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# Role of Phospholipase C in Chlorphentermine-Induced Pulmonary Phospholipidosis in Rat (42703)

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*Abstract.* Daily, oral administration of chlorphentermine (60 mg/kg) for 5 days to rats produced a significant increase in the concentration of whole lung total phospholipid as well as sphingomyelin, phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, and phosphatidylcholine. Similarly, a significant elevation in total and all individual phospholipid components was found in the lysosomal fraction of chlorphentermine-treated rat lung. In contrast, the activities of pulmonary $Na^+,K^+$-ATPase and alkaline phosphatase, enzymatic markers of membrane function, were not markedly affected by chlorphentermine treatment. The observed lung phospholipidosis was accompanied by inhibition of phospholipase C activity. Regardless of the phospholipid substrate, chlorphentermine significantly decreased pulmonary phospholipase C to approximately the same extent. Our data show that accumulation of phospholipid in whole lung and lysosomes is associated with an inhibition of phospholipase C activity. 

A disorder in phospholipid metabolism is associated with the use of certain cationic amphiphilic drugs (1). In particular, chlorphentermine is well known to display a high affinity for pulmonary tissue and produces a characteristic massive accumulation of hypertrophic alveolar macrophages (2). The observed chlorphentermine-induced morphological alterations in pulmonary tissue are associated with a marked increase in both alveolar macrophage and whole lung phospholipid content (3, 4). It has been suggested that cationic amphiphilic drugs accumulate within pulmonary lysosomes in conjunction with elevated phospholipid content (1). Studies were thus undertaken to determine whether chlorphentermine increased the phospholipid content in rat pulmonary lysosomes.

Incubation of a purified rat liver lysosomal preparation with chlorphentermine was found to result in inhibition of phospholipases A and C (5). Further, it was reported that chlorphentermine decreased lung phospholipid synthesis as evidenced by reduced incorporation of choline into phospholipids (6). Based on these observations it was suggested that chlorphentermine-induced pulmonary phospholipidosis may be related to an impaired degradation of phospholipid (5, 6). Experiments were also undertaken to examine the influence of chlorphentermine *in vivo* on the activity of rat lung phospholipase C, one of the lysosomal enzymes responsible for phospholipid catabolism.

**Methods.** Male rats of the Sprague–Dawley strain weighing approximately 200 g purchased from Canadian Breeding Farm and Laboratories Ltd., St. Constant, Quebec, were employed in this study. All animals were maintained on Purina Laboratory Chow and had free access to water throughout the course of the experiment. The animals were housed in plastic cages (six per cage) and maintained on a 12-hr light/dark cycle. Animals were administered 60 mg/kg of chlorphentermine daily by the oral route for a period of 5 days. Corresponding pair-fed controls received an equal volume (0.5 ml) of physiological saline daily for 5 days. All rats were killed by decapitation and bled 24 hr after the last drug administration. Lungs were removed and immediately frozen in liquid nitrogen. Lung tissue was kept at −60°C for subsequent biochemical assays.

Pulmonary tissue was subjected to differential centrifugation to obtain a lysosomal fraction according to the method of Hostetler *et al.* (7). Lung tissue was homogenized in 10 vol of 0.25 *M* sucrose and this fraction constitutes the homogenate utilized in this study. In lysosomal experiments, the homog-



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enate was centrifuged at 750*g* for 5 min. The pellet was discarded and the supernatant was recentrifuged at 8800*g* for 10 min. This supernatant was centrifuged for a further 10 min at 20,000*g*. The resultant supernatant obtained was discarded and the pellet was subsequently washed with 0.25 *M* sucrose. The pellet thus obtained constituted the lysosomal fraction.

The total phospholipid content (TPL) of pulmonary tissue was extracted from the homogenate and lysosomal fractions according to the procedure described by Folch *et al.* (8). The method of Rouser *et al.* (9) was used for the separation of individual phospholipid classes by thin-layer chromatography. Both total phospholipid phosphorous and individual phospholipid class phosphorous were determined by the method of Chen *et al.* (10). Tissue homogenates were assayed for $Na^+,K^+$-adenosine triphosphatase (ATPase) by the method of Schwartz *et al.* (11) and for alkaline phosphatase according to Lansing *et al.* (12). The activity of phosphatidylinositol phospholipase C was determined in homogenate according to the procedure of Lipsky and Lietman (13). In addition, the activity of phospholipase C was determined using phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylglycerol as substrates as described by Lipsky and Lietman (13). All enzyme assays were performed at 37°C and data were expressed as specific activity per milligram protein. The phospholipid content in homogenate was expressed as milligram per gram tissue (4) while in the lysosomal fraction the data were expressed as microgram per milligram protein (14). The method of Lowry *et al.* (15) was used to determine protein in homogenate and lysosomal fractions using bovine serum albumin as a standard.

Twenty-four hours after the last intubation all rats were killed by decapitation and bled. For morphology, whole lungs from each rat were fixed by immersion in Bouin's fixative and processed for light microscopy. The left lung was divided into three blocks, embedded in paraffin, and stained with hematoxylin and eosin. Three cross sections of each block were examined. All alveoli in each cross section were examined for the presence of foam cells. Semiquantitation of foam cells (FC) in pulmonary alveoli was by an arbitrary scale as follows: +++, one to four FC in many alveoli in all experimental animals; ++, one to three FC in some alveoli in most experimental animals; +, one to two FC in very few isolated alveoli in most experimental animals; −, no FC observed (18).

All reagents were of the purest grade available and dissolved in doubly glass-distilled water. Chlorphentermine (Parke-Davis, Brockville, Ontario) was dissolved in physiological saline. Precoated chromatographic plates with silica gel 80-A° Whatman type K5 were purchased from Chromatographic Specialties Ltd. (Brockville, Ontario). Lipid standards were obtained from Serdary Research Laboratories (London, Ontario). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Data were analyzed statistically using a Student's *t* test and significant differences between the mean values are indicated when the *P* value was $<0.05$.

**Results.** As reported previously (18) oral, daily chlorphentermine administration to rats at a dose of 60 mg/kg for 5 days produced an accumulation of large macrophages in lung identified as foam cells. The observed morphological alterations in pulmonary tissue produced by chlorphentermine were associated with an increase in the levels of the individual phospholipid classes in the homogenate as illustrated in Fig. 1. Chlorphentermine significantly elevated the concentration of pulmonary sphingomyelin (SP), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylcholine (PC). The highest percentage increase was in the level of pulmonary PC. A similar quantitative increase was seen in lavaged and unlavaged lung (4) and thus the unlavaged lung homogenate was used in this study. Chlorphentermine was found to produce a significant rise in all individual phospholipid classes in the lysosomes of rat pulmonary tissue (Fig. 2). It is of interest that the relative percentage increase in the individual phospholipid classes was approximately equal in the homogenate and lysosomal fractions.

Data in Fig. 3 demonstrate the influence of chlorphentermine on the total phospholipid

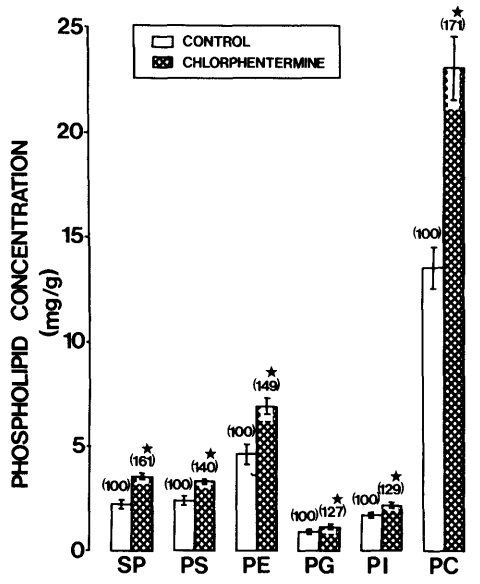


FIG. 1. Influence of chlorphentermine on rat lung phospholipid content. Each bar represents the mean ± SEM of six animals in each group. Rats were administered orally chlorphentermine (60 mg/kg/day) for 5 days. Corresponding controls received the vehicle physiological saline. *Statistically significant difference when compared with the control values ($P < 0.05$).

(TPL) content in pulmonary homogenate and lysosomal fractions. Chlorphentermine significantly increased TPL content in both the homogenate and lysosomal fraction. The quantitative rise in TPL was approximately 1.5-fold higher than that of control in either lung fraction.

The effects of chlorphentermine on the activities of pulmonary alkaline phosphatase and $Na^+,K^+$ ATPase, enzymatic markers for lung tissue toxicity (16, 17) were also determined in lung homogenate. Chlorphentermine did not significantly alter the activities of lung alkaline phosphatase and $Na^+,K^+$ ATPase (Table I). In contrast, a significant decrease was noted in phospholipase C activity, irrespective of the substrate utilized. In the case of PI-, PC-, and PS-PLC the percentage fall in enzymatic activity was to approximately 78% of control, while PE-PLC and PG-PLC were reduced to 85% of control, taking control as 100%.

**Discussion.** It has been well documented that treatment with chlorphentermine produces a marked increase in total as well as individual phospholipid classes in rat lung (18). In agreement with previous findings, chlorphentermine administration significantly elevated the levels of individual and total pulmonary phospholipids. Quantitatively the highest rise noted was in the level of pulmonary PC, the phospholipid found previously to be increased to the greatest extent (18, 19). Reasor and Kacew (4) demonstrated that even in lungs in which the alveolar macrophages were removed by lavage, chlorphentermine still produced a significant elevation in total phospholipid and PC after a 1-week treatment. It was suggested that a possible site for phospholipid accumulation within the pulmonary cell was the lysosome (1, 5). Indeed, this study is the first to clearly demonstrate that in lung lysosomes obtained from chlorphentermine-treated rats the total and individual phospholipid content was increased. It is of interest that netilmicin, a cationic amphiphilic antibiotic, elevated total and individual phospholipid content in rat renal cortex homogenate as well as in lysosomes (14). Our data support the view that

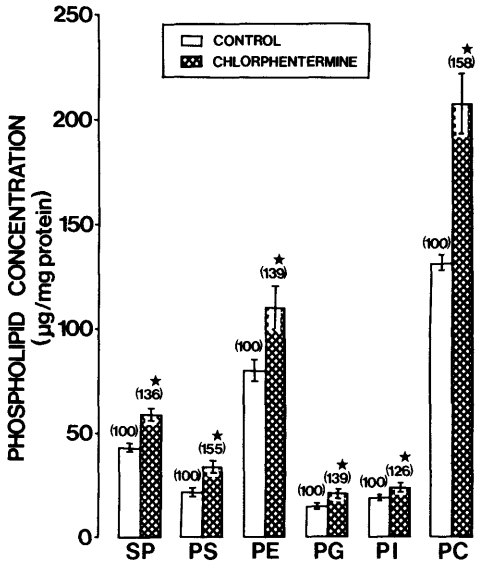


FIG. 2. Effect of chlorphentermine on pulmonary lysosomal phospholipid content. Each bar denotes the mean ± SEM of six rats in each group. For experimental details, see the legend to Fig. 1. *Statistically significant difference when compared with the control values ($P < 0.05$).

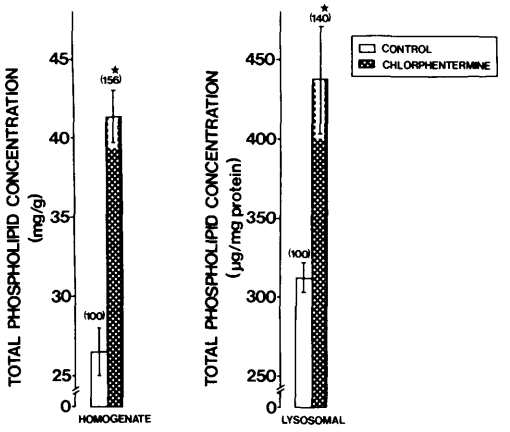


FIG. 3. Influence of chlorphentermine on total phospholipid content in whole lung and lysosomal fraction. Each bar represents the mean ± SEM of six rats in each group. For experimental details, see legend to Fig. 1. *Statistically significant difference when compared with the control values ($P < 0.05$).

chlorphentermine-induced pulmonary phospholipidosis is associated with phospholipid accumulation in lysosomes.

Morphologic and biochemical evidence of chlorphentermine-induced phospholipidosis led investigators to question the functional relevance of this phenomenon. Although Lullmann *et al.* (20) found that chlorphentermine increased pulmonary vascular pressure, Smith *et al.* (21) demonstrated an absence of hypertensive pulmonary vascular disease. In subsequent studies, chlorphentermine treatment was shown to alter glycolysis and mitochondrial function in lung (22, 23), indicating that an alteration in energy metabolism might be associated with a disruption in pulmonary transport mechanisms. It is of interest that Mehendale *et al.* (24) found that chlorphentermine interfered with the transport mechanisms associated with lung 5-hydroxytryptamine clearance and metabolism. In the present investigation chlorphentermine appeared not to markedly affect ATPase, an enzyme involved in pulmonary active transport mechanisms (17). Similarly, chlorphentermine did not significantly alter alkaline phosphatase, an enzyme associated with pulmonary phospholipid and believed to play a role in surfactant secretion (16). In contrast, lung damage produced by asbestos or cadmium was accompanied by an increase in alkaline phosphatase (25, 26). Thiourea-induced edema was associated with a fall in the activity of pulmonary ATPase (17). It would appear that chlorphentermine may alter the energy pathways within lung cells but this agent does not seem to affect plasma membrane function.

Induction of phospholipidosis by cationic amphiphilic drugs has been postulated to result from a decreased degradation of phospholipid by phospholipases (1, 5). *In vivo* administration of gentamicin was found to inhibit renal cortex phospholipase A (27). In addition, incubation of rat renal cortex with gentamicin was reported to produce inhibition of phospholipase A and C (13, 27). Hostetler (5) found that incubation of a rat liver lysosomal preparation with chlorphentermine significantly inhibited phospholipases A and C. Data in this study demonstrated that chlorphentermine *in vivo* produced a significant fall in phospholipase C. The findings that activities of phospholipase C toward different substrates were inhibited may account for the observed rise in the individual phospholipid classes. The quantitative increase in total phospholipid was greater than the inhibition of phospholipase C activity. Since chlorphentermine has been shown to

TABLE I. EFFECTS OF CHLORPHENTERMINE ON THE ACTIVITIES OF PULMONARY ATPase, ALKALINE PHOSPHATASE, AND PHOSPHOLIPASE C[a]

| Enzyme | Treatment | |
|---|---|---|
| | Control | Chlorphentermine |
| $Na^+,K^+$ ATPase | 0.93 ± 0.08 | 0.86 ± 0.08 |
| Alkaline phosphatase | 6.52 ± 0.64 | 5.81 ± 0.67 |
| PI-PLC | 140.33 ± 11.72 | 109.97 ± 10.73* |
| PC-PLC | 132.29 ± 6.01 | 104.26 ± 3.89* |
| PS-PLC | 134.57 ± 3.91 | 104.71 ± 4.19* |
| PE-PLC | 121.60 ± 6.95 | 104.35 ± 1.28* |
| PG-PLC | 129.19 ± 7.35 | 109.67 ± 2.25* |

[a] Each value is the mean ± SEM of six animals per group. Animals were administered chlorphentermine (60 mg/kg/day po for 5 days) while corresponding controls received physiological saline. The activities of Na, K ATPase and alkaline phosphatase are expressed as µmole/hr/mg protein. PLC denotes phospholipase C activity while the substrate utilized is given, e.g., PI-PLC would be phosphoinositol phospholipase C. PLC activity is expressed as nmole/hr/mg protein.

* Statistically significant difference when compared with control values ($P < 0.05$).

inhibit phospholipase A activity *in vitro*, it is likely that inhibition of this enzyme is occurring *in vivo* as well, and this may be contributing, in part, to the lack of correlation in these responses.

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