0031-3998/87/2206-0748$02.00/0
PEDIATRIC RESEARCH

Vol. 22, No. 6, 1987
Printed in U.S.A.

Peroxisomal Proliferation in Heart and Liver of Mice Receiving Chlorpromazine, Ethyl 2(5(4-Chlorophenyl)Pentyl) Oxiran-2-Carboxylic Acid or High Fat Diet: A Biochemical and Morphometrical Comparative Study

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ABSTRACT. Chlorpromazine and related drugs including trifluoperazine, clopenthixol, and fluphenazine are *in vitro* inhibitors of mitochondrial carnitine palmitoyltransferase and cytochrome c oxidase and of peroxisomal carnitine octanoyltransferase from mouse heart and liver. By contrast with 0.1% ethyl 2(5(4-chlorophenyl)pentyl) oxiran-2-carboxylic acid or 0.1% clofibrate-containing diets, the treatment of mice with 0.1% chlorpromazine-containing diet fails to induce peroxisomal proliferation in liver and heart. An 0.5% chlorpromazine-containing diet did induce peroxisomal proliferation. Inhibition of peroxisomal β-oxidation presumably via the reduction of carnitine octanoyltransferase by chlorpromazine elicits the appearance in liver of lamellar structures resembling those seen in human peroxisomal disorders and induces accumulation of very long-chain fatty acids in plasma. The peroxisomal proliferation induced by administration of high dose chlorpromazine is ascribed to its ability to depress mitochondrial fatty acid oxidation by impairing cytochrome c oxidase and carnitine palmitoyltransferase activities. (*Pediatr Res* 22: 748–754, 1987)

Abbreviations

DAB, 3,3′-diaminobenzidine
POCA, ethyl 2(5(4-chlorophenyl)pentyl) oxiran-2-carboxylic acid

Experiments with chlorpromazine, an inhibitor of peroxisomal carnitine octanoyltransferase, have led to the proposal that peroxisomal β-oxidation which was depressed by the phenothiazine in isolated hepatocytes (1) was dependent on carnitine (2). On the other hand, the inhibition of both cytochrome c oxidase and chlorpromazine palmitoyltransferase activities (2) were proposed as the cause of the reduced ketone body formation from long-chain fatty acids (1) in isolated hepatocytes incubated in the presence of chlorpromazine at concentrations between 0.4 to 1.0 mM.

In several circumstances, *in vivo* inhibition of mitochondrial fatty acid oxidation in rodents is associated with peroxisomal proliferation. The latter phenomenon mainly consists of an increased peroxisomal population and enhanced capacity of the peroxisomal β-oxidation. One example is treatment of rodents with 2-oxiran carboxylic acid derivatives such as POCA which are well-known inhibitors of hepatic carnitine palmitoyltransferase type I and which produce a peroxisomal proliferation in liver (3). Another example is given by treatment of mice with valproic acid (4, 5).

Chlorpromazine, unlike 2-oxiran carboxylates, has been reported to produce lipid accumulation but no induction of peroxisomal proliferation in rodent livers (6). This discrepancy between the *in vitro* property of chlorpromazine to inhibit mitochondrial fatty acid oxidation and the failure of the drug to increase, *in vivo*, the peroxisomal population and β-oxidation capacity is only apparent. Indeed, we demonstrate that a diet containing 0.5% (w/w) chlorpromazine causes peroxisomal proliferation in liver and heart. On the other hand, we report that the diet containing only 0.1% (w/w) chlorpromazine is unable to induce peroxisomal proliferation and we cannot conclude that this drug concentration inhibits mitochondrial fatty acid oxidation *in vivo*. In the treated animals, impairment of peroxisomal fatty acid oxidation could occur as attested by a lipid deposition in liver cells mimicking the storage of very long-chain fatty acids in tissues from patients with peroxisomal disorders and by the accumulation of the very long-chain substrates in plasma. The effects of the administration to mice of the chlorpromazine-containing diets are compared with those of a 0.1% (w/w) POCA and high fat [20% oleate-(w/w)] diets.

MATERIALS AND METHODS

Adult male NMRI mice, weighing at least 28 g, were used. Control mice were fed on a standard animal food. Treated animals were fed on a diet obtained by mixing the powdered animal food with either 0.1% clofibric acid, 0.1% POCA, 20% oleate, and 0.1 or 0.5% chlorpromazine.

Clofibric acid (2-5p(chlorophenoxy)-2-methylpropionic acid), trifluoperazine, homovanillic acid, and peroxidase type II were purchased from Sigma Chemical Co. (St. Louis, MO). The CoA derivatives of palmitic, lauric, octanoic, hexanoic, and acetic

Received March 12, 1987; accepted August 14, 1987.
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Supported by Grants of the Belgian NFWO, FNRS, and SPPS, by Grant 84/90-74 of the "Action de Recherche Concertée des Services du Premier Ministre", U.S. Public Health Services Grant AM9235, European Communities Twinning Research Program, and Grant 3.0071.83 of the Belgian FGWO. J.V. is Chargé of the Belgian FNRS.

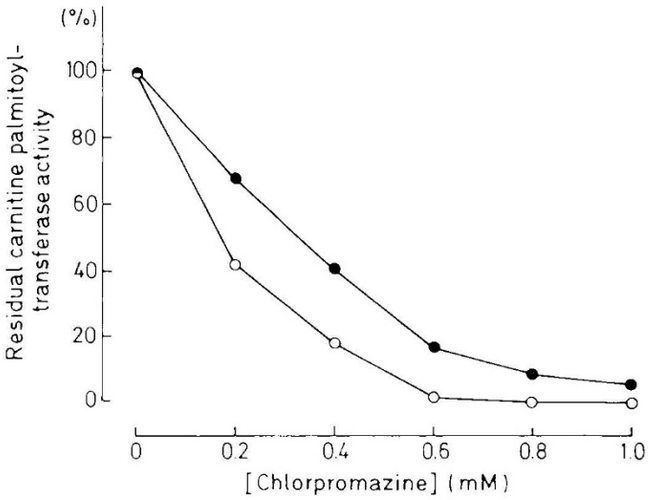

Fig. 1. Effect of chlorpromazine on the reaction catalyzed by carnitine palmitoyltransferase and monitored in mouse heart (○) and liver (●) homogenates. The activities are expressed as the percentage of the activity measured in the absence of chlorpromazine.

acids were from Pharmacia (Uppsala, Sweden); FAD, NAD were from Boerhinger Pharma (Mannheim, FRG) and other common chemicals were of analytical grade from Merck (Darmstadt, FRG).

Chlorpromazine was kindly offered by Professor G. Lambert (Department of Pharmacology, University of Louvain, Louvain, Belgium). Fluphenazine and clopenthixol were gifts from Labaz (Brussels, Belgium) and Lundbeck (Brussels, Belgium), respectively. POCA was generously given by Dr. G. Ludwig of BYK Gulden Lomberg Chemische Fabrick GmbH (Konstanz, FRG).

Previously established procedures were used for the assay of the enzymes: palmitoyl-CoA oxidase, lauroyl-CoA oxidase and glycolate oxidase (7), carnitine acyltransferases (8), cytochrome c oxidase (9), catalase and urate oxidase (10), and butyryl-CoA dehydrogenase (11). The cyanide-insensitive lauroyl-CoA oxidation was measured according to Lazarow and de Duve (12) and the oxidation by coupled mouse liver mitochondria of palmitoylcarnitine was measured as described by Van Hoof *et al.* (13). Protein was measured by the method of Lowry *et al.* (14) using bovine serum albumin as standard.

Electron microscopic studies were performed as described by Van Hoof *et al.* (13) for tissue samples without cytochemistry or Van den Branden *et al.* (15) for cytochemical investigations. For stereological studies, the sections photographed were, in order to

Table 1. *Effects of 0.1% POCA-, 0.1% clofibrate-, and 0.1% chlorpromazine-containing diets given to mice during 4 days on liver peroxisomal oxidations, catalase, and carnitine acyltransferase activities**

	Controls	0.1% clofibrate	0.1% POCA	0.1% chlorpromazine
Carnitine acetyltransferase	0.31 ± 0.06	1.07 ± 0.26	2.79 ± 0.73	0.27 ± 0.09
Carnitine octanoyltransferase	2.10 ± 0.10	3.18 ± 0.16	11.36 ± 1.04	1.56 ± 0.59
Carnitine hexanoyltransferase	2.57 ± 0.31	3.79 ± 0.37	25.73 ± 5.20	2.12 ± 0.26
Carnitine palmitoyltransferase	1.12 ± 0.13	1.45 ± 0.12	3.68 ± 0.27	0.99 ± 0.07
Lactate dehydrogenase	148 ± 8	167 ± 4	123 ± 7	130 ± 6
Catalase	2461 ± 276	4996 ± 774	9867 ± 2184	1792 ± 178
Palmitoyl-CoA oxidase	0.88 ± 0.02	4.01 ± 1.00	18.08 ± 2.00	0.94 ± 0.10
Lauroyl-CoA oxidase	1.32 ± 0.22	5.45 ± 1.12	30.73 ± 1.86	1.40 ± 0.17
Cyanide-insensitive lauroyl-CoA oxidation	1.16 ± 0.31	5.06 ± 1.14	26.55 ± 5.20	1.07 ± 0.24
Glycolate oxidase	0.44 ± 0.04	0.17 ± 0.07	0.30 ± 0.08	0.23 ± 0.05
Urate oxidase	1.07 ± 0.09	0.91 ± 0.14	0.96 ± 0.07	1.15 ± 0.06

* Enzyme activities are expressed as μmol of substrate consumed or product formed per min and per g of liver. Results are the mean activity ± SEM calculated from at least six animals.

Table 2. *Effects of 7-day administration of 0.1% POCA-, 20% oleate-, and 0.5% chlorpromazine-containing diets to mice on liver mitochondrial and peroxisomal oxidations, catalase, and carnitine acyltransferase activities**

	Controls	0.1% POCA	20% oleate	0.5% chlorpromazine
Carnitine acetyltransferase	1.74 ± 0.37	15.31 ± 4.87	4.29 ± 0.41	2.97 ± 0.35
Carnitine octanoyltransferase	10.08 ± 2.12	63.87 ± 12.09	27.28 ± 6.57	27.90 ± 0.35
Carnitine palmitoyltransferase	6.21 ± 0.95	17.20 ± 2.60	10.72 ± 1.34	13.81 ± 1.53
Butyryl-CoA dehydrogenase	9.24 ± 2.01	9.40 ± 0.92	7.09 ± 0.71	6.64 ± 0.88
Mitochondrial palmitoylcarnitine oxidation	0.25 ± 0.03	0.70 ± 0.11	0.15 ± 0.04	0.19 ± 0.04
Catalase	10.73 ± 1.40	35.00 ± 5.64	12.89 ± 2.47	10.72 ± 1.50
Palmitoyl-CoA oxidase	4.70 ± 0.39	84.42 ± 11.03	24.48 ± 4.93	24.31 ± 5.02
Lauroyl-CoA oxidase	7.24 ± 1.00	140.3 ± 18.5	32.52 ± 5.37	37.14 ± 4.38
Cyanide-insensitive lauroyl-CoA oxidation	6.05 ± 1.98	109.0 ± 13.6	27.59 ± 6.30	30.50 ± 6.87
Glycolate oxidase	2.51 ± 0.32	2.72 ± 0.40	3.28 ± 0.52	2.81 ± 0.52
Urate oxidase	5.38 ± 0.40	6.22 ± 0.83	8.06 ± 1.31	6.12 ± 0.67
Protein (mg/g liver)	192 ± 26	167 ± 15	159 ± 16	173 ± 22

* Catalase reaction excepted (U/mg protein), enzyme activities are expressed as mU/mg protein. Results are the mean activity ± SEM calculated from at least six animals.

analyse distinct cells, taken from ribbons separated by at least 20 μm. The magnification was determined with a grating replica (E. F. Fullam Inc., Schenectady, NY). A multipurpose test grid similar to that described by Weibel *et al.* (16) was used to calculate the volume fraction or membrane area. For the recording and processing of morphometrical data, an Apple II plus (Apple Computer, Inc. Cupertino, CA) was used.

Very long-chain fatty acids were measured in plasma, each

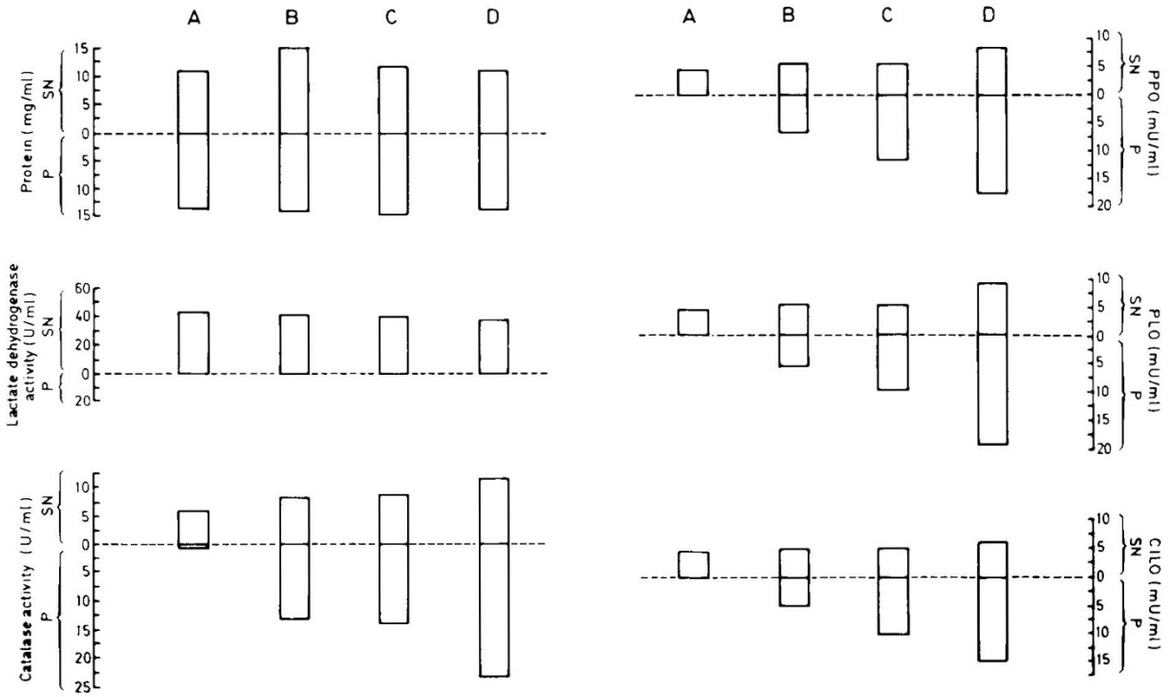

Fig. 2. Effects of various treatments of mice on the activity and sedimentability of heart peroxisomal enzymes. *A*, untreated mice; *B*, *C*, and *D*, mice receiving 4 days of the 0.1% POCA, 20% oleate, and 0.5% chlorpromazine-containing diets, respectively. The supernatant (*SN*) and pellet (*P*) fractions were obtained after high speed centrifugation (3,000,000 × *g* min) of the postnuclear supernatants (6,000 × *g* min) from, in each condition, six pooled myocardia. The values reported in histograms are the means calculated from results of three separate experiments. The enzyme activities are expressed as nmol (mU) or μmol (U) of substrate consumed or product formed per min per ml of fraction. Each (SN or P) fraction corresponds to 200 mg of myocardium/ml. *PPO*, peroxisomal palmitoyl-CoA oxidase (H_2O_2 production); *PLO*, peroxisomal lauroyl-CoA oxidase (H_2O_2 production); *CILO*, cyanide-insensitive lauroyl-CoA oxidation (NADH production).

Table 3. *Quantitative ultrastructural analysis of mouse liver hepatocytes**

	Controls	0.1% POCA	20% oleate	0.5% chlorpromazine
Fractional volume (% of cytoplasmic volume)				
Peroxisomes	1.41 ± 0.12	3.87 ± 0.41	2.35 ± 0.29	2.51 ± 0.30
Lipid droplets	0.29 ± 0.15	3.28 ± 0.92	1.57 ± 0.61	1.90 ± 0.26
Mitochondria	19.50 ± 0.93	18.53 ± 1.13	18.29 ± 1.07	15.95 ± 0.76
External surface ($\mu m^2/\mu m^3$)				
Peroxisomes	0.118 ± 0.012	0.369 ± 0.035	0.188 ± 0.028	0.313 ± 0.042
Lipid droplets	0.024 ± 0.009	0.081 ± 0.015	0.136 ± 0.075	0.153 ± 0.024
Mitochondria	1.241 ± 0.081	1.037 ± 0.056	0.984 ± 0.095	0.971 ± 0.043
Feature/μm^2				
Peroxisomes	0.063 ± 0.006	0.192 ± 0.019	0.121 ± 0.017	0.232 ± 0.035
Lipid droplets	0.010 ± 0.004	0.022 ± 0.004	0.019 ± 0.007	0.085 ± 0.010
Mitochondria	0.495 ± 0.025	0.436 ± 0.035	0.377 ± 0.027	0.375 ± 0.020
Particles/μm^3				
Peroxisomes	0.097	0.308	0.198	0.514

* The analyzed section area was 1357, 1460, 1430, and 1453 μm^2 for control, 0.1% POCA, 20% oleate, and 0.5% chlorpromazine treatments, respectively. Values are given ± SEM for analyzed sample. The estimation of particles numbers is obtained by application of the equation: $N_v = (N_a)^{3/2}/\beta(V_v)^{1/2}$, (16), where N_v is the number of particles per unit volume, N_a is the number of profiles per unit area in section, V_v is the volume fraction of particles, and β is a shape factor. Peroxisomes are considered as spheres ($\beta = 1.382$) and their population is considered homogeneous in size.

experimental group consisting of at least four animals. Their extraction was performed as described by Folch *et al.* (17) after addition of 2 μg of heptacosanoic acid as internal standard and before transmethylation with HCl 2 N in methanol at 75°C for 16 h. The gas chromatographic identification of methylesters was made by comparison of the retention times with those of known standards.

RESULTS

Biochemical Studies. In vitro Inhibition by Chlorpromazine of Several Enzyme Activities. The inhibitory effect of chlorpromazine on the activity of the total mitochondrial carnitine palmitoyltransferase from mouse heart and liver is illustrated in Figure 1. In this experiment the liver enzyme activity in the presence of 1 mM chlorpromazine amounted to 0.07 (control values 1.16) U/g tissue. The heart enzyme reaction was completely prevented by this concentration of the phenothiazine. Analogs including fluphenazine, clopenthixol, and trifluoperazine also inhibited both liver and heart carnitine palmitoyltransferases. In both organs, higher to lower inhibitions were respectively achieved by chlorpromazine, trifluoperazine, clopenthixol, and fluphenazine (not shown). Mouse liver and heart cytochrome c oxidase activities were similarly inhibited by chlorpromazine and could not be detected in the presence of more than 0.8 mM chlorpromazine. The strongest inhibition was obtained under clopenthixol: 0.15 mM clopenthixol induced complete inhibition of cytochrome c oxidase activity from liver or heart. Peroxisomal carnitine octanoyltransferase was inhibited by phenothiazines in these mouse tissues (data not shown) as previously reported for rat liver (2).

The 0.1% Chlorpromazine-Containing Diet. Administration of a 0.1% chlorpromazine-containing diet for 1 wk had little or no

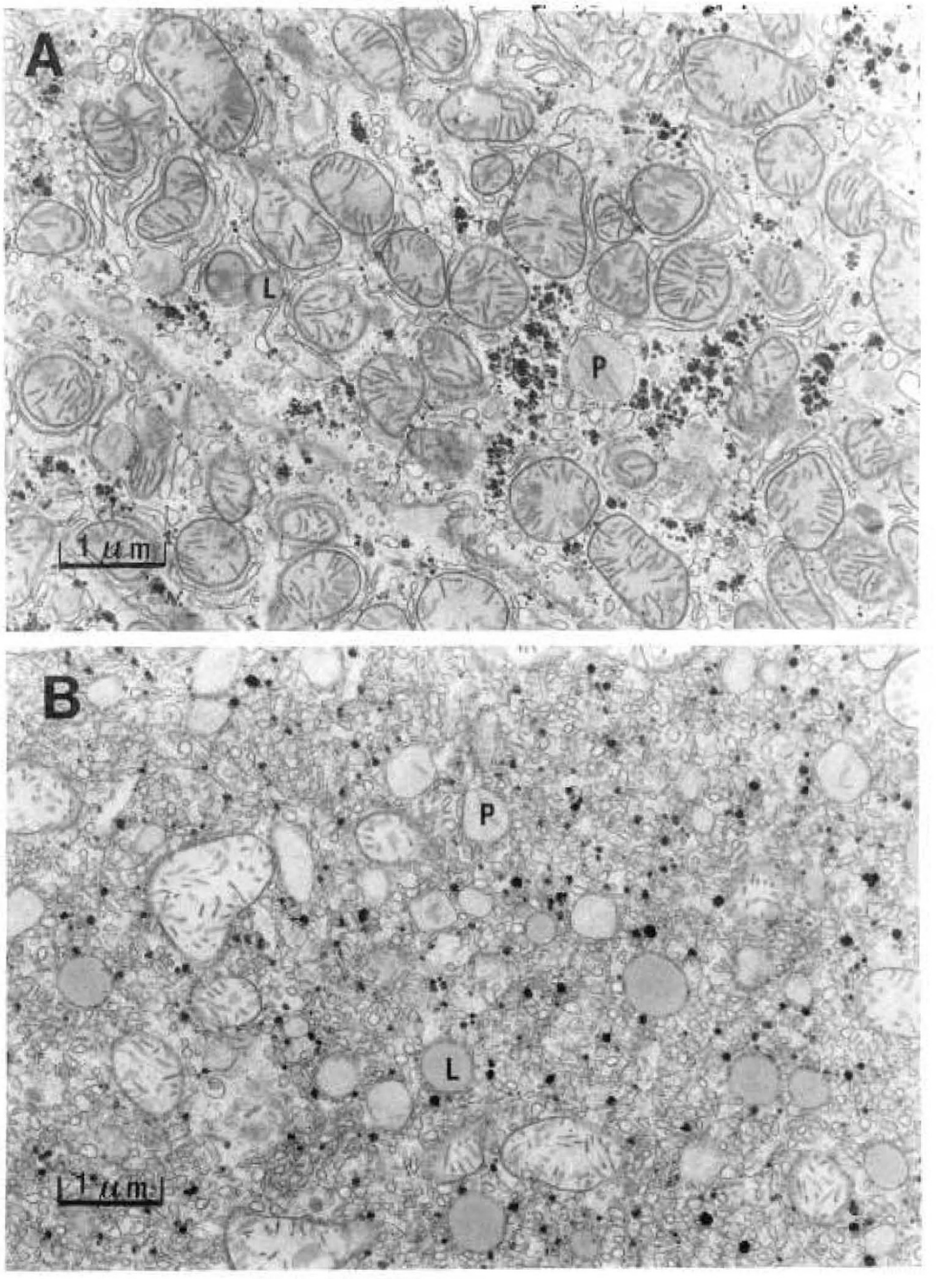

Fig. 3. Peroxisomal proliferation in hepatocytes from the 0.5% chlorpromazine-treated mice. *L*, lipid droplets; *P*, peroxisomes. *A*, control mouse liver (1 μm). *B*, chlorpromazine-treated mouse liver, 0.5% (1 μm).

effect on liver peroxisomal β-oxidation, catalase, or carnitine acyltransferase activities (Table 1). Prolonged treatment with 0.1% chlorpromazine (1 month) did not further modify these enzyme activities (not shown). By contrast, in livers from animals given the 0.1% clofibrate or 0.1% POCA-containing diet for 1 wk, an enhanced capacity of peroxisomal β-oxidation as well as increased carnitine acyltransferase activities were measured (Table 1). The administration of 0.1% chlorpromazine-containing diet failed to increase the activity of the peroxisomal β-oxidation in heart (not shown).

Administration to Mice of a 0.5% Chlorpromazine-Containing Diet. The higher dose of chlorpromazine was effective after 4 days in producing increased liver peroxisomal β-oxidizing capacity and proliferation of hepatic peroxisomes (see below). The effects of this treatment on liver enzymology were less pronounced than those obtained under 0.1% POCA treatment and of the same magnitude as those found after a high fat diet (Table 2). The hepatic enzymes were affected differently by the treatments and, in livers from the 0.5% chlorpromazine-treated mice, the activities of palmitoyl-CoA oxidase, lauroyl-CoA oxidase, and cyanide-insensitive lauroyl-CoA oxidation were increased 5-fold; those of carnitine acyltransferases were approximately 2-

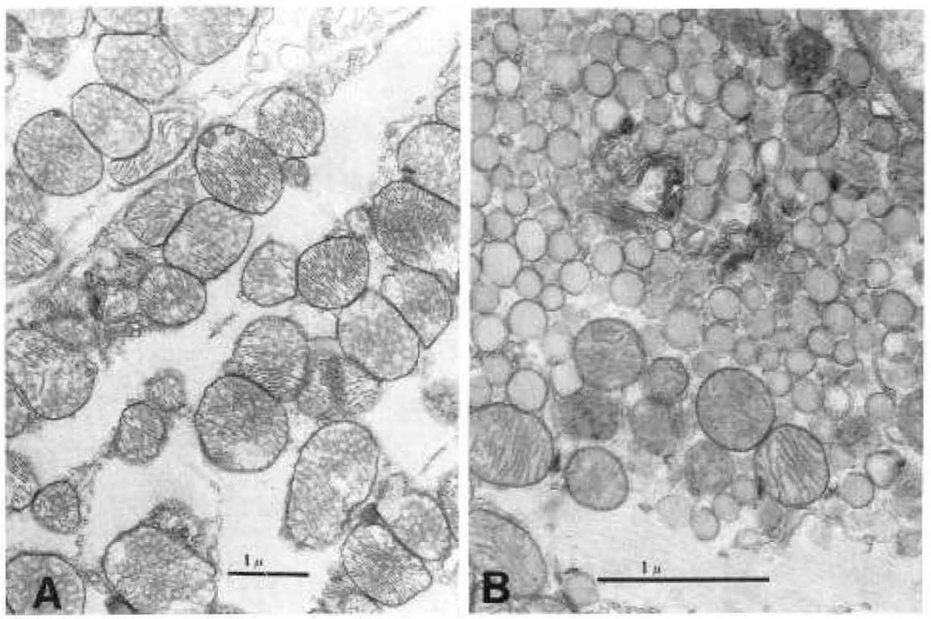

Fig. 4. Comparison between cardiomyocytes from control (*A*) and 0.5% chlorpromazine-treated (*B*) mice. Note the massive organelle proliferation characterizing the heart cell from the treated animal.

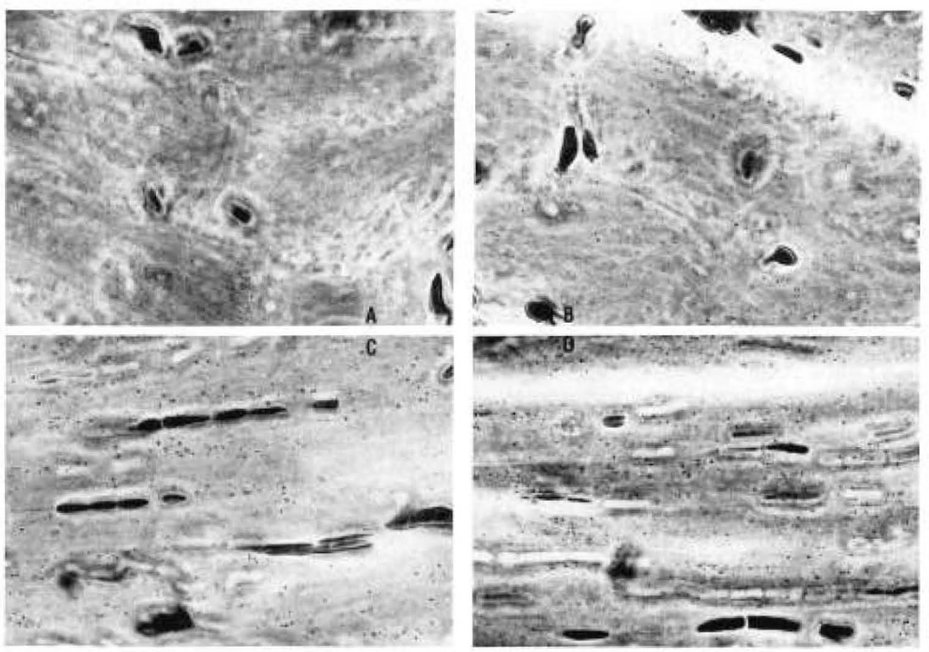

Fig. 5. Comparison between the peroxisomal proliferations induced by various treatments in myocardium. Peroxisomes are recognised by their positive response (*black labeling*) to the DAB reaction. *A*, untreated mice; *B*, 0.1% POCA-treated mice; *C*, 20% oleate-treated mice; *D*, 0.5% chlorpromazine-treated mice.

fold higher, while catalase, glycolate oxidase, urate oxidase, and butyryl-CoA dehydrogenase activities as well as mitochondrial palmitoylcarnitine oxidation were normal or decreased (Table 2).

Heart peroxisomal induction was minimal in mice receiving POCA and optimal in the animal given 0.5% chlorpromazine or high fat diets. Biochemical changes characterizing the peroxisomal proliferation in the heart were the enhancement of carnitine octanoyltransferase (not shown), catalase and peroxisomal β-oxidizing activities, and a higher proportion of these activities linked to sedimentables particles (Fig. 2).

Morphological studies. The peroxisomal population was studied by light and electron microscopy in liver and heart from 0.1% POCA-, 20% oleate-, 0.1 and 0.5% chlorpromazine-treated mice and compared with controls. During the administration of the 0.1% chlorpromazine-containing diet, an abnormal occurrence of lipid droplets was observed in liver cytosol but no significant change was induced at the level of peroxisomal population in liver and heart.

Lipid Droplets. In hepatocytes from animals receiving the 0.5% chlorpromazine-containing diet, the number of lipid droplets was increased relative to the control liver cells while a peculiar aspect of the smooth endoplasmic reticulum was noticed. The accumulation of lipid droplets was also observed in hepatocytes from 0.1% POCA- and 20% oleate-treated mice. The characteristics of this abnormal accumulation of lipid droplets are given in Table 3. Conversely to liver, no abnormal accumulation of lipid droplets was seen in cardiac cells from treated animals.

Peroxisomal Proliferation. Peroxisomes were increased in size and number in hepatocytes from treated animals (Table 3). The increase of the peroxisomal volume fraction was maximal after POCA. By contrast with peroxisomes, little or no change in the mitochondrial population could be recorded. The ratio between the peroxisomal and the mitochondrial volume fractions were equal to 0.07, 0.21, 0.13, and 0.16 in livers from the control, the 0.1% POCA-, 20% oleate-, and 0.5% chlorpromazine-treated animals, respectively. The ratio between the peroxisomal and the mitochondrial envelope surfaces also was increased after treatment (Table 3). Figure 3 illustrates the peroxisomal proliferation in liver from mice given the 0.5% chlorpromazine treatment.

In myocardial cells, increased numbers of microbodies were observed 4 days after the onset of the treatments of mice with the 0.1% POCA-, 20% oleate-, and 0.5% chlorpromazine-containing diets (Fig. 4). The peroxisomal nature of these cellular structures was strongly supported by their cytochemical reaction for catalase. Maximal peroxisome proliferation in heart cells was obtained after administration of oleate and chlorpromazine while POCA treatment was less efficient (Fig. 5).

Lamellar Structures. The 0.1 and 0.5% chlorpromazine treatments resulted in the abnormal occurrence of lamellar structures in the liver cytosol (Fig. 6). The latter material was more frequently seen in the animals receiving the lower dose of chlorpromazine. It is similar to that observed in cytosol or in lysosomes from liver or other tissues of patients suffering from peroxisomal deficiency syndromes (Fig. 6) and is classically believed to represent very long-chain fatty acid deposition (18–22). In the 0.1% chlorpromazine-treated animals, the deficient oxidation of very long-chain fatty acids was confirmed by their accumulation in plasma; the C_{26}/C_{22} ratio [ratio between cerotic (C_{26}) and behenic (C_{22}) acids] was increased 2- to 3-fold (23).

DISCUSSION

Changes induced in liver. Peroxisomal proliferation in liver from rodents in a variety of conditions has been documented extensively (12, 24). In most cases it consists of the enhancement of both peroxisomal β-oxidizing activities and peroxisomal volume fractions. In addition to the peroxisomal changes, increased ability of mitochondria to oxidize fatty acids also has been reported (25–27). Diets containing 0.1% POCA, 20% oleate, and most probably 0.5% chlorpromazine lead to the inhibition or chronic overloading of mitochondrial fatty acid oxidation. Induction of peroxisomal proliferation is a common characteristic of these animal models.

In the animals receiving a 0.1% chlorpromazine-containing diet, no peroxisomal proliferation occurs. In this case, the peroxisomal fatty acid oxidation is impaired *in vivo* as attested by the deficient oxidation of very long-chain fatty acids. Lamellar structures that are similar to the inclusions in the cerebrohepatorenal syndrome (Zellweger disease) and infantile Refsum disease livers and in adrenoleukodystrophy brain are observed in hepatocytes from 0.5% chlorpromazine-treated animals and compared with lamellar structures in a patient with deficient acyl-CoA oxidase. These structures are believed to be elicited by the well-known storage of very long-chain fatty acids in these diseases (18, 28). The shortening of very long-chain substrates has been demonstrated to be catalyzed by the peroxisomes (29, 31). Multilamellar structures also have been described in lysosomes from tissues exposed to cationic amphiphilic drugs that inhibit lysosomal phospholipid degradation (32–34). In our experiments, the increase of very long-chain fatty acids in plasma supports the idea that lamellar structures represent very long-

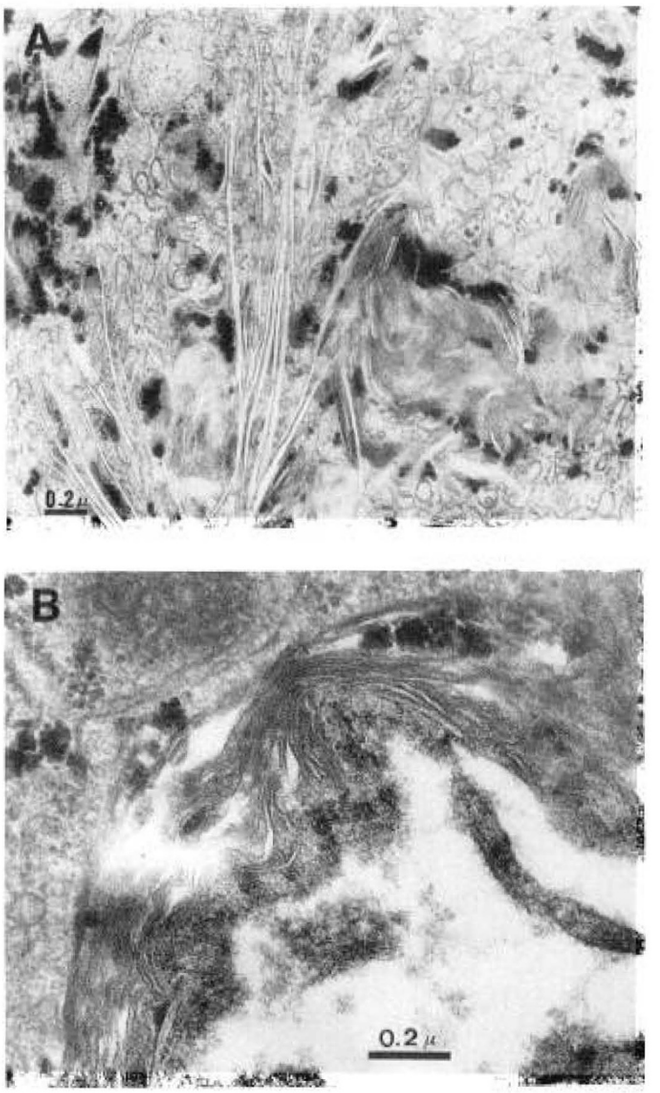

Fig. 6. Lamellar structures seen in liver cytosol from a 0.1% chlorpromazine-treated mouse (*A*) and from a patient with the acyl-CoA oxidase deficiency (*B*).

chain fatty acid deposition consecutive to the inhibition (*in vivo*) of peroxisomal fatty acid oxidation. Both mechanisms (deficient phospholipid degradation and peroxisomal fatty acid oxidation) may operate simultaneously in the genesis of the lamellar structures in chlorpromazine-treated mouse livers.

The mouse liver carnitine acyltransferases are inhibited by chlorpromazine and are not inactivated by the phenothiazine. Indeed, the activity of these enzymes measured on liver homogenates are normal and increased in mice given the 0.1 and 0.5% chlorpromazine-containing diets, respectively.

Peroxisomal proliferation also takes place in the myocardium. Both biochemical and cytochemical aspects of heart peroxisomal induction have been studied in previous works. In rodents, peroxisomal β-oxidation activity has been measured in the heart (35, 36) as well as its enhancement when partially hydrogenated fish oil or soybean oil are included in the diet (37). The morphological description of heart peroxisomes in rodents, but also in primates, has been given previously (38). Fahimi *et al.* (39) have demonstrated that ethanol in the diet was efficient enough in rats to increase myocardial catalase activity and the number of heart DAB-reactive organelles. The increase of heart peroxisomes has also been described in mice given phytol (15). With the present work, we demonstrate the parallelism that exists between the number of DAB-positive organelles and the activity and sedimentability of catalase and peroxisomal β-oxidation. As was suggested for liver, the propensity of the treatments to induce peroxisomal proliferation in the heart might be correlated to their ability to depress mitochondrial fatty acid oxidation in this tissue.

Acknowledgments. Professor François Van Hoof is gratefully acknowledged. We thank Professor G. Dacremont (Gent) for the assay of very long-chain fatty acids. The biopsies of the patient with acyl-CoA oxidase deficiency was made available by Dr. J. Scotto, Dr. B. T. Poll-Thé, and Dr. J. M. Saudubray (Paris). The authors are endebted to Than-Tuyn Müller, Annette Marcelis-Herssens, and Bernadette Wantier. Marina Pauwels prepared the cytochemical stains for light and electron microscopy.

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