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Induction of the peroxisome proliferator activated receptor by fenofibrate in rat liver

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The process of peroxisome proliferation in rodent liver by hypolipidemic compounds and related substances has recently been shown to be receptor-mediated. In the present study, we have examined the effect of oral administration of the strong peroxisome proliferator fenofibrate on the hepatic expression level of the peroxisome proliferator activated receptor (PPAR) in rats. Immunoblots of rat liver cytosols and nuclear extracts using antibodies raised against recombinant PPAR/β-galactosidase fusion proteins revealed a pronounced increase in the amount of PPAR protein in response to fenofibrate treatment. This induction could also be confirmed at the level of RNA by Northern blotting. A time-course investigation showed a delayed accumulation of mRNA in response to the treatment, starting on day 2 after a latency period of at least one day. Thus, induction of the PPAR as a response to peroxisome proliferators represents one important dimension of the pleiotropic effects of peroxisome proliferators.

PPAR; Fusion protein; Antibody; mRNA; Hypolipidemic compound

1. INTRODUCTION

Peroxisome proliferation is a process frequently observed in the livers of rats and mice in response to the administration of a specific class of non-genotoxic carcinogens, the so-called peroxisome proliferators (e.g. fibrates such as clofibrate, phthalates such as diethylhexylphthalate, tiadenol) [1]. After oral administration, these substances evoke a pleiotropic response in the main target organ liver, including an increase of the number and size of peroxisomes, induction of peroxisomal and non-peroxisomal enzymes, liver hypertrophy and hyperplasia, and, as a long-term effect, hepatocarcinogenesis [2–5]. Because of the structural diversity of the different peroxisome proliferators, the existence of a specific receptor mediating their effects was a matter of controversy for several years [6–8]. In 1990, the cloning of a gene belonging to the steroid hormone receptor family was reported and its expression product was proven to enhance the transcription of respective genes in response to the application of peroxisome proliferators [9,10]. However, no direct binding of these compounds to this new receptor has been shown until now. With respect to these findings this protein has been termed peroxisome proliferator activated receptor (PPAR). Recently, the identification of three closely related members of the hormone receptor superfamily that share extensive homology with the mouse PPAR has been reported for *Xenopus laevis* [11]. All the three new receptors seem to be stimulated in their biological activity upon addition of peroxisome proliferators to the test system.

To further investigate the signal cascade leading to the multiple effects in the liver after application of peroxisome proliferators, we decided to raise antibodies against the PPAR in order to obtain a direct specific probe for the receptor. In the present paper, we report on the preparation of a PPAR-specific antiserum and its use for the detection of PPAR protein in different preparations of rat livers. In particular, evidence for the induction of the PPAR by fenofibrate, a strong peroxisome proliferator, is presented.

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediamine tetraacetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PPAR, peroxisome proliferator activated receptor; SDS, sodium laurylsulfate.

2. MATERIALS AND METHODS

2.1. *Materials*

Fenofibrate was provided by Laboratoires Fournier (Fontaine les Dijon, France). Primer oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia, Freiburg, Germany). *Taq* polymerase was obtained from Perkin-Elmer (Überlingen, Germany). The first strand cDNA synthesis kit was purchased from Pharmacia (Freiburg, Germany). The nonradioactive DNA labeling and detection kit, 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate were obtained from Boehringer Mannheim (Mannheim, Germany). Biodyne A and Fluorotrans membranes, the second antibody and the blue marker were from Pall (Dreieich, Germany), Sigma (Deisenhofen, Germany) and Biorad (München, Germany), respectively.

2.2. *Generation of PPAR-specific antisera*

Total RNA was isolated from pooled livers of adult male BALB/c and C57BL/6N mice according to Chomczynski and Sacchi [12], and PCR [13] (thirty cycles, 1 min 94°C, 1 min 48°C, 3 min 72°C, each) was performed after mRNA selection and reverse transcription, using the oligonucleotides AACCCGGGACAATGAACCCTTTC and TTCCCGGGCGGAGTGAAGA as PPAR-specific 5'-sense- and 3'-antisense-primer. The *Eco*RI/*Kpn*I (FP 1) and *Bgl*II/*Bal*I (FP 2) restriction fragments of the amplified PPAR cDNA were fused to the *lacZ* gene of the bacterial expression vector pEX2 as detailed in Fig. 1. After transformation of these constructs into the *E. coli* strain pop 2136, the corresponding β-galactosidase/PPAR fusion proteins were obtained and purified essentially as described [14,15]. New Zealand white rabbits were immunized with the obtained preparations according to standard procedures to yield two polyclonal antisera.

2.3. *Sample preparation*

For preparation of cytosols and nuclear extracts, male rats were fed a diet containing 0.25% (w/w) fenofibrate for seven days. For preparation of RNA, male rats were fed the same diet for one to twelve days. All animals were starved 12 h before sacrifice except for those treated for one day only. Livers were excised after cervical dislocation and were either frozen immediately in liquid nitrogen to preserve them for the isolation of RNA or processed directly to obtain cytosols and nuclear extracts as follows: livers were homogenized in three volumes of homogenization buffer (20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 25 mM KCl, 1 mM PMSF, 1 mM DTT and 10% (v/v) glycerol) using 10 strokes of a drilling Teflon pestle. The obtained homogenates were centrifuged for 5 min at 1,000 × *g*. After resuspension of the pellets in homogenization buffer (three volumes), centrifugation was repeated to remove remainders of cytosol. The resulting sediments were resuspended in homogenization buffer (two volumes) containing 0.4 M NaCl, using a glass homogenizer. The suspensions were stirred gently for 20 min on ice with a magnetic stir bar followed by centrifugation at 100,000 × *g* for 1 h. The recovered supernatants representing the nuclear extracts were frozen in liquid nitrogen and stored at −80°C until use. The 1,000 × *g* supernatants of the first centrifugation were centrifuged at 10,000 × *g* for 15 min and the resulting supernatants were centrifuged at 100,000 × *g* for 1 h to yield the cytosolic fractions that were preserved and stored like the nuclear extracts. RNA was prepared from the frozen livers as detailed by Chomczynski and Sacchi [2].

2.4. *Analytical procedures*

Protein concentrations were determined according to Bradford [16]. Cytosols and nuclear extracts were subjected to SDS-PAGE on 8–25% gradient gels using the Phast System (Pharmacia). Subsequent electrotransfer to Fluorotrans membranes was performed on the same apparatus according to the manufacturers recommendations for 3 Vh. Immunodetection was carried out by the method of Towbin et al. [17] using one of the PPAR-specific antisera (1:200) as the first antibody and an alkaline phosphatase-coupled second antibody (1:1000). 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate served as chromogenic substrates for the final visualization.

Total RNAs were run on 1% denaturing agarose gels containing formaldehyde and were subsequently transferred to Biodyne A membranes by standard capillary blotting [18]. PPAR mRNA was detected by hybridization with a digoxigenin-labelled PPAR-cDNA (50 ng/ml) followed by immunostaining according to the protocol of the kit supplier. Prehybridization was carried out at 55°C for 4 h in a solution containing 10× Denhardt's (aqueous solution of Ficoll, polyvinylpyrrolidone and bovine serum albumin, 0.2% each), 2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 0.5% SDS, 0.01 M EDTA and 100 μl/ml salmon sperm DNA. After addition of the denatured probe hybridization was continued overnight under the same conditions. The filters were washed in 2 × SSC, 0.5% SDS (2 times, 15 min each) at room temperature and 0.1 × SSC, 0.5% SDS (2 times, 15 min each) at 55°C and subjected to immunostaining. Color development was allowed overnight.

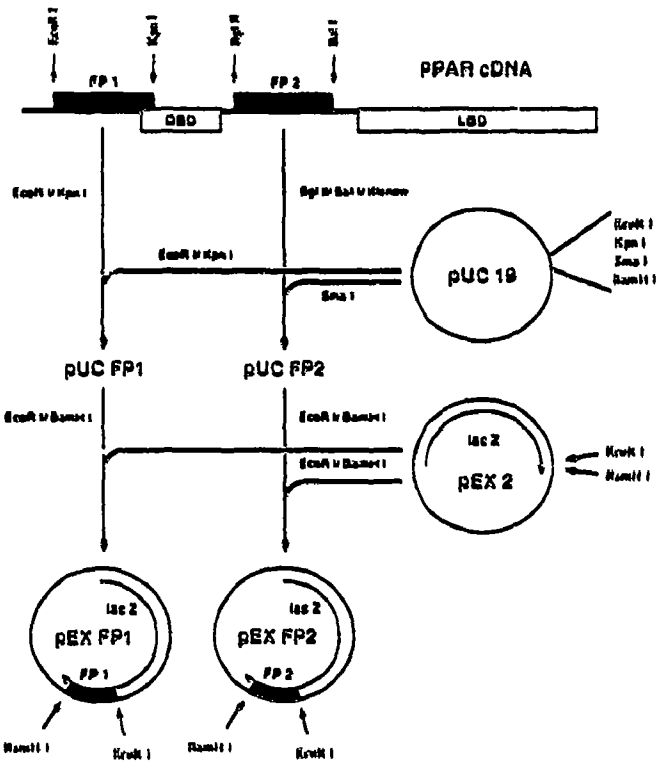

Fig. 1. Construction of the PPAR-expression vectors. Fragments FP 1 and FP 2 of the PPAR cDNA were ligated into the *Eco*RI/*Bam*HI-site located in the 3'-terminal region of the *lacZ* gene of the bacterial expression vector pEX2. To yield compatible ends both fragments were first cloned into the plasmid pUC 19. FP 1 was obtained by *Eco*RI/*Kpn*I digest of the PPAR cDNA and was set into the *Eco*RI/*Kpn*I-site of the plasmid. FP 2 was obtained by *Bgl*II/*Bal*I digest of the PPAR cDNA and was ligated into the *Sma*I-site of the plasmid after blunt-end generation using the Klenow fragment of DNA polymerase I. Separate restriction digests of both constructs with *Eco*RI/*Bam*HI resulted in fragments that could be integrated into the *Eco*RI/*Bam*HI-site of pEX2 in the correct orientation and frame to yield β-galactosidase/PPAR fusion proteins on induction. DBD, putative DNA binding domain; LBD, putative ligand binding domain.

3. RESULTS

The amplification of the PPAR cDNA starting from reverse transcribed mRNA of mouse liver resulted in the isolation of the expected 1.4 kb cDNA fragment in good yields (2–3 μg per 50 μl). The *Eco*RI/*Kpn*I-fragment (FP 1) and the *Bgl*II/*Bal*I-fragment (FP 2) of this cDNA were chosen for the expression in bacteria because both show low homology to the corresponding sequences of other members of the steroid hormone receptor family. After expression as β-galactosidase fusion proteins and subsequent purification, these preparations were used to raise antibodies in rabbits. The resulting antisera both displayed high titers towards the fusion proteins. However, only the FP 2-antiserum showed specific immunoreactions with rat liver proteins. Thus, only experiments using the FP 2-antiserum are reported in the following. Immunoblot analysis of hepatic cytosols (Fig. 2) and nuclear extracts (data not

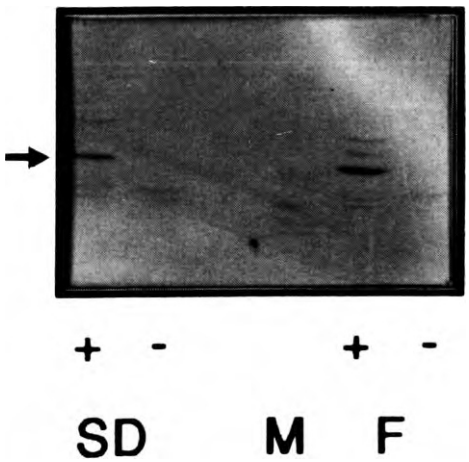

Fig. 2. Rat liver cytosols (10 μg per lane) were electrophoresed, blotted and immunostained with the FP 2 antiserum as described in Section 2. SD, male Sprague-Dawley rats; F, male Fischer rats; +, fenofibrate-treated; −, untreated; M, prestained molecular weight marker (106 kDa, 80 kDa, 49.5 kDa, 32.5 kDa, 27.5 kDa, 18.5 kDa). The arrow marks the position of the PPAR (53 kDa).

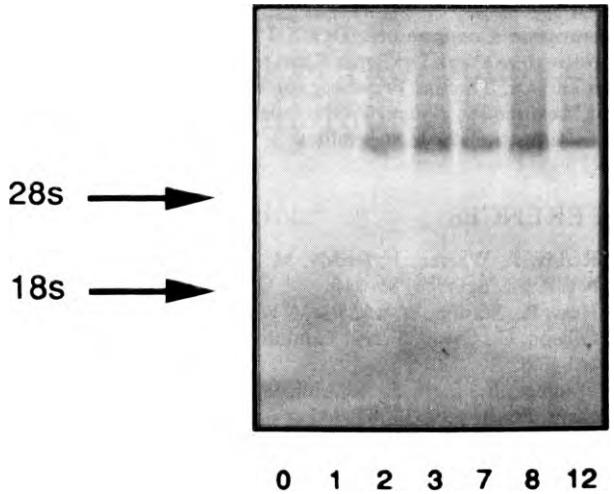

Fig. 3. Northern blot of liver RNA from fenofibrate-treated rats. Rat liver total RNA (10 μg per lane) was electrophoresed, blotted and hybridized with a digoxigenin-labelled PPAR cDNA probe as described in Section 2. The figure below each lane gives the duration of the fenofibrate treatment of the respective rat in days. The arrows mark the positions of the ribosomal RNAs.

shown) obtained from untreated rats did not show specific signals in the expected molecular weight range of 52–53 kDa. Instead, two moderately immunoreactive proteins with apparent molecular weights of about 33 and 38 kDa were detected.

In contrast, cytosols as well as nuclear extracts prepared from rats after fenofibrate treatment for one week displayed a strongly immunoreactive species with an apparent molecular weight of about 53 kDa. In addition, a number of less prominent bands appeared in the higher molecular weight range. A typical result with rat cytosol is shown in Fig. 2.

Northern blot analysis of rat hepatic RNA from animals that were treated with fenofibrate for 0–12 days indicated a low constitutive expression of PPAR mRNA in rat liver. After a latency period of at least one day the level of PPAR mRNA was significantly increased and the signal remained enhanced for all 12 days (Fig. 3). Surprisingly, we determined the size of the rat PPAR mRNA to be close to 6 kb whereas the size of mouse PPAR mRNA was reported to be 1.8 and 2 kb [9].

4. DISCUSSION

Starting from the published cDNA sequence of the mouse PPAR [9] we have generated fusion proteins and with their aid antisera against defined regions of the protein that we have designated FP 1 and FP 2. Comparison of the deduced amino acid sequence of mouse PPAR to those of the different *Xenopus* PPAR-related nuclear hormone receptors that have recently been cloned [11] revealed a sequence homology of FP 2 among the mPPAR and the orthologous *Xenopus* gene xPPARα of 82% from which an interspecies immunological cross-reaction may reasonably be expected. At the same time, the homology to the other members of this gene subfamily does not exceed 50%. Therefore, we conclude that the 53 kDa protein that strongly interacts with our FP 2 antiserum is indeed the rat PPAR. The nature of the other, less prominent signals obtained with this antiserum is yet unclear. Like the PPAR, most of them appear to be fenofibrate-treatment dependent and we thus speculate that they might represent either processed variants of the PPAR or other members of a putative PPAR-family [11].

The FP 1 protein sequence homology among mPPAR and xPPARα of only 53% suggests a lower degree of conservation of the PPAR N-terminus, which could serve as an explanation for the failure of our FP 1-antiserum (anti-mouse PPAR) to detect the rat PPAR protein.

Our present data clearly indicate the induction of the PPAR in rat liver on the level of mRNA and protein on administration of fenofibrate, a strong peroxisome proliferator, while the constitutive expression appeared to be very low. The transcription-stimulating activity of the PPAR is significantly enhanced by peroxisome proliferators as clearly demonstrated by Green and coworkers [9,10]. However, they also reported on some basal transcriptional activity of the PPAR in the absence of peroxisome proliferators that was apparently dependent on the intracellular concentration of PPAR [10]. Since the responsive elements of the different genes that are targets for the PPAR may be differentially susceptible to this receptor in the presence and absence of its ligand, the level of receptor expression could represent a second dimension in the regulation of PPAR-dependent gene transcription.

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