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Disaturated phosphatidylcholine in the pulmonary airspaces of rats treated with chlorphentermine

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A phospholipid storage disorder is induced in the lungs of laboratory animals following the repeated administration of a number of cationic, amphiphilic drugs [1–3]. While the basic mechanism responsible for the induction of the phospholipidosis is not known for certain, there is good evidence that the drugs inhibit phospholipid catabolism [4–8] leading to the increase in phospholipids in this tissue.

The most dramatic changes in the lungs are observed in the airspaces, which become filled with phospholipid-rich alveolar macrophages (AMs) [3, 9, 10] and acellular particulate material [11]. Several reports have appeared describing changes in the phospholipid content of lungs from animals treated with one such drug, chlorphentermine [12–15], but little quantitative data exist concerning phospholipids in the airspaces [16].

An important phospholipid in the lung is disaturated phosphatidylcholine (DSPC) which is the principal component of surfactant, the surface-active material which lines the alveoli and is responsible for maintaining alveolar stability at low lung volumes [17, 18]. Because of its essential role in lung function, we were interested in quantifying changes in DSPC in the airspaces of rats during the induction of phospholipidosis by chlorphentermine. To do this, rats were treated with chlorphentermine for varying periods of time, the lungs were lavaged, and the effluents were separated into a cellular fraction and an acellular particulate fraction. The total phospholipid and the DSPC content in each fraction were then determined. The results of our study are presented in this report.

Materials and methods

Chlorphentermine adminstration. Male, Long Evans hooded rats (220–225 g at the start of treatment) were treated for 1, 2 or 4 weeks with chlorphentermine (CP) hydrochloride (PreSate, Warner-Chilcott, Morris Plains, NJ) (30 mg/kg, i.p., 5 days/week). Control animals were administered vehicle (0.9% NaCl) in the same volume (1 ml/kg) as the treated rats received. Because CP is an anorectic drug, treated rats failed to gain weight as rapidly as controls. Therefore, sufficient food was withheld from the control animals so that the weight gains were comparable for both groups. The pair-fed control rats were examined after receiving vehicle for 1, 2 and 4 weeks, and the values obtained for all variables measured in this study were not different at the various time points. Therefore, control values were combined for purposes of comparison to the treatment groups.

Collection of AMs and acellular fraction (ACF). Twenty-four hours after the last injection of CP, rats were killed with an overdose of pentobarbital and exsanguinated by severing the abdominal aorta. The lungs were lavaged *in situ* using 4–5 ml vol. of calcium–magnesium-free Hanks' balanced salt solution, pH 7.4, at 37°. The lavage procedure was terminated when 80 ml of effluent had been collected since beyond this point essentially no cells or particulate material was recovered. The lavage effluent was centrifuged at 120 *g* for 10 min to pellet the AMs. The supernatant fraction, which contained no cells, was carefully removed and saved for preparation of the acellular fraction described below. The AMs were resuspended in a small volume of cold 0.9% NaCl and placed on ice. The number of AMs collected from each rat was determined by enumeration on a hemocytometer.

Pulmonary surfactant is prepared experimentally in a number of ways from lavage effluents as well as whole lung homogenates [19–22]. The common feature among these procedures is that the surfactant is in particulate form and is obtained through one or more centrifugation steps. To be consistent with these procedures, we collected the particulate fraction (ACF) of the lavage effluent by centrifuging the supernatant fraction collected in the first step above, at 105,000 *g* for 60 min. The resulting pellet was resuspended in 1 ml of cold 0.9% NaCl and termed the acellular fraction. The supernatant fraction collected in this step was discarded. Both AMs and ACF were stored frozen at −20° until used.

Analytical procedures. To measure the total phospholipid and DSPC content of each fraction, samples were extracted with chloroform–methanol (2:1, v/v) as described by Folch *et al.* [23]. One aliquot was used for the measurement of DSPC using the osmium tetroxide method described by Mason *et al.* [24]. Both total phospholipid and DSPC were quantified by measurement of lipid phosphorous using the assay of Ames and Dubin [25] following washing of the samples in 10% $Mg(NO_3)_2$ in 70% ethanol.

When appropriate, statistical analysis was performed using a one-way analysis of variance with a Student–Newman–Keuls test [26]. Statistical significance was taken to be $P < 0.05$.

Results and discussion

Administration of CP led to an apparent increase in the recovery of AMs from the lungs at all times with only the recovery at 4 weeks being significantly different from controls (Table 1).

The total phospholipid (TPL) and DSPC content of AMs (expressed on a per cell basis) increased dramatically over the 4-week treatment period (Fig. 1). After 4 weeks of CP administration, the levels of TPL and DSPC were, respectively, 20- and 35-fold greater than that found in an equivalent number of control AMs. Considering the increase in cells recovered over this period, it can be calculated that TPL and DSPC were elevated 46- and 80-fold, respectively, in this fraction.

The increases in TPL and DSPC were more pronounced in the AM fraction than in the acellular fraction (Table 1). The levels of both TPL and DSPC in the AM fraction progressively increased during the entire treatment period while the levels of these species present in the acellular fraction did not increase very much beyond 1 week of drug treatment.

From the data presented in Table 1 it can be calculated that, after 4 weeks of treatment, the TPL recovered from the airways (both fractions) increased about 30-fold while the total DSPC recovered increased about 33-fold.

DSPC is the principal phospholipid in surfactant, the surface-active material which is so important in maintaining alveolar stability. Consequently, we were interested in quantifying changes in this phospholipid in the airspaces during induction of phospholipidosis in rats by administration of CP. To evaluate changes in DSPC relative to TPL, this latter component was measured as well.

Considering the total levels recovered from the airspaces

Table 1. Effects of chlorphentermine on alveolar macrophages (AMs) and the acellular fraction (ACF) from the airways of rats

Treatment period	AMs			ACF	
	Cells/kg ($\times 10^{-7}$)	TPL*†	DSPC*†	TPL	DSPC
			(μmoles/kg body weight)		
Control	9.4 ± 1.3‡	3.0 ± 0.5	0.8 ± 0.2	2.6 ± 0.7	1.6 ± 0.4
1 Week	14.2 ± 3.6	44.6 ± 9.0	25.5 ± 4.7	23.1 ± 4.8	12.5 ± 3.3
2 Weeks	18.6 ± 2.8	100.1 ± 23.6	44.5 ± 12.1	27.9 ± 5.5	15.1 ± 2.8
4 Weeks	20.8 ± 2.6	140.1 ± 15.1	64.0 ± 6.5	32.3 ± 6.2	15.9 ± 3.3

* Abbreviations: TPL, total phospholipid; and DSPC, disaturated phosphatidylcholine.

† Values for the AM fraction correspond to that present in the total fraction and were determined by multiplying the TPL and DSPC levels per cell (Fig. 1) times the number of cells/kg body weight.

‡ Values represent means ± S.E.M. (N = 4–6).

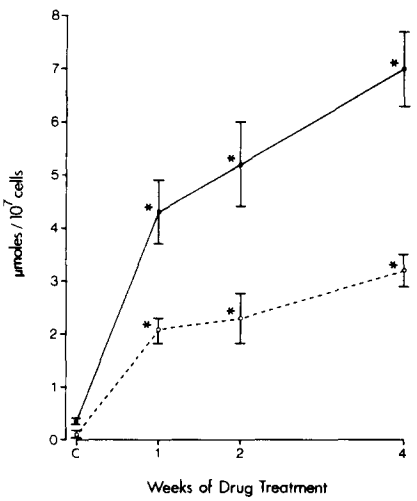

Fig. 1. Phospholipid and DSPC content of alveolar macrophages from control rats and rats treated for 1, 2 or 4 weeks with chlorphentermine. Values represent means ± S.E. of four to six preparations. Key: total phospholipid (—●—); DSPC (---○---); and (*) $P < 0.05$ compared to the control (C) preparation.

(cellular and acellular quantities), DSPC increased roughly in parallel with TPL. Each increased about 30-fold after 4 weeks of drug treatment. Both quantitatively and relatively, the largest increases in both components occurred in the AM fraction. This appeared to result from both the increase in numbers of AMs present in the airspaces as well as the increase in TPL and DSPC within the AMs. It can be calculated from the data presented in Table 1 that at all time points about 60–70% of the increase in both TPL and DSPC occurred in the AM fraction. Nevertheless, the amount of DSPC recovered from the acellular fraction after 4 weeks of treatment was nearly 10 times the level obtained from control rats. Therefore, very high levels of DSPC existed free in the airspaces during drug treatment.

The elevated DSPC levels in the airspaces may originate from either an enhanced synthesis and secretion or an impairment in degradation. Karabelnik and Zbinden [5] reported that the synthesis of phospholipids in the lung may actually be decreased with CP treatment. It has also been shown that CP can inhibit the catabolism of phospholipids by phospholipases A and C [4]. Therefore, it is reasonable to assume that the elevation in DSPC resulted principally from an inability to metabolize the quantities released into the airspaces.

In summary, we have shown that administration of chlorphentermine to rats resulted in a time-dependent increase in TPL and DSPC in the pulmonary airspaces of rats. The largest increase for both components was in the AM fraction; however, the levels of TPL and DSPC were also elevated in the ACF. The results of this study are an example of the remarkable changes that occur in the pulmonary airspaces following induction of phospholipidosis by chlorphentermine.

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Effects of the enantiomers of 5-hexyne-1,4-diamine on ODC, GAD and GABA-T activities in the rat

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(±)-5-Hexyne-1,4-diamine is a potent enzyme-activated irreversible inhibitor of mammalian ornithine decarboxylase (ODC) (EC 4.1.1.17) [1]. When injected into rats, it causes marked decreases in ODC activity and polyamine concns in several organs [2]. However, this compound is rapidly transformed *in vivo* to 4-aminohex-5-ynoic acid, a potent enzyme-activated irreversible inhibitor of 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T) (EC 2.6.1.19) and of glutamate decarboxylase (GAD) (EC 4.1.1.15) [3]. The use of specific inhibitors allowed us to identify mitochondrial monoamine oxidase (MAO) (EC 1.4.3.4) as being involved in this metabolism. When (±)-5-hexyne-1,4-diamine is given at dose sufficient to effectively reduce polyamine concns in rat organs, the consequent inhibition of 4-aminobutyrate (GABA) metabolism in the brain leads to undesirable behavioural changes [3]. The compound is therefore unsuitable as a tool for the investigation of the physiological role of polyamines.

We reported recently the asymmetric synthesis of the two enantiomers of 5-hexyne-1,4-diamine from the corresponding enantiomers of 4-aminohex-5-ynoic acids [4]. *R*-(−)-5-Hexyne-1,4-diamine was the only enantiomer responsible for the inactivation of rat liver ODC [4]. Since the mitochondrial oxidation of 5-hexyne-1,4-diamine concerns only the amino group at the 1-position [3], *R*-(−)-5-hexyne-1,4-diamine should be metabolized to *R*-(−)-4-aminohex-5-ynoic acid which was believed to have no action on GABA metabolism [5]. Therefore *R*-(−)-5-hexyne-1,4-diamine should selectively inactivate ODC. In order to confirm this hypothesis, we have investigated the effects of each of the enantiomers of 5-hexyne-1,4-diamine on the activities of ODC in the ventral prostrate and of GABA-T and GAD in the brain of rats.

Materials and methods

Chemicals. The following compounds were purchased: L-ornithine, pyridoxal-phosphate, ammonium sulfate, reduced glutathione (GSH), sucrose and buffer reagents (Merck, Darmstadt, F.R.G.); *S*-adenosyl-L-methionine, dithiothreitol, NAD^+, 4-aminobutyric acid, L-glutamate, 2-oxoglutarate (Sigma, St. Louis, MO); homovanillic acid, horseradish peroxidase, tetrasodium EDTA (Calbiochem, San Diego, CA); DL-[1-^{14}C]ornithine (sp. radioactivity 58 Ci/mole) (Radiochemical Centre, Amersham, U.K.); DL-[1-^{14}C]glutamate (50 Ci/mole) (New England Nuclear Corp., Boston, MA). (±)-5-Hexyne-1,4-diamine [1] and its pure enantiomers (cross-contamination <0.5%) were synthesized in our laboratories [4].

Animals. Male rats of the Sprague–Dawley strain (200–220 g body wt) were purchased from Charles River, France. Animals had access to standard diet and water *ad lib.* and were kept under a constant 12 hr light/12 hr dark lighting schedule. They were killed by decapitation at about the same time of day to minimize effects due to diurnal fluctuations. Drugs, dissolved in 0.9% saline, were injected intraperitoneally. Rats given saline served as controls.

Assays of enzyme activities and determination of GABA. The assays of MAO, ODC, GABA-T and GAD activities, and the measurements of whole-brain GABA concns were performed as described previously [3].

Results and discussion

As a preliminary experiment, we investigated the *in vitro* oxidation of the two enantiomers of 5-hexyne-1,4-diamine by MAO. The two enantiomers and the racemic mixture of 5-hexyne-1,4-diamine were oxidized at the same maximum velocity by the MAO preparation (not shown). Michaelis constants (K_m) were found to be 1.0 ± 0.1, 0.9 ± 0.1 and 0.8 ± 0.1 mM for *R*-(−)-, *S*-(+)- and *R,S*-(±)-5-hexyne-1,4-diamine respectively. These results suggest that both enantiomers could be oxidized *in vivo* to the corresponding enantiomers of 4-aminohex-5-ynoic acid with retention of configuration as the oxidation does not involve the asymmetric center [2]. Nevertheless *R*-(−)-5-hexyne-1,4-diamine should be a selective inhibitor of ODC *in vivo*, in spite of its possible oxidation, since it has been reported that the *S*-(+)-enantiomer of 4-aminohex-5-ynoic acid was responsible for the inhibition of mammalian GABA-T and GAD [5]. The effect of *R*-(−)-5-hexyne-1,4-diamine on GABA metabolism was investigated in the following experiments.

Single doses of 100 mg/kg of each enantiomer or of 200 mg/kg of the racemate of 5-hexyne-1,4-diamine were injected 4 hr before the killing of the animals. As expected