Mitochondrial 2,4-dienoyl-CoA reductases in the rat: differential responses to clofibrate treatment

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Abstract The effect of a peroxisome proliferator, clofibrate, on mitochondrial 2,4-dienoyl-CoA reductases was studied in rat liver. The specific activity of reductase measured with 2,4-hexadienoyl-CoA as the substrate increased 2.9-fold in the liver homogenate and 2.5-fold in the mitochondrial extract, whereas acyl-CoA oxidase activity increased 13-fold and Δ^3,Δ^2-enoyl-CoA isomerase activity, 25-fold in the homogenate. Chromatography of the rat liver homogenate on hydroxylapatite, which separates the mitochondrial isoforms (M_r 120,000 and M_r 60,000) showed that the M_r 60,000 isoform increased 3.5-fold and the M_r 120,000 isoform 6-fold. When the isoforms were assayed with 2,4-hexadienoyl-CoA and *trans*-2,*cis*-4,7,10,13,16,19-docosaheptaenoyl-CoA, the activity ratios of $C6_{2,4}$ to $C22_{2,4,7,10,13,16,19}$ were 1.5–2.1 for the both isoforms isolated from livers of either control or clofibrate-treated rats. A quantitative immunological experiment with the antibody for the 120,000 reductase in the mitochondrial extracts showed a 6.9-fold increase in the signal, confirming the observation that this isoform is induced more than the other. The mRNA levels of reductase, isomerase, and peroxisomal multifunctional enzyme (MFE) were found to rise in a parallel manner when analyzed by in situ or slot hybridizations, which suggests that the increase was mediated by the same mechanism. Peroxisome proliferators have been shown to increase the mRNA levels of MFE by inducing peroxisome proliferator-activated receptor (PPAR)-mediated expression of the corresponding gene. The short-chain Δ^3,Δ^2-enoyl-CoA isomerase and the M_r 120,000 reductase are exceptions among the mitochondrial β-oxidation proteins, which usually show only a minor response to peroxisome proliferators.—**Hakkola, E. H., J. K. Hiltunen, and H. I. Autio-Harmainen.** Mitochondrial 2,4-dienoyl-CoA reductases in the rat: differential responses to clofibrate treatment. *J. Lipid Res.* 1994. **35:** 1820–1828.

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A great deal of new information on metabolic pathways involving (poly)unsaturated fatty acids has accumulated recently. One characteristic of the auxiliary enzymes of β-oxidation that has emerged is the occurrence of multiple isoforms distributed not only in different subcellular organelles, but also within the same organelle. This has been shown for 2,4-dienoyl-CoA reductase (1) and Δ^3,Δ^2-enoyl-CoA isomerase (2), and there is evidence that it is also true of 2-enoyl-CoA hydratase 2 (β-hydroxyacyl-CoA dehydrase) (3, 4).

The physiological significance of isoforms in separate organelles can be envisaged, but their functions within the same organelle are less obvious. The only clear difference in function has been shown for the mitochondrial Δ^3,Δ^2-enoyl-CoA isomerases, where the two mitochondrial isoforms have distinct chain-length specificities (2). In the case of 2,4-dienoyl-CoA reductases, the occurrence of two mitochondrial isoforms has been documented but nothing is known about their physiological differences.

Fibrate hypolipidemic drugs and industrial plasticisers have been reported to cause peroxisomal proliferation and to induce peroxisomal enzyme activities in rodents (5). Several other proteins involved in fatty acid metabolism are also affected by the treatment, among them the microsomal cytochrome P450IVA1 (6), which is the enzyme for ω-hydroxylation of fatty acids, and the cytosolic fatty acid-binding protein (7). The increase in enzyme activities is due to increased transcription rates of the genes encoding for these proteins (8), and their induction mechanism has been partly characterized. Along with all the peroxisomal β-oxidation enzymes, mitochondrial short-chain Δ^3,Δ^2-enoyl-CoA isomerase activity (9) and the total mitochondrial of 2,4-dienoyl-CoA reductase activity are increased (10).

To understand the physiological functions of different 2,4-dienoyl-CoA reductases, we have been screening them for differences in their properties and reaction to stimuli. The present work, using chromatographic, immunological and in situ and slot hybridization techniques, shows that the mitochondrial isoform of 2,4-dienoyl-CoA reductase with a native M_r of 120,000 (reductase-120) responds

Abbreviations: RT, reverse transcription; MFE, multifunctional enzyme; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element.

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to clofibrate treatment more than does the isoform with M_r 60,000 (reductase-60).

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats were fed a standard pellet diet supplemented with 0.5% clofibrate (Orion Pharmaceutical Co., Espoo, Finland) (w/w) for 0, 1, 3, 6, and 14 days. The proposal for this research was approved by the University of Oulu Committee on Animal Experimentation.

Enzyme assays

2,4-Dienoyl-CoA reductase was assayed by spectrophotometric measurement as described (11) using 60 μM 2,4-hexadienoyl-CoA or *trans*-2,*cis*-4,7,10,13,16,19-docosaheptaenoyl-CoA as the substrates. 2,4-Hexadienoyl-CoA was synthesized from sorbic acid via the mixed anhydride system (12). Docosahexaenoyl-CoA was generously provided by Prof. Harald Osmundsen and synthesized via N-docosahexaenoyl imidazole (13). Docosahexaenoyl-CoA (60 μmol) was incubated in 0.1 M KP_i, pH 7.4, with acyl-CoA oxidase (0.3 U/ml from *Candida* species, Sigma Chemical Co., St Louis, MO) and the reaction was followed at 312 nm until completion (4 min). Acyl-CoA oxidase was removed via ultrafiltration (Ultrafree-Pf filter units LGC, cut off mol wt 10,000, Nihon Millipore Kogyo K. K., Yonezawa, Japan). The filtrate (*trans*-2,*cis*-4,7,10,13,16,19-docosaheptaenoyl-CoA in 0.1 M KP_i, pH 7.4) was supplemented with 125 μM NADPH (final concentration) and used as a medium for the reductase assay. All assays were completed within 1 h after the incubation of docosahexaenoyl-CoA with acyl-CoA oxidase. Δ^3,Δ^2-Enoyl-CoA isomerase was measured with *trans*-3-hexenoyl-CoA (9) and acyl-CoA oxidase was measured as described with dodecanoyl-CoA as the substrate (14). A unit of reductase, isomerase, or acyl-CoA oxidase was expressed in terms of μmol substrate metabolized per minute. Catalase activity was measured and the catalase activity unit was specified as described by Böck, Kramar, and Pavelka (15).

Separation of mitochondrial 2,4-dienoyl-CoA reductases by hydroxylapatite chromatography

Frozen livers (4 g) from the control and clofibrate-treated rats were homogenized 1:10 (w/v) with an Ultra Turrax tissue disrupter in 0.2 M KCl, 10 mM KP_i, and 0.5 mM dithiothreitol (Boehringer; Mannheim, Germany), pH 7.2. The 100,000-*g* supernatant was dialyzed against 20 mM KP_i and 0.5 mM dithiothreitol, pH 7.2, and applied to a 1.5 cm × 7 cm hydroxylapatite column (Bio-Rad; Richmond, CA) equilibrated with the same buffer. A linear 160 ml KP_i gradient (10 mM to 500 mM) was used to elute the bound proteins, and the reductase activity of the fractions was assayed. The flow rate was 10 ml/min.

Preparation of subcellular organelles

Mitochondria were isolated from the rats treated with clofibrate. The crude mitochondrial fraction was prepared and further purified by a self-generated Percoll density gradient as described (16). The quality of the mitochondrial preparation obtained by this method has been assessed previously (11).

To prepare peroxisomes, the samples of L-fractions were layered on a 30% Nycodenz solution and centrifuged at 130,000 *g* for 1 h. This ensures that the peroxisomes are sedimented to the bottom of the tube, leaving the other organelles at the top of the tube (17).

Immunoblotting

The proteins were separated according to molecular size by SDS-PAGE and then transferred electrophoretically to a nitrocellulose filter. After visualization of the proteins by heparin-toluidine staining, the filter was then destained in 8% acetic acid and 50% methanol before immunostaining. It was incubated for 30 min at 25°C with 3% bovine serum albumin (w/v), 10 mM Tris-HCl, and 0.9% NaCl (buffer A), pH 7.4, washed in buffer A, and incubated for 1 h with the antireductase-120 antibody (11) in buffer A at 25°C. After washing, it was incubated for 1 h with the second antibody (affinity-purified goat anti-rabbit IgG with horseradish peroxidase conjugate; Bio-Rad). The reaction was visualized with the ECL Western blotting detection system (Amersham International plc, Amersham, UK). The exposed films were scanned with a BioImage® apparatus (Millipore, Bedford, MA) for quantification.

Reverse transcription/polymerase chain reaction

The reverse transcription (RT) and polymerase chain reaction (PCR) used to prepare cDNA probes for reductase-120 and the peroxisomal multifunctional isomerase-hydratase-dehydrogenase enzyme (MFE) were carried out as described by Perkin-Elmer/Cetus with a few modifications. Total RNA (1.5 μg) isolated with an RNAgents Total RNA Isolation Kit (Promega Corporation, Madison, WI) from the liver of a rat treated with clofibrate for 14 days was used for the reactions. This was transcribed to a single-strand DNA in a 20-μl reaction containing Taq polymerase buffer (50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatine (w/v), 10 mM Tris-HCl, pH 8.3), 10 mM deoxynucleotides (each), 5 pmol of specific antisense primers (for 2,4-dienoyl-CoA reductase and MFE), murine MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD), and 20 U RNAasin (Promega). The reaction mixture was incubated for 60 min at 42°C. For PCR amplification of the product, the reaction volume was increased to 100 μl by adding Taq polymerase buffer to the

same concentration as in the reverse transcription reaction, 20 pmol of antisense primers, 30 pmol of specific sense primers, and 2.5 U of Taq DNA polymerase I (Perkin-Elmer/Cetus, Norwalk, CT). Amplification was performed by means of 35 cycles of denaturation (60 sec at 95°C), annealing (30 sec at 55°C), and extension (2 min at 72°C and a further 10 min after the 35th cycle). The products were analyzed on 0.8% agarose gels and removed from the gel with Ultrafree-MC Durapore 0.45 μm tubes (Millipore, Bedford, MA).

The primers used for the reactions were chosen from the published cDNA sequences for reductase-120 (the reductase cDNA identified by Hirose et al. (18) representing nucleotides 224–246 and 949–972 (A 5' TGACAACTTTCCTGTCCAGCCTG 3' and B 5' TACATCCCACTCCTCCTTGGTGA 3') and MFE (19) representing nucleotides 938–960 and 2156–2178 (A 5' TTTCTTTTGCGAGAGTGGGGATC 3' and B 5' AAGGCTGAATCACCAGTTTGCTGC 3').

The amplification product for the reductase-120 was digested with *Eco*RI (New England BioLabs, Beverley, MA) and *Pst*I (New England BioLabs) and the MFE product with *Hind*III (New England BioLabs) and subcloned to pGEM4Z. The DNA sequences of the amplification products were partially determined by the dideoxynucleotide sequencing method using the Sequenase enzyme (United States Biochemical Corp., Cleveland, OH) and compared with the published cDNA sequences for the corresponding proteins.

In situ hybridization using RNA probes

In order to prepare an anti-sense RNA probe, the pGEM4Z plasmids were linearized with *Sac*I (New England BioLabs) and *Sal*I (New England BioLabs) for isomerase, *Eco*RI and *Pst*I for reductase, and *Apa*I (New England BioLabs) and *Bst*BI (New England BioLabs) for MFE and ^{35}S-UTP-labeled antisense and sense RNAs were obtained with SP6 and T7 RNA polymerases using an in vitro transcription kit (Pharmacia LKB). The probes in the sense orientation were used as controls for nonspecific hybridization.

In situ hybridizations were carried out according to Hogan, Constatini, and Lacy (20) and Hoeffler et al. (21) with modifications (22). In the prehybridization treatments the deparaffinized sections were sequentially passed through 0.2 M HCl (20 min), diethylpyrocarbonate-H_2O (5 min), 1 mg/ml proteinase K (Boehringer, Mannheim) in PBS (phosphate-buffered saline: 0.02 M NaP_i, 0.15 M NaCl, pH 7.4) (15–30 min) at 37°C. After glycine treatment and washes the proteolysis was stopped by immersing the sections in 4% paraformaldehyde in PBS for 20 min. The slides were acetylated in freshly prepared 1/400 (v/v) acetic anhydride in 0.1 M triethanolamine/HCl, pH 8.0 (2 × 5 min) and in PBS (5 min). The slides were dehydrated by sequential immersion for 2 min in 30%, 50%, 75%, 95%, and 100% ethanol and air-dried before addition of the prehybridization mixture. Prehybridization buffer (10 mM dithiothreitol, 0.02% (w/v) BSA, 0.3 M NaCl, 50% formamide, 10% dextran sulfate, 1 mg/ml yeast tRNA, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinyl pyrrolidone, 5 mM EDTA, 10 mM Tris-HCl, 10 mM NaP_i pH 6.8) was applied to the sections, which were incubated at 50°C for 2–3 h. The prehybridization mixture was replaced with a hybridization solution consisting of the same mixture with the addition of the radiolabeled RNA probe (3 × 10^6 cpm). The hybridization mixture was heated to 100°C for 1 min and cooled on ice. Aliquots of 40 μl were applied to each slide and the slides were coated with coverslips and hybridized overnight at 50°C.

After hybridization, the slides were rinsed in a washing buffer consisting of the hybridization mixture without dextran sulfate or tRNA at 60°C (2 × 30 min) and in 0.5 M NaCl in 10 mM Tris-HCl, 1 mM EDTA (TE) at 37°C (15 min), digested with 40 μg/ml RNase A (Sigma) in 0.5 M NaCl-TE at 37°C (30 min) and 0.5 M NaCl-TE at 37°C (15 min), and washed in 2 × SSC (0.15 M NaCl, 0.015 M Na-citrate), pH 7.0 at 45°C (2 × 15 min), 1 × SSC at 45°C (2 × 15 min). The slides were then dehydrated in an ethanol series with increasing concentrations and air-dried. For autoradiography, they were dipped in Kodak NTB-2 nuclear track emulsion diluted 1:1 in 1% glycerol and exposed for 10 days at 4°C. The exposed slides were developed in Kodak D-19 developer

TABLE 1. Effect of clofibrate treatment on certain enzyme activities in rat liver homogenate

Parameter	Control Rats	Clofibrate Rats
Liver/body weight ratio	0.040 ± 0.002	0.056 ± 0.002[a]
Catalase (k/mg)	0.39 ± 0.09	0.49 ± 0.10
Acyl-CoA oxidase (mU/mg)	1.52 ± 0.5	20.0 ± 7.2[a]
2,4-Dienoyl-CoA reductase (mU/mg)	11.3 ± 3.7	32.5 ± 8.8[a]
Δ^3,Δ^2-Enoyl-CoA isomerase (U/mg)	0.56 ± 0.13	14.0 ± 1.7[a]

The livers were homogenized and the enzyme activities were measured as described in Materials and Methods. The values are means ± SD (n = 7).
[a] $P < 0.0001$.

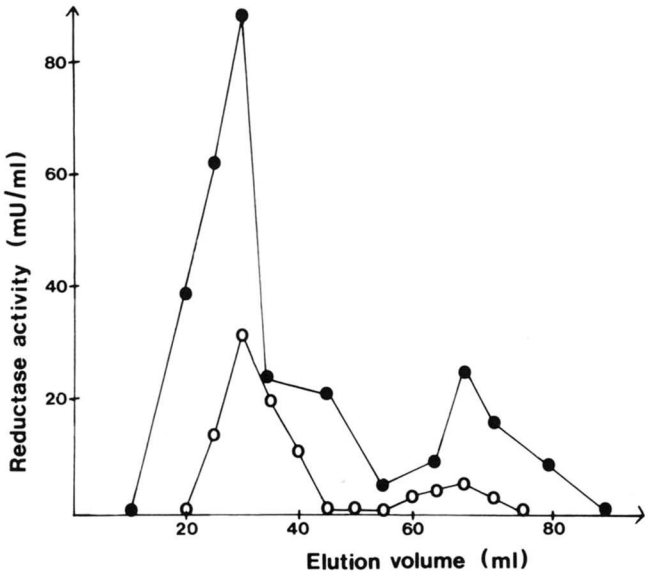


Fig. 1. Effect of clofibrate treatment on 2,4-dienoyl-CoA reductase activities in rat liver homogenates. Four g of liver from control (○) and clofibrate-treated (●) rats was homogenized as described in Materials and Methods. Both homogenates were applied to a hydroxylapatite column and eluted with a linear KP_i gradient. The peak eluting at 105 mM KP_i is reductase-60 and the peak eluting at 260 mM KP_i is reductase-120.

for 5 min at room temperature, fixed for 5 min, and counterstained with Gill's hematoxylin No. 1 (Sigma, St. Louis, MO) and eosin (Oriola, Espoo, Finland).

Slot hybridization of RNA

mRNAs were isolated from 100-mg samples of the tissues used for the in situ hybridization experiments with a QuickPrep Micro mRNA Purification Kit (Pharmacia P-L Biochemicals, Inc. Milwaukee, WI). Slot hybridization of the mRNA samples was performed according to the method described in the laboratory manual of Sambrook, Fritsch, and Maniatis (23). Approximately 0.1 μg of mRNAs isolated from the tissue samples was denatured and applied to a nitrocellulose filter in a manifold. Prehybridization and hybridization were performed at 42°C. The cDNA probes used for the hybridization (glyceraldehyde-3-phosphate dehydrogenase, Δ^3,Δ^2-enoyl-CoA isomerase, reductase-120, and MFE) were ^{32}P-labeled with a Random Primed DNA Labeling Kit (Boehringer Mannheim, Germany) according to the instructions supplied with the kit.

RESULTS

Feeding the rats with 0.5% clofibrate for 14 days caused a 2.9-fold increase in total 2,4-dienoyl-CoA reductase activity in the rat liver homogenate as measured with 2,4-hexadienoyl-CoA as substrate. Acyl-CoA oxidase activity was observed to increase 13-fold and isomerase activity 25-fold during the treatment. Catalase activity underwent a minor increase, if any (1.3-fold), and the liver–body weight ratio increased (**Table 1**). There exist reports that the rate of β-oxidation of unsaturated fatty acids in liver cells and mitochondria (24, 25) isolated from clofibrate-treated rats increases many-fold. As 2,4-dienoyl-CoA reductase is considered to be the key enzyme in the degradation of unsaturated fatty acids, it is difficult, in view of the change detected in reductase activity, to explain the observed increase in β-oxidation of polyenoyl-CoAs.

As the overall induction of 2,4-dienoyl-CoA reductase was assayed in liver homogenates and there are two mitochondrial 2,4-dienoyl-CoA reductases in the rat liver, we studied the induction further by separating the isoforms by hydroxylapatite chromatography as described previously (1). The use of such a technique was justified in this context; even when an extract of isolated peroxisomes was applied to the hydroxylapatite column, the peroxisomal reductase activity could not be detected in the KP_i gradient (data not shown). When 4 g of rat liver was extracted and applied to the column as described in Materials and Methods, reductase-120 (peak at 260 mM KP_i) represented 15% of the total activity recovered in the control rats and 22% in the clofibrate-treated rats, demonstrating that reductase-60 (peak at 105 mM KP_i) is the main activity in the liver when measured with 2,4-hexadienoyl-CoA. Estimated from the activity peaks, reductase-120 was induced 6-fold and reductase-60 3.5-fold (**Fig. 1**).

To study the substrate specificities of the mitochondrial reductases, reductase activity in fractions containing the peaks of reductase-60 and reductase-120 from the hydroxylapatite column were taken and measured with *trans-*

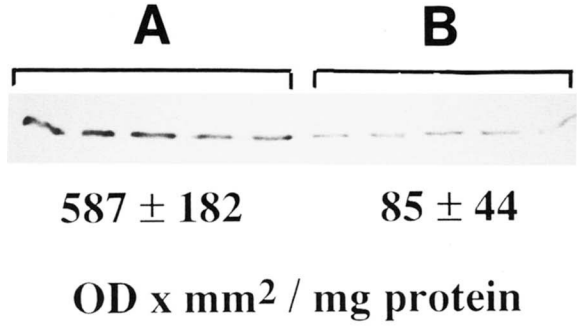


Fig. 2. Quantification of mitochondrial reductase-120 from livers of control and clofibrate-treated rats. Absorbances and areas of the bands were measured, and the mean ± SD OD × mm ^{2}/mg protein is expressed for each set of mitochondria, $P < 0.0001$. The mitochondria were isolated from five control and five clofibrate-treated rats in a Percoll density gradient and homogenized and a 100,000 *g* supernatant was prepared. A sample containing 0.1 mU of 2,4-dienoyl-CoA reductase in each preparation was immunoblotted and an antibody for reductase-120 was used for detection. Lanes 1–5 (A): mitochondria from clofibrate-treated rats. Lanes 6–10 (B): mitochondria from control rats.

TABLE 2. Signals detected in in situ hybridization experiments with rat liver tissue using probes for 2,4-dienoyl-CoA reductase, Δ^3,Δ^2-enoyl-CoA isomerase, and MFE

Time of Clofibrate Treatment	2,4-Dienoyl-CoA Reductase	Δ^3,Δ^2-Enoyl-CoA Isomerase	MFE
No treatment	−/−	−/−	−/−
1 Day	±/±	+ +/+ +	+ + +/+ + +
3 Days	+/+	+ +/+ +	+ + + +/+ + + +
6 Days	+/+	+ + +/+ + +	+ + + +/+ + + +
14 Days	+/±	+ +/+ +	+ + + +/+ + + +

Two animals were treated for the same time period and the signals detected in both are listed.

2,4-hexadienoyl-CoA and *trans*-2,*cis*-4,7,10,13,16,19-docosaheptaenoyl-CoA. The activity ratios of $C6_{2,4}/C22_{2,4,7,10,13,16,19}$ were 1.8/1 and 1.5/1 for reductase-60 and 2.1/1 and 1.8/1 for reductase-120 in clofibrate-treated rats and control rats, respectively.

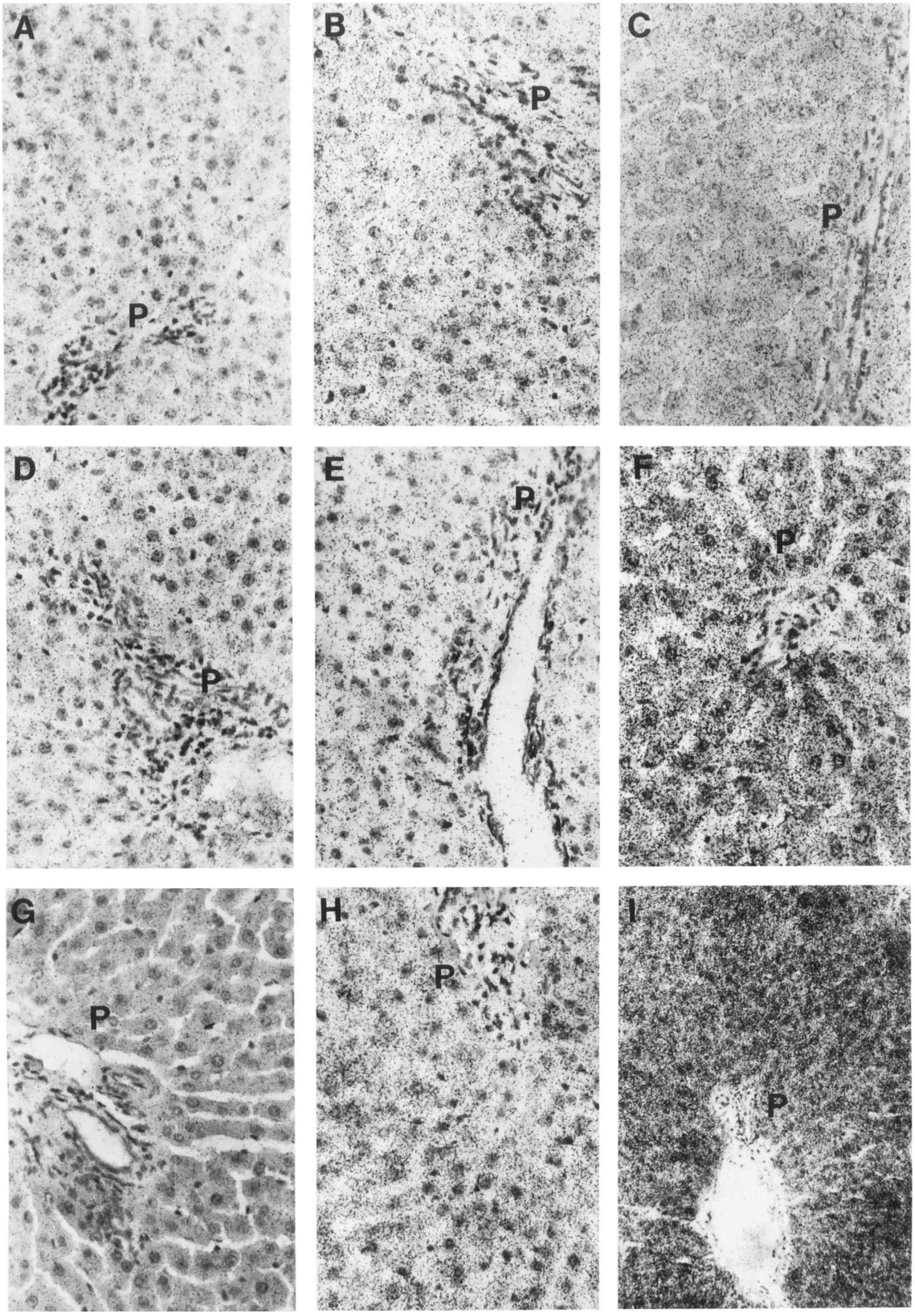
To investigate the activity increase further, mitochondria were isolated on Percoll gradients from the livers of five control and five clofibrate-treated rats. Mitochondrial protein was extracted, and it was observed that the specific activity of the reductase had increased from 8.2 ± 1.9 mU/mg protein to 20.6 ± 6.1 mU/mg protein (2.5-fold). When samples from the control and clofibrate-treated rats containing 0.1 mU of reductase were immunoblotted with antireductase-120 antibody, the signal rose 6.9-fold in the case of clofibrate treatment (**Fig. 2**). A larger proportion of the total mitochondrial reductase pool thus consisted of reductase-120 in the treated rats (Fig. 2). This finding is in agreement with the chromatographic data and suggests that reductase-120 is more sensitive to clofibrate treatment than reductase-60.

Even though several experiments were carried out with isolated peroxisomes, there did not exist clear quantitative data regarding the changes in the rate of the β-oxidation of unsaturated fatty acids during peroxisome proliferation. To study the induction and substrate specificity of the peroxisomal reductase, peroxisomes were isolated from livers of control and clofibrate-treated rats as described in Materials and Methods. As an indicator of effective clofibrate treatment, acyl-CoA oxidase activity increased from 12.8 ± 3.2 mU/mg peroxisomal protein (n = 4) to 93.4 ± 12.9 mU/mg peroxisomal protein (n = 5) (means ± SD). In the same peroxisome preparations, 2,4-dienoyl-CoA reductase activities were 21.0 ± 6.5 mU/mg peroxisomal protein ($C6_{2,4}$), 7.1 ± 2.9 mU/mg peroxisomal protein ($C22_{2,4,7,10,13,16,19}$) and 37.5 ± 14.9 mU/mg peroxisomal protein ($C6_{2,4}$) and 13.0 ± 3.3 mU/mg peroxisomal protein ($C22_{2,4,7,10,13,16,19}$) in peroxisomes from control and clofibrate-treated rats, respectively.

The effects of clofibrate on peroxisomal proteins have been shown to be due to an increase in their mRNA levels (8). To study whether this is also the case for the mitochondrial clofibrate-inducible proteins reductase-120 and short-chain Δ^3,Δ^2-enoyl-CoA isomerase, their mRNA levels were compared by in situ hybridization in rat tissue samples with those of the peroxisomal multifunctional isomerase-hydratase-dehydrogenase enzyme (MFE) (26), which has been well documented to be highly inducible (8, 27).

In in situ hybridization experiments, the mRNA levels of all the proteins studied here were low in the control animals. When the rats were fed clofibrate for 1, 3, 6, and 14 days, however, a clear increase in the levels of all the mRNAs studied was seen in the liver tissue; those in the other tissues (heart, kidney, small intestine, testis and brain) remained low. In in situ hybridization the level of induction varied considerably among mRNAs studied. The clofibrate response was already detected on day 1 (isomerase, MFE) and reached its maximum on day 3 or 6 (**Table 2, Fig. 3**). The hybridization signal seemed to be slightly stronger in the periportal than in the pericentral regions of liver, but this finding was not totally consistent. The RNA slot hybridization experiments confirmed the in situ hybridization results. mRNA isolated from the same tissue samples as used for the in situ hybridizations was hybridized with the cDNA probes (MFE, 2,4-dienoyl-CoA reductase, Δ^3,Δ^2-enoyl-CoA isomerase). A cDNA probe for glyceraldehyde-3-phosphate dehydrogenase was used as a control, as the gene for this protein is regarded as being expressed steadily in different tissues. When the filter was subsequently hybridized with

Fig. 3. In situ hybridizations to demonstrate the expression of mRNAs for 2,4-dienoyl-CoA reductase, Δ^3,Δ^2-enoyl-CoA isomerase, and MFE in hepatocytes from the periportal areas (marked with P) of rat livers. There was a slight increase in the hybridization signals in the course of the treatment for 2,4-dienoyl-CoA reductase mRNA (A–C), a moderate increase in the mRNA signals for Δ^3,Δ^2-enoyl-CoA isomerase (D–F), and an intense labeling for MFE mRNAs (G–I). The magnitudes in the images are ×265 (A–H) and ×105 (I). Light field images for 2,4-dienoyl-CoA reductase mRNAs in a rat with no clofibrate treatment (A), after 1 day of clofibrate treatment (B), and after 14 days of clofibrate treatment (C). Images for Δ^3,Δ^2-enoyl-CoA isomerase mRNAs in a rat with no clofibrate treatment (D), after 1 day of clofibrate treatment (E), and after 14 days of clofibrate treatment (F). Images for MFE mRNAs in a rat with no clofibrate treatment (G), after 1 day of clofibrate treatment (H), and after 14 days of clofibrate treatment (I).



the probes, a clear increase following the time course of the treatment was seen with all the probes except the control (**Fig. 4**). The mRNA levels of MFE were increased 15-fold, isomerase 29-fold, whereas reductase mRNAs levels were increased 7-fold.

DISCUSSION

The induction of peroxisomal proteins by peroxisome proliferators has been shown to be due to their increased transcription rates (8) and recent findings have shed some light on the actual mechanism of peroxisome proliferation. A member of a nuclear hormone receptor superfamily has been identified as a peroxisome proliferator-activated receptor (PPAR) (28, 29). After ligand binding, the receptor binds to a peroxisome proliferator-responsive element (PPRE) in the 5' flanking region of its target gene (30, 31), and thus enhances gene expression. It seems likely that the PPRE is found in the promotor regions of most genes responding to peroxisome proliferators. This induction mechanism is regarded as being responsible for the observed increase in the peroxisomal β-oxidation rate after treatment with peroxisome proliferators. In addition to the enzymes of the inducible pathway, there are other non-inducible proteins for acyl-CoA oxidase (32) and 3-ketoacyl-CoA thiolase (33) that catalyze the same reaction steps. The effect of peroxisome proliferators on the overall β-oxidation rate in the mitochondria is minimal, a 2-fold increase having been reported (34). This is probably the reason that the effect of such proliferators on mitochondria has not attracted much attention.

The chromatographic data and immunological evidence presented in this work show that the activity and protein levels of 2,4-dienoyl-CoA reductase (reductase-120) increase about 6-fold in rat liver during clofibrate treatment. The Δ^3,Δ^2-enoyl-CoA isomerase activity was also found to increase at least 10-fold, and published data suggest that this is mainly due to a rise in the level of the mitochondrial short chain Δ^3,Δ^2-enoyl-CoA isomerase protein (2, 9).

Our in situ hybridization and slot hybridization experiments show that the increase of the mitochondrial enzymes is also due to an increase in the mRNA levels of these proteins. The in situ hybridization technique does not provide information on the rate of synthesis or degradation of mRNA, but as the increase in the mRNA levels of the peroxisomal proteins is due to increased transcription rates (8) and MFE and the mitochondrial short-chain isomerase and reductase-120 seem to respond in parallel, it is likely that induction arises in the same way in both organelles. The induction was detectable in the liver, which is in agreement with previous results (35), but in situ hybridization experiments showed no response in

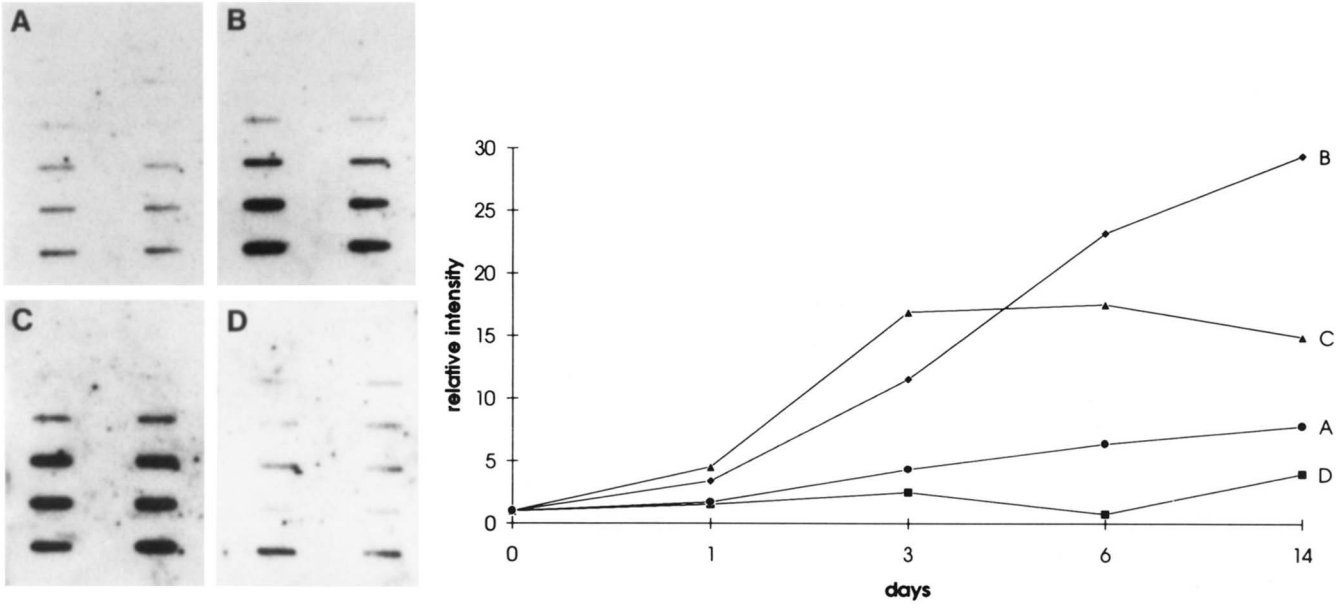


Fig. 4. Slot hybridizations with cDNA probes for 2,4-dienoyl-CoA reductase, Δ^3,Δ^2-enoyl-CoA isomerase, MFE, and glyceraldehyde-3-phosphate dehydrogenase. Left panel: mRNAs were isolated from the livers of rats treated with clofibrate for 0, 1, 3, 6, and 14 days. mRNA (0.1 μg) was applied to each well and similar samples to both columns. Line 1: mRNA from rats with no clofibrate treatment; line 2: 1 day of treatment; line 3: 3 days of treatment; line 4: 6 days of treatment; line 5: 14 days of treatment. A: 2,4-dienoyl-CoA reductase; B: Δ^3,Δ^2-enoyl-CoA isomerase; C: MFE; D: glyceraldehyde-3-phosphate dehydrogenase. Right panel: The absorbances of the signals shown in the left panel were quantified with BioImage® apparatus. For each probe the signal density on day 0 was given the value 1.0, and the values for other time points are given as relative absorbances (= absorbance at the given time point/absorbance on day 0).

other tissues tested (heart, kidney, brain, intestine, and testis) even after 14 days of clofibrate treatment (data not shown).

The identification of an inborn error involving 2,4-dienoyl-CoA reductase deficiency gives a clue to the different physiological functions of the different reductases (36). As there are very few peroxisomes in muscle, and as 17% of normal reductase activity was detected in muscle tissue of this patient, this can be taken as indirect evidence that the error was due to a deficiency in one mitochondrial isoform. This, in turn, suggests that the mitochondrial isoforms have different physiological functions, as the condition was lethal. Our previous work had shown that the mitochondrial reductase isoforms have different physical properties (pI, M_r, immunological reactivity) (1), but no clear differences were found in their catalytic activity towards C6 and C10 substrates. The present work has demonstrated that the both mitochondrial isoforms showed 1.5- to 2.1-fold higher catalytic activity towards the C6 substrate than the C22 substrate. Thus studies on substrate specificities did not reveal any clue to the possible differences in their roles in fatty acid metabolism. Furthermore, measurements for reductase activity in isolated peroxisomes showed similar specificity to substrates tested.

Only two types of experimental observations point to differences in the physiology of the mitochondrial reductases, namely *i)* the differential isoform profile in heart and liver, and *ii)* their differential responses to clofibrate treatment. 4-Pentenoate (or more precisely its catabolite 3-keto-4-pentenoyl-CoA) is a potent inhibitor of mitochondrial β-oxidation (37), and the different responses of the mitochondrial reductases to clofibrate stimulation detected here allow a re-evaluation of the metabolic effect of pent-4-enoyl-CoA on liver mitochondria isolated from control and clofibrate-treated rats (10). As clofibrate treatment induces the activity of reductase-120, this isoform could be responsible for the previously observed increased rate of pentenoate degradation at the level of 2,4-pentadienoyl-CoA, which is, in turn, reflected by the abolishment of the inhibition of fatty acid oxidation. Furthermore, the significance of the regulation of mitochondrial 2,4-dienoyl-CoA reductase activity is also demonstrated in experiments with liver mitochondria isolated from streptozotocin-induced diabetic rats. In these mitochondria 2,4-dienoyl-CoA reductase activity was stimulated 5-fold and subsequently the inhibitory effect of polyunsaturated enoyl-carnitines on mitochondrial β-oxidation was partially suppressed (38).

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