Differential regulation by a peroxisome proliferator of the different multifunctional proteins in guinea pig: cDNA cloning of the guinea pig D-specific multifunctional protein 2

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After our previous report on the cloning of two cDNA species in guinea pig, both encoding the same hepatic 79 kDa multifunctional protein 1 (MFP-1) [Caira, Cherkaoui-Malki, Hoeffler and Latruffe (1996) FEBS Lett. **378**, 57–60], here we report the cloning of a cDNA encoding a second multifunctional peroxisomal protein (MFP-2) in guinea-pig liver. This 2356 nt cDNA encodes a protein of 735 residues (79.7 kDa) whose sequence shows 83 % identity with rat MFP-2 [Dieuaide-Noubhani, Novikov, Baumgart, Vanhooren, Fransen, Goethals, Vandekerckhove, Van Veldhoven and Mannaerts (1996) Eur. J. Biochem. **240**, 660–666]. In parallel, we studied the effect of ciprofibrate, a hypolipaeic agent also known as peroxisome proliferator in rodent, on the expression of MFP-1 and MFP-2 (2.6 kb) in rats and guinea pigs. By Northern blotting analysis we demonstrated that three MFP-1-related mRNA species are expressed in the guinea-pig liver. The expression of two of them (3.5 and 2.6 kb) is slightly increased by ciprofibrate, whereas the 3.0 kb MFP-1

mRNA is, unlike the rat one, strongly down-regulated in guinea pigs treated with ciprofibrate. In a similar way, the hepatic expression of the guinea-pig 2.6 kb MFP-2 mRNA is also down-regulated in guinea pigs treated with ciprofibrate. These results demonstrate (1) that in contrast with the unique 3.0 kb MFP-1 rat mRNA, at least three hepatic MFP-1-related mRNA species are co-expressed in guinea pig; and (2) that, opposed to the accepted idea of non-responsiveness of the guinea pig to ciprofibrate, this drug affects MFP-1 and MFP-2 gene expression in this species. Also, the mRNA species for acyl-CoA oxidase and thiolase, two other enzymes of the peroxisomal β -oxidation pathway that are induced severalfold in responsive species are down-regulated in guinea pig. This paper is the first, to our knowledge, reporting the down-regulation of the expression of genes encoding enzymes involved in the peroxisomal β -oxidation of fatty acids (MFP-1) and bile acid synthesis (MFP-2) in mammals.

INTRODUCTION

Until recently, peroxisomal β -oxidation in mammals was thought to be catalysed by three enzymes: palmitoyl-CoA oxidase, a multifunctional protein (MFP-1, see further) displaying 2-enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase activity, and a 3-ketoacyl-CoA thiolase. The findings of different acyl-CoA oxidases (ACOs), multifunctional proteins and thiolases in rat liver peroxisomes, together with a study of their substrate specificity, suggest the presence of at least two separate β -oxidation systems in these organelles [1]. The first system, consisting of the above-mentioned enzymes, is involved in the degradation of straight-chain fatty acids such as very-long-chain fatty acids, eicosanoids and probably polyunsaturated fatty acids given the dienoyl-CoA isomerase activity associated with MFP-1 [2]. The second system, acting on fatty carboxylates possessing a 2-methyl branch such as pristanic acid and bile acid intermediates, consists of 2-methylacyl-CoA-specific oxidases (trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase), a second multifunctional protein (named MFP-2) [3–5] and a 58 kDa SCP-2-containing thiolase [1,6]. Although both MFP-1 and MFP-2 possess hydratase and dehydrogenase activities, the reactions show opposite stereospecificities. MFP-1 transforms 2-*trans*-enoyl-CoAs into L-3-hydroxyacyl-CoAs and

dehydrogenates the L-isomers [5,7,8]. MFP-2, in contrast, forms and dehydrogenates D-3-hydroxyacyl-CoA [5,9]. Both MFPs can hydrate 2-methyl-enoyl-CoAs [4,5,9,10]. In contrast, the 3-hydroxy isomers formed by MFP-2 and used by its dehydrogenase have the same (3*R*, 2*R*) configuration [or (24*R*, 25*R*) configuration in bile acid intermediates], implicating its role in pristanic acid degradation and bile acid synthesis [4,5]. The MFPs are structurally unrelated to each other, and their organizations are different.

The administration to rats of ciprofibrate, a potent hypolipaeic drug and peroxisome proliferator (PP), causes a marked induction of several peroxisomal enzymes including the three enzymes of fatty acid β -oxidation [11,12]. Species differences in the response sensitivity to PPs are well known. Rat and mouse are strong responders; hamster, jerboa and rabbit are intermediates; and guinea pig, cat, dog, monkey and human are considered as weak responders or non-responders [13,14]. Extensive studies have been done on the regulation of the rat peroxisomal β -oxidation enzyme's expression, but a very few experiments have been reported on the guinea pig, except for the cloning of two highly similar MFP cDNA species [15]. The corresponding mRNA species, of respective sizes 3.5 and 2.6 kb, both encode the same MFP, which is 80 % identical with the rat MFP-1.

Abbreviations used: ACO, acyl-CoA oxidase; MFP, multifunctional protein; PP, peroxisome proliferator; RACE, rapid amplification of cDNA ends; UTR, untranslated region; SCP-2, sterol carrier protein-2.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number Y13623.

Because in guinea pig, as in man, ciprofibrate only provokes weak peroxisome proliferation without any hepatomegaly, and because fibrate molecules are hypolipaeic drugs commonly used in human to prevent atherosclerosis [16], we thought that a better understanding of the species differences in the regulation of the peroxisomal β -oxidation system could help to establish safety standards in human; the new data presented here will probably contribute to starting this investigation. In the present study we treated guinea pigs and rats with ciprofibrate for 2 weeks. The drug was administered at three different plasma dosages. A new 3.0 kb MFP-1-related mRNA was detected for the first time in guinea-pig liver. In addition the 2.6 kb MFP-2 mRNA was found and its corresponding cDNA was cloned. We demonstrated that, in contrast with the accepted idea of non-responsiveness of the guinea pig to PPs, the expression of these two mRNA species was strongly (3.0 kb MFP-1) or weakly (2.6 kb MFP-2) down-regulated by ciprofibrate in a dose-dependent manner.

EXPERIMENTAL

Animals

Adult male Sprague-Dawley rats Crl:CD BR and adult male guinea pigs Crl:(HA)BR were obtained from Charles River, France. Animals were housed in an air-conditioned room maintained at $20 \pm 2^\circ\text{C}$, with $55 \pm 20\%$ relative humidity and a 12 h light/12 h dark cycle. Rats were fed with a certified rodent diet (AO4C-10), and guinea pigs with a complete diet (114C) from UAR (Villemoisson/Orge, France).

Drug and treatments

Ciprofibrate (WIN 35,833) (Sterling Winthrop) was suspended in 0.24% autoclaved gum tragacanth and administered daily to animals by gastric intubation (5 ml/kg). The drug was administered at a dosage of 3 mg/kg per day (to five rats, group B) and at three different dosages of 3, 2×10 or 2×30 mg/kg per day (to five guinea pigs per dosage: groups B, C, D respectively) during two weeks, in accordance with the conditions defined by Pacot et al. [17]. Control animals (group A) received the same volume of gum tragacanth alone.

Purification of peroxisomes

Peroxisomes were isolated from the livers of guinea pigs and rats, in the presence of 0.5 mM PMSF, as described by Cherkaoui-Malki et al. [18]. The light mitochondrial fraction was loaded on a discontinuous Nycodenz gradient supported by a Maxidenz cushion in Quickseal tubes and subjected to centrifugation at 130 000 g for 70 min in a vertical rotor (Beckman Vti50). Gradient fractions containing peroxisomes were washed in homogenization buffer at 20 000 g for 15 min and then quickly frozen in liquid nitrogen and stored at -80°C for further analysis.

Enzyme assay

Proteins were determined by the method of Bradford [19]. The peroxisomal heat-labile 2-enoyl-CoA hydratase (or crotonase) activity was assayed spectrophotometrically at 280 nm by following the hydration of the *trans* double bond for 1 min, as described by Steinman and Hill [20], in peroxisomal fractions with crotonoyl-CoA as a substrate. For the measurements of 3-hydroxyacyl-CoA dehydrogenase activities, substrate-dependent NAD^+ reduction was followed fluorimetrically with 3-L-hydroxy-octanoyl-CoA, 3-D-hydroxyoctanoyl-CoA and (24R,25R)-varanoyl-CoA (3 α ,7 α ,12 α ,24-tetrahydroxy-5 β -cholestan-26-oyl-

CoA SH) as substrates exactly as described by Dieuaide-Noubhani et al. [2].

SDS/PAGE and immunoblotting

Proteins from purified peroxisomal fractions were separated by SDS/PAGE [10% (w/v) gel]. The gels were stained with Coomassie Blue to compare total protein patterns between control and treated guinea pigs. Electrophoresis and immunostaining were performed on nitrocellulose membranes (Millipore) with a semi-dry multigel electroblotter [21]. The membrane was incubated with affinity-purified polyclonal antibody raised against rat MFP-2, as described by Dieuaide-Noubhani et al. [5]. Detection was with alkaline phosphatase-conjugated anti-(rabbit IgG) from goat.

Northern blot analysis

Total RNA was obtained from livers of control and 2 week-treated rats and guinea pigs. In each control and treated group, total RNA ($4 \times 5 \mu\text{g}$ from four animals) was electrophoresed and Northern blot analysis was performed as described [22]. Three rat cDNA probes were a gift from Dr Osumi: MFP-1 (pMJ26 corresponding to the almost complete coding sequence [23]), (ACO) [24] and thiolase [25]. Two other rat probes were used: *Eco*RI-digested MFP-2 cDNA [5] and β -actin cDNA (gift from Dr S. Alonso [26]). Two guinea-pig probes derived from the 3.3 kb MFP-1 cDNA [15] (P_1 , nt -42 to 440; P_2 , nt 2675-3232) were used (Figure 1). A fragment (nt 334-1395) of guinea-pig MFP-2 cDNA presented in this paper was also used as a probe. Hybridization was performed at 42°C overnight in 50% (v/v) formamide/5 $\times$ Denhardt's medium/5 $\times$ SSC/0.1% SDS. The filters were washed twice at 42°C for 30 min in $2 \times$ SSC/0.5% SDS and once at 65°C for 30 min in SSC/0.1% SDS. The filters were dehybridized in several volumes of boiling 0.1% SDS between each hybridization.

cDNA library screening, cloning and sequencing

The two *Eco*RI fragments of rat MFP-2 cDNA were used as probes to screen a guinea-pig adult liver cDNA λ gt 10 library (Clontech Lab., Palo Alto, CA, U.S.A.), in accordance with the manufacturer's recommendations. The cDNA inserts of positive phages were subcloned in pBluescript vector and sequenced several times on both strands by using universal primer and three specific primers (located at nt 335, 1040 and 1630 respectively along the guinea-pig MFP-2 cDNA sequence), in accordance with the manufacturer's recommendations (*fmol* DNA Sequencing System[®]; Promega).

Rapid amplification of cDNA 5' ends (RACE)

Total RNA was purified from a guinea-pig liver by the method of Cherkaoui-Malki and Caira [22]. Poly(A)⁺ RNA was prepared by the method of Sambrook et al. [27]. First-strand cDNA synthesis from mRNA was realized with a Riboclone[®] synthesis kit (Promega) and the homopolymeric tailing of cDNA with TdT and dCTP. PCR-amplification of target cDNA and the cloning of 5'-RACE product were realized with the Gibco-BRL protocol. Two specific anti-sense primers from the MFP-2 cDNA sequence were designed (primer 1, CATAATACAGTCCAGTTCCG, located at position 1040; primer 2, CCCAGTCTTCATCAC-TTATCC, located at position 355), as well as two 5'-RACE primers (primer 3, CGGAATTCGAACCTTCGGGIIGGG-IIGGGIIG, complementary to the poly(dC) tail; primer 4, CGGAATTCGAACCTTC). The two PCR amplifications

Figure 1 Schematic representation of two probes (P_1 and P_2) located along the guinea-pig (GP) MFP-1 cDNA sequence [15]

Probes were used in Northern blotting analysis of total hepatic RNA species. See the Experimental section for further details.

were performed under the following conditions: hot start, 45 s at 94 °C; 45 s at 60 °C; 2 min at 72 °C; 35 cycles and a final extension at 72 °C for 7 min. A 400 bp DNA fragment was amplified and subcloned in pGEM-T Easy® vector (Promega) in accordance with the manufacturer's recommendations and was subsequently sequenced.

RESULTS

Detection of several MFP-1-related mRNA species in guinea pig liver

In a previous study, by means of probes related to the human MFP-1, we cloned two cDNA species from guinea-pig liver [15]. The cDNA species, with sizes of 2.5 and 3.3 kb respectively, differed only in the lengths of their 3' non-coding region. The deduced amino acid sequence of the unique encoded protein was identical with that of rat MFP-1; hence this protein was considered to be the guinea-pig MFP-1. In the present study, total RNA species from the livers of control and ciprofibrate-treated guinea pigs were extracted and analysed by Northern blotting. Probes P_1 and P_2 (Figure 1), fragments of the guinea-pig 3.3 kb MFP cDNA, allowed the detection of two main mRNA species of respective sizes 3.5 and 2.6 kb (Figure 2). Regardless of

Figure 2 Northern blot analysis of total RNA species purified from guinea-pig and rat livers

Two probes, P_1 and P_2 , were obtained by PCR from the guinea-pig (GP) 3.3 kb MFP-1 cDNA (see Figure 1). Lanes A, control animals; lanes B, animals treated with ciprofibrate at 3 mg/kg per day; lane C, animals treated with ciprofibrate at 2×10 mg/kg per day; lane D, animals treated by ciprofibrate at 2×30 mg/kg per day. For details, see the Experimental section.

Figure 3 Northern blot analysis of total RNA species purified from guinea-pig and rat livers

Lanes A, control animals; lanes B, animals treated with ciprofibrate at 3 mg/kg per day; lane C, animals treated with ciprofibrate at 2×10 mg/kg per day; lane D, animals treated with ciprofibrate at 2×30 mg/kg per day. For details, see the Experimental section. Abbreviation: TH, thiolase.

the probe used, the 3.5 kb mRNA was the most expressed mRNA. As expected, probe P_2 (ranging from nt 2675 to nt 3232) detected only the longer mRNA (Figure 2). Another mRNA, whose size of 3.0 kb fits the size of the rat MFP-1, was also detected, although very faintly, by the guinea-pig P_1 probe (Figure 2); X-ray films were analysed by densitometric scanning (results not shown). Interestingly, when a rat MFP-1 cDNA was used as a probe under stringent hybridization (Figure 3b), the two 3.5/2.6 kb mRNA species were not detected (Figure 3), despite the high similarity (80 % identity) between rat and guinea-pig MFP-1s and the observed cross-reactivity. Instead, the 3.0 kb mRNA previously detected with the guinea-pig probe (Figure 2) was detected in control guinea-pig liver (group A). Because the size of this mRNA fits the size of the inducible rat MFP-1

Table 1 Peroxisomal hepatic 2-enoyl-CoA hydratase, L-specific dehydrogenase activities of MFP-1 and D-specific dehydrogenase activities of MFP2 in control and ciprofibrate-treated guinea pigs

Activities were measured in the purified peroxisomal fraction of guinea pig livers, with synthetic substrates. Animals were treated for 14 days. Most values in the table are means $\pm$ S.E.M. for duplicate measurements, which were within 10% difference. For details, see the Experimental section.

Treatment (ciprofibrate)	Substrates ...	Specific activity of MFP-1			Specific activity of MFP-2			
		Hydratase (μ mol/min per mg of protein)	L-Specific dehydrogenase (nmol/min per mg of protein)		D-Specific dehydrogenase (nmol/min per mg of protein)			
			L-3-Hydroxyoctanoyl-CoA		(24 <i>R</i> , 25 <i>R</i>)-Varanoyl-CoA		D-3-Hydroxyoctanoyl-CoA	
			Activity	Fold	Activity	Fold	Activity	Fold
Group A (control)		12 $\pm$ 2	221 $\pm$ 29	1.0	93 $\pm$ 9	1.0	251 $\pm$ 16	1.0
Group B (3 mg/kg per day)		13 $\pm$ 1	381 $\pm$ 84	1.7	132 $\pm$ 6	1.4	376 $\pm$ 26	1.5
Group C (2 $\times$ 10 mg/kg per day)		12 $\pm$ 1	392 $\pm$ 15	1.8	157 $\pm$ 15	1.7	400 $\pm$ 57	1.6
Group D (2 $\times$ 30 mg/kg per day)		12 $\pm$ 4	460 $\pm$ 155	2.1	207 $\pm$ 18	2.2	471 $\pm$ 174	1.9

Figure 4 Immunoblot of peroxisomal fractions purified from the liver of control and ciprofibrate-treated guinea pigs

The immunoblot was incubated with affinity-purified polyclonal antibody raised against rat MFP-2. Lane A, control guinea pig; lane B, guinea pig treated with ciprofibrate at 3 mg/kg per day; lane C, guinea pig treated with ciprofibrate at 2 $\times$ 10 mg/kg per day; lane D, guinea pig treated with ciprofibrate at 2 $\times$ 30 mg/kg per day. For details, see the Experimental section.

mRNA (Figure 3b), it might represent the true guinea-pig MFP-1 mRNA. Whatever the nature of this newly discovered mRNA, these results suggest the presence of more than one MFP-1-

related mRNA in the guinea pig. These results indicate that the similarity between these three guinea-pig mRNA species is high enough to allow their detection with the same guinea pig probe.

Opposite effects of ciprofibrate on the different MFP-1 mRNA levels in guinea pig

In rat, as already known, the MFP-1 mRNA level is strongly induced by ciprofibrate (Figure 2 and 3b). In guinea pig, in contrast, the 3.0 kb MFP-1 mRNA was down-regulated by ciprofibrate in a dose-dependent manner: the level of the 3.0 kb guinea-pig MFP-1 mRNA was decreased by 80% in the livers of guinea pigs treated once a day at 3 mg/kg (group B) and became undetectable in the liver of guinea pigs treated twice a day at 10 or 30 mg/kg (respectively groups C and D) (Figure 3b). However, the levels of the two 3.5 and 2.6 kb guinea-pig MFP-1 mRNA species were slightly increased by ciprofibrate in a dose-dependent manner (Figure 2) up to a 2-fold-induction, as detected by densitometric scanning (results not shown). This dual regulation further supports the idea of distinct MFP-1 proteins in guinea-pig liver.

Down-regulation by ciprofibrate of the two other enzymes involved in the β -oxidation of long-chain fatty acids in guinea pig

To investigate whether the down-regulation by ciprofibrate was limited to the 3.0 kb MFP-1 mRNA, Northern blots were analysed with probes corresponding to the inducible rat palmitoyl-CoA oxidase (ACO) and rat thiolase. The ACO probe hybridized to two mRNA species (Figure 3a). The main band (3.8 kb) had the same size as the rat ACO [24]. A minor band was also detected at 2.3 kb. The levels of the two ACO mRNA species were remarkably increased in treated rats but strongly diminished in treated guinea pigs. Whereas the 3.8 kb mRNA became undetectable in groups C and D, the 2.3 kb mRNA was only weakly decreased in all groups. The response in the 1.7 kb thiolase mRNA resembles that seen with 3.8 kb ACO mRNA: an increase in rat, but a complete down-regulation in groups C and D of the guinea pigs (Figure 3c).

Influence of ciprofibrate on MFP-1-related enzyme activities in guinea-pig hepatic peroxisomes

To attempt to correlate the detected changes in mRNA levels of MFP-1 (-related) proteins, we determined hydratase (crotonyl-

-56	atgccctaggccggcgtgaggttgtgagagggcgacggcgagcgggtgcagcc	1321	GTT TTG GAT GTC TAT GCT TAT TCT GGG GAG GAA CTT ATA TGC TAC AAT CAG TTT TCC GTG
1	M G S P L R F D G R V V L V T G A G G G	141	V L D V Y A Y S G G E E L I C Y N Q F S V
61	TTG GGC CGA GCT TAT GGC CTG GCT TTT GCA GAA AGA GGA GCA TCA GTT ATT GTG GAT GAT	1381	TTT GTT GTT GGC TCT GGG GGC TTT GGT GGA AAA CGT ACA TCA GAA AAA CTC AAG GTG GCT
121	L G R A Y A L A F A E R G A S V I V D D	461	F V V G S G G F G G K R T S E K L K V A
121	TTA GGA GGG GAC TTC AAG GGA GTT GGT AAA GGC TCC TTA GCA GCT GAT AAG GTG GTT GAA	1441	GTA GCC ATT CCA AGT AGA CCC CCT GAT GCT GTA ATA ACA GAT AAT ACC TCA CTT GAT CAG
41	L G G D F K G V G K G S L A A D K V V E	481	V A I P S R P P D A V I T D N T S L D Q
181	GAA ATA AGA AGA AAA GGT GGA AGA GCA GTG GCC AAT TAT GAT TCA GTG GAA GGA GAG	1501	GCT GCT TTG TAC CGC CTC AGT GGA GAC TGG AAC CCT TTG CAT ATT GAC CCT AAC TTT GCA
61	E I R R K K G G R A V A N Y D S V E A G E	501	A A L Y R L S G D W N P L H I D P N F A
241	AAG ATT GTG AAG GCA GCA CTG GAT GCT TTT GGA AGA ATA GAT GTT GTA GTC AAC AAT GCT	1561	AGC ATT GCA GGT TTT GAG AAG CCC ATA TTA CAT GGA TTA TGT ACT TTT GGG TTT TCT GCC
81	K I V K A A L D A F G R I D V V N N A	521	S I A G F E K P I L H G L C T F G F S A
301	GGA ATT CTG ACC CAC CGT TCT TTC AGT AGG ATA AGT GAT GAA GAC TGG GAT ATA GTC CAT	1621	AGG CAT GTT TTA CAG CAG TTT GCA GAT AAT GAT GTG TCA AAG TTT AAC GCA ATT AAG GTT
101	G I L T H R S F S R I S D E D W D I V H	541	A A L V L Q Q F A D N D V S K F K A I K V
361	AGG GTT CAT TTA TGG GGC TCA TTT CAA GTG ACC CGG GCA GCA TGG AAT CAT ATT AAG AAA	1681	CGT TTT GCA AAA CCA GTA TAT CCA GGG CAA ACT CTA AAA ACT GAG ATT TGG AAG GAA GGT
121	R V H L W G S F Q V T R A A W N H M K K	561	R F A K P V Y P G Q T L K T E M W K E G
421	CAG AAC TAT GGG AGG ATC ATT ATG ACT ACA TCG TCT GCA GGA ATA TAT GGT AAC TTT GGC	1741	AAC AGA ATT CAC TTT CAA ACC AAG ATT CTA GAA ACT GGA GAT ATT GTT ATT TCA AAT GCA
141	Q N Y G R I I M T T S S A G I Y G N F G	581	N R I H F Q T K I L E T A Y A N L A
481	CAG GCA AAT TAC AGT GCA GCA AAA TTG GGT CTT TTG GGC CTC TCA AAC ACT ATC GCA ATC	1801	TAT GTA GAT CTT GTA CCA CCA TCT GAT CTT TCA GCT AAG ACA CAC CCT GAG GGA GSG GAA
161	Q A N Y S A A K L G L L G L S N T I A I	601	Y V D L V P P S D L S A K T H P E G G E
541	GAA GGC AAG AAA AAC AAT ATC CAT TGC AAC ACT ATT GCC CCC ACT GCC GGA TCC CGG TTG	1861	CTA CAA AGT ACG CGT GTA TTT GAG GAA ATA AAT CGT AGG CTT AAG GAT ATC GGG CAC AAG
181	E G K K N N I H C N T I A P T A G S R L	621	L Q S T R V F E E I N R R L K D I G H K
601	ACT CAG ACT GTT ATT CCA GAT GAT CTT GTT CAA GCC CTA AAG CCA GAA TAT GTG CCA CCC	1921	GAG GTG AAG AAA GTA AAT GCT GTA TTT GAA TGG CAT ATT ACT AAA GGT GGA AAT ACT GCA
201	T Q T V M P D D T L K P E V A L A	641	E V K K V N A V F E W H I T K G N T A
661	CTG GTC CTT TGG CTT TGT CAC GAT AGT TGT GAG GAA AAT GGT GGC TTA TTT GAG GTT GGA	1981	GCT AAG TGG ACT ATT GAT CTG AAG AAT GGC TCT GGA AAA GTG TAC CAA GGT CCT GCT GAA
221	L V L W L C H D S C E E N G G L F E V G	661	A K W T I D L K N G S G K V Y Q G P A E
721	GCA GGA TGG ATT GCA AAA TTA CGC TGG GAG AGG ACC CTG GGA GCT ATT GTC ACA GGA AAG	2041	GGT TCT GCT GAC ACT TCC ATT TTA ATT TCA GAT GAA GAT TTC ATG GAA GTA GTC CTG GGC
241	A G W I G K L R W E R T L G A I V R R K	681	G S A D T S I L I S D E D F M E V V L G
781	AAT GAG CCA GTG ACT CCT AAG ACA GTG AAG GCT AAC TGG AAG AAG ATC TGT GAC TTC GAT	2101	AAG CTT GAC CCT CAG AAG GCA TTC TTC AGT GGC AGA CTG AAG GCC AAA GGG AAC ATC ATG
261	N E P V T P E T V K A N W K K I C D F D	701	K L D P Q K A F F S G R L K A K G N I M
841	AAT GCC AGC AAG CCG CAG AGT ATC CAA GAC TCA TGT GGT GGT ATG GTT GAA ATT TTG CAT	2161	CTG AGT GAC AAA CTT CAG ATG ATT CTT AAA GAC TAT GCC AAT CTC TGA agggatctacactg
281	N A S K P Q S I M T Q E S A C T G G M V I L H	721	L S Q K L Q M I L L E T A Y A N L *
901	AAA ATA GAT TCA GAA GAA GTT TCA ACA AAT CCT ACC AGT CAT GCC ACA TCT ACA GCA TCG	2224	ctagtgaatatagaatacttatgcttgaagattctctacaaagtatcaaaactaaatatagaagaattgcatactgc
301	K I D S E E V S T N P T S H A T S A S		
961	CCA GGA TAT GCT AGC GCT ATT GGG TAT AAG CTT CCT TCA TTC TCT CTT TCA TAT ACG GAA		
321	P G Y A S A I G Y K L P S F S P S Y T E		
1021	CTG GAC TGT ATT ATG TAT GCC CTT GGA GTG GGA GCA TCA GTG AAA GAT CCA AAA AAT TTG		
341	L D C I M Y A L G V G A S V K D P K N L		
1081	AGA TTT CTC TAT GAA GGA AGT CCT GAT TTC TCC TGT CTG CCT ACT TTT GGA GTT ATT GTA		
361	R F L Y E G S P D F S C L P T F G V I V		
1141	GCT CAG AAG TCT GTA TTT GGT GGA GGA TTA GCA GAC GTT TCT GGA CTT TCA ATC AAC TTT		
381	A Q K S V F G G G L A D V S G L S I N F		
1201	GCA AAG GTT CTT CAT GGG GAA CAA TAC TTG CAG ATA TAT AAA CCA TTT CCT AGA GCA GGA		
401	A K V L H G E Q Y L Q I Y K P F P R A G		
1261	AAA TTA AAA TGT GAA GCA GTT ATT GCT GAT ATC CTC GAT AAA GGA TCT GGC TTA GTA ATT		
421	K L K C E V I A D I L D K G S G L V I		

Figure 5 Nucleotide sequence and deduced amino acid sequence of guinea-pig MFP-2

The numbering of the nucleotides starts from the first nucleotide of the initiation methionine residue (start codon ATG). The 56 nt preceding the open reading frame are indicated by negative numbers and both 5' and 3' end regions are in lower-case letters. The presumed polyadenylation signal AATATA is indicated with a double underline. The termination codon is indicated by an asterisk. The numbering of amino acid residues is indicated below the nucleotide sequence. The short-chain alcohol dehydrogenase family signature is singly underlined, with the active site located by a dotted line. The three last C-terminal residues ANL, constituting the peroxisomal targeting signal, are in bold.

CoA as substrate) and 3-hydroxyacyl-CoA dehydrogenase (3-L-hydroxyoctanoyl-CoA as substrate) levels in purified guinea-pig liver peroxisomes (Table 1). The specific activity of the hydratase seemed to be unchanged in ciprofibrate-treated guinea pigs, even at the highest dose (2×30 mg/kg per day). In peroxisomes from rats treated with 3 mg/kg per day (a dose that resulted in equivalent ciprofibrate plasma levels to those in the guinea pigs treated with the highest dose [17]), crotonase activity was increased 12-fold, in agreement with the Northern blot analysis. In contrast with the crotonase activity, a slight increase was observed in the L-specific 3-hydroxyacyl-CoA dehydrogenase activities in the treated guinea pigs, being doubled at the highest ciprofibrate dose. At present the interpretation of these findings is not easy. The contribution of the proteins corresponding to the 3.0 kb mRNA (which is down-regulated) and to the 3.5 and 2.6 kb mRNA species (which are slightly up-regulated) to the overall peroxisomal crotonase and 3-hydroxyacyl-CoA dehydrogenase are not known. Because of proteolytic events occurring during isolation and storage, the hydratase activity might be partly lost as explained in the Introduction section. Furthermore a slight mitochondrial contamination does not affect the crotonase activity (determined as heat-labile activity) but can add to the L-specific dehydrogenase activities.

Characterization of guinea-pig MFP-2 by immunoblotting and enzyme measurement

To reveal the possible presence of MFP-2 in guinea pig and its response to PPs, peroxisomes from control and treated animals were subjected to immunoblotting with an affinity-purified antiserum directed against rat MFP-2. Three major polypeptides with respective molecular masses of 79, 66 (triplet) and 34 kDa were detected (Figure 4). A minor band around 45 kDa was visible. Although the size of rat MFP-2 and guinea-pig MFP-2 are similar, proteolysis seems to proceed differently. In rat, this antiserum recognizes a 45 kDa (C-terminal hydratase domain plus SCP-2 domain) and a 40 kDa (N-terminal dehydrogenase domain). In contrast, Qin et al. [9] showed, when using an antiserum against the hydratase domain, the presence of 66 and 34 kDa fragments. More interestingly was the fact that the Western blot revealed a 2-fold increase in the 79 kDa (and its proteolytic fragments) in the ciprofibrate-treated guinea pigs (Figure 4). Compared with rat liver peroxisomes, Coomassie staining of the gels or Ponceau staining of the blots indicated that the 79 kDa MFP-2 was much more abundant in guinea-pig liver peroxisomes than in rat peroxisomes and was one of the major peroxisomal proteins (results not shown). In contrast with those

of MFP-2, the levels of MFP-1 (or related proteins) seemed to be lower than in control rats and were not detectable with an antiserum raised against rat liver MFP-1 (results not shown). In agreement with the immunoblotting data, enzyme activity measurement with (24*R*, 25*R*)-varanoyl-CoA and D-3-hydroxyoctanoyl-CoA, two selective substrates for rat MFP-2 (and some of its proteolytic fragments) [5,8], revealed a slight but significant increase in the treated animals (Table 1). The increase, between 1.4-fold and 2.2-fold, was correlated with the estimated 2-fold increase at the MFP-2 protein level.

Cloning of the guinea-pig peroxisomal MFP-2 cDNA

To complete our study on the regulation of the expression of different MFPs in guinea pig, the guinea-pig MFP-2 cDNA was cloned. By screening 5×10^5 independent plaques of an adult guinea-pig liver cDNA λ gt10 library with the two *Eco*RI fragments of the rat MFP-2 cDNA (overlying the full-length rat cDNA), we isolated five overlapping clones. Analysis of these clones revealed a composite cDNA sequence (Figure 5) encoding the almost complete guinea-pig MFP-2. The 5' end (the 5' non-coding region and the first 334 encoding nucleotides) was amplified by reverse transcription-PCR from hepatic mRNA species (RACE protocol). Beginning with the first encountered ATG codon encoding the initiator methionine, the cDNA sequence was 83.6% identical with rat MFP-2 [5], also named 17 β -hydroxysteroid dehydrogenase type IV [28] or multifunctional enzyme II [9]. It exhibited an open reading frame of 2208 nt encoding an enzyme of 735 residues. The calculated molecular mass was 79.692 kDa, in agreement with the size detected by immunoblotting (Figure 4). The total MFP-2 cDNA sequence (2356 nt) included 56 bp in the 5' non-coding region and 92 bp in the 3' non-coding region, excluding the poly(A) tail. No perfect polyadenylation signal AATAAA was found in the 3' non-coding region, but a consensus AATATA sequence was found at position 2334, this sequence being one of the putative active polyadenylation signals [29] having 10% activity, compared with the perfect AATAAA sequence [30]. The deduced amino acid sequence shows high degree of similarity (Fasta Program, [31]) with proteins recently cloned and characterized in different mammals (although not necessarily as a protein involved in peroxisomal β -oxidation, as reflected in their names): 84% to human 17 β -hydroxysteroid dehydrogenase [32], 82% to mouse 17 β -hydroxysteroid dehydrogenase type IV [33] and 79% to pig 17 β -oestradiol dehydrogenase type IV [34]. The organization of guinea-pig MFP-2 is similar to those of the other 80 kDa proteins mentioned above. In the N-terminal domain, harbouring the D-specific 3-hydroxyacyl-CoA dehydrogenase activity, a 29-residue-long motif (residues 151–179) characteristic of the short-chain alcohol dehydrogenase family is found. The central domain represents the 2-enoyl-CoA hydratase domain, and C-terminally the amino acid sequence is similar to SCP-2 (sterol carrier protein-2). The N-terminal and central parts also display similarity with the yeast peroxisomal MFPs [35,36], which are also D-specific [35]. The guinea-pig MFP-2 ends in the tripeptide ANL (single-letter code), which might act as a peroxisomal targeting signal. Mechanisms responsible for the recognition and import of proteins into peroxisomes have been analysed in detail by Subramani's group, who were the first to show that a tripeptide sequence, SKL, at the C-terminus is necessary and sufficient for the transport of the protein into peroxisomes [37]. Amino acid substitutions within the tripeptide revealed that conserved variants such as [SAGCN]-[RKH]-[LIVMAF] can function as the targeting signal for peroxisomes in mammalian cells [38]. It

Figure 6 Northern blot analysis of total RNA purified from guinea-pig and rat livers

The rat (a) and guinea pig (GP) (b) MFP-2 cDNA probes were used successively. Lanes A, control animals; lanes B, animals treated with ciprofibrate at 3 mg/kg per day; lane C, animals treated with ciprofibrate at 2×10 mg/kg per day; lane D, animals treated with ciprofibrate at 2×30 mg/kg per day. For details, see the Experimental section.

is not clear why, despite their overall similarities in organization, the proteolytic fragments of MFP-2 in rat and guinea pig differ. Rat MFP-2 is thought to be cleaved, after import into the peroxisomes, after residue 316, and to a smaller extent after residue 312 [4], resulting in a 34 kDa D-3-hydroxyacyl-CoA dehydrogenase and a 45 kDa enoyl-CoA hydratase. However, when rat MFP-2 is expressed in *Pichia pastoris* [9], the formation of 66 kDa and 33 kDa fragments, in addition to the full-size protein, was seen. Apparently, under these conditions, the rat protein is cleaved at the same sites as those accessible in the guinea-pig counterpart. We think that the 66 and 34 kDa fragments represent MFP-2 and the 45 kDa hydratase, lacking the C-terminal SCP-2 domain, respectively.

Down-regulation of 2.6 kb MFP-2 mRNA level by ciprofibrate

By Northern blot analysis (Figures 6a and 6b), we demonstrated that the MFP-2 mRNA was expressed constitutively at a high level in the guinea-pig liver and was of the same size as the rat counterpart, being 2.6 kb. In contrast with the MFP-1-related proteins, which are encoded by at least three mRNA species, only one MFP-2 mRNA was found. Whereas in rat the MFP-2 mRNA was induced slightly (1.5-fold) by ciprofibrate, in guinea pig the MFP-2 mRNA levels were lowered by the treatments, in a dose-dependent manner: between 40% and 50% decrease in all treated groups (Figures 6a and 6b). The same results were seen with guinea pig or rat probes. It is not clear why, despite the lower mRNA levels, the D-specific 3-hydroxyacyl-CoA dehydrogenase activities were higher in ciprofibrate-treated guinea pigs.

DISCUSSION

The present report shows that, in agreement with the results obtained from rat liver [4,5,8,9] two multifunctional proteins with different stereospecificities are involved in peroxisomal β -oxidation in the guinea pig. MFP-2 is highly expressed in guinea-pig liver; this is in contrast with the situation in the rat. In contrast with current belief, we obtained evidence for the presence of more than one MFP-1 in guinea pig. Furthermore our results reveal that, in contrast with the strong induction of MFP-1 in

rat, in guinea pig the 3.0 kb MFP-1-related mRNA is down-regulated, whereas the 3.5 and 2.6 kb MFP-1 mRNA species are induced 2-fold at most. In addition the response of MFP-2 is different: a modest increase is seen in rat, whereas in guinea pig the mRNA levels are approximately one-half. Some of the most pertinent questions are discussed below.

What could be the significance of the different MFP-1-related mRNA species and their size heterogeneity?

The coding sequence and the 3' untranslated region (3' UTR) of the two MFP-1 transcripts (3.5 and 2.6 kb mRNA species) are strictly identical [15], thus indicating that the two observed mRNA species originate from the expression of a single gene, and that the variation in size might have arisen as a consequence of the differential use of several polyadenylation signals in the 3' UTR, rather than of alternative splicing (alternative use of exons) [39]. Although the appearance of multiple mRNA species as a result of alternative use of multiple polyadenylation signals is usually characteristic of the synthesis of mRNA species encoding several proteins [40], in the present case the functional significance of this size heterogeneity remains unknown; however, it is possible that the appearance of more than 1 kb of the 3' UTR within the 3.5 kb variant of MFP-1 mRNA might influence the turnover rate of this mRNA isoform. Consequently, the differential use of polyadenylation signals might be an important step in post-transcriptional regulation of peroxisomal MFP-1 synthesis. Indeed, the fact that the 3.5 kb form was the major form detected in the liver of control and treated guinea pigs could indicate that this transcript was more stable, possibly because of a long stabilizing 3' UTR. As well as these two messengers a 3.0 kb mRNA was detected for the first time in guinea-pig liver. Despite the presumed high degree of similarity, this 3.0 kb mRNA was weakly recognized by the guinea-pig probe P₁, whereas the 3.5 and 2.6 kb mRNAs hybridized to the rat MFP-1 probe at less stringent conditions (results not shown). We therefore suggest (1) that the guinea-pig 3.0 kb mRNA sequence might be more closely related to the rat 3.0 kb MFP-1 mRNA than to the 3.5 and 2.6 kb guinea-pig MFP-1 mRNA species, i.e. more than 80 % identity, and (2) that its level of expression in the liver is much lower than are those of the two others (a longer exposure time would allow better detection). Most probably all the encoded proteins have different physiological roles, presumably acting on a different set of (straight chain) fatty acid derivatives. As shown in this paper, the regulation of these MFP-1-related mRNA species by PPs is opposite senses.

Do the MFP-1-related mRNA species and MFP-2 mRNA encode truly peroxisomal proteins?

On the basis of the C-terminus, SKL, the protein encoded by the two up-regulated 3.5 and 2.6 kb MFP-1 mRNA species [15] should be imported into the peroxisomes. Although in rat liver peroxisomes the presence of only two MFPs (and their breakdown products) has so far been demonstrated [3,5,8], this does not rule out the presence of other similar proteins catalysing all or only some of the MFP reactions. The guinea-pig MFP-2 cDNA ended in the tripeptide ANL, whereas AKL is found in the counterparts in rat [5,9,28], mouse [33] and man [32], and AKI in pig [34]. Although ANL has not so far been shown to be functional as a peroxisomal-targeting signal in mammals, the sequence fits the degeneracy consensus proposed by Sommer et al. [41] for glycosomal import. Interestingly, ANL is also found at the C-terminus of catalase in rat, man and mouse.

How does ciprofibrate regulate the expression of peroxisomal enzymes in guinea pig?

In this paper we have demonstrated for the first time in a mammal that the expression of the mRNA species encoding three enzymes involved in the peroxisomal β -oxidation of the straight-chain acyl-CoAs (i.e. ACO, MFP-1 and thiolase), known to be increased severalfold by ciprofibrate in rodents, is negatively regulated in the guinea pig. Until now, guinea pigs were considered to be a moderately responsive or even non-responsive species to PPs, but our results show that appropriate doses of PP (ciprofibrate in our case) can lead to unexpected, sometimes strong, down-regulation of the expression of several genes involved in lipid metabolism. In addition the D-specific MFP-2, involved in the breakdown of bile acid intermediates and pristanic acid [4,5], was negatively regulated by ciprofibrate, at least at the post-transcriptional level (50 % decrease). In purified peroxisomes, however, a modest increase (2-fold) in the immunosignals associated with the MFP-2, as well as in the D-specific 3-hydroxyacyl-CoA dehydrogenase activities catalysed by MFP-2, was seen. The discrepancy between the mRNA levels and activities could be the result of a pleiotropic effect of PPs that might affect gene expression at different levels (i.e. transcriptional, post-transcriptional, translational and post-translational levels). Indeed, in rat liver, as well as the induced transcription of genes encoding peroxisomal β -oxidation enzymes by PPs, enhanced synthesis rates and half-lives of these proteins have also been reported previously [42]. By a similar method to ours, Corton et al. [28] have shown that the mRNA and protein levels of the rat liver 17 β -hydroxysteroid dehydrogenase IV were differentially regulated by Wy 14,643, a well-known peroxisome proliferator. They explained this discrepancy by the involvement of two distinct mechanisms of regulation. In our case the decrease in mRNA levels observed after a 15-day treatment might be due to their decreased stability and/or to a decreased transcription rate, whereas the turnover rate (proteolysis) of the proteins can be decreased in an attempt to counterbalance this transcriptional/post-transcriptional effect of ciprofibrate, leading to the observed increase in protein levels. Although other studies have demonstrated that clofibrate treatment of rats did not seem to affect the MFP-2 protein levels [3,5,9], a 1.5-fold increase in the protein levels was seen in the present study with ciprofibrate.

Guinea pigs, like humans, are known to be weakly responsive to peroxisome proliferators, i.e. they exhibit only a slight peroxisome proliferation and no increase in β -oxidation activity. Nevertheless, our present study reveals for the first time that, contrary to the accepted idea, ciprofibrate (a potent peroxisome proliferator) has a strong effect on several mRNA species encoding peroxisomal proteins in the former mammal. No down-regulation of the peroxisomal β -oxidation enzymes has ever been reported before, although it has been reported for other rat proteins such as Apo A-IV [43] or fatty acid synthase [44], two enzymes involved in lipid metabolism. It is now well known that in rat, peroxisome proliferators such as ciprofibrate stimulate the transcription of some peroxisomal enzymes by activating the peroxisome proliferator-activator receptor (PPAR), a steroid hormone nuclear receptor. The PPAR, in a heterodimeric form with retinoid X receptor, binds to a peroxisome proliferator response element located upstream of the target genes whose expression is consequently enhanced [45]. Until today, no PPARs have been discovered in guinea-pig liver. However, it would be very surprising if a PPAR did not exist in that mammal. Nevertheless, in guinea pig, some inhibition mechanisms of target gene expression might interact with PPAR to provoke a down-regulation of specific genes. Further molecular investi-

gation *in vitro* should be performed in the guinea pig to elucidate the complete mechanism underlying down-regulation by ciprofibrate. Finally, our results illustrate the complexity of the peroxisomal β -oxidation machinery implicated in hepatic lipid homeostasis.

This work was supported partly by funds from GIS Toxicologie Cellulaire-Dijon, Foundation for the Medical Research, the Regional Council of Burgundy and the Ligue Bourguignonne contre le Cancer. We also thank Sterling Winthrop (Sanofi) for their initial help.

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