Molecular cloning and further characterization of rat peroxisomal trihydroxycoprostanoyl-CoA oxidase

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The composite trihydroxycoprostanoyl-CoA oxidase cDNA sequence, derived from overlapping clones isolated via screening of two different rat liver expression libraries, consisted of 2509 bases and contained an open reading frame of 2046 bases, encoding a protein of 681 amino acids with a calculated molecular mass of 76711 Da. The reading frame and identity of the trihydroxycoprostanoyl-CoA oxidase cDNA were confirmed by the location of various tryptic peptides, obtained from the purified enzyme, in the deduced amino acid sequence. The Cterminus (His-Lys-Met) of trihydroxycoprostanoyl-CoA oxidase did not seem to interact with the C-terminal peroxisomal targeting signal 1 (PTS1) import receptor, although the tripeptide fits the rule of conserved PTS1 variants for targeting of proteins to glycosomes of Trypanosomatidae. At the protein level, tri-

INTRODUCTION

In mammals peroxisomal β -oxidation is responsible for the degradation of the major portion of the very long straight-chain fatty acids, dicarboxylic fatty acids, isoprenoid-derived 2-methyl branched fatty acids (e.g. pristanic acid) and eicosanoids (e.g. prostaglandins, leukotrienes and thromboxanes). In addition, hepatic peroxisomes catalyse the β -oxidation of the side chain of cholesterol, resulting in the formation of the primary bile acids (conversion of di- and trihydroxycoprostanic acids into cheno-deoxycholic and cholic acids, respectively) [1–3].

The first step of peroxisomal β -oxidation is catalysed by FADcontaining acyl-CoA oxidases that donate electrons directly to molecular oxygen, resulting in the formation of hydrogen peroxide. It has recently become clear that this step is catalysed by several enzymes, each enzyme displaying its own substrate specificity. Rat liver peroxisomes harbour three acyl-CoA oxidases: (1) palmitoyl-CoA oxidase [4,5], oxidizing the CoA-esters of straight-chain fatty acids, dicarboxylic fatty acids and eicosanoids [6]; (2) pristanoyl-CoA oxidase [7,8], oxidizing the CoA esters of 2-methyl branched fatty acids such as the naturally occurring pristanic acid and the synthetic 2-methylpalmitic acid but also displaying some activity towards the CoA-esters of straight-chain fatty acids [6]; and (3) trihydroxycoprostanoyl-CoA oxidase [5,9], oxidizing the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids [6,9]. Enzyme activity measurements and immunoblot analysis revealed that palmitoyl-CoA oxidase and pristanoyl-CoA oxidase are present in hepatic and extrahepatic tissues [6,8], whereas trihydroxyhydroxycoprostanoyl-CoA oxidase showed 45% identical amino acids with rat palmitoyl-CoA oxidase, whereas the identity with pristanoyl-CoA oxidase was much lower (22%). Northern analysis of multiple rat tissues revealed a signal (approx. 2.6 kb) only in liver and (although much weaker) in kidney. Dot-blot analysis of total liver RNA revealed that the mRNA for trihydroxycoprostanoyl-CoA oxidase is not induced after treatment of rats with structurally unrelated peroxisome proliferators and indicates that highly similar mRNAs are present in other mammals, including man. Immunocytochemistry showed a decrease in trihydroxycoprostanoyl-CoA oxidase protein in individual liver peroxisomes ('diluting-out effect') after treatment of rats with bezafibrate, whereas the palmitoyl-CoA oxidase labelling was significantly increased.

coprostanoyl-CoA oxidase was found only in liver [5]. Similar experiments also demonstrated that treatment of rats with fibrates causes the induction of palmitoyl-CoA oxidase in liver and some extrahepatic tissues (heart, kidney and intestinal mucosa). In contrast, pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase activities were not induced [5,7].

In human liver only two acyl-CoA oxidases have been detected: (1) palmitoyl-CoA oxidase, the substrate specificity of which resembles that of rat palmitoyl-CoA oxidase; and (2) a branchedchain acyl-CoA oxidase that oxidizes the CoA esters of 2-methyl branched fatty acids as well as those of the bile acid intermediates di- and trihydroxycoprostanic acids, which also possess a 2methyl substitution in their side chain [10]. Thus the human branched-chain acyl-CoA oxidase combines the functions of rat pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase. As with human palmitoyl-CoA oxidase, the human branched-chain acyl-CoA oxidase seems to be expressed not only in liver but also in kidney as revealed by enzyme activity measurements [10].

The presence of multiple acyl-CoA oxidases is reminiscent of the mitochondrial system, in which distinct acyl-CoA dehydrogenases are involved in the desaturation of different acyl-CoAs. The primary structure of these dehydrogenases has been unravelled, allowing the elucidation of the mutations present in various human diseases [11].

To characterize further the mammalian oxidases with different substrate specificities and to obtain insight in the evolvement of the corresponding genes during evolution, we decided to clone the cDNA species of rat pristanoyl-CoA oxidase [12], rat

Abbreviations used: PTS1, C-terminal peroxisomal targeting signal 1.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X95189.

trihydroxycoprostanoyl-CoA oxidase and human branchedchain acyl-CoA oxidase. This report describes the molecular cloning and further characterization of rat trihydroxycoprostanoyl-CoA oxidase, an enzyme consisting of two identical subunits, each of approx. 70 kDa [9].

EXPERIMENTAL

Generation of polyclonal antibodies against acyl-CoA oxidases

Palmitoyl-CoA oxidase [8] and trihydroxycoprostanoyl-CoA oxidase [9] were isolated as described before. To obtain an antiserum against the native form of trihydroxycoprostanoyl-CoA oxidase, the peak fractions of the final gel-filtration column [9] were emulsified with Freund's adjuvant and injected subcutaneously in rabbits. Boosting and preparation of the antisera were done as described by Mayer and Walker [13]. The preparation of antibodies against the 53 and 21 kDa subunits (B and C subunits respectively) of rat palmitoyl-CoA oxidase [8] and against the denatured subunit of trihydroxycoprostanoyl-CoA oxidase [9] were as described.

Amino acid sequencing of tryptic peptides

For trypsin digestion of trihydroxycoprostanoyl-CoA oxidase, 4 ml of the purified enzyme preparation containing 1 mg of protein was concentrated and the buffer was replaced by 0.4 M ammonium carbonate buffer, pH 8 (Microcon-10; Amicon). An aliquot (0.1 ml containing 250 μ g of oxidase) was digested with 50 μ g of alkylated trypsin (Promega) for 24 h at 37 °C. Subsequently the preparation was loaded on a DeltaPak C18 column (150 mm × 2 mm; 300 Å, 5 μ m; Waters). The peptides were eluted with a linear gradient of 10–90% (v/v) acetonitrile containing 0.05% (v/v) trifluoroacetic acid at a flow rate of 100 μ l/min over a period of 120 min. Selected fractions containing pure peptides were subjected to amino acid sequencing by Edman degradation in a 477A Protein Sequencer (Applied Biosystems).

Immunoblotting

Tissues to be studied were removed from decapitated rats and homogenized in 4 vol of 0.25 M sucrose/1 mM EDTA (pH 7.2)/ 0.1 % (v/v) ethanol. Aliquots of the homogenates corresponding to 25 μ g of tissue protein and purified enzymes (200 ng) were applied on ready-to-use gels (8–18 % ExcelGel SDS; Pharmacia). After electrophoresis (Multiphor II horizontal system; Pharmacia), the separated polypeptides were transferred to nitrocellulose by semi-dry blotting [14]. After blocking for 1 h in PBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween-20, blots were incubated overnight with antisera. Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL detection reagents; Amersham) after incubation with a peroxidase-labelled goat anti-rabbit IgG. For some blots, alkaline phosphatase-labelled sheep anti-rabbit IgG and Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colour development were used.

Isolation of the cDNA for rat trihydroxycoprostanoyl-CoA oxidase

For immunoscreening, XL1-Blue bacteria were transfected with 10⁶ plaque-forming units of a λ -UniZap rat liver cDNA expression library [oligo(dT) primed; Stratagene]. After induction of the fusion proteins by overnight overlay with isopropyl β -D-thiogalactoside-soaked nitrocellulose membranes, the non-specific binding sites on the membranes were blocked with 10% (v/v) newborn calf serum in Tris-buffered saline containing

0.05% (v/v) Tween-20. Positive clones were detected by incubation overnight with the primary antibody and the immune complexes were revealed by incubation with an alkaline phosphatase-labelled sheep anti-rabbit IgG and subsequent colour detection with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Eight clones, which were strongly cross-reactive in the second round with both antibodies to trihydroxycoprostanoyl-CoA oxidase, were isolated and pBluescript SK(-) plasmids containing specific cDNA inserts were prepared after subcloning in vivo. Sequencing was done by the dideoxynucleotide chaintermination method [15] after subcloning of different parts of the cDNA into pBluescript SK(+) or by using oxidase-specific primers. Translation of the cDNA sequences and subsequent alignment with the rat palmitoyl-CoA oxidase amino acid sequence [16] revealed that the longest clone started at position 214 of rat palmitoyl-CoA oxidase. For completion of the whole open reading frame, 106 plaques of a random-primed and oligo(dT)-primed λ -ZAP rat liver cDNA library (Stratagene) were screened by hybridization with the radioactively labelled 5' EcoRI fragment of the longest trihydroxycoprostanoyl-CoA oxidase clone (clone 3a; see Figure 1). Twenty intensely crosshybridizing clones were further purified and their plasmid DNA was isolated after subcloning in vivo and characterized by PCR with a vector primer in the sense direction and trihydroxycoprostanoyl-CoA oxidase-specific primers in the reverse direction. The clones yielding the longest PCR fragments in the 5' direction were sequenced.

Isolation of a partial cDNA for human palmitoyl-CoA oxidase

A cDNA encoding human palmitoyl-CoA oxidase (88 % identical with its rat counterpart) was isolated by immunoscreening 10⁶ plaque-forming units of a λ -UniZAP human liver cDNA library [oligo(dT) primed; Stratagene] by using a polyclonal rabbit antiserum directed against the A subunit of rat palmitoyl-CoA oxidase, which also recognizes the B and C subunits. The screening was performed as described above. Six clones that in the second screening round also cross-reacted with antibodies against the 23 and 51 kDa subunits of rat palmitoyl-CoA oxidase were further purified. Their plasmid DNA was isolated after subcloning *in vivo* and sequenced. The longest clone comprised 2098 bases of the human palmitoyl-CoA oxidase cDNA sequence [17–19], starting at base 82 in the open reading frame and ending at base 2180 in the 3' untranslated region. Plasmid DNA from this clone was used for all hybridization experiments.

RNA preparation for dot-blots

The animals (mouse, rat, hamster, guinea pig and rabbit) were anaesthetized with Nembutal; the livers were excised and immediately freeze-clamped in liquid nitrogen. Frozen liver tissue from dogs was kindly provided by Dr. Van de Werf (Department of Cardiology, University of Leuven, Leuven, Belgium). As a human sample, frozen tissue from a non-transplanted lobe of a liver prepared for transplantation was used. Approval was granted by the Institutional Ethics Committee.

For the isolation of total RNA, 100 mg of each frozen tissue was ground in liquid nitrogen and subsequently homogenized in 1 ml of TRIzol Reagent (Gibco BRL Life Technologies) with a Polytron tissue homogenizer. All subsequent steps for the extraction of total RNA were performed in accordance with the manufacturer's specifications.

The differences in mRNA content in the total RNA preparations were estimated after hybridization of dot-blots [20] (15 μ g of total RNA for each dot) with a human actin cDNA probe

(Clontech) and scanning of the blot with a PhosphorImager (Molecular Dynamics). Subsequently, new dot-blots were made containing the same amount of mRNA as present in 15 μ g of total RNA from a male Wistar rat. Hybridizations of the dot-blots were done in parallel with radiolabelled cDNA probes for trihydroxycoprostanoyl-CoA oxidase, palmitoyl-CoA oxidase and β -actin.

Northern analysis

For radioactive hybridization, 200 ng of trihydroxycoprostanoyl-CoA oxidase cDNA (combined plasmids derived from clones 319 and 5a) was labelled for 3 h by random priming (ReadyToGo dCTP labelling kit; Pharmacia). A rat multiple-tissue Northern blot (Clontech) was hybridized in accordance with the manufacturer's instructions except that 1.5% instead of 2% (w/v) SDS was used in the hybridization solution and that the membrane was washed under more stringent conditions ($0.1 \times$ SSC, 0.5% SDS, 68 °C) to avoid cross-hybridization with other acyl-CoA oxidases.

Post-embedding immunocytochemistry

Livers of Sprague–Dawley rats were fixed by perfusion of the anaesthetized animals via the portal vein [21] with 0.25 % (v/v) glutaraldehyde/0.2 M sucrose/0.1 M Pipes buffer (pH 7.4). Tissue sections (100 μ m) were cut with a microslicer (Dosaka EM Co.) and embedded in London resin white (soft grade; London Resin Co.). Ultrathin sections (50–70 nm; Reichert-Jung ultramicrotome) were collected on Formvar-coated nickel grids and processed for Protein A–gold (15 nm particles) immunogold electron microscopy [22]. For comparison, liver sections of control and bezafibrate-treated animals were incubated in parallel with the antisera directed against native trihydroxycoprostanoyl-CoA oxidase or the C subunit of palmitoyl-CoA oxidase. Incubated sections were contrasted with uranyl acetate and lead citrate and inspected in a Philips 301 electron microscope.

RESULTS AND DISCUSSION

Characterization and identification of the cDNA encoding rat liver trihydroxycoprostanoyl-CoA oxidase

The cDNA sequence for the complete open reading frame encoding rat trihydroxycoprostanoyl-CoA oxidase was obtained by three consecutive screenings of two different rat liver libraries. All oxidase clones, the restriction sites of the cDNA that were used for subcloning, and the sequencing strategy are summarized in Figure 1. The complete cloned cDNA spanned 2509 bases. Three methionine residues were present in the same reading frame at the translation start region (ATG at positions -13 to -15, 1 to 3 and 13 to 15). However, only the ATG at position 1 to 3 fitted Kozak's rule of 'conserved nucleotides' [23] with an A at -3 and a G at +4. The use of this start codon would result in an open reading frame of 2046 bases encoding a protein of 681 amino acids with a nominal molecular mass of 76711 Da. The consensus sequence for polyadenylation, AATAAA, was found at position 2292, 13 bases upstream of the poly(A) tail. Finally, a stretch of 20 G was found in the 3' trailer at position 2120 (Figure 2).

The higher calculated nominal mass of the deduced trihydroxycoprostanoyl-CoA oxidase protein than that of rat palmitoyl-CoA oxidase (74.6 kDa) was confirmed by SDS/PAGE and immunoblotting of the purified enzymes. In 10% homogeneous gels, the protein (estimated molecular mass 77.6 kDa) ran just behind the A subunit of palmitoyl-CoA oxidase (estimated molecular mass 75.8 kDa) (results not shown). The previously reported molecular mass of 69.6 kDa for the trihydroxycoprostanoyl-CoA oxidase subunit [9] was based on its migration in gradient gels.

The identity of the trihydroxycoprostanoyl-CoA oxidase protein with the translated open reading frame of the cloned cDNA was confirmed by mapping the trihydroxycoprostanoyl-CoA oxidase peptides to the translated cDNA sequence (Figure 2). All peptide sequences obtained after trypsin cleavage of the purified enzyme were found in the deduced amino acid sequence.

Does trihydroxycoprostanoyl-CoA oxidase interact with the peroxisomal targeting signal 1 (PTS1) receptor?

The last three residues of trihydroxycoprostanoyl-CoA oxidase are His-Lys-Met, in comparison with Ser-Lys-Leu in palmitoyl-CoA oxidase. Most peroxisomal matrix proteins contain a Cterminal tripeptide that functions as a peroxisomal targeting signal. The tripeptide consists of Ser-Lys-Leu or a conserved variant in which Ser can be replaced by Ala or Cys, and Lys by Arg, His or Gln [12,24,25]. Although for engineered luciferase constructs His can also substitute for Ser, and Met for Leu, in glycosomal (trypanosomes) protein import experiments [26], the combination His-Lys-Met does not match any of the known Cterminal targeting sequences in mammals. Also our experimental results suggest that the C-terminus of trihydroxycoprostanoyl-CoA oxidase does not interact with the PTS1 receptor. Neither the purified oxidase nor a synthetic peptide consisting of the Cterminal 13 amino acids (CYIRPLMLGWRHKM; the underlined cysteine residue was introduced to allow coupling of the peptide to albumin) bound to the bacterially expressed biotinylated human PTS-1 receptor [27]. In contrast with trihydroxycoprostanoyl-CoA oxidase, purified rat palmitoyl-CoA oxidase (Ser-Lys-Leu) [16] or pristanoyl-CoA oxidase (Ser-Gln-Leu) [12] bound to the receptor (results not shown). To study the interaction of trihydroxycoprostanoyl-CoA oxidase and the PTS1 receptor in vivo, the C-terminal portion of trihydroxycoprostanoyl-CoA oxidase (starting at position 366), fused to the Gal4 DNA-binding domain, was tested in a two-hybrid system. After co-transformation of the yeasts with a plasmid encoding a fusion protein of the activation domain linked to the PTS1 receptor [27], no blue colonies emerged. In control experiments, with the C-terminal part of palmitoyl-CoA oxidase fused to the Gal4 DNA-binding domain, blue colonies were obtained (results not shown). Taken together, these results indicate that His-Lys-Met is most probably not a PTS1 targeting signal and suggest that trihydroxycoprostanoyl-CoA oxidase is targeted to peroxisomes by hitherto unidentified N-terminal or internal sequences or by co-translocation with another peroxisomal matrix protein [28,29].

Two separate gene duplication events in the mammalian acyl-CoA oxidase tree

Computer alignment of the deduced amino acid sequence of trihydroxycoprostanoyl-CoA oxidase with those of palmitoyl-CoA oxidase [16] and pristanoyl-CoA oxidase [12] revealed that trihydroxycoprostanoyl-CoA oxidase shares considerably more amino acid identity with palmitoyl-CoA oxidase (45 %) than with pristanoyl-CoA oxidase (22 %) (Figure 3), despite the fact that trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase are both active on 2-methyl branched compounds, the bile acid intermediates and 2-methyl-substituted fatty acids respectively [6]. These observations, together with the finding that pristanoyl-CoA oxidase shares an amino acid identity with the other mammalian oxidases that is as low as with the yeast acyl-CoA oxidase [12], indicates that the pristanoyl-CoA oxidase



Figure 1 Cloning and sequencing strategy of rat trihydroxycoprostanoyl-CoA oxidase

The complete cDNA sequence, shown in Figure 2, was generated by the alignment of sequences obtained from distinct clones. The starting position of the clones, indicated by numbers in (\mathbf{a}), is shown by arrowheads (for clones 319 and 133a both start and end positions are marked). The polylinker, which connects the oligo(dT)-primed clones to the multicloning site of the pBluescript SK(-) phagemid vector, is also indicated. The combined sequence spans 2509 bases with 197 bases in the 5' leader sequence, an open reading frame of 2046 bases (double line) and a 3' trailer sequence of 266 bases. Start (ATG) and stop (TGA) codons, the cleavage sites of restriction enzymes, which were used for subcloning, and the polyadenylation site (AATAAA) are also shown in (\mathbf{a}). (\mathbf{b}) The size and position of the *Eco*RI fragment of clone 3a, used for plaque hybridization to obtain the missing 5' end. (\mathbf{c}) The positions of specific PCR primers; (\mathbf{d}) the sequences obtained in the sense and anti-sense directions. Because of a stretch of 20 G bases (Poly G) near the polyadenylation site, the polymerase reaction in the sense direction was halted.

-197	(CCATAGCGAAGACTTCATGAAGACTGTCCCAGGCATGCT	1021 CAG ACG CAG CAG CAG CAG AAA CTC CTT CCT CAG CTT GCT GTG AGC TAT GCC TTC CAC TTC ACG 341 Gin Thr Gin Gin Gin Lys Leu Leu Pro Gin Leu Ala Val Ser Tyr Ala Phe His Phe Thr										
-158	GTGACACAAACTACAGAAGGTGGGAAAAGATCTTTGTGGT	CAAACCATCCGGACCTTGGCTACCGAGACAGAACAATAC	1081 GCC ACC AGC CTC TCA GAA TTC TTC CAC AGC TCC TAC AGT GCT ATT CTG AAG AGA GAC TTC 361 Ala Thr Ser Leu Ser Glu Phe Phe His Ser Ser Tyr Ser Ala Ile Leu Lys Arg Asp Phe										
-79	TGACCGCATTCACTCATACACAGTTCTCGGCACCTCCCAG	TECTCAGAGCAGACCCTCAAGGAGATGAGCAGATCCAGG	1141 AGC CTC CTG CCT GAG CTC CAT GCA TTG AGC ACT GGT ATG AAG GCC ACG TTT GCA GAC TTC 381 Ser Leu Leu Pro Glu Leu His Ala Leu Ser Thr Gly Met Lys Ala Thr Phe Ala Asp Phe										
1	ATG GGG AGC CCA ATG CAC CGA GTG TCC CTG (GGG GAC CAC TGG AGC TGG CAA GTG CAC CCG	1201 TGT GCC CAG GGC GCC GAG ATC TGT CGC AGA GCT TGC GGG GGC CAT GGC TAC TCA AAG CTG										
1	Met Gly Ser Pro Met His Arg Val Ser Leu (Gly Asp His Trp Ser Trp Gln Val His Pro	401 Cys Ala Gin Gly Ala Glu Ile Cys Arg Arg Ala Cys Gly Gly His Gly Tyr Ser Lys Leu										
61	GAC ATA GAC AGC GAA AGG CAC TCA CCG TCC TA AGD TIE ASP Ser Glu Arg His Ser Pro Ser I	TTC AGT GTG GAG CGA CTT ACC AAC ATC CTT	1261 AGC GGC CTG CCG ACA CTG GTT GCT CGA GCA ACA GCC TCT TGC ACA TAT GAG GGT GAG AAT										
21		Phe Ser Val Glu Arg Leu Thr Asn Ile Leu	421 Ser Gly Leu Pro Thr Leu Val Ala Arg Ala Thr Ala Ser Cys Thr Tyr Glu Gly Glu Asn										
121	GAT GGA GGC CTC CCA AAC ACC GTG CTG CGA AASp Gly Gly Leu Pro Asn Thr Val Leu Arg	AGA AAA GTC GAA AGC ATC ATA CAA AGT GAC	1321 ACG GTG CTC TAC CTG CAA GTG GCC AGG TTT CTG ATG AAG AGC TAT CTG CAG GCT CAA GCG										
41		Arg Lys Val Glu Ser Ile Ile Gln Ser Asp	441 Thr Val Leu Tyr Leu Gin Val Ala Arg Phe Leu Met Lys Ser Tyr Leu Gin Ala Gin Ala										
181	CCA GTG TTT AAT TTG AAG AAG CTT TAC TTC /	ATG ACC CGA GAG GAG CTA TAT GAG GAT GCG	1381 TCC CCA GGC GCC ACA CCA CAG AAG CCT CTC CCT CAG TCC GTC ATG TAT ATT GCC ACA CAA										
61	Pro Val Phe Asn Leu Lys Lys Leu Tyr Phe P	Met Thr Arg <mark>Giu Giu Leu Tyr Giu Asp Ala</mark>	461 Ser Pro Gly Ala Thr Pro Gln Lys Pro Leu Pro Gln Ser Val Met Tyr Ile Ala Thr Gln										
241	ATT CAA AAG AGA TTC CAT CTC GAG AAG CTA (GCC TGG AGC CTG GGC TGG TCA GAA GAT GGT	1441 AGG CCA GCC AGG TGC TCA GCC CAG ACT GCA GCT GAC TTC CGC TGC CCA GAT GTC TAT ACC										
81	Ile Gin Lys Arg Phe His Leu Glu Lys Leu)	Ala Trp Ser Leu Gly Trp Ser Glu Asp Gly	481 Arg Pro Ala Arg Cys Ser Ala Gln Thr Ala Ala Asp Phe Arg Cys Pro Asp Val Tyr Thr										
301	CCT GAA CGC ATT TAT GCT AAC AGA GTC CTT Pro Glu Arg Ile Tyr Ala Asn Arg Val Leu	GAT GGA AAC GTC AAC TTA AGC TTA CAT GGT	1501 ACA GCC TGG GCA TAT GTG TCT ACC AGG CTC ATA AGA GAT GCA GCA CAC CGT ACA CAG ACC										
101		Asp Gly Asn Val Asn Leu Ser Leu His Gly	501 Thr Ala Trp Ala Tyr Val Ser Thr Arg Leu Ile Arg Asp Ala Ala His Arg Thr Gin Thr										
361	GTT GCC ATG AAT GCT ATC CGA AGC CTG GGC	TCA GAT GAA CAG ATT GCT AAA TGG GGC CAA	1561 CTC ATG AAG TCC GGG GTT GAC CAG CAT GAT GCC TGG AAT CAA ACT ACT GTC ATC CAC CTT 521 Leu Net Lys Ser Gly Yal Asp Gln His Asp Ala Trp Asn Gln Thr Thr Val IIc His Leu										
121	Val Ala Met Asn Ala Ile Arg <u>Ser Leu Gly</u>	Ser Asp Glu Gln Ile Ala Lys Trp Gly Gln											
421	CTC TGC AAA AAC TTC CAA ATC ATC ACA ACA TLeu Cys Lys Asn Phe Gin Ile Ile Thr Thr	TAC GCC CAG ACA GAG CTG GGA CAC GGG ACA	1621 CAG GCT GCT AAG GCT CAC TGC TAC TTC ATC ACT GTG AAG AAT TTC AAG GAA GCT GTG GAG										
141		Tyr Ala Gin Thr Giu Leu Giy His Giy Thr	541 Gin Ala Ala Lys Ala His Cys Tyr Phe Ile Thr Val Lys Asn Phe Lys Giu Ala Val Giu										
481	TAC CTA CAG GGC CTG GAG ACT GAA GCC ACC Tyr Leu Gln Gly Leu Glu Thr Glu Ala Thr	TAT GAT GAA GCC AGG CAG GAG CTT GTG ATA	1681 AAA CTA GAC AAG GAA CCA GAG ATT CAG CGT GTG CTC CAA CGC CTC TGT GAC CTC TAT GCC										
161		Tyr Asp Glu Ala Arg Gln Glu Leu Val Ile	561 Lys Leu Asp Lys Glu Pro Glu Ile Gln Arg Val Leu Gln Arg Leu Cys Asp Leu Tyr Ala										
541	CAC AGC CCT ACG ATG ACT TCC ACC AAG TGG THIS Ser Pro Thr Met Thr Ser Thr Lys Trp	TGG CCT GGG GAC TTG GGA TGG TCG GTC ACC	1741 TTA CAC GGT GTT CTG ACT AAC TCA GGG GAC TTT CTG CAT GAT GGC TTC CTG TCT GGG GCC										
181		Trp Pro Gly Asp Leu Gly Trp Ser Val Thr	581 Leu His Giy Val Leu Thr Asn Ser Giy Asp Phe Leu His Asp Giy Phe Leu Ser Giy Ala										
601	CAT GCT GTG GTC CTA GCC CAG TTG ACC TGC THis Ala Val Val Leu Ala Gln Leu Thr Cys	TTA GGA GTC CGG CAC GGC ATG CAC GCC TTC	1801 CAG GTG GAC ATG GCC AGA GAA GCC TTC CTA GAC CTG CTT CCC TTG ATC CGG AAG GAT GCC										
201		Leu Gly Val Arg His Gly Met His Ala Phe	601 G1n Val Asp Met Ala Arg G1u Ala Phe Leu Asp Leu Leu Pro Leu Ile Arg Lys Asp Ala										
661	ATT GTG CCC ATT CGG AGC CTA GAG GAT CAC	ACC CCA CTG CCA GGA ATC ACA GTT GGG GAC	1861 ATC TTG TTA ACC GAT GCT TTT GAC TTC TCG GAC CAT TGT TTA AAC TCG GCA CTT GGC TGT										
221	Ile Val Pro Ile Arg Ser Leu Glu Asp His	Thr Pro Leu Pro Gly Ile Thr Val Gly Asp	621 I]e Leu Leu Thr Asp Ala Phe Asp Phe Ser Asp His Cys Leu Asn Ser Ala Leu Gly Cys										
721	ATA GGC CCC AAG ATG GGT TTG GAA CAC ATA I	GAC AAT GGC TTC CTG CAA CTG AAC CAC GTG	1921 TAT GAT GGA CAC GTC TAC GAA CGC CTG TTT GAG TGG GCT CAG AAG TAC CCA GCC AAT ACT										
241	Ile Gly Pro Lys Met Gly Leu Glu His Ile	Asp Asn Gly Phe Leu Gin Leu Asn His Val	641 Tyr Asp Giy His Val Tyr Giu Arg Leu Phe Giu Trp Ala Gin Lys <u>Tyr Pro Ala Asm Thr</u>										
781 261	CGG GTT CCC AGA GAA AAC ATG CTC AGT CGC Arg Val Pro Arg Glu Asn Met Leu Ser Arg	TIT GCA GAG GTC TTG CCA GAT GGT ACC TAC Phe Ala Glu Val Leu Pro Asp Gly Thr Tyr	1981 CAG GAG AAC CCT GCC TAT AAG AAG TAT ATC CGA CCA CTG ATG CTC GGC TGG AGA CAC AAG 661 GIn Gin Asm Pro Ala Tyr Lys Lys Tyr I'e Arg Pro Leu Met Leu Gly Trp Arg His Lys										
841	CAG AGG CTT GGG ACG CCA CAG AGC AAT TAT	CTT GGC ATG TTG GTG ACC CGG GTG CAG CTG	2041 ATG TGA AAAGTCAAAGGATTTGGGACCGAGAAGCACCACGGCCTTACTATGGCACATATACATAGAGAATTTAAAGC										
281	Gin Arg Leu Giy Thr Pro Gin Ser Asn Tyr	Leu Gly Met Leu Val Thr Arg Val Gln Leu	681 Met ***										
901	CTG TGT AAA GGA ATC CTA CCC TCC CTC CAG	AAG GCT TGC ATC ATT GCC ACG CGC TAC TCA	2118 ACCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC										
301	Leu Cys Lys Gly Ile Leu Pro Ser Leu Gin	Lys Ala Cys Ile Ile Ala Thr Arg Tyr Ser											
961	GTA ATC CGC CAT CAG TCT CGA CTT CGG CCC	AGT GAC CCA GAG GCA AAA ATC CTG GAA TAC	2197 GTCTACTATTGAGCATGTTTGAAACTTTCCCTTGTCCATCTATAGCATGTATTTGGCTAAATGCTAAAATTTTTGTTTT										
321	Val Ile Arg His Gln Ser Arg Leu Arg Pro	Ser Asp Pro Glu Ala Lys Ile Leu Glu Tyr											
			2276 ACATACAGGAAAAGCTAATAAACTTGTCAGTTACAAA										

Figure 2 Nucleotide and deduced amino acid sequence of rat trihydroxycoprostanoyl-CoA oxidase

The nucleotide sequence, numbered in the 5' to 3' direction and starting at the methionine start codon, was generated from overlapping sequences as depicted in Figure 1. Numbers at the left of the sequences indicate the appropriate nucleotide or amino acid position. Nucleotides in the 5' non-coding region are indicated by negative numbers. Amino acid residues are numbered starting at the start codon; the stop codon is marked ***. The regions for which peptide sequences were obtained are doubly underlined. The polyadenylation signal (AATAAA) and the poly-G region are singly underlined.

gene diverged early in evolution from a common ancestral gene followed later by a divergence of the palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase genes.

Stretches of very conserved sequences between trihydroxycoprostanoyl-CoA oxidase and palmitoyl-CoA oxidase were found mainly in the middle part of the proteins (trihydroxycoprostanoyl-CoA oxidase residues 146–280, 308–360, 384–396, 406–418 and 434–453). Because this part of palmitoyl-CoA oxidase corresponds to the B subunit (51 kDa), this probably explains why the antibody raised against the denatured form of

rthcox racox1 rprcox	M G	S F	M .	H R - M	V S G S]L 0]S 5	D	H W]s w]d s	Q - V	V H M N L W	P P S	D I D[L D I] D R] P	S E K E K G	 P I	- s	 A Y	R R R	HS AS AR	LP A LA	S F T F S F	S N N	V E P E S K	R[L I E[T T M	NI HI LF	L L W	D G D G D G	G L S I - Ç	ED	45 28 40
rthcox racoxl rprcox	N T N T V L	VI R F DF	RR	R K R E K T	V E I E I F	S I N L S T	I	QS LN EN	D P D P D P	V D L	F N F Q F A	L H R	K K E D P -	L Y I -	Y F N F	M C L C F (r R r R G A	E E S C D I	2 L 2 R 2 P	Y E Y E L E] D V К	A I A V E R) 0 K E	K R K S L N	F H A T F L	H L M R R	EK VK CK	L K R	AW MR VF	E Y E Y	G 7 G 7 G	95 78 85
rthcox racoxl rprcox	WS IS FF	E D H	G E E	P E E I D M	R I M W L K	Y F P N P	, K	L Y K I	- A L A L V	N N L	R V F V M N	L E C	DG PV LG]N G M	V N L N Y D	LS YS WS	S L S M S L	HG F- AN	G V I K	A M - I C V	N P L	A I T L H M	R L L	S L N Q V F	6 9 6 1 6 9	D T T	 I I	- G	 S G	- E - A S E	2 0 1 1 1 1	134 119 139
rthcox racox1 rprcox	I A Q E H F	K V K V K V	IG IX L	Q L R P E K	C K S Q I Y	N F E I N I	Ē	I I I I I F		Y Y F	AQ AQ AL	T T T	E L E M E L	G G S	H G H G H G	T I T I	ί Η L I T	QG RG KA	Г Г Г	E T E T R T	LE T T	A T A T A H	Y Y Y	D P D P	A R K T A T		E L E F E F	V I I	IH LN LH	S P S P S P	T T D	184 169 189
rthcox racox1 rprcox	M T V T F E	S S A	K K	W W W W F W	P G P G V G	D I G I N I	, G , G , G	W S K T K T	V S N A T	н]н н	A V A I A V	V V V	L A L A F A	000	L - L - L Y	T (I (T)	L Q D	G V G E G Q	RCC	H G Y G R G	M L L	H A H A H S	F		P I P I Q I	R R R	S L E I D P	E G K	DH TH TL	T F K F L F	P L P L	233 218 239
rthcox racox1 rprcox	P C P C P C	: I 1 : I 1 : V 1		G D G D G D	I G I G M G	P H	(H F (L	G L G Y G Q	E H E H N G	I M L	D N D N D N	GG	F L Y L F A	о к н	L N M D F H	н[] N] К[]	/ R 7 R 7 R	V F I F I F	PR R R R	E N E N Q N	M M L	L S L M L D	R K	FA YA TG	E V Q V N V	/ L / K / T	PD PD SE	G G	TY TY HY	Q F V F H T	R L (- [P	283 267 289
rthcox racox1 rprcox	G 1 F H	P (P I	2 S S Z R	N Y N K Q R	L - L I L 0	λ.	Y S L	G M G T G S	L V M V L S	T F S	R V V R G R	Q S I	L L F L S I		K G G N S I	I I A I S I	5 P 0 7 V	S I S I N I	Q S K	K A K A L A		I I T I I I	A A A	TR IR IR	Y S Y S F S	SV SA SA	IR VR TR	H R R	QS QS QF	R L E 1 G F	R K T	329 311 339
rthcox racox1 rprcox	P S Q S D F	E I	E	AK PQ IP	I L I L V L	E Y D F E Y	Q Q P	T Q T Q L Q	Q Q Q Y Q W	K K	L L L F L L	P P P	QL LL YL	A A A	V S T A A A	Y J Y J Y J	A F A L	- H - H D H	I F I F I F	T A V G S K	T R T	SL YM IF	S K L	E F E T D L	F H Y L I E	- - -	 Q R	- A	S S R I G K	Y S N E V G	A S T	374 358 388
rthcox racox1 rprcox	IL IC TV	K H QC TC	D D R	FS LS QS	LL EL SG	P F P F	L	H A H A H A	L S L T L A	T A S	G M G L A G	ĸ	АТ <u>А</u> - Р-	F F L	A D T S	F (W 1 W 1	A 7 A 7 A	0 - N A 0 F	6 6 6 6	AE I E I Q		CR CR CR	R M E	AC AC VV	G G G G G G	H H H	G Y G Y G Y	S S L	K L H S A M	S G S G N R	L I F	423 407 437
rthcox racox1 rprcox	P 1 P N G E		V A V R N	R A T F D N	T A T P D P	S A N	T T T	Y E F E Y E	G E G E G E	N N N	TV TV NV	L M L	YL ML LQ		V A T A T S	R E R E N Y	ΓL ΓL	M K M K L S	S [] [] [] [Y L Y D L E	Г О Н	A Q V R P L	A S Q	5 P 3 - D G	G A A -	- -	P Q 	K K	PL LV HF	P Q G G T S	S M P	473 451 482
rthcox racox1 rprcox	V M V S L M	Y Y T T	A N N N	T O D L F L	R P P S E A	A F Q F Y F	C C C C C C	S A Q P I L		v k	A V F M	W A	- T P T S S	A M X	A D V D A D	F H I N	R C I S D	P I L E S E	V G A	YT LT PL] T E A	A W A Y A Y	λ[Κ R	V L R L L	S T A A V C	R R Y	L I L V L L	R E R	D A I A E S	A H A K H Q	R	517 501 532
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rthcox racox1 rprcox	EI AV SI	Q F Q F		LQ LR LG	R L N L R L			Y A Y S Y G	L H L Y L W	G G C	V L I S L S	T Q Q	N S K G H T	G G A	DF DF LL	L H L H Y H	H D G G G	G F S I G Y] L] I [] I	S G T G S G	A A E	Q V Q L Q T	D I S G	MA Q V KA	R E N A M E	A R D	FL IL A I	D E L	L L L L M L	PI TI CV	-	615 598 632
rthcox racoxl rprcox	I F I F L F	X [] P 1 D []	A A A A	I L V A V A	L I L V L V	D / D / D /	F	D F D F A F	S I K I S I	H M F	CL TL VL	G	S A S V S P	L L I	G C G R G R	Y I Y I A	D G D G D W	HV NV RI	/ Y / Y / Y	E B E N K N	L L L	F- F- WA	- - A	 V L	 0 0	2 - 2 S	 G V	- - L	E W E W E R	A Q A F A A	K K W	655 638 682
rthcox racox1 rprcox	Y P S P W P	ANL	.	T Q T E	EN VH EF	P E T Z	Y Y N	K K H K K S	Y I H L V A	R K N	PL PL RL] M Q K	L G S K S Q	W L L	R Н 	к м 	1															681 661 70(

Figure 3 Alignment of the three peroxisomal rat acyl-CoA oxidases

The amino acid sequences of rat trihydroxycoprostanoyl-CoA oxidase (rthcox), rat palmitoyl-CoA oxidase (racox1) [16] and rat pristanoyl-CoA oxidase (rprcox) [12] were aligned by computer [Pile Up program (gap penalty of 3) at the Belgian EMBL Node in Brussels]. Identical and homologous amino acids are boxed. The numbers at the right of each row indicate the amino acid positions of the individual oxidases. The palmitoyl-CoA oxidase, corresponding to mRNA type II [16], was not included in the alignment because it is derived by differential splicing of each again from the same palmitoyl-CoA oxidase gene [40]. The site of proteolytic cleavage of palmitoyl-CoA oxidase is marked by an arrow.

trihydroxycoprostanoyl-CoA oxidase cross-reacts weakly with the B subunit of palmitoyl-CoA oxidase (results not shown) and vice versa [9]. Interestingly, the region where the palmitoyl-CoA oxidase subunit A is cleaved into subunits B and C (cleavage between Val⁴⁶⁸ and Ala⁴⁶⁹) [16] was not homologous. It should be noted that in contrast with palmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase (and also pristanoyl-CoA oxidase) is not cleaved after import into peroxisomes, because immunoblotting experiments with rat liver homogenates or isolated peroxisomes revealed only a single band, corresponding to the uncleaved protein. A stretch of amino acids was also conserved in the C-terminal part of trihydroxycoprostanoyl-CoA oxidase (residues 610–657), but the extreme C-terminus was completely different (see above).

Tissue distribution of trihydroxycoprostanoyl-CoA oxidase mRNA

Northern analysis of a multiple tissue blot from rat revealed that the size of trihydroxycoprostanoyl-CoA oxidase mRNA is approx. 2.6 kb (Figure 4a), suggesting that the cloned trihydroxycoprostanoyl-CoA oxidase cDNA (2509 bases) is almost complete. Because all clones for trihydroxycoprostanoyl-CoA oxi-



Figure 4 Rat multiple-tissue Northern blot analysis

A rat multiple-tissue Northern blot (Clontech), containing approx. 1 μ g of mRNA per lane, was hybridized with a probe for trihydroxycoprostanoyl-CoA mRNA (**a**, **b**; exposure times of 2.5 and 12 h respectively). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. After being stripped, the membrane was reprobed for palmitoyl-CoA oxidase mRNA (**c**; exposure time 24 h) and actin mRNA (**d**; exposure time 1 h). Signals corresponding to palmitoyl-CoA oxidase mRNA became visible in spleen (lane 3) and in testis (lane 8) after a longer exposure (3 days) of the blot (results not shown). As revealed by the levels of actin mRNA (**d**) the signals for trihydroxycoprostanoyl-CoA oxidase and palmitoyl-CoA oxidase in liver (lane 5) are underestimated. The two distinct bands on the Northern blot after hybridization with the actin probe (1.8 and 2.0 kb) represent different mRNAs of distinct β -actin isoforms. The smaller isoform is present mainly in muscle tissue. The positions of markers (sizes given in kb) are shown at both sides of the blots.



Figure 5 Immunoblot analysis of different rat tissues

Proteins (50 μ g), present in homogenates of different rat tissues, were separated by SDS/PAGE followed by immunoblotting with the antiserum against the native trihydroxycoprostanoyl-CoA oxidase as described in the Experimental section (a). For comparison, duplicate blots were incubated with antiserum against palmitoyl-CoA oxidase, either against the 51 kDa B subunit (b) or against the 23 kDa C subunit (c). Lane 1, spleen; lane 2, lung; lane 3, testis; lane 4, cerebellum; lane 5, cerebrum; lane 6, intestinal mucosa; lane 7, liver; lane 8, kidney; lane 9, heart; lane 10, skeletal muscle. The migration of the molecular mass standards, indicated in kDa, is given at the right of the figure.

dase, which were obtained from the oligo(dT)-primed library, ended at the same position (base 2310) and a polyadenylation signal (AATAAA) was located 13 bases upstream of a poly(A) sequence (see above), the missing part of the cDNA must be from the 5' leader sequence. The size of the rat palmitoyl-CoA oxidase



Figure 6 Dot-blot analysis of hepatic RNA from different species

Total RNA was isolated from livers of the animals listed below and aliquots, each containing the same amount of mRNA relative to the amount present in 15 μ g of total RNA from male Wistar rat (lane 2), were dot-blotted in triplicate as described in the Experimental section. The dot-blots were hybridized with a probe for the mRNA for trihydroxycoprostanoyl-CoA oxidase (a), palmitoyl-CoA oxidase (b) and β -actin (c). The following animals were used: male Sprague-Dawley rat (lane 1), male Wistar rat (lane 2), male Wistar rat given a diet containing 0.3% (v/w) clofibrate for 14 days (lane 3), female Wistar rat (lane 4), hamster (lane 5), mouse (lane 6), rabbit (lane 7), guinea pig (lane 8), dog (lane 9) and man (lanes 10 and 11). The calculation of the mRNA content of the human hepatic RNA preparation was complicated by the fact that its mRNA content, estimated via the actin mRNA levels, was more than 100-fold higher than that of the liver of a male Wistar rat. This might be because a human β -actin cDNA probe was used for the hybridization. Therefore two different amounts of total RNA were spotted for the human preparation, one according to the calculations for the β -actin signal (lane 10) and one corresponding to 15 µg of total RNA according to absorbance measurements (lane 11). Trihydroxycoprostanoyl-CoA oxidase mRNA and palmitoyl-CoA oxidase mRNA were present in all species investigated, but in different relative quantities. Apparently the palmitoyl-CoA oxidase mRNA levels vary between distinct rat strains.

mRNA was approx. 3.7 kb, which is in agreement with the results obtained by Miyazawa et al. [16]. The difference in length between the palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase mRNA species is mainly due to the shortening of the 3' untranslated region, which extends as much as 1681 bases for palmitoyl-CoA oxidase, whereas it is only 266 bases for trihydroxycoprostanoyl-CoA oxidase.

Trihydroxycoprostanoyl-CoA oxidase mRNA was present in large quantities in liver (exposure time 2.5 h; Figure 4a). Only after longer exposure (12 h; Figure 4b) of the blot was a weak signal observed in kidney, but not in the other tissues (exposure times up to 3 days). These results suggest that the trihydroxycoprostanoyl-CoA oxidase gene is not transcribed in extrahepatic tissues with the exception of kidney. The results agree with earlier enzyme activity determinations [5,7,30] and the immunoblot analysis of tissue homogenates shown in Figure 5a. The palmitoyl-CoA oxidase gene, in contrast, was transcribed in most tissues examined (Figure 4c). The highest mRNA levels were found in liver, heart and kidney. On immunoblot analysis liver, kidney and intestinal mucosa displayed the strongest signals (Figures 5b and 5c), in agreement with previous reports [8,31].

Dot-blot analysis of total liver RNA with trihydroxycoprostanoyl-CoA oxidase cDNA probes revealed cross-hybridization in all species examined, including man (Figure 6a).

Trihydroxycoprostanoyl-CoA oxidase mRNA is not induced by treatment of rats with different hypolipidaemic agents

Whereas palmitoyl-CoA oxidase mRNA was markedly induced in liver by treatment of male rats with clofibrate, trihydroxycoprostanoyl-CoA oxidase mRNA levels remained unchanged (Figure 6). In a second series of experiments, we tested whether other structurally unrelated peroxisome proliferators known to induce different subsets of (peroxisomal) proteins [32,33] would exert any effect on the transcription of the trihydroxycoprostanoyl-CoA oxidase gene. No increases in hepatic trihydroxycoprostanoyl-CoA oxidase mRNA levels were visible after treatment of rats with bezafibrate (an analogue of clofibrate), BM 15.766 (a hypocholesterolaemic piperazine derivative



Figure 7 Dot-blot analysis of hepatic RNA from control rats and rats treated with peroxisome proliferators

Total hepatic RNA was isolated from control male Sprague–Dawley rats (lane 1) or rats treated via gastric intubation for 14 days with 75 mg/day per kg of BM 15.766 (lane 2), of bezafibrate (lane 3) or of BM 17.0249 (lane 4) and dot-blotted as described in the legend to Figure 6. After hybridization with probes for trihydroxycoprostanoyl-CoA oxidase (**a**), palmitoyl-CoA oxidase (**b**) and β -actin (**c**), blots were exposed to film.



Figure 8 Immunoelectron microscopic localization of trihydroxycoprostanoyl-CoA oxidase in rat liver sections

Ultrathin liver sections from a control rat (**a**) and a bezafibrate-treated rat (**b**) were processed in parallel with a post-embedding Protein A–gold method and the antibody raised against the native form of trihydroxycoprostanoyl-CoA oxidase. Selected areas (shown at the same magnification; scale bars represent 250 nm) of pericentral hepatocytes are presented showing specific labelling of the peroxisomal matrix with gold particles. Peroxisomal cores or other cell organelles such as mitochondria (MI), smooth endoplasmic reticulum (SER), lysosomes and nuclei are negative. Most peroxisomes in the control liver exhibit a high labelling density (**a**). After bezafibrate treatment, a strong proliferation of peroxisomes and SER was seen. Individual peroxisomes were enlarged and a decrease in overall labelling density for trihydroxycoprostanoyl-CoA oxidase was observed (**b**).

inhibiting 7-dehydrocholesterol-5,7-reductase [32]) or BM 17.0249 (2,2,13,13-tetrachlorotetradecanedioic acid [33]). In contrast, palmitoyl-CoA oxidase mRNA was strongly induced by bezafibrate and BM 17.0249, whereas treatment with BM 15.766 did not lead to higher palmitoyl-CoA oxidase mRNA levels (Figure 7). The lack of an increase in palmitoyl-CoA oxidase mRNA despite marked peroxisome proliferation after BM 15.766 treatment is in agreement with earlier results obtained by enzyme activity measurements and immunoblotting [34].

Immunocytochemistry with the post-embedding Protein A– gold method revealed a significant labelling of peroxisomes for trihydroxycoprostanoyl-CoA oxidase (Figure 8). Other cell organelles such as nuclei, mitochondria, lysosomes or endoplasmic reticulum remained negative. After bezafibrate treatment, peroxisomes were enlarged and increased in number. A decrease in the labelling density for trihydroxycoprostanoyl-CoA oxidase was observed (Figure 8). In contrast, the labelling density for palmitoyl-CoA oxidase was significantly increased (results not shown), confirming earlier results by Beier et al. [35].

Conclusion

The present findings show: (1) that the amino acid sequence of trihydroxycoprostanoyl-CoA oxidase is more related to the sequence of palmitoyl-CoA oxidase than to that of pristanoyl-CoA oxidase, despite the fact that both trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase are active towards 2methyl branched carboxylates (the bile acid intermediate trihydroxycoprostanic acid and 2-methyl branched fatty acids such as pristanic acid respectively) [6]; and (2) at the mRNA level that the trihydroxycoprostanoyl-CoA oxidase gene is transcribed only in liver and - to a smaller extent - in kidney and that it is not induced by treatment of rats with structurally unrelated peroxisome proliferators. Because bile acid synthesis is essentially a hepatic function, it is not surprising that trihydroxycoprostanoyl-CoA oxidase is not expressed in extrahepatic tissues. The significance of a possible low expression in kidney remains to be elucidated. Further studies of the promoter regions of the acyl-CoA oxidase genes will help to clarify the differences in tissuespecific expression. A study of the trihydroxycoprostanoyl-CoA oxidase promoter (and pristanoyl-CoA oxidase promoter) will also be required to explain why, in contrast with rat palmitovl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase (and also pristanoyl-CoA oxidase) is not induced by treatment of the animals with peroxisome proliferators. Peroxisome proliferators stimulate the transcription of responsive genes [36,37] via the peroxisome proliferator activated receptor, which binds as a heterodimeric complex with the retinoic X receptor to direct repeat sequences in the promoter region of the genes [38,39]. The absence of such responsive elements in the promoter might be one of the possible explanations for the non-inducibility of trihydroxycoprostanoyl-CoA oxidase (and pristanoyl-CoA oxidase).

We thank Mrs. C. Brees, Mrs. P. Van Rompuy (Leuven) and Mrs. A. Appel (Heidelberg) for skilful technical assistance, Dr. M. Andries (Laboratory of Cell Pharmacology, Leuven) for peptide synthesis, Dr. P. Holvoet (Laboratory of Vascular and Molecular Biology, Leuven) for the use of the PhosphorImager, Dr. A. Völkl (Department of Anatomy and Cell Biology II, Heidelberg) for the gift of the antibody to palmitoyl-CoA oxidase (A subunit), and Dr. J. Pill (Research Laboratories, Boehringer Mannheim, Mannheim, Germany) for the BM 15.766 and BM 17.0249 compounds. This study was supported by the Fonds voor Geneeskundig Wetenschappelijk Onderzoek and the Geconcerteerde Onderzoeksacties van de Vlaamse Gemeenschap. E. B. was partly supported by a grant from the Deutsche Forschungsgemeinschaft.

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Received 25 April 1996/28 June 1996; accepted 23 July 1996

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