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Short Communication

AMINOACETONE METABOLISM BY SEMICARBAZIDE-SENSITIVE AMINE OXIDASE IN RAT AORTA

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Abstract—High speed (105,000 *g*/60 min) membrane fractions from rat aorta homogenates metabolized the aliphatic amine aminoacetone (AA) to methylglyoxal (MG) with a K_m of 19 ± 3 μM, and V_{max} of 510 ± 169 nmol MG/hr/mg protein. This deaminating activity appears to be due to a semicarbazide-sensitive amine oxidase (SSAO), which is associated with smooth muscle cells in blood vessels of the rat and other species. AA was a competitive inhibitor (K_i of 28 ± 6 μM) of the metabolism of benzylamine, a synthetic amine often used as an assay substrate for SSAO. AA is produced endogenously from mitochondrial metabolism of threonine and glycine, and thus could be a physiological substrate for SSAO, whereas the production of MG by SSAO could have cytotoxic implications for cellular function.

Key words: methylglyoxal; aliphatic amines; benzylamine; smooth muscle

Mammalian blood vessels contain an amine oxidase enzyme which metabolizes the synthetic amine BZ†, and which is sensitive to inhibition by 1 mM semicarbazide and related "carbonyl" reagents. This SSAO can be distinguished from mitochondrial MAO which also metabolizes BZ, by being insensitive to inhibition by certain MAO-selective inhibitors [1, 2]. Also SSAO appears to be found predominantly on the plasma membrane especially in smooth muscle cells which are the major site of SSAO in blood vessels [3, 4]. Although SSAO in some species has modest deaminating activity towards certain physiologically-occurring **aromatic** amines (e.g. tyramine, dopamine, tryptamine, β-phenylethylamine), the enzyme in human vasculature is poor at degrading these aromatic amines, and the identity of the physiological substrate(s) for SSAO and its possible importance not only in vascular smooth muscle but also in other cell types (e.g. adipocytes, chondrocytes) where it occurs, remains to be determined. In this respect, such species-related differences in the substrate specificity of SSAO have further complicated attempts to resolve this issue [1].

Recently, however, SSAO was shown to deaminate *in vitro* some endogenously-occurring **aliphatic** amines, producing aldehyde metabolites with potentially cytotoxic properties. For example, MA, a dietary amine which is also produced *in vivo* by cellular metabolism of creatinine, sarcosine and adrenaline, is deaminated to formaldehyde by SSAO in vascular and adipose tissue homogenates from man and other species [5–10]. Furthermore, urinary MA excretion was enhanced after administration of SSAO inhibitors to rats [11]. We have also shown that AA, an aliphatic amine product of the mitochondrial metabolism of glycine and threonine is deaminated to MG by SSAO in membrane fractions from human umbilical artery, with an apparent K_m (92 μM) for metabolism by the human enzyme being much lower than K_m values of other monoamines previously considered as possible physiological substrates [12]. AA is also a substrate for SSAO in bovine lung microsomal fractions [13] and these recent results with "membrane-bound" SSAO are of interest in relation to earlier reports on the solubilization and purification of an "aminoacetone oxidase" from goat liver [14] and the existence of a soluble SSAO in bovine and goat plasma capable of metabolizing AA [15, 16]. In order to gain more information about the potential for AA being a potential physiological substrate for SSAO in various species, the current study has examined the characteristics of deamination of this amine by membrane fractions obtained from the rat aorta.

Materials and Methods

Materials. AA hydrochloride was prepared as described previously [12]. DNP, MG (40% aqueous solution), methylglyoxal dimethyl acetal, reduced glutathione (GSH), glyoxalase I (grade IV from yeast) and pargyline hydrochloride were purchased from the Sigma Chemical Co. (Poole, U.K.). Propargylamine hydrochloride was obtained from the Aldrich Chemical Co. (Gillingham, U.K.). The hydrochlorides of l-deprenyl (selegiline) and MDL 72145 [(E)-2-(3,4-dimethoxyphenyl)-3-fluoro-allylamine] were gifts from Prof. J. Knoll, Semmelweis University (Budapest, Hungary) and Dr M. G. Palfreyman, Merrell Dow Research Institute (Cincinnati, OH, U.S.A.), respectively. [7-^{14}C]BZ hydrochloride (51 mCi/mmol) was obtained from Amersham International (Amersham, U.K.).

Methods: (1) *Tissue homogenates.* Male Sprague–Dawley rats (weighing 350–550 g) were obtained from our Departmental Breeding Colony, Biomedical Services Unit, University of Dundee. In order to obtain adequate amounts of tissue for preparing membrane fractions for use in each experiment, it was necessary to pool aortae from four to six rats at a time involving a total wet weight of around 200 mg. This tissue was homogenized in 1 mM potassium

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† Abbreviations: SSAO, semicarbazide-sensitive amine oxidase; MAO, monoamine oxidase; BZ, benzylamine; MA, methylamine; AA, aminoacetone; DNP, dinitrophenylhydrazine; MG, methylglyoxal.

phosphate buffer pH 7.8 (100 mg tissue/3 mL) and centrifuged at 600 *g* for 10 min. The supernatants were then centrifuged (105,000 *g* for 1 hr at 4°) to prepare membrane fractions which were suspended in the same buffer in a volume of 100 mg original tissue/3 mL for those experiments in which MG formation from AA was estimated. These membrane fractions generally had a protein content of around 0.2–0.4 mg/mL determined by the method of Lowry *et al.* [17]. However, these fractions were used at a further 5-fold dilution in the radiochemical assays for studying inhibitory effects of AA upon [^{14}C]BZ metabolism. These different dilutions were chosen after carrying out preliminary studies with each type of assay to determine conditions for ensuring linearity of product formation with time and protein concentration.

(2) *Estimation of MG formation from AA.* This method is described fully elsewhere [12]. Briefly, assays contained 100 μL membrane fraction, 300 μL 1 mM potassium phosphate buffer (pH 7.8), and 100 μL AA solutions prepared in 0.2 M potassium phosphate buffer (pH 7.8) in order to give required final assay concentrations. Assays were incubated for 60 min at 37°, and resulting MG formation was estimated spectrophotometrically by the absorbance at 550 nm of its DNP adduct. This was achieved by reference to separate standard curves which were obtained by derivatizing standard concentrations of MG as above in the presence of equivalent amounts of these membrane fractions, giving an estimated extinction coefficient for the adduct of 4.35×10^4 cm^{-1} M^{-1} in this series of experiments.

Towards the conclusion of these experiments, we became aware of the fact that commercially-supplied aqueous solutions of MG may sometimes contain other aldehydes and carbonyl-containing impurities which could interfere with the adduct formation or, indeed, derivatize with DNP to render the standard curves inaccurate. Consequently, we carried out additional calibration experiments using high purity MG, prepared immediately before use by hydrolysing 200 μL MG dimethyl acetal in 5 mL 5% (v/v) H_2SO_4, and the stock solution thus prepared (theoretically 330 mM for complete hydrolysis) was then estimated by a spectrophotometric assay method which follows its conversion to *S*-D-lactoylglutathione in the presence of glyoxalase I and GSH, as described by others [18, 19]. In two separate samples of freshly-prepared MG, this analysis gave estimated concentrations of 324 and 289 mM, respectively. Similarly, the 40% (v/v) commercial solution of MG was diluted by 500-fold to give solutions nominally of 325 mM, which in two separate experiments were estimated as 306 and 324 mM by the glyoxalase assay. DNP-derivatization was carried out with diluted samples of each of these solutions in the presence of rat aortic membranes, in order to prepare standard curves in which the absorbances measured were related back to the glyoxalase-based determinations of the MG concentrations in the stock solutions used. Individual estimates of extinction coefficients for the DNP-adduct calculated from each experiment were 4.2 and 4.4 ($\times 10^4$) cm^{-1} M^{-1} with freshly-prepared MG, and 3.5 and 4.3 ($\times 10^4$) cm^{-1} M^{-1} with commercial MG. These results suggest that there is little evidence that the extinction coefficients vary markedly whether using freshly-prepared or commercially-obtained MG, indicating that our original calibration experiments with Sigma MG should be valid.

The effects of inhibitor drugs upon AA deamination were studied by preparing these drugs at required concentrations in 1 mM potassium phosphate buffer (pH 7.8), and these were included in assays initially containing 100 μL membrane fraction and 300 μL inhibitor solution. Control assays contained all constituents except inhibitor drug. Assay tubes in these experiments were preincubated for 20 min at 37°, before subsequent addition of AA and continuation of the assay as described above.

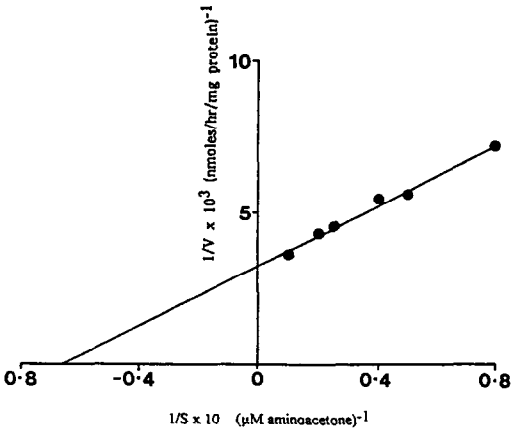

Fig. 1. Representative Lineweaver–Burk plot for AA (12.5–100 μM) metabolism to MG by a high-speed membrane fraction from several pooled rat aortae. Each point is the mean of triplicate determinations.

(3) *Effects of AA upon* [^{14}C]*BZ metabolism.* The kinetics of the interaction between AA and [^{14}C]BZ metabolism was studied by radiochemical assay as described previously [12].

(4) *Data analysis.* Kinetic constants (K_m, V_{max} and K_i) for AA and BZ were estimated by non-linear regression computer analysis using the "EZ-fit" programme from the Higher Education National Software Archive.

Results

AA was converted to MG by high-speed membrane fractions of rat aorta. Kinetic constants for metabolism were obtained by determining rates of AA metabolism at concentrations from 12.5 to 100 μM. Figure 1 shows a representative Lineweaver–Burk plot for one preparation. Mean (± SE) kinetic constants derived from computer programme analysis of data from four different preparations, were 19 ± 3 μM (K_m) and 510 ± 169 nmol MG produced per hour per milligram protein (V_{max}).

The effects of various amine oxidase inhibitors (at 1 mM) upon the deamination of 100 μM AA in three different membrane preparations were also studied (Table 1). These drugs are principally irreversible inhibitors, and their use at concentrations which would produce complete inhibition of MAO and/or SSAO [12] involved an initial preincubation step (20 min at 37°) with the membrane fractions before addition of the substrate. It can be seen that the MAO-selective inhibitors pargyline and l-deprenyl did not inhibit AA metabolism. However, enzyme activity was essentially abolished by propargylamine and MDL 72145. Although the latter would inhibit any MAO activity present, they are also effective inhibitors of SSAO, and in view of the inactivity of pargyline and deprenyl as inhibitors, the results suggest that SSAO is the enzyme responsible for AA metabolism here.

The final series of experiments studied the influence of AA upon the ability of membrane fractions to metabolize BZ, a well-established substrate for SSAO in the rat aorta [20]. The metabolism of BZ (2–20 μM) was determined in the absence and presence of AA (10, 20 and 40 μM). AA proved to be a competitive inhibitor of BZ metabolism, as indicated in the Lineweaver–Burk plot in Fig. 2 showing results from a representative experiment. Computer-based analysis gave a K_i (μM) for AA of 28 ± 6 μM. K_m and V_{max} values for BZ in the absence of AA were 6.6 ± 0.7 μM

Table 1. Inhibitor effects upon aminoacetone metabolism in rat aortic membrane preparations

Inhibitor	Specific activity (nmol MG/hr/mg protein) Prep. 1	Prep. 2	Prep. 3	Mean activity (% of control)
Control	1178	290	431	100
Propargylamine	0	0	42	2
MDL 72145	0	0	42	2
Pargyline	1141	327	403	99
Deprenyl	1178	342	325	97

Membrane preparations were preincubated with 1 mM inhibitors for 20 min before assay of 100 μM aminoacetone metabolism.

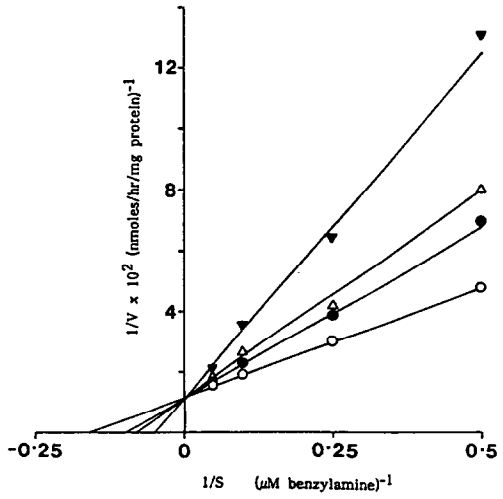

Fig. 2. Representative Lineweaver–Burk plot showing competitive inhibition of [^{14}C]BZ metabolism by AA in a high-speed membrane fraction from several pooled rat aortae. Final AA (μM) concentrations were 0 (○), 10 (●), 20 (△), 40 (▼). Each point is the mean of triplicate determinations.

and 79 ± 15 nmol/hr/mg protein (mean ± SE) from five different preparations.

Discussion

AA was metabolized by high speed membrane fractions of rat aorta to a product whose DNP-derivative had absorbance properties consistent with those of MG. The appearance of this product was prevented by preincubating the membrane fractions with enzyme inhibitors having a spectrum of activity which strongly suggested that MG is produced by oxidative deamination of AA, carried out by a SSAO, which has a widespread distribution in the vasculature of many species [1]. Although DNP is reported to form derivatives with various endogenous carbonyl containing compounds (e.g. glyceraldehyde, dihydroxyacetone phosphate) produced by pathways of sugar metabolism [21], it seems unlikely that membrane fractions isolated from the rat aorta should contain or should spontaneously produce significant quantities of these, but if so, our blank assays where membranes are incubated without AA before carrying out the DNP derivatization should compensate for this possibility [12].

The physiological function and possible role of SSAO in metabolizing endogenous amines is still unclear [1]. This enzyme is usually characterized by its activity towards the non-physiological amine BZ as a high affinity substrate. Many earlier studies have found, as also seen here, that SSAO in rat blood vessel homogenates has a particularly low K_m (around 5 μM) for BZ, compared with SSAO in various other species, and the differences in values obtained has contributed to evidence for the existence of species-related differences in the properties of the enzyme [1]. This metabolism of BZ (studied by radiochemical assay), was inhibited competitively by AA with a K_i value of 28 μM, quite similar to the apparent K_m (19 μM) determined separately (by colorimetric method) for AA as a substrate for rat aortic SSAO. The K_m for AA as a substrate for rat SSAO found here is much lower than K_m values (around 100 μM) previously determined for SSAO in human umbilical artery and bovine lung [12, 13], again reinforcing evidence for heterogeneity in the biochemical properties of this enzyme between species. Previous studies have indicated that the K_m for AA metabolism by the soluble SSAO in goat plasma was 40 μM, whereas an even lower K_m of 9 μM was reported for a purified "aminoacetone oxidase" from goat liver [14, 16].

The idea that metabolism by SSAO of aliphatic amines of environmental and/or physiological origin could produce vascular damage or dysfunction has arisen from a recognition of the potential toxicity of various aliphatic aldehydes (and also of hydrogen peroxide), which are products of oxidative deamination by SSAO. For example, the administration of the industrial aliphatic amine allylamine to laboratory animals produces cardiovascular lesions which can be prevented by SSAO inhibitors and which have been attributed to the metabolism of allylamine to the highly toxic aldehyde acrolein by SSAO primarily in the smooth muscle of blood vessels [22, 23]. Cytotoxic effects of allylamine can also be demonstrated upon cultured aortic smooth muscle cells and these actions are similarly prevented by SSAO inhibitors [24, 25]. Human cultured endothelial cells which do not contain SSAO activity themselves, become sensitive to cytotoxic injury upon exposure to methylamine if SSAO in human umbilical artery homogenate or plasma is included in the culture medium to convert methylamine to formaldehyde. This has suggested that endothelial damage in blood vessels could result from the metabolism of endogenous aliphatic amines to aldehydes either by the plasma enzyme or by the membrane-bound SSAO in the sub-endothelial smooth muscle of the vascular wall [26].

While SSAO can convert AA *in vitro* to MG, another potentially cytotoxic aldehyde capable of producing tissue dysfunction by reacting with cellular macromolecules [21], it still remains to be investigated if this represents a significant metabolic route for MG formation *in vivo*. The

major source of endogenous MG is assumed to be from glucose metabolism, via triose phosphate formation [21]. Although L-threonine can be converted to AA by the mitochondrial enzyme L-threonine dehydrogenase, Ohmori *et al.* [27] found little evidence for significant amounts of MG being produced when incubating threonine with rat liver mitochondria, and concluded that threonine is a minor precursor for MG in mammalian tissues. However, it should be noted that AA is not a substrate for the MAO enzyme found in mitochondria [12], and since SSAO does not appear to be associated with this subcellular site [1], and the liver is a poor source of SSAO anyway, a deamination route for MG formation from threonine is unlikely to have been detected under the experimental conditions used.

The present study showing that rat aortic SSAO metabolizes AA to MG, extends our previous findings with SSAO in tissues of bovine and human origin [12, 13]. In all of these cases, it is of interest that SSAO has a lower apparent K_m (suggesting a higher affinity) for AA than for methylamine, another endogenously-occurring aliphatic amine previously proposed as a possible physiological substrate for SSAO. The possibility that SSAO can metabolize aliphatic amines such as these *in vivo* to produce physiologically- and/or toxicologically-active products is clearly of interest for further investigation.

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