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CHARACTERIZATION OF HUMAN SERUM AND UMBILICAL ARTERY SEMICARBAZIDE-SENSITIVE AMINE OXIDASE (SSAO)

SPECIES HETEROGENEITY AND STEREOISOMERIC SPECIFICITY

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Abstract—Semicarbazide-sensitive amine oxidases (SSAOs) are located in cardiovascular smooth muscle, cartilage and brown adipose tissues of different species, including human. The enzyme is also present in blood, and its activity appears to be altered under certain pathological conditions. SSAOs from both human umbilical arteries and serum were partially purified, and some of their biochemical properties were investigated. Both human artery and blood SSAO exhibited very similar substrate preference, lack of stereospecificity catalyzing the deamination of pro-*R* and pro-*S* benzylamine-deuterated enantiomers, and were very sensitive towards (*E*)-2-(4-fluorophenethyl)-3-fluoroallylamine (MDL-72974A). It was concluded that circulating serum SSAO is identical to the SSAO from vascular tissues. Human SSAO exhibited distinctly different properties in comparison to bovine and rat SSAOs.

Key words: semicarbazide-sensitive amine oxidase (SSAO); monoamine oxidase (MAO); serum amine oxidase; SSAO inhibitor; umbilical artery; stereospecificity

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 the smooth muscle cells of blood vessels [1–3]. Although SSAO is able to deaminate several of the same monoamines as does MAO (EC 1.4.3.4), it is distinctly different from MAO. MAOs (MAO-A and MAO-B) are well known to be flavine-containing enzymes located on the outer membrane of mitochondria in almost all mammalian tissues [4]. SSAO has not yet been purified to homogeneity, and its properties are largely unknown. The enzyme is sensitive to semicarbazide and thus has been considered to be a copper-pyridoxal (PLP)- [5] or pyrroloquinoline quinone (PQQ)- [6] enzyme. 6-Hydroxydopa was found recently to be the correct prosthetic group for BSAO [7]. It is, however, unclear whether SSAOs possess the same cofactor as BSAO. SSAO oxidizes biogenic amines, such as 2-phenylethylamine, *p*-tyramine and dopamine, but seems to be inactive towards 5-HT and β-hydroxylated catecholamines, such as noradrenaline [8]. Both SSAO and MAO readily deaminate aliphatic amines [9, 10]. The physiological function of SSAO is still unclear. The high SSAO activity found in cardiovascular tissues suggests that this enzyme may play an important role in these tissues. The enzyme may be at least partly involved in the deamination of circulating biogenic amines from endogenous and exogenous sources [11]. Methylamine can be readily deaminated by SSAO but not by MAO [10]. The urinary excretion of methylamine is increased in rats treated with SSAO inhibitors [12], and it has been considered to be an endogenous substrate [13].

Allylamine, an industrial chemical, is a relatively specific cardiovascular toxin that causes extensive and progressive vascular and myocardial lesions in several mammalian species [14]. It is now known that allylamine can be metabolized to acrolein by vascular SSAO [15]. Acrolein acts as a distal toxin responsible for the cellular damage caused by allylamine intoxication. The SSAO inhibitor semicarbazide provides protection against the progressive damage caused by allylamine [16].

Some biochemical properties of human aorta SSAO have been reported [17, 18]. It is not yet clear, however, what the source of serum SSAO is or whether it is the same enzyme that is present in the vascular smooth muscles. Serum amine oxidase activities have been found to be increased in diabetic patients [19] and elevated in toxin-induced diabetic rats and sheep [20, 21]. Alteration of blood SSAO activities in other pathological conditions has also been reported [22, 23]. The deamination of methylamine by SSAO is particularly interesting, since formaldehyde is produced during the reaction [24]. This product is well known to be extremely reactive and toxic. Uncontrolled endogenous formation of formaldehyde via deamination of methylamine by SSAO has been shown to be potentially capable of causing cytotoxicity and may induce endothelial damage that could subsequently cause

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† Abbreviations: SSAO, semicarbazide-sensitive amine oxidase; MAO, monoamine oxidase; and BSAO, bovine serum amine oxidase.

atherosclerosis [25]. We have investigated some properties of the partially purified human serum and umbilical artery SSAO, and found that the properties of the enzymes from both serum and vascular tissues are quite similar. Bovine and rat SSAOs have also been compared with the human enzyme.

MATERIALS AND METHODS

Preparation of SSAO. The enzyme was partially purified from both human umbilical artery (provided by the Department of Obstetrics and Gynaecology, Royal University Hospital, Saskatoon) and serum (obtained from the Red Cross, Saskatoon). Arteries were dissected from umbilical cords and the fat tissue was removed carefully; the arteries (10 g fresh weight) were then rinsed thoroughly with saline, sliced into small pieces, and homogenized with a Polytron homogenizer (PT-10-35, at setting 5 for four periods of 10 sec on ice) in chilled 0.01 M phosphate buffer (pH 6.8) (1:10, w/v). The crude homogenates were centrifuged at 110,000 *g* for 30 min. The supernatants were applied to a DEAE column (2.6 × 20 cm) equilibrated with 0.01 M phosphate buffer (pH 6.8). SSAO was eluted with a linear gradient of sodium chloride (0.1 to 0.4 M) at a flow rate of 60 mL/hr. The fractions containing SSAO were pooled and concentrated by ultrafiltration using an Amicon Filtrator. The enzyme preparations were further purified by gel filtration using a column (1.5 × 90 cm) of Sephacryl S-300. The pooled fractions of SSAO from the Sephacryl column eluates were concentrated by ultrafiltration, and the enzyme was stored at −20°. Serum (200 mL) was also purified through DEAE and Sephacryl S-300 columns using chromatographic procedures similar to those described above for the preparation of tissue SSAO. Twenty-three- and forty-two-fold purifications were obtained with respect to the umbilical artery and serum SSAO.

Synthesis of stereospecific deuterated benzylamine. *R*-[α-$^2H_1$]- and *S*-[α-$^2H_1$]Benzylamine were synthesized as previously described [26]. Briefly, benzaldehyde[$^2H_1$] was prepared from protio benzaldehyde via the dithiane with butylithium and deuterium oxide. Enzymatic reduction of the aldehyde was carried out using horse liver alcohol dehydrogenase, NAD^+ and ethanol in pH 7.4 phosphate buffer. Reaction of the alcohol, i.e. (+)-*S*-benzyl alcohol[α-$^2H_1$], with *p*-toluenesulfonyl chloride (retention of configuration), gave the tosylate, which was treated with an aqueous-acetone solution of sodium azide (inversion of configuration). The azide was reduced with lithium aluminum hydride (retention of configuration). The resulting amine (*R*-form) was converted into its hydrochloride salt by treatment of an ether solution of the amine with ethanolic hydrogen chloride. (+)-*S*-Benzyl alcohol[α-$^2H_1$] was treated with phosphorus tribromide at −40° (inversion of configuration) and allowed to warm to 20°. After 4 min the bromide was isolated, converted to the azide (inversion), and reduced to the amine (*S*-form) as described above.

Amine oxidase assay. A fluorometric method was used for the estimation of SSAO activities [4]. The procedure was based on the formation of an intense fluorophore formed between homovanillic acid and the hydrogen peroxide released during the oxidation of the amines. The crude SSAO preparations were incubated at 37° 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 2 μg homovanillic acid and 0.82 U horseradish peroxidase. The developed fluorescence intensity was measured in a spectrophotofluorometer (Aminco–Bowman) at an excitation wavelength of 310 nm and an 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 method of Bradford [27] with bovine serum albumin as standard.

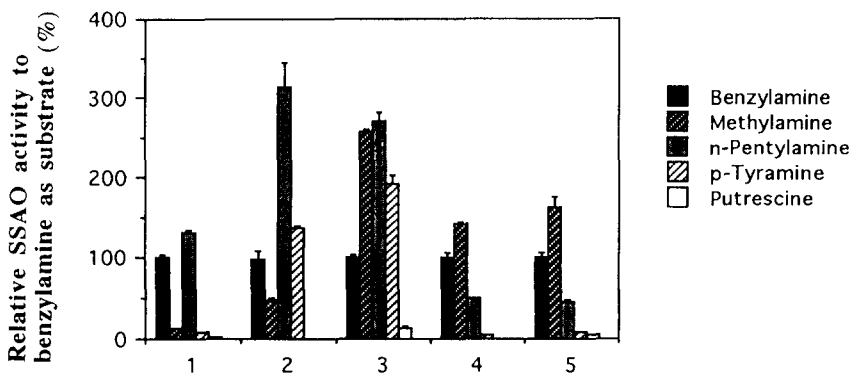

Fig. 1. Comparison of serum and vascular tissue SSAO activities towards different substrates. Enzyme activities were measured by a fluorometric method. The substrates (1×10^{-4} M) used were benzylamine, methylamine, *n*-pentylamine, *p*-tyramine and putrescine. Relative enzyme activities towards different substrates (in comparison with benzylamine) are presented. SSAO activities towards benzylamine were 2.13 ± 0.04, 0.044 ± 0.0004, 0.05 ± 0.001, 0.86 ± 0.05 and 0.025 ± 0.003 nmol/mg protein/min for BSAO (1) and SSAO obtained from bovine aorta (2), rat aorta (3), human umbilicals (4) and human serum (5), respectively. Each value is the mean ± SEM of 3 independent experiments.

Table 1. Kinetic parameters and deuterium isotope effects of human artery and serum SSAOs with respect to deamination of stereospecific α-deuterated benzylamine

	Undeuterated BZ		R-[α-$^2H_1$]BZ		S-[α-$^2H_1$]BZ	
SSAO	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Artery	239 ± 15	1.55 ± 0.35	193 ± 3	1.93 ± 0.13	204 ± 12	2.16 ± 0.11
Serum	25 ± 3	2.79 ± 0.90	20 ± 2	2.62 ± 1.04	19 ± 1	3.66 ± 0.71

The partially purified SSAOs from the human umbilical artery and serum were incubated with different concentrations of undeuterated, R-[α-$^2H_1$]- and S-[α-$^2H_1$]-benzylamine (BZ). V_{max} (maximal velocities, pmol/mg protein/min) and K_m (Michaelis–Menten constant, 1×10^{-4} M) values and the statistics were obtained from the equations described by Wilkinson [29]. Each value is the mean ± SEM of 3 independent experiments.

Animals and materials. Rat aortae were collected from Wistar male rats (200 g). Benzylamine, methylamine, homovanillic acid, BSAO, horseradish peroxidase, and semicarbazide were purchased from Sigma (St. Louis, MO, U.S.A.); [7-^{14}C]benzylamine was obtained from Amersham (Oakville, Ontario, Canada). Clorgyline [*N*-(2,4-dichlorophenoxy-*n*-propyl)-*N*-methylpropargylamine·HCl] was obtained from May & Baker Ltd. (Dagenham, U.K.). (*E*) - 2 - (4 - Fluorophenethyl) - 3 - fluoroallylamine·HCl (MDL-72974A) and (*E*)-2-(3′,4′ - dimethoxyphenyl) - 3 - fluoroallylamine·HCl (MDL-72145) were gifts from the Marion-Merrell-Dow Research Institute (Cincinnati, OH, U.S.A.).

RESULTS AND DISCUSSION

As can be seen in Fig. 1, the relative enzyme activities towards different substrates (i.e. with respect to the activity towards typical SSAO substrate benzylamine as 100%) of the partially purified SSAO obtained from human arteries and serum were compared with the activities of the rat aorta SSAO and bovine amine oxidases. The specific enzyme activity of the human serum SSAO was relatively low due to high concentrations of serum protein. The substrate preference of this human circulating SSAO appeared to be very similar to that of the umbilical artery SSAO. The substrate preference for human SSAO, however, was quite different from that of the enzymes from other species. BSAO, for example, possessed very little activity towards methylamine, whereas rat aorta SSAO preferentially catalyzed the deamination of methylamine and *p*-tyramine. Human SSAO did not appear to oxidize *p*-tyramine.

Amine oxidases are well known to possess stereospecificity in the cleavage of the hydrogen (or deuterium) atom from the α-carbon of the substrates [26, 28]. We have investigated the stereospecificity of the deamination of the stereoisomers of α-deuterated benzylamine, i.e. its pro-*R* and pro-*S* enantiomers, catalyzed by human artery and serum SSAO. As can be seen in Table 1, the deamination of both enantiomers by human artery and serum SSAO was assessed kinetically. No appreciable deuterium isotope effect with respect to SSAO from either source was observed.

During the enzymatic deamination of monoamines, three types of stereospecific abstraction of hydrogen atoms from the α-carbon of the amines have been observed [26, 28]: (a) enzymes such as the mammalian mitochondrial monoamine oxidases catalyze the cleavage of pro-*R* hydrogen from the α-carbon; (b) rat aorta, hog kidney and pea seedling amine oxidases remove the pro-*S* hydrogen from the α-carbon; and (c) BSAO, although it also exhibits a 2-fold deuterium isotope effect, has no absolute stereospecificity. SSAOs from both human artery tissue and serum did not exhibit the deuterium isotope effect and stereospecificity; therefore, they cannot be categorized in any of the above groups. This observation also suggests that the cleavage of the hydrogen atom from the α-carbon is probably not the rate-limiting step in the deamination of benzylamine catalyzed by human SSAO.

Several haloallylamine compounds have been found to exhibit potent inhibitory activity towards monoamine oxidase and SSAO [30, 31]. We have compared the *in vitro* effectiveness of two fluoroallylamine inhibitors toward different SSAOs. As can be seen in Fig. 2, MDL-72974A was generally more potent than MDL-72145 in inhibiting SSAO activities. Both human artery and serum SSAOs were more sensitive towards MDL-72974A than MDL-72145 by a factor of over 1000 in their IC_{50} values. BSAO was less sensitive towards MDL-72974A, but more sensitive towards MDL-72145 in comparison to the effects on human SSAO. MDL-72974A was about 10-fold more potent than MDL-72145 in the inhibition of rat and bovine SSAO activities. These inhibitors appear to be very useful in the study of SSAO heterogeneity.

It is interesting that the chemical structures of MDL-72974A and MDL-72145 are rather similar and that the fluoroallylamine moiety is directly involved in the mechanism-based inhibition of amine oxidases. It is yet to be established whether the substitution of the benzene ring or the aliphatic side chain is responsible for the specificity of the interaction between the enzymes and inhibitors. It is clear that in order to answer this question SSAOs would have to be purified and cloned, and the primary structure and the cofactor elucidated. The low concentration of the enzyme molecule and its apparent instability have hampered such attempts to

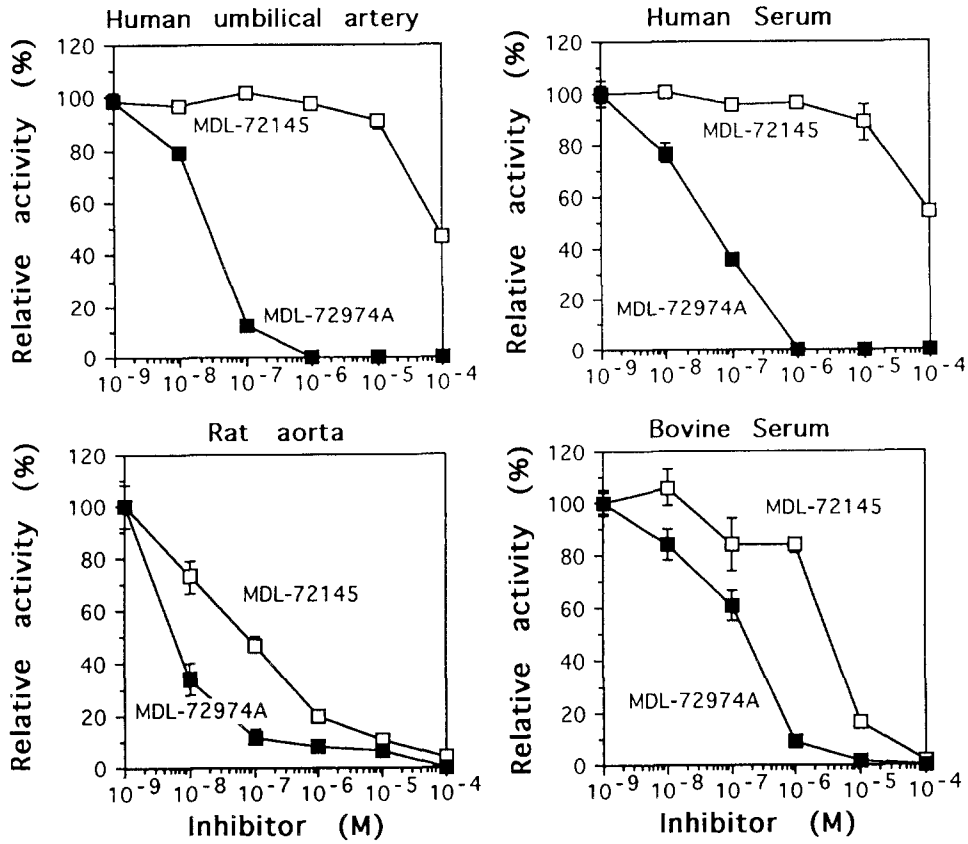

Fig. 2. Effects of MDL-72974A and MDL-72145 on SSAO from different serum and tissues. SSAO activity towards benzylamine (5×10^{-4} M) was measured. The conditions for the enzyme assays are indicated in the legend of Fig. 1. Each value is the mean ± SEM of 3 independent experiments.

date. Uncritical generalization of the properties of different SSAOs, i.e. based on the properties of the well known BSAO, may be inappropriate.

In conclusion, according to substrate preference, stereospecificity and sensitivity to inhibitors our results indicate that SSAOs exist in heterogenous forms among the different species. Human serum SSAO appears to be identical to the cardiovascular tissue SSAO.

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