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Differences in the Formation of PPARα-RXR/*aco*PPRE Complexes between Responsive and Nonresponsive Species upon Fibrate Administration

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ABSTRACT

Peroxisome proliferator-activated receptor-α (PPARα) is responsible for the hypolipidemic, peroxisome proliferation and carcinogenic effects of fibrates. Rats and mice are responsive, but guinea pigs and primates are resistant to the proliferative and carcinogenic effects of these drugs, but the hypolipidemic effect is still manifest. It is not yet clear whether humans should be considered unresponsive, and there is concern about the long-term safety of fibrates. We present molecular evidence for the reported resistance of human cells to peroxisome proliferation by describing a deficient interaction of nuclear extracts from human cells with an acyl-CoA oxidase (ACO)-peroxisome proliferator response element probe upon fibrate addition. Electrophoretic mobility shift assay analysis showed that ciprofibrate elicited a concentration-dependent increase in the binding of nuclear extracts from cells of rat (Morris) and human (HepG2) origin to an ACO-peroxisome proliferator response element probe, although in HepG2 cells the increase was of marginal statistical significance. In Morris cells, the increase was more marked than in HepG2 cells (4-fold versus 1.5-fold at 0.2 mM ciprofibrate), and maximal binding was achieved earlier in Morris (30 min) than in HepG2 cells (3 h). Morris cells responded to the addition of ciprofibrate by increasing the levels of ACO mRNA, whereas HepG2 did not. The ratio between PPARβ/PPARα mRNAs was higher in HepG2 cells than in Morris cells (3.2 versus 1.9), pointing to an antagonizing effect of PPARβ on PPARα activity. These results were obtained in untransfected cells expressing their own basal set of receptors. We also provide evidence of the translocation of PPARα from the cytosol to the nucleus upon activation by ciprofibrate.

Peroxisome proliferator-activated receptors (PPARs) comprise a type of nuclear receptor deeply involved in the maintenance of lipid and glucose homeostasis (Smith, 1996). They recognize specific sequences termed peroxisome proliferator response elements (PPREs) in the promoter regions of target genes. The PPRE is a direct repeat of a consensus core sequence of TGACCT separated by one nucleotide. Furthermore, PPARs require the participation, as a heterodimerization partner, of another nuclear transcription factor, 9-*cis* retinoic acid receptor (RXR; Lemberger et al., 1996). One of the three receptor subtypes, PPARα, is directly responsible for the hypolipidemic (Peters et al., 1997a), peroxisome proliferation (Lee et al., 1995) and liver carcinogenic effects (Peters et al., 1997b) of a chemically diverse group of compounds known as peroxisome proliferators. The carcinogenic effect in rats seems to be produced as a long-term consequence of the peroxisome proliferation elicited by these compounds, which include drugs widely used in human therapy such as hypolipidemic fibrates (Bentley et al., 1993).

Rats and mice are extremely responsive to the effects elicited by PPARα activation upon fibrate administration. Other rodent (guinea pig) and nonrodent species (such as humans and other primates) are resistant or nonresponsive to the proliferative and carcinogenic effects of these drugs, but the hypolipidemic effect is still manifest (Bentley et al., 1993, Cattley et al., 1998). There is no conclusive evidence that humans are not responsive to peroxisome proliferation. Although the indirect experimental data reported until now suggests that humans should be included among the nonresponsive species, the issue remains controversial and concern has been expressed about the long-term safety of drugs such as fibrates (Cattley et al., 1998).

Peroxisome proliferators produce different responses

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator response element; RXR, 9-*cis* retinoic acid receptor; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-polymerase chain reaction; ACO, acyl-CoA oxidase; HB, hypotonic buffer; EMSA, electrophoretic mobility shift assay; APRT, adenosyl phosphoribosyl transferase.

among species despite that cells from nonresponsive species express functionally active PPARα and RXR that heterodimerize and interact with the PPREs present in target genes (Mukherjee et al., 1994; Varanasi et al., 1996; Tugwood et al., 1998). However, most of the data on the functionality of PPARs was obtained from cells transfected with various PPRE constructs and different expression vectors for PPARα and RXR. Such an experimental approach produces deep modifications in the basal expression and activities of constitutive receptors compared with untransfected cells, making the extrapolation of these results to physiological conditions controversial. To circumvent this shortcoming, we have characterized the interaction of nuclear extracts, obtained from rat liver or untransfected cells from rat and human origin treated with fibrates, with a specific PPRE probe. We demonstrate differences in the strength of binding and temporal pattern of interaction of nuclear extracts from rat and human cells with the PPRE probe. These differences could contribute to the different response of these species to peroxisome proliferator administration. Furthermore, although it has been reported in cotransfection experiments that the transcription factor Sp1 manifests transactivation synergism with the PPAR/RXR heterodimer (Krey et al., 1995), we show that Sp1 is not involved in the establishment of these differences. Finally, for the first time in untransfected cells, we present experimental evidence of the migration of PPARα from the cytosol to the cell nucleus upon administration of a specific PPARα agonist.

Materials and Methods

Drugs and Cell Culture. Drugs were purchased from Acofarma (bezafibrate, Barcelona, Spain), or kindly provided by ICI-Farma (clofibrate; Pontevedra, Spain) and Sanofi Winthrop Research Division (ciprofibrate, Malvern, PA). HepG2 human hepatoma cells (European Collection of Cell Cultures, Salisbury, UK) and rat Morris 7800 C1 hepatoma cells (obtained from Dr. H. Sorensen, University of Oslo, Norway) were grown in Ham's F-12 medium supplemented with 7% fetal calf serum (Gibco-BRL; Paisley, Scotland), 100 U/ml penicillin, and 100 mg/l streptomycin (Sigma, St. Louis, MO). Cells were seeded at a density of 7×10^5 (HepG2) or 9×10^5 (Morris) cells/100-mm-diameter plate. Incubation with clofibrate, ciprofibrate, or bezafibrate began at least 32 h after plating. Stock solutions of these compounds were made in dimethyl sulfoxide (DMSO; Sigma). The concentration of DMSO in the culture medium varied between 0.5 and 1%, depending on the concentration of fibrate used; corresponding control plates were supplemented with the same concentration of DMSO. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Mossman, 1983). Cell assays involving drug addition were performed at drug concentrations and incubation times that provided a cell viability of at least 90%.

Animals and Treatments. Eighteen male Sprague-Dawley rats from Letica Scientific Instruments (Barcelona, Spain), weighing ~130 g each, were maintained on a 12-h light/dark cycle under conditions of constant humidity and temperature (22 ± 2°C) and were fed with a standard Panlab diet for 5 days before the beginning of the experiments. The animals were processed in three groups of six, with two control and four bezafibrate-treated rats in each group. The animals received a single oral dose (69 mg/100 g b.wt.) of bezafibrate suspended in carboxymethyl cellulose-Tween (0.5–0.1%; Sigma). The oral dose of bezafibrate was calculated according to the

TABLE 1

Ratios of mRNA levels for PPARα, PPARβ, and RXRα in Morris 7800 C1 and HepG2 cells

Specific mRNA levels were determined as described in *Materials and Methods*. Results are the mean ± S.E. of three different assays performed in duplicate.

Ratio	Morris 7800 C1 Cells	HepG2 Cells
PPARβ/PPARα	1.89 ± 0.21	3.2 ± 0.22[a]
PPARβ/RXRα	0.36 ± 0.05	7.6 ± 2.14[b]
PPARα/RXRα	0.19 ± 0.03	2.4 ± 0.58[c]

[a] $P = .013$ versus Morris cells.
[b] $P = .028$ versus Morris cells.
[c] $P = .019$ versus Morris cells.

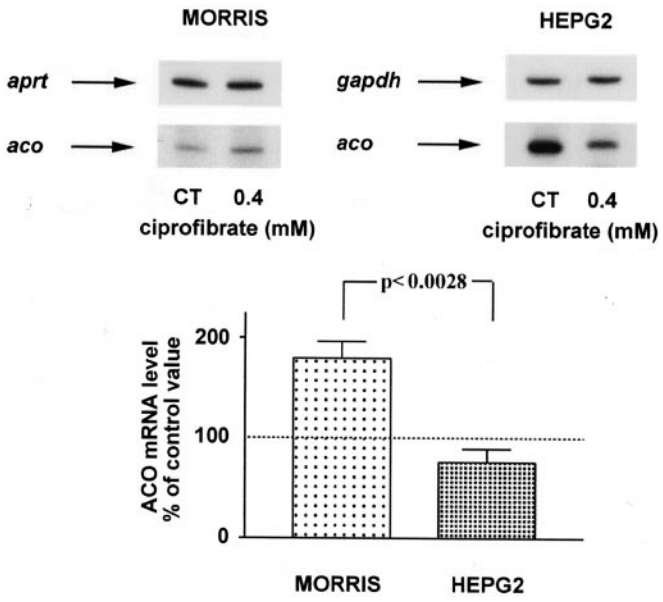

Fig. 1. Changes in ACO mRNA levels in Morris 7800 C1 and HepG2 cells upon incubation with ciprofibrate. Hepatoma cells were incubated for 3 h with 0.4 mM ciprofibrate. A, autoradiography of a representative RT-PCR assay showing the ACO signal for control (CT) and ciprofibrate-treated cells, and the corresponding signals for the APRT (Morris 7800 C1) or GAPDH (HepG2) mRNA used as a reference. B, percentage of change of hepatoma cell ACO mRNA levels regarding CT values (100%). Values are the mean ± S.E. of four different experiments.

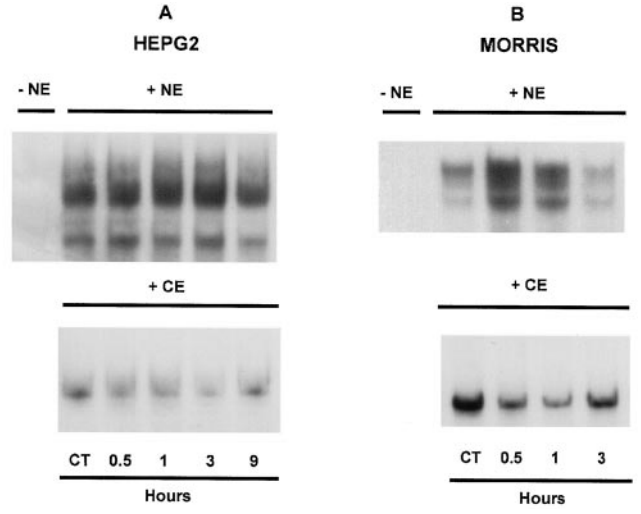

Fig. 2. Time-dependent effect of ciprofibrate on the interaction of nuclear and cytosolic extracts from Morris 7800 C1 and HepG2 cells with an ACO-PPRE probe. EMSAs were performed by incubating a specific ACO-PPRE probe with nuclear (NE) or cytosolic (CE) extracts obtained from HepG2 and Morris 7800 C1 cells and incubated with 0.2 mM ciprofibrate at different times. An autoradiography of a representative EMSA with extracts obtained from HepG2 (A) and Morris 7800 C1 (B) cells is shown. Experiments were repeated at least twice.

daily drug intake described previously in feeding experiments (Alegret et al., 1994). After 7, 15, 30, 60, and 180 min of bezafibrate administration two animals of each group were sacrificed by cervical dislocation (between 8:00 and 9:00 AM) and liver samples were taken and processed immediately to obtain nuclear extracts. Animals treated for 15 days were fed on a diet containing 0.45% bezafibrate; liver nuclear extracts were obtained as described. All procedures were conducted in accordance with the principles and guidelines established by the University of Barcelona Bioethics Committee as stated in Law 5/1995, 21 July, from the Generalitat de Catalunya.

RNA Isolation. Total RNA was isolated with the Ultraspec reagent (Biotecx Laboratories, Houston, TX). RNA was measured by its absorbance at 260 nm. The integrity of RNAs was assessed by electrophoresis in 1% agarose gels, containing 2% formaldehyde, followed by ethidium bromide staining.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). mRNA levels were determined by RT coupled to PCR. The RT reaction (final volume 20 μl) was performed for 1 h at 37°C with 1 [rat acyl-CoA oxidase (ACO), rat and human PPARα, PPARβ, and RXRα], 1.5 (human ACO), or 0.5 μg (rat liver ACO) as the starting total RNA. The reaction mixture also contained 125 ng of random hexamers (Promega, Madison, WI); 20 U RNAsin (Promega); 200 U M-MLV-RT (Gibco-BRL) in 75 mM KCl, 3 mM $MgCl_2$, 10 mM dNTPs (Sigma), and 50 mM Tris-HCl, pH 8.3. The PCR reaction (50 μl) was carried out with an aliquot of 5 μl of the RT reaction and 0.5 μg of each of the specific primers (Boehringer Mannheim, Indianapolis, IN), 200 μM dNTPs, 1 U *Taq* DNA polymerase (Gibco-BRL), and 0.25 μCi of [α-^{32}P]dATP (Amersham Pharmacia) in 20 mM Tris-HCl, pH 8.5, and 2.5 mM $MgCl_2$. Amplification was carried out by 34 (human ACO), 30 (rat PPARα, PPARβ, and RXRα), 27 (human PPARα, PPARβ, and RXRα), 20 [human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rat liver ACO], or 21 (rat ACO) cycles of PCR. Each cycle consisted of sequential denaturation at 90°C for 1 min, annealing at 60°C (or 58°C for rat ACO) for 1 min 15 s, and elongation at 72°C for 1 min 50 s, followed by a final elongation step at 72°C for 5 min. The primers used were the following: for human *aco*, 5′-GCCCAGGTGAAGCCTGATGGA-3′ and 5-GACTGGTGCCTCACAGCGCTG-3′; for human *pparα*, 5′-GGAAAGCCCACTCTGCCCCCT-3′ and 5′-AGTCACCGAGGAGGGGCTCGA-3′; for human *pparβ*, 5′-GAGCAGCCACAGGAGGAAGCC-3′ and 5′-CCGTCACAGCCCATCTGCAGT-3′; for human *rxrα*, 5′-CCAAACATTTCCTGCCGCTCG-3′ and 5′-CCGTCACAGCCCATCTGCAGT-3′; for human *gapdh*, 5′-CAGTCCATGCCATCACTGCCA-3′ and 5′-AGGTGGAGGAGTGGGTGTCGC-3′; for rat *aco*, 5-ACTATATTTGGCCAATTTTGTG-3′ and 5′-TGTGGCAGTGGTTTCCAAGCC-3′; for rat *pparα*, 5′-GGCTCGGAGGGCTCTGTCATC-3′ and 5′-ACATGCACTGGCAGCAGTGGA-3′; for rat *pparβ*, 5′-GAGGAAGTGGCCACGGGTGAC-3′ and 5′-CCACCTGAGGCCCCATCACAG-3′; for rat *rxrα*, 5′-GCTCTCCAACGGGTCGAGGCT-3′ and 5′-TGGGTGTGGTGGGTACCGACA-3′; and for rat *aprt*, 5′-AGCTTCCCGGACTTCCCCATC-3′ and 5′-GACCACTTTCTGCCCCGGTTC-3′. To avoid unspecific annealing, cDNA and *Taq* DNA polymerase were separated from primers and dNTPs by using a layer of paraffin (Fluka); in this way, reaction components contact only when paraffin fuses (59°C). Rat adenosyl phosphoribosyl transferase (APRT) and human GAPDH were used as controls to normalize the results. For each set of samples both genes, problem and control, were amplified together, except for human ACO. In the latter case, however, the same RT reaction product was used for the amplification of human ACO and GAPDH genes. Five microliters of each PCR reaction mixture was subjected to electrophoresis in 5% polyacrylamide gel in 1× TBE (Sigma). Gels were dried, autoradiographed, and quantified by image analysis (Vilbert-Lourmat version 4.6).

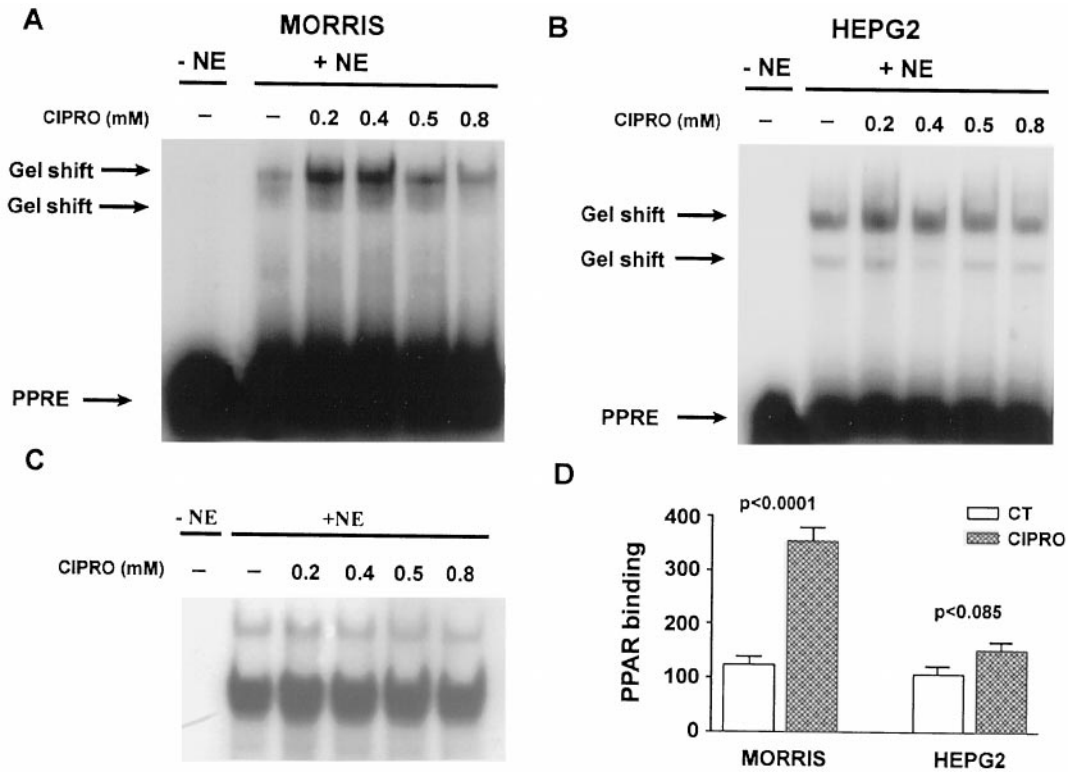

Fig. 3. Concentration-dependent effect of ciprofibrate on the interaction of nuclear extracts from Morris 7800 C1 and HepG2 cells with an ACO-PPRE probe. EMSA was performed by incubating a specific ACO-PPRE probe with nuclear extracts (NE) obtained from cells incubated with different ciprofibrate (CIPRO) concentrations for 30 min (Morris 7800 C1) or 3 h (HepG2). An autoradiography of a representative EMSA with an ACO-PPRE probe and nuclear extracts obtained from Morris 7800 C1 (A) or HepG2 (B) cells is shown. C, autoradiography of EMSA with an unrelated Oct1 probe with NE obtained from Morris 7800 C1 cells. Two different assays were performed. D, percentage of change in the intensity of the main shifted band with respect to control (CT) values for the two types of hepatoma cells studied incubated with 0.2 mM ciprofibrate. Each column represents the average of the results obtained in four independent samples. $P < .0001$ for Morris versus HepG2 cells values.

Nuclear and Cytosolic Extracts. Nuclear and cytosolic cell extracts were obtained from monolayer cell cultures by the method of Dignam et al. (1983). Morris and HepG2 cells were harvested and washed at 800*g* in hypotonic buffer (HB: 15 mM Tris-HCl, pH 7.4; 15 mM NaCl; 60 mM KCl; 0.5 mM EDTA; and 1 mM phenylmethylsulfonyl fluoride; Sigma). Cytosolic extracts and nuclei were prepared by washing pelleted cells at 1000*g* in 200 μl of HB containing Triton X-100 (0.025% for Morris 7800 C1 and 0.05% for HepG2 cells). Supernatant constituted the cytosolic extract. Pelleted nuclei were washed twice in HB at 1000*g*. Nuclear proteins were extracted by adjusting KCl concentration to 360 mM. After 30 min under shaking, chromatin was pelleted by centrifugation for 30 min at 100,000*g* and nuclear extracts (supernatant) were frozen in liquid nitrogen. Cytosolic and nuclear extracts were stored at −80°C. All steps were performed at 4°C. Protein concentration was determined according to Bradford (1976). The integrity of nuclei preparation was assessed by standard flow cytometry with propidium iodide. Flow cytometrical histograms of DNA fluorescence displayed sharp peaks (coefficient of variance <3.5%) with absence of shoulders, indicating very low aggregation and/or disintegration. The percentage of nuclear debris was 0.8.

When isolating nuclei from tissue, 2 g of rat liver was homogenized in 20 ml of homogenization buffer (10 mM HEPES, pH 7.6; 2 M sucrose; 15 mM KCl; 1 mM EDTA; 10% glycerol; 0.15 mM spermine; 0.5 mM spermidine; 0.5 mM dithiothreitol; and 0.5 mM phenylmethylsulfonyl fluoride; Sigma). Ten milliliters of this homogenate was loaded on top of 4-ml pads of homogenization buffer in SW 30 tubes. Samples were centrifuged at 40,000*g* for 60 min and pelleted nuclei were washed in 5 ml of HB at 1,000*g*. Preparation of nuclear extracts continued by extraction in 360 mM KCl and centrifugation (30 min, 100,000*g*) as described above.

Electrophoretic Mobility Shift Assay (EMSA) and Supershift Analysis. Gel mobility shift assays were carried out with nuclear and cytosolic extracts from rat liver and hepatoma cells. Extracts (2 μg) were incubated for 15 min on ice in 25 mM Tris-HCl, pH 8, containing 60 mM KCl, 1 mM $MgCl_2$, 5% glycerol, and 2 μg of poly(dI-dC) in a final volume of 20 μl. Approximately 20,000 cpm (0.5–1 ng) of ^{32}P end-labeled probe, either PPRE, Oct1 (Pharmacia), or Sp1f, was then added and the incubation was on ice for a further 30 min. Supershift assays were performed by adding, during the last 15 min of incubation, preimmune sera, polyclonal antibody against PPARα (kindly provided by Dr. J. K. Reddy, Northwestern University Medical School, Chicago, IL), or RXRα (Santa Cruz Biotechnology, Santa Cruz, CA) at various concentrations. The PPRE-ACO probe was obtained from the annealing of single-stranded comple-

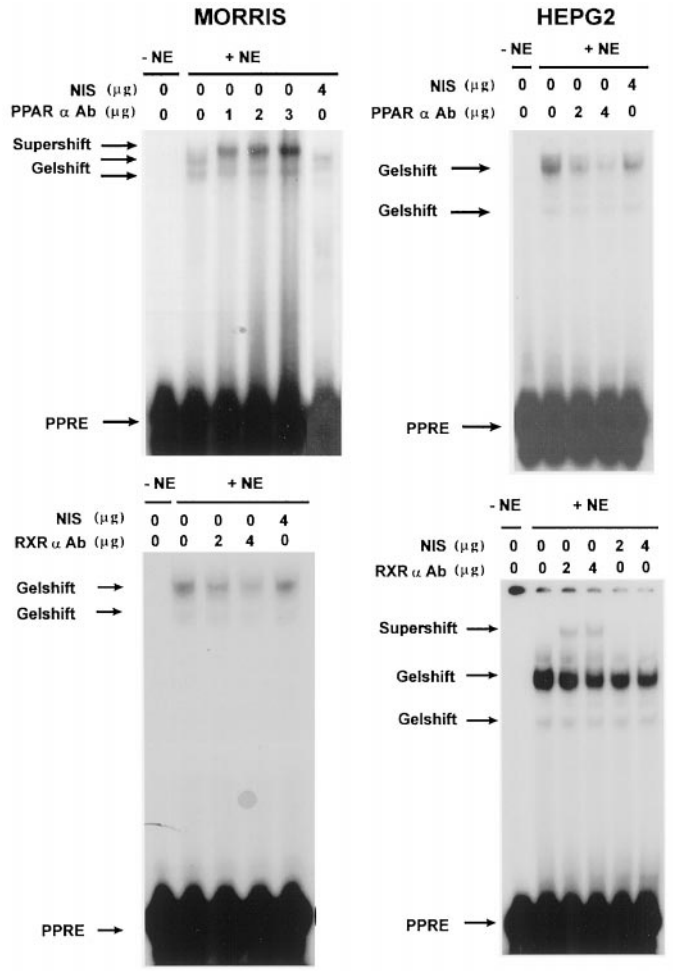

Fig. 4. PPARα and RXRα proteins participate in the formation of the pattern of shifted bands. Autoradiographs of representative electrophoretic mobility super-shift assays performed by incubating a specific ACO-PPRE probe with nuclear extracts (NE) obtained from untreated HepG2 (right) or Morris 7800 C1 (left) cells are shown. Nonimmune sera, polyclonal antibody against PPARα (top) or RXRα (bottom) were added at the indicated concentrations during the last 15 min of incubation. Experiments were repeated at least twice.

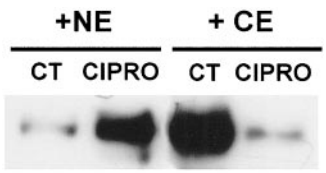

Fig. 5. PPARα migrates from the cytosol to the cell nucleus upon addition of ciprofibrate. Representative Western blot of PPARα protein in nuclear (NE) and cytosolic (CE) extracts from Morris 7800 C1 cells treated with 0.2 mM ciprofibrate (CIPRO) for 30 min. Twenty micrograms of protein from each extract was loaded in each lane. Two different assays were performed.

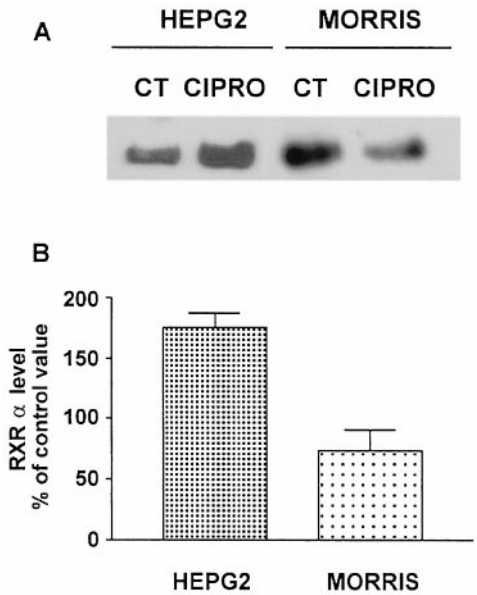

Fig. 6. Changes in the nuclear amount of RXRα protein in Morris 7800 C1 and HepG2 cells after incubation with 0.2 mM ciprofibrate. A, representative Western blot of RXRα protein in nuclear extracts of cells treated with 0.2 mM ciprofibrate for 30 min (Morris 7800 C1) or 3 h (HepG2) and in nuclear extracts of the corresponding control (CT) cells. Twenty micrograms of protein was loaded in each lane. B, percentage of change in the intensity of the RXRα band with respect to CT values (100%) for Morris (−3–150%, 95% CI, NS) and HepG2 (124–227%, 95% CI, $P = .009$) hepatoma cells. Each column represents the mean ± S.E. of the results obtained in three independent samples.

mentary oligonucleotides (Boehringer Mannheim) spanning nucleotides −629 to −606 of the rat ACO gene (Tugwood et al., 1992). The following sequences were used: upper strand, 5′-CGAACGTGACCTTTGTCCTGGTCC-3′; and lower strand, 5′-GGACCAGGACAAAGGTCACGTTCG-3′. The Sp1f probe (Noé et al., 1997) contains the most proximal GC box in the hamster dihydrofolate reductase promoter. These double-stranded oligonucleotides were end-labeled with T4 polynucleotide kinase and γ-[^{32}P]ATP. Protein-DNA complexes were resolved by electrophoresis at 4°C on a 5% polyacrylamide gel in 0.5× TBE.

Western Blot Analysis. Nuclear and cytosolic extracts (20 μg) from Morris 7800 C1 and HepG2 cells were subjected to SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (1970). Proteins were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA) by using the HEP-1 electrotransfer system (Applied Genetechnology Systems GmbH). Immunodetection was performed by the BM chemiluminescence kit (Boehringer Mannheim). Primary antibodies against PPARα (kindly provided by Dr. J. K. Reddy), RXRα, and Sp1 (Santa Cruz Biotechnology) were used at dilutions of 1:1000 (anti-PPARα and RXRα) and 1:150 (Sp1). Equal loading of protein in each lane was verified by Coomassie blue staining.

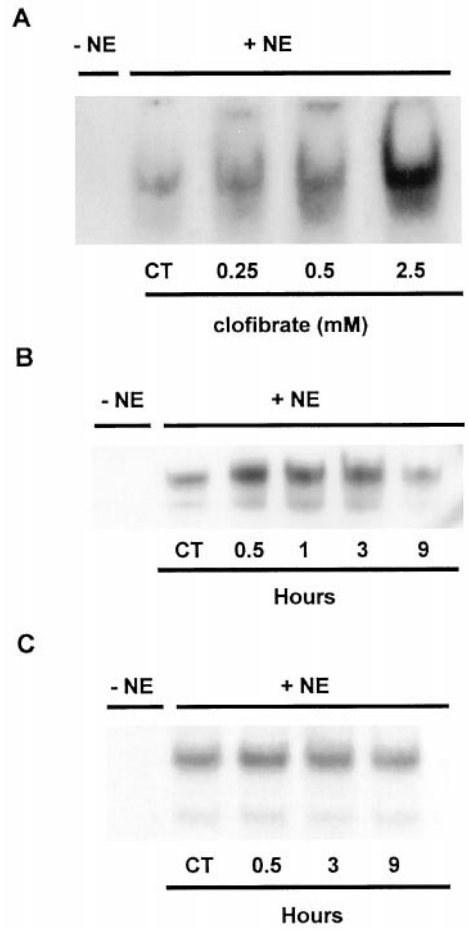

Fig. 7. Ciprofibrate effects on the interaction of nuclear extracts from Morris 7800 C1 and HepG2 cells with an ACO-PPRE probe are shared by other fibrates. Autoradiographs of representative experiments showing A) concentration- (at 30-min incubation) and B) time-dependent (at 2.5 mM) effects with regard to control (CT) cells on EMSA performed with an ACO-PPRE probe and nuclear extracts (NE) of Morris 7800 C1 cells incubated with clofibrate. C, time-dependent effect of 0.6 mM bezafibrate on EMSA performed with an ACO-PPRE probe and NE from HepG2 cells. Two different assays were performed.

Data Analysis. Results are expressed as mean ± S.E., and their statistical significance was evaluated, when appropriate, with Student's *t* test.

Results

To gain further insight into the molecular mechanisms responsible for the reported different response of rat Morris and human HepG2 hepatoma cells to peroxisome proliferators (Sohlenius et al., 1995; Stang et al., 1995), we determined their relative levels of PPARα, PPARβ, and RXRα mRNA, and characterized the binding of nuclear proteins to a specific ACO-PPRE probe. Furthermore, we incubated these cells with ciprofibrate, a potent peroxisome proliferator, and subsequently determined the effect on 1) ACO mRNA levels; 2) binding of nuclear and cytosolic proteins to a specific ACO-PPRE probe, and 3) nuclear content of PPARα and RXR. Additionally, we determined the in vivo response of ACO mRNA levels and the binding of nuclear proteins to a specific ACO-PPRE probe in livers obtained from bezafibrate-treated rats.

Differential Effect of Ciprofibrate Addition to Morris and HepG2 Cells on ACO mRNA Levels. The induction of the fatty ACO gene is widely accepted as a marker of peroxisome proliferation (Bentley et al., 1993). The addition of 0.4 mM ciprofibrate to the incubation medium of Morris cells resulted in a significant induction of ACO mRNA levels with respect to the levels attained in HepG2 cells cultured in the same conditions (Fig. 1). This effect of ciprofibrate on Morris cells was maximal after 3 h of incubation with ciprofibrate, whereas with HepG2 cells, no increase in the levels of ACO mRNA was observed over a 24-h incubation with this drug (data not shown).

Levels of PPARα, PPARβ, and RXRα mRNA in Untreated Morris and HepG2 Cells. There is a very good correlation between PPARα mRNA and protein levels (Lemberger et al., 1996b). Palmer et al. (1998) suggested that the low levels of PPARα mRNA, and thus protein, present in human samples and HepG2 cells are insufficient to compete effectively with other proteins that bind to PPREs. In our experimental conditions, although the ratio between PPARα/RXRα mRNAs was higher in HepG2 cells than in Morris cells, the ratio between PPARβ/RXRα and PPARβ/PPARα mRNAs also was higher in HepG2 cells than in Morris cells (Table 1), pointing to a higher relative expression of the PPARβ isoform in human cells than in cells of rodent origin.

Effect of Ciprofibrate on Binding of Nuclear and Cytosolic Proteins from Morris and HepG2 Cells to an ACO-PPRE Probe. Nuclear and cytosolic extracts, prepared from Morris and HepG2 cells incubated with 0.2 mM ciprofibrate, were subjected to gel-shift analysis with an ACO-PPRE probe. Ciprofibrate triggered a transient increase in the intensity of the shifted bands for the two cell lines studied when using nuclear extracts (Fig. 2). However, in Morris cells the increase in the binding was more marked than in HepG2 cells and furthermore, maximal binding was achieved earlier in Morris (30 min) than in HepG2 cells (3 h). Conversely, with cytosolic extracts the intensity of the shifted bands was decreased in the presence of ciprofibrate in both cell lines. The concentration-dependent effect of ciprofibrate on the binding of nuclear extracts to the ACO-PPRE probe was assayed at incubation times giving maximal response in each

cell line (30 min for Morris cells and 3 h for HepG2 cells). Although the maximum increase in the binding of nuclear extracts obtained from HepG2 cells was 1.5-fold at 0.2 mM ciprofibrate and attained a marginal statistical significance, an almost 4-fold increase at 0.2 mM ciprofibrate was obtained with nuclear extracts from Morris cells (Fig. 3D). The change in the binding of nuclear extracts elicited by ciprofibrate was specific for the ACO-PPRE probe because the binding of the same nuclear extracts from ciprofibrate-treated Morris cells to an unrelated DNA probe, Oct1 (Noé et al., 1997), was not modified (Fig. 3C). As shown by supershift assays performed with specific antibodies (Fig. 4), PPARα and RXRα proteins participate in the formation of the pattern of shifted bands obtained after incubation of nuclear extracts from Morris and HepG2 cells with an ACO-PPRE probe.

Ciprofibrate Effects on Content of Nuclear PPARα and RXRα in Morris Cells. The reciprocal changes observed in the intensity of the shifted bands of nuclear and cytosolic extracts in the presence of an ACO-PPRE probe after ciprofibrate addition (Fig. 2) suggested the possibility of changes in the cellular distribution of PPARα and RXRα proteins elicited by the drug. Indeed, in Morris cells, a clear increase in the nuclear content of PPARα protein was detected after a 30-min incubation with 0.2 mM ciprofibrate, whereas its presence in cytosolic extracts was reduced (Fig. 5). For the RXRα protein, incubation of Morris cells with 0.2 mM ciprofibrate resulted in a 30% reduction in its nuclear content, but the change did not reach statistical significance (Fig. 6). In the same conditions, the amount of RXRα protein in nuclear extracts from HepG2 cells was significantly increased by 70%.

Ciprofibrate Effects Are Shared by Other Fibric Acid Derivatives. Clofibrate, another fibric acid derivative, modified in a concentration- and time-dependent manner the binding of nuclear extracts obtained from Morris cells with an ACO-PPRE probe (Fig. 7, A and B). Furthermore, this effect was accompanied by a 2-fold increase in the ACO mRNA levels after a 9-h incubation with 1 mM clofibrate (data not shown). When similar experiments were performed with HepG2 cells incubated with clofibrate (data not shown) or bezafibrate, the changes in band intensity were smaller and they were maximal after a 3-h incubation (Fig. 7C), reproducing the effects observed after ciprofibrate addition. In these conditions, no change in HepG2 ACO mRNA levels was observed.

In Vivo Effect of Bezafibrate on Binding of Liver Nuclear Extracts to an ACO-PPRE Probe. Nuclear extracts obtained from rat liver bound specifically to the ACO-PPRE probe, producing a pattern of retarded bands in gel-shift assays (Fig. 8A). The treatment of rats with bezafibrate produced an increase in the intensity of the shifted bands, which was maximal after 15 min. The gel-shift corresponding to the maximal increase in binding is shown in Fig. 8B. The increase in the binding produced by bezafibrate was specific for the ACO-PPRE probe because the binding of the same nuclear extracts to the Oct1 probe was not modified. The increase in binding to the ACO-PPRE probe, similar to the one observed in Morris cells after ciprofibrate addition, was followed by a 1.6-fold induction in rat liver ACO mRNA levels after 30 min of bezafibrate administration (Fig. 9).

Sp1 Transcription Factor Is Not Involved in the Different Response to Ciprofibrate of Morris and HepG2 Cells. Because Krey et al. (1995) had described a synergistic effect of Sp1 with the PPAR/RXR heterodimer in the activation of the *aco* promoter, we explored whether Sp1 expression or binding could be involved in the different response of Morris and HepG2 cells to ciprofibrate. Ciprofibrate addition to both Morris and HepG2 cells did not modify either the amount of Sp1 present in nuclear extracts, or the intensity of the retarded bands obtained after incubation of nuclear extracts and a Sp1 DNA probe (Fig. 10). Thus, Sp1 seems not to be involved in the establishment of the reported differences between rat and human cells.

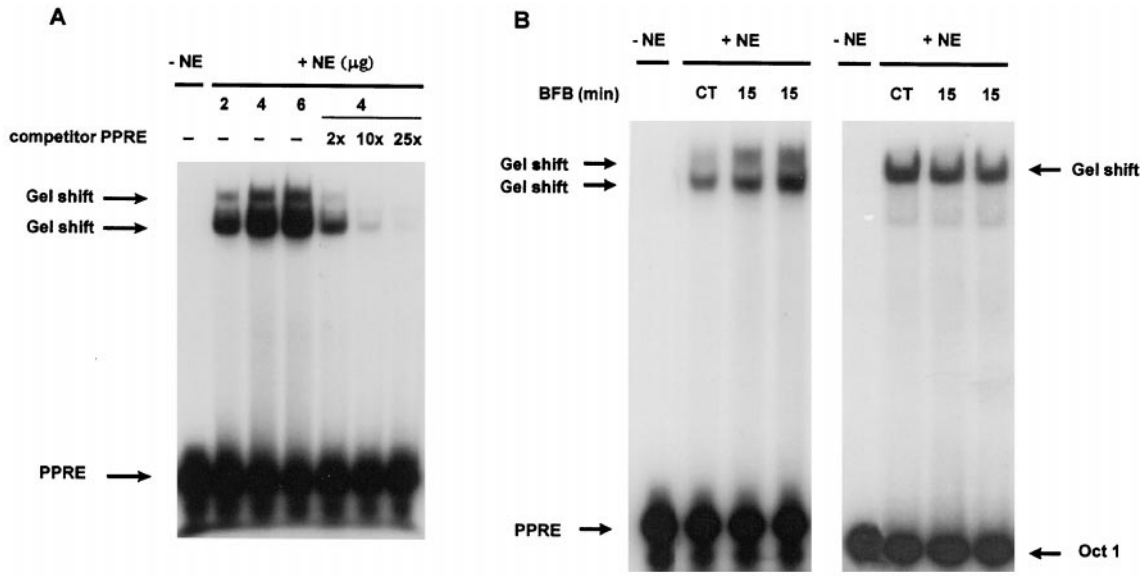

Fig. 8. Effect of bezafibrate on the interaction of an ACO-PPRE probe with hepatic nuclear extracts (NE) obtained from treated Sprague-Dawley rats. A, autoradiography showing the binding of increasing amounts of rat hepatic NE to an ACO-PPRE probe. Also shown is the competition produced by the addition of different excess-fold of the unlabeled probe. Assays were performed in duplicate. B, autoradiography of a representative EMSA performed with liver NE obtained from control (CT) and treated rats after 15 min of bezafibrate administration (69 mg/100 g b.wt.), incubated with a specific ACO-PPRE probe or an unrelated Oct1 probe.

Discussion

In this study, we show that upon incubation with fibrates, nuclear extracts from rat Morris cells bind to an ACO-PPRE probe earlier and more intensely that nuclear extracts from human HepG2 cells. This effect depends both on the concentration of fibrate added and the time of incubation. Furthermore, we show that in the two cell lines, PPARα and RXRα are the transcription factors responsible for the binding of nuclear extracts to the ACO-PPRE probe. Although in both cell lines PPARα and RXRα heterodimerize and interact with their specific response elements, we show that in cells of human origin the increase in nuclear binding triggered by PPARα agonists is weak and unable to produce a proliferative response, as reflected by the lack of induction of the levels of ACO mRNA in HepG2 cells. However, the rapid and intense increase in binding of nuclear extracts from rat hepatoma cells results in an induction of ACO mRNA. This response is very similar to that which we observed in rats after fibrate administration. Therefore, the lack of peroxisome proliferation in liver human cells after fibrate addition is related to the inability of PPAR-RXR complexes to increase their interaction with the PPRE present in the *aco* promoter.

Several transcription factors, such as Sp1 (Krey et al., 1995), chicken ovoalbumin upstream promoter transcription factor (Miyata et al., 1993), hepatocyte nuclear factor-4 (Winrow et al., 1994), thyroid receptor (Chu et al., 1995), LXRα (Miyata et al., 1996), and TAK1/TR4 (Yan et al., 1998), as well as coactivator and integrator proteins (DiRenzo et al., 1997), can modulate the transcriptional activity of PPARs, at least in cells cotransfected with these factors. Thus, a differential interaction of one or several of these proteins in Morris or HepG2 cells upon addition of fibrates could be responsible for the observed differences. However, the absence of changes in the pattern of retardation bands in both types of cells after incubation with fibrates argues against this possibility. In fact, our results on Sp1 abundance in the cell nucleus and its invariable binding to a specific probe exclude this transcription factor as one of the elements involved in the different response of rat Morris and human HepG2 cells to peroxisome proliferators.

The characteristics of the response element, their specific sequence, spatial disposition, and extended 5′ sequence are also determinants in the selectivity and transactivation efficiency of PPARs (Palmer et al., 1995; Ijpenberg et al., 1997; Nakshatri and Bhat-Nakshatri, 1998). We used an identical ACO-PPRE probe for all the experiments performed either with nuclear extracts from rat or human origin, thus minimizing the possible influence of PPRE characteristics in the differences observed.

Human and rat PPARα have a high degree of similarity in their DNA (98%) and ligand (94%)-binding domains, although they are not exactly identical (Mukherjee et al., 1994). It has been reported that a single amino acid change in the ligand-binding domain of PPARα profoundly alters its transcriptional activity (Hsu et al., 1995). Thus, the inherent differences in the amino acid sequences of the PPARα from both species is probably one of the key elements involved in the weak increase in the interaction of HepG2 nuclear extracts with the ACO-PPRE probe, and consequently in the absence of ACO mRNA induction after fibrate addition. The use in our experimental conditions of a fixed PPRE probe to analyze its interaction with nuclear extracts obtained from fibrate-treated cells expressing their own constitutive pool of receptors strongly supports this hypothesis. In agreement with these results, a cDNA for PPARα has been isolated recently from human samples that does not encode a functional PPAR (Palmer et al., 1998). Palmer et al. (1998) further suggested that low levels of PPARα expression in human liver may be at the basis of human unresponsiveness to peroxisome proliferation. Our results indicate that although PPARα levels may be important in determining the response to peroxisome proliferation, it might not be the only determinant factor, given that HepG2 cells remain unresponsive to peroxisome proliferation despite possessing a higher ratio between PPARα/RXRα than Morris cells. Although it can be argued that part of the PPARα mRNA detected may correspond to a recently described truncated form of PPARα with dominant negative activity (Gervois et al., 1999), it is worth mentioning that, in addition, the ratios between PPARβ/RXRα and PPARβ/PPARα mRNAs were higher in HepG2 cells than in Morris cells. This finding is interesting because PPARβ has been reported to be a physiological antagonist of

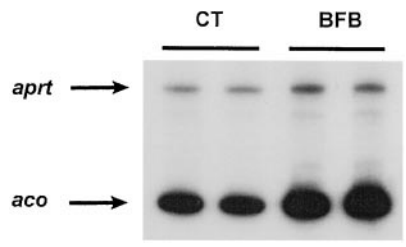

Fig. 9. Effect of bezafibrate administration on rat liver ACO mRNA levels. Autoradiography of a representative RT-PCR assay showing the ACO signal for control (CT) and bezafibrate-treated (BFB) rats (69 mg/100 g b.wt.) after 30 min of drug administration, and the corresponding signals for the APRT mRNA used as a reference. Each line corresponds to a different animal. Fold induction for the ACO signal is 1.4 and 1.7 for the two BFB-treated rats.

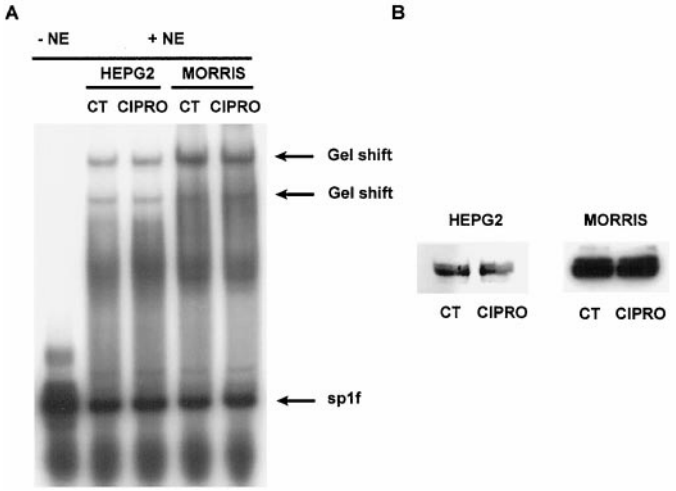

Fig. 10. Sp1 is not involved in the different response to ciprofibrate of Morris 7800 C1 and HepG2 cells. A, autoradiography of a representative EMSA performed by incubating a specific Sp1 probe with nuclear extracts (NE) obtained from control (CT) cells or cells incubated with 0.2 mM ciprofibrate (CIPRO) for 30 min (Morris 7800 C1) or 3 h (HepG2). Two different assays were performed. B, representative Western blot of Sp1 protein in NE of CT cells or cells incubated with 0.2 mM CIPRO for 30 min (Morris 7800 C1) or 3 h (HepG2). Twenty micrograms of protein from each extract was loaded in each lane. Two different assays were performed.

PPARα (Jow and Mukherjee, 1995). A higher expression of the PPARβ isoform in human cells than in cells of rodent origin, at least for the expression of PPARα and its common partner RXRα, could imply an abolition of PPARα activity in HepG2 cells by PPARβ.

RXRα also has been shown to modulate the activity of PPARs; for example, PPARγ, another member of this family of transcription factors (Smith, 1996; Lemberger et al., 1996a), interacts with corepressors NCoR and SMRT in solution, but not when bound to the ACO-PPRE as a heterodimeric complex with RXRα (Yan et al., 1998). The modification in the nuclear content of RXR after fibrate addition, increased in HepG2 and marginally decreased in Morris cells, points also to the involvement of RXR in the mechanism leading to the different response between rat and human cells to peroxisome proliferators.

As a rule, PPARs are classified as subtype II nuclear receptors, exhibiting among other characteristics, a nuclear localization irrespective of their activation state (Schoonjans et al., 1996). Huang et al. (1995) challenged this assumption demonstrating that in CV1 cells transfected with a PPARα construct, the unbound form of this transcription factor was present in the cytosol and migrated to the nucleus upon addition of a PPARα-agonist. However, the overexpressed levels of PPARα in transfected cells could have altered the relative subcellular distribution of this transcription factor. Our results constitute the first experimental evidence for the translocation of PPARα in untransfected cells, being present in the cytosol in the resting state, and migrating to the nucleus after activation with appropriate agonists. In this regard, it has been shown that PPARs are able to bind a 72-kDa heat shock protein (Alvares et al., 1990; Huang et al., 1994). Heat shock proteins are known to act as intracellular chaperones (Watowich and Morimoto, 1988) that play a role in the functionality of glucocorticoid and progesterone receptors (Sánchez et al., 1990; Rexin et al., 1991).

In summary, we present evidence at the molecular level for the reported resistance of human cells to peroxisome proliferation, by describing a deficient interaction of nuclear extracts from human cells with an ACO-PPRE probe upon fibrate addition. Interestingly, these results were obtained in untransfected cells expressing their own basal set of receptors, thus without modification of their proportion of endogenous transcription factors. Also, in untransfected cells, we provide experimental evidence of the translocation of PPARα from the cytosol to the nucleus upon activation by specific agonists.

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References

Alegret M, Ferrando R, Vázquez M, Adzet T, Merlos M and Laguna JC (1994) Relationship between plasma lipids and palmitoyl-CoA hydrolase and synthetase activities with peroxisomal proliferation in rats treated with fibrates. *Br J Pharmacol* **112:**551–556.

Alvares K, Carrillo A, Yuan PM, Kawand H, Morimoto RI and Reddy JK (1990) Identification of cytosolic peroxisome proliferator binding protein as a member of the heat shock protein Hsp 70 family. *Proc Natl Acad Sci USA* **87:**5293–5297.

Bentley P, Calder I, Elcombe C, Grasso P, Stringer D and Wiegand H-J (1993) Hepatic peroxisome proliferation in rodents and its significance for humans. *Food Chem Toxicol* **31:**857–907.

Bradford MM (1976) A rapid sensitive method for quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal Biochem* **72:**248–254.

Cattley RC, DeLuca J, Elcombe C, Fenner-Crisp P, Lake BG, Marsman DS, Pastoor TA, Popp JA, Robinson DE, Schwetz B, Tugwood J and Wahli W (1998) Do peroxisome proliferating compounds pose a hepatocarcinogenic hazard to humans?. *Reg Toxicol Pharmacol* **27:**47–60.

Chu R, Madison LD, Lin Y, Kopp P, Rao MS, Jameson JL and Reddy JK (1995) Thyroid hormone (T3) inhibits ciprofibrate-induced transcription of genes encoding β-oxidation enzymes: Cross talk between peroxisome proliferators and T3 signaling pathways. *Proc Natl Acad Sci USA* **92:**11593–11597.

Dignam JD, Lebovitz RM and Roeder R (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* **11:**1475–1479.

DiRenzo J, Söderström M, Kurokawa R, Ogliastro M-H, Ricote M, Ingrey S, Hörlein A, Rosenfeld MG, Glass CK (1997) Peroxisome proliferator-activated receptors and retinoic acid receptors differentially control the interactions of retinoid X receptors heterodimers with ligands, coactivators and corepresors. *Mol Cell Biol* **17:**2166–2176.

Gervois P, Pineda I, Chinetti G, Grötzinger T, Dubois G, Fruchart J-C, Fruchart-Najib J, Leitersdorf E and Staels B (1999) A truncated human peroxisome proliferator-activated receptor α splice variant with dominant negative activity. *Mol Endocrinol* **13:**1535–1549.

Hsu M-H, Palmer CNA, Griffin KJ and Johnson EF (1995) A single amino acid change in the mouse peroxisome proliferator-activated receptor α alters transcriptional responses to peroxisome proliferators. *Mol Pharmacol* **48:**559–567.

Huang Q, Alvares K, Chu R, Bradfields CA and Reddy JK (1994) Association of peroxisome proliferator-activated receptor and Hsp 72. *J Biol Chem* **269:**8493–8497.

Huang Q, Yeldandi AV, Alvares K, Ide H, Reddy JK and Rao MS (1995) Localization of peroxisome proliferator-activated receptor in mouse and rat tissues and demonstration of its nuclear translocation in transfected CV-1 cells. *Int J Oncol* **6:**307–312.

Ijpenberg A, Jeannin E, Wahli W and Desvergne B (1997) Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (PPAR)/Retinoid X receptor heterodimer binding to DNA. *J Biol Chem* **272:**20108–20117.

Jow L and Mukherjee R (1995) The human peroxisome proliferator-activated receptor (PPAR) subtype NUC1 represses the activation of hPPARα and thyroid hormone receptors. *J Biol Chem* **270:**3836–3840.

Krey G, Mahfoudl A and Wahli W (1995) Functional interactions of peroxisome proliferator-activated receptor, retinoid-X receptor and Sp1 in the transcriptional regulation of the acyl-Coenzyme-A oxidase promoter. *Mol Endocrinol* **9:**219–231.

Laemmli HK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond)* **227:**680–685.

Lee SS-T, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernández-Salguero PM, Westphal H and González FJ (1995) Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferation. *Mol Cell Biol* **15:**3012–3022.

Lemberger T, Desvergne B and Wahli W (1996a) Peroxisome proliferator-activated receptors: A nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol* **12:**335–363.

Lemberger T, Saladin R, Vazquez M, Assimacopoulos F, Staels B, Desvergne B, Wahli W and Auwerx J (1996b) Expression of the peroxisome proliferator-activated receptor α gene is stimulated by stress and follows a diurnal rhythm. *J Biol Chem* **271:**1764–1769.

Miyata KS, McCaw SE, Patel HV, Rachubinski RA and Capone JP (1996) The orphan nuclear hormone receptor LXRα interacts with the peroxisome proliferator-activated receptor and inhibits peroxisome proliferator signaling. *J Biol Chem* **271:**9189–9192.

Miyata KS, Zhang B, Marcus SL, Capone JP and Rachubinski RA (1993) Chicken ovoalbumin upstream promoter transcription factor (COUP-TF) binds to a peroxisome proliferator-responsive element and antagonizes peroxisome proliferator-mediated signaling *J Biol Chem* **268:**19169–19172.

Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Meth* **65:**55–63.

Mukherjee R, Jow L, Noonan D and McDonnell DP (1994) Human and rat peroxisome proliferator activated receptors (PPARs) demonstrate similar tissue distribution but different responsiveness to PPAR activators. *J Steroid Biochem Mol Biol* **3/4:**157–166.

Nakshatri H and Bhat-Nakshatri P (1998) Multiple parameters determine the specificity of transcriptional response by nuclear receptors HNF-4, ARP-1, PPAR, RAR and RXR through common response elements. *Nucleic Acids Res* **26:**2491–2499.

Noé V, Chen C, Alemany C, Nicolás M, Caragol I, Chasin LA and Ciudad CJ (1997) Cell-growth regulation of the hamster dihydrofolate reductase gene promoter by transcription factor Sp1. *Eur J Biochem* **249:**15–20.

Palmer CNA, Hsu M-H, Griffin KJ and Johnson EF (1995) Novel sequence determinants in peroxisome proliferator signaling. *J Biol Chem* **270:**16114–16121.

Palmer CNA, Hsu M-H, Griffin KJ, Raucy JL and Johson EF (1998) Peroxisome proliferator activated receptor-α in human liver. *Mol Pharmacol* **53:**14–22.

Peters JM, Cattley RC and González FJ (1997a) Role of PPARα in the mechanism of action of the nongenotoxis carcinogen and peroxisome proliferator Wy-14,643. *Carcinogenesis* **18:**2029–2033.

Peters JM, Hennuyer N, Staels B, Fruchart J-C, Fievet C, Gonzalez FJ and Auwerx J (1997b) Alterations in lipoprotein metabolism in peroxisome proliferator-activated receptor α-deficient mice. *J Biol Chem* **272:**27307–27312.

Rexin M, Busch W and Gehring U (1991) Protein components of the nonactivated glucocorticoid receptor. *J Biol Chem* **266:**24601–24605.

Sánchez ER, Hirst M, Scherrer LC, Tang H, Welsh MJ, Harmon JM, Simons SS Jr, Ringold GM and Pratt WB (1990) Hormone-free mouse glucocorticoid receptors overexpressed in Chinese hamster ovary cells are localized to the nucleus. *J Biol Chem* **265:**2123–20130.

Schoonjans K, Staels B and Auwerx J (1996) Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. *J Lipid Res* **37:**907–925.

Smith SA (1996) Peroxisomal proliferator activated receptors (PPARs): Molecular targets for hypolipidaemic agents and insulin sensitisers. *Pharmacol Rev Commun* **8:**57–64.

Sohlenius AK, Wigren J, Bäckström K, Andersson K and DePierre JW (1995) Synergistic induction of acyl-CoA oxidase activity, an indicador of peroxisome proliferation, by arachidonic acid and retinoic acid in Morris hepatoma 7800 C1 cells. *Biochim Biophys Acta* **1258:**257–264.

Stang H, Kovacs W, Böck P and Kremser K (1995) Differential induction of peroxisomal enzymes by hypolipidaemics in human (HepG2) and rat (MH1C1) hepatoma cell lines. *Eur J Clin Chem Clin Biochem* **33:**775–783.

Tugwood JD, Holden PR, James NH, Prince RA and Roberts RA (1998) A peroxisome proliferator-activated receptor-alpha (PPARα) cDNA cloned from guinea-pig liver encodes a protein with similar properties to the mouse PPARα: implications for species differences in responses to peroxisome proliferators. *Arch Toxicol* **72:**169–177.

Tugwood JD, Isseman I, Anderson RG, Bundell KR, McPheat WL and Green S (1992) The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl-CoA oxidase gene. *EMBO J* **11:**433–439.

Varanasi U, Chu R, Huang Q, Castellon R, Yeldandi AV and Reddy JK (1996) Identification of a peroxisome proliferator responsive element upstream of the human peroxisomal fatty acyl-coenzyme A oxidase gene. *J Biol Chem* **271:**2147–2155.

Watowich SS and Morimoto RI (1988) Complex regulation of heat shock- and glucose-responsive genes in human cells. *Mol Cell. Biol* **8:**393–405.

Winrow CJ, Marcus SL, Miyata KS, Zhang B, Capone JP and Rachubinski RA (1994) Transactivation of the peroxisome proliferator-activated receptor is differentially modulated by hepatocyte nuclear factor-4. *Gene Expression* **4:**53–62.

Yan ZY, Karam WG, Staudinger JL, Medvedev A, Ghanayem BI and Jetten AM (1998) Regulation of peroxisome proliferator activated receptor α-induced transactivation by the nuclear orphan receptor TAK1/TR4. *J Biol Chem* **273:**10948–10957.

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