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Semicarbazide-sensitive Amine Oxidase from the Smooth Muscles of Dog Aorta and Trachea: Activation by the MAO-A Inhibitor Clorgyline

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Abstract—Semicarbazide-sensitive amine oxidase (SSAO) has been identified in the dog trachea and aorta smooth muscles. The dog SSAO is blocked by hydrazine inhibitors. SSAOs from several different vascular smooth muscle sources, such as the rat and bovine aorta, and human umbilical artery, as well as the bovine plasma, are insensitive to the MAO-A inhibitor clorgyline; the dog SSAO on the other hand is significantly activated by clorgyline. Two methods, i.e. radioenzymatic and fluoroemetric methods, have been applied to substantiate this clorgyline-induced activation. The activation was detected with respect to the deamination of different substrates, such as benzylamine, β-phenylethylamine and longer carbon chain aliphatic amines, but not with respect to methylamine. The clorgyline effect is reversible, non-competitive and time-independent; it depends on electrostatic and hydrophobic interactions between clorgyline and hydrophobic regions of the dog SSAO enzyme.

Semicarbazide-sensitive amine oxidase (SSAO, EC 1.4.3.6) is an enzyme or group of enzymes residing predominantly in the plasma membrane of vascular smooth muscle cells, such as in blood vessels and heart tissue (Wibo et al 1980; Lewinsohn 1981; Lyles & Singh 1985). Although SSAO is able to deaminate several of the same monoamines as does monoamine oxidase (MAO), it is distinctly different from MAO. MAOs (MAO-A and MAO-B) are well known to be flavine enzymes located on the outer membrane of mitochondria in almost all mammalian tissues (Yu 1986). SSAO has been previously considered to be a pyridoxal (PLP)- (Buffoni 1980) or pyrroloquinoline quinone (PQQ) (Lobenstein-Verbeek et al 1984) dependent copper enzyme. Recent work, however, has shown that 6-hydroxydopa is probably the correct prosthetic group (Jane et al 1990). SSAO has little in common with diamine oxidase, because it does not catalyse the deamination of diamines (Yu 1990). SSAO is insensitive to selective MAO inhibitors, such as clorgyline (Clarke et al 1982), and so this inhibitor has usually been applied to inactivate MAO activity in studies of SSAO.

SSAO oxidizes biogenic amines, such as β-phenylethylamine, *p*-tyramine and dopamine, but seems to be relatively inactive towards 5-HT and β-hydroxylated catecholamines, such as noradrenaline (Elliott et al 1989a). Aliphatic amines are readily deaminated by both rat mitochondrial MAO (Yu 1989) and aorta SSAO (Yu 1990).

Recently it has been shown that SSAO in the isolated perfused mesenteric artery can be related to the tyramine pressor effect (Elliott et al 1989b) and SSAO is at least partly involved in the deamination of circulating biogenic amines. Methylamine has been identified as an endogenous substrate of SSAO and its urinary excretion is increased in rats treated with SSAO inhibitors (Lyles & McDougall 1989). The high SSAO activity found in cardiovascular tissues suggests that this enzyme might play an important role in these tissues. Allylamine, an industrial chemical, is a relatively specific cardiovascular toxin which causes extensive and progressive vascular and myocardial lesions in several mammalian species (Boor & Hysmith 1987); it is now known that allylamine can be metabolized to acrolein by vascular SSAO (Nelson & Boor 1982). Acrolein acts as a distal toxin responsible for the cellular damage caused by allylamine intoxication. The SSAO inhibitor semicarbazide (but not specific MAO inhibitors) provides protection against the progression of damage caused by allylamine (Boor & Nelson 1980).

Regarding the distribution of SSAO in different species the literature presents diverse results with activities being reported in the rat bone (Andree & Clarke 1981), interscapular brown adipose tissue (Barrand et al 1984) small intestine (Suzuki & Matsumoto 1987) and cartilage (Lyles & Bertie 1987) as well as in human uterus (Ryder et al 1979). Human aorta SSAO is known to possess different kinetic parameters (Suzuki & Matsumoto 1984; Precious & Lyles 1988) than that of the rat aorta SSAO (Precious et al 1988). In this study, we report the identification of SSAO in dog vascular smooth muscle tissues and its activation by the MAO-A inhibitor clorgyline.

Materials and Methods

Materials

Tracheal and aorta smooth muscles were collected from native Taiwanese Tugo and from Canadian Huskie dogs; aorta and veins were obtained from bovine (International Packers, Saskatoon); Wistar male rats were also used as a source of aorta and tracheal tissues. Benzylamine, β-phenylethylamine, *n*-heptylamine, methylamine, β-octylglucoside, homovanillic acid, horse-radish peroxidase, semicarbazide and pargyline were purchased from Sigma (St Louis, MO, USA); [7-^{14}C]benzylamine was obtained from Amersham

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(Oakville, Ontario, Canada). Clorgyline [*N*-(2,4-dichlorophenoxy-*n*-propyl)-*N*-methylpropargylamine HCl] was obtained from May and Baker Ltd (Dagenham, UK) and deprenyl [phenylisopropyl-methylpropargylamine HCl] was a gift from Professor J. Knoll (Budapest, Hungary). All other chemicals were of analytical grade.

Preparation of enzymes

Tissues (dog tracheal smooth muscle and aorta from dog, rat and sheep) were dissected, sliced into small pieces and homogenized with a Polytron homogenizer (PT-10-35, setting 5 for four periods of 5 s on ice) in chilled 0·01 M phosphate buffer (pH 6·8). The crude homogenates were centrifuged at 800 *g* for 10 min and the supernatants further centrifuged at 32 000 *g* for 30 min. The supernatant fractions were used for the enzyme analyses. In some later studies, partially purified SSAOs from the dog trachea were used. The supernatant fractions as described above were further precipitated with ammonium sulphate (60% saturation). The precipitates were resuspended in the homogenizing buffer, dialysed against the same buffer and stored at −20°C until used. The enzyme was stable under these conditions for at least 3 months.

Determination of SSAO activities

Two methods, radioenzymatic and fluorometric, were used for the estimation of SSAO activities. The radioenzymatic procedure using [^{14}C]benzylamine as substrate followed the procedure previously described for monoamine oxidase (Yu 1986). The enzyme preparations were incubated at 37°C for 30 min in the presence of benzylamine (5×10^{-5} M, 0·2 μCi) in a final volume of 200 μL 2 M citric acid. The oxidized products were extracted into 1 mL toluene:ethyl acetate (1:1, v/v), of which 600 μL was then transferred to a counting vial containing 10 mL Omnifluor (New England Nuclear, Boston, USA). Radioactivity was assessed by liquid scintillation spectrometry (Beckman LS-7500).

The fluorometric method was based on the formation of an intense fluorphore formed from homovanillic acid and the hydrogen peroxide released during the oxidation of the amines (Yu 1990). The crude SSAO preparations were incubated at 37°C for 10 min in the presence of benzylamine in a total volume of 200 μL of 0·05 M phosphate buffer (pH 7·5) containing 50 μg homovanillic acid and 0·82 units of horseradish peroxidase. The developed fluorescence intensity was measured in a spectrofluorometer (Aminco-Bowman) at an excitation wavelength of 315 nm and emission wavelength of 425 nm. The enzyme reactions were linear both with respect to time (for at least 15 min) and the amount of enzyme used in both measurement procedures.

Protein concentrations were determined by the Bradford method (Bradford 1976) with bovine serum albumin as standard. Kinetic parameters were analysed according to Wilkinson (1961).

Results

As can be seen from Fig. 1A using [^{14}C]benzylamine as substrate, the dog aorta SSAO in the supernatant fractions (32 000 *g*, 30 min) of the crude homogenates were inhibited by semicarbazide with an EC50 of 10^{4} M. Clorgyline, on the other hand, enhanced the enzyme activity by up to 250%

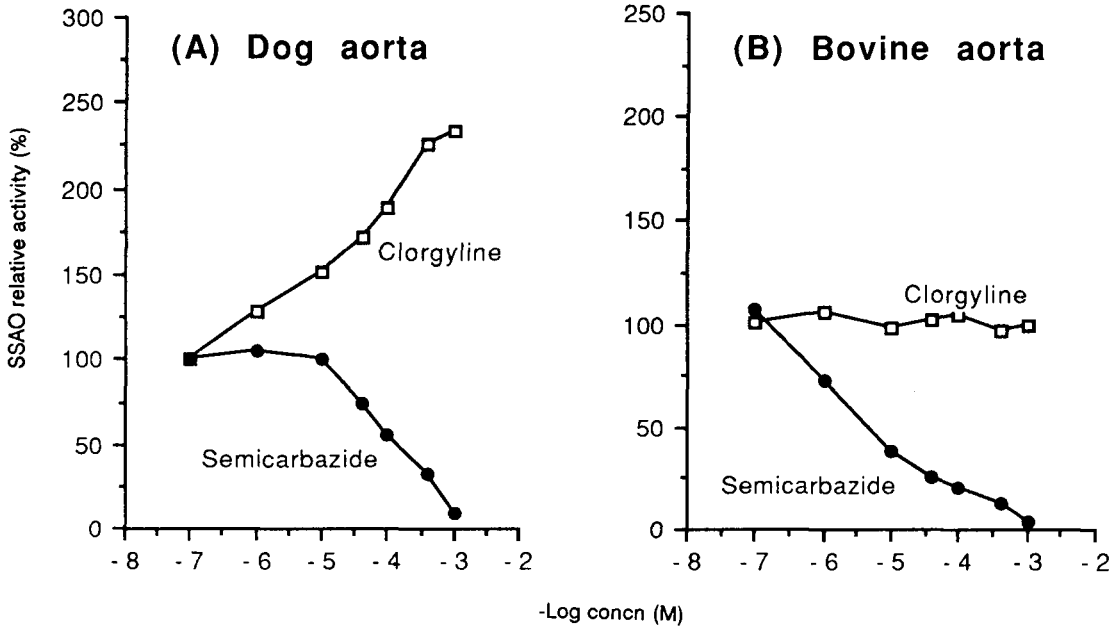

FIG. 1. Inhibition or activation by clorgyline and semicarbazide of the deamination of benzylamine by dog (A) and bovine (B) aorta SSAO. The enzymes were preincubated with clorgyline (□) and semicarbazide (●) for 10 min at ambient room temperature (21°C) and the enzyme reactions initiated by adding [^{14}C]benzylamine (5×10^{-4} M). After incubation at 37°C for 30 min the radioactively labelled products were extracted and assessed. Activities are expressed as percentages of corresponding experiments conducted in the absence of inhibitor. Each point is the mean of two experiments using different enzyme preparations.

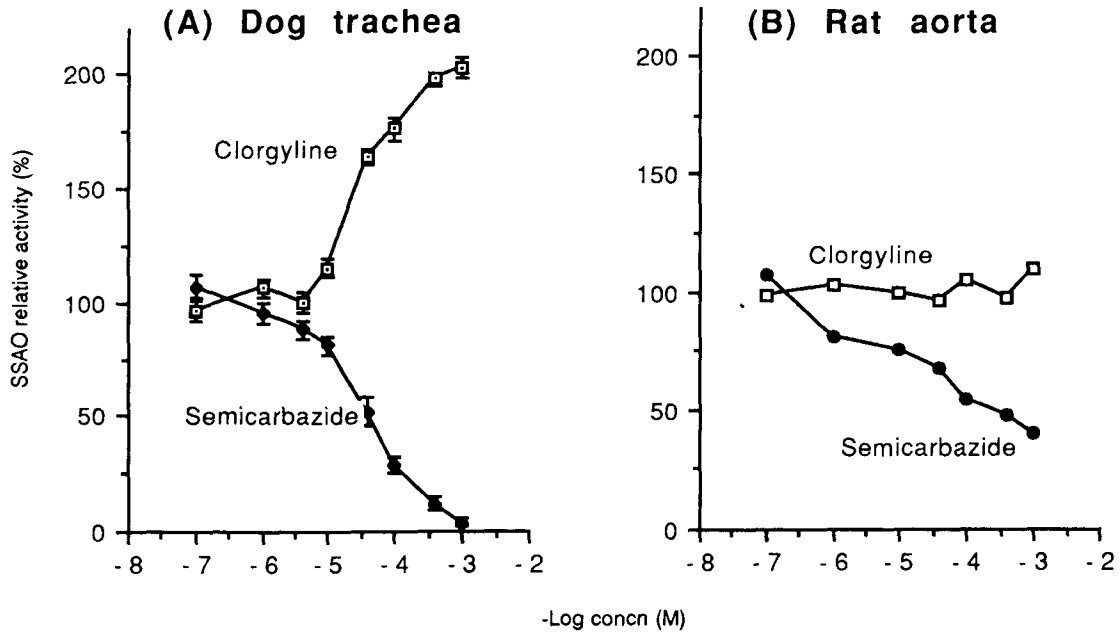

FIG. 2. Inhibition or activation by clorgyline and semicarbazide of the deamination of benzylamine by dog (A) and rat (B) trachea SSAO. The enzymes were preincubated with clorgyline (□) and semicarbazide (●) for 10 min at ambient room temperature (21°C) and the enzyme reactions initiated by adding benzylamine (5×10^{-4} M) followed by further incubation at 37°C for 20 min. The production of hydrogen peroxide was assessed by a fluorometric method. Activities are expressed as percentages of corresponding experiments conducted in the absence of inhibitor. Each point is the mean of two experiments using different enzyme preparations.

of the control at a concentration of 10^{-3} M. Clorgyline was also found to activate partially purified dog aorta and tracheal SSAOs to a similar extent to that of the crude dog enzyme (results not shown). SSAO obtained from the bovine aorta (Fig. 1B), however, was not affected by clorgyline up to 10^{-3} M. Such activation was also not detected in smooth muscle SSAO preparations isolated from rat trachea and aorta, neither was it seen for bovine vein nor human umbilical arteries using the same analytical procedure (results not shown).

Using the fluorometric procedure, which measures deamination by a different principle (i.e. estimation of the formation of H_2O_2 in the oxidative deamination), the effect of clorgyline and semicarbazide on the dog tracheal and rat aorta SSAO is shown in Fig. 2. Both enzymes were sensitive to semicarbazide. The activation of the dog tracheal SSAO by clorgyline was demonstrated. Clorgyline does not effect the rat aorta SSAO (Fig. 2B). Using this fluorometric method we have also observed that clorgyline can increase dog aorta SSAO activity, but did not affect SSAOs from other tissues (i.e. from rat trachea and human umbilical artery). These findings confirmed the results of the radioenzymatic method.

Other propargylamine compounds, such as the MAO-B inhibitors pargyline and deprenyl, were also shown to activate dog SSAO. The magnitudes of the pargyline and deprenyl activation, however, are lower than that induced by clorgyline. At a concentration of 10^{-3} M deprenyl and pargyline, for example, induced an activation of only 30 and 35%, respectively.

The kinetic parameters of the deamination of benzylamine catalysed by the dog tracheal SSAO in the absence or presence of clorgyline are shown in Table 1. As can be seen the V_{max} values increased, but the K_m values were unaffected by clorgyline.

When an enzyme preparation was treated for 20 min with clorgyline (10^{-3} M) at ambient temperature followed by separation of the enzyme on a small Sephadex G-25 column (Pharmacia PD-10 disposable column), the original level of enzyme activity was recovered after the clorgyline was removed. The activation effect of clorgyline on dog SSAO is, therefore, reversible. The activation is also time-independent, since incubation of SSAO with clorgyline for up to one hour did not affect the degree of activation.

The extent of activation was diminished in the presence of the non-ionic detergent β-octylglucoside. As can be seen from Fig. 3, while β-octylglucoside does not effect the enzyme activity at concentrations up to 1%, it decreased

Table 1. Effect of clorgyline on the kinetic parameters of dog tracheal smooth muscle SSAO with respect to deamination of benzylamine.

Clorgyline	V_{max} (nmol min^{-1} mg^{-1})	K_m (1×10^{-6} M)
0	0·552 ± 0·014	6·50 ± 0·58
5×10^{-5} M	0·647 ± 0·035*	8·32 ± 0·77
1×10^{-3} M	0·850 ± 0·070*	7·97 ± 0·65

SSAO activity was estimated in the presence of benzylamine (1, 2, 4, 10, 20, 40, 100 and 200 μM) using a radioenzymatic method. Each value was the mean ± s.e. of three determinations. Statistical estimation is according to Wilkinson (1961). *$P < 0·01$ compared with the control value which was obtained in the absence of clorgyline.

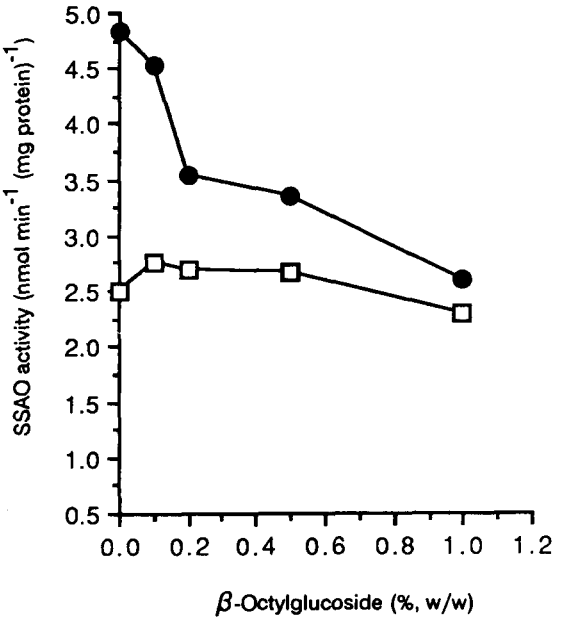

FIG. 3. Effect of β-octylglucoside on the clorgyline-induced action of dog aorta SSAO. The enzyme was pretreated with 10^{-3} M clorgyline at room temperature for 30 min. Both clorgyline-activated (●) and the untreated control (□) enzymes were further incubated in different concentrations of the non-ionic detergent β-octylglucoside at ambient temperature (21°C) for 20 min before addition of benzylamine substrate.

drastically the clorgyline-induced activation with increasing concentrations of the detergent.

The clorgyline-induced activation of SSAO isolated from both dog aorta and tracheal tissues was also observed with respect to the deamination of aliphatic amines, such as *n*-pentylamine and *n*-heptylamine and to a lesser extent β-phenylethylamine. The deamination of methylamine by SSAOs from both dog tissues, however, was not activated by clorgyline (results not shown). In this study a saturated substrate concentration was applied. The dog aorta SSAO activity was increased to 184 and 133% by clorgyline (10^{-4} M) with respect to *n*-pentylamine and 2-phenylethylamine. The dog tracheal SSAO was also found to be increased considerably to 206% by clorgyline in the oxidative deamination of *n*-heptylamine.

Discussion

Clorgyline is a propargylamine derivative, possessing high specific inhibitory activity towards MAO-A (see review by Yu 1986). Several compounds containing the propargylamine moiety are known to be reaction-based inhibitors, which are metabolized initially by MAO-A or MAO-B and then the product is covalently linked to the 8a-CH_3 of the riboflavin moiety at the active site of the MAOs. Other amine oxidases, such as rat aorta SSAO and bovine serum amine oxidase are inhibited by semicarbazide but not by propargyl inhibitors and probably do not possess flavine as a cofactor. These amine oxidases are usually unaffected by propargylamine MAO inhibitors. The propargyl inhibitors are, therefore, used to inactivate MAO in SSAO preparations. As is shown in this paper, however, clorgyline activates the dog tracheal and aortic smooth muscle SSAO. In order to confirm that this clorgyline-induced activation was a novel reaction, and not the result of artefact formation, such as may be caused by altering solvent extraction or any unidentified chemical interactions with the labelled benzylamine, we have identified the radioactively labelled product, namely benzaldehyde by thin layer chromatography (using C-18 reverse phase thin layer with 60% acetonitrile as elution solvent), and showed that the formation of the [^{14}C]-benzaldehyde was enhanced. Furthermore the activation phenomenon was also indicated by a fluorometric procedure, in which the production of hydrogen peroxide during the deamination was assayed. It is questioned whether or not any unknown components in the enzyme preparation might be involved in SSAO activation induced by clorgyline. Such a component was not detected, since partially purified dog SSAOs could also be activated by clorgyline. There is thus no doubt that the deamination of benzylamine by the dog SSAO was truly activated by clorgyline.

We have analysed SSAO from two Canadian Huskie dogs (anaesthetized with pentobarbitone) as well as four Taiwanese Tugo dogs (unanaesthetized) and the SSAO from the trachea of all these animals was activated to a similar degree in the presence of clorgyline during deamination with benzylamine as substrate. Such a finding rules out any possible effect from the anaesthetic agent. This enhancement of SSAO activity by clorgyline was not detected with respect to SSAOs isolated from several smooth muscle tissues or from bovine serum. The results support, therefore, the suggestion that SSAO is heterogenous in different species and perhaps in different tissues.

It has recently been reported that unsedimented monoamine oxidase isolated from rat brain and liver is also activated by clorgyline (Azam et al 1990). Such an activation appears to be quite different from that observed here for dog SSAO. In Azam's study with soluble rat liver amine oxidase 5-hydroxytryptamine (5-HT) was used as a substrate. Dog SSAO is unable to deaminate 5-HT. Furthermore, the soluble rat liver amine oxidase was activated by clorgyline at lower concentrations (i.e. 10^{-7}–10^{-5} M) than found in our study; it was also inhibited when the concentrations were increased. Clorgyline thus acts as an activator in an apparently different deamination system.

Although the increase of the V_{max} value and the unchanged K_m value by clorgyline (see Table 1) suggest that the mode of activation is noncompetitive, the mechanism of the activation is not clear. It is interesting to note that at relatively higher benzylamine concentrations, i.e. over 2×10^{-4} M, substrate inhibition occurred in the absence of clorgyline but when clorgyline was included in the incubation mixture, substrate inhibition was no longer detected. As a result, the degree of activation was more pronounced when the enzyme assay was conducted at higher benzylamine concentrations. That the non-ionic detergent β-octylglucoside could abolish this activation, suggests that clorgyline perhaps possesses an electrostatic or hydophobic interaction with one or more regions of the dog SSAO. This would reduce the hindrance of the binding of the substrate with the enzyme. The binding of clorgyline with SSAO is probably not very tight, as shown by

its ready separation by gel filtration (with Sephadex G-25) in the absence of detergent. Only certain enzymes, such as the dog SSAO or soluble rat liver amine oxidase, possess clorgyline binding sites and such interaction can subsquently activate the deaminase activities.

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