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Evidence for in Vivo Scavenging by Aminoguanidine of Formaldehyde Produced via Semicarbazide-Sensitive Amine Oxidase-Mediated Deamination

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ABSTRACT

Aminoguanidine (AG) is capable of preventing advanced protein glycation and inhibiting the activity of enzymes with carbonyl groups as cofactors, such as nitric-oxide synthase (NOS) and semicarbazide-sensitive amine oxidase (SSAO). The hydrazide moiety of AG can also interact with different endogenous carbonyl metabolites and potentially harmful endogenous aldehydes. Aldehydes can be generated via different pathways, such as lipid peroxidation (malondialdehyde and 4-hydroxynonenal), oxidative deamination (aldehydes), and carbohydrate metabolism (methylglyoxal). Formaldehyde and methylglyoxal are produced via SSAO-catalyzed deamination of methylamine and aminoacetone, respectively. An increase in SSAO-mediated deamination is known to be associated with various vascular disorders, such as diabetic complications. The present study demonstrates that AG is not only capable of rapidly interacting with aldehydes in vitro but also scavenging aldehydes in vivo. The AG-formaldehyde adducts were traced, and their structures were elucidated by high-performance liquid chromatography-mass spectrometry. AG has also been shown to block formaldehyde-induced β-amyloid aggregation. Thus, AG can be an aldehyde scavenger in addition to blocking advanced glycation and inhibition of SSAO and NOS activity. Such reactions may contribute to its pharmacological effects in the treatment of vascular disorders associated with diabetic complications and other disorders.

Aminoguanidine (AG) possesses a nucleophilic hydrazine (-$NHNH_2$) and a guanidine [-NHC(=NH)NH_2] moiety, which enable it to scavenge dicarbonyl compounds (Thornalley, 2003). AG effectively blocks protein glycation, and it prevents the formation of advanced glycation end products (AGEs) in vitro and in vivo (Brownlee et al., 1986; Skamarauskas et al., 1996). Information on AG and AGEs has generated the idea of a strategy to break the existing AGE cross-links (Vasan et al., 2003). Mounting evidence indicates that AG is quite effective in preventing diabetic complications (Cameron and Cotter, 1996; Friedman et al., 1997; Forbes et al., 2004), arterial stiffening (Corman et al., 1998; Chang et al., 2006), atherosclerosis (Panagiotopoulos et al., 1998), stroke (Zhang et al., 1996), kidney damage (Soulis et al., 1996; Bolton et al., 2004), and even ischemic cerebral neurodegeneration (Zimmerman et al., 1995) in animal studies. AG has significant clinical implications in the treatment of disorders associated with advanced glycation. Although the potential side effects of AG are of serious concern (Oturai et al., 1996; Freedman et al., 1999), AG serves as a prototype agent for future drug development aiming to prevent the formation of AGEs (Thornalley, 2003).

AG is also capable of inhibiting enzymes that possess carbonyl groups as cofactors, such as nitric-oxide synthase (NOS) (Alderton et al., 2001) and semicarbazide-sensitive amine oxidase (SSAO) (Yu and Zuo, 1997). Both enzymes may be involved in a cytotoxic cascade. SSAO is located on the outer cell surface of vascular smooth muscles and endothelial cells (Yu et al., 2003), and it circulates in the blood, probably as result of shedding from the vasculatures (Abella et al., 2004). An increase in serum SSAO activities was found in patients with diabetic complications, vascular disorders, and heart failure (Yuen et al., 1987; Ishizaki, 1990; Boomsma et al., 1995, 1997; Garpenstrand et al., 1999). Increased se-

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ABBREVIATIONS: AG, aminoguanidine; AGE, advanced glycation end product; SSAO, semicarbazide-sensitive amine oxidase; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; Fmoc-Cl, *N*-(9-fluorenyl)methoxycarbonyl chloroformate; DNPH, 2,4-dinitrophenylhydrazine; HPLC, high-performance liquid chromatography; MDL-72974A, (*E*)-2-(4-fluorophenetyl)-fluoroallylamine; $A\beta_{1-40}$, β-amyloid; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; TMA, tetramethylammonium chloride; ThT, thioflavine T; ANOVA, analysis of variance; FA, formaldehyde.

rum SSAO has been identified as an independent prognostic risk factor for heart patients (Boomsma et al., 2000).

Methylamine and aminoacetone are endogenous substrates for SSAO (Yu, 1990; Deng and Yu, 1999). The deaminated products include formaldehyde and methylglyoxal, respectively, as well as hydrogen peroxide and ammonium (Yu and Zuo, 1993; Yu et al., 2002, 2003). Formaldehyde and methylglyoxal are extremely reactive and capable of forming Schiff's bases with free amino or amide groups of proteins (Gubisne-Haberle et al., 2004), and subsequently, they form methylene bridges and produce irreversible cross-linked complexes between proteins and single-stranded DNA (Bolt, 1987). This was thought to contribute to protein misfolding, associated with many chronic pathological conditions (Yu et al., 2003). Selective SSAO inhibitors have been shown to protect SSAO-mediated toxicity in vitro (Yu and Zuo, 1993) and to prevent atherogenesis (Yu and Zuo, 1996; Yu et al., 2002) and lipopolysaccharide-induced inflammation in animal models (Yu et al., 2006). AG, at concentrations many times lower than required for blocking advanced protein glycation, has been shown to inhibit SSAO in vitro and in vivo (Yu and Zuo, 1997). In this study, we investigate whether AG scavenges free endogenous aldehydes in addition to inhibiting SSAO activity.

Materials and Methods

Materials. Glycine, pyridine, acetic anhydride, acetone, tetramethylammonium chloride, boric acid, sodium hydroxide, phosphorus pentoxide, citric acid, phosphoric acid, hydrochloric acid, acetic acid, sodium acetate, ethanol, diethyl ether *N*-(9-fluorenyl)methoxycarbonyl chloroformate (Fmoc-Cl), and 2,4-dinitrophenylhydrazine (DNPH) were purchased from Sigma-Aldrich (St. Louis, MO). [^{14}C]Methylamine and [^{14}C]AG were obtained from American Radiolabeled Chemicals (St. Louis, MO) and Moravek Biochemicals (Brea, CA), respectively. HPLC-grade acetonitrile, methanol, hexane, and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Unless stated otherwise, all reagents were of analytical grade. MDL-72974A was kindly provided by Marion-Merrell Dow (Cincinnati, OH). β-Amyloid (Aβ_{1-40}) was purchased from BioSource International (Camarillo, CA), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and methylglyoxal were from Sigma-Aldrich. Formaldehyde was obtained from BDH (Toronto, ON, Canada). Methanol (10–15%), which prevents polymerization, is included in the 37% formaldehyde solution. Microfluor black plates (96-well) for the fluorometry were purchased from Dynex Technologies (Chantilly, VA).

Animals. Male CD1 Swiss White mice weighing 30 g were used in the experiments. The animals were housed in hanging wire cages with free access to food and water on a 12-h light/dark cycle (lights on at 6:00 AM) at a temperature of 19–20°C. The experimental protocol has been designed according to the guidelines of the Canadian Council on Animal Care, and it was approved by University of Saskatchewan Animal Care Committee. Mice were treated with 200 μl of saline i.p. or 100 mg/kg aminoguanidine i.p., and subsequently, 1 h later, with 10 mg/kg formaldehyde i.p. (in 200 μl of saline). After the last injection, mice were placed in metabolic cages for urine collection. To substantiate the formation of formaldehyde-protein adducts derived from deamination of methylamine, radioactively labeled [^{14}C]methylamine (5 μCi; 100 μl) was administered via tail vein intravenous injection after pretreatment with aminoguanidine or saline. Animals were sacrificed after 3 days, and the dissected tissues (n = 3–5) were further divided into three parts for independent analyses for radioactive residuals

Urine Collection. Mice were placed in metabolic cages (Nalgene, Rochester, NY) for urine collection for a period of 24 h. The urine-collecting vessels were positioned over Styrofoam containers filled with dry ice, thereby freezing the urine immediately after excretion. During urine collection, the animals were allowed free access to tap water, but food was withheld.

Derivatization of Aminoguanidine with Fmoc. For the derivatization, 500 μl of 0.8 M potassium-borate buffer, pH 10, was added to 1.0-ml samples and vortexed for 60 s. One milliliter of Fmoc-Cl reagent solution (10 mM in acetonitrile) was then added to the buffered samples and vigorously vortexed for 1 min. The reaction was terminated by extraction of excess reagent (Fmoc-Cl), and its hydrolysis product Fmoc-OH, and acetonitrile with 5.0 ml of hexane. The top hexane layer was discarded, and this procedure was repeated twice. The potassium-borate buffer was neutralized by addition of 0.1 ml of 20% (v/v) acetic acid. Aliquots (250 μl) of these samples were subjected to HPLC analysis.

Chromatography. The HPLC system was composed of a Shimadzu solvent delivery module (LC-10 AD*vp*; Shimadzu, Kyoto, Japan), a Shimadzu autoinjector (SIL-10AD*vp*), a Shimadzu DGU-14A degasser, and a reverse phase HPLC column [Beckman Ultrasphere IP C18 5-μm column (4.6 × 250 mm); Beckman Coulter, Fullerton, CA]. A Shimadzu SPD-10A*vp*UV-visible detector was used for spectrophotometric detection. For determination of the aminoguanidine, a tertiary gradient system, based on previous methods for analysis of amino acids (Kazachkov and Yu, 2005), was used. Solvent A was 20 mM citric acid containing 5 mM tetramethylammonium chloride (TMA), adjusted to pH 2.85 with 20 mM sodium acetate containing 5 mM TMA. Solvent B was 80% (v/v) 20 mM sodium acetate solution containing 5 mM TMA adjusted to pH 4.5 with concentrated phosphoric acid and 20% (v/v) 100% methanol. Solvent C was 100% acetonitrile. The flow rate was maintained at 1.4 ml/min throughout the analysis. Separation was performed at a column temperature of 25°C. Absorbance was measured at 265 nm.

Determination of Urinary Aldehydes. A previously described HPLC-spectrophotometric method (Yu and Deng, 1998) was used for determination of aldehydes. Aldehydes were derivatized with DNPH, and propionaldehyde was used as an internal standard. During the course of the investigation, we found that formaldehyde-AG adduct was cleaved at pH 2.0 (which is used in the conventional DNPH procedure), so both free and released formaldehyde were detected. At pH 5.5, DNPH derivatization proceeded well, but AG-formaldehyde adducts did not cleave; therefore, only free formaldehyde was detected. Consequently, the derivatization procedure was conducted under two conditions, namely 1) 10 mM DNPH prepared in 2 N HCl and 2) DNPH dissolved in distilled water containing 50% acetonitrile. The derivatization was conducted in 25-ml screw-capped tubes containing aliquots of urine (0.2 ml), 200 nM propionaldehyde, and 200 μM DNPH in HCl solution, pH 2.0, or in 50 mM phosphate buffer, pH 5.5, in a total volume of 5 ml, which was mixed and incubated at 37°C for 10 min. The hydrazone products were vigorously extracted twice with 10 ml of pentane. The pentane extracts were evaporated at 40°C under a nitrogen stream in a water bath, and the dried precipitates were carefully dissolved in 500 μl of acetonitrile. Aliquots (20 μl) of the concentrated samples were subjected to HPLC analysis. Elution was isocratic with a mobile phase containing 49% acetonitrile in water at a flow rate of 1.0 ml/min. Spectrophotometric detection at 330 nm was conducted using a Shimadzu SPD-10A*vp*UV-VIS detector.

Distribution of Residual Radioactivity in Tissues following Administration of [^{14}C]Methylamine. Three days after treatment with [^{14}C]methylamine, mice were sacrificed, and radioactive residual activities in different tissues were analyzed. The dissected tissues were homogenized in 0.2 M phosphate buffer, pH 7.5 [1:20 (w/v)]. Aliquots of the homogenates were transferred to counting vials containing 25 μl of Solvable (PerkinElmer, Waltham, MA) and 10 ml of aqueous counting scintillation fluid (Amersham Biosciences, Piscataway, NJ). Radioactivities were assessed by liquid scintillation spectrometry (LS6500 multipurpose liquid scintillation counter; Beckman Coulter).

HPLC-Mass Spectrometry. The products following interaction between aminoguanidine and formaldehyde were analyzed by electrospray mass spectrometry (Quattro Ultima; Micromass, Manchester, UK). Aminoguanidine (0.2 mM) was incubated in 1 mM formaldehyde for 2 h at 37°C. Mass spectrometric analysis was conducted in both positive and negative ion MS1-mode (*m/z* 50–850). The source temperature was 120°C, and the capillary voltage was 2.53 kV with a cone voltage of 45 V.

Interactions of β-Amyloid$_{1-40}$ with Aldehydes. Freshly prepared seed-free Aβ_{1-40} at 1 mg/ml was incubated with different concentrations of aminoguanidine (2–50 mM) in the presence or absence of 1 mM formaldehyde in 20 mM phosphate-buffered saline, pH 7.4, in 0.2 ml of Eppendorf tubes at 37°C without shaking or pipetting. A seed-free monomer solution of Aβ_{1-40} peptide was pretreated immediately before each experiment. Aβ_{1-40} was dissolved in 100% HFIP at 1 mg/ml, and the sample was incubated in a water bath sonicator at 4°C for 2 h. The HFIP was removed under a gentle stream of nitrogen. The treated Aβ_{1-40} crystals were dissolved in NANOpure water (Barnstead, Dubuque, IA) and used immediately. The purity of Aβ monomers (free of oligomers) was ensured by an assessment using an oligomer specific antibody. The final Aβ concentration was determined using a Bradford protein assay (Bio-Rad, Hercules, CA).

Thioflavine T Fluorometry of β-Sheet Formation of β-Amyloid. ThT fluorescence assays reveal the early stage of Aβ_{1-40} aggregation, i.e., β-sheet formation (Chen et al., 2006). Aβ (final concentration at 10 μg/ml) was incubated in the presence or absence of aldehydes in a total reaction mixture of 200 μl (in 50 mM glycine-NaOH buffer, pH 9.0). Aliquots of the reaction solution were transferred to black Microfluor plates at different time intervals for fluorescence readings. Fluorescence was monitored at an excitation wavelength of 450 nm and an emission wavelength of 482 nm using a Spectra Max Gemini XS fluorescence reader (Molecular Devices, Sunnyvale, CA).

Statistics. The results were assessed using a one-way analysis of variance (ANOVA) followed by multiple comparisons (Newman-Keuls test). The null hypothesis used for all analyses was that the factor had no influence on the measured variable. Significance was accepted at the >95% confidence level.

Results

Interaction of Aminoguanidine with Formaldehyde. Figure 1 summarizes the results of interactions between AG and the aldehydes, as measured by spectrometry. The reactions are saturable and the absorption spectra following the interactions altered in a time- and dose-dependent manner. The spectral change seems more pronounced with respect to methylglyoxal compared with formaldehyde. The absorption spectra with regard to AG-methylglyoxal, but not AG-formaldehyde, adducts were detected in the visible wavelength range following prolonged incubation. However, the spectrophotometric analysis does not reveal those products without spectral alteration.

The interaction between formaldehyde and AG is slightly affected by pH in favor of alkaline conditions (data not shown). The rates of the disappearance of the AG after incubation of the two reagents at pH 7.4 were assessed. Analysis of the AG-Fmoc derivative by HPLC indicated, the major AG-Fmoc peak dramatically diminished after interaction with formaldehyde (Fig. 2). Interestingly, at least four additional peaks were noted in the chromatogram, representing unidentified formaldehyde-AG adducts. These products retain at least one free amino group to interact with Fmoc-Cl.

Figure 3 shows an example of the scavenging of formaldehyde by AG in vitro. This experiment measured the disappearance of formaldehyde after incubation with increasing amounts of AG. The reaction rates are dependent upon the concentrations of AG and the ratios of formaldehyde to AG.

Effect of AG on the Formation of Formaldehyde-Protein Adducts following Administration of [^{14}C]Methylamine in Mice. Intraperitoneal administration of [^{14}C]methylamine causes a long-lasting radioactive residual accumulation in different tissues of mice (Fig. 4). This has been previously shown to be due to a blockade of the conversion of methylamine to formaldehyde, which cross-links with proteins (Yu and Deng, 1998). The radioactive deposition can be effectively reduced by pretreatment with both a selective SSAO inhibitor and AG. Interestingly, AG is significantly more effective in the inhibition of the radioactive deposition compared with the highly selective and potent SSAO inhibitor MDL-72974A, which is more potent in blocking SSAO activity in vivo than AG. Subsequent experiments indicate that AG is capable of scavenging formaldehyde, which may be due to incomplete inhibition of deamination of methylamine, in addition to its inhibitory effect on SSAO.

Effect of AG on Urinary Formaldehyde Excretion. Urinary formaldehyde was analyzed using a DNPH derivatization/HPLC procedure. The derivatization was initially

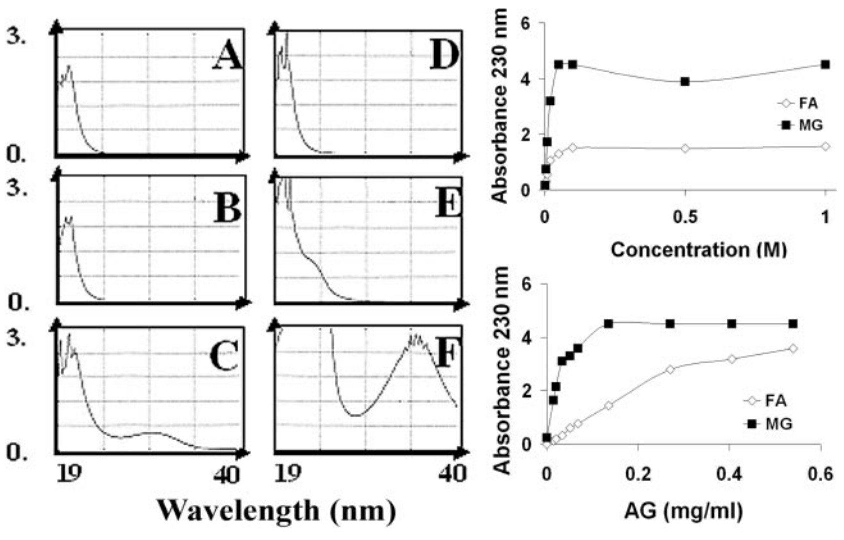


Fig. 1. Change in absorption spectra of aminoguanidine following interactions with formaldehyde and methylglyoxal. AG (1 mM) was incubated in 0.05 M phosphate buffer, pH 7.5 (A and D), 4 mM formaldehyde (B and E), or 0.4 mM methylglyoxal (C and F) at 25°C. A, B, and C are spectra measured at zero time; and D, E, and F are spectra measured after 20 min of incubation. G, adducts formation of AG with formaldehyde or methylglyoxal at different concentrations measured at 230 nm. H, Adducts formation AG at different concentrations with formaldehyde or methylglyoxal at 0.2 mM.

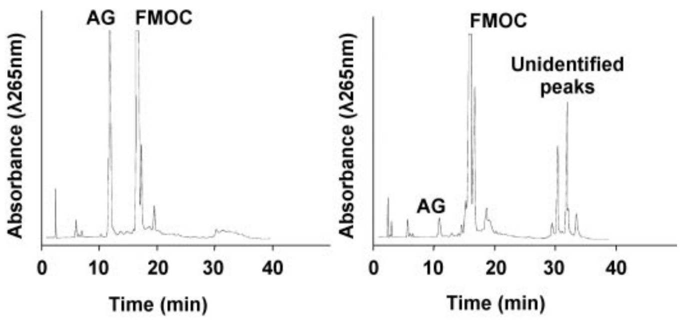


Fig. 2. HPLC separation of Fmoc derivatives of aminoguanidine and its formaldehyde adducts. The Fmoc derivatization procedure and HPLC conditions are described under *Materials and Methods*. The chromatograph profiles Fmoc derivatives of AG preincubated in the absence (left panel) and presence (right panel) of formaldehyde.

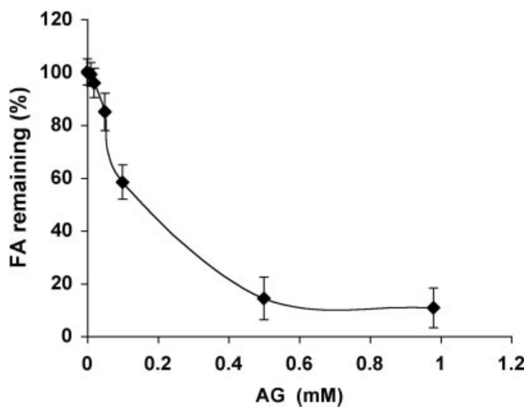


Fig. 3. Dose-dependent scavenge of formaldehyde by aminoguanidine in vitro. Formaldehyde (2×10^{-2} mM) was incubated in the presence of different concentrations of AG at pH 7.5 and 37°C for 2 h. The remaining formaldehyde was assessed using a DNPH/HPLC procedure.

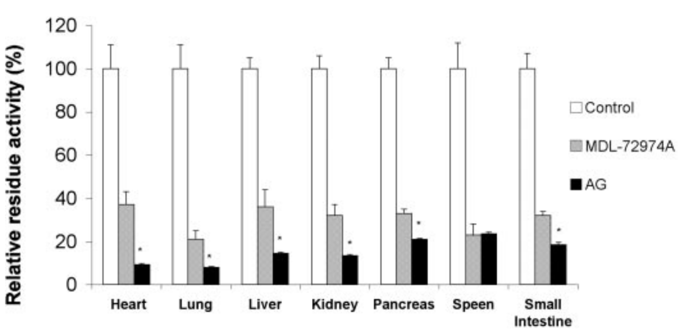


Fig. 4. The effect of aminoguanidine and SSAO inhibitor on the residual radioactivity in different mouse tissues after administration of [^{14}C]methylamine. The animals were pretreated with saline or 100 mg/kg i.p. AG 1 h before administration of [^{14}C]methylamine (5 μCi; in 100 μl of saline, via tail vein injection). The residual radioactivity in different tissues was assessed 72 h after administration of the labeled methylamine. Values are means ± standard error of the mean of at least three animals. Statistical comparison in different groups of experiments was performed using a one-way analysis of variance, followed by Newman-Keuls multiple comparisons; *, $p < 0.01$ in comparison with the effect between AG and MDL-72974A.

conducted at pH 2.0, but it was subsequently revised to pH 5.5. Figure 5 summarizes the results obtained using both methods. At pH 2.0, the urinary formaldehyde increased following injection of formaldehyde, but prior treatment with AG did not reduce formaldehyde excretion as expected. In contrast, a substantial increase was detected in the group treated with both formaldehyde and AG, in comparison with

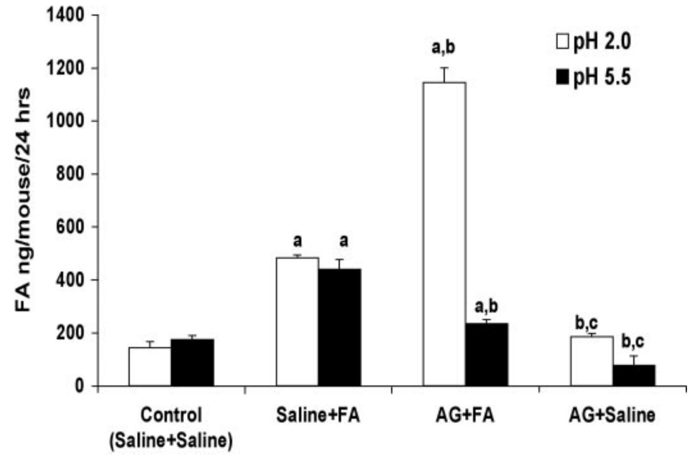


Fig. 5. Urinary excretion of formaldehyde in mice following administration of aminoguanidine and formaldehyde. Experiment details are described under Animals in *Materials and Methods*. DNPH derivatization was conducted either at pH 2.0 (open bars) or at pH 5.5 (closed bars). Data represent mean ± S.E. ($n = 5$). [a], $p < 0.01$ in comparison with saline control; [b], $p < 0.01$ in comparison between pH 2 and 5.5; and [c]$p < 0.01$ in comparison with FA-treated control.

the animals treated with only formaldehyde. This puzzling result was eventually resolved recognizing that AG-formaldehyde would deconjugate at low pH. When the DNPH derivatization procedure was conducted at pH 5.5, AG was shown to be capable of significantly reducing urinary formaldehyde levels, either with or without the treatment of formaldehyde. Details about the rationale of this experiment and discussion related to pH are presented under *Discussion*.

Radioactive Tracing of [^{14}C]AG following Interaction with Formaldehyde in Vitro and in Vivo. The interaction between AG and formaldehyde was also traced using carbon-14-labeled AG both in vitro and in vivo. The [^{14}C]AG and formaldehyde adducts were revealed using the same Fmoc/HPLC procedure as described above. As shown in Fig. 6A, the HPLC fractions were collected, and the radioactivity in each fraction was determined. AG exhibits a major peak at fraction 10 to 11 along with a minor peak at around fractions 19 to 20. After interaction with formaldehyde, the AG peak disappeared, and instead, a considerable amount of activity was detected in the solvent front. In addition, several new peaks containing radioactivity were detected in fractions 31 and 33 as well as in the solvent front.

For in vivo studies, [^{14}C]AG (10 mg/kg containing 5 μCi/mouse) was administered to the mice, and constituents in the urine were derivatized with Fmoc-Cl and subsequently analyzed by HPLC. As shown in Fig. 6B, the chromatographic profile of the AG-formaldehyde adducts seems to be quite similar to that observed in the in vitro experiment.

Structural Analysis. To characterize the product of the reaction, AG was incubated with formaldehyde for 2 h and then analyzed by mass spectrometry. As shown in Fig. 7, the absolute major ion has a mass of 87.0876, which corresponds to the aminoguanidine methylene ion with positive ionization. A number of minor spikes of larger ion masses were not identified

Effect of AG on Formaldehyde-Induced β-Amyloid$_{1-40}$ β-Sheet Formation. Figure 8 reveals the effect of AG on the formation of amyloid β-sheet in vitro by a ThT fluorometry. Formaldehyde significantly enhances the

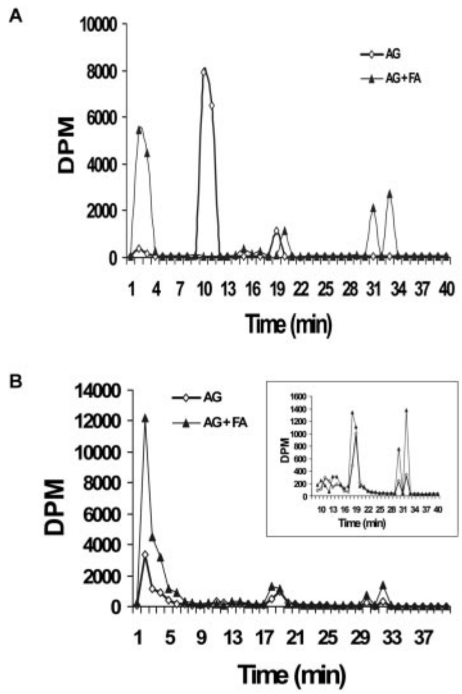


Fig. 6. Tracing the formation of aminoguanidine-formaldehyde adducts in vitro and in vivo using [14C]aminoguanidine. A, 0.2 mM [14C]AG was incubated with 1 mM formaldehyde at 37°C for 2 h, and the products were assessed with Fmoc/HPLC procedure. B, [14C]AG (10 mg/kg; 5 μCi) was administered via intraperitoneal injection to mice, and 24-h urine was collected for the analyses of AG, and its metabolites and adducts.

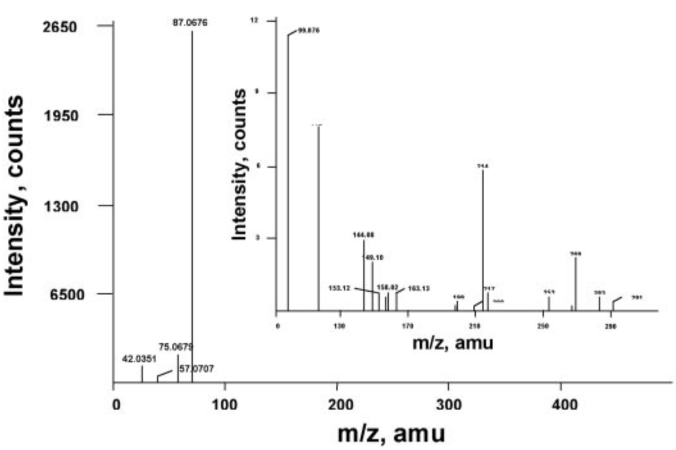


Fig. 7. Identification of the structure of aminoguanidine-formaldehyde adducts by mass spectrometry. Mass spectrometry clearly indicates that the major ion is aminoguanidine methylene with a mass of 87.0876. Details of ions of higher *m/z* are included within the figure.

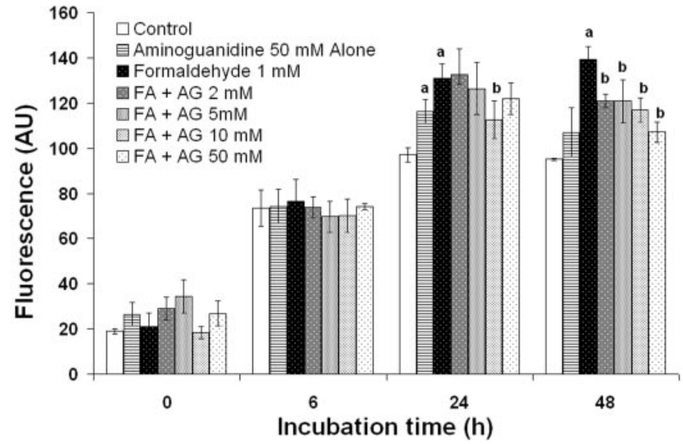


Fig. 8. Effect of aminoguanidine on formaldehyde-induced β-sheet formation in vitro. Aβ (1 mg/ml) was incubated in 1 mM FA in the absence or presence of different concentrations of AG for 48 h. The reaction mixtures (200 μl) were then incubated in the presence of 2 mM ThT (in 50 mM glycine-NaOH buffer, pH 9.0); fluorescence was measured (excitation, 450 nm; emission, 480 nm) using a SpectraMax fluoro-plate reader (Molecular Devices). Data represent mean $\pm$ S.D. of a representative experiment of three. [a], $p < 0.01$ in comparison with untreated Aβ control; and [b], $p < 0.01$ in comparison with Aβ in the presence of formaldehyde but absence of AG.

β-sheet formation. AG by itself does not affect the formation of amyloid β-sheets, but it effectively blocks the formaldehyde-induced induction of β-amyloid folding.

Discussion

AG, a prototype α,β-dicarbonyl scavenger, prevents protein glycation, which leads to accumulation of AGEs, and it is implicated to various age-related disorders (Thornalley, 2003). The hydrazine moiety of AG also reacts with other carbonyl groups, including physiologically important constituents (such as pyridoxal and some quinone compounds as enzyme cofactors) as well as with potentially harmful free aldehydes.

Formaldehyde and methylglyoxal, derived from SSAO-mediated deamination of methylamine and aminoacetone, respectively, in the vicinity of the vascular surface, have drawn considerable interest related to diabetic complications (Yu et al., 2003). Formaldehyde forms a Schiff's base between basic amino acid residues (Lys and Arg) of proteins (Gubisne-Haberle et al., 2004), and it induces Aβ aggregation (Chen et al., 2006). Formaldehyde, along with other reactive aldehydes, i.e., malondialdehyde and 4-hydroxynonal generated via lipid peroxidation, may contribute to protein misfolding particularly on the vascular surface.

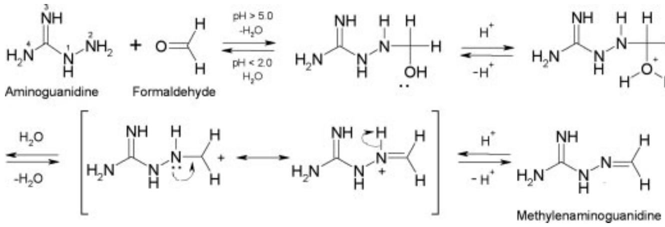
In the present study, we demonstrate that AG not only rapidly interacts with formaldehyde and methylglyoxal in vitro but also is capable of scavenging aldehydes in vivo. When [14C]methylamine was administered to mice, long-lasting radioactive protein residual activities were detected in all tissues. As shown in Fig. 4, both selective SSAO inhibitors and AG dramatically reduced such formation of radioactive residues. This is clearly a result of production of formaldehyde-protein adducts due to SSAO-mediated deamination of methylamine (Yu et al., 2006). Interestingly, AG, a less potent SSAO-inhibitor, is significantly more effective than the highly potent SSAO inhibitor MDL-72974A in blocking the adduct formation (Fig. 5). This result suggests that in addition to inhibition of SSAO, AG can also interact with formaldehyde due to incomplete inhibition of SSAO-mediated deamination of methylamine.

To substantiate whether AG is capable of scavenging formaldehyde in vivo, AG was administered to the animals, and the excretion of formaldehyde was assessed. The initial result was completely unexpected, namely, the urinary formaldehyde level was significantly increased rather than decreased. We conducted a number of experiments and ruled out the possibility that AG blocks formaldehyde metabolism, i.e., via aldehyde dehydrogenase (data not shown). In the initial experiment, the analyses of formaldehyde were conducted using a commonly used procedure, i.e., DNPH derivatization in strong acidic conditions followed by HPLC detection. We finally found that at such a low pH, the bond between AG and formaldehyde (of the AG-formaldehyde adducts) can be broken and thus formaldehyde would be released. We also observed that pH lower than 5.5 was required for DNPH to interact with aldehydes; yet, under such conditions, the AG-formaldehyde adducts would not be cleaved. When urine samples from the AG treated mice were analyzed with DNPH derivatization at pH 5.5, AG significantly reduced formaldehyde excretion. This is direct evidence that AG scavenges formaldehyde in vivo. The increase in formaldehyde levels under acidic conditions (i.e., below pH 2.0) is due to deconjugation of AG-formaldehyde. This is strong evidence that AG scavenges the aldehyde and that the conjugated form of adducts is excreted under physiological pH.

In an attempt to elucidate the structure of the AG-formaldehyde adducts, [^{14}C]aminoguanidine tracing was used both in vitro and in vitro, with subsequent identification by HPLC. We observed at least four new peaks were present in the HPLC chromatographic profile in the in vitro as well as the in vivo experiments (Fig. 6). AG possesses two primary amino groups, which occupy positions 2 and 4 (see Scheme 1). Both amino groups can react with formaldehyde. As indicated in the scheme, the amino group at position 2 preferentially reacts with formaldehyde.

After the incubation of AG and formaldehyde for 2 h, a mass spectrum analysis was conducted. The major ion mass is 87.0876, corresponding to the aminoguanidine methylene ion with positive ionization. In the presence of excessive formaldehyde, the second amino group at position 4 of aminoguanidine may also be involved. Thus, the aminoguanidine methylene adduct remains reactive for AG. This may lead to a second AG molecule and to further polymerization, leading to formation of heterogeneous, long-chain, and ring-closed molecular products. The analysis of these heterogeneous products became quite difficult. As shown in Fig. 6, we observed a substantial increase in radioactivity in the solvent front. These unidentified hydrophilic products of the AG-formaldehyde interaction were not derivatized by Fmoc. Ring cyclization may occur. Unfortunately, attempts to elucidate the structure of the compounds present in this fraction using HPLC-MS were unsuccessful.

AG is reported to be able to cross the blood-brain barrier and prevent cerebral ischemia and neurodegeneration (Mahar Doan et al., 2000), and AGE has been shown to be related to β-amyloid polymerization (Münch et al., 1997). We have observed that formaldehyde derived from SSAO-mediated deamination can enhance β-amyloid oligomerization, and we propose a role for SSAO in the formation of β-amyloid plaques on the cerebral vascular surface (Chen et al., 2006). The present data show AG is able to abolish formaldehyde-induced Aβ aggregation. The interactions between aldehydes and AG are substantially faster than between formaldehyde and proteins. Harmful aldehydes could be generated from other sources, such as malondialdehyde and 4-hydroxynonenal, as a result of lipid peroxidation (Esterbauer et al., 1991). This suggests that the beneficial effect of AG, as shown previously in animal disease models (for review, see Thornalley, 2003), may in part be due to prevention of aldehyde-induced cytotoxicity and protein misfolding.



Scheme 1. Interaction of aminoguanidine and formaldehyde.

In conclusion, AG effectively blocks advanced glycation. It also interacts with physiological important carbonyl groups, such as enzyme cofactors as well as free harmful aldehydes. The clinical implications are complicated. However, it is perhaps interesting to note that AG may be quite useful under acute circumstances of aldehyde intoxication.

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