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Prostaglandin synthase dependent aldrin epoxidation in hepatic and extrahepatic tissues of rats

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The oxidation of lipophilic chemicals is an important step in the formation of toxic, mutagenic and carcinogenic metabolites. Often the formation of such metabolites can be attributed to cytochrome P-450 dependent monooxygenases (MO). However, many organs and tissues possess low cytochrome P-450 content [1, 2]. In these cases an alternative oxidative pathway mediated by prostaglandin endoperoxide synthase (PES) might be more important. PES consists of a cyclooxygenase which catalyzes the bisdioxygenation of arachidonic acid to prostaglandin G_2 (PGG_2). In a second step a reduction by a hydroperoxidase to prostaglandin H_2 (PGH_2) takes place. In *in vitro* studies a number of xenobiotics served as reducing cofactors for the hydroperoxidase activity, being co-oxidized in this process [3].

In the present investigation we compared the oxidation of aldrin to dieldrin in extrahepatic and hepatic tissues. Aldrin was found to be a suitable substrate since it is converted to dieldrin, a stable non-reactive metabolite which can be analyzed by gas chromatography [4]. We tried to identify the pathway by which aldrin is metabolized in liver, lung, seminal vesicles and in a subcutaneous granulation tissue. In liver high monooxygenase and low PES activities have been reported [5]. The opposite proportion of activities was found in seminal vesicle microsomes [3]. The latter were therefore used in our study as a reference for PES-dependent activation of xenobiotics. In lung both activities are present, but they are distributed in different cell types [6]. The subcutaneous granulation tissue was included into this study because it serves as a convenient target tissue for the demonstration of several genetic endpoints, including gene mutations, after exposure of rats to the test compounds [7].

The MO and PES catalyzed aldrin epoxidations were distinguished by the addition of NADPH, a cofactor of cytochrome P-450 dependent monooxygenases, indomethacin, a specific inhibitor of cyclooxygenase, and arachidonic acid, the precursor of prostaglandins.

Materials and methods

Aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-5,8-exo-dimethanonaphthalene) and dieldrin (1,2,3,4,10,10-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octa-hydro-1,4-endo-5,8-exo-dimethanonaphthalene), purity > 99%, were from Riedel-de-Haen, Seelze, F.R.G. NADPH, arachidonic acid and indomethacin were purchased from Sigma Chemical Co., St. Louis, MO. *n*-Hexane (zur Rückstandsanalyse) was from E. Merck, Darmstadt, F.R.G.

Animals used were random bred Sprague–Dawley male rats (210–250 g, SIV 50 Ivanovas Kissleg, F.R.G.) kept under standard conditions (12 hr light–dark cycle, diet No 890 from NAFAG, Gossau, Switzerland).

To recover liver, lung, seminal vesicle and granulation tissue microsomes tissues were removed and rinsed in phosphate buffer (PBS) solution, chopped and homogenized with a glass/Teflon potter homogenizer (4% w/v) in cold 0.15 M KCl (pH 7.4). The homogenates were centrifuged at 9000 *g* for 20 min, and the resulting pellets were discarded. The supernatants were centrifuged at 100,000 *g* for 60 min to obtain the microsomal pellet. The microsomal pellet was suspended in 0.15 M KCl (pH 7.4) to a protein concentration of 10 mg/ml and stored at −70°. The protein determination was performed according to Bradford *et al.* [8].

For recovering intact granuloma cells the granulation tissue was initiated by the injection of 25 ml germ free air into the subcutaneous scapular area of rats. After 4 days the growing tissue was dissected out and dissociated enzymatically into single cells with collagenase-dispase according to standardized protocols described elsewhere [7].

Hepatocytes were recovered by liver perfusion according to the method of Berry and Friend [9] with slight modifications [10]. Analysis was performed within 4 hours after hepatocyte isolation.

The aldrin epoxidation assay was that described by Wolff [4]. The analysis was further improved by the application of glass capillary gas chromatography. The incubation contained 0.1–0.7 mg microsomal protein in 0.15 M KCl at 37°. NADPH (2 mM), 10 μM arachidonic acid and/or 10 μM indomethacin were added, followed 2 min later by the addition of 50 μM aldrin (final concentration). The total volume was 1 ml. After 10 min the reaction was stopped by the addition of 5 ml ice-cold *n*-hexane. Aldrin and dieldrin were extracted by vigorous shaking of the hexane–buffer mixture. After freezing the aqueous phase the hexane extract was decanted into stoppered glass tubes of 10 ml capacity and stored at −20° until analysis. Without further cleaning 1 μl aliquots of the hexane extract were injected on column into the capillary gas chromatograph.

2×10^6 granuloma cells in 990 μl serum-free Dulbecco's modified Eagle medium or 2×10^5 hepatocytes in 990 ml serum-free Williams medium E were incubated in glass vials (Packard Instruments, Downers Grove, IL) at 37° in a shaking water bath. Five μl ethanol, 5 μl indomethacin (10 μM final concentration) or 5 μl arachidonic acid (10 μM final concentration) were added 10 min prior to adding 5 μl aldrin (50 μM final concentration). After an incubation of 5 min with hepatocytes and 30 min with granuloma cells the reaction was stopped by the addition of 5 ml ice-cold *n*-hexane and dieldrin extracted as described above.

Quantitative analysis of dieldrin was carried out with a Carlo Erba capillary gas chromatograph (model fractovap 4160) equipped with a ^{63}Ni-electron-capture detector HT-25 and a temperature programmer LT Mod. 430. The temperature program was 60° for 1 min after injection, then 50°/min up to 185° and 10°/min up to 240°. The compounds in the hexane extract were separated on a glass capillary column (19 m × 0.32 mm) coated with SE-54 as the stationary phase (film thickness 0.15 μM). The carrier gas was hydrogen (0.2 kg/cm^2, linear velocity 0.5 m/s) and the make up gas nitrogen (1.2 kg/cm^2). The temperature of the detector was 275°. The dieldrin content was determined according to the peak heights calibrated with known amounts of dieldrin and by peak analysis with a Hewlett-Packard Integrator Model 3390 A using external dieldrin standards.

Results

Aldrin epoxidation activities of microsome preparations from liver, lung, seminal vesicle and granulation tissue with and without addition of NADPH, indomethacin and arachidonic acid are summarized in Table 1. In liver microsomes, dieldrin was efficiently formed in the presence of NADPH, whereas arachidonic acid had no effect on the aldrin epoxidation activity. In the lung NADPH and arachidonic acid both stimulated the aldrin epoxidation. The arachidonic acid induced activity was inhibited with indomethacin by 70%. In seminal vesicle and granulation tissue no NADPH dependent activity was found but arachidonic

Table 1. Stimulation and inhibition of aldrin epoxidation in microsomes of liver, lung, seminal vesicle and granulation tissue by indomethacin (IM), arachidonic acid (AA) and NADPH

	pmol dieldrin / mg micros. protein × min
Liver	n.d.
+ NADPH	2300 ± 500
+ AA	n.d.
+ AA + IM	n.d.
Lung	n.d.
+ NADPH	35.0 (28.0, 42.0)
+ AA	1.2 (1.0, 1.4)
+ AA + IM	0.4 (0.3, 0.5)
Seminal vesicles	n.d.
+ NADPH	n.d.
+ AA	10.5 ± 2
+ AA + IM	1.4 ± 1.4
Granulation tissue	n.d.
+ NADPH	n.d.
+ AA	2.2 (1.6, 2.8)
+ AA + IM	0.7 (0.4, 1.0)

n.d.: assayed but not detectable.

2 mM NADPH, 100 μM arachidonic acid (AA), 10 μM indomethacin (IM). Mean value ± SEM when N = 3, or mean value of two experiments.

acid stimulated the aldrin epoxidation. Indomethacin inhibited this activity significantly, by about 90% in seminal vesicle and 70% in granulation tissue microsomes.

In Table 2 the aldrin epoxidation activity and the effects of arachidonic acid or indomethacin in freshly isolated granuloma cells and hepatocytes is summarized. The arachidonic acid and indomethacin concentrations used had no effects on the viability of the cells (as measured by trypan blue exclusion). In hepatocytes the two chemicals had no effect, whereas arachidonic acid stimulated and indomethacin significantly inhibited the aldrin epoxidation in granuloma cells.

Discussion

The conversion of aldrin to dieldrin by microsomes and cells can be quantified in extrahepatic tissue using the improved glass capillary gas chromatography method. The limit of detection was 1 pg of dieldrin. The results presented in Table 1 demonstrate that aldrin is metabolized by microsome preparations from all four tissues but in different proportions by MO and PES dependent pathways. In the liver, aldrin is epoxidized by NADPH-dependent mixed function oxidases only. In lung the MO-dependent aldrin epoxidation is approximately 1.5% of that of liver.

Table 2. Stimulation and inhibition of aldrin epoxidation in freshly isolated hepatocytes and granulation tissue cells

	(N)	pmol dieldrin / 10^6 cells × min
Freshly isolated granulation tissue cells	7	0.07 ± 0.03
+ AA	4	0.35 ± 0.22*
+ IM	4	0.01 ± 0.01†
Freshly isolated hepatocytes	3	890 ± 100
+ AA	2	900
+ IM	2	940

10 μM arachidonic acid (AA), 10 μM indomethacin (IM).

Mean value ± SEM. *$P < 0.005$, †$P < 0.001$. Student's *t*-test.

Arachidonic acid and indomethacin modulated the aldrin epoxidation in lung, seminal vesicle and granulation tissue microsomes. This strongly suggests that aldrin might serve as a cofactor of the prostaglandin hydroperoxidase. As expected, seminal vesicle microsomes contain the highest PES dependent activity. Therefore, this preparation is suitable as a reference system for cooxidation-dependent aldrin epoxidation. In lung microsomes the PES-dependent activity was 11.5% and in granulation tissue 21% of that of the seminal vesicle microsomes.

From these findings it is concluded that aldrin is not a specific substrate for MO-dependent oxidases. Therefore, it can be used as a model compound to determine the epoxidation of olefins with the possibility of separating the MO and PES dependent activities. Comparing the contribution of the two oxidative systems in the four organs chosen, measurable activities in both systems were found only in the lung. The ratio of PES to MO dependent activities was 1:30. This is explained by the heterogeneity of cell types in this tissue. Clara cells have a high activity of cytochrome P-450 dependent monooxygenases and alveolar type II cells are enriched in prostaglandin synthase activities [6].

In the experiments with freshly isolated hepatocytes and granuloma cells, the cofactors necessary for the enzyme reactions are provided by the cells themselves. When the calculation was based on the number of cells, it was found that hepatocytes metabolized aldrin with a 10,000-fold higher activity than granuloma cells. Taking protein content as a basis of calculation (10^6 hepatocytes = 1300 μg protein: 10^6 granulation tissue cells = 160 μg proteins) the metabolizing capacity of hepatocytes was still 1000-fold higher than that of granuloma cells.

As expected from results obtained with microsomes, the epoxidation occurred in hepatocytes exclusively by MO and in granulation tissue cells by PES dependent pathways. The importance of PES dependent epoxidations of xenobiotics has already been demonstrated in mutagenicity tests *in vivo* using the "granuloma pouch assay". In this system in which the test compounds are injected directly into a subcutaneous air pouch lined with granuloma cells, benzo(*a*)pyrene [11] and aflatoxin B_1 [12] induced gene mutation at the 6-thioguanine locus [7]. Most likely the low metabolic PES dependent activity found in granulation tissue was sufficient to form reactive mutagenic species *in vivo* in individual cells. Both chemicals, 7,8-dihydro-7,8-dihydrobenzo(*a*)pyrene [3, 13] and aflatoxin B_1 [14, 15] were reported to be epoxidized by PES dependent pathways *in vitro*.

In summary the aldrin epoxidation was completely NADPH-dependent in liver microsomes and hepatocytes. In lung microsomes two pathways are involved. The NADPH-dependent activity was 1.5% and the arachidonic acid dependent aldrin epoxidation 0.3% of the activity found in liver. In seminal vesicle microsomes, in granulation tissue microsomes and cells, aldrin epoxidation was stimulated by arachidonic acid and inhibited by indomethacin. These results suggest that aldrin was epoxidized by a prostaglandin synthase mediated pathway as an alternative enzyme to the cytochrome P-450 dependent monooxygenases in extrahepatic tissues.

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Effect of 9-alkyl derivatives of 6-methylthioguanine on brain specific binding of [^{3}H]diazepam

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Both biochemical and pharmacological evidence support the hypothesis that [^{3}H]benzodiazepine binding to high affinity central benzodiazepine receptors may mediate some of the action of benzodiazepines [1]. Since various findings have suggested purines as putative endogenous ligands for benzodiazepine receptors [2, 3], we have studied the effect of synthetic purines and purine nucleosides on [^{3}H]diazepam binding with rat brain membranes [4, 5]. Among these analogs, 6-*n*-pentyldithioguanine had the highest potency, with a K_i value of 0.92 μM. In the present study we found that some 9-alkyl derivatives of 6-methylthioguanine were capable of inhibiting high affinity brain specific binding of [^{3}H]diazepam in the nanomolar range.

Methods

Male Wistar rats (200–250 g) were obtained from the Animal Unit at the University of British Columbia. The animals were decapitated, brain tissues were homogenized in 20 vol. of ice-cold 0.32 M sucrose in a Teflon–glass homogenizer, and membranes were prepared as described previously [5]. Specific binding of [^{3}H]diazepam was assessed in an incubation volume of 0.2 ml containing 25 mM sodium phosphate buffer, pH 7.4, membrane protein (*ca.* 0.082 mg/assay) and 6.2 nM [*methyl*-^{3}H]diazepam (0.1 μCi/assay). Incubation with or without the addition of purine derivatives was carried out at 0° for 20 min and was terminated by rapid filtration under vacuum through glass-fiber filters (Whatman GF/B). These were washed three times with 5 ml of cold 0.05 M sodium phosphate buffer, pH 7.4, and then counted for radioactivity. Specific binding of [^{3}H]diazepam was defined as that displaced by 3 μM diazepam and represented about 97% of the total binding. Protein content was determined by the method of Lowry *et al.* [6].

IC_{50} Values were assessed graphically at five different concentrations of purine derivatives in duplicate and expressed as means. K_i values were calculated according to the formula: $K_i = IC_{50}/(1 + [L]/K_d)$. $[L]$ equals concentration of ligand.

[*Methyl*-^{3}H]diazepam (sp. act. 80.3 Ci/mmole) was purchased from New England Nuclear. Unlabeled diazepam was a gift from Hoffmann-La Roche Ltd., Vaudreuil, Quebec. Various 9-alkyl derivatives of 6-methylthioguanine were synthesized according to Noell and Robins [7].

Results and discussion

The abilities of various 9-alkyl derivatives of 6-methylthioguanine (structure shown in Fig. 1) to replace specifically bound [^{3}H]diazepam were tested by incubating the

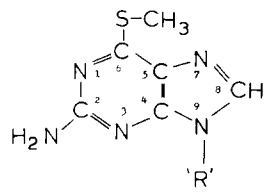

Fig. 1. Structure of 9-alkyl-6-methylthioguanine.

compounds in the standard binding mixture. The results presented in Table 1 show that 9-alkyl derivatives of 6-methylthioguanine were much more active than 6-methylthioguanine which showed a K_i value of 16 μM as an inhibitor of [^{3}H]diazepam binding [4]. The most potent compound, 9-*n*-pentyl-6-methylthioguanine, had an IC_{50} of 0.2 μM with a K_i of 0.082 μM. When the 6-methylthio group of 9-alkyl-6-methylthioguanine was replaced by a 6-thio

Table 1. Inhibition of [^{3}H]diazepam binding to rat cortical membranes by 6-methylthioguanine 9-alkyl derivatives

Compound	IC_{50} (μM)	K_i (μM)
9-Methyl-6-methylthioguanine	10	4.1
9-*n*-Propyl-6-methylthioguanine	0.25	0.10
9-*n*-Pentyl-6-methylthioguanine	0.2	0.082
9-*n*-Propyl-6-thioguanine	5	2.05
9-*n*-Pentyl-6-thioguanine	5	2.05

Data represent the mean values for four to six separate experiments with standard deviations of less than 10%. K_i values were calculated according to the formula: $K_i = IC_{50}/(1 + [L]/K_d)$.