Vasoactive effects of methylamine in isolated human blood vessels: role of semicarbazide-sensitive amine oxidase, formaldehyde, and hydrogen peroxide

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Conklin, D. J., H. R. Cowley, R. J. Wiechmann, G. H. Johnson, M. B. Trent, and P. J. Boor. Vasoactive effects of methylamine in isolated human blood vessels: role of semicarbazide-sensitive amine oxidase, formaldehyde, and hydrogen peroxide. Am J Physiol Heart Circ Physiol 286: H667–H676, 2004; 10.1152/ajpheart.00690.2003.—It is hypothesized that methylamine (MA) and semicarbazide-sensitive amine oxidase (SSAO) activity are involved in the cardio-vascular complications in human diabetics. To test this, we determined the acute vasoactive effects of MA (1–1,000 μmol/l) in uncontracted and norepinephrine (NE; 1 μmol/l)-precontracted human blood vessels used for coronary artery bypass grafts [left internal mammary artery (LIMA), radial artery (RA), and right saphenous vein (RSV)]; 2) tested whether MA effects in LIMA and RSV were dependent on SSAO activity using the SSAO inhibitor semicarbazide (1 mmol/l, 15 min); 3) determined the effects of MA metabolites formaldehyde and hydrogen peroxide in LIMA and RSV; 4) tested whether the MA response was nitric oxide, prostaglandin, or hyperpolarization dependent; 5) measured the LIMA and RSV cGMP levels after MA exposure; and 6) quantified SSAO activity in LIMA, RA, and RSV. In NE-precontracted vessels, MA stimulated a biphasic response in RA and RSV (rapid contraction followed by prolonged relaxation) and dominant relaxation in LIMA (mean ± SE, %relaxation: 55.4 ± 3.9, n = 30). The MA-induced relaxation in LIMA was repeatable, nontoxic, and age independent. Semicarbazide significantly blocked MA-induced relaxation (%inhibition: 82.5 ± 4.8, n = 7) and SSAO activity (%inhibition: 98.1 ± 1.3, n = 26) in LIMA. Formaldehyde (%relaxation: 37.3 ± 18.6, n = 3) and H2O2 (%relaxation: 55.6 ± 9.0, n = 9) at 1 mmol/l relaxed NE-precontracted LIMA comparable with MA. MA-induced relaxation in LIMA was nitric oxide, prostaglandin, and possibly cGMP independent and blocked by hyperpolarization. We conclude that vascular SSAO activity may convert endogenous amines, like MA, to vasoactive metabolites; coronary artery bypass grafts; diabetes; H2O2.

A CURRENT HYPOTHESIS STATES that chronic methylamine (MA) exposure induces vascular injury and promotes vascular disease, including atherosclerosis, in humans via semicarbazide-sensitive amine oxidase-mediated (SSAO) metabolism of MA to injurious metabolites: formaldehyde, H2O2, and ammonia (NH3) (19, 50–52). Recent clinical and experimental studies support such a relationship. Altered plasma MA levels, MA excretion, and elevated plasma SSAO activity are present in human diseases associated with chronic vascular pathology [e.g., diabetes mellitus and uremia (Refs. 3, 5, 6, 27, and 49; for reviews, see Refs. 19 and 51)]. In the case of Type I diabetes, SSAO plasma levels increase at the onset of disease (6) and plasma SSAO activity positively correlates with the amount of glycosylated hemoglobin, an indicator of the severity of complications in human diabetics (5, 43). Similarly, plasma SSAO activity is elevated within 2 wk after streptozotocin-induced diabetes in rats (22). Thus much circumstantial evidence links MA levels and SSAO activity to the development of vascular pathology in diabetic humans, and recently the suggestion has been made that therapeutic inhibition of SSAO could slow the progression of vascular disease (17, 19, 51, 53).

MA is a ubiquitous primary amine derived from a multitude of sources and is preferentially metabolized by SSAO compared with other amine oxidases (16, 33, 47). Methylamine is both an exogenous (present in cigarette smoke and wine and foods) and an endogenous amine, and it is a metabolic end product of diverse compounds, including epinephrine, carbamoylase, creatine, nicotine, and sarcosine (35, 38, 48). MA metabolism in rats and humans appears to be due largely to SSAO activity (16, 34). For example, MA excretion is elevated in rats after the administration of SSAO inhibitors (34) and in humans after the consumption of creatine, certain fish and seafood, and some fruits and vegetables (38). Moreover, MA is metabolized by vascular homogenates, including the rat aorta and human umbilical artery, to formaldehyde (9, 39). Finally, SSAO inhibitors prevent MA toxicity in cultured endothelial cells (47). Thus present evidence supports the concept that MA, exogenous and endogenous, is converted to metabolites, formaldehyde and H2O2, by endogenous SSAO activity.

The plasma and tissue forms of the SSAO (EC 1.4.3.6) enzymes are distinct from the monoamine oxidases (MAO), diamine oxidases, and polyamine oxidases (33). The copper-containing SSAs share common features including insensitivity to MAO inhibitors (e.g., clorgyline, deprenyl), preference for aliphatic amines and the aromatic benzylamine, and inhibition by carbonyl-containing compounds, such as semicarbazide, for which the current name is derived (33, 47). SSAO activity is present in all mammalian cardiovascular tissues tested, including plasma/serum, the aorta, and the heart with the most concentrated SSAO activity present in the mammalian aorta, including those of the human and rat (11, 13, 14, 32, 33, 37, 39, 44). This high level of SSAO activity in the cardiovascular tissues implies functionality, although a specific function for the SSAO enzyme has yet to be established (7, 33, 51).

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To better understand the contribution of MA and vascular SSAO activity to human vascular physiology and pathophysiology, we studied the acute vascular effects of MA in isolated human blood vessels of patients undergoing coronary artery bypass surgery. We tested whether MA-induced vascular effects were dependent on blood vessel SSAO activity by using the SSAO inhibitor semicarbazide. Additionally, we assessed the role of age, endothelial function, and contribution of nitric oxide and prostaglandins in the MA effects in the left internal mammary artery (LIMA). Finally, we measured the SSAO activity present in the three human blood vessel types examined, the LIMA, radial artery (RA), and right saphenous vein (RSV).

**Materials and Methods**

**Human Subjects and Blood Vessels**

Research involving humans and animals in this study adhered to the ethical principles put forth in federal guidelines and the American Physiological Society “Guiding Principles in the Care and Use of Animals” and was approved by the local Institutional Review Board and Institutional Animal Care and Use Committee. Consenting adult humans [age (means ± SE): all, 62.8 ± 1.8 yr; men, 59.9 ± 2.4 yr (~77% of total); women, 71.9 ± 1.9 yr] undergoing coronary artery bypass graft (CABG) surgery at Luther Hospital/Midfolf Clinic (Eau Claire, WI) between 2000 and 2003 were the source of the blood vessels (Institutional Review Board No. T-4028). Unused sections of the LIMA, left and right RA, and RSV were placed in lactated Ringer solution and refrigerated (4°C) at the hospital. Vessels were retrieved between 4 and 16 h after surgery, cleaned of blood, staples, thread, and extraneous tissue, and placed in fresh physiological saline solution (PSS) with glucose (pH 7.4, 4°C). All vessel experiments were begun within 24 h of surgical removal.

**Vascular Ring Physiology**

The human blood vessel segments used were free of overt trauma and relatively free of luminal thrombi and adventitial hematomas. The majority of vessels of each vessel type were uniform in size, but there was variation, and the exact location from where each vessel segment was removed was unknown (e.g., ankle vs. knee region of the RSV). The segment ends were trimmed, and approximately 2- to 3-mm segments (“rings”) were cut. Rings were hung on stainless steel hooks in PSS bubbled with 20% O2–5% CO2 (balance N2) at 37°C. One hook was connected to an isometric strain-gauge transducer (Kent Scientific; Litchfield, CT), whereas the other was attached to a fixed support rod. Transducer signals were fed into a PowerLab analog-to-digital converter and recorded on a personal computer using Chart software (version 3.4.9; IWorx; Dover, NH).

Rat thoracic aortas (TA) were taken from CO2-euthanized 12- to 14-wk-old male Sprague-Dawley rats and ~1-yr-old male Wistar rats, placed in cold PSS, and treated as previously reported (13).

All rings were subjected to the same four initial steps in sequence. Rings were equilibrated to a specified tension for 30 min in the bath [1 g for the RSV and rat TA or 3 g for the LIMA and RA (15)]. Rings were stimulated with 100 mmol/l potassium (HI K+)-PSS to test for viability. Rings were washed three times with PSS over 30 min and reequilibrated to resting tension (3X PSS). Rings were contracted with norepinephrine (NE; 1 or 10 μmol/l, control NE) and then stimulated with ACh (1 μmol/l) to test for the presence of an EDRF [nitric oxide (NO)] response before experimentation [i.e., a contraction and relaxation (C/R) cycle].

Following the standard protocol, each ring was assigned one of four experimental protocols.

**Protocol 1.** Unstimulated rings were exposed to 1 mmol/l MA, formaldehyde, or H2O2 (10 min) or cumulative MA, formaldehyde, or H2O2 concentrations (1, 10, 100, and 1,000 μmol/l), followed by a C/R cycle.

**Protocol 2.** NE-precontracted rings were exposed to 1 mmol/l MA, formaldehyde, or H2O2 (10 min) or cumulative MA concentrations (1, 10, 100, and 1,000 μmol/l) before or after ACh.

**Protocol 3.** Semicarbazide (1 mmol/l, 15 min)-pretreated rings were contracted with NE, followed by exposure to 1 mmol/l MA and then ACh addition (in LIMA and RSV only due to availability).

**Protocol 4.** Nω-nitro-ω-arginine methyl ester (L-NAME; 200 μmol/l, 20 min), indomethacin (Indo; 100 μmol/l, 20 min), or L-NAME + Indo-pretreated rings were stimulated with a C/R cycle, followed by exposure to 1 mmol/l MA (in LIMA only).

After all treatments and 3x PSS washouts, rings were precontracted with HI K+, followed by exposure to ACh (1 μmol/l) and then sodium nitroprusside (SNP; 100 μmol/l). ACh and SNP exposure assessed vessel responsiveness to endogenous and exogenous NO, respectively. To test for MA hyperpolarization, a subset of HI K+ -precontracted rings were exposed to MA, formaldehyde, or H2O2 (1 mmol/l, 10 min) followed by ACh and SNP addition (in LIMA only). The experimental duration was typically 4–6 h.

Vessel contractions were normalized as a percentage of the control NE contraction. Vessel relaxations were calculated as the percent reduction of the agonist-induced contractions (i.e., NE or HI K+). Concentration-concentration-response curves were used to intercalulate the apparent effective concentration producing 50% relaxation (NE-precontracted vessels) or contraction (uncontracted and NE-precontracted vessels; %responses were pooled). Relaxation half-times (t1/2; in s) were calculated for MA (1 mmol/l) and ACh (1 μmol/l) relaxations in NE-precontracted LIMA and for SNP (100 μmol/l) relaxations in HI K+ -precontracted LIMA.

**cGMP ELISA Assay**

To ascertain whether cGMP was involved in MA relaxation, we used longer segments of LIMA and RSV (0.5–1.0 cm, 100–150 mg total) exposed to MA or SNP. The percent relaxation and cGMP level for each vessel were quantified. Briefly, vessel segments were contracted with HI K+ solution, followed by 3x PSS and a C/R cycle (as above). Next, they were precontracted with 1 or 10 μmol/l NE and, when stable, exposed to MA (1 mmol/l) or SNP (100 μmol/l). After the response was allowed to plateau (20–40 min), vessels were wrapped in aluminum foil, frozen in liquid nitrogen, and stored at −80°C until analysis. Unstimulated LIMA and RSV (n = 5 vessels, patient-matched) vessels were used for baseline measurement of cGMP.

Vessel segments were minced on ice and cold homogenized in 0.1 mol/l HCl (25–30 strokes, 0.1 g vessel wet wt/1 ml HCl). A homogenate subsample (50 μl) was frozen for protein analysis, and the remaining samples were centrifuged (14,000 g, 20 min). Supernatants were processed for cGMP analysis according to the ELISA kit manufacturer’s instructions (DirectCyclic GMP Kit, Assay Designs; Ann Arbor, MI) using the acetylated and overnight incubation protocols. Protein was determined with the Bio-Rad Protein Dye Concentration kit (Bio-Rad; Hercules, CA) using BSA as the standard (Sigma; St. Louis, MO).

**SSAO Assay**

Standard assay protocols for measurement of SSAO activity radiometrically using [14C]benzylamine hydrochloride (BZA; 1 μmol/l; 59 μCi/ml, Amersham; Rockford, IL) as the substrate were followed (13, 32). Blood vessel segments were homogenized in Sorensen’s Na+–K+-PBS (0.1 M, pH 7.8) at a ratio of 1 g of sample per 30 ml of buffer using a hand-held glass homogenizer. The homogenate was centrifuged, and 30 μl of supernatant were used in each assay. The SSAO final assay volume was 245 μl with [14C]BZA in PBS (1 μmol/l) and deprenyl in PBS (1 μmol/l). After a 30-min incubation at 37°C, the assay was stopped by the addition of 2 mmol/l citric acid.
SSAO activity was measured in homogenized human LIMA (means ± SE patient age: 66.1 ± 2.0 yr, n = 27), RA (58.1 ± 2.5 yr, n = 12), and RSV (66.4 ± 0.6 yr, n = 5) with or without semicarbazide (1, 10, 100, or 1,000 μmol/l, 20-min preincubation) at 37°C. Protein was determined with the Bio-Rad Protein Dye Concentrate reagent using BSA in saline as the standard (Sigma). SSAO activity was calculated as nanomoles of BZA substrate metabolized per 30 min per milligram of protein. Semicarbazide inhibition was calculated as a percentage of the control SSAO activity (i.e., without semicarbazide = 100%).

**Chemicals and Solutions**

PSS was composed of the following (in mmol/l): 130 NaCl, 4.7 KCl, 1.17 MgSO₄·7H₂O, 1.18 KH₂PO₄, 14.9 NaHCO₃, 2.0 CaCl₂, and 5.0 glucose; pH 7.4. HI K⁺-PSS was composed of the following (in mmol/l): 34.7 NaCl, 100 KCl, 1.17 MgSO₄·7H₂O, 1.18 KH₂PO₄, 14.9 NaHCO₃, 2.0 CaCl₂, and 5.0 glucose; pH 7.4. All other chemicals were purchased from Sigma and dissolved in distilled water except for Indo, which was dissolved in 0.1 mol/l NaHCO₃ in 0.1 N NaOH.

**Statistics**

Values are reported as means ± SE. Statistical comparisons between two groups were performed with Student’s paired or unpaired t-tests and between more than two groups using one-way ANOVA with posttest comparisons using the Student-Newman-Keuls test (SigmaStat, SPSS; Chicago, IL). Statistical significance was assumed at P ≤ 0.05.

**RESULTS**

**MA, Formaldehyde, and H₂O₂ Effects in Isolated Human Blood Vessels and Rat TA**

In preliminary tests, MA (1 mmol/l, 10 min) exposure in NE-precontracted human vessels produced biphasic responses, i.e., initial contraction, followed by prolonged relaxation, in RA and RSV and predominately strong relaxation in LIMA (4 of 30 LIMA were weakly contracted; Fig. 1A). MA-induced contractions in RA and RSV were significantly greater than those in LIMA, whereas MA-induced relaxations were significantly greater in LIMA and RA than in RSV (Fig. 1A and Table 1).

Unlike human blood vessel responses to 1 mmol/l MA, NE-precontracted rat TA were minimally affected by MA, and these responses appeared further blunted in TA of older rats. In contrast, vigorous biphasic responses to 1 mmol/l H₂O₂ were present in both young and old rat TA alike and were similar to the 1 mmol/l H₂O₂–induced responses in human LIMA (Table 1). Formaldehyde (1 mmol/l, 10 min) produced either minimal contraction or relatively strong relaxation, whereas no biphasic responses were observed in any blood vessel tested (Table 1). Formaldehyde-induced relaxations were slow in onset and prolonged in duration, and overt toxicity was seen in only 2 of
18 rat TA and in none of the 6 human vessels exposed, i.e., toxicity was the near-complete loss of a tension response to HI K+ after 3 × PSS washout after the formaldehyde exposure. Vessel sensitivities to MA, formaldehyde, or H2O2 were comparable between toxicants and between vessels (Fig. 1C and Table 2), RA were significantly more sensitive to MA-induced contraction than to MA-induced relaxation, whereas LIMA were more sensitive to H2O2-induced relaxation compared with RSV (Table 2). Similarly, RSV were more sensitive to MA-induced relaxation than to H2O2-induced relaxation (i.e., Fig. 1, B and C, and Table 2). Of the six human vessels tested (3 LIMA and 3 RSV), no vessel responded to formaldehyde at <1 mmol/l (note the same apparent EC50 values for all formaldehyde-induced responses; Table 2).

**MA Relaxation in Isolated Human LIMA**

Because the MA-induced relaxation was the dominant response in the LIMA and because LIMA was the most frequently obtained blood vessel type, we further studied mechanisms of the MA relaxation in LIMA only. MA exposure (1 mmol/l, 10 min) inhibited or reduced NE contractions equally in unstimulated or NE-precontracted LIMA (means ± SE; % reduction of NE-contraction: MA pre-NE, 53 ± 7; MA post-NE, 61 ± 5, n = 5 and 9 vessels, respectively; Fig. 2). The MA-induced relaxation in NE-precontracted LIMA was repeatable, reversible, and nontoxic. In NE-precontracted LIMA, a second MA relaxation was indistinguishable from the first, and a third MA relaxation also was elicited (means ± SE; % relaxation: first relaxation, 72.0 ± 7.5; second relaxation, 60.8 ± 12.6; third relaxation, 82.1, n = 5, 5, and 1 vessels, respectively). MA exposure had no observable long-lasting inhibitory or toxic effects in any vessel.

**Table 1. Contraction or relaxation responses in NE-precontracted isolated human blood vessels and the rat thoracic aorta after 10-min exposure to 1 mmol/l MA, formaldehyde, or H2O2**

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>MA</th>
<th>Formaldehyde</th>
<th>H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction</td>
<td>0.6 ± 0.3 (30)</td>
<td>3.7 ± 3.7 (3)</td>
<td>15.8 ± 5.3 (9)*</td>
</tr>
<tr>
<td>Relaxation</td>
<td>55.4 ± 3.9 (30)</td>
<td>37.3 ± 18.6 (3)</td>
<td>55.6 ± 9.0 (9)</td>
</tr>
<tr>
<td>RSV</td>
<td>17.4 ± 3.8 (21)</td>
<td>2.6 ± 2.6 (3)</td>
<td>25.4 ± 5.6 (8)</td>
</tr>
<tr>
<td>Relaxation</td>
<td>20.6 ± 4.3 (21)</td>
<td>31.7 ± 15.9 (3)</td>
<td>6.5 ± 3.2 (8)</td>
</tr>
<tr>
<td>Rat thoracic aorta</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction</td>
<td>12.5 ± 7.2 (4)</td>
<td>2.1 ± 2.1 (4)</td>
<td>41.2 ± 11.5 (4)*</td>
</tr>
<tr>
<td>Relaxation</td>
<td>8.3 ± 8.3 (4)</td>
<td>37.5 ± 21.6 (4)</td>
<td>50.8 ± 18.2 (4)</td>
</tr>
</tbody>
</table>

 Values are means ± SE [in %change from norepinephrine (NE; 1 μmol/l)-precontracted tension]; numbers in parentheses are numbers of vessels. The young Sprague-Dawley rats were 18–20 wk old, and the old Wistar rats were 1 yr old. MA, methylamine; LIMA, left internal mammary artery; RSV, right saphenous vein. *Significant difference between value with asterisk and other toxicant values for same vessel and same response or significant difference only between values with asterisks for same vessel (P < 0.05).

**Table 2. Apparent effective concentrations producing EC50 in isolated human blood vessels to cumulative concentrations (1–1,000 mmol/l) of MA, formaldehyde, and H2O2**

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>MA</th>
<th>Formaldehyde†</th>
<th>H2O2</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction</td>
<td>Not observed</td>
<td>315 (1)</td>
<td>175 ± 58 (5)</td>
</tr>
<tr>
<td>Relaxation</td>
<td>230 ± 30 (8)</td>
<td>315 ± 0 (2)</td>
<td>162 ± 41 (9)</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction</td>
<td>65 ± 48 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Relaxation</td>
<td>280 ± 40 (3)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RSV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Contraction</td>
<td>200 ± 52 (6)</td>
<td>315 (1)</td>
<td>220 ± 38 (8)</td>
</tr>
<tr>
<td>Relaxation</td>
<td>230 ± 28 (5)*</td>
<td>315 ± 0 (2)</td>
<td>315 ± 0 (4)*</td>
</tr>
</tbody>
</table>

Values are means ± SE (in μmol/l interpolated from cumulative response curves of uncontracted and NE-precontracted blood vessels); numbers in parentheses are numbers of vessels. Uncontracted vessel experimental data were not observably different from sensitivity data of contraction in NE-precontracted vessels and thus were pooled (where performed). RA, radial artery; ND, not determined. *Significant difference between values with asterisks for same vessel (P < 0.05). †The apparent EC50 for formaldehyde was derived from responses at 1 mmol/l only; thus the value may be a gross underestimate of the true EC50.
responses in RSV without effect on subsequent reactivity (data not shown; n = 2).

**ACh, Nitric Oxide, and Prostanoids in MA-induced Relaxation in LIMA**

ACh-induced relaxations were most often present in LIMA compared with RA or RSV (ACh addition usually produced small contraction in RSV; data not shown). The percent MA-induced relaxation was vessel specific and, in general, was associated with the ACh response, i.e., LIMA had the strongest ACh and MA relaxations, RSV had the strongest MA contractions and the weakest ACh and MA relaxations, and RA produced both strong MA contractions (equal to RSV) and strong ACh and MA relaxations (equal to LIMA; see Fig. 1A).

Despite the general positive association between ACh-induced relaxation and MA-induced relaxation observed across vessels, these two responses were not correlated within LIMA.

In LIMA, the MA relaxation, on average, was greater than two times stronger than the ACh-induced relaxation regardless of the order of addition (i.e., means ± SE; ACh added before or after MA: pre-MA ACh, 25 ± 6%; post-MA ACh, 14 ± 6%, n = 21 and 20 vessels, respectively). More specifically, the percent MA relaxation was not correlated with the percent ACh relaxation or with patient age (a variable that was significantly correlated with the percent ACh relaxation in LIMA; Fig. 4A).

L-NAME pretreatment (200 μmol/l, 20 min) in a subset of LIMA rings possessing a relatively strong ACh relaxation significantly inhibited the ACh-induced relaxation but had no effect on the MA-induced relaxation or with patient age (a variable that was significantly correlated with the percent ACh relaxation in LIMA; Fig. 4A). L-NAME pretreatment (200 μmol/l, 20 min) in a subset of LIMA rings possessing a relatively strong ACh relaxation significantly inhibited the ACh-induced relaxation but had no effect on the MA-induced relaxation or with patient age (a variable that was significantly correlated with the percent ACh relaxation in LIMA; Fig. 4A). L-NAME pretreatment (200 μmol/l, 20 min) in a subset of LIMA rings possessing a relatively strong ACh relaxation significantly inhibited the ACh-induced relaxation but had no effect on the MA-induced relaxation (Fig. 4A). In addition, neither Indo alone (100 μmol/l, 20 min) nor L-NAME + Indo pretreatment affected the MA-induced relaxation in NE-precontracted LIMA (Fig. 4C). Furthermore, the mean relaxation t½ for MA-induced relaxation was significantly longer in duration than the t½ for either ACh-induced relaxation in

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**Fig. 3.** A: representative tracings of responses of control and semicarbazide (Semi; 1 mmol/l, >15 min)-pretreated isolated LIMA rings from one patient to NE (1 μmol/l), MA (1 mmol/l, 10 min), and ACh (1 μmol/l). B: Semi significantly blocked MA-induced relaxation but had no effect on NE contraction and ACh relaxation in LIMA rings (n = 8–9). Values are means ± SE and are presented as a percentage of the control NE-induced contractions; n, number of vessels. *Significant difference from MA control.
SSAO AND METHYLAMINE-INDUCED VASCULAR RELAXATION

Table 3. Vascular responses were intact after MA exposure or after Semi + MA exposure in isolated human LIMA

<table>
<thead>
<tr>
<th></th>
<th>MA alone</th>
<th>Semi + MA</th>
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<tbody>
<tr>
<td>HI K⁺</td>
<td>102±23</td>
<td>127±24</td>
</tr>
<tr>
<td>ACh</td>
<td>20±11</td>
<td>16±7</td>
</tr>
<tr>
<td>SNP</td>
<td>90±11</td>
<td>83±11</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 7 vessels. High potassium (HI K⁺; 100 mmol/l) contractions and ACh (1 μmol/l) relaxations and sodium nitroprusside (SNP; 100 μmol/l) relaxations were performed in LIMAs after prior exposure to MA (1 mmol/l, 10 min) or semicarbazide pretreatment plus MA (Semi + MA, 1 mmol/l, 15 min) and three bath changes with physiological saline solution. HI K⁺ contractions were calculated as a percentage of the control NE contraction; ACh and SNP relaxations were calculated as the percent reduction in the HI K⁺ contraction.

Role of cGMP in MA-induced Relaxation in Human LIMA

Because most vascular relaxations depend on cGMP production in vascular smooth muscle cells, we investigated the role of cGMP in MA-induced relaxation in LIMA and RSV. SNP (100 μmol/l) stimulated a statistically significant three-fold increase in cGMP levels (in pmol/mg protein) in NE-precontracted RSV, and the cGMP level was also increased in SNP-stimulated LIMA (%relaxation: 100; cGMP: 1.520, n = 1 vessel) but not in RA (%relaxation: 73.9; cGMP, 0.099, n = 1 vessel) (Table 4). However, 1 mmol/l MA exposure failed to significantly elevate cGMP in either NE-precontracted LIMA or RSV despite producing significant, long-lasting relaxations (Table 4). A strong positive correlation was observed in LIMA between the MA-induced %relaxation and the %B/B₀ value (i.e., used to interpolate cGMP levels; r² = 0.70, n = 9), but a weaker relationship was present when the picomole cGMP levels were normalized to protein levels (r² = 0.48, n = 9; data not shown).

Human Blood Vessel SSAO Activity, Patient Age, and SSAO Inhibition

Mean SSAO activities were similar among all three human blood vessel types (Fig. 5A). For the age range examined, SSAO activity was not correlated with patient age for any vessel (Fig. 5B) but was significantly correlated between patient-matched LIMA and RSV (r² = 0.76, P = 0.054, n = 5 vessels; data not shown) and similarly inhibited by semicarbazide (1, 10, 100, and 1,000 μmol/l) in all three blood vessel types.

Fig. 4. Relationships among MA (1 mmol/l, 10 min)-induced relaxation, ACh (1 μmol/l)-induced relaxation, and age in isolated NE (1 mmol/l)-precontracted LIMA (n = 20) were investigated. A: percent MA-induced relaxation was not correlated with the percent ACh-induced relaxation (r² = 0.0001) or patient age, although ACh relaxation and patient age were significantly correlated (r² = 0.23, P = 0.014). B: LIMA were pretreated with the nitric oxide synthase inhibitor N⁵-nitro-L-arginine methyl ester (1-NAME; 200 μmol/l, 20 min), indomethacin (Indo; 100 μmol/l, 20 min), or 1-NAME + Indo. 1-NAME pretreatment significantly reduced ACh-induced relaxation in a subset of LIMA (n = 9) with strong ACh-induced relaxations (ACh control: ≥15% relaxation) but had no effect on MA-induced relaxation (MA + 1-NAME). Values are means ± SE; n, number of vessels. *Significantly different from all other treatments.
types (Fig. 5; IC<sub>50</sub>: LIMA, 16.7 ± 19.5 μmol/l; RA, 21.2 ± 11.2 μmol/l; RSV, 14.7 ± 13.3 μmol/l, n = 25, 10, and 5 vessels, respectively).

DISCUSSION

This is the first report of the acute vasoactive effects of MA in isolated human blood vessels. Several of our findings clearly appear at odds with the proposed role of MA as a potent vascular toxicant in humans. We find the most prominent effect of MA in isolated human blood vessels is a generally robust yet benign relaxation. This relaxation, highly expressed in LIMA, is dependent on vascular SSAO activity and, more importantly, is reversible and repeatable. It is quite distinct from the SSAO-dependent, yet quite injurious, actions of allylamine, a well-known cardiovascular toxicant, or from effects of the α,β-unsaturated aldehyde acrolein in isolated rat TA, rat coronary arteries, and human blood vessels, where both agents produce vasospasm in rat coronary arteries (12, 13). Our present findings in isolated human blood vessels, while somewhat surprising, suggest that vascular SSAO activity (and possibly MA) may be a source of vasoactive signaling molecules that generally relax blood vessels.

**MA Responses in Human CABG Vessels Are Dependent on Vascular SSAO Activity**

MA effects in isolated human LIMA are clearly dependent on vascular SSAO activity. Data from several experiments support this conclusion. The pretreatment of isolated LIMA with semicarbazide (1 mmol/l, 30–40 min; SNP concentration: 100 μmol/l, 30–40 min). Relaxations were calculated as the percent reduction in the NE-precontracted tension. Control vessels were 5 matched sets of LIMAs and RSVs that were not subjected to NE precontraction. *Significantly greater cGMP level compared with control and MA-exposed RSVs (P < 0.05).

Table 4. Vascular relaxations and cGMP levels after MA or SNP exposure in NE-precontracted human LIMA and RSV

<table>
<thead>
<tr>
<th></th>
<th>%Relaxation</th>
<th>[cGMP], pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.128±0.034</td>
<td>0.034</td>
</tr>
<tr>
<td>+MA, &lt;50% relaxation</td>
<td>34.1±7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>+MA, &gt;50% relaxation</td>
<td>87.8±5.0</td>
<td>0.227±0.066</td>
</tr>
<tr>
<td>RSV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.118±0.028</td>
<td>0.028</td>
</tr>
<tr>
<td>+MA</td>
<td>57.2±6.2</td>
<td>10.1±0.30</td>
</tr>
<tr>
<td>+SNP</td>
<td>100±4.0</td>
<td>0.322±0.065*</td>
</tr>
</tbody>
</table>

Values are means ± SE; numbers in parentheses are numbers of vessels. MA concentration: 1 mmol/l, 30–40 min; SNP concentration: 100 μmol/l, 30–40 min. Relaxations were calculated as the percent reduction in the NE-precontracted tension. Control vessels were 5 matched sets of LIMAs and RSVs that were not subjected to NE precontraction. *Significantly greater cGMP level compared with control and MA-exposed RSVs (P < 0.05).

Moreover, semicarbazide pretreatment and posttreatment inhibition of MA relaxation in LIMA precludes a nonspecific mechanism of action of MA [e.g., MA does not directly block adrenergic receptors (31)].

We show here, for the first time, that three human blood vessels used as CABG vessels possess similar levels of rela-

Fig. 5. A: Semicarbazide-sensitive amine oxidase (SSAO) activity was similar between the homogenized human blood vessels (LIMA, n = 26; RA, n = 12; and RSV, n = 5). B: SSAO activity in human blood vessels was not correlated with the age of the patient (R<sup>2</sup> = 0.0002). C: Semicarbazide inhibition of SSAO activity in homogenized human LIMA (n = 26), RA (n = 12), and RSV (n = 5) was concentration dependent; however, no differences between vessels in the Semicarbazide concentrations producing 50% inhibition (IC<sub>50</sub>) were detected. Blood vessel homogenates were preincubated with Semicarbazide (1, 10, 100, and 1,000 μmol/l; 20 min) and assayed at 37°C. Values are means ± SE and are presented as a percentage of control vessel SSAO activity (i.e., without Semi); n, number of vessels.
tively abundant, age-independent (age range = 45–81 yr) amine oxidase activity that is inhibited by semicarbazide in a concentration-dependent manner (Refs. 13 and 37; although our reported inhibition in human saphenous vein appears more sensitive, IC50 = 500 vs. 14.7 μmol/l, Ref. 37 vs. present study, respectively). Recently, we found similarly abundant SSAO activity in homogenized human coronary arteries of accident victims aged 7–71 yr (13). Human aortic SSAO activity was ~10 times more concentrated than in human coronary arteries, LIMA, RA, RSV, and other human blood vessels but was also age independent (23, 32). Similarly the age of rats (12–14 vs. 52 wk) had little effect on TA responses to MA exposure in our present study. So whereas age and perhaps atherosclerosis (23) appear to have little effect on SSAO expression in the blood vessel wall, the factors important in the regulation of overall SSAO expression (e.g., genetic, diabetes, environmental, etc.) remain to be identified (5, 6, 19, 51).

Dependence on SSAO activity is a hallmark of allylamine cardiovascular toxicity in vivo, in isolated blood vessels, and in cultured cardiovascular cells (2, 8, 52). The cellular toxicity of MA, in contrast, has a more complicated history because MA toxicity is orders of magnitude less toxic than allylamine in cultured cardiovascular cells (14, 30, 52). Whereas allylamine and MA are relatively similar substrates for homogenized rat aortic SSAO activity [Km = 145.2 and 246.7 μmol/l, respectively (47)], human blood vessels have strikingly different Km values for MA [e.g., cerebral microvessels, 22 μM (Ref. 11); umbilical artery, 832 μM (see Ref. 33 for a review)]. While the disparity in toxicity between these two amines is likely due to the metabolism of allylamine to acrolein and MA to the less toxic formaldehyde, it is likely that affinity and catalytic rates and, thus, specificity of various SSAOs for different amines vary dramatically from site to site (11, 33, 51). However, biological activity is not predictable solely based on substrate availability and Km. In our present study, for example, rat aortic responses were minimal in the presence of 1 mmol/l MA despite the fact that the rat aorta possessed high SSAO activity and an adequate affinity (9, 32). Nonetheless, substrate specificity and distribution of SSAOs are likely important to the potential contribution of MA to differential cardiovascular pathology in humans [e.g., atherosclerosis, retinopathy, nephropathy, and coronary artery disease (51)].

**Mechanism of Action of MA Responses in Human LIMA**

Because effects of MA in LIMA and RSV appear dependent on SSAO activity, it follows that the effects of MA are likely due to one or more of MA’s metabolites: formaldehyde, H2O2, and NH3. The MA responses in LIMA and RSV were similar, qualitatively and quantitatively, to both formaldehyde- and H2O2-induced responses. For example, H2O2 (1 mmol/l) pretreatment inhibits subsequent NE contractions in rabbit aorta, whereas in precontracted vessels H2O2 induces contractions, relaxations, or biphasic responses dependent on the blood vessel type (25, 28, 40). More specifically, H2O2 induced biphasic responses in rat TA and human RA and RSV that are similar in appearance to our MA responses in isolated RA and RSV (Ref. 28 and present study). In the arteries, however, weak contractions are similar for MA and formaldehyde exposures, whereas H2O2 is much more efficacious. Notably, vasospasm occurs more often in human RA and RSV compared with LIMA, and our general reactivity findings are consistent with these data (10, 24). Additionally, MA, formaldehyde, and H2O2 effects generally are reversible at 1 mmol/l in the rabbit aorta, rat TA, and human blood vessels (Refs. 25, 28, and 40 and present study). Finally, the apparent EC50 for MA, formaldehyde, and H2O2 in all three human vessels are very similar (approximate range = 200–300 μmol/l), although formaldehyde responses are only observed at 1 mmol/l. Thus we conclude that both formaldehyde and H2O2 produced by SSAO metabolism of MA contribute to the MA-induced vascular responses in human LIMA.

MA-, formaldehyde-, and H2O2-induced relaxation in LIMA may be dependent on vascular smooth muscle cell membrane hyperpolarization via activation of K+ channels because HI K+ precontraction decreases their ability to stimulate relaxation. Hyperpolarization is used in H2O2-induced relaxation of the rat aorta and porcine coronary artery (4, 28), and K+ channels may act as redox-sensitive targets for H2O2 as proposed in the mechanism of hypoxic pulmonary vasoconstriction and hypoxia-induced vasodilation (see Ref. 1 for a review). Moreover, H2O2 is considered an EDHF in human mesenteric arteries but not in the carbachol-induced relaxation in human RA (21, 36). In addition, the relaxation (and generally low toxicity) observed with 1 mmol/l formaldehyde in human blood vessels, while surprising, is not without precedent because formaldehyde (660 μmol/l) relaxes NE-precontracted but not 25 mmol/l KCl-depolarized rabbit aortas by inhibition of Ca2+ influx and NE inactivation in vitro (42). However, we cannot rule out that the strong depolarizing stimulus used in the present study (i.e., 100 mmol/l KCl) may have inhibited relaxation pathways stimulated during NE-precontraction by MA, formaldehyde, and H2O2.

Regardless of which specific metabolite is involved, MA-induced relaxation clearly appears independent of endothelial NO- or prostanooid release in human LIMA. This conclusion is supported by the observation that inhibition of endothelial nitric oxide synthase activity with L-NAME significantly reduced the ACh relaxation but had no effect on the MA relaxation. Yet, if the endothelium is involved, it perhaps is releasing an EDHF in response to MA metabolites. Hamilton et al. (21) propose that blood vessels with reduced EDRF capacity compensate with enhanced EDHF production. Because no inverse relationship was observed between ACh and MA relaxations in LIMA from patients with significantly reduced EDRF, we favor the more likely role of formaldehyde or H2O2 generated at the vascular smooth muscle cell plasma membrane [i.e., the location of SSAO (44)] acting as autocrine and/or paracrine factor(s).

It is unclear whether MA-induced relaxations are dependent on increased cGMP. While the MA-induced relaxation in RSV appears cGMP independent, there is a weak positive association between cGMP levels and the %relaxation to MA in LIMA. However, even though many relaxations are mediated by cGMP, it is possible that the formation of formaldehyde and/or H2O2 at the vascular smooth muscle cell plasma membrane directly relax(es) vessels by activation of K+ channels, thiol oxidation, inhibition of Ca2+ influx, adrenergic inactivation, or some combination of these mechanisms.
Implications for Human Health: Pharmacological and Toxicological Considerations

In the present study, MA at 1–1,000 μmol/l had no easily observable adverse effects in isolated human blood vessels. However, it is clear that massive MA exposure can be lethal in humans. As a result of an accidental spill of purified liquid MA, 35 Chinese persons of 7–71 yr of age and a nearly equal male-to-female distribution were hospitalized 7 to 8 h postexposure with 6 resulting fatalities (46). Overt toxicity in these male-to-female distribution were hospitalized 7 to 8 h postexposure with 6 resulting fatalities (46). Overt toxicity in these patients included significant cardiovascular symptoms of tachycardia and low or “unmeasurable” blood pressure and pulse. These symptoms are consistent with the scenario of severe systemic hypotension, accompanied by reflex tachycardia, declining cerebral perfusion, and ultimate coma (10 of 35 people suffered light to deep coma). On the basis of the MA-induced prolonged and robust relaxation in LIMA, RA, and RSV blood vessels in our study, one might predict severe hypotension would follow a systemic, high-level MA dose [assuming the MA relaxation occurs in the resistance vessels—a testable hypothesis that is supported by the ubiquitous presence of SSAO activity in human conductance and resistance blood vessels (11, 13, 23, 32, 39)]. With the exception of an acute toxic exposure, reaching a toxic level dosage of MA in humans is unlikely. Although MA is present in a variety of common exogenous sources, including wine, cigarette smoke, a variety of foods, and as a metabolite of nicotine, it is unlikely that anyone could reach acute toxic doses by these paths (35, 38, 48), because the estimated normal human plasma [MA] = <1–5 μmol/l and the uremic human plasma [MA] = 10–20 μmol/l (3, 45). Even though [MA] in plasma is elevated in diabetic humans, especially those with chronic renal dysfunction, it is unlikely to exceed 50 μmol/l (27, 49). Some red wines possess up to 5 mg/l MA (35), which could elevate plasma MA levels by ~30 μmol/l in a 70-kg male assuming the consumption of 1 liter of wine and 100% absorption. Our data suggest that exposure of isolated human blood vessels to 80 μmol/l MA would produce a very modest vascular relaxation.

It has been suggested that treatment of human diabetics with a SSAO inhibitor, such as aminoguanidine, may provide vascular protection by inhibiting SSAO activity, diminishing amine metabolism, subsequent aldehyde and adduct formation, and reducing advanced glycation end products (AGEs) (17, 20, 48). However, it is unclear how a SSAO inhibitor would affect the variety of cardiovascular and noncardiovascular pools of SSAO activity, including adipose and gastrointestinal tract smooth muscle, where the function of SSAO also remains undetermined (18). For example, the SSAO protein homolog vascular adhesion protein-1 (VAP-1) expressed in endothelial and vascular smooth muscle cells mediates lymphocyte binding (26, 41). Whereas previous studies detect little to no SSAO activity in endothelial cell cultures (50), purified human and bovine brain microvessels possess concentrated SSAO activity with relatively high affinity for MA (11). Thus endothelial cell VAP-1 may contribute to overall vascular SSAO enzymatic activity and also to nonenzymatic functions associated with amine binding, a pathway we cannot rule out in the present study. Moreover, there are no specific inhibitors for each tissue-specific pool of SSAO activity (Refs. 17 and 37 and L. Sayre, unpublished observations), and thus we should tread cautiously because the effects of SSAO inhibition or overexpression of SSAO in developing rats are detrimental to vascular tissue (19, 29) and remain unknown in the adult, although treatment of Parkinson’s patients with carbidopa or hydralazine may indicate limited systemic toxicity (for reviews, see Refs. 19 and 51).

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GRANTS

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