

Short and long term influence of phenothiazines on liver peroxisomal fatty acid oxidation in rodents

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Evidence is given that phenothiazines depress hepatic peroxisomal fatty acid oxidation *in vivo*. After oral administration to rats thioridazine and chlorpromazine inhibit peroxisomal β -oxidation, evaluated by H_2O_2 production, during 2 weeks. In mice, this effect could not be demonstrated. However, in both species VLCFA are increased after short and long term drug administration. Electron microscopy reveals the presence of membranous structures in liver cytoplasm or lysosomes. The inhibition by thioridazine of peroxisomal β -oxidation does not lead to hepatic peroxisome proliferation. The activities of enzymes related to fatty acid breakdown are not increased and liver peroxisomes are microscopically normal.

Thioridazine; Chlorpromazine; Peroxisome; β -Oxidation; VLCFA

1. INTRODUCTION

The question raised by the existence of a β -oxidation system in mammalian liver peroxisomes [1] is the partitioning of fatty acids between peroxisomal and mitochondrial β -oxidations. An impairment of the former system resulting in the accumulation of VLCFA in body fluids and tissues exists in patients with peroxisomal diseases [2]. Although phenothiazines were demonstrated in rodent liver to inhibit peroxisomal β -oxidation [3,4], no animal model for the chronic depression of this route has been studied.

The aim of the present work is to investigate whether thioridazine and chlorpromazine, two phenothiazines, inhibit peroxisomal β -oxidation in

normal rats and mice when they are administered chronically through the diet. Changes in the peroxisomal fatty acid breakdown are evaluated through fluctuations of the H_2O_2 produced by fatty acyl-CoA oxidase which catalyzes the first step of this pathway. Proliferation of peroxisomes, peroxisomal β -oxidizing capacity and accumulation of VLCFA are also studied.

2. MATERIALS AND METHODS

Adult male Wistar rats (200–300 g) and NMRI mice (25–30 g) were fed *ad libitum* on a standard laboratory animal meal (AO4-UAR, Epinau, France). Treated animals were fed on a diet containing thioridazine doses ranging from 0.1 to 0.5% (w/w) and 0.1% (w/w) chlorpromazine. In some experiments β -oxidation was stimulated for 2 days by feeding margarine only.

Thioridazine-hydrochloride was a gift from Sandoz Pharmaceuticals; chlorpromazine was obtained from Serva.

Peroxisomal β -oxidation was evaluated by

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Abbreviations: T, thioridazine; CPZ, chlorpromazine; RCA, residual catalase activity; VLCFA, very long chain fatty acid; LCFA, long chain fatty acid

measuring RCA after inhibition of the catalase- H_2O_2 complex with aminotriazole [5]. Aminotriazole (1 g/kg in 0.9% NaCl) and methanol (3.5 mmol/kg in rats and 3.2 mmol/kg in mice) were administered simultaneously by intraperitoneal injection. Rats were killed by decapitation 2 h after administration of aminotriazole-methanol, mice after 1 h. Catalase activity was assayed in total liver homogenate by the titanium oxysulfate method [6]. A lower RCA reflects a higher H_2O_2 production, i.e. higher activity of peroxisomal β -oxidation.

Carnitine acetyltransferases [7], lauroyl-CoA and glycolate oxidases [8], D-amino acid oxidase [9] and cytochrome P-450 [10,11] were assayed by established procedures. One unit of enzyme activity is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate/min.

VLCFA were analyzed in plasma. Their extraction was performed as in [12] after addition of 2 μg heptacosanoic acid as internal standard and before transmethylation with 2 M HCl in methanol at 75°C for 16 h. The gas chromatographic identification of the methyl esters was made by comparison of their retention time with reference retention times.

Each experimental group consisted of at least 4 animals. Results are expressed as the mean \pm SE. For statistical analysis the Mann-Whitney test was used [13].

Liver slices of 0.5 mm thickness were fixed at room temperature in 4% formol-calcium buffered with cacodylate for 24 h. Cryostat sections were stained for catalase which visualizes the peroxisomes [14]; for electron microscopy they were postosmicated and embedded in Epon.

3. RESULTS

3.1. Phenothiazines and weight gain

At a dose of 0.1% thioridazine, weight gain in rats is diminished as compared to matched controls. At doses of 0.25% and 0.5% rats seem to lose weight. However, after a short period of weight loss, rats receiving a 0.25% thioridazine diet are back to their initial weight after about 3 weeks. From then on, the weight gained curve parallels the control curve with a constant difference of about 100 g.

Table 1

Influence of a thioridazine diet on peroxisomal β -oxidation in rat liver

Conditions	RCA (U_B /g of liver)	Total catalase (U_B /g of liver)
Control diet	43 \pm 1 a	85 \pm 5
0.1% T diet, 1 week	50 \pm 6 b	—
0.25% T diet, 1 week	59 \pm 8 c	—
0.5% T diet, 1 week	62 \pm 3 d	—
0.25% T diet, 2 weeks	51 \pm 5 e	111 \pm 35 (NS)
0.25% T diet, 3 weeks	41 \pm 7 f	—
0.25% T diet, 8 weeks	29 \pm 3 g	86 \pm 8 (NS)
2 days of fat diet	35 \pm 2 h	96 \pm 1 (NS)
3 weeks 0.25% T + 2 days of 0.25% T fat diet	27 \pm 10 i	71 \pm 7 (NS)
3 weeks 0.25% T + 2 days 0.5% T	55 \pm 12 j	95 \pm 2 (NS)

$P < 0.01$: a vs d, g; $P < 0.05$: a vs c, e, h; NS: a vs b, f, j and h vs i

3.2. Phenothiazines and peroxisomal fatty acid oxidation

In rats (table 1), peroxisomal fatty acid breakdown is inhibited in animals receiving thioridazine at doses larger than 0.1% after 1 week. Good inhibition is obtained using a 0.25%

Table 2

Influence of a 0.1% chlorpromazine diet on peroxisomal β -oxidation in rat and mouse liver

Conditions	RCA (U_B /g of liver)
Rats	
control	43 \pm 1
CPZ 1 week	60 \pm 9
	($p < 0.01$)
CPZ 8 weeks	48 \pm 5
	(NS)
Mice	
control	41 \pm 4
CPZ 1 week	47 \pm 6
	(NS)
CPZ 8 weeks	41 \pm 4
	(NS)

Table 3

Influence of thioridazine and chlorpromazine on VLCFA concentration (ng/ml) in plasma from rats and mice

Conditions	Rat		Mouse	
	C26:C22	C24:C22	C26:C22	C24:C22
Control	0.323 ± 0.013	2.413 ± 0.027a	0.069 ± 0.024	0.571 ± 0.084
2 weeks T	0.196 ± 0.010	2.975 ± 0.066b	—	—
8 weeks T	0.298 ± 0.039	3.248 ± 0.191c	—	—
1 week CPZ	0.257 ± 0.110	2.861 ± 0.110d	0.131	0.719
6 weeks CPZ	0.304 ± 0.019	2.872 ± 0.057e	0.160	0.834

$P < 0.05$: a vs b, c; $P < 0.01$: a vs d, e. Values for CPZ-treated mice were measured on pooled samples from 4 animals

thioridazine diet. At thioridazine doses higher than 0.25%, toxic effects of the drug become apparent (sleepiness, low food intake, diarrhea). A 0.25% thioridazine diet was chosen for further experiments. With this diet the inhibitory effect disappears after 2 weeks. After 8 weeks we even observe a significantly increased H_2O_2 production.

Inhibition by the drug cannot be restored when β -oxidation is first stimulated by fat feeding or by increased drug doses. Total liver catalase activity is not changed by a 0.25% thioridazine diet.

Cytochrome P-450 is not changed after 3 weeks of a 0.25% thioridazine diet (45.8 ± 6.4 nmol/g of liver in thioridazine-treated rats and $46.9 \pm$

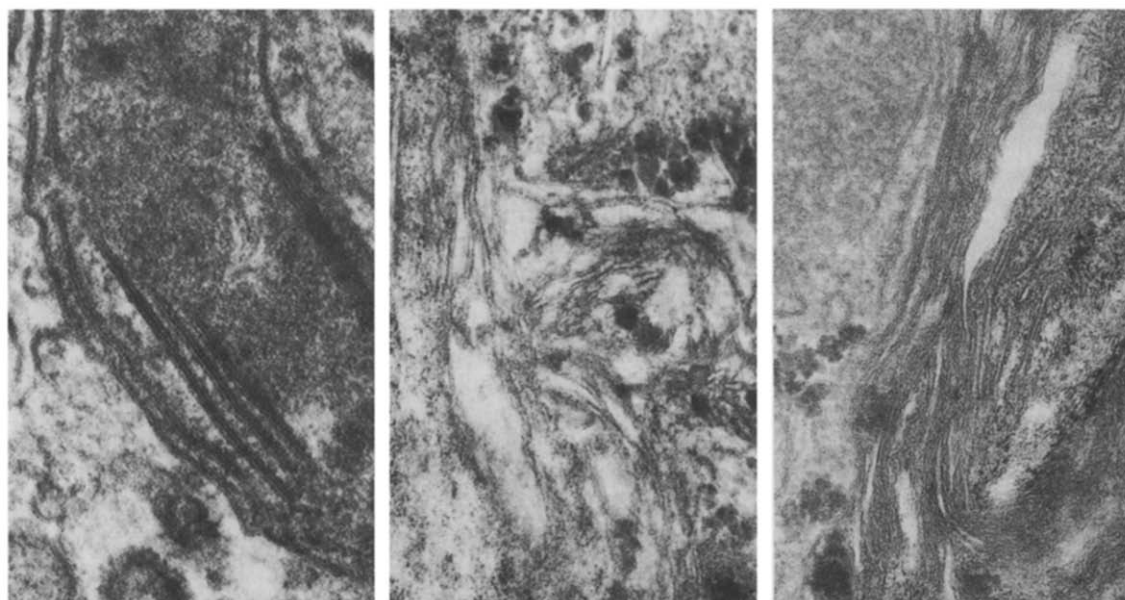


Fig.1. Electron micrographs of membranous lamellae in liver cells. (A) Rigid lamellae in Kupffer cell lysosomes of rats treated with thioridazine for 2 weeks; chlorpromazine induces identical structures. They are presented as 3 parallel lines limiting 2 electronlucent spaces. The distance between the outer lines is about 110 Å. (B) Lamellae with irregular spacing in parenchymal cells of chlorpromazine-treated rats (8 weeks). They are reminiscent of lamellae seen in human peroxisomal diseases. (C) Irregularly spaced lamellae in liver parenchymal cells of a patient with pseudo-neonatal adrenoleukodystrophy [20].

6.3 nmol/g of liver in control rats).

A 0.1% chlorpromazine diet inhibits peroxisomal β -oxidation in rat liver after 1 week. After long term administration of chlorpromazine (8 weeks) this inhibitory effect has disappeared (table 2).

The plasma C26:C22 ratio is diminished after 1 and 2 weeks of chlorpromazine or thioridazine diet; after 8 weeks it is back to normal. The C24:C22 ratio is increased both after 1, 2 and 8 weeks of drug administration (table 3).

Electron microscopy of liver reveals the presence of membranous lamellae in lysosomes of non-parenchymal cells (thioridazine and chlorpromazine) as well as in the cytoplasm of the parenchyma at the rim of fat droplets (chlorpromazine) (fig.1). In analogy with the human peroxisomal diseases, such material is believed to represent VLCFA deposition [15]. These lamellae are not seen in livers from untreated animals.

In mice the inhibitory effect of chlorpromazine cannot be shown after 1 and 6 weeks of drug diet. Nevertheless, increased plasmatic C26:C22 and C24:C22 ratios were present after 1 and 6 weeks of chlorpromazine diet (table 3).

3.3. Phenothiazines and peroxisomal proliferation

After 2 (not shown) and 8 weeks of a 0.25% thioridazine diet enzymes related to peroxisomal β -oxidation are not significantly induced in rat liver (table 4).

Liver sections do not show light microscopic changes of peroxisomal number after short and long term thioridazine or chlorpromazine diets in rats and mice.

4. DISCUSSION

Our results demonstrate that the short term administration of phenothiazines to rodents can depress liver peroxisomal β -oxidation. Nevertheless it is clear that this effect, assayed by peroxisomal H_2O_2 production, is only transitory (rats) or not detectable (mice). The long term administration of phenothiazines does not depress overall peroxisomal fatty acid oxidation and can even stimulate it. How can we explain that, in the latter conditions, VLCFA which are specifically oxidized by peroxisomes, accumulate in tissues (trilamellar structures) and in body fluids (increased plasmatic

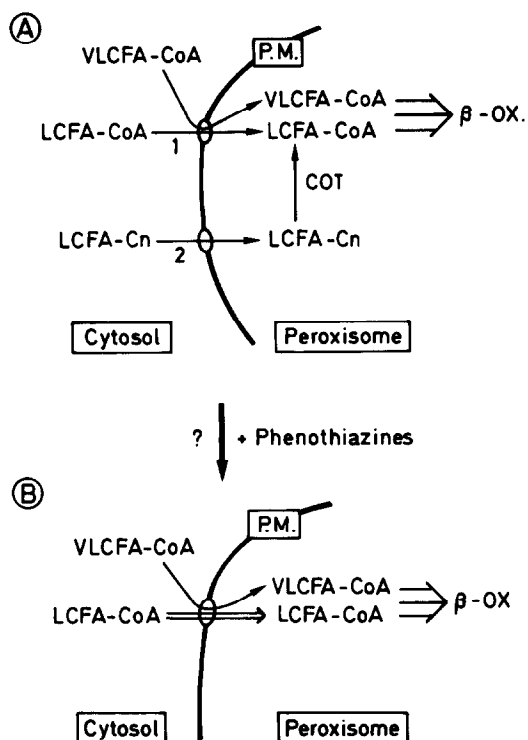
Table 4

Activities of several peroxisomal enzymes, carnitine acyltransferases and butyryl-CoA dehydrogenase in livers from thioridazine-treated (8 weeks) and untreated rats

Enzymes	+ T	Untreated
D-Proline oxidase	2344 \pm 568	2096 \pm 312
Glycolate oxidase	854 \pm 150	1014 \pm 58
Lauroyl-CoA oxidase	1138 \pm 52	1048 \pm 64
Cyanide insensitive lauroyl-CoA oxidation	1457 \pm 97	1356 \pm 118
Carnitine acetyltransferase	642 \pm 150	632 \pm 96
Carnitine octanoyl- transferase	1515 \pm 302	1412 \pm 156
Carnitine palmitoyl- transferase	1252 \pm 110	1304 \pm 126
Butyryl-CoA dehydrogenase	1512 \pm 56	1687 \pm 138

Activities are expressed as mU/g of tissue. Values are not significantly different in treated and untreated rats

VLCFA) from animals given phenothiazines? One must absolutely distinguish the flux of fatty acids through peroxisomal β -oxidation (which represents the sum of the peroxisomal acyl-CoA oxidizing reactions expressed per time unit) from the activity of this pathway on the various classes of fatty acids. The former is estimated by the measurement of RCA which mainly depends on the H_2O_2 produced by the fatty acyl-CoA oxidase and is not (in mice) or slightly (in rats) inhibited by phenothiazines in vivo. The second could be modified by the drugs according to scheme 1. The bulk of physiological fatty acids are LCFA and evidence has previously been given that the entry of these substrates into peroxisomes can be carnitine-dependent [16]. LCFA can also enter these organelles in the form of acyl-CoA esters [17]. The inhibition by phenothiazines of COT [16] directly impairs carnitine-dependent entry of LCFA into peroxisomes. We suggest that the latter change, even if it does not or poorly inhibit the flux of fatty acids through peroxisomal β -oxidation, could dramatically affect the proportions of the various classes of fatty acids which are oxidized by peroxisomes especially VLCFA which must normally enter peroxisomes in the form of acyl-CoA esters since they are not substrates for carnitine acyltransferase. Abnormal competition



between long chain acyl-CoA and very long chain acyl-CoA for the peroxisomal membranal transport of acyl-CoA moieties could take place under the administration of phenothiazines.

We have as yet no explanation why in rats RCA is temporarily increased and not in mice. We remarked that chlorpromazine is more toxic to mice than to rats. None of the experimental rats died during a 0.1% chlorpromazine diet, while mortality was high in mice (13 out of 20 mice). Perhaps intoxication causes diminished food intake. We demonstrated earlier that fasting causes an increased flux of fatty acids through the peroxisomal oxidase [5]; this might mask a partial inhibition.

Recently Vamecq et al. [18] have shown the presence of trilamellar structures in the liver of mice treated with a 0.1% chlorpromazine diet for 6 weeks. In rats, we find membranous inclusions which are not completely identical. After 8 weeks of a chlorpromazine diet they are very similar to those in livers from 2 patients with peroxisomal

Scheme 1. Hypothetic influence of the phenothiazine dependent inhibition of carnitine octanoyltransferase (COT) on the peroxisomal handling of very long chain acyl-CoA esters (VLCFA-CoA). (A) Evidence has previously been given that LCFA can enter peroxisomes in the form of acyl-CoA (pathway 1) or acylcarnitine (pathway 2) esters which are abbreviated LCFA-CoA and LCFA-Cn, respectively. One of the functions of COT is to allow peroxisomal oxidation of LCFA-Cn by conversion to the corresponding CoA derivatives. In this representation it is assumed that the transmembranal transport mechanisms for the acyl-CoA esters are different from those involved in the peroxisomal entry of the acylcarnitine moieties. P.M., peroxisomal membrane; β -OX., peroxisomal β -oxidation system. (B) The inhibition of COT must prevent or inhibit the acylcarnitine-mediated mode of entry of LCFA into the peroxisome. In these conditions, only the acyl-CoA-mediated mode of entry for LCFA remains possible. This is expected to interfere with the handling by peroxisomes of VLCFA possibly at the level of entry of VLCFA-CoA in these organelles. Phenothiazines can act by shifting the peroxisomal oxidation of fatty acids from situation A to situation B. This change is associated (isolated hepatocytes [3] and short term influence of phenothiazines in rats) or not (short term influence of phenothiazines in mice and long term influence in rats and mice) with a decreased flux of fatty acids through the peroxisomal β -oxidation assayed by H_2O_2 production. Other comments are given in the text.

diseases: a patient with the pseudo-Zellweger syndrome [19] and a patient in whom a diagnosis of pseudo-neonatal adrenoleukodystrophy was made [20] (fig.1). They are reminiscent of the inclusions in adrenal cortex cells and brain macrophages found in human peroxisomal diseases [21-24]. They differ however from the trilamellar inclusions seen in livers from phytanic oxidase deficiency or genuine CHRS patients [14].

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