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PEROXISOMAL FATTY ACID OXIDATION IS SELECTIVELY INHIBITED BY PHENOTHIAZINES IN ISOLATED HEPATOCYTES

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SUMMARY: The production of hydrogen peroxide by isolated hepatocytes in response to lauric, palmitic and oleic acids, a measurement of peroxisomal fatty acid oxidation, is inhibited by phenothiazines under conditions in which ketone body production, a measurement of mitochondrial fatty acid oxidation, does not reveal inhibition of mitochondrial activity. This novel finding provides a pharmacological tool for the study of peroxisomal function in whole cells. The mechanism of this effect of phenothiazines, detected in hepatocytes from rats treated with a peroxisome proliferation inducing drug, is not yet known.

Fatty acid β-oxidation in liver cells occurs in two different subcellular compartments, mitochondria and peroxisomes (1,2). The functional implications of this double localization are not yet established. Only mitochondria oxidize butyrate (2-4) and peroxisomes apparently are responsible for the oxidation of very long chain fatty acids, C_{22} or longer (5,6) however, for the bulk of saturated and unsaturated fatty acids employed by the cell for structural and energetic purposes, both organelles are effective in performing oxidative degradation (2). The evidence presently available suggests that palmitate and oleate are oxidized mainly in mitochondria in the livers from normal rats and also from rats treated with drugs that induce a large increase in the amount of peroxisomal β-oxidation enzymes (7,8) yet the activity of peroxisomes is always present and their relative contribution has not been established.

To determine the relative role played by mitochondria and peroxisomes, specific inhibitors would be of great value. Cyanide and (+)-octanoyl carnitine have been employed as mitochondrial inhibitors in observations with isolated hepatocytes (7), but the inhibition of carnitine acyl transferases should not be considered specific for mitochondria since the enzymes are

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also present in peroxisomes (8) and cyanide, in metabolic studies with intact cells, will affect mitochondrial fatty acid oxidation and also other cell functions. For peroxisomal fatty acid oxidation, so far there has been no description of specific inhibitors.

We started a search for specific peroxisomal inhibitors that might help in the study of the extent of the peroxisomal contribution to fatty acid oxidation and eventually, shed some light on the regulatory mechanisms involved. Isolated hepatocytes constitute an adequate model for these studies; fatty acid induced generation of hydrogen peroxide reflects the activity of peroxisomes (4,7,9) ketone body production that of mitochondria (10) and there are several procedures to evaluate the effect of the potential inhibitors on cell viability. Our observations led us to the finding that phenothiazine derivatives apparently are selective inhibitors of peroxisomal fatty acid oxidation in isolated hepatocytes from rats which, to enhance the system, had been treated with a peroxisome proliferation inducing drug (1,11). This finding is a pharmacological phenomena and as such provides a valuable tool for the study of peroxisomal function; but in addition, phenothiazines have a well established value in the pharmacological evaluation of some general regulatory mechanisms in cells (12) which might also apply to peroxisomes. A preliminary communication of these results has appeared (13).

MATERIAL AND METHODS

Male Sprague Dawley rats (150-250g) fed ad libitum with standard rat chow containing Nafenopin 1g per kg, were employed. The drug was administered for 2 to 3 weeks prior to the experiments. Isolated rat hepatocytes were prepared, approximately at 10 A.M., by the method of Berry and Friend (14) as modified by Krebs et al. (15). The rats were anesthetized with nembutal. Krebs-Henseleit bicarbonate buffer (16) containing 11 mM glucose, without calcium and 65 U/ml of collagenase was employed to disaggregate the cells. After washing, the cells were resuspended in collagenase-free buffer containing 2.5 mM $CaCl_2$. Cell viability, estimated as trypan blue exclusion, was 86.3 $\pm$ 5.7 average percent $\pm$ S.D. Preparations with less than 80% viability were not employed. For the incubations, approximately 15 mg of cells were suspended in 2.5 ml containing 120 mM NaCl, 4.8 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 50 mM methanol, 10 mM semicarbazide, 0.18 mM defatted bovine serum albumin (17) and 0.54 mM fatty acid sodium salt. The phenothiazines were added dissolved in methanol. The incubations were performed in duplicate at 37°C in standard glass scintillation vials under O_2:CO_2 (95:5, v/v) in a shaking bath, 100 strokes/min.

The generation of H_2O_2 was estimated from the peroxidatic generation of formaldehyde trapped as its semicarbazone (4,7). The incubation was stopped

with 1 ml of 30% w/v thrichloroacetic acid; after cooling in ice, the supernatant was collected by centrifugation. Formaldehyde was measured in 1 ml of supernatant (18). Acetoacetate and β-hydroxybutyrate were determined in 0.75 ml of supernatant, after neutralization, by the method of Williamson (19). Values for the production of ketone bodies (acetoacetate plus β-hydroxybutyrate) and hydrogen peroxide, correspond to measurements after incubation in the presence of substrate, corrected for blank values of non-incubated vials containing substrate. Protein was determined employing a standard procedure (20). Collagenase Type I, 130-178 U/mg, was obtained from Worthington. Substrates, coenzymes, β-hydroxybutyrate dehydrogenase, bovine serum albumin, hydrazine hydrate and chlorpromazine from Sigma Chemical Co. Thioridazine and fluphenazine were gifts from Sandoz Pharmaceuticals and E.R. Squibb and Sons, respectively. Nafenopin was a gift from Ciba-Geigy.

RESULTS

The generation of hydrogen peroxide by isolated hepatocytes in response to lauric acid decreases when chlorpromazine is present in the incubation medium, Fig. 1. This effect is proportional to the dose employed in the range 0.1-0.5 mM when 15 mg wet weight of cells per vial are employed. At these concentrations there is no detectable decrease in ketone body production.

At a constant dose of chlorpromazine, the inhibitory effect is more marked when fewer cells per vial are employed, Fig. 2. This result is to be expected in a crude system with a drug that interacts unspecifically with

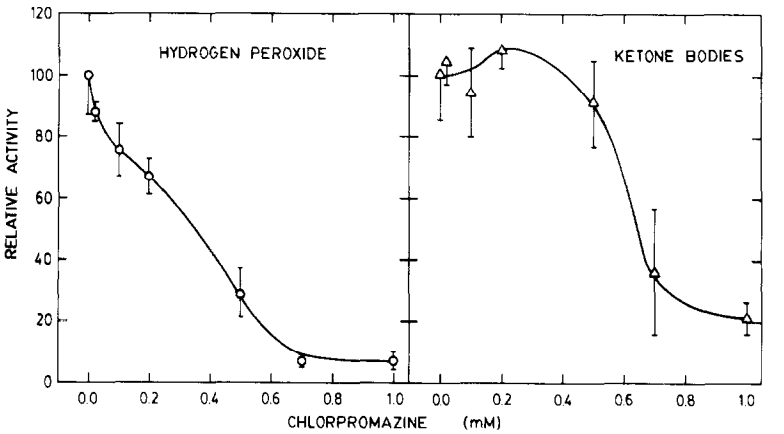

Figure 1 Chlorpromazine effects on the generation of hydrogen peroxide, a peroxisomal product, and ketone bodies, mitochondrial products. Isolated hepatocytes from nafenopin treated rats, incubated with lauric acid as substrate. Relative values represent the mean from 3 to 6 separate determinations ± S.D. The values in the absence of chlorpromazine in nmoles of product x min^{-1} x mg^{-1} protein, were 11.92 ± 1.62 and 14.44 ± 2.09 for hydrogen peroxide and ketone bodies, respectively.

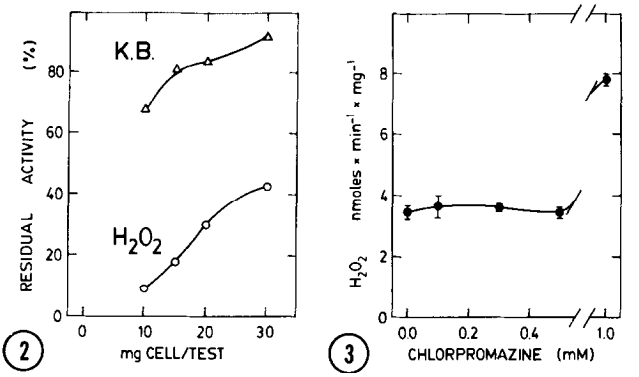

Figure 2 Cell concentration dependence of the effect of 0.2 mM chlorpromazine on laurate induced hydrogen peroxide and ketone body production by isolated hepatocytes.

Figure 3 Hydrogen peroxide generation in isolated hepatocytes by urate oxidase, a peroxisomal enzyme, at different concentrations of chlorpromazine. Conditions as described in Material and Methods, employing 1.0 mM uric acid instead of fatty acid as substrate.

membranes and proteins. If the inhibitory effect observed is the consequence of the specific interaction of chlorpromazine with some cell component, the effective inhibitory dose would be lower for a purified system.

To control for the possible interference of chlorpromazine with the coupled reaction employed to trap and detect hydrogen peroxide produced in peroxisomes, observations were made in which laurate, the substrate for the peroxisomal enzyme fatty acyl-CoA oxidase was replaced by urate, substrate for the peroxisomal enzyme urate oxidase (21). The results, shown in Fig. 3, indicate that chlorpromazine, at concentrations up to 0.5 mM with 15 mg of cells per vial, will not inhibit the detection procedure employed. At higher concentrations an increase in urate oxidase mediated hydrogen peroxide production is observed. This increase is paralleled by decreased cell viability measured by trypan blue staining of damaged cells and release of two cytosolic enzymes, lactate dehydrogenase and phosphoglucomutase (results not shown). The increase in apparent urate oxidase activity with cell damage, would correspond to the suppresion of permeability barriers between the enzyme and urate.

The inhibition of peroxisomal laurate oxidation by chlorpromazine is also observed with palmitate and oleate as substrates (Table I). With these

TABLE I Influence of chlorpromazine (CPZ) on the production of hydrogen peroxide and ketone bodies induced in isolated hepatocytes by different fatty acids

	Hydrogen peroxide		Ketone bodies	
	Control	CPZ	Control	CPZ
	(nmoles x min^{-1} x mg^{-1} protein)			
Lauric acid (n=11)	11.1 ± 1.9	8.3 ± 1.3***	15.0 ± 1.2.	16.6 ± 2.3*
Palmitic acid (n=9)	2.7 ± 1.2	1.2 ± 0.4**	12.4 ± 1.5	11.0 ± 1.3*
Oleic acid (n=7)	2.2 ± 0.8	1.2 ± 0.4**	11.9 ± 1.0	12.3 ± 0.5*

Incubations were performed in the presence or absence of chlorpromazine 0.2 mM with 15 mg wet weight of cells as described under Material and Methods. Each value represents the mean ± S.D. *, non-significant difference with the control; **, $0.001 < P < 0.005$; ***, $P < 0.001$.

substrates, the inhibitory effect of 0.2 mM chlorpromazine on hydrogen peroxide production is more marked, decreasing it by one half, yet ketone body production remains constant. These results also show that laurate is a better peroxisomal substrate; it generates acetyl units at approximately one third the rate of mitochondria. The peroxisomal activity on palmitate and oleate is low, only one tenth of the mitochondrial, so low that it is difficult to detect it, with this assay procedure, in hepatocytes from normal rats.

Other phenothiazines are also inhibitory for peroxisomal activity. Fluphenazine, an example of the piperazine series and thioridazine, an example of the piperidine series, are as effective or more effective than chlorpromazine, when tested at 0.2 mM concentration (Table II).

TABLE II Influence of phenothiazine derivatives on the production of hydrogen peroxide and ketone bodies induced by lauric acid in isolated hepatocytes.

	Hydrogen peroxide	Ketone bodies
	(nmoles x min^{-1} x mg^{-1} protein)	
Control	11.0 ± 2.2	15.1 ± 1.4
Chlorpromazine (0.2 mM)	8.0 ± 1.3**	16.0 ± 2.3*
Fluphenazine (0.2 mM)	7.8 ± 1.6**	13.1 ± 1.7*
Thioridazine (0.2 mM)	4.0 ± 2.2***	11.3 ± 3.1**

Incubations were performed as described under Material and Methods. Each value represents the mean ± S.D. of 8 separate determinations. *, non-significant difference with the control; **, $0.001 < P < 0.005$; ***, $P < 0.001$.

DISCUSSION

The metabolic products employed to detect the activity of peroxisomes and mitochondria are hydrogen peroxide and ketone bodies. Hydrogen peroxide has been shown to be a specific product of peroxisomal activity in subcellular fractions, isolated cells and perfused liver (4,7,9,21). In some cases (4,7) its production is measured by accumulation of formaldehyde generated by coupled peroxidation of methanol. Ketone bodies are mitochondrial products; peroxisomes apparently do not make them (4,7,9). However, in the interpre- peroxisomal function, is the possibility of a calmodulin mediated regulatory step. Phenothiazine derivatives are well known inactivators of calmodulin (12).

tation of our results, it should be considered that ketone bodies are not the only product of mitochondrial fatty acid oxidation. Our result could also be explained if the assumption is made that phenothiazines simultaneously raise the fraction of acetyl-CoA channeled to ketone bodies and at the same time inhibit mitochondrial fatty acid oxidation. This and other possibilities are currently under study and our results give no indication of reduced mitochondrial activity.

Several alternatives exist to explain the inhibitory effect of phenothiazines on peroxisomal fatty acid oxidation. The following should be considered and are presently under evaluation: Fatty acyl-CoA oxidase is a flavoprotein with FAD non-covalently bound (22); phenothiazines have been shown to displace FAD from flavoprotiens (23). Phenothiazine derivatives, under different experimental conditions, interact with cell membranes and labilize them at the higher concentrations we employed (24); in this case the specificity for peroxisomes would require additional explanations. Phenothiazines bind strongly to proteins, for example albumin (25) and could compete with substrates; in fact, the study of this property led us to the finding reported here. Free radical products have also been implicated in the action of phenotiazines (26). Another mechanism which could account for the specificity and would raise most interesting possibilities for regulation of the

The various mechanisms by which phenothiazine derivatives might exert the effect described in this article, suggest that studies with subcellular particles or purified enzymes may be difficult to interpret. It should be established if the inhibition takes place through interference with catalytic or regulatory aspects of the subcellular system responsible for fatty acid oxidation in peroxisomes.

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