Thyromimetic mode of action of peroxisome proliferators: activation of 'malic' enzyme gene transcription

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Peroxisome proliferators induce thyroid-hormone-dependent liver activities, e.g. 'malic' enzyme, mitochondrial glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase, S14 [Hertz, Aurbach, Hashimoto and Bar-Tana (1991) *Biochem. J.* **274**, 745–751]. Here we report that the thyromimetic effect of peroxisome proliferators with respect to 'malic' enzyme results from transcriptional activation of the 'malic' enzyme gene, mediated by binding of the peroxisome proliferator activated receptor (PPAR α)/retinoid X receptor (RXR α) heterodimer to a 5'-flanking enhancer of the 'malic' enzyme promoter. The

enhancer involved is distinct from the thyroid hormone response element of the 'malic' enzyme promoter and is partly homologous with that which mediates transcriptional activation of peroxisomal acyl-CoA oxidase by peroxisome proliferators. Hence transcriptional activation of thyroid-hormone-dependent liver genes by xenobiotic or endogenous amphipathic carboxylates collectively defined as peroxisome proliferators is mediated by a transduction pathway similar to that involved in transcriptional activation of peroxisomal β -oxidative genes and distinct from that which mediates thyroid hormone action.

INTRODUCTION

Amphipathic carboxylates known collectively as peroxisome proliferators (PPs) (e.g. fatty acids, xenobiotic aryloxyalkanoic acids, xenobiotic substituted dicarboxylic acids) induce liver peroxisomal β -oxidation as well as microsomal cytochrome *P*-450₄-catalysed ω -oxidation of long-chain fatty acids (reviewed in [1]). Induction of these activities involves transcriptional activation of peroxisomal β -oxidative genes [2,3] and cytochrome *P*-450₄ [4] mediated by binding of the peroxisome proliferator activated receptor (PPAR α) [5] or the PPAR α /retinoid X receptor (RXR α) heterodimer [6] to defined 5'-flanking enhancers of these genes [3,4,7].

In addition to regulating fatty acid β/ω -oxidation, PPs induce a calorogenic effect which, like that exerted by thyroid hormones [8–10], is characterized by a substantial reduction in liver phosphate and redox potentials [11]. Furthermore xenobiotic PPs induce in euthyroid and hypothyroid rats, or in rat hepatocytes cultured in thyroid-hormone-free media, liver activities classically considered to be thyroid-hormone-dependent, e.g. 'malic' enzyme, mitochondrial glycerol-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase and S14 [12,13]. The hepatic thyromimetic activity of PPs is not mediated by modulating plasma thyroid hormone levels or by PP binding to the thyroid hormone nuclear receptor [11,12]. Thus thyroid hormone binding to isolated liver nuclei or nuclear extract was competitively displaced by some but not all PPs capable of inducing the above liver activities [12]. Furthermore, in contrast with the thyromimetic effect induced by PPs in liver cells, no PP-induced increase in growth hormone was observed in cultured GH1 pituitary cells [12] and no increases in heart 'malic' enzyme or mitochondrial glycerophosphate dehydrogenase were observed in PP-treated animals under conditions where thyroid hormones were active [11], thus leaving open the question concerning the transduction pathway mediating the liver-specific thyromimetic effect induced by PPs.

To elucidate further the mode of action of PPs as inducers of liver thyroid-hormone-dependent genes, the induction of 'malic' enzyme by these agents has now been further analysed in order to define the elements of the transduction pathway that mediate the liver thyromimetic activity of PPs. Liver 'malic' enzyme was chosen as a model system because its established response to thyroid hormone is well characterized in terms of the ligand, receptor and response element involved [14–18].

MATERIALS AND METHODS

Animals

Euthyroid male albino rats weighing 150–200 g were fed with laboratory powdered chow diet containing the PPs specified. Food intake remained unaffected by the PPs added to the chow diet as specified. For hormonal induction, animals were injected intraperitoneally with 15 μ g of tri-iodothyronine (T_3)/100 g body weight per day [12]. Animal studies were conducted in accord with humane animal care principles and procedures.

Cultured cells

Primary rat hepatocytes were isolated as previously described [12] and cultured on 60 mm Falcon culture dishes in RPMI 1640 medium containing 10% charcoal-treated fetal calf serum, 100 m-units/ml insulin, 10 μ g/ml cortisol, 50 μ g/ml streptomycin sulphate and 50 μ g/ml penicillin G. CV-1 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco).

'Malic' enzyme activity

Liver homogenate was prepared in 4 vol. of 0.25 M sucrose. 'Malic' enzyme activity (EC 1.1.1.40) was assayed in the 100 000 g cytosolic fraction as previously described [12].

Abbreviations used: AOX, acyl-CoA oxidase; CAT, chloramphenicol acetyltransferase; PP, peroxisome proliferator; PPAR α , peroxisome proliferator activated receptor α ; PPRE, peroxisome proliferator response element; RXR α , retinoid X receptor α ; T_3 R, thyroid hormone receptor; T_3 , tri-iodothyronine; TRE, thyroid hormone response element; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol.

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RNA preparation and analysis

Rat liver total RNA was prepared as previously described [12] and 25 µg was subjected to Northern-blot analysis using Gene-Screen membranes (DuPont–NEN). Rat 'malic' enzyme mRNA was determined using the 1000 bp *EcoRI* restriction fragment of pME6 [14] ³²P-labelled by the random-priming method.

Run-on transcription

Rat liver nuclei were isolated as described [19] and stored at –70 °C in storage buffer containing 50 % (w/v) glycerol, 50 mM Hepes, pH 7.8, 1 mM MgCl₂, 4 mM MnCl₂ and 0.1 mM EDTA. Transcription assays were carried out in 0.2 ml of buffer containing 25 % glycerol, 50 mM Hepes, pH 7.5, 50 mM NaCl, 2.5 mM MgCl₂, 0.05 mM EDTA, 5 mM diethiothreitol (DTT), 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP, 2 mM phosphocreatine, 2 µg creatine kinase, 40 units of RNasin, 0.1 mM PMSF and 250 µCi of [³²P]UTP, using nuclei samples amounting to 4.0 absorbance units (260 nm). Incubation was carried out at 26 °C for 30 min; 20 units of RNase-free DNase I was then added and then 30 µg of proteinase K. Newly synthesized [³²P]RNA from three pooled liver nuclei preparations was extracted and hybridized for 48 h at 42 °C with either 1 µg of 'malic' enzyme cDNA (*Bam*HI + *Bgl*II) restriction fragments of pMEB [16] or 2 µg of peroxisomal acyl-CoA oxidase (AOX) cDNA (pMJ125) [20] affixed to Nytran in 1 ml of 50 % formamide containing 50 mM Tris/HCl, pH 8.0, 10 mM EDTA, 6 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 1 % SDS, 0.12 % Ficoll, 0.12 % polyvinylpyrrolidone, 0.12 % BSA and 300 µg of salmon sperm DNA. The extent of hybridization was normalized to the signal obtained with β-actin cDNA. Hybridization to puc19 served as background. The washed blots were autoradiographed and quantified by densitometry.

Transfection assays

Primary rat hepatocytes cultured for 6 h as described above were washed in serum-free medium and transfected for 16 h with 15 µg of the reporter plasmid, 3 µg of the respective expression plasmid DNAs and 50 µg of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulphate in 4 ml of serum-free medium. After transfection the cells were washed and treated with the respective ligands for an additional 48 h and then scraped off, sonicated and centrifuged to remove cell debris. Chloramphenicol acetyltransferase (CAT) activity normalized by protein content was determined as described by Jacoby et al. [21].

CV-1 cells cultured as described above were plated on to 25 cm² flasks in DMEM supplemented with 10 % charcoal-treated fetal calf serum. The following day the medium was replaced and cells were transfected with CsCl-purified plasmid DNA added by calcium phosphate precipitation. The β-galactosidase expression vector pRS GAL (2 µg) added to each precipitate served as an internal control for transfection. Cells were transfected for 6 h, washed and treated with ligands added as 1000 × stocks in DMSO for 42 h. Cell extracts were prepared by freeze-thawing and assayed for β-galactosidase and CAT activities. Transfection efficiencies were normalized to β-galactosidase activity.

DNase I footprinting

The 169 bp fragment containing the (–590/–422)ME promoter sequence was prepared by cleavage of the (–590/–422)ME-TK-CAT plasmid with either *Hind*III or *Bam*HI depending on

the strand to be labelled. The DNA was then labelled by incorporating [α -³²P]dCTP using the Klenow fragment of DNA polymerase I. The labelled DNA was then digested with either *Bam*HI or *Hind*III to yield the respective strands labelled at one end. End-labelled 169 bp fragments were isolated by agarose-gel electrophoresis. DNA-binding reactions were carried out in 50 µl containing 5.5 mM Hepes, pH 7.4, 1.4 mM MgCl₂, 84 mM KCl, 0.55 mM DTT, 5.5 % glycerol and 3 µg of poly(dI-dC). Some 25 µg of COS-7 extract transfected with either control or expression plasmids for PPARα and RXRα were added where stated. The mixture was incubated at 0 °C for 20 min. Labelled DNA (5 × 10⁴ c.p.m., ~2 ng) was added and the mixture was further incubated at room temperature for 15 min. DNase digestion was carried out by adding 5 µl of a mixture containing 1 unit/ml DNase I and 40 mM CaCl₂ and the mixture was further incubated at 0 °C for 3 min. Digestion was terminated by adding 100 µl of stop buffer containing 100 mM Tris/HCl, pH 7.6, 15 mM EDTA, 100 mM NaCl, 0.3 % SDS, 30 µg/ml salmon sperm DNA and 100 µg/ml proteinase K. After incubation at 37 °C for 30 min, samples were extracted with phenol/chloroform and precipitated with ethanol. The pellets were resuspended in 9 µl of formamide-containing buffer and loaded on to a 6 % polyacrylamide/8M urea sequencing gel. DNase I cleavage sites that were protected from digestion were identified using chemical cleavage sequencing reactions of the same labelled fragments.

Gel mobility-shift assays

Gel mobility-shift assays were carried out with *in vitro* synthesized PPARα and RXRα [22] or with whole COS extracts overexpressing PPARα and RXRα. Binding was carried out in 20 µl of Hepes, pH 7.9, containing 50 mM KCl, 1 mM DTT, 2.5 mM MgCl₂, 10 % glycerol and 1 µg of poly(dI-dC). The binding reaction mixture was preincubated on ice for 30 min and then 0.1 ng of the respective [³²P]oligonucleotide was added and the reaction was further incubated at room temperature for another 20 min. The reaction was subjected to electrophoresis on pre-cooled prerun (30 min, 200 V) polyacrylamide gel. Protein–DNA complexes were resolved on a 5 % non-denaturing polyacrylamide gel in 0.5 × Tris/borate/EDTA. Electrophoresis was carried out at 300 V at 4 °C. Gels were dried and autoradiographed at –70 °C.

For the preparation of COS extracts overexpressing PPARα and/or RXRα, COS-7 cells were seeded into 100 cm² flasks in DMEM supplemented with 10 % fetal calf serum. The following day, cells were transfected for 5 h with 20 µg of pSG5, pSG5-PPARα, pSG5-RXRα or both expression plasmids added by calcium phosphate precipitation, washed, glycerol-shocked and incubated for 48 h. After incubation, cells were harvested, washed with PBS and lysed by three cycles of freezing–thawing in 100 µl/plate lysis buffer (600 mM KCl, 10 mM Tris/HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 1 µg/ml pepstatin and 1 µg/ml leupeptin). The lysate was centrifuged at 20000 g for 15 min and the supernatants divided into aliquots and stored at –70 °C.

For the preparation of *in vitro* synthesized PPARα or RXRα, DNAs of mouse PPARα and human RXRα cloned in pSG5 were linearized by cleavage with *Xba*I for *in vitro* transcription with T7 RNA polymerase (Stratagene). Translation in rabbit reticulocyte lysates was as directed by the supplier (Promega).

Molecular probes and plasmid constructs

'Malic' enzyme mRNA was determined using the 1 kb *EcoRI* restriction fragment of the recombinant DNA pME6 [14].

Peroxisomal AOX mRNA was probed by using the PMJ125 plasmid [20] as previously described [12]. The (–882/+1)ME-CAT construct consisting of the 882 bp 5'-flanking region of the 'malic' enzyme gene and 5'-deletions of this fusion construct were prepared as previously described [15,18]. (–467/+1)ME-CAT and (–444/+1)ME-CAT 5'-deletion constructs were prepared by fusing synthetic oligonucleotides containing the (–467/–427)ME or (–444/–427)ME promoter sequences to (–426/+1)ME-CAT obtained by the *NspV* restriction site at –426. (–590/–422)ME-TK-CAT was prepared by inserting the (–590/–422)ME promoter sequence cut by *ScaI*(–590) and *NspV*(–426) into a modified pBLCAT3 [23] containing a *NurI* and a *NspV* restriction site in its polylinker. A *SphI*–*XbaI* restriction fragment containing the 'malic' enzyme *ScaI*–*NspV* promoter sequences was inserted into pBLCAT2 [23]. ME-PPRE-TK-CAT was prepared by inserting the synthetic oligonucleotide encompassing the (–465/–437)ME promoter sequence (5'-ACTGGACCTGTGCCCTTAACACCTTTT-3'), flanked by *HindIII* (5') and *BamHI* (3') restriction sites, into pBLCAT2. (–287/–261)ME-TK-CAT was prepared as previously described [18]. PSG5-mPPAR α expression plasmid [7] was obtained from S. Green (ICI). c-erbA α expression plasmid [24] was obtained from H. Towle (University of Minnesota, Minneapolis, MN, U.S.A.). pSG5-RXR α expression plasmid was constructed by inserting the *EcoRI* fragment of RS-hRXR α [22] (R. Evans, Howard Hughes Medical Institute, La Jolla, CA, U.S.A.) into the *EcoRI* site of pSG5.

Statistics

Significance of differences was analysed by the Mann–Whitney *U* test.

RESULTS

The induction of liver 'malic' enzyme activity by two different PPs, an aryloxyalkanoic fibrate drug [25] (bezafibrate) and a substituted non-oxidizable long-chain dioic acid [26] (Medica 16), was accounted for by a daily increase (1–3 days) in liver 'malic' enzyme mRNA content (Table 1). Liver 'malic' enzyme activity and mRNA induced by bezafibrate were higher than those induced by Medica 16 (Table 1) and similar in extent to that induced by thyroid hormone treatment [12,16].

The increase in 'malic' enzyme activity and mRNA induced by PPs could be accounted for by a respective increase in 'malic' enzyme transcription rate as verified in rat liver nuclei of rats

Figure 1 Transcriptional activation of the 'malic' enzyme gene by PPs

Run-on transcription rates of 'malic' enzyme and peroxisomal AOX genes were determined as described in the Materials and methods section in isolated liver nuclei from non-treated rats or rats treated for 5 days with either 0.25% (w/w) Medica 16 or 0.2% (w/w) bezafibrate in their diet or injected daily with 15 μ g of T_3 /100 g body weight. Newly synthesized 32 P-labelled RNA from nuclei pooled from three rats was hybridized with 'malic' enzyme (ME), AOX or β -actin cDNA and the relative densities are indicated on the left. One representative experiment out of three independent ones is shown.

treated for 5 days with T_3 , Medica 16 or bezafibrate (Figure 1). Bezafibrate was again more effective than Medica 16 and of the same potency as T_3 . Induction of 'malic' enzyme transcription by PPs is in line with our previous findings on the increase in liver S14 transcription rate induced by an aryloxyalkanoic fibrate (Nafenopin) and Medica 16 [12]. It is noteworthy, however, that liver peroxisomal AOX transcription remained unaffected in T_3 -treated animals [12] (Figure 1), thus indicating that whereas PPs are thyromimetic, thyroid hormones do not act as PPs. Hence different transduction pathways must be involved in transcriptional activation exerted by thyroid hormones and PPs.

To identify elements of the transduction pathway involved in transcriptional activation of the 'malic' enzyme gene by PPs and in the light of the role played by PPAR α in mediating transcriptional activation of peroxisomal genes by PPs, the putative role of PPAR α in mediating transcriptional activation of the 'malic' enzyme gene by PPs was studied by following the expression of a CAT reporter plasmid promoted by the 882 bp 5'-flanking region of the 'malic' enzyme gene and transfected into cultured primary rat hepatocytes together with an expression vector for either PPAR α or T_3 R (Figure 2). Expression of the (–882/+1)ME-CAT construct was enhanced about 4-fold by PPAR α even in the absence of added PPs and further enhanced about 3-fold in the presence of added fibrate drugs. The increase in expression as a function of PPAR α and PPs was similar to that

Table 1 Liver 'malic' enzyme activity and mRNA induced by PPs

Liver 'malic' enzyme activity and 21S mRNA were determined as described in the Materials and methods section in rats treated for 1–3 days with either 0.25% (w/w) Medica 16 or 0.2% (w/w) bezafibrate in their diet. mRNA results are presented in densitometric units relative to values for untreated rats (day 0). Values are means $\pm$ S.D. ($n = 3$). *Significantly different from the respective untreated value: $P < 0.05$.

Day	'Malic' enzyme activity (m-units/mg of protein)		'Malic' enzyme mRNA (densitometric units)	
	Medica 16	Bezafibrate	Medica 16	Bezafibrate
0	22.0 $\pm$ 2.8	22.0 $\pm$ 2.8	1.0 $\pm$ 0.1	1.0 $\pm$ 0.1
1	18.0 $\pm$ 2.3	31.0 $\pm$ 3.8*	3.0 $\pm$ 1.8	19.0 $\pm$ 6.0*
2	25.5 $\pm$ 4.7	55.6 $\pm$ 9.0*	5.2 $\pm$ 0.9*	27.0 $\pm$ 5.0*
3	35.0 $\pm$ 5.0*	50.5 $\pm$ 9.0*	5.0 $\pm$ 0.7*	27.0 $\pm$ 3.0*

Figure 2 Activation of the 'malic' enzyme promoter by PPAR α in cultured primary hepatocytes

Cultured rat hepatocytes were transfected as described in the Materials and methods section with (–882/+1)ME-CAT construct and co-transfected with either c-erbA α or pSG5-PPAR α expression plasmids; transfected cells were then incubated in the absence or presence of T_3 (2×10^{-7} M), Nafenopin (1×10^{-4} M) or Wy14,643 (1×10^{-4} M) as indicated.

Figure 3 Transactivation of ME-CAT constructs by PPARα/RXRα

Top, CV-1 cells were transfected as described in the Materials and methods section with a series of 5' deletions of the (–882/+1)ME-CAT gene (8 µg) and co-transfected with the parent vector pSG5 (4 µg), pSG5-PPARα (2 µg), pSG5-RXRα (2 µg) or both expression vectors for PPARα and RXRα as indicated. After transfection, cells were treated with 0.1% (v/v) of the DMSO vehicle (□), 200 µM bezafibrate (▨) or 130 µM Medica 16 (■). CAT activity is presented as its fold induction, where the activity of the (–882/+1)ME-CAT construct transfected with pSG5 only and incubated in DMSO vehicle is taken as 1.0. One representative experiment of four independent ones, each carried out in duplicate, is shown. Bottom, CV-1 cells were transfected with a series of 5' deletions of the (–590/+1)ME-CAT construct (8 µg) and co-transfected with pSG5, pSG5-PPARα, pSG5-RXRα or both expression vectors as described above. CAT activity is normalized to the activity of the (–590/+1)ME-CAT construct co-transfected with pSG5. One representative experiment of four independent ones, each carried out in duplicate. Fold induction (mean ± S.D. for the four experiments concerned, 3rd column) represents CAT activity for each reporter plasmid induced by PPARα/RXRα as compared with pSG5, normalized to the respective activity of the (–315/+1)ME-CAT plasmid. *Significantly different from the respective value for the (–315/+1)ME-CAT plasmid: *P* < 0.05.

due to T₃R (c-erbAα) and T₃. Activation of the 'malic' enzyme gene by PPARα in cultured hepatocytes may indicate that, like other members of the steroid/thyroid hormone receptor superfamily [24], endogenous transcription factors sequestered within the chromatin may become inaccessible to ectopic transfected promoters. T₃-induced expression of the (–882/+1)ME-CAT construct was unaffected by added PPARα, and PP-induced expression of this construct was unaffected by c-erbAα (not shown), thus indicating that liver transcriptional activation of the 'malic' enzyme gene by thyroid hormone and PP was mediated by two different superfamily receptors converging on to the 'malic' enzyme gene promoter.

To define the response element for PPARα within the 'malic' enzyme promoter and to investigate whether it is distinct from the c-erbAα enhancer, transcriptional activation of the 'malic' enzyme gene by PPs was studied in CV-1 cells transfected with deleted 5'-flanking sequences of the 'malic' enzyme gene promoter bound to a CAT reporter and co-transfected with expression vectors for PPARα, RXRα or both. Transcriptional activation of the (–882/+1)ME-CAT construct required both PPARα and RXRα and was further promoted by either beza-

fibrate or Medica 16 (Figure 3, top). Transcriptional activation by PPARα/RXRα was similarly observed using the (–590/+1)ME-CAT construct. However, the (–315/+1)ME-CAT plasmid harbouring the thyroid hormone response element (TRE) at (–281/–261) [17] and the (–177/+1)ME-CAT plasmid were significantly less active. Thus fold induction of CAT activity induced by PPARα/RXRα/Medica 16 as compared with pSG5 (Figure 3, top) amounted to 15 ± 6, 18 ± 6, 2 ± 1 and 1.8 ± 0.5 for the (–882/+1)ME-CAT, (–590/+1)ME-CAT, (–315/+1)ME-CAT and (–177/+1)ME-CAT plasmids respectively, indicating that the putative PP enhancer was located within the (–590/–315) 5'-flanking sequence of the 'malic' enzyme promoter, being distinct from TRE. Progressively deleting 5' sequence of the (–590/+1)ME-CAT construct within the –590/–315 stretch resulted in a gradual decrease in CAT activity in response to PPARα/RXRα with no further significant change due to deletions downstream of –352 (Figure 3, bottom).

The enhancer sequence for PPARα/RXRα transactivation was further characterized by constructing the (–590/–422)ME-TK-CAT plasmid consisting of the respective 'malic' enzyme promoter sequence in front of a heterologous thymidine kinase

Table 2 Transactivation of (–590/–422)ME-TK-CAT construct by PPAR α and RXR α

CV-1 cells were transfected as described in the Materials and methods section with the (–590/–422) ME-TK-CAT reporter construct (8 μ g) and co-transfected with pSG5 (2 μ g), pSG5-PPAR α (1 μ g), pSG5-RXR α (1 μ g) or both pSG5-PPAR α and pSG5-RXR α expression vectors as indicated. CAT activity is presented as its fold induction, where the activity of the reporter construct co-transfected with pSG5 is taken as 1.0. Results are means $\pm$ S.D. for four independent experiments, each carried out in duplicate. *Significantly different from the respective pSG5 value: $P < 0.05$.

Expression plasmid	CAT activity (fold induction)
pSG5	1.0
PPAR α	1.7 $\pm$ 0.25
RXR α	4.5 $\pm$ 2.1
PPAR α /RXR α	20.7 $\pm$ 5.0*

promoter linked to a CAT reporter gene. As shown in Table 2, PPAR α /RXR α activated CAT expression promoted by the (–590/–422)ME promoter sequence in front of a heterologous promoter, thus indicating that the (–590/–422)ME promoter sequence harboured a putative response element mediating 'malic' enzyme transactivation by PPAR α /RXR α . It is noteworthy that transcriptional activation by PPAR α /RXR α in the absence of added ligand and the relatively moderate increase induced by added ligand (1.5-fold, Figure 3, top) is similar to that previously reported for the expression of other genes induced by PPAR α [7] and may either reflect the constitutive inductive capacity of PPAR α or the availability of an endogenous PPAR α activator. Ligand-dependence could be better shown by using a mutated PPAR α (PPAR α -G) [4] having a lower affinity for ligand and therefore being somewhat more dependent on added exogenous ligand [27]. Using PPAR α -G/RXR α instead of PPAR α /RXR α for activating the (–882/+1)ME-CAT construct resulted in 3.0-fold ligand-dependent activation (not shown).

Binding sites for the PPAR α /RXR α heterodimer within the (–590/–422)ME promoter sequence were analysed by DNase I protection using the (–590/–422)ME promoter sequence and looking for protection exerted by COS-7 extracts overexpressing PPAR α and RXR α . As shown in Figure 4, a single PPAR α /RXR α protection site was observed within the (–590/–422)ME promoter sequence consisting of nucleotides –458 to –441. In addition, a DNase I-hypersensitive site dependent on the addition of COS extract overexpressing PPAR α /RXR α was observed at nucleotide –434 of the upper strand (Figure 4). DNase I protection within the (–590/–422)ME promoter sequence was competed for by unlabelled oligonucleotide consisting of the PPAR α /RXR α -binding site of the peroxisomal AOX promoter [7] (Figure 4, lanes 6 and 13).

Binding of the PPAR α /RXR α heterodimer to the putative (–462/–441)ME-PP response element (PPRE) was analysed by gel retardation in the presence of PPAR α , RXR α or both, derived either from COS cells transfected with the respective expression plasmids or produced *in vitro* by translation in rabbit reticulocytes (Figure 5). (–462/–441)ME-PPRE was retarded only in the presence of the PPAR α /RXR α heterodimer (Figure 5, top, lanes 4 and 8), thus supporting the requirement for both receptors in transfection studies as outlined above (Figure 3, Table 2). Binding of the heterodimer to (–462/–441)ME-PPRE could be shifted by an anti-PPAR α serum (Figure 5, top). PPAR α /RXR α binding to ME-PPRE could be competitively

Figure 4 DNase I footprint analysis of the (–590/–422)ME promoter sequence

The (–590/–422)ME promoter sequence labelled at the 3' end of the upper and lower strands was subjected to DNase I footprint analysis as described in the Materials and methods section in the presence of extracts from COS-7 cells transfected with either the pSG5 parent plasmid or overexpressing PPAR α and RXR α . G corresponds to a Maxam-Gilbert G sequencing. Lanes 1, 7, 8, 14, no added extract; lanes 2, 3, 9, 10, in the presence of added pSG5 extracts; lanes 4, 5, 11, 12, in the presence of added PPAR α /RXR α extracts; lanes 6, 13, in the presence of added PPAR α /RXR α extracts together with 40 ng of AOX-PPRE. The footprinted (–462 to –441)ME sequence of the upper strand is denoted. The footprinted (–442 to –463)ME sequence of the lower strand is indicated by a line with numbers indicating boundaries. The arrowhead on the upper strand corresponds to a hypersensitive site at nucleotide position –434.

displaced by AOX-PPRE [3,7] showing a 25-fold higher affinity for PPAR α /RXR α than ME-PPRE (Figure 5, bottom). It is noteworthy that the (–462/–441)ME promoter sequence which binds PPAR α /RXR α consists of a (–462/–450) direct repeat (DR-1) motif (GGACCT G TGCCT) which is partially homologous to the (–550/–558) PPRE sequence (TGACCT T TGTCTCT) of the peroxisomal AOX promoter [3,7] followed downstream by a (–446/–441) half-site ACACCT motif. Interaction between the (–462/–450) direct repeat and the (–446/–441) half-site motif in promoting PPAR α /RXR α

Figure 5 Mobility shift of ME-PPRE by PPAR α and RXR α

Top, Gel mobility-shift assays of ³²P-labelled (–465/–437)ME-PPRE were carried out as described in the Materials and methods section using PPAR α , RXR α or both transcribed and translated in rabbit reticulocytes (lanes 1–4) or using whole COS-7 extracts transfected as described in the Materials and methods section with expression vectors for PPAR α , RXR α or both as indicated (lanes 5–13). Specificity of binding to ME-PPRE was analysed by adding unlabelled ME-PPRE (lane 9, 25-fold excess), AOX-PPRE (lane 10, 2.5-fold excess) or (–467/–445)ME (lane 11, 25-fold excess). The position of the PPAR α /RXR α heterodimer is indicated by an arrow. Polyclonal antisera prepared against PPAR α (Ab) or non-immune serum (NI) were included in the binding assay as indicated. Bottom, Binding of ³²P-labelled ME-PPRE to COS-7 extracts overexpressing PPAR α and RXR α was competed with by increasing concentrations of unlabelled AOX-PPRE ($\blacktriangle$), (–465/–437)ME ($\bullet$) or (–467/–445)ME ($\blacksquare$).

binding to (–462/–441)ME-PPRE could be shown by analysing the binding affinity of PPAR α /RXR α to the (–465/–437)ME promoter sequence as compared with the (–467/–445)ME sequence. As shown in Figure 5 (bottom) the binding affinity of PPAR α /RXR α to the (–465/–437)ME promoter sequence was 8 times higher than its binding to the (–467/–445)ME sequence, indicating that the direct repeat and the flanked half site may complement each other in promoting PPAR α /RXR α binding to ME-PPRE.

PPAR α /RXR α -induced transactivation promoted by (–462/–441)ME-PPRE was confirmed by constructing the (–465/–437)ME-TK-CAT plasmid consisting of ME-PPRE in front of a heterologous thymidine kinase promoter. As shown in Table 3, ME-PPRE-TK-CAT expression was transactivated by PPAR α /RXR α in line with PPAR α /RXR α binding to ME-PPRE as verified above (Figures 4 and 5).

Table 3 Transactivation of ME-PPRE-TK-CAT construct by PPAR α and RXR α

CV-1 cells were transfected as described in the Materials and methods section with the (–465/–437)ME-TK-CAT construct (8 μ g) and co-transfected with pSG5 (2 μ g), pSG5-PPAR α (1 μ g), pSG5-RXR α (1 μ g) or both pSG5-PPAR α and pSG5-RXR α expression vectors as indicated. CAT activity is presented as its fold induction, where the activity of the reporter construct co-transfected with pSG5 is taken as 1.0. Results are means $\pm$ S.D. of three independent experiments, each carried out in duplicate. *Significantly different from the respective pSG5 value: $P < 0.05$.

Expression plasmid	CAT activity (fold induction)
pSG5	1.0
PPAR α	0.5 $\pm$ 0.04
RXR α	1.5 $\pm$ 0.2
PPAR α /RXR α	8.0 $\pm$ 1.0*

Figure 6 Upstream ‘malic’ enzyme promoter sequences affecting transactivation by PPAR α /RXR α

CV-1 cells were transfected as described in Materials and methods section with (–590/–422)ME-TK-CAT, (–465/–437)ME-TK-CAT, (–287/–261)ME-TK-CAT or TK-CAT reporter plasmids (8 μ g) and co-transfected with pSG5-PPAR α (1 μ g) and pSG5-RXR α (1 μ g) expression plasmids. Fold CAT activity represents CAT activity induced by PPAR α /RXR α as compared with pSG5. The results are means $\pm$ S.D. for three independent experiments, each carried out in duplicate. *Significantly different from the respective value for TK-CAT: $P < 0.05$.

The role played by sequences upstream of (–462/–441)ME-PPRE in enhancing PPAR α /RXR α -induced ME-PPRE-mediated transactivation of the ‘malic’ enzyme gene was first indicated in transfection studies using the (–590/+1)- and (–467/+1)-ME promoter sequences (Figure 3, bottom) and was further confirmed by comparing the transactivation efficiency of (–465/–437)ME-TK-CAT and (–590/–422)ME-TK-CAT constructs. As shown in Figure 6, CAT expression was more efficiently promoted by the (–590/–422)ME construct, thus indicating that sequences within the (–590/–465)ME promoter could contribute to PPAR α /RXR α -induced transactivation mediated by (–462/–441)ME-PPRE. It should be noted, however, that no binding of PPAR α /RXR α to (–590/–465)ME promoter sequences was observed in DNase I-protection studies (Figure 4).

DISCUSSION

The thyromimetic activity of PPs has been previously verified in terms of the calorigenic effect and the liver activities induced by PPs as compared with thyroid hormone. Both induce a strongly oxidized liver cytosolic and mitochondrial redox state with a concomitant decrease in mitochondrial phosphate potential [11].

Furthermore, liver enzyme activities classically considered to be thyroid-hormone-dependent were all found to be similarly induced by PPs [12]. The liver thyromimetic effect of PPs was here investigated in order to characterize further the transduction pathway concerned.

The present study demonstrates that, like thyroid hormone, the induction of liver 'malic' enzyme by PPs may be accounted for by transcriptional activation of the 'malic' enzyme gene. Thus the increase in activity was correlated with an increase in 'malic' enzyme mRNA as well as in the rate of transcription of the 'malic' enzyme gene as verified by run-on transcription assays. Transfection and binding studies reported here further indicate that transcriptional activation of liver 'malic' enzyme gene by PPs is mediated by binding of the PPAR α /RXR α heterodimer to a distinct (–462/–441)ME-PPRE partly homologous with that of peroxisomal AOX-PPRE (Figures 4 and 5). The 'malic' enzyme example may thus add to previously reported liver activities induced by PPs, namely peroxisomal β -oxidative genes [1,5–7] and cytochrome *P*-4504 [1,4], all being affected by a common transduction pathway consisting of PPAR α /RXR α binding to the respective PPREs. Hence, rather than being considered as inducers of β/ω -oxidative genes, amphipathic carboxylates collectively defined as PPs should be classified as pleiotropic modulators of transcription of a variety of genes not necessarily related to fatty acid oxidation.

In spite of the general similarity between transcriptional activation of liver 'malic' enzyme and peroxisomal AOX by PPs, several differences are noteworthy. ME-PPRE consists of a DR-1 (GGACCT G TGCCCT) partly homologous to AOX-PPRE (TGACCT T TGTCCT) [3,7] followed, however, downstream by an ACACCT half-site motif (Figure 4). The direct repeat and the half-site motif complement each other in enhancing PPAR α /RXR α binding to ME-PPRE (Figure 5, bottom). The contribution made by the flanking half-site of (–462/–441)ME-PPRE to transactivation by PPAR α /RXR α still remains to be clarified by respective mutational analysis of ME-PPRE. 'Malic' enzyme could be similar in this respect to cytochrome *P*-4504 A6 where transactivation by PPAR α of the Z element, consisting of an AOX-PPRE-like sequence, was found to depend on an adjacent AGTTCA half-site [4]. Also, and in contrast with AOX-PPRE [7], transcriptional transactivation by PPAR α /RXR α binding to (–462/–441)ME-PPRE is affected by upstream (–590/–465)ME promoter sequences (Figures 3, bottom, and 6) which, however, do not bind PPAR α /RXR α (Figure 4). Enhancement of PPAR α /PPRE-mediated transactivation by distant upstream sequences has similarly been reported for cytochrome *P*-4504 A6 [4]. It is still left to be investigated whether the upstream sequences concerned are engaged in binding transcription factors other than PPAR α /RXR α which may enhance transcriptional transactivation initiated by PPAR α /RXR α binding to ME-PPRE. Also, these studies remain to be complemented by studying the chromatin context of the endogenous 'malic' enzyme gene as well as the role played by additional regulatory sequences of the 'malic' enzyme promoter not present in the transfected promoter constructs used here.

While this paper was being prepared for publication, the activation of 'malic' enzyme gene expression by PPs was independently analysed and reported by Castelein et al. [28]. PPAR α /RXR α activation of 'malic' enzyme gene expression by PPs was claimed by these authors to be mediated by the (–338/–326)ME promoter element. Within the framework of studying the homologous 'malic' enzyme promoter, their claim was based on 2.1-fold PPAR α /RXR α -activated expression of (–435/+39)ME-CAT, whereas the shorter (–325/+39)ME-CAT construct was inactive (Figure 3A of ref. [28]). This

activation by PPAR α /RXR α is indeed similar to that observed by us using the (–444/+1)ME-CAT as compared with the (–315/+1)ME-CAT construct (Figure 3, bottom). However, it should be noted that PPAR α /RXR α activation of the (–352/+1)ME-CAT construct was only 1.1-fold higher than that of the (–315/+1)ME-CAT construct (Figure 3, bottom) and definitely could not account for the 4–10-fold transcriptional activity of the (–590/–315)ME-CAT construct as compared with the (–315/+1)ME-CAT construct (Figure 3, bottom). Since the longest homologous 'malic' enzyme promoter studied by Castelein et al. [28] did not exceed the (–490/+39)ME sequence (Figures 2 and 3 of [28]), and as transcriptional transactivation by PPAR α /RXR α binding to (–462/–441)ME-PPRE is strongly affected by upstream (–590/–465)ME promoter sequences (Figures 3, bottom, and 6), Castelein et al. [28] could not but overlook induction by PPAR α /RXR α mediated by 'malic' enzyme promoter sequences upstream of the (–338/–326)ME promoter element. Hence most of the transcriptional activity of the homologous 'malic' enzyme promoter is accounted for by the (–462/–441)ME-PPRE being complemented by upstream sequences within the (–590/–465)ME promoter (Figures 3 and 6). This conclusion concerning the homologous 'malic' enzyme promoter is further corroborated by analysing transcriptional activation by PPAR α /RXR α of 'malic' enzyme promoter sequences in front of the heterologous TK promoter. The (–590/–422)ME promoter sequence activates transcription in the context of the TK heterologous promoter independently of the (–338/–326)ME element (Figure 6). Furthermore the 8-fold activation by PPAR α /RXR α of (–465/–437)ME-CAT observed here (Table 3) is significantly higher than the 2-fold activation of (–338/–326)ME-TK-CAT shown by Castelein et al. [28] (Figure 3B) and confirmed by us. The lower activity of the (–338/–326)ME element claimed by Castelein et al. [28] indeed required its tandem repeat in front of TK in order to yield transcriptional activity in excess of the intrinsic activity observed with the TK promoter *per se* ([28], Figure 4). Hence the weaker response of the (–338/–326)ME element to PPAR α /RXR α than the (–465/–437)ME element both in the context of the homologous 'malic' enzyme promoter and the heterologous TK promoter may indicate that the (–462/–441)ME-PPRE is primarily involved in transactivating the 'malic' enzyme promoter. The putative interaction between superfamily receptors (e.g. HNF-4, COUP, PPAR α /RXR α and others) that may bind to the (–338/–326)ME TGACCT motif and PPAR α /RXR α bound to (–462/–441)ME-PPRE still remains to be investigated.

The present study clearly indicates that transcriptional activation of liver 'malic' enzyme gene by PPs is mediated by a transduction pathway which differs from that involved in thyroid hormone action, both in terms of the receptors and the promoter elements concerned. Transcriptional activation of liver 'malic' enzyme by the two distinct T₃R/TRE and PPAR α /PPRE transduction pathways may serve as a model case for other liver genes previously reported to be induced by both thyroid hormone and PPs [12]. The physiological or evolutionary rationale for the convergence of these two transduction pathways in promoters of liver thyroid-hormone-dependent genes is at present unknown. The two transduction pathways could perhaps be synergistically linked by auxiliary elements common to both or compete for shared transcription factors. Preferential binding of RXR α to PPAR α rather than to T₃R has recently been reported [29]. Also, PPAR α has been recently reported to heterodimerize in solution with T₃R β , resulting in dominant negative transcriptional regulation of (DR-4) TRE or a positive effect on (DR-2) TRE [30].

The therapeutic use of thyroid hormones as hypolipidaemic/calorigenic agents is confounded by their positive inotropic/chronotropic effect on the heart. Previous attempts to distinguish between hepatic and cardiac effects of thyroid hormones by searching for thyroid hormone analogues with a differential hepatic/cardiac efficacy [31] have essentially failed because the liver and heart transduction pathways for thyroid hormones are essentially identical. However, as the thyromimetic activity of PPs and that of thyroid hormone are mediated by distinct transduction pathways, the tissue specificity of each may differ as well. Indeed, no increase in growth hormone mRNA was induced by PPs in cultured pituitary cells under conditions where thyroid hormone was effective [12]. Similarly, mitochondrial glycerol-3-phosphate dehydrogenase and 'malic' enzyme could be induced in liver by both treatment modes, whereas the two respective cardiac activities were induced by T_3 only, while Medica 16 was without effect [11]. The liver-specific thyromimetic activity of Medica 16 taken together with its failure to induce cardiac thyromimetic activities may be exploited for its use as a selective thyromimetic agent without cardiac toxicity.

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