Aldrin Epoxidase Activity in Developing Rabbit Lung

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Summary

Pulmonary aldrin epoxidase activity was determined in 2-, 4-, 6-, 8-, 12-, and 16-week-old male rabbits using isolated perfused lung preparations and *in vitro* incubation techniques. In the perfusion experiments, it was determined that aldrin epoxidase activity increased with age up to 12 weeks of age. Although there was an increase in the weight of lungs with age, this did not appear to be related to the increase in aldrin epoxidation. *In vitro* experiments carried out using the same substrate to tissue ratio failed to exhibit an age-related increase in the aldrin epoxidase activity. Extrapolated values for aldrin epoxidation from *in vitro* experiments yielded much higher values than realized from perfusion experiments. Accessibility of the substrate to the enzymatic sites, saturation of the accessible enzyme system, and limiting availability of the cofactor necessary for the oxidative reaction remain as the possible mechanisms for the discrepancy between perfusion and *in vitro* incubation studies.

Speculation

Pulmonary aldrin epoxidase activity determined using isolated perfused lung preparations does not correlate with *in vitro* results obtained from incubations fortified with cofactors. However, the ratio of the two may become a useful predictive factor to obtain realistic contribution of an organ to the metabolism of xenobiotics.

Because of their direct contact with the environment, the lungs are constantly exposed to innumerable environmental pollutants. These agents, which are often detrimental, can gain entry into the lungs either through direct exposure by inhalation or indirectly through the vascular system. A number of xenobiotics have been reported to be accumulated by the lungs (21). Of these, halogenated compounds constitute a diverse group of man-made chemical agents which have wide and extensive industrial use and consequently a wide environmental distribution. Investigations of the mechanisms of uptake and disposition of halogenated xenobiotics by the lung have been infrequent. There are numerous halogenated solvents which exhibit hepatotoxicity (4) upon exposure of animals to their vapors, indicating their uptake and subsequent redistribution from the lung. Among the chlorinated compounds reported to be accumulated in the lungs are: chlorpromazine (23, 24), chlorcyclizine (1, 11), and chlorphentermine (14). Mechanisms of uptake, metabolism, and disposition of these and other chlorinated compounds by the lungs remain largely unexplored.

Interactions of epoxides (halogenated or nonhalogenated) with the lung are of particular interest. Many carcinogenic agents may be metabolized to epoxides. These epoxides are thought to be the carcinogenic forms of the carcinogens (6). In addition, epoxides have been found to act as mutagens (3, 8), to act as arylating agents that react with proteins and nucleic acids *in vivo* (5, 12), and to cause malignant transformations of tissue culture cells (5). An example of a known interaction of epoxides with the lung is the pulmonary necrosis caused by bromobenzene, epoxide metabolites of which bind readily in the lung (22).

The presence of epoxidase activity in the lung tissue was indirectly suggested by a report of the formation of hydroxylated metabolites of polycyclic hydrocarbons (6). Formation of an unstable epoxide intermediate is assumed in the hydroxylation of the compounds containing two vicinal unsubstituted carbon atoms (10). The first direct demonstration of epoxidase activity in lung tissue was reported by Mehendale *et al.* (19).

Aldrin and its epoxide, dieldrin (Fig. 1), are chlorinated cyclo-diene pesticides extensively used in the past as pesticide sprays. Using isolated perfused rabbit lung preparations (19), it was demonstrated that aldrin from perfusate can be accumulated in the lungs, where it is metabolized to its epoxide, dieldrin. This epoxide is in turn accumulated by the lungs. There are two distinct phases to aldrin accumulation in the lungs from the circulating perfusate (19). Although aldrin was readily metabolized to dieldrin by the isolated perfused lung, dieldrin itself was not metabolized further. In the liver, which is the primary site of aldrin metabolism, dieldrin is metabolized by epoxide hydrase to give two isomeric diols which are subsequently conjugated (10, 16). The lack of pulmonary dieldrin epoxide hydrase activity was consistent with other reports of undetectable levels of this enzyme activity in the lung tissue (9, 20), although epoxide hydrase has been detected in human and hamster lungs (2, 3, 7).

The objective of this study was to determine the epoxidase activity in developing rabbit lungs using aldrin as a substrate. Aldrin epoxidase was measured utilizing (1) an isolated perfused lung model; and (2) *in vitro* incubation of subcellular preparations. The immediate objective was to determine the course of development of aldrin epoxidase activity in the lung. The second objective was to determine if the enzyme activity measured by *in vitro* incubations may be compared to data from intact lung perfusions.

MATERIALS AND METHODS

ANIMALS

Male New Zealand White rabbits were used for this study, in view of relevant background information available on pulmonary aldrin epoxidation in this strain of rabbits (18). Animals ranging from 2 to 16 weeks (adult) of age were used in these studies. Animals were maintained in the University of Mississippi Medical Center animal facilities until immediately prior to use.

SUBCELLULAR PREPARATION AND INCUBATION PROCEDURE

The procedure described by Matsubara *et al.* (15) was used for preparing the lung homogenates. Animals were anesthetized with pentobarbital sodium (60 mg/kg IV) which was mixed with heparin (1000 IU/kg). Lungs were removed and perfuse washed with warm (0.9% w/v) saline to wash out blood and any free pentobarbital. They were then placed in the preparation medium (0.154 M KCl and 0.05 M Tris-HCl buffer, pH 7.4). After excess connective tissue was removed, the lungs were placed in (1:4 w/v) measured volume of buffer (0.154 M KCl:0.05 M Tris-HCl, pH 7.4) and homogenized with a Teflon-glass type Potter-Elvehjem homogenizer. The homogenate was centrifuged at 400 × g for 10 min, and

Fig. 1. Structures of aldrin and dieldrin.

the supernatant fluid was recentrifuged at $9000 \times g$ for 10 min to remove nuclear and heavy mitochondrial fractions, respectively. The $9000 \times g$ supernatant was used for incubation with aldrin. The reaction mixture contained 2.0 ml Tris-HCl buffer (0.05 M, pH 7.4), 1.0 ml of enzyme, and 2 μ moles of NADPH (or equivalent generating system). Reaction was initiated by the addition of 0.06 μ mole aldrin in 5 μ l of ethanol. The amount of substrate used was 2.5 μ moles/10 g lung, which was found to be the saturation dose in a previous study (18). The reaction mixture was incubated in 25 ml Erlenmeyer flasks at 37°C in a shaking bath for 30 min. The reaction was terminated by adding 2 ml of (9:1) hexane:acetone mixture.

ISOLATED PERFUSED LUNG PREPARATIONS

The perfusion apparatus was the same used previously in this laboratory (18, 19) and has been described in detail previously.

Animals were anesthetized with pentobarbital sodium (60 mg/kg IV) which was mixed with heparin (1000 IV/kg). The trachea was exposed by a longitudinal ventral incision on the neck and clamped after the lungs were fully inflated. The diaphragm and the rib cage were incised exposing the heart and lungs, which were carefully removed intact. During cannulation, the tissues were bathed in warm physiologic saline. The pulmonary artery was cannulated with a 2-inch length of PE 360 tubing prefilled with the perfusate to avoid possible air emboli. The pulmonary vein was cannulated with 3-inch length of PE 360 tubing via an incision in the left ventricle. The trachea was cannulated with a 2-inch length of PE 330 tubing. The cannulated lung preparation was perfused with warm saline for approximately one min to remove the blood from the vascular system. The washed and blotted preparation was weighed along with the cannulae and the clamps. Weight of the lungs was obtained at the end of the experiment by weighing the lung preparation as above and by subtracting the weight of cannulae, clamp, etc., from the total weight. Cannulated lungs were connected to the preprimed apparatus, and perfusion was established through pulmonary artery along with artificial ventilation (18). Experiments were initiated after the initial equilibration period of 10 min by introducing 2.5 μ moles aldrin per 10 g lung in 50 μ l of ethanol.

EXTRACTION AND ANALYSES

The reaction mixtures from the *in vitro* experiments, or the perfusate, and the lung homogenates from the perfusion experiments were extracted with (9:1) mixtures of pesticide quality hexane:acetone as described previously (18). Aldrin and dieldrin was analyzed by a gas-chromatograph fitted with an electron capture detector as previously described (17). Protein concentrations were determined using the biuret method or the method of Lowry *et al.* (13). Aldrin epoxidase activity was expressed as μ mole dieldrin formed per mg protein or per g lung tissue per 30 min.

RESULTS

PERFUSION EXPERIMENTS

A time-course study was undertaken to determine the time of maximal production of dieldrin by isolated perfused rabbit lung preparations (Fig. 2). Lung preparations obtained from 12-week-old rabbits were utilized in this series of experiments. Experiments were initiated by the addition of 2.5 μ moles of aldrin to 100 ml

perfusate and terminated at indicated time points. Aldrin and dieldrin concentrations were determined in perfusate and lung homogenate samples. Production of dieldrin by these lung preparations proceeded at a maximal rate during the first 15 min, showed a curvilinear response between 15 and 30 min, and appeared to level off thereafter (Fig. 2). Hence, all other experiments were terminated at 30 min.

Figure 3 depicts the pharmacokinetics of aldrin and dieldrin in a typical recirculating isolated perfused lung preparation. Aldrin is taken up by the lung in a biphasic manner: the concentration of aldrin initially decreases rapidly followed by a slower decline. Aldrin accumulated in the lung is metabolized to dieldrin, some of which appears in the perfusate. The concentration of dieldrin

Fig. 2. Time-course of dieldrin formation by isolated perfused rabbit lung preparations. Total dieldrin produced by the lung preparations was determined by summation of dieldrin found in the perfusate and with lung homogenates in experiments terminated at indicated time points. Ordinate, dieldrin production either as μ moles per whole lung or as μ mole/g of lung tissue. Points, 3 to 5 individual determinations with 12-week-old rabbit lungs. Results are mean $\pm$ S.E.

Fig. 3. Pharmacokinetics of aldrin in the perfusate. Aldrin and dieldrin were assayed in perfusate samples taken at indicated time points. Ordinate, μ moles of aldrin ($\blacktriangle$), dieldrin ($\triangle$), or total aldrin equivalents ($\circ$) per ml of perfusate; abscissa, time in min. Also shown in the graph is dieldrin ($\bullet$) accumulation in the lung. The results presented are those obtained with 12-week-old rabbits.

Fig. 4. Aldrin epoxidase activity in developing rabbit lungs. Aldrin epoxidase activity is expressed as μ moles of dieldrin produced by isolated perfused lung preparations per g lung tissue per 30 min on the ordinate and the abscissa shows age of rabbits. Value at 2 weeks is significantly different from all other values except 4 week value. Four-week value is significantly different from all values except 2 and 16 week values. Six-week value is significantly different from 12-week value. $P < 0.05$. Results are mean $\pm$ S.E. of 3 to 5 individual determinations.

in the perfusate increases steadily throughout the duration of these experiments (Fig. 3). After the initial 10 min, the curve representing the sum of aldrin and dieldrin in the perfusate levels off, suggesting that any aldrin taken up by the lung via the second phase of aldrin uptake is metabolized, and an equivalent quantity of dieldrin is returned to the perfusate. Total quantity of aldrin and dieldrin retained by the lung is represented by the dashed line in Figure 3. This also reached a steady-state after the initial 10 min.

Aldrin epoxidase activity of intact lung preparations obtained from 2- to 16-week-old rabbits was determined in similar perfusion experiments. Figure 4 illustrates the development of pulmonary aldrin epoxidase activity with age. Total dieldrin produced by the lung was calculated as the sum of dieldrin present in the perfusate and of that in the lungs at the end of 30 min perfusion. Aldrin epoxidase activity increased up to 12 weeks and appeared to decline slightly thereafter. Thus, lungs from 12-week-old rabbits exhibited maximal epoxidase activity. Weight of lungs also increased sharply between 4 and 8 weeks and increased at a much slower rate thereafter.

IN VITRO EXPERIMENTS

Aldrin was incubated with lung homogenates and various centrifugation fractions to determine the distribution of aldrin epoxidase activity (Table 1). Maximal activity was obtained in 9000 $\times$ g supernatant fraction. Hence, all *in vitro* experiments were conducted using 9000 $\times$ g supernatant fraction of lung homogenates. In some experiments, the NADPH generating system was omitted to determine aldrin epoxidation in the absence of added cofactors. In the absence of added cofactor, only minimal production of dieldrin was observed ($0.017 \pm 0.002 \mu\text{mole/g/30 min}$). Additional incubation experiments conducted with omission of the components of the NADPH generating system indicated that

addition of NADP is essential to obtain any appreciable rates of aldrin epoxidation with 9000 $\times$ g supernatant fractions. Additional experiments with lung homogenates have also indicated that addition of NADP is essential for the epoxidation of aldrin in intact lung (Mehendale, unpublished data).

Aldrin epoxidation was measured *in vitro* in preparations of 9000 $\times$ g supernatant fractions of lungs obtained from male rabbits of age 2 to 16 weeks (Table 2). When the activity was expressed as μ moles/g of lung tissue, all lung preparations exhibited same level of activity with the exception of 6-week-old lung preparations which showed decreased activity. Incubations from 8- and 12-week-old lungs yielded the highest specific activity. In terms of the total potential of the lungs (Table 3), aldrin epoxidation increases with age by virtue of the increased lung

Fig. 5. Change in weight of lungs with age. Ordinate, wet weight of lungs in g; abscissa, age of rabbits. Results are mean $\pm$ S.E. of 7 to 9 animals.

Table 1. Aldrin epoxidase activity in subcellular fractions of rabbit lung¹

Lung fraction	Dieldrin produced ($\mu\text{moles/g lung/30 min}$)
Homogenate	0.174 ± 0.001^2
400 $\times$ g pellet	0.132 ± 0.003
400 $\times$ g supernatant	0.211 ± 0.003
9000 $\times$ g pellet	0.114 ± 0.005
9000 $\times$ g supernatant	0.205 ± 0.003

¹ Aldrin epoxidase activity was determined in triplicate incubations of various centrifugation fractions obtained from 12-wk-old animals.

² Mean $\pm$ S.E. of three determinations.

Table 2. Aldrin epoxidase activity in developing rabbit lung¹

Age (wk)	Dieldrin produced	
	$\mu\text{moles/g lung/30 min}$	$\text{nmoles/mg protein/30 min}$
2	0.21 ± 0.006^2	2.80 ± 0.20
4	0.18 ± 0.008	2.28 ± 0.09
6	0.16 ± 0.007^3	2.35 ± 0.33
8	0.18 ± 0.009	3.23 ± 0.16
12	0.20 ± 0.01	3.12 ± 0.36
16	0.18 ± 0.015	2.48 ± 0.09

¹ Aldrin epoxidase activity was determined in 9000 $\times$ g supernatant fractions of perfused-washed lungs. Incubation techniques are as described in the text.

² Mean $\pm$ S.E. of at least 3 animals.

³ Statistically different from all other values. $P < 0.05$.

Table 3. Aldrin epoxidation in rabbit lung

Age (wk)	Aldrin epoxidase activity (μ moles dieldrin/lung/30 min)	
	Intact lungs, perfusion experiments ¹	<i>In vitro</i> experiments extrapolated ²
2	0.068 $\pm$ 0.01 ³	0.49 $\pm$ 0.06
4	0.081 $\pm$ 0.01	0.60 $\pm$ 0.03
6	0.34 $\pm$ 0.04	1.39 $\pm$ 0.22
8	0.38 $\pm$ 0.01	1.78 $\pm$ 0.09
12	0.68 $\pm$ 0.02	2.17 $\pm$ 0.25
16	0.62 $\pm$ 0.05	2.30 $\pm$ 0.24

¹ Aldrin epoxidase activity was determined in isolated perfused lung preparations as described in the text. Total dieldrin produced was calculated by adding the quantity of dieldrin present in the perfusate and in the lung at the termination of the experiments.

² Amount of dieldrin formed in *in vitro* incubations as in Table 1 was equivalent to dieldrin production for 250 mg of lung tissue. Total dieldrin per lung was calculated from the lung weight.

³ Mean $\pm$ S.E.

tissue reflected in increased wet weight of lungs with age (Fig. 5). The ratio of aldrin epoxidation measured by perfusion of whole lung to that measured by *in vitro* incubation was examined (Table 3). For 2- and 4-week lungs, the ratio is 7, whereas for other lungs it approximates 4.

DISCUSSION

Results of the perfusion experiments indicate that pulmonary aldrin epoxidase activity increases with the age of rabbits. This does not appear to be a function of the wet weight of lung tissue because lung weight increased from 2 to 16 weeks of age. The results of perfusion studies cannot be corroborated by the *in vitro* experiments because age-related increase in aldrin epoxidase activity was not observed in *in vitro* incubation experiments. Often, results obtained from *in vitro* incubations of tissue preparations are extrapolated to the whole organ to assess the metabolism of a given chemical by the intact organ. In the present study, production of total dieldrin from aldrin by the intact perfused lung preparations can be calculated by the summation of dieldrin present in the perfusate and that present in the lung. Similarly, total dieldrin production by the whole organ can be calculated by extrapolation of the *in vitro* results. As may be noted in Table 3, the values derived at by two techniques are remarkably different. For 2- and 4-week-old lungs, there is a 7-fold difference, and for others, the factor appears to stay between 3 to 4. The possibility of using such a factor to predict the *in vivo* activity is raised by these results. Alternatively, it may be more reliable to use intact, isolated perfused organs to assess the contribution of these organs toward the biotransformation of a particular xenobiotic.

In attempting to explain the reasons for the differences between the perfusion and the *in vitro* studies, two major factors may be considered. Firstly, transport of the substrate and accessibility to the enzyme may be different. Aldrin appears to be taken up by the pulmonary tissue by diffusion (18), and hence, the uptake process may not be limiting. However, compartmentalization of the substrate within the lung tissue may explain the inability of the perfused lung preparations to metabolize aldrin to dieldrin to the extent predicted by the *in vitro* studies. Saturation of the enzyme sites or limiting availability of cofactor may be expected to result in reduced epoxidation of aldrin. Although saturation of all the enzyme sites within the lung seems unlikely, it is possible that the substrate may have easy access to only a portion of the enzyme sites, resulting in an apparent saturation of enzyme. On the other hand, availability of the cofactor may become limiting, hence, an apparent saturation of the epoxidase system. Recently, Thurman *et al.* (25) concluded that availability of cofactor can become limiting, thus affecting the rate of drug biotransformation reactions by intact perfused liver preparations. Whatever the

mechanism, it is apparent that quantitative extrapolations of drug biotransformations from *in vitro* studies to *in vivo* situations may not be reliable.

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- This research was supported by a grant from the Mississippi Lung Association Research and Medical Education Fund.
- Received for publication May 15, 1979.
- Accepted for publication June 13, 1979.