La Jolla, CA). Recombinant human PKCα and PKCδ were purchased from Calbiochem (San Diego, CA). Poly-deoxyinosinic-deoxy-cytidylic acid {poly[d(I-C)]} was purchased from Roche (Basel, Switzerland). DNA retardation and Tris-glycine gels and anti-V5 and anti-V5−HRP conjugated antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-RXRα antibodies were purchased from Santa Cruz (Santa Cruz, CA). Anti-PKCα antibodies were purchased from AbCam (Cambridge, MA). Anti-PKCδ antibodies were purchased from Biomol. Liver samples from wild-type and PPARα null mice were a generous gift from J. Peters (Pennsylvania State University). Other chemicals and reagents were of the highest available grade.

Plasmids. The construction of pBK-CMV-rPPARα (pBK-PPARα) was previously described (*30*). UAS luciferase (pFR-luciferase) was purchased from BD Biosciences Clontech (Palo Alto, CA). pRL-TK (phRL-TK) and pRL-CMV (phRL-CMV) were purchased from Promega. pACO-(−581/−471)G.Luc (PPRE-luciferase), which contains the PPRE from the ACO gene upstream of the luciferase reporter gene, was supplied by J. Tugwood (Central Toxicology Laboratories, Zeneca Pharmaceuticals, Maccelsfield, U.K.) and has been previously described (*31*). The MBP−PPARα protein was generated by subcloning full-length rat PPARα cDNA (a gift from F. Gonzalez, National Cancer Institute, Bethesda, MD) into pMAL-c2X (New England Biolabs, Beverly, MA). pcDNA3.1/V5-His-rPPARα was generated by cloning full-length rat PPARα cDNA into pcDNA3.1/V5-HisA (Invitrogen). HD-Luciferase, which contains a functional PPRE from the peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase gene, was a generous gift from D. Waxman (Boston University, Boston, MA). Protein kinase C α, δ, ϵ, and β were a generous gift from Y. Ono (Kobe University, Kobe, Japan). PKCζ was a generous gift from R. Farese (University of South Florida, Tampa, FL). The pGL3-$(J_{wt})_3$TK-luciferase (pGJ3TK), a construct containing three repeats of the consensus PPARα response element (5′-AGGTCAAAGGTCA-3′), was a generous gift from R. Evans (*32*).

Cell Culture and Reporter Assays. COS-1 cells were maintained in α minimum essential medium (Sigma), 8% FBS (HyClone), and 1% penicillin and streptomycin (P/S) (Sigma). The stably transfected FaO cell line (*33*) and the wild-type FaO cell line were maintained in D-MEM, 5% FBS, and 1% P/S. Immortalized hepatocytes from PPARα null ($MuSH^{-/-}$) or wild-type ($MuSH^{+/+}$) mice were previously described (*34*) and were maintained in α-MEM, 5% FBS, 1% P/S, and 0.1 μM dexamethasone at 34 °C. All other cells were maintained at 37 °C. For transient transfections, cells were plated in 24-well dishes and transfected at approximately 80% confluency. A LipofectAMINE transient transfection procedure was employed according to the manufacturer's instructions (Life Technologies, Inc., Manassas, VA) with 2 μg of total DNA and 1.5 μL of Lipofectamine per well. Cells were transfected for either 6 or 24 h and were allowed to recover for at least 6 h in a standard medium prior to treatment. Treatments were carried out for 6 or 24 h, as listed in each figure legend. Treatments included DMSO (0.1%), Wy-14,643 (50 μM), PMA (1 μM), Go6976 (20 μM), HBDDE (100 μM), Rottlerin (6 μM), Staurosporine (163 nM), PD98059 (25 μM), and A23187 (1 μM).

Transfected cells were harvested, and the amount of luciferase was measured using the luciferase single (if pcDNA3.1-v5His/βgal was used as the transfection control) or dual luciferase (if pRL-TK or pRL-CMV were used) assay system (Promega). Luciferase samples were measured in a TD20/20 luminometer (Turner Designs, Sunnyvale, CA).

In Vitro Phosphorylation Assays. For reactions involving PKC purified mix (Promega), phosphorylation of the MBP−PPARα protein was performed by incubating 20 μg of MBP−PPARα protein in 20 mM HEPES (pH 7.4), 1 mM DTT, 10 mM $MgCl_2$, 1.7 mM $CaCl_2$, 600 μg/mL phosphatidylserine, and 0.15 mM [^{32}P]ATP (1 mCi of [^{32} P]ATP/reaction) with 1 μL of purified PKC (Promega) in a total

volume of 60 μL. For reactions involving purified recombinant PKCα or PKCδ (Calbiochem), 3.5 μg of pMal-PPARα and equimolar concentrations of each other construct were incubated in 20 mM HEPES (pH 7.4), 10 mM magnesium acetate, 1 mM DTT, 100 μM ATP, 5 μCi of [^{32}P]ATP, 100 μg/mL phosphatidylserine, and 20 μg/mL diacylglycerol (*35*). After a 30 min incubation at 30 °C, the proteins were purified with amylose resin and subjected to SDS−PAGE analysis and autoradiography.

In Vivo Labeling. COS-1 cells were transfected with pcDNA3.1/V5-His-rPPARα, corresponding single mutants, or an empty vector as described in Cell Culture and Reporter Assays. After recovery, the cells were washed with phosphate-free medium and incubated with 5 mL of phosphate-free medium with 5% dialyzed FBS for 1 h and identical medium with 2.5 mCi of [^{32}P]orthophosphate for 4 h in the presence or absence of vehicle (DMSO, 0.1%), Wy-14,643 (50 μM), PMA (1 μM), and/or Go6976, Rottlerin, A23187, and PD98059 at doses listed in the transfection method section. The cells were washed with phosphate-buffered saline, scraped from the plate, and suspended in 1 mL of NP-40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), and 1% NP-40 with phosphatase inhibitors such as 10 mM sodium pyrophosphate, 20 mM sodium molybdate, 10 mM sodium fluoride, 0.4 mM sodium vanadate, 100 μM okadaic acid, and a 1:100 dilution of mammalian protease inhibitor cocktail (Sigma catalog no. P8340)]. After a 20 min incubation at 4 °C, the cells were centrifuged at 10000*g* and 4 °C for 20 min. The supernatant was transferred to 100 μL of Protein−S sepharose pre-equilibrated with NP-40 lysis buffer and incubated on a rocker for 30 min at 4 °C. The resin was pelleted by centrifugation at 5000*g* and 4 °C for 1 min, and the supernatant was transferred to 100 μL of Protein G−Sepharose pre-equilibrated with NP-40 lysis buffer and bound to anti-V5 antibody. The samples were incubated on a rocker at 4 °C for at least 1 h, centrifuged for 1 min at 4 °C and 5000*g*, and washed three times with NP-40 lysis buffer. Proteins were eluted from the resin using 100 μL of 2× sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.05% bromophenol blue, and 5% (v/v) β-mercaptoethanol]. Proteins were resolved on a 4 to 20% Novex Tris-glycine gel, transferred to a PVDF membrane, and subjected to Western blot analysis. Afterward, the gels were analyzed by autoradiography for detection of incorporation of [^{32}P]orthophosphate into PPARα. The film of the Western blot and autoradiography was scanned and analyzed with OptiQuant (Packard, 1998). Values obtained for the level of phosphate incorporation were divided by values from the Western blot to determine the relative increase in the level of phosphate incorporation.

Co-Immunoprecipitation. For co-immunoprecipitation studies, 20 μL of ^{35}S-labeled in vitro-translated PPARα constructs was incubated with 5 μg of MBP−RXRα protein in Tris-buffered saline and 0.5% BSA for at least 1 h. The resin was washed five times with RIPA buffer (phosphate-buffered saline with 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) and eluted with a maltose solution [20 mM Tris (pH 7.4), 200 mM NaCl, 1 mM EDTA, 10 mM β-mercaptoethanol, 1 mM NaN_3, and 10 mM maltose]. Eluted samples were subjected to SDS−PAGE and autoradiography.

Gel Shift Assay. One microliter of in vitro-translated pcDNA3.1/v5-His constructs was incubated with 0.1 μg of MBP−RXRα protein in 15 μL of 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 0.5% CHAPS, and 6.83 ng/μL poly(dI/dC). The DNA probe for the peroxisome proliferator response element CNPPRE was 5′-CAAAACTAGGTCAAAGGTCA-3′. The DNA probe for the noncompeting element MeD was 5′-GTGTTAGAGGGCACAGGTCC-3′. The probe was radiolabeled with 50 μCi of [^{32}P]ATP (3000 Ci/mmol) using 10 units of polynucleotide kinase. For the supershift assays, 100 ng of anti-RXRα (Santa Cruz) or 1 μg of anti-V5 (Invitrogen) was added. Equivalent amounts of in vitro-translated protein were added to each well.

Data Analysis. All statistical analyses were performed using Minitab 12.2 (Minitab Inc., State College, PA, 1998).

RESULTS

Wy-14,643 and PMA Enhance the Activity of PPARα by Affecting PKC. To investigate the effect of phorbol esters on PPARα-mediated transcription, we transfected COS-1 cells with the HD-luciferase construct, which contains a physiological PPRE from the HD gene, and PPARα. Both Wy-14,643 and PMA induced reporter enzyme activity, while cotreatment with both chemicals caused a stronger response (Figure 1A). To determine whether PMA could affect the response at physiological levels of PPARα, we utilized the rat hepatoma cell line FaO, which had been stably transfected with PPRE-luciferase. PMA enhanced the Wy-14,643-mediated response in a dose-dependent manner (Figure 1B). A functionally inactive isoform of PMA, 4α-PMA, did not affect the Wy-14,643 response.

The effect of various PKC inhibitors on PPARα-mediated transcription was investigated. Chemical inhibitors of PKCα (Go6976 and HBDDE), PKCδ (Rottlerin), or all PKCs (Staurosporine) were used. COS-1 cells transfected with HD-luciferase exhibited significant inhibition in Wy-14,643-induced, PPRE-driven reporter activity in the presence of these inhibitors (Figure 2A). To test whether PKC isoforms themselves could enhance PPARα activity, COS-1 cells were transfected with pcDNA3.1/V5-His-rPPARα, HD-luciferase, and either PKCα, -β, -γ, -δ, -ϵ, or -ζ expression plasmids. Many of the PKC constructs increased the level of basal and Wy-14,643-induced activation of HD-luciferase (Figure 2B). In the absence of transfected PPARα, renilla luciferase was unaffected (data not shown).

Mutation of PPARα at Consensus Phosphorylation Sites Blocks PPARα Phosphorylation and Activity. To determine whether PPARα is directly phosphorylated in response to Wy-14,643 or PMA, COS-1 cells were transfected with PPARα and treated with these chemicals in the presence of [^{32}P]orthophosphate. Immunoprecipitation analysis demonstrates an increased level of phosphorylation of PPARα by both chemicals (Figure 3). To demonstrate the importance of PKC specific phosphorylation, all four PKC consensus phosphorylation sites were converted to alanine (tetraA) or glutamic acid (tetraE). COS-1 cells were transfected with pBK-PPARα tetraA or tetraE and either HD-luciferase (Figure 4A) or pGJ3TK (data not shown). In both reporter systems, the wild-type PPARα was responsive to Wy-14,643 while the mutants were nonresponsive, giving values similar

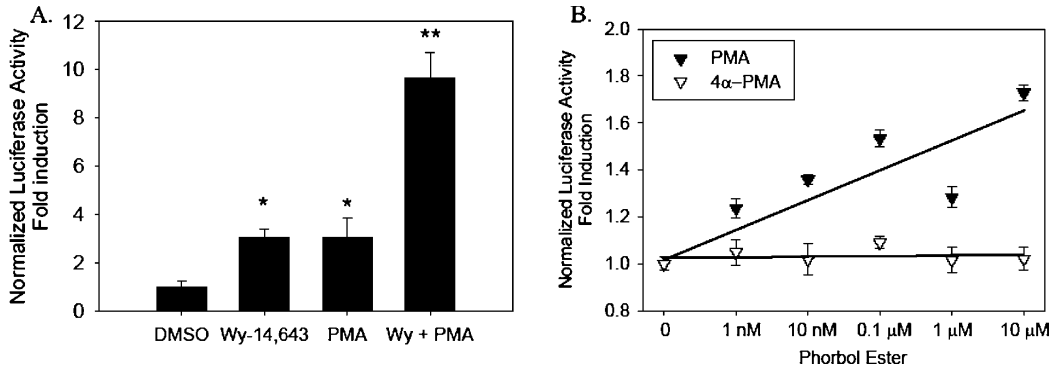

FIGURE 1: Phorbol esters enhance PPARα activity. (A) COS-1 cells were transfected with pBK-PPARα, HD-luciferase, and pcDNA3.1-v5His-LacZ. After recovery, cells were treated with DMSO (0.1%), Wy-14,643 (50 μM), PMA (1 μM), or both Wy-14,643 and PMA for 24 h. Luciferase activity was corrected for transfection efficiency as described in Experimental Procedures. Asterisks depict values that are significantly different from those of DMSO-treated cells, while two asterisks indicate that the value is significantly different from that for the single treatment ($p < 0.05$, Dunnett's t test, Minitab). (B) FaO rat hepatoma cells stably transfected with PPRE-luciferase were treated for 24 h with Wy-14,643 (50 μM) in the absence or presence of increasing concentrations of PMA or the inactive 4α-PMA. Luciferase activity was corrected for transfection efficiency as described in Experimental Procedures.

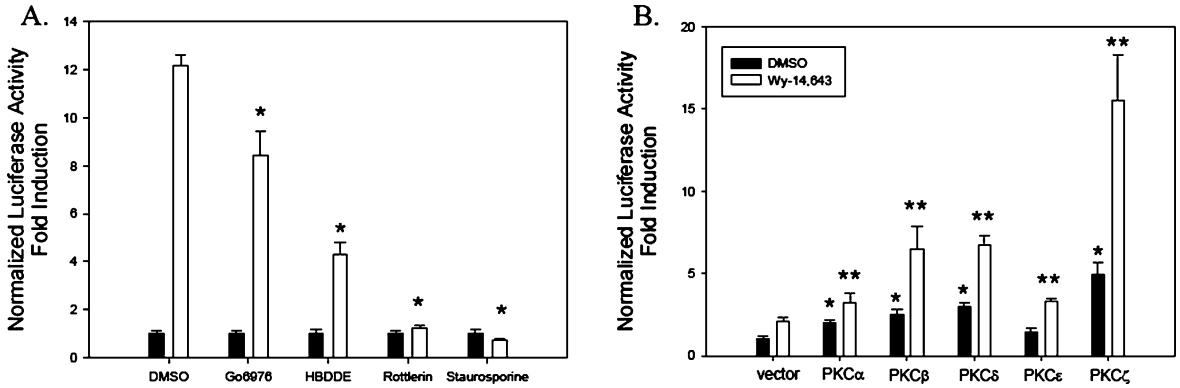

FIGURE 2: Protein kinase C modulates PPARα activity. (A) COS-1 cells were transfected with pBK-PPARα, HD-luciferase, and pRL-CMV. After recovery, cells were treated for 24 h with vehicle (DMSO, 0.1%, black bars), Wy-14,643 (50 μM, white bars), and either vehicle (DMSO, 0.1%) or a PKC inhibitor. Luciferase values were corrected for transfection efficiency and protein recovery (see Experimental Procedures). Bars are standardized to the DMSO-treated control. Asterisks depict values significantly different from those of the Wy-14,643-treated cells in the absence of inhibitor ($p < 0.05$, Dunnett's t test, Minitab). (B) COS-1 cells were transfected with pcDNA3.1/v5/His-PPARα, HD-luciferase, pcDNA3.1-v5His-lacZ, and either vector (pcDNA3.1/v5/His-empty) or the indicated PKC construct. After recovery, cells were treated with vehicle (DMSO, 0.1%, black bars) or Wy-14,643 (50 μM, white bars) for 24 h. Luciferase values were corrected for transfection efficiency and protein recovery. Single asterisks indicate a significant difference from the value for vector-transfected cells treated with DMSO, while double asterisks indicate a significant difference from the value for vector-transfected cells treated with Wy-14,643 ($p < 0.05$, Dunnett's t test, Minitab).

to that of the empty pBK vector. To determine which of these sites might be important, site-directed mutagenesis was used to mutate these sites individually in the pcDNA3.1/V5-His-rPPARα construct. Each single mutant demonstrated a significant decrease in the level of HD-luciferase activation in response to Wy-14,643 treatment, although each of the mutants retained some function (Figure 4B). Each mutant or wild-type PPARα was in vitro translated or expressed as a GFP-tagged fusion protein in vivo in COS-1 cells, and in each case, there is no evidence suggesting that translation or stability is affected by these mutations (data not shown).

Mutations at T129 and S179 Affect the Function of PPARα. Since the consensus PKC phosphorylation sites are within the regions of the protein responsible for heterodimerization and DNA binding, we examined whether these functions were affected. In vitro-translated protein from PPARα and PPARα mutant constructs were tested for their ability to be coprecipitated with pMal-RXRα using amylose resin (Figure 5). Mutations in T129 and S179 reduced the level of RXR association. Similarly, these mutations reduced the extent of association of PPARα with RXRα in a mammalian two-hybrid assay (data not shown). Next, the ability of PPARα to bind to a consensus PPARα binding site was tested using an electromobility shift assay. Of the four mutants that were tested, T129A had the strongest defect in the ability to bind DNA (Figure 6). The other mutations had little effect on DNA binding in vitro.

The Level of PPARα Phosphorylation Is Reduced by Mutation of Potential PKC Phosphorylation Sites. To investigate the potential for direct phosphorylation of PPARα by PKC, bacterially expressed MBP−PPARα protein was incubated with a mix of purified PKC isoforms in the presence of radioactive ATP and increasing concentrations of the enzyme. PPARα was directly phosphorylated by PKC in a dose-dependent fashion (Figure 7A). Because previous inhibitor studies in Figure 2 suggested a potential role for PKCα and PKCδ, we tested their ability to phosphorylate PPARα in vitro. The level of PKCα phosphorylation at T129, S142, and S179 was lower than that of the wild-type protein (Figure 8B). Phosphorylation by PKCδ was inhibited by mutating sites S110, T129, and S179 (Figure 8C). Both enzymes had reduced efficacy in phosphorylating T129A and

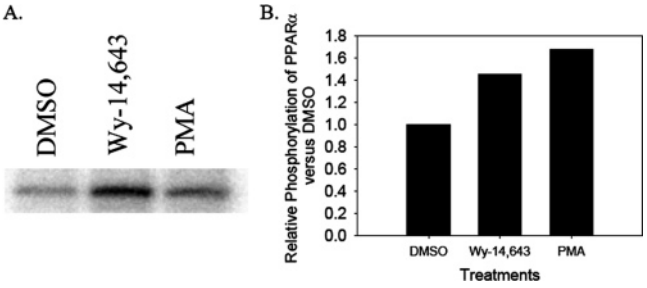

FIGURE 3: Mutation of PPARα at consensus PKC phosphorylation lowers its response to Wy-14,643. (A) COS-1 cells were transfected with either pBK-PPARα, pBK-PPARα mutated at serine 110, 142, and 179 and threonine 129 to all alanines (tetraA) or glutamic acids (tetraE), or empty vector and HD-luciferase and pRLTK. After recovery, cells were treated with vehicle (DMSO, 0.1%, black bars) or Wy-14,643 (50 μM, white bars) for 24 h. Luciferase values were corrected for transfection efficiency and protein recovery. Single asterisks indicate a significant difference from values of PPARα-transfected cells treated with DMSO, and double asterisks indicate a significant difference from PPARα-transfected cells treated with Wy-14,643. (B) COS-1 cells were transfected with either pBK-PPARα or pBK-PPARα individually mutated at each of the four sites listed in panel A (S110A, T129A, S142A, and S179A) or empty vector and HD-luciferase and pcDNA3.1-v5His-lacZ. After recovery, cells were treated with vehicle (DMSO, 0.1%, black bars) or Wy-14,643 (50 μM, white bars) for 6 h. Luciferase values were corrected for transfection efficiency and protein recovery. Asterisks represent significant differences as in panel A.

S179A mutants. Interestingly, S110A and S142A were differentially phosphorylated by the two isoforms. To determine whether these mutants are phosphorylated within cells, wild-type PPARα or PPARα mutated at one of four potential phosphorylation sites was transfected into COS-1 cells treated with PMA in the presence of [^{32}P]orthophosphate. The level of PMA-induced phosphorylation was lower in three of the mutants: T129A, S142A, and S179A (Figure 8).

DISCUSSION

Protein phosphorylation is an important mechanism by which transcription factors (reviewed in ref *36*), including nuclear receptors (reviewed in ref *37*), are regulated at the post-translational level. Of the nuclear receptors, the estrogen receptor (ER) has been the most characterized and is phosphorylated in response to estradiol treatment (*38*−*40*) or in the absence of exogenous ligand (*38*, *41*, *42*). In addition, phosphorylation can both inhibit and activate the ER, depending on the residue being affected. For example, phosphorylation at serine 104, 106, and 118 reduces estrogen activity (*40*). Phorbol ester treatment induces phosphorylation at serine 118, and MAPK-dependent phosphorylation of this serine enhanced transcriptional activation activity (*43*, *44*). In the case of human ERα, cdk7, MAPK, p90rsk1, Akt, and PKA are capable of phosphorylating this receptor and affecting transcriptional activity (*37*). The most common kinases involved in regulating nuclear receptor activity include MAPK, cdk7/cyclin H, JNKs, and PKA. Protein kinase C directly phosphorylates at least three nuclear receptors, RARα1 [S157 (*45*)], VDR [S51 (*46*)], and PPARα (ref *29* and this work).

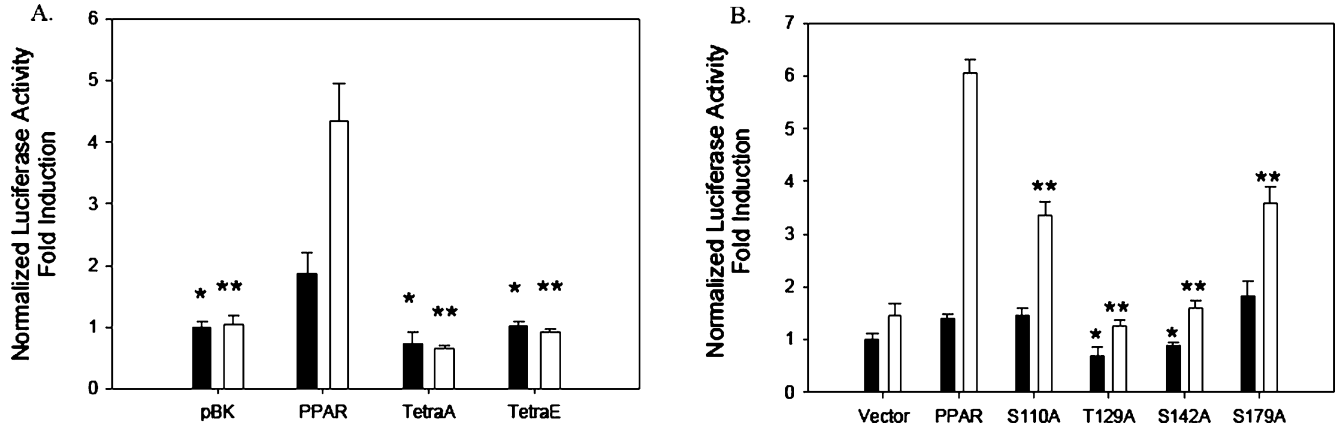

FIGURE 4: PPARα is phosphorylated in COS-1 cells following treatment with PMA or Wy-14,643. (A) COS-1 cells were transfected with pcDNA3.1-v5His-PPARα and recovered overnight. The cells were cultured in phosphate-free medium for 1 h and then cultured with [^{32}P]orthophosphate for 4 h in the presence of either DMSO (0.1%), Wy-14,643 (50 μM), or PMA (1 μM). After treatment, cells were lysed, precleared with Protein G−Sepharose, and purified with anti-V5 antibody and Protein G−Sepharose. Protein was eluted with SDS sample buffer. Proteins were separated on a 12% Tris-glycine gel, dried, and exposed to film. (B) Each band was measured using OptiQuant. Identical gels were used for Western blot analysis to determine the total amount of PPARα present in the sample, and this value was used to normalize the phosphate incorporation. The data are representative of duplicate experiments.

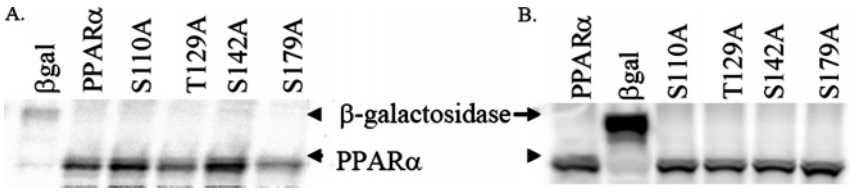

FIGURE 5: Mutation of PPARα at T129 or S179 prevents heterodimerization with RXRα. (A) pcDNA3.1-v5-His-PPARα, pcDNA3.1-v5-His-PPARα mutants, or β-galactosidase was translated in vitro using [^{35}S]methionine as described in Experimental Procedures. These proteins were incubated with MBP−RXRα protein in TBS+ and pulled down using amylose resin. Each sample was washed, eluted with SDS sample buffer, and subjected to SDS−PAGE analysis. The resulting gel was dried, and the radiolabeled proteins were visualized by autoradiography. (B) To confirm equal translation efficiency, an equal amount of each translated protein from panel A was separately resolved by SDS−PAGE. The resulting gel was dried, and the radiolabeled proteins were visualized by autoradiography.

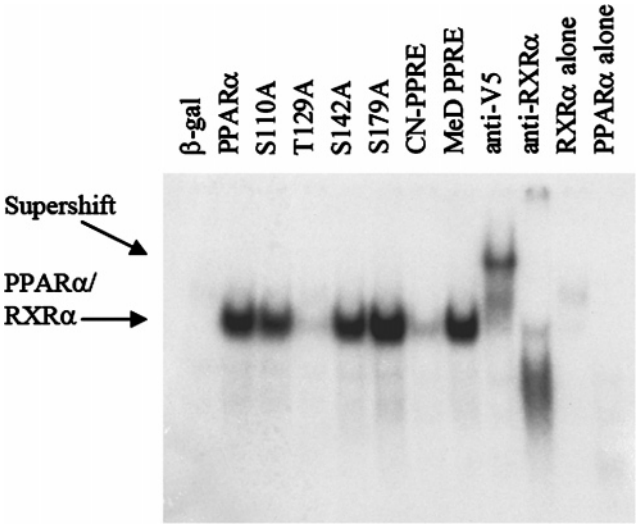

FIGURE 6: Mutation at T129 ablates the DNA binding ability of PPARα. The electromobility shift assay was used to test DNA binding of in vitro-translated pcDNA3.1-v5-His-PPARα or the -PPARα mutants and MBP−RXRα protein as described in Experimental Procedures. Cold competitor (CN-PPRE), noncompetitor (MeD PPRE), or antibodies were used to show the specificity of DNA binding.

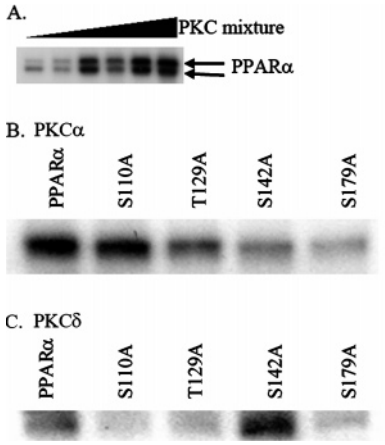

FIGURE 7: PPARα is directly phosphorylated by PKC in vitro. (A) Bacterially expressed PPARα was incubated in the presence of a PKC mixture and [^{32}P]ATP for 30 min at 30 °C as described in Experimental Procedures. Increasing amounts of enzyme were added. Afterward, the products were denatured, separated on an 8% Tris-glycine gel, dried, and exposed to film. (B) Bacterially expressed full-length PPARα, full-length PPARα mutated at one of four potential sites, or empty vector was phosphorylated in vitro as described for panel A, except that recombinant PKCα was used instead of the PKC mixture. (C) Like panel B, except that recombinant PKCδ was used as the kinase.

PPARγ, a closely related ortholog of PPARα, is regulated extensively by phosphorylation. PPARγ phosphorylation is regulated through many pathways: growth factors EGF (*47*) and PDGF (*48*), inflammatory molecules TNFα (*49*), PGF2α (*50*), and 13-HODE (*51*), and tumor promoter TPA (*47*). An aspartate mutation of serine 112 of PPARγ2 decreases ligand binding activity and slows coactivator recruitment (*52*). Although not as extensively examined, PPARα is also regulated by kinase signaling cascades. Insulin treatment of transfected CV-1 cells or rat hepatocytes increases the level of phosphorylation of PPARα (*19*) at serine 12 and 21 in a MAPK-dependent manner and augments PPARα transcriptional activity (*20*). Activation of p38 MAPK increases reporter activity in transfected CV-1 cells (*53*). In FaO rat hepatoma cells, treatment with ciprofibrate induces phosphorylation of PPARα (*54*). In the study presented here, we show that PPARα phosphorylation is enhanced by the potent ligand Wy-14643 as well. Statins modulate phosphorylation of PPARα through geranylated proteins, downregulating PPARα (*21*). Activation of PKA had the opposite effect, enhancing activation of PPARα by treatment with Wy-14,-643 (*24*).

Several observations led to the hypothesis that PKC could directly phosphorylate and affect the activity of PPARα and that cross-talk between these two pathways exists. First, as mentioned above, RARα and VDR are direct targets of PKC, and other nuclear receptors are affected by PKC activation, including the estrogen and progesterone receptors (*36*). Second, PKC inhibitors affect the ability of PPs to regulate gene expression. Several PKC inhibitors block Wy-14,643-mediated induction of fos and jun, immediate early genes critical for hepatomegaly (*25*). Similarly, blocking PKC in mice with dietary glycine inhibits Wy-14,643-mediated cell proliferation while having no effect on peroxisome proliferation (*55*, *56*). Third, PPARα activators increase PKC activity in vivo, although PPs do not serve as direct activators of PKCs (*57*). The mechanism for this activation is unclear but may involve cellular ATP or calcium levels (*58*) or cellular respiration (*59*).

PPARα itself has been shown to be regulated by particular PKC isoforms (*29*). PPARα-mediated basal and Wy-14,643-induced transcription is inhibited by the classical PKC inhibitor Ro 31-8220. In human PPARα, serine 179 and 230 were shown to be critical for the Wy-14,643-mediated induction of transcription, and mutation of these sites ablated the activity of human PPARα (*29*). Interestingly, mouse and rat PPARα proteins contain an alanine residue at position 230 in place of the serine present in the human PPARα protein. Mutation of mouse PPARα at S179 did not completely ablate transcriptional activity in our studies, suggesting a fundamental difference between rodent and human PPARα.

From the data presented herein, it is clear that the cross-talk between these two important pathways is complex and may involve many PKC isozymes and multiple sites within PPARα. Importantly, the level of PPARα phosphorylation was increased by PMA, an activator of conventional and nonconventional PKCs. In these studies, chemical inhibitors were utilized to determine which PKC isozymes may be involved. For example, at the concentrations that were used, Go6976 inhibits PKCα/β, HBDDE inhibits PKCα/γ, Rottlerin inhibits PKCδ, and Staurosporine is a broad-spectrum kinase inhibitor (*60*−*63*). The level of PMA-induced PPARα phosphorylation was decreased by Staurosporine, Rottlerin, and Go6976, indicating that PKCα, -β, and -δ are potentially involved. This is reinforced by the fact that PKCα and -δ can phosphorylate PPARα in vitro.

The increase in the degree of PPARα phosphorylation by activation of PKC is associated with an increase in PPARα transcriptional activity, as assessed with several PPRE-driven or chimeric reporter assay systems. PMA increases basal PPARα activity (that seen in the absence of an exogenous ligand) and strengthens the response seen with the potent PP Wy-14,643. All PKC chemical inhibitors were associated with little effect on basal levels of PPARα activity with a

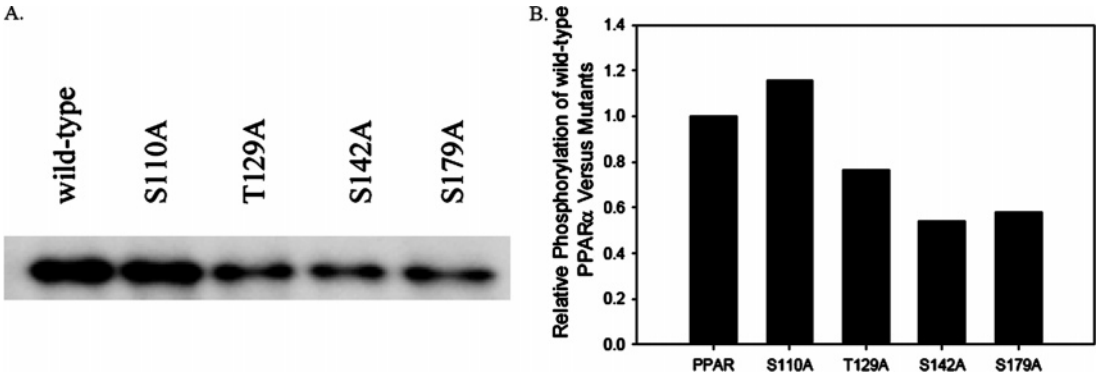

FIGURE 8: Mutation of PPARα at T129, S142, and S179 reduces the level of phosphorylation of transfected PPARα in transfected cells. (A) COS-1 cells were transfected with pcDNA3.1-v5His-PPARα or an identical construct mutated at one of four consensus PKC phosphorylation sites for 24 h and recovered overnight. The cells were cultured in phosphate-free medium for 1 h and then cultured with [^{32}P]orthophosphate for 4 h in the presence of PMA (1 μM). The cells were lysed, precleared over Protein G−Sepharose, purified over Protein G−Sepharose with anti-V5 antibody, and eluted with sample buffer. Proteins were separated on a 12% Tris-glycine gel, dried, and exposed to film. (B) Each band in panel A was assessed using OptiQuant, and values for radioactive phosphate incorporation were corrected by Western blot values.

significant decrease in PP-induced reporter activity. Conversely, overexpression of various PKC isozymes had variable effects, depending on the reporter assay that was used, for both basal and PP-induced activity. Whether this inconsistency is the result of artifacts of overexpression or if this points to a more complicated role of specific PKC isozymes in regulating PPARα activity is unclear. Nonetheless, multiple PKCs can affect PPARα activity and phosphorylation.

The PKC-dependent phosphorylation of both RARα1 and VDR occurs within the DNA binding and hinge regions (C- and D-domains, respectively), as is the case with PPARα. Similarly, PKA phosphorylates ERα at S236 within the C-domain (*64*). In these instances, phosphorylation results in a decrease in level of binding of the nuclear receptor to DNA and is an important means of transcriptional repression (*37*). Likewise, in vitro phosphorylation of PPARα with PKC inhibits RXR heterodimerization and PPRE binding. Activation of PKC within the context of gene expression has been associated with an increase in PPARα transcriptional activity, which is unlike that seen for RARα and VDR. Two possibilities for explaining these dichotomous observations exist. First, downstream targets of PKC may in turn phosphorylate PPARα at a site that results in transcriptional activation. This site may be stoichiometrically favored or is dominant in activity to that of the PKC sites within PPARα. Support for this hypothesis comes from the fact that the MAPK sites at S12 and S21 increase PPARα activity, and our studies have shown that inhibiting MAPK activity with PD98059 decreases the level of phorbol ester-induced PPARα phosphorylation. Second, PKC may be affecting activity of another protein that enhances PPARα activity such as coactivators. Once again, this effect would need to be dominant over PKC-dependent decreases in the level of PPARα binding to DNA. Numerous PPARα coactivators such as SRC-1, PGC-1, and p300/CBP are phosphorylated by MAPKs and PKA (*37*), resulting in increased activity of the transcriptional complex. Conversely, phosphorylation of p300 at S89 by PKCδ results in a decrease in histone acetyl transferase activity and a diminished activity of the complex (*65*). Coupled with the fact that the level of PPARα phosphorylation increases with PMA treatment, this observation makes the first possibility more likely than the coactivator-dependent theory of PKC activation of PPARα.

Taken together, the data presented herein show that PPARα is a direct target of PKC and that activation of this kinase is associated with improved PPARα transcriptional ability. The cross-talk between these two important pathways is complex with multiple isozymes and sites potentially playing a role.

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