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Effect of Pyridoxamine on Semicarbazide-sensitive Amine Oxidase Activity of Rabbit Lung and Heart

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Abstract—Rabbit lung and heart show clorgyline-resistant benzylamine oxidase activity which is sensitive to semicarbazide (SSAO) and α-amino-guanidine. This SSAO activity is inhibited by pyridoxamine with an IC_{50} of $6{\cdot}3 \times {}^{-6}$ M for lung and of $1{\cdot}1 \times 10^{-5}$ M for heart, the inhibition being non-competitive and only partially reversed by dialysis at 4°C. Semicarbazide, α-aminoguanidine and pyridoxamine show a similar time-dependent type of inhibition of rabbit lung and heart SSAO.

Roth & Gillis (1975) reported that intact perfused rabbit lung contains three forms of amine oxidases, two of which are analogues of the A and B forms of mitochondrial monoamine oxidase (MAO), the third one being similar to the enzyme found in plasma. This third type is resistant to the irreversible MAO inhibitor clorgyline but sensitive to the carbonyl reagent semicarbazide, and while it was previously referred to as "clorgyline-resistant amine oxidase", it is now generally known as "semicarbazide-sensitive amine oxidase" (SSAO). A similar enzyme was found in rat lung (Andree & Clarke 1982; Danh et al 1984).

It is now recognized that the lung is a site for the uptake, accumulation and/or metabolism of numerous endogenous and exogenous or xenobiotic chemicals (Bend et al 1985); the presence there of SSAO, which is able oxidatively to deaminate many amines, may thus be of considerable physiological importance. No diamine oxidase (DAO) activity was detected in rat or rabbit lung by Rao et al (1986), whereas a high activity of this kind was found in the lungs of patients with small-cell carcinoma of the lung (Baylin et al 1975).

Our aim in this paper was to confirm the presence of semicarbazide-sensitive benzylamine oxidase in the rabbit lung, to describe this enzyme in the heart and to study the inhibition of both enzymes by pyridoxamine, which is an inhibitor of the copper-containing amine oxidases like rabbit plasma benzylamine oxidase (Buffoni et al 1985).

Materials and Methods

Preparation of tissues

Rabbits were killed by cervical dislocation. The lung and heart were rinsed thoroughly in saline (0·9% NaCl w/v), blotted dry and weighed. The tissues were thoroughly minced and homogenized in 0·25 M sucrose containing 0·01 M Na-K phosphate buffer at pH 7·4 by the use of a Teflon glass homogenizer with a tissue (g): buffer (mL) ratio of 1:10. Crude homogenates were centrifuged at 600 *g* for 15 min and the decanted supernatants were centrifuged again at 12 000 *g* for 20 min to remove mitochondria.

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Enzyme assay

Determination of the SSAO activity. Activities were determined radiochemically by the method of McCaman et al (1965) using benzylamine as substrate. Unless stated otherwise, 50 μL of clorgyline 6×10^{-3} M was preincubated with 100 μL of homogenate, 50 μL of catalase (21 U mL^{-1}) and 50 μL of Na-phosphate buffer M/15 at pH 7·4 for 30 min at 37°C in air before the addition of 50 μL of [^{14}C] benzylamine (1 mM) (total volume 0·3 mL). Deamination products were extracted with 1 mL of ethyl acetate after the addition of 100 μL of 3M hydrochloric acid. 500 μL of the ethyl acetate extract was taken for liquid scintillation counting in Instagel (15 mL). Quench correction was obtained by channel ratio.

Determination of the diamine oxidase activity. The diamine oxidase activity was determined by the method of Lehmann et al (1974). The assay conditions were: 100 μL of *o*-dianisidine solution (2·5 mg mL^{-1}), 100 μL of peroxidase (2 U mL^{-1}), 100 μL of sample, 100 μL of substrate solution either 10 mM putrescine or 10 mM histamine and Na-phosphate buffer M/15 at pH 7·4 to the final volume of 0·9 mL. The reaction was carried out at 37° C in air generally for 120 min and stopped with 2 mL of 4·5 M sulphuric acid.

Protein determination

Protein content was obtained by the method of Lowry et al (1951).

Kinetic analysis

The effect of pyridoxamine, semicarbazide and α-aminoguanidine on the deamination of [^{14}C]benzylamine was studied in the 12 000 *g* supernatant of rabbit lung and heart homogenates in which MAO activity was inactivated by prior incubation (30 min) with 10^{-3}M clorgyline (final concn). The concentrations of benzylamine used were: 1, 5, 10, 16·6, 42, 166 μM. Reaction rates were followed for 10 min. Linearity of the reaction with time and protein concentrations was ensured in all assays. Apparent K_m and V_{max} values were calculated by computer program according to Wilkinson (1961).

Materials

[^{14}C]Benzylamine hydrochloride was purchased from ICN Pharmaceuticals Inc. (Irvine, California, USA), ethyl-l-

[^{14}C]phenylethylamine hydrochloride was from NEN (Boston, Mass. USA).

Benzylamine hydrochloride and bovine plasma albumin were purchased from Sigma (St. Louis, MO, USA), semicarbazide hydrochloride and 3·3′-dimethoxybenzidine dihydrochloride (dianisidine) were from Merck (Darmstadt, W. Germany). Horseradish peroxidase and catalase were obtained from Boehringer (Mannheim, W. Germany). The other reagents were standard laboratory reagents of analytical grade. Male rabbits (New-Zealand (2–3 kg)) were obtained from the S. Morini breeding colony (S. Polo d'Enza, Reggio E., Italy).

Results

SSAO

Owing to the complexity of factors influencing the selectivity of substrates for SSAO, MAO-A and MAO-B, our assay method required validation. According to the accepted criteria, SSAO are resistant to inhibition by clorgyline at 10^{-3}M, whereas MAO-A and MAO-B are completely inhibited by this concentration; our observations generally concurred with those findings.

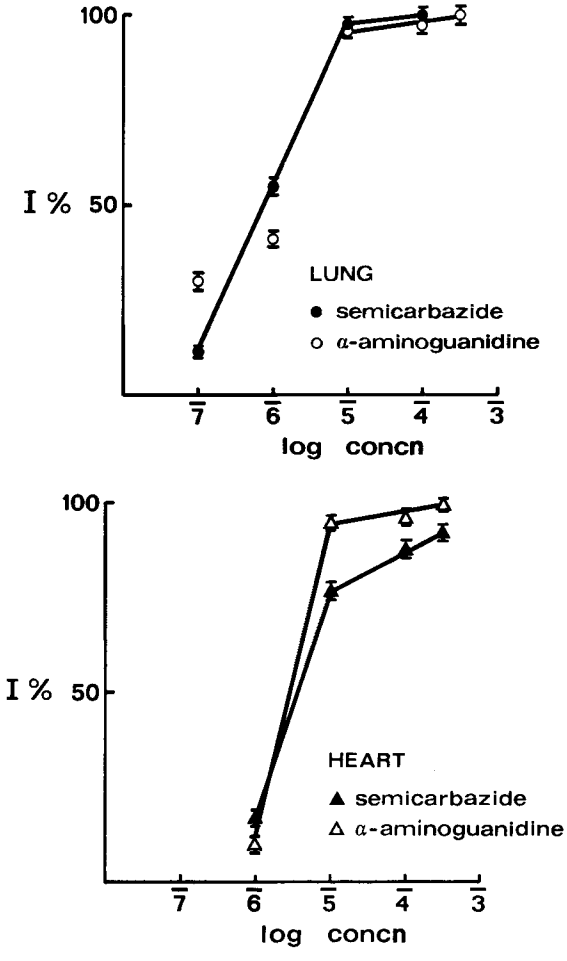

FIG. 1. Effect of α-aminoguanidine and semicarbazide on the SSAO activity of rabbit lung and heart (12000 *g* supernatant). Values are means ± s.e. (n = 4). The activity was assayed after 30 min of preincubation with 1 mM clorgyline both in the presence and in absence of semicarbazide or α-aminoguanidine. The activity in the lung in the presence of clorgyline alone was 0·27 ± 0·003 nmol mg^{-1} min^{-1} and in the heart 0·09 ± 0·003 nmol mg^{-1} min^{-1}.

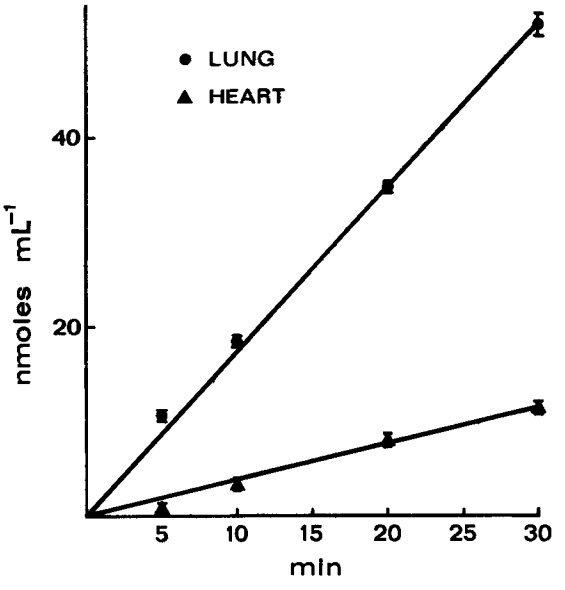

FIG. 2. Time dependence linearity of clorgyline resistant benzylamine oxidase activity (SSAO) of rabbit lung and heart (12000 *g* supernatant). The values are mean ± s.e. (n = 4). 1 mM clorgyline was preincubated for 30 min before the addition of 0·17 mM [^{14}C]benzylamine (final concentration). Lung protein = 6·5 mg mL^{-1}; heart protein = 3·74 mg mL^{-1}.

The clorgyline-resistant activity found in both lung and heart is inhibited by semicarbazide and α-aminoguanidine thus showing it to be a semicarbazide-sensitive oxidase (Fig. 1); this activity is linearly time-dependent up to 30 min (Fig. 2).

The effect of the two inhibitors is preincubation time-dependent (Fig. 3) and only partially reversed by dialysis (Table 1).

DAO

To show that the observed enzymic activity was at least partially independent of the presence of DAO activity, the effects of putrescine and histamine on the oxidation of [^{14}C]benzylamine by lung and heart homogenates in the presence of 1 mM clorgyline were studied (competition experiments). Neither substance inhibited the oxidation of [^{14}C]benzylamine in lung and heart 12000 *g* supernatants in the presence of 1 mM clorgyline at different concentrations between 3–330 μM. Only histamine at 10 mM inhibited the oxidation of benzylamine by 33% (lung enzymic activity: nmol mg^{-1} min^{-1} 0·33 ± 0·005 s.e. in the absence of histamine, 0·22 ± 0·005 in the presence of 10 mM histamine, n = 4, $P < 0·05$), and at this concentration is a substrate of SSAO (Table 2).

β-Phenylethylamine oxidation

β-Phenylethylamine was a good substrate of rabbit lung and heart SSAO, with a higher K_m and a lower V_{max} than benzylamine (Table 3). The observed differences in the K_m values are statistically significant ($P < 0·05$); those in V_{max} are not.

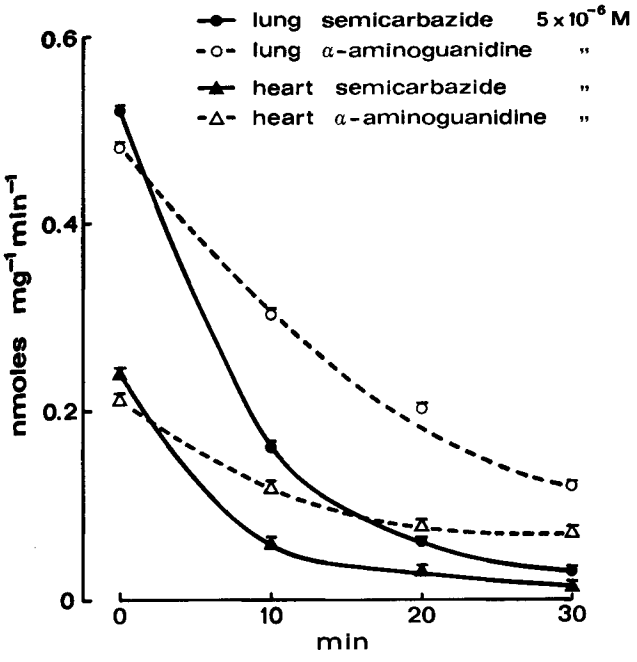

FIG. 3. The effect of preincubation time on inhibition of rabbit lung and heart SSAO (12000 *g* supernatant) by semicarbazide and α-aminoguanidine. Values are mean ± s.e. (n = 4). 1 mM clorgyline was preincubated for 30 min before the addition of 0·17 mM [^{14}C]benzylamine. The effects of α-aminoguanidine and semicarbazide were determined after various preincubation times of SSAO with these inhibitors.

Table 1. Effect of dialysis on inhibition of SSAO by semicarbazide and α-amino guanidine. Method: dialysis was carried out against M/15 Na phosphate buffer at pH 7·4 (10 times the solution volume) for 4 days × 4 changes at 4° C. (mean ± s.e. of 4 different experiments).

	% Inhibition before and after dialysis			
	Lung		Heart	
	Before	After	Before	After
Semicarbazide 6×10^{-5} M	100	90 ± 1	98·5 ± 0·5	88 ± 1
α-Aminoguanidine 6×10^{-5} M	96·5 ± 0·5	94 ± 1	91·5 ± 0·5	88 ± 1

Table 2. Oxidation of histamine, putrescine, β-phenylethylamine and benzylamine by rabbit lung (12000 *g* supernatant) in presence of 1 mM clorgyline. 1 mM clorgyline was preincubated for 30 min before addition of substrates. Values are means ± s.e. (n = 4).

Substrates	(mM)	Activity (nmol mg^{-1} min^{-1})
Histamine	10	0·05 ± 0·002
Histamine	1	0
Putrescine	10	0
Putrescine	1	0
Benzylamine	1	0·08 ± 0·003
β-Phenylethylamine	1	0·04 ± 0·004

See determination of the diamine oxidase activity in Methods. The peroxidase method was used for all substrates. This method gives lower values in comparison with the isotopic one also because it needs a longer incubation time with the substrate.

Table 3. Comparison between K_m and V_{max} of benzylamine and β-phenylethylamine obtained with rabbit lung and heart (12000 *g* supernatant) in presence of 1 mM clorgyline. Values are means ± s.e. of 6–8 experiments.

	Lung [K_m (μM)]	Heart [K_m (μM)]	Lung [V_{max} (nmol mg^{-1} min^{-1})]	Heart [V_{max} (nmol mg^{-1} min^{-1})]
Benzylamine	12·7 ± 4	10·3 ± 3	0·36 ± 0·03	0·15 ± 0·015
β-Phenylethylamine	39·8 ± 4	124·6 ± 9	0·26 ± 0·02	0·13 ± 0·012

Pyridoxamine as inhibitor of rabbit lung and heart SSAO

The inhibition of lung and heart SSAO by pyridoxamine was preincubation time-dependent (Fig. 4) like those of semicarbazide and α-aminoguanidine, and was only partially reversed by dialysis (Table 4) whereas the inhibition of the benzylamine oxidase activity of the plasma was reversed by dialysis.

The inhibition of SSAO by pyridoxamine is presented in Fig. 5. In these experiments pyridoxamine was preincubated with the enzyme for 30 min. The IC_{50} was $6·3 \times 10^{-6}$ M for lung and $1·1 \times 10^{-5}$ M for heart SSAO.

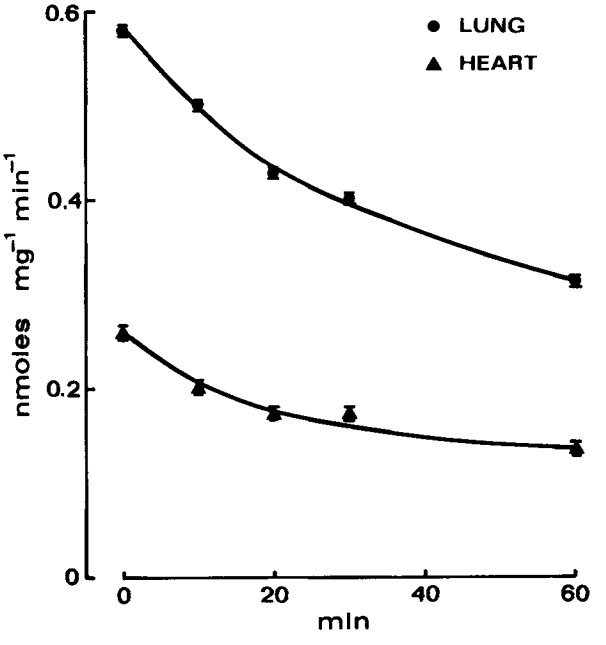

FIG. 4. Effect of preincubation time on inhibition of rabbit lung and heart SSAO (12000 *g* supernatant) by 10^{-5} M pyridoxamine. Values are mean ± s.e. (n = 4). 1 mM clorgyline was preincubated for 30 min before the addition of 0·17 mM [^{14}C]benzylamine. Pyridoxamine was preincubated for the time indicated in the Figure.

Table 4. Effect of dialysis on inhibition of SSAO and of rabbit plasma benzylamine oxidase (BzAO) by pyridoxamine. Method: dialysis was carried out against M/15 Na phosphate buffer at pH 7·4 (10 times the solution volume) for 4 days × 4 changes at 4°C.

	% Inhibition before and after dialysis	
	Before	After
Plasma (BzAO)	53	4
Lung	98	57
Heart	100	46

Table 5. Effect of pyridoxamine on apparent Michaelis-Menten constants of rabbit lung and heart SSAO (12000 *g* supernatant) in presence of 1 mM clorgyline. Pyridoxamine and clorgyline were preincubated for 30 min. All experiments were done with the same preparation of SSAO. This lung preparation is more active than the others, but has a higher K_m. Each value is mean ± s.e. of 4 experiments.

	Lung		Heart	
	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})	K_m (μM)	V_{max} (nmol mg^{-1} min^{-1})
Control	24·6 ± 1·2	0·81 ± 0·016	11·8 ± 0·72	0·30 ± 0·005
Pyridoxamine				
10^{-5} M	24·9 ± 1·7	0·62 ± 0·02	12·4 ± 0·6	0·22 ± 0·003
$1·7 \times 10^{-5}$ M	15·1 ± 1*	0·34 ± 0·017	—	—
$3·3 \times 10^{-6}$ M	—	—	12·3 ± 0·6	0·24 ± 0·008
$1·7 \times 10^{-6}$ M	—	—	12·9 ± 1·5	0·29 ± 0·019

* $P < 0·05$

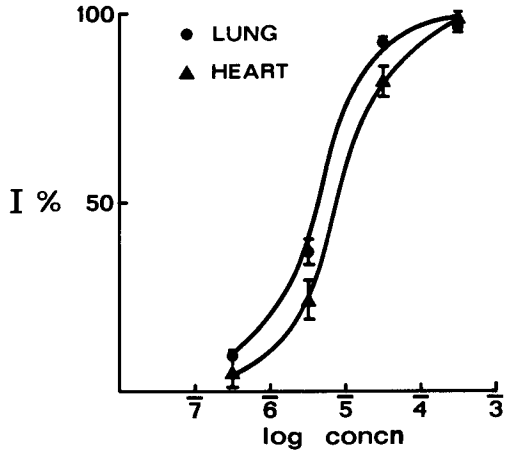

FIG. 5. Effect of pyridoxamine on SSAO activity of rabbit lung and heart (12000 *g* supernatant). Values are mean ± s.e. (n = 4). The activity was assayed after 30 min of preincubation with 1 mM clorgyline either in the presence or in the absence of pyridoxamine. The activity in the lung in the presence of clorgyline alone was 0·351 ± 0·006 and in the heart was 0·101 ± 0·005 nmol mg^{-1} min^{-1}.

Kinetics of pyridoxamine inhibition of rabbit lung and heart SSAO

The effect of pyridoxamine after 30 min of preincubation in the presence of 1 mM clorgyline was studied at six different benzylamine concentrations (1, 5, 10, 16·6, 42, 166 μM). Under these conditions the effect of pyridoxamine is mainly observed on the V_{max}, which is decreased, whereas the K_m remains within the limits of experimental error with the exception of lung SSAO where K_m is decreased by the $1·7 \times 10^{-5}$ M concentration. A similar observation has been reported (Buffoni et al 1985) for pig plasma benzylamine oxidase. The inhibition appears to be mainly of the non-competitive type (Table 5). It is interesting that the s.e. between the apparent SSAO kinetic constants of different preparations is about 20% of the mean, whereas with the same preparation it is lower.

Discussion

Rabbit lung and heart show a benzylamine oxidase activity which is resistant to 1 mM clorgyline and inhibited by semicarbazide and α-aminoguanidine, thus placing it in the class of semicarbazide-sensitive amine oxidase (SSAO). This enzymic activity also acts on β-phenylethylamine with a higher K_m and a lower V_{max} than benzylamine. Histamine at 10 mM is also observed to be partially oxidized by the enzyme, and in fact benzylamine oxidation in the lung is reduced by histamine at this concentration. No diamine oxidase activity was observed in the lung, and this is in agreement with the results of Rao et al (1986). The oxidation of histamine by SSAO is probably an alternative process taking place only at high concentrations.

We have confirmed here that pyridoxamine inhibits rabbit lung and heart SSAO as well as rabbit plasma benzylamine oxidase, as previously shown (Buffoni et al 1985). This inhibition strongly resembles those by α-aminoguanidine and semicarbazide, all three inhibitors needing a preincubation time and their inhibition being only partially reversed by dialysis at 4° C.

Kinetic analysis showed pyridoxamine to be a non-competitive inhibitor of rabbit lung and heart SSAO after 30 min of preincubation. It might be suggested that pyridoxamine acts as substrate for the enzyme followed by product-induced non-competitive inhibition. A small amount of H_2O_2 production was obtained by the peroxidase method with pure pig plasma benzylamine oxidase using pyridoxamine as substrate (data not shown). Semicarbazide is also a non-competitive inhibitor of tissue SSAO and has been tentatively classed as a suicide inhibitor in view of the fact that the initial competitive inhibition changes to a non-competitive one after 20 min incubation (Andree & Clarke 1982).

The sensitivity of SSAO to pyridoxamine, semicarbazide and α-aminoguanidine suggests that these enzymes belong to the same class of plasma benzylamine oxidase.

Acknowledgements

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