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Biochemical Pharmacology 61 (2001) 741–748

Biochemical Pharmacology

2-Bromoethylamine as a potent selective suicide inhibitor for semicarbazide-sensitive amine oxidase

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Received 28 March 2000; accepted 8 August 2000

Abstract

Semicarbazide-sensitive amine oxidase (SSAO) catalyzes the deamination of methylamine and aminoacetone to produce toxic aldehydes, i.e. formaldehyde and methylglyoxal, as well as hydrogen peroxide and ammonia. An increase of SSAO activity was detected by different laboratories in patients suffering from vascular disorders, i.e. diabetes and myocardial infarction. The enzyme has been suggested to play a role in vascular endothelial damage and atherogenesis. To date, there are no selective SSAO inhibitors. In the present study, 2-bromoethylamine (2-BrEA) was found to be a highly effective and selective inhibitor of SSAO obtained from different sources. The inhibition was irreversible and time dependent. It was competitive when the enzyme was not preincubated with the inhibitor, but became noncompetitive after incubation of the enzyme with 2-BrEA. The aldehyde trapping agent *o*-phenylenediamine was capable of preventing 2-BrEA-induced inhibition of SSAO activity. An aldehyde product was detected to be an initial product of 2-BrEA after it was incubated with SSAO. The inhibition, therefore, is mechanism-based. The SSAO inhibitory effects of eight structural analogues of 2-BrEA were assessed. It was concluded that a bromine atom at the beta position is quite important for exerting high potency of SSAO inhibition. The inhibition of SSAO activity by 2-BrEA was also demonstrated *in vivo*. It increased the urinary excretion of methylamine, an endogenous substrate for SSAO, in mice. 2-BrEA can be employed as a very useful tool in the investigation of SSAO.

Keywords: Amine oxidase; SSAO; Enzyme inhibitor; Formaldehyde; Methylamine

1. Introduction

SSAO is an enzyme or group of enzymes sensitive to semicarbazide and related hydrazine compounds [1]. They are distributed in different tissues [2], particularly in vascular smooth muscles and adipose tissue [2,3]. Methylamine and aminoacetone are recognized to be physiological substrates for SSAO enzymes [4–6] and give rise to the deaminated products formaldehyde and methylglyoxal, respectively.

$$CH_3NH_2 + O_2 + H_2O \xrightarrow{SSAO} HCHO + H_2O_2 + NH_3$$

$$CH_3COCH_2NH_2 + O_2 + H_2O \xrightarrow{SSAO} CH_3COCHO + H_2O_2 + NH_3$$

Methylglyoxal has been found to be increased in diabetes and is considered to be involved in protein glycation and associated diabetic vascular disorders [7]. Interestingly, SSAO activity has been reported to be increased in the serum of diabetic patients [8–10], and also in the blood and kidney of diabetic rats (streptozotocin-treated) [11]. Plasma SSAO was also found to be elevated in patients diagnosed with congestive heart failure [12]. It has been proposed that the increased SSAO-mediated deamination of methylamine and aminoacetone may be related to diabetic complications [13,14].

A physiological role for the function of SSAO has been proposed. The enzyme was found to be associated with the GLUT-4 glucose transporter and involved in the signaling of glucose uptake in adipocytes [15,16]. It was found that a vascular adhesion protein, VAP-1 [17], which plays a role in leukocyte trafficking and is up-regulated in some chronic

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Abbreviations: SSAO, semicarbazide-sensitive amine oxidase (EC 1.4.3.6); MAO, monoamine oxidase (EC 1.4.3.4); BZ, benzylamine; 2-BrEA, 2-bromoethylamine; MDL-72974A, (*E*)-2-(4-fluorophenethyl)-3-fluoroallylamine HCl; and o-PD, *o*-phenylenediamine.

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inflammatory conditions, appears to be identical to SSAO [18]. A new proposed function of this old enzyme clearly warrants more investigation.

Up to now there is no selective SSAO inhibitor available for research. Semicarbazide and hydrazide compounds are not very useful, because they can non-selectively inhibit a variety of enzymes. Several β-fluoro compounds have been reported to be highly potent SSAO inhibitors, but these inhibitors are also potent MAO inhibitors [19]. 2-BrEA and 3-bromopropylamine were reported to be capable of inhibiting guinea pig lung tissue-bound SSAO activity [20]. In the present investigation, we assessed whether several structurally related halogenated amines inhibited human, rodent, and rabbit SSAO. The mode of action of 2-BrEA inhibition was investigated.

2. Materials and methods

2.1. Materials

2-BrEA, 2-chloroethylamine, 2-fluoroethylamine, 2-methoxyethylamine, 2-bromobenzylamine, and semicarbazide were purchased from the Sigma Chemical Co. *N*-(2-Chloroethyl)-*N*-2-bromoethylamine was obtained from RBI. [7-^{14}C]Benzylamine hydrochloride (59 mCi/mmol) was purchased from Amersham Life Science (Amersham International). All other chemicals were of analytical grade.

2.2. Synthesis of 2-bromo-1-aminopropane and 2-bromo-1-aminopentane hydrobromide

1-Amino-2-propanol was brominated with 48% hydrobromic acid by heating at 140° for 6 hr, and then the excess hydrobromic acid was distilled. The product was isolated from the very viscous residue by repeated trituration in acetone to give a white crystalline product, m.p. 157–159°. The mass spectrum was consistent with the title compound.

n-Butyryl cyanide was prepared by heating a mixture of *n*-butyryl chloride and cuprous cyanide at 110° for 64 hr. The resulting keto nitrile was extracted with ether and then reduced with lithium aluminum hydride to 1-amino-2-pentanol. The hydrobromide salt of the amino alcohol was brominated with PBr_3, and the title compound was isolated by precipitation of its oxalate salt from the ether extract of the basified reaction mixture. Its identity was confirmed by mass spectrometry.

2.3. Animal experiments

Male CD1 Swiss white mice were supplied by the Animal Resource Centre, University of Saskatchewan. The animal studies were in strict accordance with the guidelines established by the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee. The mice were housed in hanging wire cages with free access to food and water on a 12-hr light/dark cycle (lights on at 6:00 a.m.) at a temperature of 19–20°.

2.4. Preparation of human SSAO

Enzyme was prepared from human umbilical arteries (provided by the Department of Obstetrics and Gynecology, Royal University Hospital). 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 800 *g* for 10 min, and the low-speed supernatants were further centrifuged at 110,000 *g* for 30 min. The resulting low- and high-speed supernatants and the membrane fractions were stored at −70° and used as the enzyme samples in this study. The properties of both soluble and membrane-bound SSAOs were quite similar. The present data are from soluble SSAO, unless otherwise specified.

2.5. Determination of SSAO and MAO activities

SSAO activity was determined by a radioenzymatic procedure using ^{14}C-labelled benzylamine as substrate, following our previously described procedure [21]. The SSAO enzyme preparations were preincubated with clorgyline (1×10^{-4} M) at room temperature for 20 min to ensure that any MAO activity, if present, was inactivated completely. Then the enzyme was incubated in the presence of benzylamine (5×10^{-5} M, 0.1 μCi) in a final volume of 200 μL at 37° for 30 min. The enzyme reaction was terminated by adding 200 μL of 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 of Omnifluor (New England Nuclear). Radioactivity was assessed by liquid scintillation counting (Beckman LS-7500). One unit of enzyme activity is defined as 1 nmol of product formed per min per mg of protein.

The radioenzymatic assay for MAO used ^{14}C-labelled substrates. The enzyme preparations were incubated at 37° for 30 min in the presence of the MAO-A substrate serotonin (5×10^{-4} M, 0.1 μCi) and the MAO-B substrate β-phenylethylamine (1×10^{-5} M, 0.1 μCi) in a final volume of 200 μL. The reactions were terminated by the addition of 200 μL of 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 of Omnifluor. Radioactivity was assessed by liquid scintillation spectrometry (Beckman LS-7500).

2.6. Determination of methylamine

Urinary methylamine was determined using an HPLC-fluorometric procedure [22]. Isopropylamine was added as an internal standard to the urine immediately after its collection, and the samples were stored at −70° until used. Urine samples (0.5 mL) were pre-purified by application to a small column of CG-50 amberlite (0.5 × 2.5 cm). The column was rinsed with 10 mL water and the amines were eluted with 2 mL of 1 M HCl. The samples (100 μL) were derivatized with *o*-phthaldialdehyde (OPA; 100 μL) under alkaline conditions (pH 10.4), and 50 μL of each was separated in a Shimadzu HPLC system (Sil-9A auto injector) equipped with a pre-column derivatization program. The fluorescent amine derivatives were separated in an analytical Ultrasphere I.P. column packed with octadecyl-bonded spherical 5-μm silica particles (250 × 4.6 mm i.d.) (Beckman). The column was eluted with 55% methanol at a flow rate of 1.0 mL/min, using an SSI 222B solvent delivery system (State College). For quantitative assessment, a programmable fluorescence detector (Hewlett Packard, HP1046A) with excitation at 360 nm and emission at 445 nm was employed. The signals of peak area were integrated using a Spectra-Physics SP-4290 integrator. The retention times for methylamine and isopropylamine are 11.9 and 29.2 min, respectively. The recovery of methylamine and isopropylamine from the ion exchange chromatography was approximately 80%. The peak ratios of methylamine/isopropylamine (internal standard) were used for the calculation of urine levels of methylamine.

2.7. Detection of aldehyde as a deaminated product of 2-BrEA by HPLC

o-PD has been used previously as a trapping agent for methylglyoxal produced from the SSAO-mediated deamination of aminoacetone [23]. Rabbit serum SSAO was used in this experiment. The serum (50 μL) was dialyzed in 0.1 mM potassium phosphate buffer (pH 7.5) for 2 hr. 2-BrEA was incubated with serum (*ca.* 60 mg protein) in a total of 200 mL of 50 mM potassium phosphate buffer containing 200 mM o-PD at 37° for 30 min. The enzymatic reaction was terminated by adding 100 mL of 0.6 M perchloric acid. The mixture was then centrifuged at 15,000 *g* for 10 min. The o-PD-aldehyde derivative in the supernatant was assessed by HPLC analysis. The HPLC system was composed of a Shimadzu LC10AT HPLC pump, a WISP 712B Auto-injector (Waters, Millipore), and a Spectra-physics recorder. The separation was performed using a reversed-phase Ultrasphere I.P. analytical column (150 × 4.6 mm i.d., Beckman). Elution was isocratic with 20 mM phosphate buffer, pH 4.6, containing 0.3 mM sodium 1-octanesulfonate and 20% acetonitrile, at a flow rate of 1.0 mL/min. Spectrophotometric detection at 254 nm was conducted using a Lambda-Max model 481 LC spectrophotometer (Waters, Millipore).

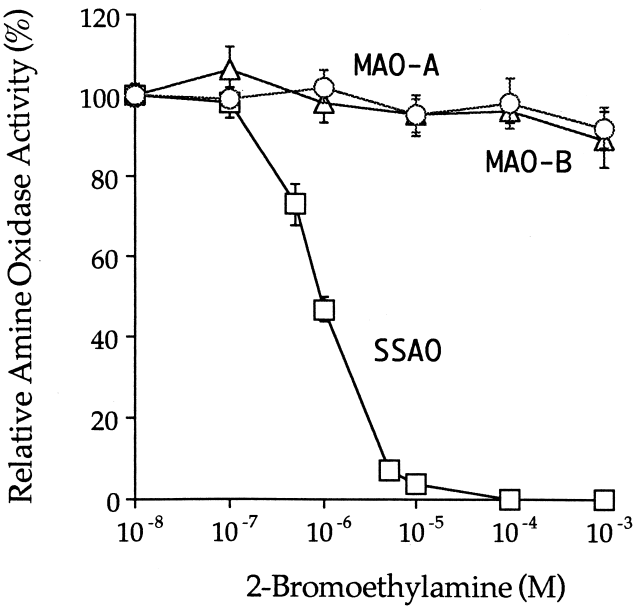

Fig. 1. Effect of 2-BrEA on human umbilical artery SSAO and rat mitochondrial MAOs. Enzyme activities were measured by a radioenzymatic method using benzylamine, serotonin, and β-phenylethylamine as substrates for SSAO, MAO-A, and MAO-B, respectively. The SSAO preparations were preincubated with clorgyline (1×10^{-5} M) in order to block MAO activities. For assessment of the inhibitory effect, the enzymes were preincubated in the presence or absence of different concentrations of 2-BrEA for 20 min at room temperature before the addition of substrates. The specific activities of the untreated control enzymes were 0.35 ± 0.03, 9.23 ± 0.80, and 7.44 ± 0.63 nmol/min/mg protein for SSAO, MAO-A, and MAO-B, respectively. Each value is the mean ± SEM of at least three independent experiments.

2.8. Statistics

Results were assessed using ANOVA followed by Newman–Keuls multiple comparisons. In general, the null hypothesis used for all analyses was that the factor has no influence on the measured variable, and significance was accepted at the $>$ 95% confidence level.

3. Results

3.1. Inhibition by 2-BrEA of SSAO and MAO activities

As can be seen in Fig. 1, 2-BrEA was quite effective at inhibiting human umbilical SSAO activities *in vitro.* The EC_{50} value was estimated to be 1 μM, and its potency was equivalent to that of semicarbazide. The potency of inhibition by 2-BrEA on mouse aorta and rabbit serum SSAO was comparable to the effect on human SSAO (results not shown). Neither MAO-A nor -B was affected by 2-BrEA up to 1 mM.

The effects of 2-BrEA on mouse aorta SSAO and brain MAO activities were also demonstrated in an *in vivo* experiment. Figure 2 shows a dose-dependent inhibition by

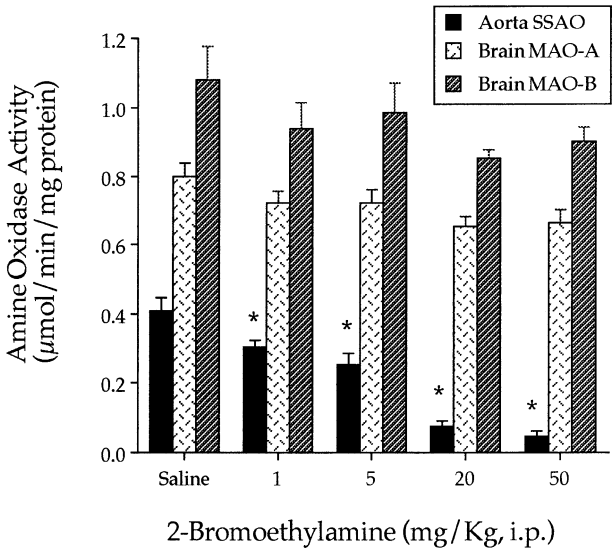

Fig. 2. Inhibition by 2-BrEA of mouse aorta SSAO and brain MAO activities *in vivo*. Mouse aorta and brain tissues were collected 3 hr after the i.p. administration of different doses of 2-BrEA. Values are means ± SEM of five animals at each dose. Key: (*) $P < 0.01$, compared with the saline controls.

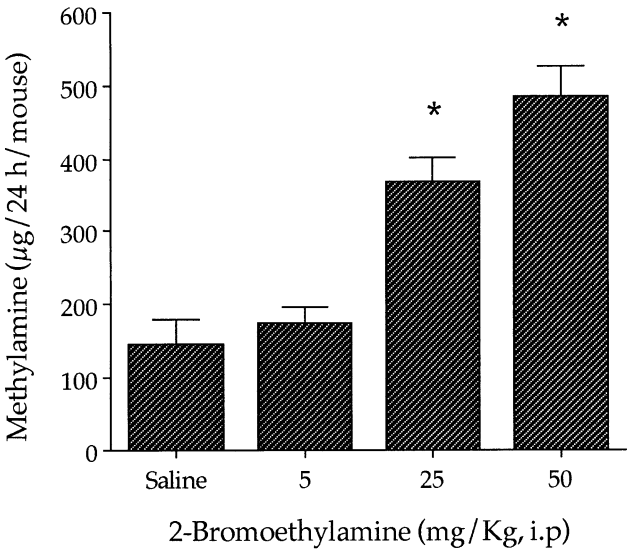

Fig. 3. Effect of 2-BrEA on urinary methylamine levels in mice. 2-BrEA (5, 25, and 50 mg/kg) and saline were administered i.p. to mice, and then urine was collected for 24 hr. Methylamine was assessed by an HPLC-fluorometric procedure as described in "Materials and methods." Values are means ± SEM of the urine collections from three cages, each containing three mice at each dose. Key: (*) $P < 0.01$, compared with the saline controls.

2-BrEA. Inhibition of SSAO by a single i.p. injection of 2-BrEA as low as 1 mg/kg was detected. 2-BrEA at 20 mg/kg inhibited over 80% of the aorta SSAO activity. On the contrary, MAO activities were unchanged following administration of 2-BrEA. Whether or not 2-BrEA can pass the blood–brain barrier is unclear.

3.2. Effect of 2-BrEA on urinary excretion of methylamine

Methylamine is an endogenous SSAO substrate [21]. Inhibition of SSAO by 2-BrEA caused a 3-fold increase of urinary methylamine levels at a dose of 50 mg/kg (Fig. 3). Although one-third of the SSAO activity was inhibited by 2-BrEA at 5 mg/kg, it was insufficient to change the oxidative deamination of methylamine *in vivo*.

3.3. Effect of 2-BrEA and its structural analogues on SSAO activity

It would be interesting to know whether modification of the chemical structure of 2-BrEA would alter the inhibition of SSAO and MAO-B. The 2-bromo substitution was replaced by different halogen and methoxy groups. In addition, aliphatic groups of different chain lengths were attached to the β-position of 2-BrEA. Amines with 2-bromobenzene moieties were also employed (see Fig. 4). The effects of these compounds on human umbilical SSAO activity *in vitro*, i.e. IC_{50}, are summarized in Table 1.

2-BrEA, the parent compound, exhibited the most potent inhibitory effect on human umbilical SSAO activity. Substitution of the bromine with a chlorine or fluorine atom substantially reduced the inhibitory activity. The ability of 2-bromo-1-aminopropane to inhibit SSAO activity was also less potent. If bromine was substituted at the 3-carbon position, i.e. 3-bromopropylamine, its inhibitory effect was reduced dramatically. The 2-bromobenzene derivatives exhibited a weak effect on SSAO activity in comparison to 2-BrEA.

3.4. Mode of inhibition of 2-BrEA

The inhibition was clearly time dependent (Fig. 5), since preincubation of SSAO with 2-BrEA increased the inhibition.

After incubation of human umbilical SSAO with 2-BrEA, the enzyme sample was subjected to gel filtration using a small Sephadex G-25 column. That the SSAO activity could not be recovered after incubation with 2-BrEA indicates that the inhibition was irreversible (Fig. 6).

The mode of inhibition was further revealed by enzyme kinetics (Fig. 7). Interestingly, if 2-BrEA and the substrate BZ were co-incubated with the enzyme, the inhibition appeared to be competitive. Following preincubation of SSAO with 2-BrEA, the inhibition became noncompetitive.

To determine whether or not the inhibition might be due to chemical interaction of the aldehyde product with the enzyme, we conducted an experiment including an aldehyde trapping agent, o-PD. As can be seen in Fig. 8, o-PD by itself slightly inhibited SSAO activity up to 0.2 mM. However, in the presence of 2-BrEA (2 μM), which inhibited SSAO activity by 90%, o-PD was able to at least partially protect the enzyme against 2-BrEA-induced inhibition.

That 2-bromoacetaldehyde was probably involved in the

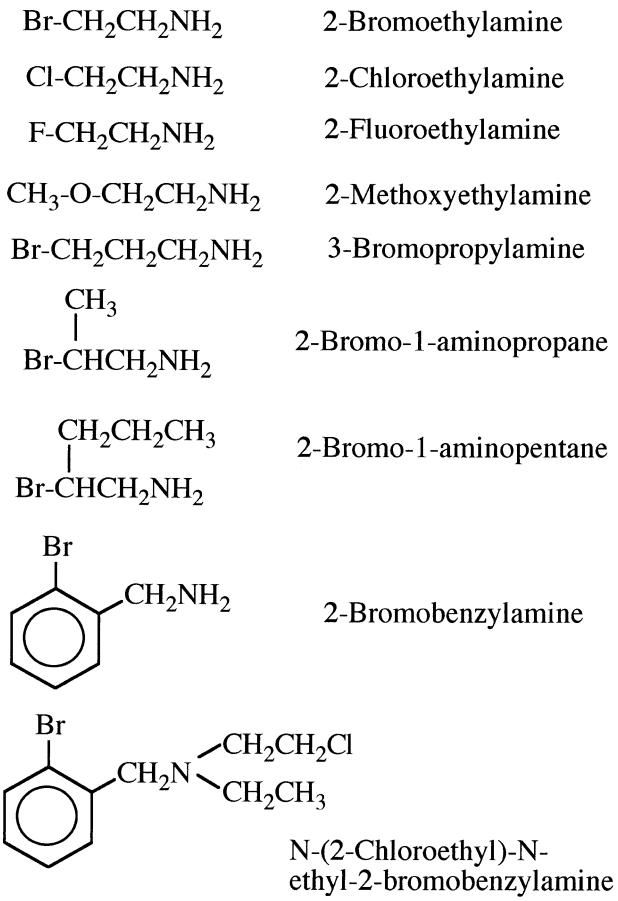

Fig. 4. Structural analogues of 2-bromoethylamine.

inhibition of SSAO activity was further confirmed by direct detection of the reaction product by HPLC. As can be seen in Fig. 9C, a trace amount of an aldehyde product, i.e. a peak with a retention time at 6.3, was detected when 2-BrEA was incubated with rabbit serum SSAO and o-PD. Such an o-PD reaction product was not formed if o-PD was incubated with 2-BrEA or the SSAO enzyme preparation alone (Fig. 9, panels A and B, respectively).

Table 1
Effect of 2-bromoethylamine (2-BrEA) and its structural analogues on human umbilical artery SSAO activity *in vitro*

Compounds	IC_{50}	
	SSAO	MAO-B
2-Bromoethylamine	1×10^{-6} M	$>1 \times 10^{-3}$ M
2-Chloroethylamine	6×10^{-5} M	$>1 \times 10^{-3}$ M
2-Fluoroethylamine	1×10^{-2} M	
2-Methoxyethylamine	$>1 \times 10^{-3}$ M	
3-Bromopropylamine	1×10^{-3} M	
2-Bromo-1-aminopropane	3×10^{-5} M	$>1 \times 10^{-3}$ M
2-Bromo-1-aminopentane	6×10^{-5} M	
2-Bromobenzylamine	1×10^{-4} M	
N-(2-Chloroethyl)-*N*-ethyl-2-bromobenzylamine	2×10^{-4} M	$>1 \times 10^{-3}$ M

Results are the averages of at least three independent experiments for each compound, estimated using a series of concentrations of the inhibitors according to Lineweaver–Burk plots.

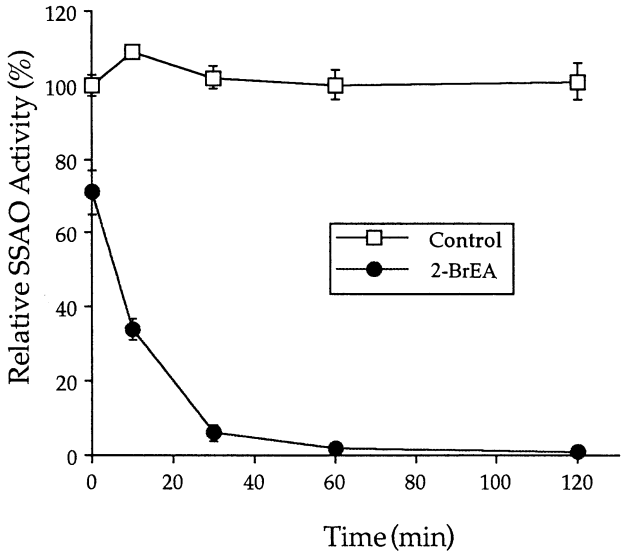

Fig. 5. Time-dependent inhibition by 2-BrEA of human umbilical SSAO activity. The enzyme was preincubated with 2-BrEA (1×10^{-5} M), as indicated on the figure; the substrate benzylamine was then added, and the mixture was further incubated for 5 min at 37°. The specific activities were 0.33 ± 0.04 and 0.24 ± 0.09 nmol/min/mg protein for the untreated and 2-BrEA-treated SSAO, respectively. Each value is the mean ± SEM of at least three independent experiments.

4. Discussion

SSAO was discovered serendipitously over three decades ago during investigation of MAO. SSAO activity is not inhibited by typical MAO inhibitors, such as clorgyline and deprenyl [24]. For a long time, neither its endogenous substrate nor its physiological function was understood. It became clear that SSAO could readily metabolize methylamine and aminoacetone, which are well known to be synthesized *in vivo*. Overproduction of toxic aldehydes due to increased SSAO-mediated deamination was proposed to be potentially hazardous in certain pathological conditions, such as diabetic complications [25]. It was reasonable to suggest that reduction of SSAO-mediated deamination may be beneficial in these conditions [13,26].

We found that 2-BrEA is indeed an effective inhibitor of SSAO from different sources, i.e. human umbilical vascular tissues, and rabbit and rodent serum. This is consistent with the finding of its inhibitory effect towards guinea pig SSAO [20]. It is interesting to note that up to 1 mM 2-BrEA did not inhibit either MAO-A or -B. To the best of our knowledge, this is the first selective SSAO inhibitor that does not also have an inhibitory effect on MAO. The inhibition was time dependent, noncompetitive, and irreversible. Inhibition of SSAO activity *in vivo* was also demonstrated. Indeed, 2-BrEA induced a dose-dependent increase of urinary meth-

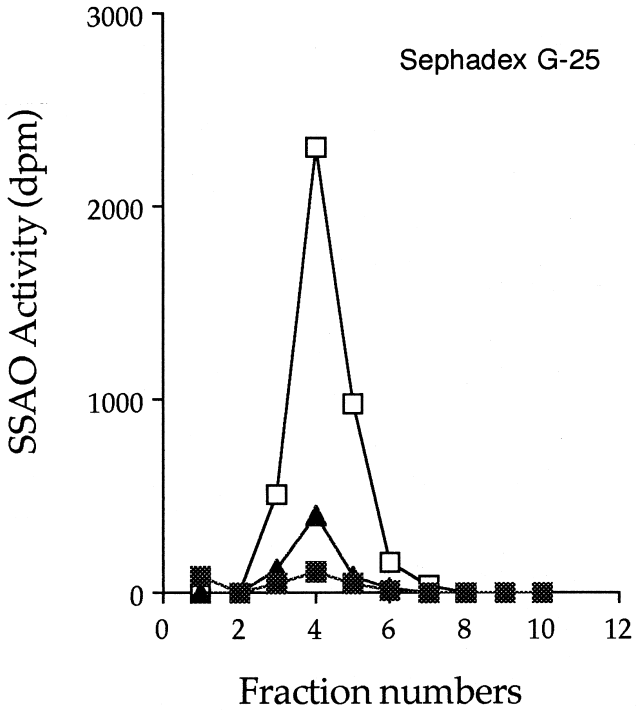

Fig. 6. Assessment of irreversible inhibition of SSAO by 2-BrEA. Human umbilical SSAO was incubated with 2-BrEA (1×10^{-5} M) for 30 min and then separated, using a small Sephadex G-25 column (PD-10, Pharmacia). SSAO was incubated with 2-BrEA at concentrations of 1×10^{-4} M (■) and 1×10^{-5} M (▲) or without an SSAO inhibitor (□). SSAO activity was determined in the eluted fractions. Fractions 2–4 represent protein with molecular weight > 10,000. The figure represents a typical result from three independent experiments.

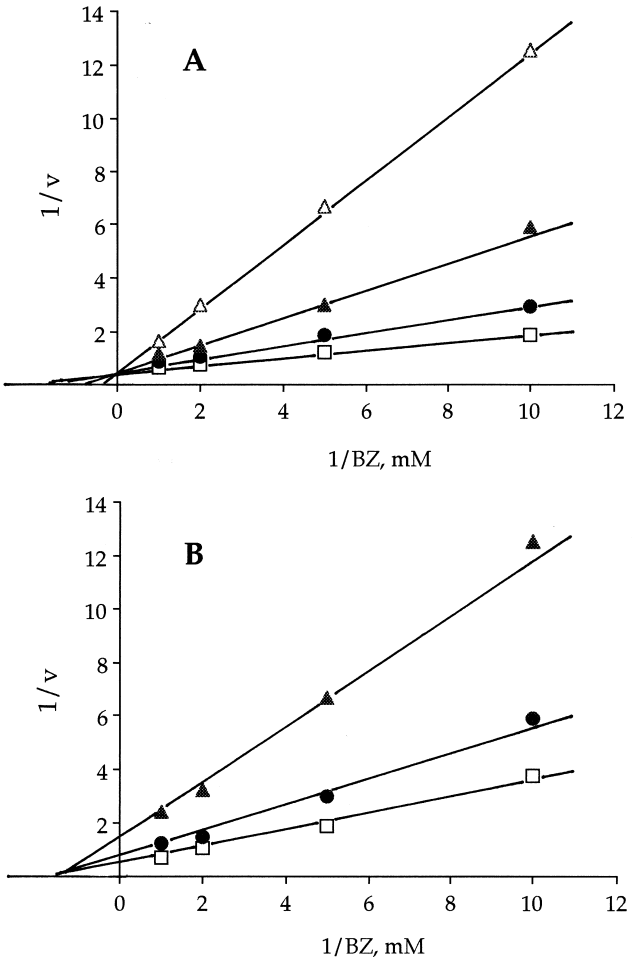

Fig. 7. Kinetic assessment of 2-BrEA inhibition of human umbilical SSAO activity. Lineweaver–Burk plots represent results with respect to enzyme preincubated with 2-BrEA (B) or without preincubation (A). The concentrations of 2-BrEA were: 5×10^{-6} M (●), 1×10^{-5} M (▲), and 2×10^{-5} M (Δ); the open squares (□) represent the controls. For SSAO assays, [^{14}C]benzylamine (BZ) (from 5×10^{-5} to 1×10^{-4} M) was used as the substrate.

ylamine, which is known to be an endogenous substrate for SSAO but not for MAO. 2-BrEA could be a useful tool for the investigation of SSAO.

We also investigated whether modification of the chemical structure of 2-BrEA could alter its inhibitory potency. Replacement of the bromine moiety of 2-BrEA with fluorine or chlorine, or with methoxyl groups, drastically reduced its SSAO inhibitory activity. Substitution of bromine at the 2-carbon position is quite important, since the inhibitory effect of 3-bromopropylamine was decreased dramatically. Since SSAO has been shown to possess a high affinity for longer chain aliphatic groups [5], two structural analogues of 2-BrEA with an increased number of carbon chains were synthesized and assessed. Unfortunately, such modification did not seem to enhance its ability to inhibit SSAO activity.

It is interesting that ethylamine is known to be a substrate for SSAO [5], yet, attaching a bromine moiety at the beta position rendered the molecule an inhibitor of the enzyme. In fact, we were able to detect an aldehyde product, presumably 2-bromoacetaldehyde, as a deaminated product of 2-BrEA. Furthermore, the aldehyde trapping agent o-PD was able to partially protect the enzyme against 2-BrEA. The reaction rate of aldehydes with o-PD is relatively slow at 37°; therefore, its protective effect is limited. These results suggest that 2-BrEA is converted to 2-bromoacetaldyde, which, assisted by the strong electron withdrawing property of the bromine atom, interacts with SSAO and irreversibly inhibits the enzyme. This conclusion was also supported by the enzyme kinetic experiments. Therefore, 2-BrEA is a typical suicide inhibitor. More work on the mechanism of action of 2-BrEA is required. It was shown previously that the mechanism-based inhibition of MAO by phenelzine also leads to the production of phenylacetaldehyde in the initial phase of the enzyme reaction [27].

It has been reported that 2-BrEA could alter mouse renal ultrastructures [28]. In fact, it was used as a nephrotoxin to produce lesions of renal papillary necrosis in laboratory animals [29]. In those studies, however, a rather large dose, i.e. 300 mg/kg, was employed. Interestingly, 2-BrEA-induced lesions are associated with the renal vasculature,

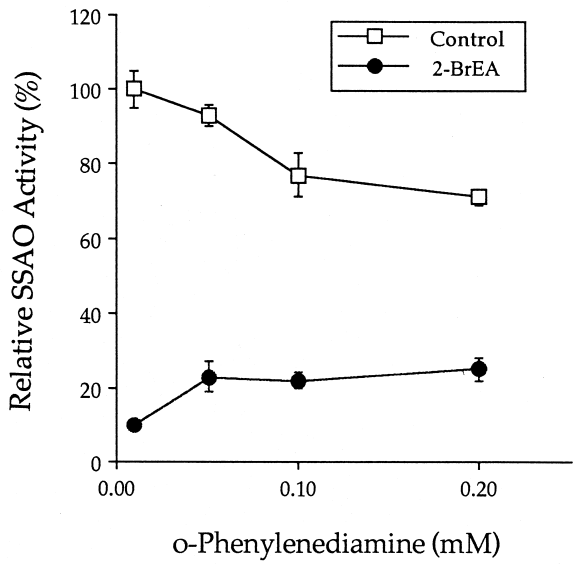

Fig. 8. Effect of o-PD on 2-BrEA-induced inhibition of human umbilical SSAO. o-PD (from 5×10^{-5} to 2×10^{-4} M) was included in the enzyme incubation mixture with 1×10^{-5} M 2-BrEA (●) or without 2-BrEA (□). The specific activities were 0.32 ± 0.02 and 0.03 ± 0.01 nmol/min/mg protein for the untreated and 2-BrEA-treated SSAO, respectively. Each value is the mean ± SEM of at least three independent experiments.

where SSAO is located. It is quite possible that the endothelial damage is caused by the generation of 2-bromoacetaldehyde via deamination of 2-BrEA, before it inactivates the SSAO activity completely. SSAO is well known to be responsible for the bioactivation of allylamine, i.e. by production of acrolein, which induces severe vascular damage [30]. However, 2-BrEA at a low dose is sufficient at inhibiting SSAO activity and does not seem to cause any apparent toxicity. Whether 2-BrEA or its structurally modified analogues have any pharmaceutical implications remains to be established. Nevertheless, the highly selective inhibition of SSAO by 2-BrEA could be a very useful tool in the study of this enzyme.

Acknowledgments

The authors wish to thank Lisa Shira-Bock and Yuchen Wang for technical assistance, Dr. D. A. Durden for mass-spectrometric analyses, and the Medical Research Council of Canada and Saskatchewan Health for their continuing financial support.

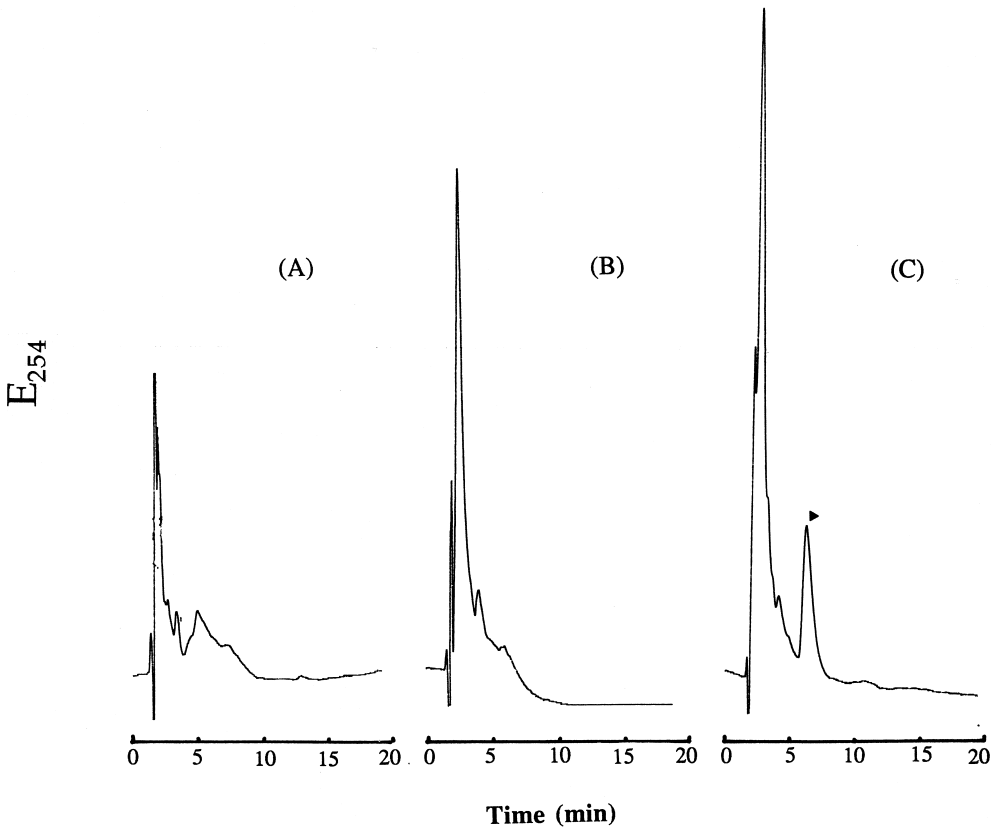

Fig. 9. HPLC detection of aldehyde product generated from SSAO-mediated deamination of 2-BrEA. 2-BrEA (1×10^{-5} M) was incubated with rabbit serum SSAO (C). Blanks are those incubations in the absence of enzyme (A) or 2-BrEA substrate (B). Details of the HPLC conditions are indicated in "Materials and methods."

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