Contribution of Serum and Cellular Semicarbazide-Sensitive Amine Oxidase to Amine Metabolism and Cardiovascular Toxicity

D. J. Conklin, S. D. Langford, and P. J. Boor*

Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0609

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Semicarbazide-sensitive amine oxidase (SSAO) plays a role in the in vivo and in vitro toxicity of several environmental and endogenous amines. We investigated the role of SSAO as a component of cell culture medium (through addition of fetal calf serum (FCS)) compared to intracellular SSAO in the in vitro cytotoxicity of three amines and metabolites. Smooth muscle cells and beating cardiac myocytes were grown in 96-well plates and exposed to various concentrations and combinations of FCS in medium, amines (allylamine, AA; benzylamine, BZA; and methylamine, MA), and amine metabolites (aldehydes: acrolein, benzaldehyde, and formaldehyde; hydrogen peroxide, H2O2; ammonia, NH3). Amine and amine metabolite cytotoxicity was quantified by monitoring cell viability. SSAO activity was measured in FCS, cardiovascular cells, or rat plasma by a radioenzymatic assay using [14C]BZA. Our data show that AA and its aldehyde metabolite, acrolein, were the most toxic compounds to both cell types. However, AA toxicity was FCS-dependent in both cell types, while BZA, MA, and amine metabolite (i.e., aldehydes, H2O2, and NH3) cytotoxicity showed little FCS dependence. In these experiments, medium containing 10% FCS had a calculated amine metabolic capacity that was 30- to 50-fold that of the cultured smooth muscle cellular content in a single well of a 96-well plate. Our study demonstrates that SSAO in FCS contributes to amine metabolism and cytotoxicity to rat cardiovascular cells in vitro and how critical it is to evaluate serum for its role in mechanisms of amine toxicity in vitro and in vivo. © 1998 Society of Toxicology.

Key Words: SSAO; amines; vascular metabolism; cytotoxicity; vascular smooth muscle cells; allylamine; benzylamine; methylamine.

The semicarbazide-sensitive amine oxidase (SSAO; EC 1.4.3.6) enzymes are amine oxidases distinct from known monoamine oxidases that share several common features, including insensitivity to monoamine oxidase inhibitors (e.g., clorgyline and deprenyl), predilection for aliphatic amines as substrates, and inhibition by carbonyl-containing compounds, such as semicarbazide, from which the current name is derived (Lyles, 1995; Yu, 1990). The role of plasma- and tissue-bound SSAO in amine metabolism and toxicity in mammals has been the subject of recent intense interest (see review by Lyles, 1995, 1996; Yu et al., 1997).

A wide range of SSAO activity is measured in bovine, equine, porcine, rodent, and human plasma and serum (Lewinson et al., 1978; Lyles, 1996). While the highest SSAO activity is present in the aorta of mammals, including humans and rats (Lewinson et al., 1978), the source of plasma SSAO, presumably a secreted form, is unknown. The demonstration of elevated plasma and tissue SSAO in diabetics and experimental rat models of diabetes has prompted recent interest in the role of SSAO in human vascular disease (Boomsma et al., 1995; Hayes and Clark, 1990). Plasma SSAO is increased in humans with late-stage diabetes and correlates with the levels of glycosylated hemoglobin, an indicator of the severity of vascular disease in diabetics (Boomsma et al., 1995; Thorntalley et al., 1996). Plasma SSAO is elevated in patients with congestive heart failure and further elevated in patients with concomitant diabetes (Boomsma et al., 1997). These observations suggest a clinical relevance of circulating SSAO in the development of cardiovascular diseases.

The physiological function of SSAO is unknown, but a role in endogenous amine metabolism is supported by experimental data that show decreased endogenous amine metabolism following administration of the SSAO inhibitors, semicarbazide or hydralazine, to rats (Elliot et al., 1989; Lyles and McDougall, 1989) and metabolism of the trace endogenous amine, methylamine, by vascular homogenates (Boor et al., 1992; Precious et al., 1988; Yu and Zuo, 1993). Several endogenous amines have been proposed as substrates for SSAO, but perhaps the most extensive studies have defined a role for vascular SSAO in the metabolism of the exogenous, unsaturated 3-carbon aliphatic amine, allylamine (AA, 3-aminopropene). AA is a specific cardiovascular toxin used to model myocardial necrosis and atherosclerosis (Boor et al., 1979; Boor and Hysmith, 1987; Will et al., 1971). Administration of semicarbazide, a relatively specific SSAO inhibitor, blocks AA toxicity.
**RESULTS**

**Comparative Amine Cytotoxicity**

Allylamine. AA was significantly more toxic (>100–1000×) than BZA or MA to rat smooth muscle cells in medium with 10% FCS (Fig. 1). AA was toxic to VSMC and beating cardiac myocytes with similar LC50s in the presence of medium with 10% FCS (Figs. 2A and 2B, Table 1). AA cytotoxicity to all cardiovascular cells was dependent on the % FCS in the medium with little AA cytotoxicity occurring at <2.5% FCS (LC50 > 10 mM; Fig. 2, Table 1; data from 2.5% FCS experiments not shown). Similarly, there was little change
FIG. 1. Comparative cytotoxicity of allylamine (AA), benzylamine (BZA), and methylamine (MA) in cultured adult rat aortic vascular smooth muscle cells following 24 h exposure in medium with 10% fetal calf serum in 96-well plates as determined by MTT absorbance. Points represent means ± SE of four wells (standard error was less than 5% for each point). Experiments were repeated at least three times with different passages with similar results.

FIG. 2. Dependence of allylamine cytotoxicity to cultured (A) adult rat aortic vascular smooth muscle cells and (B) neonatal rat beating cardiac myocytes on the concentration of fetal calf serum (FCS) in the culture medium as determined by MTT absorbance. Points represent means ± SE of four wells (A) and of a single well (B) per % FCS and per allylamine concentration. Experiments were repeated at least three times with different passages (adult rat aortic vascular smooth muscle cells) or with three different primary cell lines (neonatal rat beating cardiac myocytes) with similar results.

in the LC50 following prolonged incubation (48–96 h) of AA in VSMC in medium with 10% or 1.1% FCS (data not shown). Following 8 h exposure, AA cytotoxicity was evidenced by extensive plasma membrane blebbing and cell lysis in all cell types.

Benzylamine. BZA was toxic to VSMC only at relatively high concentrations following 24 h exposure in medium with 10% FCS (LC50 = 1–10 mM; Fig. 1, Table 1). BZA cytotoxicity to VSMC occurred within 2–6 h and showed little dependence on the % FCS in the medium (Table 1). However, a threefold increase in the LC50 of BZA was observed in VSMC in 10% FCS between 24 and 48 h exposure (data not shown).

Methylamine. MA was the least toxic of all amines to VSMC following 24 h exposure in medium with 10% FCS (LC50 = 100–250 mM; Fig. 1, Table 1). Moreover, MA cytotoxicity in VSMC was not dependent on the % FCS in the medium (Table 1). MA cytotoxicity to VSMC occurred <4 h after exposure.

Amine Metabolite Toxicity

Acrolein was by far the most toxic of the amine metabolites, while benzaldehyde was nontoxic to VSMC at 100 mM (Fig. 3, Table 1). Formaldehyde cytotoxicity was ~10-fold less than that of acrolein and ~10-fold greater than that of H2O2 to VSMC (Fig. 3, Table 1). Ammonia was the next to the least toxic of the amine metabolites with an LC50 > 50 mM in VSMC (Fig. 3, Table 1). Aldehyde (acrolein, benzaldehyde, and formaldehyde) and H2O2 cytotoxicity to VSMC following 24 h exposure was independent of the concentration of FCS in the medium (Table 1). In general, amine metabolite cytotoxicity to VSMC occurred within 2–4 h after exposure. The effect of medium concentration of FCS on ammonia cytotoxicity to VSMC was not tested.

Semicarbazide-Sensitive Amine Oxidase Metabolic Capacity and Activity

SSAO metabolic capacity in cell culture medium was highly correlated with the medium concentration of FCS (Fig. 4). Aliquots of medium with FCS metabolized [14C]BZA substrate resulting in high DPM per sample. However, medium with FCS had relatively low SSAO activity when normalized to protein content (Table 2). Male Wistar rat plasma also contained significant SSAO metabolic capacity, but significantly less than that present per microliter of FCS (Table 2). The SSAO metabolic capacity for the medium (with 10% FCS) in a single well of a 96-well plate was significantly greater (~40–400 fold) than that present in the VSMC or beating cardiac myocytes in a single well (Table 2). These calculations were based on the SSAO metabolic capacity per well volume and the
**TABLE 1**

Lethal Concentrations of Amines and Amine Metabolites Producing 50% Reduction (LC50, μM) in Rat Vascular Smooth Muscle Cell MTT Absorbance in 96-Well Plates Following 24 h Exposure

<table>
<thead>
<tr>
<th>Compound</th>
<th>0.1</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allylamine</td>
<td>&gt;10,000 (5)</td>
<td>17.7 ± 3.2 (6)</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>5,275 ± 634 (4)</td>
<td>4,960 ± 370 (5)</td>
</tr>
<tr>
<td>Methylamine</td>
<td>35,000 ± 7,369 (5)</td>
<td>24,000 ± 3,162 (4)</td>
</tr>
<tr>
<td>Acrolein</td>
<td>6.6 ± 1.1 (3)</td>
<td>6.8 ± 1.8 (7)</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>&gt;100,000 (3)</td>
<td>&gt;100,000 (3)</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>54 ± 13 (3)</td>
<td>63 ± 19 (3)</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>468 ± 158 (4)</td>
<td>351 ± 10 (6)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>ND</td>
<td>23,714 ± 2,784 (7)</td>
</tr>
</tbody>
</table>

Note. Abbreviations; FCS, fetal calf serum; DMEM, Dulbecco’s modified eagle’s medium; ND, not determined. Values, means ± SE from rat aortic and neonatal rat heart vascular smooth muscle cells. (n), number of plates used in determining LC50. No statistically significant differences were observed between compound LC50s in 0.1 and 10% FCS (except for allylamine).

**FIG. 4.** Semicarbazide-sensitive amine oxidase metabolic capacity and calculated well concentration in the culture medium (DMEM) containing varying concentrations of fetal calf serum (FCS) or of cardiovascular cells used in this study. SSAO capacity was measured using [14C]BZA as substrate (1 μM) and incubating for 1 h at 37°C. Estimates of well concentration are calculated from SSAO metabolic capacity measurements and the medium volume of a single well. Values represent means ± SE from duplicate samples. Similar results were obtained in duplicate experiments.

**FIG. 3.** Comparative cytotoxicity of amine metabolites (acrolein, benzaldehyde, formaldehyde, hydrogen peroxide, and ammonia) in cultured adult rat aortic vascular smooth muscle cells in medium with 10% fetal calf serum as determined by MTT absorbance. Points represent means ± SE of four wells. Experiments were repeated at least three times with three different passages with similar results.

**DISCUSSION**

Several recent studies have implicated the heterogeneous plasma and tissue-bound forms of SSAO in endogenous and exogenous amine metabolism, toxicity, and the development of cardiovascular disease in vivo (Boor et al., 1987, 1990; Lyles and McDougall, 1989; Strolin Benedetti and Dostert, 1994; Yu and Zuo, 1996; Yu et al., 1997). Surprisingly, our data show that FCS in culture medium contains a high level of SSAO metabolic capacity, which figures prominently in amine metabolism and cytotoxicity, specifically that of allylamine (AA), in vitro.

**Comparative Amine Cytotoxicity**

In our study, AA was the most toxic of the three amines tested. There was a surprising 3 and 4 order of magnitude...
greater sensitivity of cultured VSMC to AA compared with BZA and MA. These data suggest that either (1) an extremely large difference in SSAO affinity exists for these different amines, or (2) smooth muscle cells have greatly varying sensitivity to amine metabolites. The former seems unlikely with regards to vascular smooth muscle SSAO, since all these amines are readily metabolized by vascular homogenates with $K_m$'s in the 1-250 μM range (Boomsma et al., 1992; Lyles, 1995, 1996; Precious et al., 1988; Yu, 1990; Yu and Zuo, 1993).

We know that AA cytotoxicity in vitro is dependent on its metabolism by SSAO, since SSAO inhibitors, like semicarbazide, prevent cell death (Boor and Nelson, 1980; Ramos et al., 1988). However, until our present study, the previous assumption has been that the source of the SSAO was the smooth muscle cell. In the present study, AA cytotoxicity to VSMC and beating cardiac myocytes was dependent on the concentration of FCS in the culture medium (see Figs. 2A and 2B). Cytotoxicity of the other two amines, however, was not FCS-dependent, suggesting that FCS was preferentially influencing AA effects, presumably via AA-oxidative deamination by SSAO.

These data suggest that earlier reports of AA toxicity in rat VSMC and beating cardiac myocytes are due to the presence of FCS in medium and not due to the direct effects of AA mediated solely by intracellular SSAO (Ramos et al., 1988; Ramos and Thurlow, 1993; Toraason et al., 1989). In fact, Ramos and Thurlow (1993) suggest as much by noting significantly diminished AA cytotoxicity in rat VSMC when FCS was absent from the culture medium. Furthermore, in the present study, serum reduction completely protected beating myocytes from AA toxicity (Fig. 2B). In contrast to our study, Hysmith and Boor (1988) performed an AA cytotoxicity study using porcine aortic VSMC exposed to AA in buffered saline without FCS present. They found toxic sensitivity in porcine aortic VSMC to AA similar to that in rat VSMC, which suggests a direct effect of AA on porcine aortic VSMC. This may represent a real species difference in smooth muscle cellular SSAO affinity, activity, location, or metabolite detoxification capacity or pathways.

While our study clearly implicates a contribution of FCS to AA cytotoxicity, this is apparently not the case for MA. Much interest has focused on MA as a potential physiological substrate for SSAO. Furthermore, MA metabolism has been suggested as a possible mechanism in the development and progression of vascular disease associated with diabetes and stress (Boomsma et al., 1995, 1997; Thornalley et al., 1996; Yu and Zuo, 1993, 1996; Yu et al., 1997). These studies report elevated urinary MA, elevated plasma SSAO, and a correlation between plasma SSAO values and glycosylated hemoglobin, an indicator of the severity of vascular disease in diabetics (Boomsma et al., 1995, 1997; Kapeller-Adler and Toda, 1932; Thornalley et al., 1996).

Two independent studies—the present study and that of Yu and Zuo (1993)—have shown that SSAO in FCS does not readily metabolize MA in vitro. On the other hand, Yu and Zuo (1993) have shown that human serum SSAO is capable of metabolizing MA (i.e., 3.6 nmol hr$^{-1}$ ml$^{-1}$). Furthermore, incubation of human umbilical vein endothelial cells in human serum with MA resulted in concentration-dependent cytotoxicity that was inhibited by a specific SSAO inhibitor, MDL 72145 (Yu and Zuo, 1993). Therefore, it appears that SSAO from differing serum sources may vary dramatically in amine affinity and metabolic capacity. However, in the case of BZA, which is a preferred substrate for SSAO ($K_m$: 1–10 μM) and is used to measure SSAO activity (Lyles, 1995, 1996; Yu and Zuo, 1993), the low relative BZA toxicity to VSMC must be dependent on something besides the affinity for BZA of SSAO present in FCS or VSMC.

**Comparative Amine Metabolite Cytotoxicity**

Whatever the amine substrate, SSAO oxidative deamination results in formation of an aldehyde, H$_2$O$_2$, and ammonia. The reactive aldehyde, acrolein, is formed from AA metabolism by SSAO, and is believed to be the distal toxin in vivo (Boor, 1983; Boor et al., 1987, 1990). Our results from the comparative toxicity of the amine metabolites would agree with the hypothesis that acrolein is the distal toxin for AA, and—except in the case of benzaldehyde—aldehydes are the most toxic of the amine metabolites. Moreover, the cytotoxicity of metabolites had only slight FCS dependence, further supporting the idea that AA cytotoxicity, but not the cytotoxicity of its metabolites, is at least dependent on FCS.

It is likely that BZA cytotoxicity in smooth muscle cells was due to H$_2$O$_2$ formation and not benzaldehyde formation, which was surprisingly nontoxic. In contrast, formaldehyde and H$_2$O$_2$ are both more toxic than the parent amine, MA, which is notably nontoxic. Formaldehyde is probably the distal toxin when endothelial cells are exposed to MA and when concentrated umbilical artery SSAO is supplemented in the culture medium (Yu and Zuo, 1993). Our data show, however, that MA is not metabolized sufficiently by smooth muscle cells or FCS in culture medium to produce either formaldehyde or H$_2$O$_2$ in significant, cytotoxic concentrations.

**SSAO Activity and Amine Metabolic Capacity**

In our study, cellular SSAO activity varied with the cultured rat cardiovascular cell type, much as they did in our earlier study of cultured porcine vascular-derived cells (Hysmith and Boor, 1987). In that study, AA cytotoxicity was positively correlated with cellular SSAO activity (VSMC > fibroblasts > endothelial cells), which was not true in the present study where VSMC and beating myocytes were equally sensitive to AA cytotoxicity, although beating cardiac myocytes contained only 1/10th of the SSAO activity of VSMC. These data further implicate the role of SSAO contained in FCS to AA cytotoxicity.

The SSAO contained in FCS has been largely overlooked in
regard to its contribution to amine metabolism and toxicity in vitro. Calculating specific activity obscures the metabolic capacity and, moreover, says nothing about amine affinity. In our study, two points were striking: (1) 10% FCS in the medium in a well of a 96-well plate had ~30- to 40-fold greater SSAO metabolic capacity than that of the VSMC and ~400 times greater than beating cardiac myocytes, and (2) the SSAO activity ratio of FCS/smooth muscle cells was ~0.1. These calculations confirm that enzyme activity per se does not reflect the contribution made to total amine metabolic capacity in an in vitro system.

At a low concentration of FCS in the culture medium all three amines (AA, BZA, and MA) were relatively nontoxic to VSMC. Perhaps a critical SSAO metabolic capacity is necessary to generate a significant metabolite load rapidly enough to induce lethal injury. This critical metabolite load probably depletes detoxification pathways (especially glutathione), induces lipid peroxidation, increases reactive oxidant species, induces oxidant stress, and eventually depletes and reduces ATP stores and production (Awasthi and Boor, 1993; Blicharski and Lyles, 1991; Meier and Issels, 1995; Misra et al., 1995; Ramos and Thurlow, 1993). In support of this hypothesis, AA cytotoxicity is visually evident within 8 h after addition of AA in medium with 10% FCS. Furthermore, we calculated that smooth muscle cells (i.e., in a single well of a 96-well plate) in our study had ~1/100th the SSAO capacity of the homogenized umbilical artery added to cultured endothelial cells to achieve MA cytotoxicity (Yu and Zuo, 1993). Cellular SSAO metabolic capacity in a single well of a 96-well plate is clearly too low to generate a sufficient metabolite load, whereas both 5 or 10% FCS produces a metabolite load high enough to cause toxicity (compare the LC50 of acrolein with the theoretical metabolite concentrations; ~10 μM is needed for cytotoxicity; see Fig. 4).

In Vivo Considerations

Rat plasma also contains a relatively high SSAO metabolic capacity. The total metabolic capacity of whole blood (i.e., red blood cells + plasma) SSAO is ~3.5 mmol BZA/h. The calculated total SSAO activity in a single rat aorta, however, is ~15–20 mmol BZA/mg protein/h (where one 100-mg aorta at 1% protein could metabolize ~15–20 mmol BZA/h). Total blood SSAO metabolic capacity, therefore, is ~20% of the total SSAO metabolic capacity of the aorta. Recently, we observed that SSAO metabolic capacity in rat plasma is significantly depressed when the lysyl oxidase and SSAO inhibitor, β-aminopropionitrile (βAPN), is given simultaneously with AA in vivo (D. J. Conklin et al., unpublished observation). The plasma SSAO depression by βAPN is far greater than the inhibition of aortic SSAO observed previously (Trent and Boor, 1994). In combined AA + βAPN treatment, the necrotizing effect of AA on the rat myocardium is remarkably reduced (Kumar et al., 1990). In view of these in vivo and in vitro observations, we suggest, therefore, that SSAO in rat plasma contributes, in part, to the rapid myocardial necrosis that occurs with AA intoxication (Boor et al., 1979).

Conclusions

In summary, our study demonstrates that SSAO in FCS contributes to amine metabolism and cytotoxicity to rat cardiovascular cells in vitro. Similar studies have identified other FCS components that also contribute to amine cytotoxicity. The cytotoxicity of 2-(3-aminopropylamino)ethanethiol (WR-1065) to cultured Chinese hamster ovary cells is due to its metabolism by a Cu2+-dependent amine oxidase, presumed to be a polyamine oxidase, in the culture medium (Meier and Issels, 1995). Similarly, our study demonstrates how critical it is to evaluate serum for its contribution to amine cytotoxicity both in vitro and in vivo.

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