Biochemical Pharmacology, Vol. 58, pp. 1001–1008, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00182-3

Phosphorylation of Peroxisome Proliferator-Activated Receptor α in Rat Fao Cells and Stimulation by Ciprofibrate

Patricia Passilly, Hervé Schohn,‡ Brigitte Jannin,* Mustapha Cherkaoui Malki,* Daniel Boscoboinik,* Michel Dauça‡ and Norbert Latruffe*‖*

*Université de Bourgogne, Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Gabriel, 21000 Dijon and ‡Université Henri Poincaré Nancy I, Laboratoire de Biologie Cellulaire du Développement, Faculté des Sciences, 54506 Vandoeuvre-les-Nancy, France

ABSTRACT. The basic mechanism(s) by which peroxisome proliferators activate peroxisome proliferator-activated receptors (PPARs) is (are) not yet fully understood. Given the diversity of peroxisome proliferators, several hypotheses of activation have been proposed. Among them is the notion that peroxisome proliferators could activate PPARs by changing their phosphorylation status. In fact, it is well known that several members of the nuclear hormone receptor superfamily are regulated by phosphorylation. In this report, we show that the rat Fao hepatic-derived cell line, known to respond to peroxisome proliferators, exhibited a high content of PPARα. Alkaline phosphatase treatment of Fao cell lysate as well as immunoprecipitation of PPARα from cells prelabeled with [^{32}P] orthophosphate clearly showed that PPARα is indeed a phosphoprotein *in vivo*. Moreover, treatment of rat Fao cells with ciprofibrate, a peroxisome proliferator, increased the phosphorylation level of the PPARα. In addition, treatment of Fao cells with phosphatase inhibitors (okadaic acid and sodium orthovanadate) decreased the activity of ciprofibrate-induced peroxisomal acyl-coenzyme A oxidase, an enzyme encoded by a PPARα target gene. Our results suggest that the gene expression controlled by peroxisome proliferators could be mediated in part by a modulation of the PPARα effect via a modification of the phosphorylation level of this receptor. BIOCHEM PHARMACOL **58**;6:1001–1008, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. PPARα phosphorylation; rat Fao cells; ciprofibrate stimulation

PPARs¶ are members of the nuclear hormone receptor superfamily [1]. So far, four different PPARs have been identified (for a review see [2]). These transcription factors were subsequently shown to be activated by a variety of molecules such as hypolipidaemic drugs, long-chain fatty acids (for PPARα), and prostaglandins or thiazolidinediones (for PPARγ) and to stimulate the gene expression of numerous enzymes involved in lipid metabolism (for a review see [3]). It was shown that many compounds or metabolites are able to activate PPARs via a classical ligand–receptor binding [4, 5]. Moreover, the basic mechanism(s) by which PPARs are activated may also involve phosphorylation–dephosphorylation processes. Indeed, it has been demonstrated that the activity of several members of the nuclear hormone receptor superfamily are regulated by phosphorylation (for a review see [6]). Recently, several authors have shown that PPARγ, which is essential for adipocyte differentiation, is phosphorylated *in vivo* [7–10]. Shalev *et al.* [11] demonstrated that PPARα is also phosphorylated in adipocytes. On the other hand, it is known that some peroxisome proliferators change the phosphorylation level of proteins by modifying the activity of protein kinases [12–17]. We have previously shown that ciprofibrate increases the phosphorylation status of phosphoproteins in the rat Fao hepatic-derived cell line [18]. Since peroxisome proliferators such as hypolipidaemic drugs stimulate the gene expression of numerous enzymes involved in lipid metabolism and as they activate PPARα [19], it was important to examine whether PPARα was phosphorylated in Fao cells and to determine whether peroxisome proliferators were able or not to modulate the phosphorylation status of PPARα. The present study shows for the first time that PPARα is phosphorylated in rat Fao hepatic-derived cells and that its phosphorylation level is increased by ciprofibrate, a well-known peroxisome proliferator. This change in the phosphorylation level of PPARα could modulate the transcriptional control of this receptor by hypolipidaemic drugs, since acyl-CoA oxidase, a peroxisomal marker, is affected by protein phosphatase inhibitors.

‖ Corresponding author: Prof. N. Latruffe, Université de Bourgogne, Laboratoire de Biologie Moléculaire et Cellulaire, Faculté des Sciences Gabriel, 6 Boulevard Gabriel, 21000 Dijon, France. Tel. (33) 3 80 39 62 37; FAX (33) 3 80 39 62 50; E-mail: latruffe@u-bourgogne.fr

¶ *Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; PPAR, peroxisome proliferator-activated receptor; mPPAR, mouse PPAR; RIPA buffer, radioimmune precipitation buffer; ECL, enhanced chemiluminescence; and acyl-CoA, acyl-coenzyme A.

Received 23 April 1998; accepted 23 March 1999.

MATERIALS AND METHODS

Chemicals

Ciprofibrate (2-[4-(2,2-dichlorocyclopropyl)-phenoxy]-2-methylpropanoic acid) was a gift from Sanofi Winthrop. Wy-14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio]-acetic acid) was kindly provided by Wyeth Laboratories. Protease inhibitors, okadaic acid, sodium orthovanadate, alkaline phosphatase (from bovine intestinal mucosa, type XXX-L), and protein A (Sepharose CL-4B) were purchased from Sigma Chemical Co. The ECL detection system and the [^{32}P] orthophosphate (specific activity 10 mCi/mL) and [^{35}S] methionine–cysteine (specific activity 1000 Ci/mL) solutions were purchased from Amersham. Ham's F12 medium, DMEM, and fetal bovine serum were provided by GIBCO-BRL (Life Technologies).

Cell Culture

The Fao cell line, a subclone of the clonal line H4IIEC3 [20] derived from the Reuber H35 rat hepatoma [21], was kindly supplied by J. Deschatrette (INSERM, Paris). It was routinely cultured in Ham F12/DMEM medium (1v:1v) supplemented with 5% fetal bovine serum at 37° in a humidified atmosphere of 10% CO_2/90% air. The media were supplemented with 125 IU/mL specillin G and 125 μg/mL streptomycin. Cells were treated with 500 μM ciprofibrate or 50 μM Wy-14,643 added from a DMSO solution to fresh medium (0.1% final concentration of solvent). Control cells were cultured in medium containing only 0.1% (v/v) DMSO.

Production of Anti-PPAR Antibodies

Two anti-PPAR antibodies were used: an anti-PPARα antibody specific for the PPARα isoform (antibody specificity is described in the Results) and an anti-PPAR antibody which recognizes the three PPAR subtypes as previously reported [22]. The anti-PPARα antibody was raised against the amino acid sequence 45SSGSFGFTEY QY56-COOH mapping at the amino terminus of the human PPARα. This sequence differs from that of the rat PPARα by three amino acids. Several experiments were performed with a rabbit polyclonal antibody raised against the 16 carboxy-terminal amino acids of the rat PPARα. As this sequence is well conserved in rPPARβ and rPPARγ, respectively, the polyclonal antibody (called anti-PPAR in the text) recognizes the three PPAR subtypes as previously reported [22]. The synthetic peptides were coupled to the keyhole limpet hemocyanin used as a carrier according to the carbodiimide method [23] for the amino terminal peptide, and to the glutaraldehyde procedure [24] for the carboxy-terminal peptide. Polyclonal antibodies against each peptide–protein conjugate were raised by subcutaneous injections into rabbits using standard procedures.

Immunoblotting with Anti-PPARα Antibody

After incubation with ciprofibrate or solvent, cells were washed three times with cold Tris-buffered saline (TBS) and lysed in RIPA buffer (10 mM Tris–HCl pH 7.2, 150 mM NaCl, 0.5% (v/v) Nonidet P40, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS, 2 mM EDTA, and 50 mM NaF) supplemented with leupeptin (5 μg/mL), antipain (5 μg/mL), chymostatin (5 μg/mL), aprotinin (5 μg/mL), E64 (1 mg/L), and sodium vanadate (5 mM). The cell lysate was forced through a 25-gauge syringe (15 times) and centrifuged for 15 min at 16,000 *g*. After determination of protein concentration of the samples using a BioRad kit [25], an aliquot of protein (30 μg) was applied to a 10% SDS-PAGE. After transfer onto an Immobilon P membrane (Sigma), blots were blocked for one hour with 5% (w/v) non-fat milk powder in TBST (Tris–HCl pH 8, 0.138 M NaCl, 0.05 % Tween 20) and then incubated for 1 hr with the anti-PPARα antibody diluted to 1:500 with TBST and supplemented with 1% (w/v) BSA. The blots were washed three times for 10 min each in TBST containing 0.5 M NaCl and were incubated with peroxidase-labeled anti-rabbit immunoglobulin antibody (Amersham ECL kit) at a 1:5000 dilution for 45 min. The bands labeled with the antibody were visualized using an ECL Western blotting detection kit by exposure to x-ray films (RPN2103H from Amersham).

Alkaline Phosphatase Treatment of Cell Lysates

Alkaline phosphatase treatment was performed as previously described [26]. Proteins (30 μg) extracted from Fao cells were treated or not with 6 units of bovine intestine alkaline phosphatase (Sigma) in 100 mM Tris buffer, pH 8, containing 1 mM $MgCl_2$ and protease inhibitors in a final volume of 15 μL for 1 hr at 37°. The reaction was stopped by addition of 15 μL of 2-fold concentrated protein sample buffer [27]. Samples were subjected to 10% SDS-PAGE and PPARα was analyzed by immunoblotting as described previously.

^{35}S or ^{32}P In Vivo *Labeling of PPARα*

The *in vivo* labeling of PPARα in Fao cells was performed as reported by Passilly *et al.* [18]. The Fao cell line was grown in 25-cm^2 flasks to 80–90%. Cells were then cultured overnight (13–15 hr) in a methionine-and cysteine-free DMEM for the ^{35}S *in vivo* labeling, with phosphate-free DMEM for the ^{32}P *in vivo* labeling. Then, cell monolayers (approx. 5×10^6 cells) were washed three times with 1.5 mL methionine/cysteine-free DMEM or phosphate-free DMEM and labeled with either 200 μCi/mL [^{35}S] methionine–cysteine or with 30 μCi of [^{32}P] orthophosphate, at 37° for 2 hr. Subsequently, 500 μM ciprofibrate or 50 μM Wy-14,643 was added and incubated for 4 hr. Cells were then washed six times with cold Tris-buffered saline (TBS) solution and harvested using the RIPA lysis buffer.

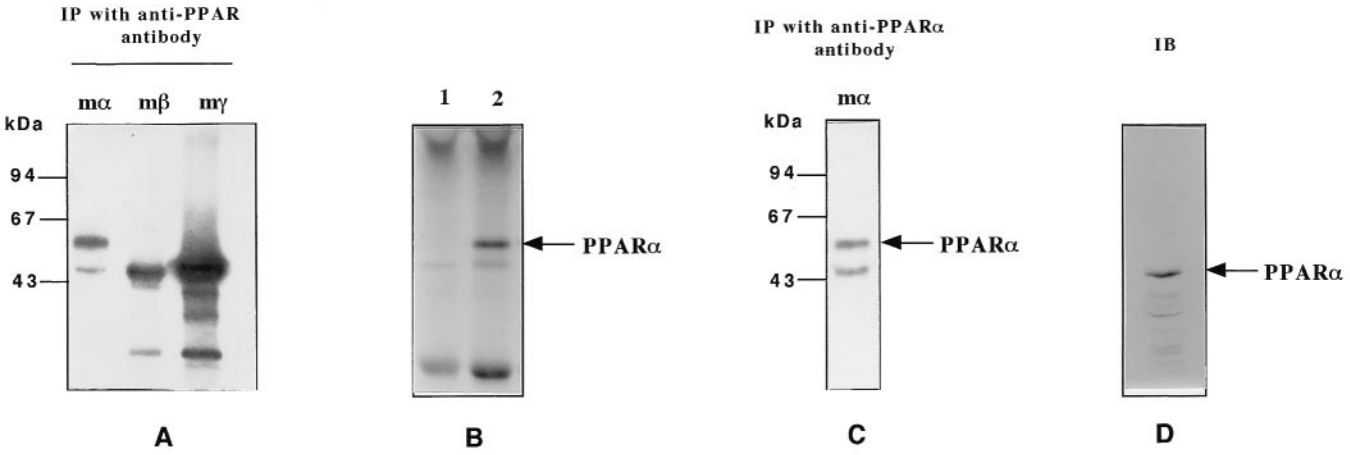

FIG. 1. Characterization of the anti-PPARα antibody and evidence for the high expression of PPARα in the rat Fao hepatic-derived cell line. (A) Recognition of mPPARα, β, and γ by the anti-PPAR antibody. Immunoprecipitation assay (IP) of *in vitro*-translated mouse [^{35}S] PPARα, β, and γ followed by SDS-PAGE (10%). The gel was processed for fluorography and subjected to autoradiography. mPPARα (mα); mPPARβ (mβ); mPPARγ (mγ). (B) *In vitro*-translated [^{35}S] mPPARα was submitted to SDS-PAGE (10%) (lane 2). The gel was processed for fluorography and subjected to autoradiography. Unprogramed lysate was used as a control (lane 1). (C) Immunoprecipitation assay (IP) of *in vitro*-translated mouse [^{35}S]mPPARα with the anti-PPARα antibody followed by SDS-PAGE (10%). The gel was processed for fluorography and subjected to autoradiography. mPPARα: mα. (D) Immunoblotting of PPARα from Fao cells. Fao cell lysates were obtained as described in Materials and Methods. Proteins (30 μg) were separated by SDS-PAGE (10%) and immunoblotted (IB) with the anti-PPARα antibody.

Immunoprecipitation of ^{35}S- or ^{32}P-Labeled PPARα

Lysates from labeled cells were passed through a 25-gauge syringe (15 times) and centrifuged for 15 min at 16,000 *g*. The supernatant was collected and protein concentrations were determined as above before immunoprecipitation assays. These assays were performed by incubating the cell lysates with the anti-PPARα antibody for 1 hr at 4°. Then, 10 mg of protein A Sepharose beads (Sigma) were added for 3 hr under continuous rotation at 4° to collect the immune complexes. Immunoprecipitates were then collected by centrifugation and washed three times with cold RIPA lysis buffer supplemented with 0.5% (w/v) BSA and three more times with cold RIPA lysis buffer alone. The washed beads were then resuspended in Laemmli sample buffer and the samples were boiled for 5 min. Immunoprecipitated proteins were then run on SDS-10% acrylamide gels. Labeled proteins were revealed by autoradiography with Kodak film (XO-Mat from Kodak). Autoradiograms were scanned with a Shimadzu densitometer (CS9000).

In Vitro *Transcription–Translation of PPAR cDNA Clones*

In vitro translation of mouse PPARαpSG5, PPARβpSG5, and PPARγpSG5 was performed using a transcription/translation system (TnT™ Coupled Reticulocyte Lysate System, Promega) as recommended by the manufacturer. To obtain [^{35}S]-labeled PPAR proteins, [^{35}S] methionine was added to the TnT™ kit.

Fluorometric Assay of Peroxisomal Palmitoyl-CoA Oxidase Activity

In order to investigate the biological role of induced PPARα phosphorylation in Fao cells, we undertook to look at the expression of acyl-CoA oxidase, an enzyme encoded by a PPARα target gene. To this end, the activity of palmitoyl-CoA oxidase was measured in Fao cells treated for 24 hr with or without 500 μM ciprofibrate in the presence or absence of phosphatase inhibitors (50 nM okadaic acid together with 10 μM sodium orthovanadate). This activity was assayed by the fluorometric measurement of the H_2O_2 (hydrogen peroxide) produced, adapted to cell line samples [28] according to the method previously reported [29]. H_2O_2 (hydrogen peroxide) reacts with homovanillic acid in the presence of peroxidase to give a fluorescent dimer. Experiments were carried out as described above; however, following centrifugation, the cells were resuspended in MOPS (3-(*N*-morpholino)propanesulfonic acid) buffer and sonicated 3 × 10 sec on ice. The cell homogenate was stored at −70° until analysis.

RESULTS

Expression of PPARα in the Rat Fao Hepatic-Derived Cell Line

As a first step in the expression analysis of the PPARα isoform in the rat hepatic-derived cell line, we verified that the signals obtained were specific to this nuclear receptor. Two polyclonal antibodies, raised against the NH_2 and COOH terminus of the PPARα isoform, respectively, were used in this study. As previously reported [22], the anti-

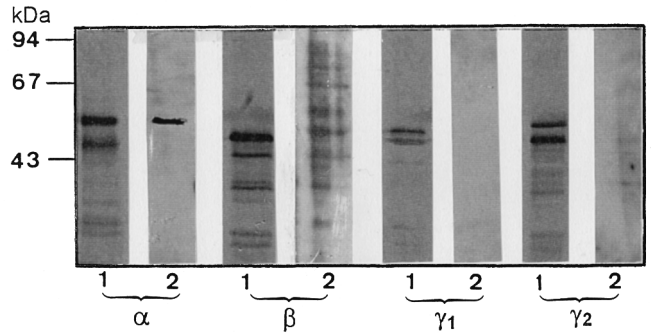

FIG. 2. Specificity of anti-PPARα antibody. Mouse PPARα pSG5, PPARβ pSG5, PPARγ$_1$ pSG5, and human PPARγ$_2$ pSG5 plasmids were *in vitro*-translated using reticulocyte lysate and [^{35}S] methionine. Translated products were submitted to SDS-PAGE (10%). The gels were either subjected to autoradiography (lane 1) or processed by Western blotting and ECL (lane 2) according to the manufacturer's protocol (Boehringer Mannheim) using the anti-PPARα antibody (diluted 1:500).

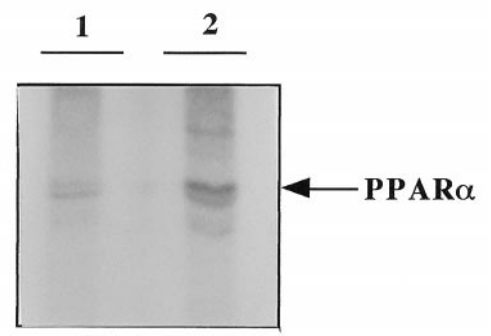

FIG. 3. PPARα is a phosphoprotein in Fao cells as shown by ^{32}P labeling. ^{32}P *in vivo* labeling of PPARα. Fao cells were metabolically labeled with [^{32}P] orthophosphate (30 μCi) for 2 hr and then cultured in medium containing 0.1% DMSO for 4 hr. Cells were lysed with RIPA buffer as described in Materials and Methods and PPARα was immunoprecipitated with the anti-PPARα antibody (lane 2), run on SDS-PAGE (10%). Preimmune serum was used as negative control (lane 1). The ^{32}P gels were fixed with 40% methanol and 3.7% formaldehyde for 30 min followed by immersion in a 10% ethanol, 4% glycerol solution. Dried gels were subjected to autoradiography.

PPAR antibody raised against the 16 carboxy-terminal amino acids of rat PPARα recognized the three PPAR subtypes in the immunoprecipitation assays (Fig. 1A). This was in agreement with the fact that in rodents, 12 and 11 of the last 16 amino acids of PPARα are conserved in PPARβ and PPARγ, respectively. The molecular mass of the translated mPPARα was around 52 kDa (Fig. 1, A and B), while the molecular masses of the β and γ forms were 46 and 48 kDa, respectively (Fig. 1A). The lower band observed in Fig. 1A (line mα) was not related to PPARα, since this band was present in the unprogramed lysate (Fig. 1B, lane 1). The antibody raised against the amino terminus of the human PPARα immunoprecipitated the *in vitro*-translated mouse PPARα (Fig. 1C). The test for cross reactions showed that the anti-PPARα antibody was specific to PPARα. Indeed, PPARγ isotypes were not recognized by this antibody and PPARβ gave a non-specific labeling (Fig. 2).

The expression of PPARα was then investigated in the rat Fao hepatic-derived cell line, a good cellular model suited to the study of the effect of peroxisome proliferators on gene expression regulation of numerous enzymes involved in lipid metabolism [18, 28, 30, 31]. By Northern blotting, Poirier *et al.* [32] detected both the mRNA for PPARα and PPARβ, but not PPARγ, in Fao cells. However, up to now, no Western blot analysis of the PPARα isoform has been carried out in Fao cells. Thus, we investigated the presence of this nuclear receptor in the Fao cell line by immunoblotting with the anti-PPARα antibody. As shown in Fig. 1D, the anti-PPARα antibody recognized a major polypeptide from Fao cell extracts exhibiting an electrophoretic mobility virtually the same (52 kDa) as that found for mPPARα in the immunoprecipitation assays (Fig. 1C). The 52 kDa band was also recognized with the polyclonal anti-PPAR antibody directed against the well-conserved carboxy terminus of PPARα.

Phosphorylation of PPARα in the Rat Fao Hepatic-Derived Cell Line

Fao cells were labeled with [^{32}P] orthophosphate and thereafter PPARα was immunoprecipitated with the anti-PPARα antibody. After SDS-PAGE analysis, the autoradiogram obtained (Fig. 3, line 2) shows a radiolabeled band with a molecular mass of 52 kDa. By comparison with the result presented in the Fig. 1D, it may be concluded that the 52 kDa polypeptide corresponds to the phosphorylated PPARα isotype. In contrast, this 52 kDa band was almost absent when we immunoprecipitated with the preimmune serum (Fig. 3, line 1). This confirms the specificity of the immune serum. To confirm the above results, Fao cells were harvested using RIPA lysis buffer and the total extracted proteins were subjected to bovine intestine alkaline phosphatase treatment. The immunoblot analysis using the anti-PPARα antibody (Fig. 4) shows that this treatment shifted the 52 kDa PPARα band to one with higher mobility (50 kDa), which is in agreement with a change from a phosphorylated PPARα form to a dephosphorylated one.

Effect of Peroxisome Proliferators on the Phosphorylation Level of PPARα

In order to study the effect of peroxisome proliferators on the PPARα phosphorylation level, Fao cells were labeled with [^{32}P] orthophosphate. Then, they were treated for 4 hr with 500 μM ciprofibrate and PPARα was immunoprecipitated with the anti-PPARα antibody. As can be seen in Fig. 5, an increase in the phosphorylation of the 52 kDa PPARα band was observed. By scanning, this increase factor was estimated to be 1.7 ± 0.3 compared to the control band. This increase is statistically significant. The time–course was performed from 0 to 4 hr and shows the highest activation at 4 hr of incubation [18]. To analyze whether

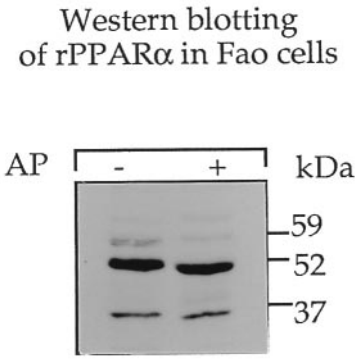

FIG. 4. PPARα is a phosphoprotein in Fao cells as shown by alkaline phosphatase treatment. Treatment of cell lysates with bovine intestine alkaline phosphatase (AP). Fao cell lysates obtained as described in Materials and Methods were treated for 1 hr at 37° with or without 6 units of bovine intestine alkaline phosphatase. Proteins (30 μg) from untreated (−) and treated (+) lysates were separated by SDS-PAGE (10%) and immunoblotted with the anti-PPAR antibody as described in Materials and Methods under "Production of Anti-PPAR Antibody". The experiments were repeated three times.

this increase was due or not to an induction of PPARα synthesis by peroxisome proliferators, Fao cells were labeled for 4 hr with [^{35}S] methionine–cysteine in the presence of 500 μM ciprofibrate, and PPARα then immunoprecipitated. Compared to controls, no significant variation was observed in the PPARα band intensity (Fig. 6), indicating that no PPARα synthesis changes occurred during treatment with the peroxisome proliferator.

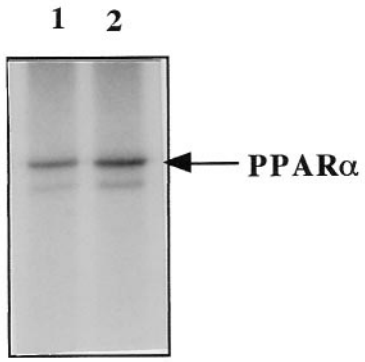

FIG. 5. Increase in the PPARα phosphorylation level by ciprofibrate. Fao cells were metabolically labeled with [^{32}P] orthophosphate (30 μCi) for 2 hr and then treated with ciprofibrate (500 μM) for 4 hr (lane 2). Control cells were cultured in medium containing 0.1% DMSO (lane 1). Cells were lysed with RIPA buffer as described in Materials and Methods and PPARα was immunoprecipitated with anti-PPARα antibody, run on SDS-PAGE (10%). The ^{32}P gels were fixed with 40% methanol and 3.7% formaldehyde for 30 min followed by immersion in a 10% ethanol, 4% glycerol solution. The assays (usually duplicated) were repeated three times and the difference is statistically significant ($P < 0.01$).

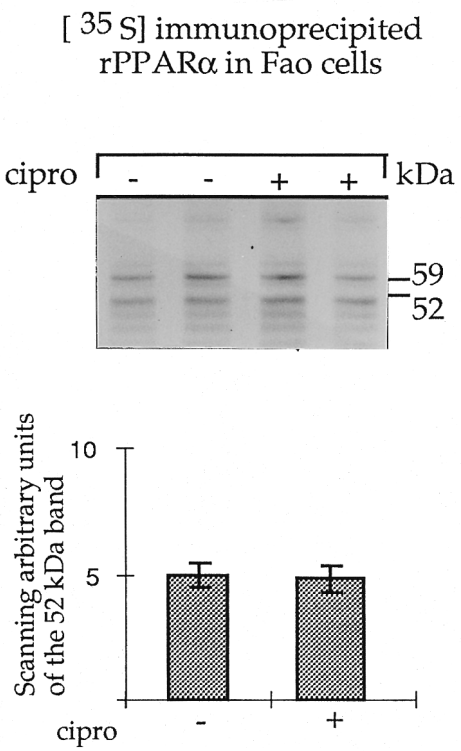

FIG. 6. No increase in PPARα level by ciprofibrate. Fao cells were metabolically labeled with ^{35}S (200 μCi/mL) for 2 hr and then treated with ciprofibrate (500 μM) for 4 hr. Control cells were cultured in medium containing 0.1% DMSO. Cells were lysed with RIPA buffer as described in Materials and Methods and PPARs were immunoprecipited with the anti-PPAR antibody as described in Materials and Methods under "Production of Anti-PPAR Antibody", and run on SDS-PAGE (10%). The ^{35}S gels were processed for fluorography. Dried gels were subjected to autoradiography. The assays (−−) and (++) represent duplicated samples. They were repeated twice and the difference is statistically not significant. Cipro: ciprofibrate.

Effect of Phosphatase Inhibitors on Ciprofibrate-Induced Peroxisomal Acyl-CoA Oxidase Activity

In order to investigate the functional effect of induced PPARα phosphorylation in Fao cells, we measured palmitoyl-CoA oxidase activity in Fao cells treated for 24 hr with or without 500 μM ciprofibrate in the presence or absence of phosphatase inhibitors (50 nM okadaic acid together with 10 μM sodium orthovanadate). These concentrations were chosen in the light of previous studies of Shalev *et al.* [13] in transiently transfected CV1 cells. Cell viability after 24 hr of treatment was checked by measuring the activity of glutamate dehydrogenase, a non-dependent phosphorylation encoded gene. The specific activities of this enzyme were found to be unaffected by treatment (data not shown). Figure 7 presents the results of a representative experiment. As expected, a strong increase in palmitoyl-CoA oxidase activity (about 8-fold) was observed in Fao cells treated by ciprofibrate only. Moreover, we showed that phosphatase inhibitor treatment led to a decrease of at least 25% in acyl-CoA activity in Fao cells treated by the peroxisome proliferator. Conversely, no significative effect of phospha-

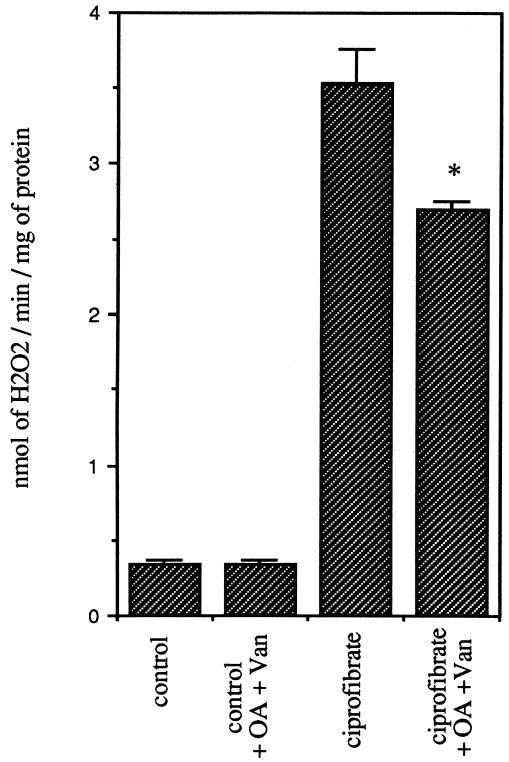

FIG. 7. Decrease in palmitoyl-CoA oxidase activity in Fao cells treated with phosphatase inhibitors. Specific activities of palmitoyl-CoA oxidase after treatment of Fao cells for 24 hr without (control) or with 500 μM ciprofibrate in the presence or absence of 50 nM okadaic acid (OA) and 10 μM sodium orthovanadate (Van). The activities were assayed as described in Materials and Methods and the specific activities were calculated after determination of the protein concentration of the cell homogenates. The figure is representative of two separate experiments, each one using duplicate flasks for each treatment; each bar represents the mean ± SEM. *Statistically significant ($P < 0.01$). H_2O_2: hydrogen peroxide produced by the biochemical reaction catalyzed by the peroxisomal acyl-CoA oxidase.

tase inhibitors was observed in basal expression of acyl-CoA oxidase (cells untreated by ciprofibrate).

DISCUSSION

It is well known that in rodent liver, peroxisome proliferators greatly enhance gene expression of some key enzymes involved in peroxisomal lipid metabolism, such as acyl-CoA oxidase [33]. This enhancement involves the activation of PPARs, which heterodimerize with retinoic X receptor (RXR) and bind to a response element, the PPRE (peroxisome proliferator response element). Furthermore, several genes are activated by this mechanism (for a review see [3]. At present, it has become clear that there is a gene activation specificity according to the PPAR isotype and the PPAR-activating ligand. For instance, hypolipidaemic drugs activate PPARα, which in turn alters the transcription of genes encoding enzymes involved in lipid metabolism (mitochondrial and peroxisomal β-oxidation path-

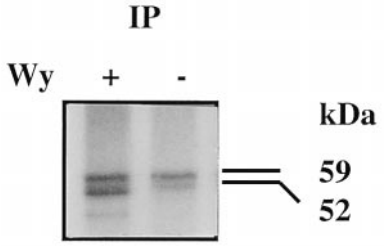

FIG. 8. Increase in the PPARα phosphorylation level by Wy-14,643. Fao cells were metabolically labeled with [^{32}P] orthophosphate (30 μCi) for 2 hr and were then treated with Wy-14,643 (50 μM) for 4 hr. Control cells were cultured in medium containing 0.1% DMSO. Cells were lysed with RIPA buffer as described in Materials and Methods and PPARs were immunoprecipited (IP) with the anti-PPAR antibody as described in Materials and Methods under "Production of Anti-PPAR Antibodies", run on SDS-PAGE (10%). The ^{32}P gels were fixed with 40% methanol and 3.7% formaldehyde for 30 min followed by an immersion in a 10% ethanol, 4% glycerol solution. The assays (−−) and (++) represent duplicated samples. They were repeated twice and the difference is statistically significant. Wy:Wy-14,643.

ways) and of enzymes of detoxication (cytochrome P-4504A1 and epoxide hydrolase) [34, 35]. On the other hand, PPARγ is mainly involved in pre-adipocyte differentiation and requires specific prostaglandins or antidiabetic drugs (thiazolidinediones) for activation [36, 37]. Recently, it was shown that PPAR isoforms (α and γ) are phosphorylated *in vivo* in adipocytes [7–11]. Due to the low PPARα content of adipose cells in comparison with the PPARα content of rat hepatocytes or hepatic-derived cell lines, we investigated the PPARα phosphorylation status in the rat Fao hepatic-derived cell line. We have reported herein that this rat cell line exhibits a high content in PPARα. By ^{32}P *in vivo* cell labeling or by alkaline phosphatase treatment of cell lysate, the phosphorylation status of PPARα was demonstrated. In addition, we show for the first time that the PPARα phosphorylation level is enhanced by cell exposure to ciprofibrate. An increase in the PPARα ^{32}P-phosphorylated band intensity was also obtained after cell treatment for 4 hr with 50 μM Wy-14,643, a strong peroxisome proliferator of the non-fibrate family also known to activate PPAR [38] (Fig. 8). The observed effect of peroxisome proliferators on PPARα phosphorylation is in agreement with the stimulation of protein phosphorylation in Fao cells previously reported [18]. The results obtained by measuring the acyl-CoA oxidase activity in Fao cells treated by ciprofibrate and phosphatase inhibitors would suggest that the process of the phosphorylation/dephosphorylation of PPARα is implicated in the regulation of PPARα transcriptional activity. Moreover, the decrease in acyl-CoA oxidase activity could result from a down-regulation.

Taken together, our results are consistent with the fact that gene expression modulation by peroxisome proliferators could be the consequence of two synergistic mechanisms: one through a direct activating effect of peroxisome

proliferators on PPARα acting as ligands; the other a possible down-regulation via the signal transduction pathway leading to PPARα phosphorylation. A similar down-regulation of PPARγ$_2$ phosphorylation has been reported in the differentiation of murine fibroblasts to adipocytes by using PPARγ$_2$ mutant at the Ser114 phosphorylation site [39]. These proposed mechanisms could be responsible for the control of peroxisomal β-oxidation enzyme activity as well as that of the toxicity and detoxication processes of cells exposed to peroxisome proliferators. From our data, it may be supposed that PPARα phosphorylation follows the formation of the ciprofibrate PPARα complex acting as a transcription factor. It is possible that, after ligand binding, the receptor undergoes a conformational change which allows increased exposure to kinase(s) as described for estrogen receptor. Thus far, we do not know if the transcriptional effect of PPARα results in mutual ligand binding and phosphorylation or in a cooperativity (or antagonism) between these two phenomena.

This work was supported by grants from l'ARC, the Comités de Bourgogne et de Meurthe et Moselle, la Ligue Bourguignonne contre le Cancer, le Comité Lorraine de la Fondation de la Recherche Médicale and le Conseil Régional de Bourgogne. We thank Dr. Valérie Nicolas-Frances for helpful discussion, Dr. Roberta Ricciarelli (Bern) for advice concerning the immunoprecipitation technique, and Arnaud Bianchi and Jean Marie Keller for their skilful assistance. We are grateful to Dr. Walter Wahli (Lausanne, Switzerland) for providing the mouse PPARα, β, and γ cDNA pSG5 plasmids, to Dr. Stephen Green (Macclesfield, England) for providing the mouse PPARα plasmid, and to David R. Bell (Nottingham, England) for testing his mPPARα antibody.

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