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The Protein Kinase C Signaling Pathway Regulates a Molecular Switch between Transactivation and Transrepression Activity of the Peroxisome Proliferator-Activated Receptor α

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Peroxisome proliferator-activated receptor (PPAR) α is a nuclear receptor implicated in several physiological processes such as lipid and lipoprotein metabolism, glucose homeostasis, and the inflammatory response. PPARα is activated by natural fatty acids and synthetic compounds like fibrates. PPARα activity has been shown to be modulated by its phosphorylation status. PPARα is phosphorylated by kinases such as the MAPKs and cAMP-activated protein kinase A. In this report, we show that protein kinase C (PKC) inhibition impairs ligand-activated PPARα transcriptional activity. Furthermore, PKC inhibition decreases PPARα ligand-induction of its target genes including PPARα itself and carnitine palmitoyltransferase I. By contrast, PKC inhibition enhances PPARα transrepression properties as demonstrated using the fibrinogen-β gene as model system. Finally, PKC inhibition decreases PPARα phosphorylation activity of hepatocyte cell extracts. In addition, PPARα purified protein is phosphorylated *in vitro* by recombinant PKCα and βII. The replacement of serines 179 and 230 by alanine residues reduces the phosphorylation of the PPARα protein. The PPARα S179A-S230A protein displays an impaired ligand-induced transactivation activity and an enhanced transrepression activity. Altogether, our data indicate that the PKC signaling pathway acts as a molecular switch dissociating the transactivation and transrepression functions of PPARα, which involved phosphorylation of serines 179 and 230. (*Molecular Endocrinology* 18: 1906–1918, 2004)

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR α (PPARα) is a ligand-activated transcription factor of the nuclear receptor superfamily. PPARα is mainly expressed in liver, heart, kidney, muscle, and cells from the vascular wall (1, 2). PPARα transactivation function occurs after heterodimerization with the retinoic X receptor (RXR) (3) and binding to specific DNA sequences called peroxisome proliferator response element (PPRE) constituted of a direct repeat of the AGGTCA sequence separated by one or two nucleotides, named respectively DR1 and DR2 (4). Natural PPARα ligands are fatty acids (FAs), FA derivatives generated via the cyclooxygenase pathway or oxidized phospholipids from oxidized low-density lipoprotein. The fibrates, a widely used class of hypolipemic drugs, are synthetic ligands (5). PPARα is implicated in the regulation of numerous physiological functions. First, PPARα is a major controller of lipid and lipoprotein metabolism. PPARα agonist treatment increases high-density lipoprotein and decreases plasma low-density lipoprotein and very low-density lipoprotein levels (6). PPREs were identified in several genes coding for β-oxidation enzymes such as carnitine palmitoyltransferase I (CPT-I), FA binding proteins, FA transport proteins, and proteins implicated in the reverse cholesterol transport pathway (7–9). Moreover, PPARα stimulates its own expression (10). Recently, it was shown that PPARα displays antiinflammatory properties by repressing major inflammatory pathways, such as nuclear factor-κB, activator protein-1 (AP-1), and also CAAT/enhancer binding protein (C/EBPβ), which controls hepatic fibrinogen-β gene expression (11–13).

In addition to ligand regulation of PPARα activity, it was demonstrated that posttranslational modifications can also modify the activities of this nuclear receptor. For instance, PPARα is phosphorylated by the MAPKs and the protein kinase A (PKA) pathways (14–17). Several studies have revealed that this phosphorylation occurs mainly in the N-terminal A/B part of

Abbreviations: AF, Activating function; AP-1, activator protein-1; CPT-I, carnitine palmitoyltransferase I; Ct, crossing threshold; DAG, diacylglycerol; DnPKC, dominant-negative PKC; FA, fatty acid; FCS, fetal calf serum; hPPARα, human PPARα; LBD, ligand binding domain; Me_2SO, dimethylsulfoxide; PKA, protein kinase A; PKC, protein kinase C; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, retinoic X receptor; TK, thymidine kinase; wt, wild-type.

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PPARα. The consequence of this phosphorylation is an increase of the activating function (AF)-1.

The protein kinases C (PKC) are signal transducers implicated in the phosphorylation of several nuclear receptors including retinoic acid receptor α1 and vitamin D receptor (18–22). PKC isoenzymes are classified in three groups on the basis of their structure and ability to bind diacylglycerol (DAG) and calcium (Ca^{2+}) (23). The classical PKC (cPKC α, βI, βII, and γ) display a physiological requirement for DAG and need Ca^{2+} for activation. The novel PKC isotypes (nPKCδ, ε, η, μ, and θ) require DAG for activity. By contrast, the atypical PKC (aPKCζ and ι/τ) are insensitive to DAG and Ca^{2+}. Only PKCα, βI, βII, δ, ε, and ζ seem to be ubiquitous isoenzymes and are found in most tissues. PKCγ expression is largely restricted to the central nervous system and spinal cord. PKCη is strongly expressed in skin and lung. PKCθ is predominantly present in skeletal muscle and, to a lower extent, in lung, spleen, skin and brain. PKCμ has been found in numerous tissues and is strongly expressed in thymus and lung. Given the plethora of substrates, numerous functions have been attributed to PKC. Among many functions, PKCs are involved in receptor desensitization, in modulating membrane structure, in regulating transcription, in mediating immune responses and in regulating cell growth. Several PKC inhibitors have been identified and classified according to their site of interaction with the PKC protein. Inhibitors of the regulatory domain can target the DAG binding site, whereas inhibitors of the catalytic domain are directed to either the substrate site or ATP-binding site. Ro 31-8220 that belongs to the class of binsindolylmaleimides, is an ATP-binding site competitive inhibitor. Ro 31-8220 inhibits specifically classical PKC isoenzymes (24, 25).

It was recently shown that PKC control PPARα expression in rat hepatocytes (26). However, to date, no information is available whether human PPARα is regulated by the PKC pathway. Here, we have investigated the effect of PKC on the activity of PPARα in human hepatocytes. We report that inhibition of PKC activity leads to a decrease of the ligand-activated transcriptional activity of PPARα. As a consequence, the induction of PPARα target genes, such as CPT-I and PPARα itself, is abolished. By contrast, inhibition of PKC activity increases the transrepression properties of PPARα as demonstrated for fibrinogen-β promoter activity and mRNA expression. The inhibition of PKC is associated with a decrease in kinase activities in hepatic cells that phosphorylate PPARα. Finally, it is shown that PPARα protein is phosphorylated by PKCα and βII *in vitro*. The replacement of the two potential PKC phosphorylation sites at serines 179 and 230 by alanine residues in the PPARα protein reduces more than half the *in vitro* phosphorylation of PPARα by PKCα and βII. The reduction in the phosphorylation of the mutant PPARα S179A-S230A protein is associated with a loss of function for the ligand-induced transcriptional activity and a gain of function for the transrepression activity. These data demonstrate, for the first time, that the PKC pathway enhances PPARα transactivation activity and inhibits its transrepression function in human liver cells and that the serines 179 and 230 in the PPARα protein are involved in these activities.

RESULTS

The Ligand-Induced Transcriptional Activity of PPARα Is Regulated by PKC Activity

To determine whether PKC modulate the transcriptional activity of PPARα, HuH-7 cells were transfected with a reporter vector containing six copies of the J site PPRE of the apolipoprotein A-II gene promoter (J6-TK-Luc) and pSG5 or pSG5 human PPARα (hPPARα). The transfected cells were treated or not with the PKC inhibitor Ro 31-8220 for 2 h before activation with the synthetic PPARα agonists Wy 14,643 or GW7647. In the absence of Ro 31-8220, the transcriptional activity of PPARα was induced 1.5- and 2-fold by Wy 14,643 and GW7647, respectively. Pretreatment of the cells with Ro 31-8220 blocked the induction of PPARα transcriptional activity by either compounds (Fig. 1). To determine that the effect of PKC inhibition occurs via PPARα ligand binding domain (LBD), HuH-7 cells were transfected with an expression vector for a chimeric protein consisting of the DNA binding domain of the *Saccharomyces cerevisiae* Gal4 transcription factor fused to the LBD of PPARα (Gal4-PPARα-LBD) and a reporter vector containing five

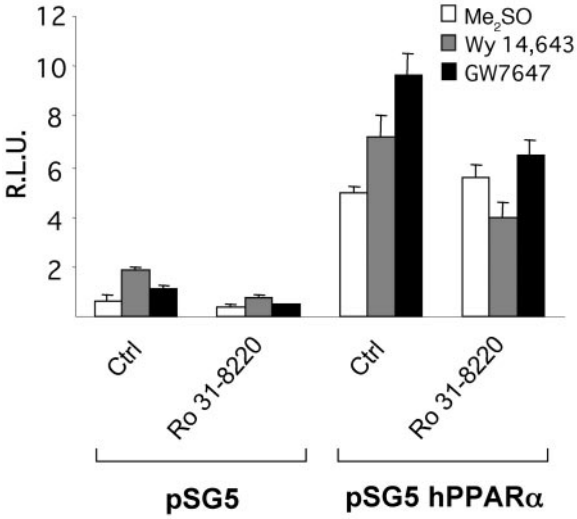

Fig. 1. PKC Inhibition Decreases Ligand-Induction of PPARα Transactivation Activity

HuH-7 cells were transfected with the pSG5hPPARα expression vector or the control pSG5 vector and the reporter vector J6-TK-Luc. After 24 h of transfection, cells were treated with or without Ro 31-8220 (1 μM) for 2 h before addition of Wy 14,643 (50 μM) or GW7647 (100 nM) for 24 h. RLU, Relative light units; Ctrl, control.

response elements for the Gal4 protein (Gal5-TK-Luc). As expected, both ligands induced the reporter activity. By contrast, treatment of cells with Ro 31-8220 decreased the ligand-induction of the reporter vector by 50% for both compounds (Fig. 2A). Furthermore, cotransfection of HuH-7 cells with an expression vector for a dominant-negative form of PKC (DnPKC) and the Gal4-PPARα-LBD expression vector decreased the activation of the Gal5-TK-Luc reporter vector by Wy 14,643 and by GW7647 (Fig. 2B). As a control, we have tested the functionality of the Ro 31-8220 compound and the DnPKC expression vector on a promoter known to be regulated by PKC. In this experiment, HuH-7 cells were cotransfected with the AP1-TK-Luc reporter construct and treated with the PKC activator phorbol myristate acetate in the presence of Ro 31-8220 or the DnPKC expression vector. These data (not shown) indicate that Ro 31-8220 compound and DnPKC expression vector inhibit efficiently PKC activity in human hepatic cells. To determine whether the PPARα N terminus may be sensitive to PKC inhibition, HuH-7 cells were transfected with an expression vector for a chimeric protein consisting of the DNA binding domain of the Gal4 transcription factor fused to N terminus of PPARα and the reporter vector Gal5-TK-Luc. The results indicate that Ro 31-8220 and DnPKC have no effect on the activity of the N-terminal part of the PPARα protein (Fig. 2, C and D). As controls, we have also tested the effect of PKC inhibition on promoter nonregulated by PPARα such as the TK promoter. As expected, no effect was observed (data not shown). These data suggest that PKC can modulate PPARα activation by its ligands in a DNA-independent manner acting via the PPARα LBD.

The PKC Signaling Pathway Influences Basal and Ligand-Induced PPARα Target Gene Expression

To demonstrate the physiological relevance of PKC inhibition on PPARα activity, the influence of PKC inhibition on basal and ligand-induced expression of PPARα target genes expression was analyzed. Therefore, HepG2 cells were pretreated with or without Ro 31-8220 for 2 h before the treatment with Wy 14,643 or GW7647 for 24 h. RNA was extracted and the expression of two well-characterized PPARα target genes, CPT-I and PPARα (7, 8, 10), was measured by quantitative PCR. As expected, treatment with both agonists alone resulted in a significant activation of CPT-I gene expression (Fig.

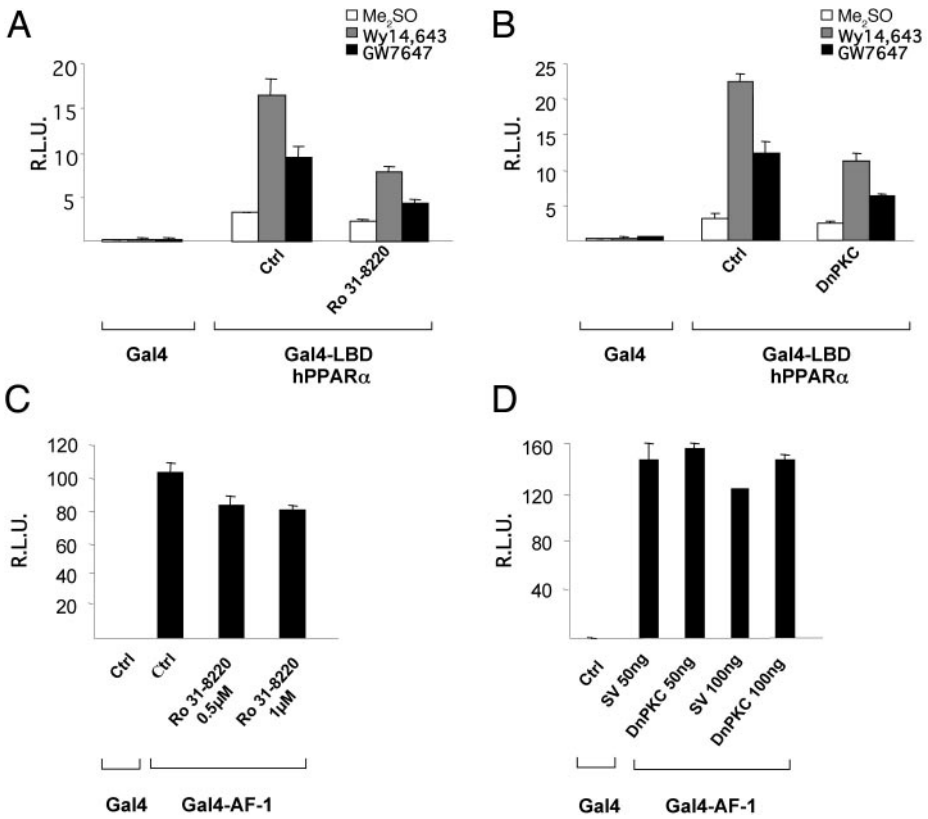

Fig. 2. Inhibition of PKC Decreases PPARα Ligand-Induction via the PPARα-LBD

HuH-7 cells were transfected with the reporter vector Gal5-TK-Luc, the Gal4-hPPARα-LBD expression vector or the control Gal4 vector and treated with or without Ro 31-8220 (1 μM) (A) or cotransfected with the DnPKC expression vector or with the control p Simian Virus 40 (pSV40) vector (B). After 24 h of transfection, cells were treated with Wy 14,643 (50 μM) or with GW7647 (100 nM) for 24 h. HuH-7 cells were transfected with the reporter vector Gal5-TK-Luc, the Gal4-AF-1 expression vector or the control Gal4 vector and treated with or without Ro 31-8220 (1 μM) (C) or cotransfected with the DnPKC expression vector or with the control pSV40 vector (D). RLU, Relative light units; Ctrl, control.

3A), whereas a smaller induction of PPARα mRNA was observed with both compounds (Fig. 3B). However, pretreatment of cells with Ro 31-8220 not only decreased basal mRNA level of PPARα and CPT-I, but also blocked the activation of these genes by both PPARα ligands (Fig. 3). These results demonstrate that modulation of PKC activity influences basal and ligand-induced PPARα target genes expression.

PKC Inhibition Increases the Repression of Fibrinogen-β Expression by PPARα

In addition to regulating gene expression via PPRE, PPARα can also repress inflammation response genes in a PPRE-independent manner. To test the effect of PKC on the transrepression activity of PPARα, the influence of Ro 31-8220 on the repression of fibrinogen-β gene expression by Wy 14,643 or GW7647 was tested in HepG2 cells. Because PKC inhibition interferes with the IL-6-induced signaling pathway, these experiments were performed on basal fibrinogen-β mRNA expression. Treatment with both Wy 14,643 and GW7647 decreased basal fibrinogen-β mRNA levels slightly (Fig. 4). Treatment with Ro 31-8220 also slightly decreased basal fibrinogen-β mRNA levels (Fig. 4). However, combined treatment with Ro 31-8220 and either PPARα ligand resulted in a stronger inhibition of fibrinogen-β mRNA levels (Fig. 4). These results suggest that PKC inhibition enhances the fibrinogen-β expression repression by PPARα.

PKC Inhibition Enhances the Transrepression Properties of PPARα

To study whether PKC inhibition modulates the transrepression properties of PPARα, the activity of the −400-bp proximal region of the human fibrinogen-β promoter (−400 hFib-β-TK-Luc), shown to be negatively regulated by PPARα, was tested in transfection experiments (12). Cotransfection with PPARα decreased fibrinogen-β promoter activity by 44% (Fig. 5), an effect that was enhanced by addition of ligand, similar as shown by Gervois *et al.* (12). Treatment of HuH-7 cells with Ro 31-8220 in the absence of the PPARα expression vector induced a slight decrease of reporter vector activity ($P = 0.0435$), whereas Wy 14,643 and GW7647 did not influence promoter activity in the absence of PPARα ($P > 0.05$) (Fig. 5). Interestingly, in the presence of PPARα, treatment with the PKC inhibitor induced a further 43% decrease of the

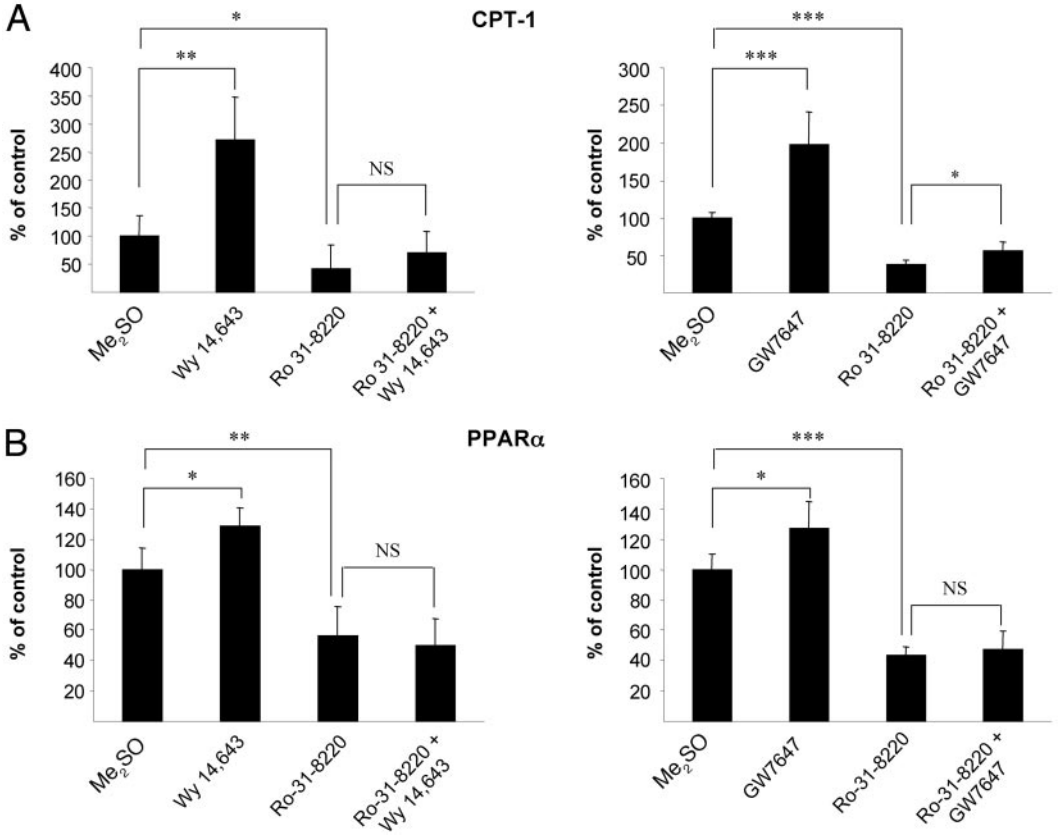

Fig. 3. Inhibition of PKC Decreases Basal and PPARα Ligand-Induced Expression of PPARα and CPT-I

HepG2 cells were treated with Ro 31-8220 (2 μM) for 2 h before activation with Wy 14,643 (50 μM) or GW7647 (100 nM) for 24 h. Then, RNA was extracted and the expression of CPT-I (A) and PPARα (B) were measured by quantitative PCR. Statistical analysis was performed using Student's *t* test. *, $0.01 < P < 0.05$; **, $0.0001 < P < 0.01$; ***, $P < 0.0001$. NS, Not significant.

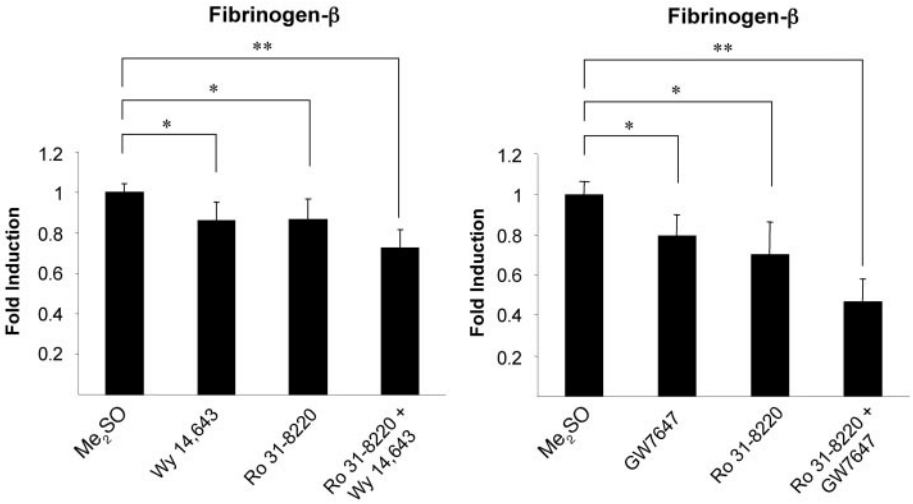

Fig. 4. Inhibition of PKC Potentiates the PPARα Ligand-Induced Repression of Fibrinogen-β mRNA Expression

HepG2 cells were treated with Ro 31-8220 (2 μM) for 2 h before addition of Wy 14,643 (50 μM) or GW7647 (100 nM) for 24 h. Then, RNA was extracted and fibrinogen-β expression was measured by quantitative PCR. Statistical analysis was performed using Student's *t* test. *, $0.01 < P < 0.05$; **, $0.0001 < P < 0.01$.

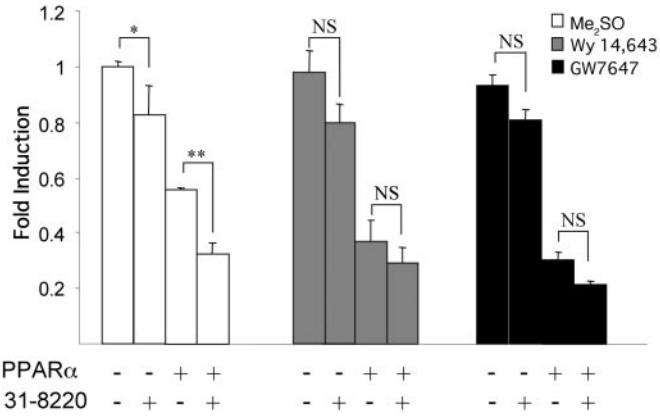

Fig. 5. Inhibition of PKC Increases the Ligand-Independent Transrepression Property of PPARα

HuH-7 cells were transfected with the reporter −400 hFib-β-TK-Luc and pSG5hPPARα expression vectors or the control pSG5 vector. After 24 h of transfection, cells were treated with Ro 31-8220 (1 μM) for 2 h before activation with Wy 14,643 (50 μM) or GW7647 (100 nM) for 24 h. Statistical analysis was performed using Student's *t* test. *, $0.01 < P < 0.05$; **, $0.0001 < P < 0.01$. NS, Not significant.

reporter activity ($P = 0.0044$) (Fig. 5). By contrast, Ro 31-8220 did not enhance fibrinogen-β promoter repression in the presence of the PPARα ligands. These results suggest that PKC inhibition increases the ligand-independent transrepression properties of PPARα, but does not influence the response to ligands (Fig. 5).

Overexpression of a DnPKC Enhances the Transrepression Properties of PPARα

To demonstrate directly that PKC inhibition modulates PPARα transrepression properties, HuH-7 cells were cotransfected with the −400 hFib-β-TK-Luc reporter vector, the DnPKC expression vector with or without the PPARα expression vector. Cotransfection of the DnPKC expression vector has no significant effect on the activity of the reporter vector ($P > 0.05$), whereas in the presence of Wy 14,643 or GW7647 a significant effect was observed ($P = 0.0342$ and $P = 0.0184$, respectively) (Fig. 6). In the presence of PPARα, a 29% decrease of the reporter activity was obtained. Interestingly, cotransfection of PPARα with the DnPKC expression vector induced a further 41% decrease of reporter activity in the absence of ligand ($P = 0.0003$) (Fig. 6). However, in the presence of PPARα, no additional inhibitory effect of the DnPKC was observed in cells treated with Wy 14,643 and GW7647 contrary to what we have observed in untreated cells. These observations suggest that PKC inhibition increases

the transrepression activity of PPARα, on the human fibrinogen-β promoter, in a ligand-independent manner.

The Replacement of Serines 179 and 230 in the PPARα Protein Results in a Protein which Has Lost Its Ligand-Induced Transcriptional Activity

We have identified by computer analysis five potential phosphorylation sites in the PPARα protein (Fig. 7A). By directed mutagenesis, we have replaced, in the PPARα protein, serine residues by alanine residues and the amino acid threonine by a valine residue individually or by pairs. We have tested all these mutant PPARα proteins for their transactivation activities in a reporter assay. HuH-7 cells were transfected with the reporter vector J6-TK-Luc and wild-type (wt) hPPARα or mutant hPPARα expression vectors. Our results show that only the double mutant PPARα S179A-S230A protein has completely lost its PPRE-dependent activation by the ligand (Fig. 7B) similarly to the

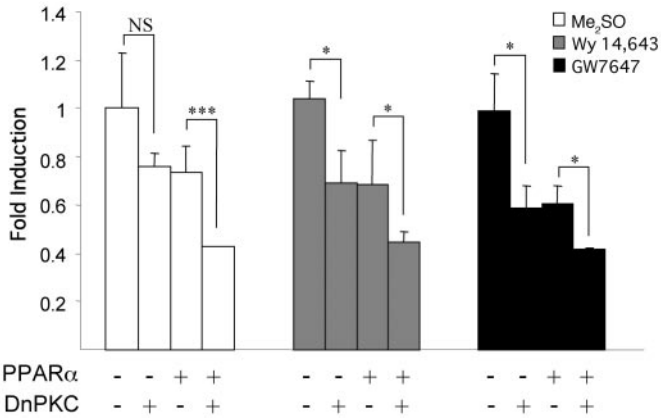

Fig. 6. Overexpression of DnPKC Increases the Transrepression Properties of PPARα

HuH-7 cells were transfected with the reporter vector −400 hFib-β-TK-Luc and pSG5hPPARα expression vectors or the control pSG5 vector in the presence of the DnPKC expression vector or the control pSV40 vector. After 24 h of transfection, cells were treated with Wy 14,643 (50 μM) or GW7647 (100 nM) for 24 h. Statistical analysis was performed using Student's *t* test. *, $0.01 < P < 0.05$; **, $0.0001 < P < 0.01$; ***, $P < 0.0001$. NS, Not significant.

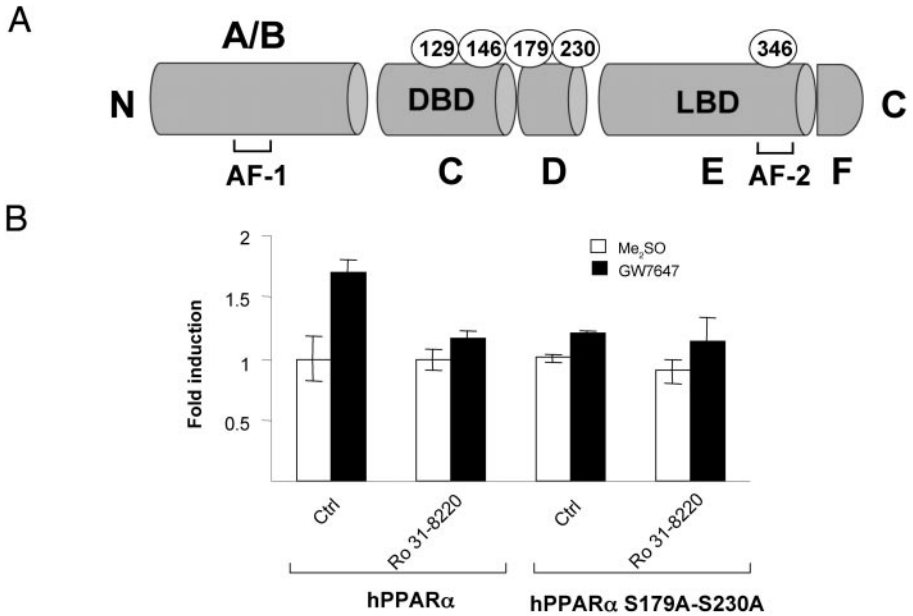

Fig. 7. The Ligand-Induced Transcriptional Activity of PPARα Is Abolished when the Serines 179 and 230 Are Replaced by Alanine Residues in the PPARα Protein

A, Localization of potential PKC phosphorylation sites in PPARα using the NetPhos 2.0 server. B, HuH-7 cells were transfected with pSG5hPPARα wt or pSG5hPPARα S179A-S230A and the reporter vector J6-TK-Luc. After 24 h of transfection, cells were treated with or without Ro 31-8220 (1 μM) for 2 h before the addition of GW7647 (100 nM) for 24 h. DBD, DNA binding domain; Ctrl, control.

effect of the PKC inhibitor, Ro 31-8220. This suggests that the serines 179 and 230 in the PPARα protein are crucial for the ligand-induced transcriptional activity of the protein and that these amino acids could be targets for PKC.

The Replacement of Serines 179 and 230 in the PPARα Protein Results in a Protein with Enhanced Transrepression Activity

To test the transrepression properties of the PPARα S179A-S230A protein compared with the PPARα wt protein, HuH-7 cells were transfected with the −400 hFib-β-TK-Luc and increasing amounts of the pSG5hPPARα wt or pSG5hPPARα S179A-S230A (Fig. 8A). The results indicate that at lower doses of the transfected PPARα expression vectors, the PPARα S179A-S230A protein exhibits enhanced transrepression activity compared with the PPARα wt protein. This gain of function is not due to a difference in the expression of wt and S179A-S230A proteins as shown in Fig. 8B. Cell extracts from transfected cells were subjected to immunoblotting using a specific PPARα antibody. The results show that the PPARα S179A-S230A protein is expressed at lower level than the wt protein arguing that the increase of the transrepression activity is not the result of a higher expression of the mutant protein. We have also tested the expression of the PKCα in these cell extracts by immunoblotting. Our results indicate that the expression of PPARα do not influence the expression of PKCα. Altogether, these results suggest that serines 179 and 230 in the PPARα protein play a role in the transrepression properties and need to be dephosphorylated for optimal repression activity of the protein.

PPARα Is Phosphorylated by PKCα and βII and the Replacement of Serines 179 and 230 by Alanine Residues in the PPARα Protein Reduces Its Phosphorylation by PKC

To demonstrate that PPARα is a target for PKC, the purified PPARα protein was incubated with HepG2 protein extracts from cells treated or not with Ro 31-8220. The results obtained show that PPARα protein is

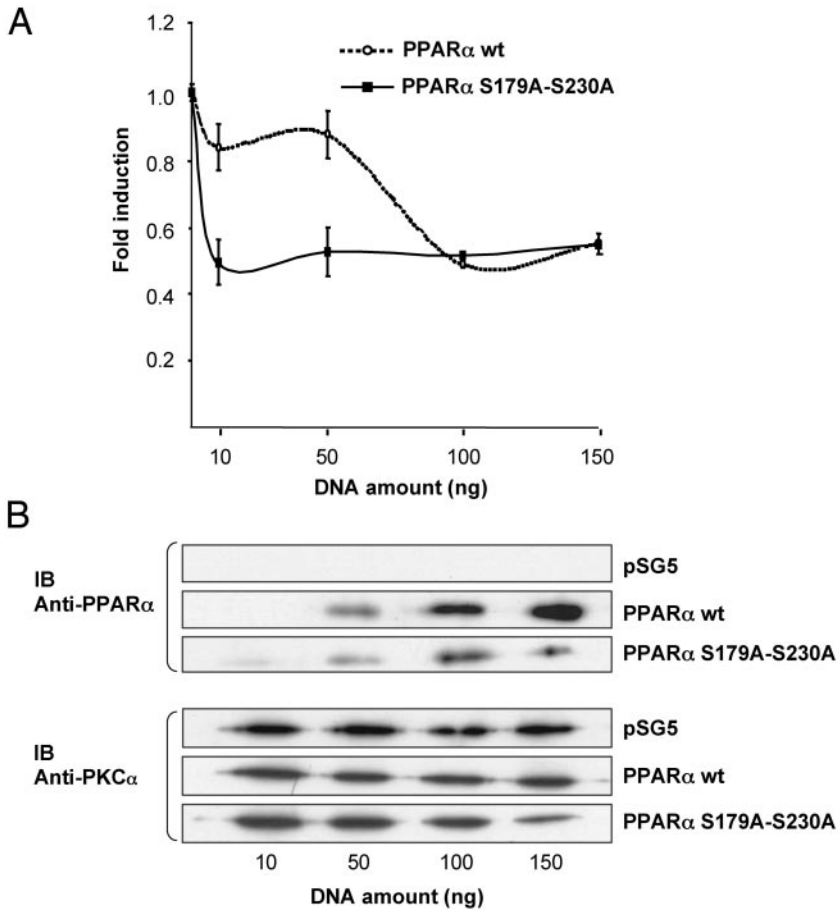

Fig. 8. The Transrepression Activity of PPARα Is Enhanced when the Serines 179 and 230 Are Replaced by Alanine Residues in the PPARα Protein

HuH-7 cells were transfected with the −400 hFib-β-TK-Luc and increasing amounts of the pSG5hPPARα wt or pSG5hPPARα S179A-S230A. After 24 h of transfection, the luciferase activity was measured in lysates of transfected cells (A). The PPARα and PKCα protein levels were measured in the same lysates by immunoblotting (IB) (B).

phosphorylated by kinases from HepG2 cell extracts and that treatment of cells with Ro 31-8220 decreases this phosphorylation (Fig. 9). This result suggests that PPARα could be directly phosphorylated by PKC. To verify this hypothesis, an *in vitro* kinase test of purified PPARα protein was performed using recombinant classical PKC isotypes that are the targets for Ro 31-8220. PPARα protein is phosphorylated by PKCα and βII (Fig. 10). To test whether the serines 179 and 230 in PPARα are targets for phosphorylation by PKC, we have produced and purified the PPARα S179A-S230A proteins from bacteria. We have realized an *in vitro* kinase test by incubating the PPARα proteins with PKCα (Fig. 10A) or PKCβII (Fig. 10B) in the presence of [γ-^{32}P]ATP. The result indicates that the PPARα S179A-S230A is 2.5 times less phosphorylated than the wt by the classical PKC. Thus, our data suggest that the serines 179 and 230 are the major phosphorylation sites for classical PKC and that these serines are crucial for the transactivation and transrepression activities of the PPARα protein.

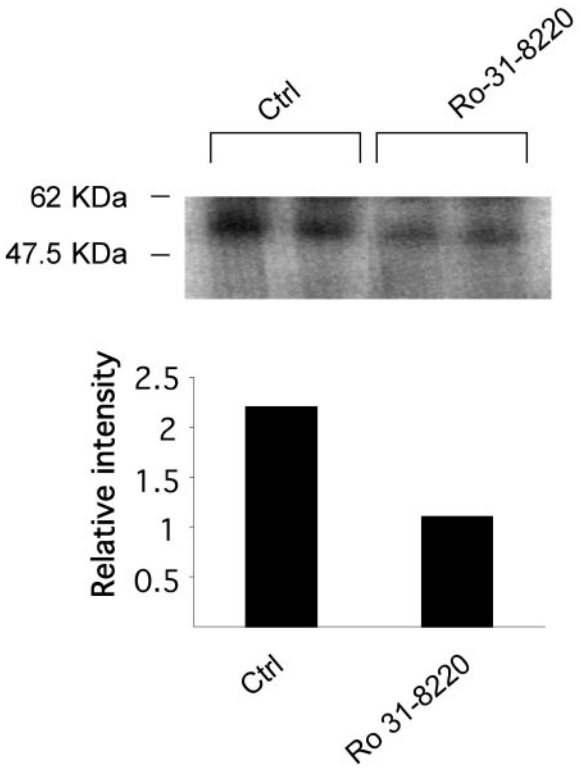

Fig. 9. PKC Inhibition Decreases PPARα Phosphorylation Activity

HepG2 cells were treated with Ro 31-8220 (2 μM) for 2 h. Then, cell extracts were incubated with purified PPARα protein and [γ-^{32}P] ATP. PPARα protein was immunoprecipitated, analyzed by SDS-PAGE and the gel was autoradiographed. Ctrl, Control.

DISCUSSION

The activity of several nuclear receptors is under control of signal transduction pathways. Like numerous other nuclear receptors (27), PPARα can be phosphorylated by different kinases including the MAPK, p38, and PKA (14–17). The transcription activation domain AF-1 in the N-terminal A/B region of PPARα harbors two consensus MAPK sites that are phosphorylated in response to insulin and both sites are necessary for the insulin-induced transcriptional activation. The effect of this posttranslational modification of PPARα is an increase of its transcriptional activity independently of the presence of ligand (AF-1 activity) (16). p38 kinase-mediated phosphorylation of PPARα results in an enhanced ligand-dependent activation due to improved cooperation with the transcriptional coactivator PGC-1 (15). *In vitro* kinase assays demonstrated that p38 kinase phosphorylates serine residues located in the N-terminal A/B domain of the PPARα protein. PKA activators stimulate PPARα activity in a ligand-independent manner. However, cotreatment with PKA activators and PPARα ligands increases this activation, suggesting that PKA stabilizes the binding of the liganded PPARα to the DNA (17). PKCs have been shown to phosphorylate and control the activity of numerous nuclear receptors (18–20, 22). However, so far a direct regulation of PPARα by the PKC pathway has not been demonstrated. In our report, we demonstrate by using a pharmacological PKC inhibitor and by overexpressing a DnPKC protein that PKC activity is necessary for optimal induction of PPARα transcriptional activity by its ligands. The reduction of PPARα activity was observed not only on full-length PPARα but also on a chimeric construct containing the Gal4 DNA binding domain fused to the C-terminal part of PPARα containing the D (hinge region), E (LBD), and F domains. These data suggest that the target sites for PKC could be localized in the C-terminal part of PPARα via which PKC influences its ligand-dependent transcriptional activity. The fact that the N-terminal part of the PPARα protein is not regulated by PKC reinforce this idea. This observation provides the first evidence of a regulation of the PPARα activity by kinases via the C-terminal part of the protein. Indeed, the previous identified phosphorylation sites are located in the N-terminal A/B part of PPARα (15, 16). Moreover, we show that PKC inhibition increases the transrepression properties of PPARα in a ligand-independent manner as determined using the fibrinogen-β gene as model. Finally, we show in an *in vitro* kinase assay that PKC inhibition, in HepG2 cells, is associated with a decrease of PPARα protein phosphorylation and that purified PPARα is phosphorylated by the classical recombinant PKC α and βII. Altogether, these results suggest that PPARα transactivation and transrepression activities are regulated by the classical PKC. PPARα contains five potential phosphorylation sites that might be phosphorylated by the classical PKC but only the muta-

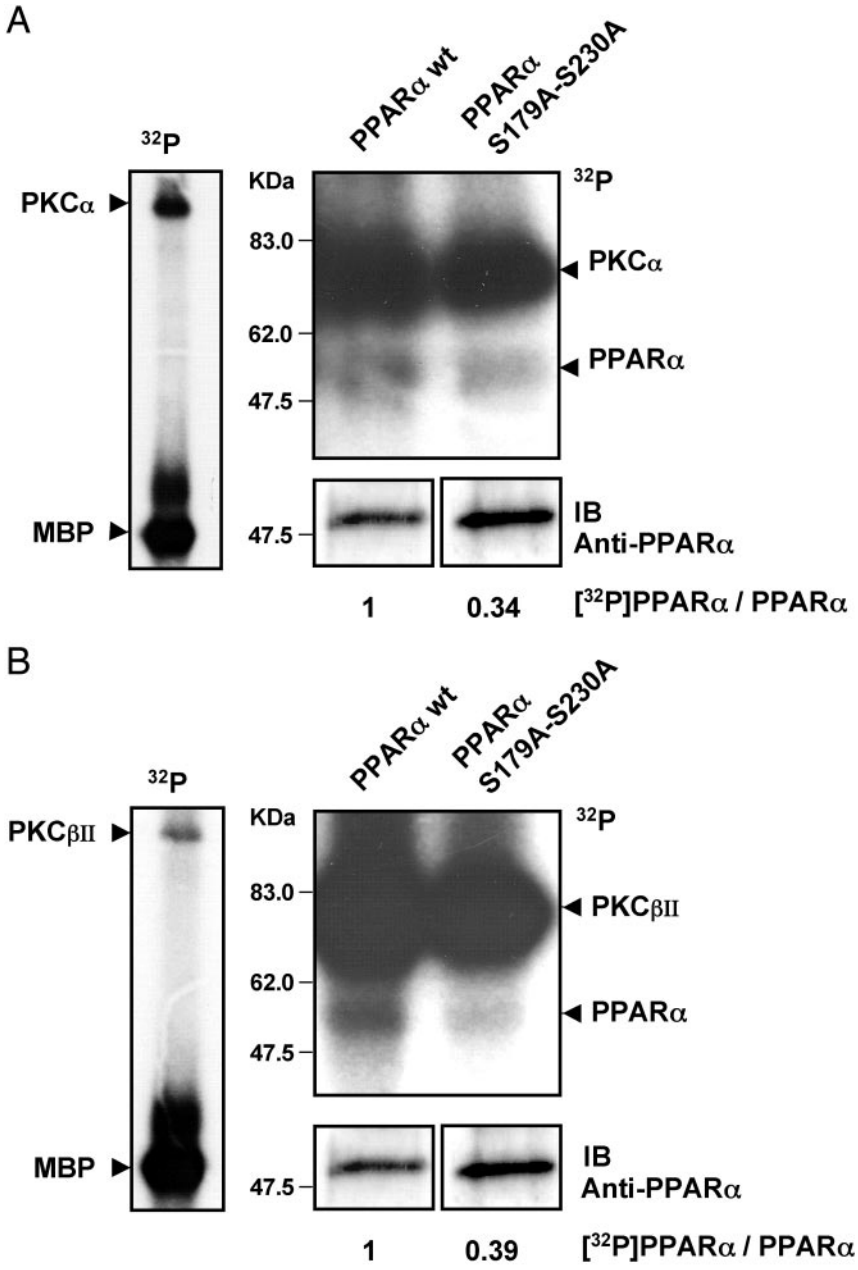

Fig. 10. PPARα Is Phosphorylated *in Vitro* by PKCα and βII and the Phosphorylation of the PPARα S179A-S230A Protein Is Reduced Compared with the wt Protein

Purified PPARα proteins wt and S179A-S230A was incubated with PKCα (A) or PKCβII (B) in the presence of [γ-^{32}P]ATP. PPARα was analyzed by SDS-PAGE and autoradiographed. The phosphorylated PPARα signals were quantified by scanning densitometry and reported to the amount of proteins used in the kinase test as determined by immunoblotting (IB anti-PPARα). The relative values are indicated. The phosphorylation of myelin basic protein protein by PKCα and PKCβII was analyzed as a control. MBP, Myelin basic protein.

tion of the two serines (serines 179 and 230) in the PPARα protein located in the domain D modulates the transactivation and transrepression activities leading to a loss of function and a gain of function of the protein, respectively. In addition, the phosphorylation of the mutant PPARα protein S179A-S230A by classical PKC is reduced compared with the wt PPARα protein. It has been shown that the domain D may be involved in the recruitment of some cofactors. The role of phosphorylation in this case could be to regulate protein-protein interactions.

Interestingly, both Ser-Thr PKC kinases and PPARα are lipid-activated signaling pathways. When activated, PKC bind to DAG and phosphatidylserine (PS) in membranes anchoring the enzyme in a subcellular compartment. Unsaturated FAs, such as oleic and

arachidonic acids, known activators of PPARα, can synergize with DAG and PS in activating the classical PKC. Saturated FAs are ineffective. To date, it is not clear whether FA and eicosanoid effects on PPARα activity are exclusively mediated through their binding to PPARα. *In vitro* assays to identify natural PPARα ligands have shown that not all FAs that activate PPARα in reporter gene assays demonstrated direct binding to PPARα (28). Thus, it can be suggested that FAs modulate PPARα activity via two synergistic pathways.

Peroxisome proliferators, especially hypolipidemic drugs such as fibrates and acyl-coenzyme A derivatives, strongly activate PKC. The identity of the PKC isozymes targeted by peroxisome proliferators in rat hepatocytes remain to be elucidated. However, such activity has been reported only to occur in rat, but not in human hepatocytes (29). Moreover, in our study, we have used two structurally different PPARα ligands to be sure that observed effects of these compounds are not due to an eventual PKC regulation. One of the downstream effects of PKC activity in rat hepatocytes is to increase PPARα gene expression (26). In human hepatoma HepG2 cells, we observed a decrease of the basal expression level of PPARα mRNA in the presence of the PKC inhibitor Ro 31-8220, which extends the results of Yaacob *et al.* (26) to human cells. This decrease of PPARα mRNA expression is associated with a decrease of the basal expression level of CPT-I showing that PKC influence the activity of PPARα in part by controlling its expression level also in human hepatocytes. Moreover, treatment with Ro 31-8220 dramatically reduced the ligand-induction of PPARα target gene expression. Thus, PKC may control PPARα activity by regulating its expression level and by controlling the response of PPARα to its ligands.

In addition to PPRE-mediated transcriptional regulation, PPARα also represses transcription of acute phase response proteins, like fibrinogen-β, in liver (30, 31). Repression of basal and IL-6-induced fibrinogen-β mRNA expression occurs via negative interference between PPARα and C/EBP-β pathway (12). To evaluate the effect of PKC inhibition on the PPARα transrepression property, we have studied basal fibrinogen-β expression because IL-6-induction of fibrinogen-β mRNA expression is dependent on PKC (data not shown and Ref. 32). Interestingly, PKC inhibition increased the PPARα ligand-induced repression of the fibrinogen-β mRNA expression, whereas treatment with either PPARα agonist or Ro 31-8220 only slightly repressed fibrinogen-β mRNA levels. To demonstrate the implication of PKC in PPARα regulation of fibrinogen-β gene expression, transient transfection experiments using the −400 proximal human fibrinogen-β promoter were performed. As previously shown (12), overexpression of PPARα decreased the activity of the human fibrinogen-β promoter and this reduction was further enhanced by the PPARα ligands. Because the inhibition of the fibrinogen-β promoter by PPARα ligands was observed in the absence of the PKC inhibitor, it appears that PKC inhibition is not required for transrepression by PPARα ligands. Indeed, PKC inhibition enhanced ligand-independent PPARα protein transrepression activity, whereas the ligand-induced repression is not influenced. These data demonstrate, for the first time, a regulation of PPARα transrepression activity by a kinase signaling pathway.

In conclusion, this study demonstrates the implication of the PKC pathway in the regulation of PPARα activity and provides, for the first time, a specific regulation mechanism allowing a switch from transactivation to transrepression activity (Fig. 11). These results suggest that under conditions of PKC inhibition, PPARα acts as a transrepression factor and displays enhanced antiinflammatory properties. In contrast, when PKC are active, PPARα switch to transactivation factor regulating PPRE-dependent genes (Fig. 11). Further investigations are necessary to identify the physiological factors controlling PPARα activity via the PKC pathway.

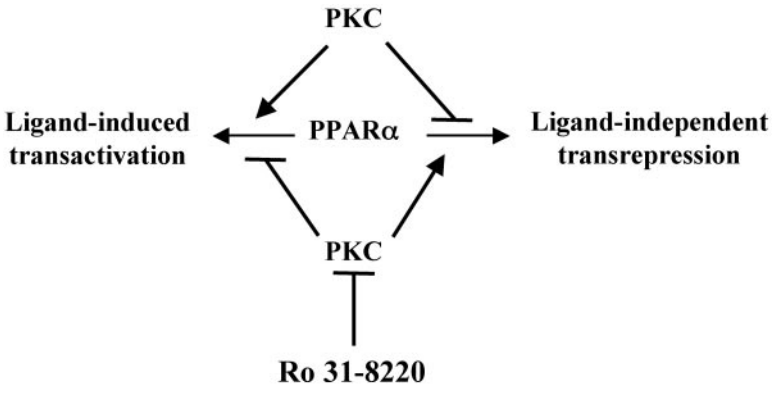

Fig. 11. Mechanism Proposed for the Regulation of PPARα Transactivation and Transrepression Activities by the PKC Pathway

Under conditions of PKC inhibition, ligand-independent PPARα transrepression properties are strongly enhanced, whereas its ligand-induced transactivation function is reduced.

MATERIALS AND METHODS

Materials

DMEM and fetal calf serum (FCS) were purchased from Invitrogen Life Technologies (Cergy-Pontoise, France), and Ultroser was obtained from BioSepra (Cergy-Saint-Christophe, France). Glutamine, gentamycine, sodium pyruvate, and nonessential amino acids were purchased from Invitrogen Life Technologies. Wy 14,643 was from Chemsyn Science Laboratories (Lenexa, KS), and GW7647 was kindly provided by Peter Brown (Glaxo-SmithKline, New York, NY). Ro 31-8220 was from Calbiochem (Meudon, France). ExGen 500 was purchased from Euromedex (Souffelweyersheim, France). HepG2 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA) and the human hepatoma cell line HuH-7 was originally established by Nakabayashi *et al.* (33). The expression vector for the DnPKC was kindly provided by Peter Parker (Imperial Cancer Research Fund, London, UK) (34). The plasmid Gal4-hPPARα was constructed by Delerive *et al.* (11). The pSG5hPPARα and J6-TK-Luc were described previously (35). The plasmid −400 hFib-β-TK-Luc was constructed by Gervois *et al.* (12). The plasmid Gal4-AF-1 was constructed by PCR-amplifying the hPPARα A/B domain (amino acids 1–101) using pSG5hPPARα as template. The AP-1-TK-Luc (Stratagene, Amsterdam, The Netherlands) contains three copies of an AP-1 enhancer. The efficiency of transfection was monitored using the control plasmid pSV-βGal or the pRL-Null vector (Promega France, Charbonnières-les-Bains, France). Purified PKC isotypes were purchased from Euromedex.

Transfection Experiments

HuH-7 cells were grown in 24-well plates in DMEM containing 10% FCS, 2 mM glutamine, 25 μg/ml gentamycine, 1 mM sodium pyruvate, and nonessential amino acids. Cells were transfected with 10 ng of J6-TK-luciferase reporter (36) and 10 ng of pSG5hPPARα expression vector or with 30 ng of Gal5-TK-Luc and 10 ng of Gal4-hPPARα-LBD using ExGen 500 according to the manufacturer's protocol. For human fibrinogen-β promoter experiments, cells were transfected with 200 ng of −400 hFib-β TK-Luc (12) and 100 ng of pSG5hPPARα expression vector using ExGen 500 as previously described. After 24 h, the cells were treated, in DMEM containing 2% Ultroser, 2 mM glutamine, and 25 μg/ml gentamycine, with PKC inhibitor for 2 h. Then 50 μM Wy 14,643, 100 nM GW 7647, or dimethylsulfoxide (Me_2SO) were subsequently added with or without PKC inhibitor for 24 h. Cell extracts were prepared and luciferase and β-galactosidase activities was measured as previously described (35).

Immunoblotting Analyses

Expression of transfected PPARα cDNA was monitored using antihuman PPARα mouse monoclonal antibody directed against the N terminus part of PPARα (amino acids 4–96) generously provided by Dr. Kodama (Perseus Proteomics, Inc., Tokyo, Japan). Cells were rinsed in ice-cold PBS and lysed in passive lysis buffer (Promega France). The proteins were subjected to SDS-PAGE electrophoresis, transferred to nitrocellulose and probed with the anti-PPARα antiserum or the anti-PKCα antiserum (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) as the primary antibody and detected using the ECL (enhanced chemiluminescence) (Amersham Pharmacia Biotech, Orsay, France).

RNA Analysis

HepG2 cells were grown in DMEM supplemented with 10% FCS, 2 mM glutamine, 25 μg/ml gentamycine, 1 mM sodium pyruvate, and nonessential amino acids. Cells were treated, in DMEM containing 2% Ultroser, 2 mM glutamine, and 25 μg/ml gentamycine, with 2 μM of Ro 31-8220 for 2 h. Then, 50 μM Wy 14,643, 100 nM GW 7647, or Me_2SO were added with or without PKC inhibitor for 24 h. RNA extraction was performed using TRIzol (Invitrogen) reagent according to the manufacturer's protocol. Reverse transcription was realized with 1 μg RNA using random hexamer primers and Moloney murine leukemia virus-reverse transcriptase (Invitrogen Life Technologies). RNA levels were measured by quantitative PCR using the Brilliant SYBR Green QPCR Master Mix (Stratagene) on the MX 4000 detection system (Stratagene) and as primers for human CPT-I , 5′-ACA GTC GGT GAG GCC TCT TAT GAA and 5′-TCT TGC TGC CTG AAT GTG AGT TGG, for human fibrinogen-β, 5′-CTG AAA GAC CTG TGG CAA AA and 5′-TCC AGG ATT GAA CGA AGC ACA CG and for hPPARα, 5′-GGT GGA CAC GGA AAG CCC AC and 5′-GGA CCA CAG GAT AAG TCA CC. As control, cyclophilin mRNA was measured using 5′-GCATAC GGG TCC TGG CAT CTT GTC C sense and 5′-ATG GTG ATC TTC TTG CTG GTC TTG C antisense primers. For each primer pair, the linearity of the reaction was confirmed to have a correlation coefficient of at least 0.98 and a PCR efficiency of 100% over the detection area by measuring a 10-fold dilution curve with cDNA isolated from HepG2 cells. Samples were analyzed in duplicate in two independent runs. Crossing threshold (Ct) values, defined as the cycle number in which the detected fluorescence exceeds the threshold value, were determined for CPT-I and fibrinogen-β and normalized to the Ct of cyclophilin using the following equation: relative values $= 2^{-(\text{ct target gene} - \text{ct cyclophilin})}$

Kinase Assay

Production of the purified PPARα protein was performed with the Impact T7 system from Ozyme (Montigny le bretonneux, France) using the pTyb12 expression vector. PPARα protein was expressed in bacteria [*Escherichia coli* BL21(DE3)pLysS] as a fusion protein with the self-cleavage intein protein and a chitin binding domain. The expression of the fusion protein was induced for 3 h with isopropyl-β-D-thiogalactopyranoside 1 mM at room temperature. Bacteria were harvested and lysed in a lysis buffer [50 mM Tris HCl (pH 8), 150 mM NaCl, 0.1 mM EDTA, and 0.1% Triton X-100]. After centrifugation of the lysate, the supernatant was loaded on the chitin column. After washes, the column was equilibrated in cleavage buffer [50 mM Tris HCl (pH 8), 150 mM NaCl, 0.1 mM EDTA, and 30 mM dithiothreitol] and left at 4 C for 48 h. In the presence of dithiothreitol, the intein undergoes specific self-cleavage that releases PPARα resulting in a single column purification of the PPARα protein. After verification of the purified PPARα protein functionality by EMSAs (data not shown), this protein was used in a kinase assay. For this test, HepG2 cells were treated for 2 h with 2 μM Ro 31-8220 in DMEM containing 2% Ultroser, 2 mM glutamine, and 25 μg/ml gentamycine. Cells were lysed in a kinase buffer [25 mM HEPES (pH 7.5), 100 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM EGTA, 0.25 mM EDTA, 0.1% Nonidet P-40, and 10 mM NaF]. Purified PPARα protein (500 ng) was incubated with cell extracts (5 μg) in the presence of [γ-^{32}P] ATP (5 μCi) for 30 min at 30 C in reaction buffer [50 mM HEPES (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 20 μM ATP, 500 μM EGTA, 25 mM β-glycerophosphate, and 1 mM orthovanadate]. PPARα protein was immunoprecipitated with PPARα antibodies (Santa Cruz Biotechnology). After four washes with 1 ml of RIPA (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 0.5% Nonidet P-40, 0.5% desoxycholate, 0.1% sodium dodecyl sulfate), 1 ml RIPA-1 M NaCl, 1 ml RIPA-TNE (vol/vol) [TNE, 10 mM Tris HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA], 1 ml TNE, samples were boiled in Laemmli buffer and loaded on 10% SDS-PAGE. After the run, the gel was treated for 30 min in neutral fixator (40% methanol, 3.5% paraformaldehyde) where all phosphoproteins are preserved, washed with 10% EtOH, 4% glycerol and dried. The gel was autoradiographed.

Oligonucleotide-Directed Mutagenesis

The cDNA encoding mutant PPARα proteins were generated using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Point mutations were introduced in the hPPARα cDNA using *Pfu* DNA polymerase, double-stranded DNA plasmid and two synthetic complementary oligonucleotide primers containing the desired mutation. The mutated cDNAs have been completely sequenced.

In Vitro Phosphorylation Test

Phosphorylation test of the PPARα protein was performed by using purified PKCα,and βII (Euromedex) according to the manufacturer's protocol. PPARα protein (~200 ng) was incubated with 10 ng of purified PKC, the lipid activator and [γ-^{32}P] ATP in the appropriate buffer. After 30 min at 30 C, the labeled proteins were analyzed by SDS-PAGE. After the run, the gel was treated 30 min in neutral fixator (40% methanol, 3.5% paraformaldehyde), washed with 10% EtOH, 4% glycerol and dried. The gel was autoradiographed. Myelin basic protein (Euromedex) was used as a control substrate for phosphorylation by PKCα and PKCβII.

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