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DIFFERENCES IN THE BIOCHEMICAL PROPERTIES OF ALDRIN EPOXIDASE, A CYTOCHROME P-450-DEPENDENT MONOOXYGENASE, IN VARIOUS TISSUES

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Abstract—Aldrin epoxidase, a cytochrome P-450-dependent monooxygenase, was studied in the lung and kidney of male rats. The sensitivity of the liver enzyme activity to different chemicals *in vitro* was influenced by the treatment of the animals with phenobarbital or methylcholanthrene. These results confirm that more than one form of cytochrome P-450 supports aldrin epoxidase in the liver. The lung and kidney aldrin epoxidase activity was not modified by the administration of chemical inducers to the rats. *In vitro*, the lung and kidney aldrin epoxidase activities were activated by tetrahydrofurane and progesterone, respectively. The results obtained from the lung and kidney indicate that one single species of cytochrome P-450, associated with aldrin epoxidase, exists in these organs, but it may be a different type, or regulated in a different manner in these tissues.

Insecticides such as aldrin are commonly used in agriculture. Given the widespread reliance upon these chemicals, their relative safety must be assured. Knowledge concerning these insecticides and particularly information related to their metabolism and regulation are vital. Insecticides, like most drugs entering a living organism, are first metabolized by microsomal cytochrome P-450-dependent monooxygenases [1, 2].

Many laboratories have demonstrated the existence of multiple forms of cytochrome P-450 in various animal species, including man [3, 4]. The exact number of these haemoproteins has not been established and could range from a few species to hundreds or even thousands of units [5].

Among the various monooxygenases, one usually distinguishes those which are inducible by phenobarbital (called cytochrome P-450 or P-450-dependent enzymes in this report), and those which are inducible by polycyclic aromatic hydrocarbons (cytochrome P_1-450 or P_1-450-dependent enzymes) [6, 7]. These classes of monooxygenases differ not only with respect to their response to different inducers, but also with respect to their physiological and biochemical properties. Among the P_1-450-dependent enzymes and other more specifically induced species, aryl hydrocarbon hydroxylase and ethoxyresorufin deethylase can be assayed in both hepatic and extrahepatic tissues by very sensitive techniques [8, 9]. Conversely, the activity of the cytochrome P-450-dependent enzymes is difficult to measure in extrahepatic tissues. Aldrin epoxidase, however, seems to be an exception, as a highly sensitive assay can be used for measuring its activity in various animal tissues [10] or in cell cultures [11].

Few studies have been published on the biochemical properties of aldrin epoxidase (for examples see [10–14]). Despite the fact that studies on *in vitro* inhibition by SKF 525 A* and 7,8-benzoflavone have been inconclusive [10], this enzyme has been classified in the P-450 monooxygenase group [10] due to its inducibility by phenobarbital and its inhibition by methylcholanthrene. These results were confirmed by Wolff *et al.* [14] from a monooxygenase system reconstituted from purified rat liver microsomal cytochrome P-450 or P-448, NADH-cytochrome P-450 reductase, dilauroylphosphatidylcholine and cholate. The values of V_m and K_m obtained under these particular experimental conditions indicated that aldrin is a highly selective substrate for cytochrome P-450 and a poor substrate for cytochrome P-448. In this respect it behaves similarly to different endogenous substrate metabolizing monooxygenases, such as cholesterol-7α-hydroxylase or progesterone-16α-hydroxylase [15].

The purpose of this study was to investigate the activity of aldrin epoxidase in various rat tissues and to compare its biochemical properties in hepatic and extrahepatic tissues.

MATERIALS AND METHODS

Aldrin, dieldrin and pDDT were purchased from Riedel-de-Haan (Seelze, F.R.G.); enzymes and co-factors were from Boehringer (Mannheim, F.R.G.); 3-methylcholanthrene from Fluka (New Ulm, F.R.G.); metyrapone from Aldrich (Beerse, Belgium); 7,8-benzoflavone from UCB (Brussels, Belgium). Parconazole and ketoconazole, two drugs with an imidazole ring, were a gift from Dr. H. Van den Bossche from Janssen Pharmaceutica (Beerse, Belgium). Other chemicals and solvents were of

* Abbreviations: MC: 3-methylcholanthrene; PB: phenobarbital; pDDT: 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)-ethane; SKF 525 A: diethylaminoethyl-2,2-diphenyl-valerate.

analytical grade and were obtained from Merck (Darmstadt, F.R.G.).

Treatment of animals. All of the experiments were performed on 200 g male Sprague–Dawley rats obtained from the Centre des Oncins (Lyon, France). They were kept at a constant temperature of 22° with free access to food (UAR A04, Villemoison, France) and tap water. The adrenalectomized rats received saline as drinking water throughout the entire experiment. Inductions were performed by i.p. injection of phenobarbital (80 mg/kg in saline), methylcholanthrene (20 mg/kg in peanut oil) or dexamethasone (10 mg/kg in peanut oil) for four consecutive days. The animals were sacrificed 24 hr (MC and PB) or 4 hr (dexamethasone) after the last injection.

Preparation of the enzymes. The animals were killed by decapitation. The organs were removed and immediately plunged in cold isotonic KCl. The liver, kidney and lung were then pressed through a metallic disc perforated with 1.5 mm diameter holes to eliminate connective tissues. Skin was frozen in liquid nitrogen and reduced to a powder in a metallic mortar as described elsewhere [16].

For each organ, the resulting pulp was diluted with 4 parts of a Tris (0.01 M)–sucrose (0.25 M) buffer (pH 7.4) and homogenized in a Potter–Elvejhem tube with a Teflon pestle. The homogenate was centrifuged for 10 min at 10,000 *g* in a refrigerated Sorvall (RC 2B) and the supernatant was stored at −20°; it was used as a source of enzymes.

Aldrin epoxidation assay. The method used was that described by Wolff *et al.* [10] with some modifications. The incubation medium (1 ml) contained Tris buffer, pH 7.4 (100 μmole), glucose-6-phosphate (3 μmole), NADP (0.5 μmole), NAD (0.5 μmole), glucose-6-phosphate dehydrogenase (1 unit), bovine serum albumin (1 mg) and $MgCl_2$ (5 μmole); aldrin (in 20 μl of methanol) and homogenate concentration varied as a function of the tissue in which the enzymatic activity was measured (Table 1). At the end of the incubation, unreacted aldrin and synthesized dieldrin were extracted by 5 ml of hexane containing 0.1–2 μg of pDDT (chosen as an internal standard). The GLC separation was performed following injection of 2 μl of the hexane extract on a conventional column (1 m long, 2 mm diameter coated with OV 1 (1%) on Chromosorb W AW DMCS, 100–120 mesh) in a Packard model 419 equipped with a ^{63}Ni-electron capture detector. Injector, column and detector were heated at 265°, 210° and 265°, respectively. The carrier gas was purified nitrogen. Retention times for aldrin, dieledrin and pDDT were about 145, 250 and 360 sec, respectively.

The concentration of dieldrin formed was calculated on the basis of the ratio of the heights of the peaks corresponding to dieldrin and pDDT. A calibration curve was made under the same conditions as for the assays.

Other assays. Aryl hydrocarbon hydroxylase was measured according to a method described elsewhere [17]. The protein concentration of the tissue preparation was determined according to the method of Lowry *et al.* [18], using bovine serum albumin as a standard.

RESULTS

Optimization of the aldrin epoxidase assay

For each organ involved in our study, we first selected the optimal conditions for the enzymatic assay, i.e. saturating substrate concentrations, and linear response of the enzymatic activity with respect to the tissue homogenate concentration and the duration of incubation. These conditions varied slightly for each tissue and are summarized in Table 1. The highest enzymatic activity was found in the liver followed by the lung, kidney and intestine. The activity was below our detectable level for the skin, testes and thymus. Therefore we focused our attention on the activity of aldrin epoxidase in the liver, lung and kidney throughout the remainder of our study.

Effect of various inducers on liver and lung aldrin epoxidase activity in normal and adrenalectomized rats

The effect of inducers was studied both in normal and adrenalectomized animals. Indeed, different experimental facts supported the participation of glucocorticoids in the control of various cytochrome P-450-dependent hepatic monooxygenases. In the rat, the circadian rhythm of the cholesterol-7α-hydroxylase activity was directly controlled by the corticoids [15, 19]. In the rat hepatoma culture, glucocorticoids also led to the expression of several steroid hydroxylases [20]. In the primary fetal rat

Table 1. Optimized experimental conditions for the assay of aldrin epoxidase in various organs

Organ	Protein concentration of $S_{10,000g}$ (mg/ml)	$S_{10,000g}$ added (μl/ml)	Incubation time (min)	Substrate concentration (μM)	Measured activity (pmole/min × g fresh organ)
Liver	27.1	10	10	44	55.1 ± 2.1
Lung	16.0	200	15	11	3.0 ± 0.7
Kidney	22.1	100	15	11	1.7 ± 0.4
Intestine	12.8	250	20	11	0.12 ± 0.10
Skin	4.6	500	30	11	<0.02
Testes	11.6	500	30	11	<0.02
Thymus	15.3	500	30	11	<0.02

The experimental procedure is described in Materials and Methods. The activities of aldrin epoxidase correspond to the mean ± S.D. of the results obtained from five animals.

Table 2. Effect of adrenalectomy and/or administration of phenobarbital, methylcholanthrene and dexamethasone on aldrin epoxidase activities in liver, lung and kidney

Pretreatment		Aldrin Epoxidase		
		Liver	Lung	Kidney
None	No	55.1 ± 2.1	3.0 ± 0.7	1.7 ± 0.4
None	Phenobarbital	129.6 ± 13.2*	3.1 ± 0.9	1.8 ± 0.6
None	Methylcholanthrene	17.9 ± 2.8*	2.8 ± 0.3	1.7 ± 0.7
None	Dexamethasone	23.8 ± 5.6*	2.1 ± 0.5	1.6 ± 0.5
Adrenalectomy	No	11.1 ± 4.3	1.5 ± 0.7	1.4 ± 0.7
Adrenalectomy	Phenobarbital	45.6 ± 10.9*	1.7 ± 0.6	1.6 ± 0.4
Adrenalectomy	Dexamethasone	11.3 ± 3.6	1.2 ± 0.3	1.5 ± 0.5
Adrenalectomy	Phenobarbital + dexamethasone	54.6 ± 13.9*	1.3 ± 0.5	1.8 ± 0.7

Adrenalectomy was performed 3 days before the administration of the inducers (see Materials and Methods). The activities of aldrin epoxidase (pmole/min × g organ) correspond to the mean ± S.D. of the results obtained from five animals.

* $P < 0.01$ for control rats (no chemical treatment).

liver cell culture, the addition of dexamethasone to the medium modified the haemoprotein species present in the cells from a P_1-450 to a P-450 type; therefore, aldrin epoxidase, which was undetectable in the cells, became inducible by phenobarbital in the presence of the corticoid [11].

In accordance with Wolff *et al* [10], the rat liver aldrin epoxidase activity was induced by phenobarbital (PB) and inhibited by methylcholanthrene (MC) and dexamethasone (Table 2). The same chemicals, however, did not affect the enzymatic activity in the lung (Table 2). In adrenalectomized animals, the constitutive aldrin epoxidase activity level was significantly diminished in the lung and liver, and was not affected in the kidney. However, the hepatic enzyme was still very well induced by phenobarbital. Dexamethasone alone did not modify the aldrin epoxidase activity. As in the normal rat, the lung and kidney enzymes were not significantly affected by any of the treatments in the adrenalectomized animals (Table 2).

These results show that if the adrenal glands played a role in the control of the expression of the liver monooxygenases, this function could not be attributed exclusively to the glucocorticoids. This observation could be linked to the particular experimental conditions used in our study, but might also indicate that other hormones—in addition or not to the glucocorticoids—were involved in the regulation of these enzymes.

Effect of various chemicals on the activity of aldrin epoxidase in vitro

The effect of various monooxygenase inhibitors

Table 3. Inhibiting effect of various chemicals on rat liver aldrin epoxidase activity *in vitro*

Compounds	Concentration (M)	Aldrin epoxidase activity		
		No treatment	Phenobarbital	3-Methylcholanthrene
Metyrapone	10^{-5}	74–88	53–62	82–97
	10^{-4}	49–55	25–34	52–76
7,8-Benzoflavone	10^{-5}	92–101	81–88	89–99
	10^{-4}	75–89	50–68	71–81
Tetrahydrofurane	10^{-2}	92–100	98–119	105–123
	10^{-1}	84–98	94–108	96–106
Progesterone	10^{-4}	43–63	80–91	36–54
	10^{-3}	24–30	52–70	14–26
Parconazole	10^{-5}	25–43	51–57	24–37
	10^{-4}	0–1	1–4	0–1
Ketoconazole	10^{-5}	69–89	79–91	70–82
	10^{-4}	40–51	70–78	36–45

The effect of various compounds on aldrin epoxidase was determined under standard conditions (Table 1) on hepatic $S_{10,000g}$ from control, PB-treated and MC-treated rats. Metyrapone, 7,8-benzoflavone and progesterone were dissolved in 10 μl of dimethylformamide prior to their addition to the medium. The other chemicals were soluble in water. The results are expressed as a percentage of the activity of three control assays conducted in the presence of the solvent alone. The experiment was run three times and the range of the results is given for each compound and all experimental conditions.

on the aldrin epoxidase were studied *in vitro*. The chemicals tested included classical monooxygenase inhibitors (metyrapone, 7,8-benzoflavone and tetrahydrofurane), an endogenous substrate of the liver monooxygenase (progesterone), and a group of imidazole derivatives which selectively inhibited the PB-induced monooxygenase (parconazole and ketoconazole).

Tetrahydrofurane, phenobarbital and dexamethasone did not affect the hepatic aldrin epoxidase activity *in vitro* (Table 3). At 10^{-4} M, 7,8-benzoflavone, a selective inhibitor of the P_1-450-dependent monooxygenase, had a limited effect on the control rat liver enzyme and a slightly more pronounced inhibitory action on the PB-induced enzyme. On the other hand, all the P-450 inhibitors strongly affected the aldrin epoxidase activity in both the control and induced livers. Parconazole was particularly efficient as it completely inhibited the enzyme activity at a concentration of 10^{-4} M, i.e. about twice as much as the substrate concentration. Progesterone was also a good inhibitor, but contrarily to metyrapone, its effect on the PB-induced enzymes was weaker. In this respect, if we compare the action of the various chemicals on the control and induced enzymatic activity, it is striking that the inhibition pattern was very similar for the control and the MC-treated rat liver enzyme and different for the PB-induced enzyme.

In the lung and kidney, the various inhibitor effects were identical regardless of the pretreatment administered to the rats (MC or PB induction). Consequently, only the results obtained from control rat tissues are shown (Table 4).

In the lung, metyrapone, progesterone and parconazole had an inhibitory effect on aldrin epoxidase, whereas 7,8-benzoflavone had no effect at all. On the other hand, tetrahydrofurane was a good activator of the enzyme, and ketoconazole less so.

In the kidney, all the chemicals tested had an inhibitory effect which was similar to that observed in the control liver, except progesterone, which surprisingly had a three-fold activating effect on the enzyme.

To demonstrate that the activating effect of tetrahydrofurane on the lung enzyme and progesterone on the kidney enzyme was not influenced by the standard conditions of the assays which varied from one tissue to another (Table 1), we conducted an experiment in which the incubation conditions were identical for the three organs (Table 5). In this experiment, only the enzyme concentrations varied according to the tissue; the other factors (substrate concentration, incubation time) remained constant. Moreover, the dilution of the enzymatic preparations was such that the quantity of dieldrin formed was about the same for each sample. Table 5 shows a great difference between the three tissues according to their sensitivity to tetrahydrofurane and progesterone; these variations were not due to the particular incubation conditions.

These activating effects of progesterone and tetrahydrofurane were not only tissue-specific, but also enzyme-specific. Aryl hydrocarbon hydroxylase, another microsomal monooxygenase which can be measured in extrahepatic tissues, was either inhibited or uninfluenced by these two compounds, regardless of the tissue or the concentration of the chemical (data not shown).

DISCUSSION

At the present stage, our study further supports the hypothesis that aldrin epoxidase is a cytochrome P-450-dependent monooxygenase as opposed to the cytochrome P_1-450- or P-448-dependent enzymes. We have not only confirmed the published results on the action of the inducers on the liver *in vivo*, but have also demonstrated a selective pattern of inhibition by various chemicals *in vitro*. In this respect, the imadazole derivatives are of particular interest as at concentrations equivalent to one-fifth that of the substrate, they inhibit 50% of the aldrin epoxidase activity. The results on the inhibition *in*

Table 4. Inhibiting effect of various chemicals on rat lung and kidney aldrin epoxidase activity *in vitro*

Compounds	Concentration (M)	Aldrin epoxidase activity (% of control)	
		Lung microsomes of normal rat	Kidney microsomes of normal rat
Metyrapone	10^{-5}	90–99	91–102
	10^{-4}	25–33	78–90
7,8-Benzoflavone	10^{-5}	96–103	100–108
	10^{-4}	85–96	72–86
Tetrahydrofurane	10^{-2}	106–121	87–98
	10^{-1}	210–282	70–79
Progesterone	10^{-4}	57–78	340–430
	10^{-3}	45–57	255–310
Parconazole	10^{-5}	60–71	34–39
	10^{-4}	4–8	21–30
Ketoconazole	10^{-5}	92–99	52–70
	10^{-4}	102–152	28–39

See footnote to Table 3.

Table 5. Comparison of the effects of progesterone and tetrahydrofurane on liver, lung and kidney aldrin epoxidase activity under similar incubation conditions

Compounds	Concentration (M)	Aldrin epoxidase activity		
		Liver	Lung	Kidney
Tetrahydrofurane	10^{-2}	100 ± 8	97 ± 12	99 ± 10
	10^{-1}	102 ± 10	224 ± 28	83 ± 11
Progesterone	10^{-6}	88 ± 7	82 ± 9	106 ± 11
	10^{-5}	61 ± 8	78 ± 4	265 ± 27
	10^{-4}	49 ± 6	70 ± 7	325 ± 38

The effects of various compounds on aldrin epoxidase activity were determined under similar conditions (incubation time: 15 min; aldrin concentration: 11 μM) on liver, lung and kidney $S_{10,000g}$ (200 μl/ml) from control rats. Dilution of the $S_{10,000g}$ in the medium was as follows: lung 1/20; kidney 1/10; liver 1/1000. Results represent mean ± S.D. from five different assays.

vitro also seem to indicate that different monooxygenases are involved in aldrin oxidation in the liver. The inhibition patterns vary both quantitatively and qualitatively as a function of the origin of the enzymatic preparation; for example, the PB-induced enzyme as compared to the control sample. One could therefore postulate that PB induces the synthesis of cytochrome P-450 species that are different from those present in untreated animals.

The high sensitivity of the gas chromatography assay allowed us to measure the enzyme activity in extrahepatic tissues. After the liver, the most active organ is the lung followed by the kidney. This observation is similar to that on the tissue distribution of two other cytochrome P-450-dependent monooxygenases, aminopyrine-*N*-demethylase and benzphetamine-*N*-demethylase [21]. Although these enzymatic activities could only be accurately measured in the liver, they were also detected in the lung and the intestine [21].

The fact that the lung and kidney aldrin expoxidases are not affected by PB and MC indicates that if cytochrome P-450-dependent enzymes are present in extrahepatic tissues, they are not regulated by the same mechanisms. It is also possible that the extrahepatic aldrin epoxidase is supported by haemoproteins other than those in the liver, and is controlled differently.

Moreover, these liver, lung and kidney monooxygenases react differently with respect to the effect of progesterone and tetrahydrofurane. This latter chemical, which has no effect on liver or kidney epoxidase activities, activates the lung enzyme remarkably well. The concentration required (10^{-1} M) to obtain this activating effect implies that the effect may be non-specific with respect to the lung microsomal membranes. Nevertheless, the tissue specificity of this effect implies that lung aldrin epoxidase is different. The enzymatic specificity has been well established because the behaviour of aldrin epoxidase is different from that of aryl hydrocarbon hydroxylase, which is not activated by tetrahydrofurane regardless of the tissue investigated (data not shown).

The action of progesterone is even more surprising. This steroid, which is normally metabolized by cytochrome P-450, should behave as a competitive inhibitor with respect to the other substrates which are metabolized by this type of cytochrome. Progesterone is normally an inhibitor of aldrin epoxidase in the liver and lung. Progesterone strongly activates this enzyme in the kidney, and hence its behaviour is quite unexpected and inexplicable. This action of progesterone is quite specific with respect to the tissue investigated and the enzyme measured. Aryl hydrocarbon hydroxylase, which can be measured in the kidney, is slightly inhibited by progesterone (data not shown).

The existence of various agents which activate microsomal monooxygenases *in vitro* has been demonstrated [22, 23]. These agents include a wide range of chemicals such as betamethasone, 7,8-benzoflavone, ethyl isocyanide, metyrapone, acetone, aniline, volatile anaesthetics or polyamines. Their effects are often slight and have been attributed to action either on the electron transfer chain by facilitating electron flow, or on the cytochrome P-450s by removing endogenous inhibitors or competitive substrates [23].

In the case of aldrin epoxidase, the activating effects of progesterone and tetrahydrofurane are much more pronounced and are quite tissue- and enzyme-specific. Therefore, it is possible that these activators do not act on the electron-transfer chain, but rather on the cytochrome P-450s. Thus, our results indicate that aldrin epoxidase is catalysed by several forms of cytochrome P-450 in the liver because the inhibition pattern observed for various products varies after induction with phenobarbital. In the kidney and lung, only one form of cytochrome P-450 may be linked to aldrin epoxidase; nevertheless, these forms may not be identical and may also be different from those found in the liver.

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