

# Mercaptoethylguanidine and Guanidine Inhibitors of Nitric-oxide Synthase React with Peroxynitrite and Protect against Peroxynitrite-induced Oxidative Damage\*

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Nitric oxide (NO) produced by the inducible nitric-oxide synthase (iNOS) is responsible for some of the pathophysiological alterations during inflammation. Part of NO-related cytotoxicity is mediated by peroxynitrite, an oxidant species produced from NO and superoxide. Aminoguanidine and mercaptoethylguanidine (MEG) are inhibitors of iNOS and have anti-inflammatory properties. Here we demonstrate that MEG and related compounds are scavengers of peroxynitrite. MEG caused a dose-dependent inhibition of the peroxynitrite-induced oxidation of cytochrome  $c^{2+}$ , hydroxylation of benzoate, and nitration of 4-hydroxyphenylacetic acid. MEG reacts with peroxynitrite with a second-order rate constant of  $1900 \pm 64 \text{ M}^{-1} \text{ s}^{-1}$  at 37 °C. In cultured macrophages, MEG reduced the suppression of mitochondrial respiration and DNA single strand breakage in response to peroxynitrite. MEG also reduced the degree of vascular hyporeactivity in rat thoracic aortic rings exposed to peroxynitrite. The free thiol plays an important role in the scavenging effect of MEG. Aminoguanidine neither affected the oxidation of cytochrome  $c^{2+}$  nor reacted with ground state peroxynitrite, but inhibited the peroxynitrite-induced benzoate hydroxylation and 4-hydroxyphenylacetic acid nitration, indicating that it reacts with activated peroxynitrous acid or nitrogen dioxide. Compounds that act both as iNOS inhibitors and peroxynitrite scavengers may be useful anti-inflammatory agents.

Nitric oxide (NO)<sup>1,2</sup> a free radical produced by a family of isoenzymes termed nitric-oxide synthases (NOS), has been im-

plicated in a variety of physiological and pathophysiological processes. The cytotoxic effects of NO are mediated in part by peroxynitrite (ONOO<sup>-</sup>),<sup>3</sup> a reactive oxidant species formed from NO and superoxide at an almost diffusion-controlled rate (1–4). Although the biological activity and decomposition of peroxynitrite are very much dependent on cellular or chemical environment (concentration of proteins, thiols, glucose, and carbon dioxide and the ratio of NO to superoxide) (1–11), peroxynitrite is now generally considered a more toxic species than either NO or superoxide anion alone (12–19). The cytotoxic processes triggered by peroxynitrite include initiation of lipid peroxidation (5, 15, 16), inhibition of mitochondrial respiration (5, 12, 17–19), inhibition of membrane pumps (20), depletion of glutathione (21), and damage to DNA (22–25) with subsequent activation of poly(ADP-ribose) synthetase and concomitant cellular energy depletion (25–27).

Under certain conditions, NO produced by each of the three major isoforms of NOS can react with superoxide to form peroxynitrite. The constitutive, endothelial NOS isoform mainly serves physiological functions and is necessary for maintaining normal vascular functions, but under conditions of hypoxia/reoxygenation and ischemia/reperfusion injury and in atherosclerosis, peroxynitrite formed from endothelial NOS-derived NO can initiate cytotoxic processes (28–33). Similarly, during neuroinjury in response to excitatory amino acid stimulation, NO produced by the brain NOS isoform and/or by the endothelial NOS isoform present in the neurons can combine with superoxide to produce cytotoxic amounts of peroxynitrite (34–36). The inducible isoform of NOS (iNOS), expressed in macrophages, vascular smooth muscle cells, cardiac myocytes, and other cell types, has been implicated in the development of cellular energetic and vascular contractile failure during conditions of immunostimulation, inflammation, and various forms of circulatory shock (37, 38). iNOS-derived NO has been shown to form peroxynitrite, and the latter species has been implicated in the pathogenesis of tissue injury under inflammatory conditions (4, 10, 24–26, 39–44).

Considering the physiological functions of these NOS isoforms, inhibition of the activity of the constitutive isoforms of NOS (endothelial and brain), while preventing the formation of peroxynitrite, may also result in undesirable effects, such as vasospasm, enhanced platelet and neutrophil adhesion to the vascular endothelium, or alterations in central nervous system functions. Consequently, when peroxynitrite is formed from constitutive NOS-derived NO, scavenging of peroxynitrite would be preferred to inhibition of NO synthesis. On the other hand, selective inhibition of iNOS, by preventing excessive NO

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<sup>1</sup> The abbreviations used are: NO, nitric oxide; NOS, nitric-oxide synthase; iNOS, inducible nitric-oxide synthase isoform; AETU, aminomethylisothiourea; MEG, mercaptoethylguanidine; GED, guanidinoethyl disulfide; DTPA, diethylenetriaminepentaacetic acid; 4-HPA, 4-hydroxyphenylacetic acid; SNAP, S-nitroso-N-acetyl-DL-penicillamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

<sup>2</sup> The IUPAC recommended nomenclature for nitric oxide, peroxynitrite anion, and peroxynitrous acid is nitrogen monoxide, oxoperoxonitrate(1-), and hydrogen oxoperoxonitrate.

<sup>3</sup> The term peroxynitrite refers to the sum of both peroxynitrite anion (ONOO<sup>-</sup>) and peroxynitrous acid (ONOOH).

and peroxynitrite formation, has been shown to provide distinct therapeutic benefits in a number of inflammatory conditions. Selective iNOS inhibitors, such as aminoguanidine, S-methylisothiourrea, aminoethylisothiourrea (AETU), and L-N<sup>6</sup>-(1-iminoethyl)lysine, have been shown to have marked protective effects in a variety of local and systemic inflammatory disorders (42, 44–53).

We have recently reported the spontaneous rearrangement of aminoalkylisothiourrea NOS inhibitors in aqueous solutions to form mercaptoalkylguanidines (53). Some of the mercaptoalkylguanidines are potent inhibitors of NOS, with selectivity toward the inducible isoform (53). For example, AETU may rearrange to MEG (and a small percentage to the cyclic NOS inhibitor aminothiazoline). Oxidation of MEG yields guanidinoethyl disulfide (GED), which is a potent iNOS-selective inhibitor in its own right (46, 47). In this paper, we describe our findings demonstrating that MEG and related compounds are potent scavengers of peroxynitrite. Based on these data, we propose that the combined mode of action (selective inhibition of iNOS and scavenging peroxynitrite) is responsible for the marked protective effects of MEG and other guanidines in pathophysiological conditions associated with iNOS expression and peroxynitrite formation.

#### EXPERIMENTAL PROCEDURES

**Measurement of Peroxynitrite-induced Oxidation of Cytochrome  $c^{2+}$** —The peroxynitrite-dependent oxidation of cytochrome  $c^{2+}$  was measured as described (54, 55). Cytochrome  $c$  was reduced by sodium dithionite immediately before use and purified by chromatography on Sephadex G-25 using 100 mM potassium phosphate containing 0.1 mM DTPA, pH 7.2, as the elution buffer. The concentration of cytochrome  $c^{2+}$  was determined spectrophotometrically at 550 nm in the same buffer ( $\epsilon_{550} = 21 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Cytochrome  $c^{2+}$  oxidation (50  $\mu\text{M}$ ) yields upon addition of peroxynitrite (25  $\mu\text{M}$  initial concentration after mixing) were assessed by incubation of reaction mixtures in 100 mM potassium phosphate containing 0.1 mM DTPA, pH 7.2, at 22 °C for 3 min in the absence or presence of MEG or related compounds (1  $\mu\text{M}$  to 3 mM). Oxidation of cytochrome  $c^{2+}$  was followed at 550 nm using a Beckman DU 640 spectrophotometer. In control, reverse-order experiments, we confirmed that the various compounds used in this study did not interfere with the spectrophotometric measurements at the above wavelength. Moreover, in control experiments, we confirmed that the compounds tested do not reduce cytochrome  $c^{3+}$ .

**Measurement of Peroxynitrite-induced Hydroxylation of Benzoate**—The peroxynitrite-dependent hydroxylation of benzoate was measured as described (55, 56). Briefly, peroxynitrite (100  $\mu\text{M}$  initial concentration after mixing) was added to a buffer containing 1 mM sodium benzoate in 100 mM potassium phosphate containing 0.1 mM DTPA, pH 7.2, at 25 °C in the absence or presence of MEG or related compounds (1  $\mu\text{M}$  to 3 mM). After a 3-min incubation at 22 °C, fluorescence was measured using a Perkin-Elmer fluorometer (Model LS50B) at an excitation wavelength of 300 nm and emission wavelength of 410 nm (slit widths of 2.5 and 3.0 nm, respectively). In control, reverse-order experiments, we confirmed that the various compounds used in this study did not interfere with the assay at the above wavelengths.

**Nitration Reactions**—Nitration of 4-hydroxyphenylacetic acid (4-HPA) in the presence of MEG or aminoguanidine was carried out at pH 7.55 and 37 °C in 50 mM phosphate. In a typical experiment, a solution containing 2 mM 4-HPA, 100  $\mu\text{M}$  DTPA, 50 mM phosphate buffer, and 0–20 mM MEG or aminoguanidine was rapidly mixed with peroxynitrite (0.6 mM initial concentration after mixing) by vortexing. After 5 min, pH was measured and then adjusted to 10–11 by adding 1 M NaOH. The absorbance at 430 nm was then read. The concentration of the 3-NO<sub>2</sub>-4-HPA formed was calculated using  $\epsilon_{430} = 4400 \text{ M}^{-1} \text{ cm}^{-1}$  (57, 58).

**Stopped-flow Experiments**—Peroxynitrite decomposition in the presence of MEG, GED, aminoguanidine, and guanidine was studied by stopped-flow spectroscopy at 302 nm (Applied Photophysics SF.17MV) with a dead time of <2 ms (15). The kinetics of peroxynitrite decomposition was fitted to a first-order equation by nonlinear regression. A typical run consisted of 400 points collected over more than nine half-lives so at least 99.9% of the peroxynitrite disappeared. The final pH was measured at the outlet.

**Cell Culture**—J774 macrophages were cultured in Dulbecco's modi-

fied Eagle's medium supplemented with 3.5 mmol/liter L-glutamine and 10% fetal calf serum as described (25). Cells were cultured in 96-well plates (200  $\mu\text{l}$  of medium/well) or in 12-well plates (3 ml of medium/well) until confluence. Cells were pretreated with various concentrations of MEG (1–300  $\mu\text{M}$ ) for 10 min, followed by the addition of peroxynitrite (1 mM), and mitochondrial respiration and DNA strand breakage were measured at 1 h. In control, reverse-order experiments, MEG was added 10 min after the addition of peroxynitrite. In another set of experiments, cells were exposed to the NO compound S-nitroso-N-acetyl-DL-penicillamine (SNAP, 3 mM) for 24 h in the absence or presence of various concentrations of MEG (10–300  $\mu\text{M}$ ), and respiration was measured at 24 h.

**Measurement of Mitochondrial Respiration**—Cell respiration was assessed by the mitochondrion-dependent reduction of MTT to formazan (25). Cells in 96-well plates were incubated at 37 °C with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration, and the cells were solubilized in Me<sub>2</sub>SO (100  $\mu\text{l}$ ). The extent of reduction of MTT to formazan within cells was quantitated by measurement of the absorbance at 550 nm.

**Determination of DNA Single Strand Breaks**—The formation of strand breaks in double-stranded DNA was determined by the alkaline unwinding method as described previously (25). Under the conditions used, in which ethidium bromide binds preferentially to double-stranded DNA, the percentage of double-stranded DNA (dsDNA) may be determined using the following equation: % dsDNA =  $100 \times ((F(P) - F(B))/(F(T) - F(B)))$ , where  $F(P)$  is the fluorescence of the sample,  $F(B)$  is the background fluorescence (*i.e.* fluorescence due to all cell components other than double-stranded DNA), and  $F(T)$  is the maximum fluorescence.

**Organ Bath Experiments in Aortic Rings**—Thoracic aortas from rats were cleared of adhering periadventitial fat and cut into rings of 3–4-mm width. Rings were placed in Krebs solution and exposed to peroxynitrite (1 mM) or a vehicle control in the presence or absence of 300  $\mu\text{M}$  MEG. Following a 30-min incubation, rings were analyzed for isometric contractility. The rings were mounted in organ baths (5 ml) filled with warmed (37 °C), oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs solution, pH 7.4, consisting of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 2.5 mM CaCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, and 11.7 mM glucose in the presence of 10  $\mu\text{M}$  indomethacin. Isometric force was measured with isometric transducers (Kent Scientific Corp., Litchfield, CT), digitized using a MacLab A/D converter (AD Instruments, Milford, MA, and stored and displayed on a Macintosh personal computer. A tension of 1 g was applied, and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min (27). Concentration-response curves to norepinephrine (10<sup>-9</sup> to 10<sup>-5</sup> M) were then obtained. In another set of experiments, the effect of MEG on the relaxant effect of the NO donor SNAP was studied. In these experiments, rings were taken from the animals, mounted for the measurement of isometric tension, treated with MEG (1 mM) or vehicle for 10 min, precontracted with norepinephrine (10<sup>-6</sup> M), and then treated with increasing concentrations of SNAP.

**Materials**—Dulbecco's modified Eagle's medium and fetal calf serum were obtained from Life Technologies, Inc. SNAP was purchased from Calbiochem. AETU and related aminoalkylisothiourreas were prepared as described previously (46). MEG and mercaptopropylguanidine were prepared from AETU and APTU, respectively, as described previously (46). Peroxynitrite was synthesized as described previously (1, 15, 16). Cytochrome  $c$  (from bovine heart) and all other chemicals were from Sigma-Aldrich Co., (St. Louis, MO).

**Statistical Evaluation**—All values in the figures and below are expressed as means  $\pm$  S.E. or as standard error of the mean of  $n$  observations. The numbers presented in the oxidation assays represent pooled data from  $n = 6$ –12 determinations obtained on at least 3 different experimental days. Student's unpaired  $t$  test was used to compare means between groups, using the Bonferroni correction for multiple comparisons. A  $p$  value less than 0.05 was considered statistically significant.

#### RESULTS

**MEG and Related Compounds Reduce Peroxynitrite-induced Oxidations**—As previously reported (54–57), peroxynitrite induced a significant oxidation of cytochrome  $c^{2+}$  and hydroxylation of benzoate. These two targets for peroxynitrite-mediated oxidation react with different reactive intermediates, such as ground state peroxynitrite (cytochrome  $c^{2+}$  (54)) and activated peroxynitrous acid (benzoate (55)). Although the reactive

TABLE I  
Inhibition by mercaptoalkylguanidines and reference compounds of the peroxynitrite-induced oxidation of cytochrome  $c^{2+}$  and hydroxylation of benzoate

| Compound                     | EC <sub>50</sub>    |          |
|------------------------------|---------------------|----------|
|                              | Cytochrome $c^{2+}$ | Benzoate |
|                              | $\mu\text{M}$       |          |
| MEG                          | 50                  | 500      |
| SEG <sup>a</sup>             | 70                  | 200      |
| N-Methyl-MEG                 | 200                 | 400      |
| N,N'-Dimethyl-MEG            | 300                 | 700      |
| S-Methyl-MEG                 | 5000                | 1300     |
| GED                          | 30,000              | 5000     |
| MPG                          | 200                 | 500      |
| N,N'-Dimethyl-MPG            | 300                 | 900      |
| S-(Dimethylaminopropyl)-AETU | 10,000              | 8000     |
| Aminoguanidine               | >30,000             | 10,000   |
| Glutathione                  | 300                 | 600      |
| Cysteine                     | 160                 | 200      |
| Penicillamine                | 60                  | 1000     |

<sup>a</sup> SEG, selenoethylguanidine; MPG, mercaptopropylguanidine.

intermediates were different, both oxidant processes studied were still dose-dependently inhibited by MEG. The EC<sub>50</sub> values differed in the two assays mostly due to the different reaction chemistries involved (see "Discussion"). Thus, comparisons of potency of the different tested compounds in absolute terms should be performed only for each assay (vertical comparison in Table I), whereas comparisons within assays (horizontal comparison in Table I) should be used only to give an idea of the preferred reaction mechanisms. In this study, we employed both assays to draw conclusions regarding the nature of the different scavenging mechanisms of MEG and related guanidines during peroxynitrite-induced oxidant processes.

MEG inhibited the oxidant processes in both assays with high potency (Fig. 1 and Table I). S-Methyl-MEG and the disulfide dimer of MEG, GED, also inhibited the oxidation of cytochrome  $c^{2+}$  and the hydroxylation of benzoate, but their potency was much weaker when compared with that of MEG (Fig. 1 and Table I). In particular, it is important to note that MEG was ~1000 times more potent than S-methyl-MEG or GED as an inhibitor of peroxynitrite-induced oxidation of cytochrome  $c^{2+}$ . L-Arginine-based NOS inhibitors, such as N<sup>G</sup>-methyl-L-arginine, did not inhibit the oxidative processes (data not shown).

We have compared the potencies of MEG and a number of related mercaptoalkylguanidines with the potencies of other compounds containing a free thiol (Table I). We have observed that, in both assays, the potencies of the free thiol-containing compounds were approximately 2 orders of magnitude greater than those that cannot undergo a rearrangement to yield free thiols (46), such as S-(dimethylaminopropyl)-AETU (Table I) and S-(dimethylaminoethyl)-AETU (data not shown). Similarly, alkylation of the sulfur (S-methyl-MEG) or its oxidation to disulfide (GED) reduced the effectiveness of the compounds as scavengers of peroxynitrite. These reductions were more pronounced in the cytochrome  $c^{2+}$  assay and less pronounced in the benzoate hydroxylation assay (Fig. 1 and Table I), in agreement with the different oxidation mechanisms involved (54, 55). Thus, the free thiol appears to be crucial to the inhibitory effect of mercaptoalkylguanidines on the peroxynitrite-induced oxidation of cytochrome  $c^{2+}$ . MEG, as a scavenger of peroxynitrite, had comparable potency to other thiol-containing scavengers, namely glutathione, cysteine, and penicillamine (Table I) and also cysteamine, cysteine ethyl ester, and cysteine methyl ester (data not shown).

Replacement of the sulfur with selenium yielded comparable inhibitory potency to MEG in all assays used, in line with the concept that seleno compounds can also scavenge peroxynitrite

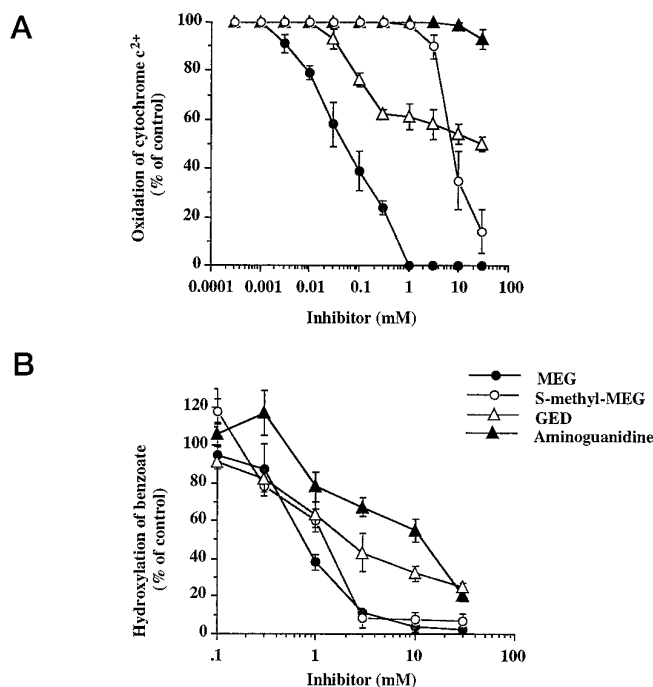


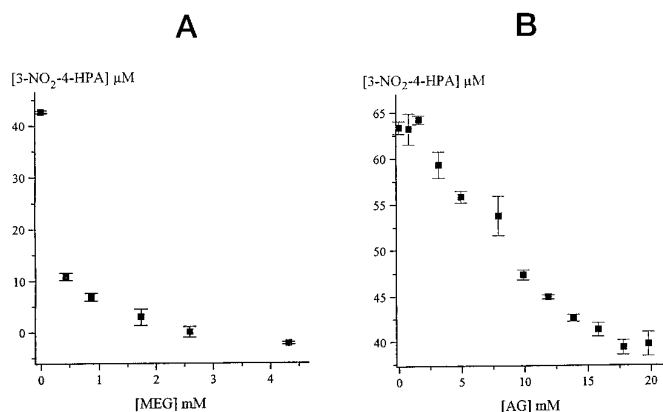
FIG. 1. Effect of MEG, GED, S-methyl-MEG, and aminoguanidine on the peroxynitrite-induced oxidation of cytochrome  $c^{2+}$  (A) and hydroxylation of benzoate (B). 100% represents values in the presence of peroxynitrite and in the absence of inhibitors. Data are expressed as means  $\pm$  S.E. ( $n = 6$ –12 determinations).

(58, 59). In most instances, N-methylation of MEG or mercaptopropylguanidine tended to reduce the potency when compared with the parent compound (Table I).

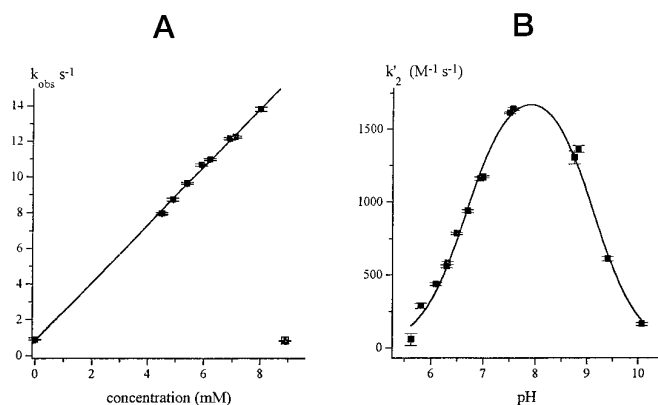
Aminoguanidine caused a modest, dose-dependent reduction of the peroxynitrite-induced hydroxylation of benzoate. However, aminoguanidine exhibited negligible potency against the peroxynitrite-induced oxidation of cytochrome  $c^{2+}$  (Fig. 1 and Table I).

**Effect of Guanidines on Nitration Reactions**—MEG and aminoguanidine (less potently) caused a dose-dependent inhibition of the nitration of 4-HPA by peroxynitrite (Fig. 2). The yield of 3-NO<sub>2</sub>-4-HPA, measured as percent of initial peroxynitrite concentration, decreased from 10.7% to 6.7% ([aminoguanidine] = 20 mM) and 0% ([MEG] = 0.5 mM) (Fig. 2), representing a 37% and 100% inhibition, respectively. On the other hand, aminoguanidine up to 5 mM failed to protect cysteine from peroxynitrite-mediated oxidation (data not shown).

**Kinetics of Oxidation of MEG by Peroxynitrite**—In the presence of a 10–25-fold excess of MEG 2HBr, the disappearance of peroxynitrite followed pseudo first-order kinetics when monitored at 302 nm by stopped-flow spectroscopy. Under these conditions, the spontaneous decomposition of peroxynitrite appeared as a non-zero intercept in a plot of  $k_{\text{obs}}$  versus [MEG] (Fig. 3A). The slope of this plot yields the apparent second-order rate constant for the reaction at a given pH. The pH dependence of the second-order rate constant (Fig. 3B) follows a bell-shaped curve, as previously observed for the reaction of cysteine with peroxynitrite anion (15). Thus, the data shown in Fig. 3 suggest that MEG readily reacts with peroxynitrite anion. Indeed, the rate constant for this reaction as a function of hydrogen ion concentration obeys the following equation:  $k_2' = k_2(K_{a1}/(K_{a1} + [H^+]))([H^+]/(K_{a2} + [H^+]))$ , where  $k_2'$  is the apparent rate constant at a given pH,  $k_2$  is the second-order rate constant of the reaction of MEG with peroxynitrite anion,  $K_{a1}$  is the ionization constant of peroxynitrous acid, and  $K_{a2}$  would correspond to the ionization constant of the sulfhydryl group of MEG. The best fit of the data shown in Fig. 3 gives  $k_2$



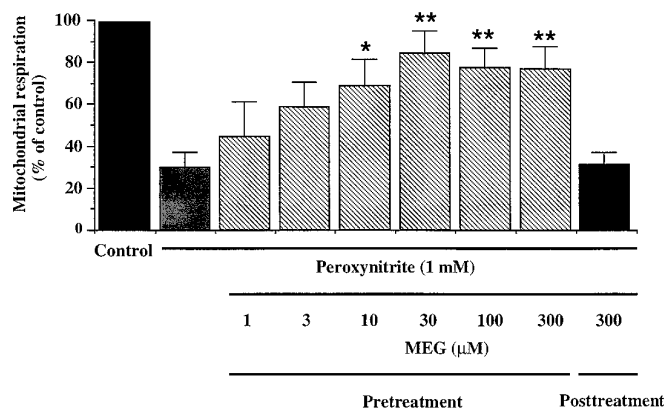
**FIG. 2. MEG and aminoguanidine inhibit the peroxynitrite-mediated nitration of 4-HPA.** Nitration experiments were performed in 50 mM potassium phosphate containing 100  $\mu$ M DTPA, pH 7.5, at 37  $^{\circ}$ C in the presence of 2 mM 4-HPA. The initial peroxynitrite concentration was 0.6 mM. Each data point represents the mean  $\pm$  S.E. of four determinations.



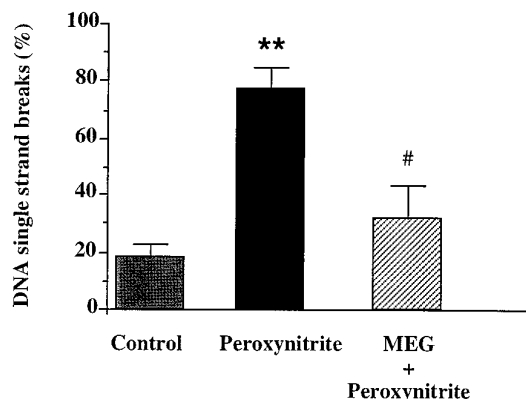
**FIG. 3. Rate constants for the reaction of peroxynitrite with MEG.** A, pseudo first-order rate constants for peroxynitrite decomposition shown as a function of MEG concentration ( $\blacksquare$ ). An apparent second-order rate constant was determined from the slope of the plot. No reaction was detectable with either GED ( $\blacktriangle$ ) or bromide anion ( $\square$ ) in the same concentration range. B, pH dependence. Peroxynitrite (0.2–0.4 mM) was added to 2.8 mM MEG in 50 mM potassium phosphate containing 100  $\mu$ M DTPA at different pH values at 37  $^{\circ}$ C. Data have been corrected for spontaneous decomposition of peroxynitrite. Each data point represents the mean  $\pm$  S.E. of 8–10 determinations.

$= 1900 \pm 64 \text{ M}^{-1} \text{ s}^{-1}$  with  $\text{p}K_{a1} = 6.7 \pm 0.04$  and  $\text{p}K_{a2} = 9.1 \pm 0.05$ . The  $\text{p}K_a$  of peroxynitrous acid at 37  $^{\circ}$ C is 6.8 (15). The apparent  $\text{p}K_a$  at 9.1 should correspond to the ionization of the thiol group of MEG. In contrast to MEG, GED, aminoguanidine, guanidine, and bromide did not accelerate the rate of peroxynitrite decomposition, indicating that they did not react in a second-order process with peroxynitrite.

**MEG Reduces Peroxynitrite-induced Oxidative Injury in Cultured Cells**—In agreement with the oxidation studies, we found that MEG inhibited the suppression of mitochondrial respiration in response to authentic peroxynitrite in J774 cells (Fig. 4). When MEG was applied to the cells shortly after exposure to peroxynitrite, no protection was observed (Fig. 4). As previously reported (22–27), peroxynitrite caused a marked increase in DNA single strand breakage in the J774 cells (Fig. 5). This effect was also reduced by pretreatment of the cells with MEG (Fig. 5). GED and aminoguanidine also reduced the suppression of mitochondrial respiration in response to peroxynitrite, but their potency was less than that of MEG (data not shown). In these assays, a significant fraction of peroxynitrite would react extracellularly before entering the cell (27). Thus, the protection by MEG in this assay is likely to represent a com-



**FIG. 4. Effect of MEG (10, 30, 100, and 300  $\mu$ M) on the peroxynitrite-induced (1 mM) suppression of mitochondrial respiration in J774 cells.** Data are expressed as percent of mitochondrial respiration in untreated (control) cells. Data are expressed as means  $\pm$  S.E. ( $n =$  six to nine wells). There was a significant ( $p < 0.01$ ) suppression of mitochondrial respiration in response to peroxynitrite. MEG was added either 10 min prior to or 10 min after peroxynitrite. \* and \*\*, significant protective effect of MEG against peroxynitrite-induced suppression of mitochondrial respiration ( $p < 0.05$  and  $p < 0.01$ , respectively).



**FIG. 5. Effect of MEG (300  $\mu$ M) on the peroxynitrite-induced (1 mM) DNA single strand breakage in J774 cells.** Data are expressed as percent of maximal DNA single strand breakage in vehicle-treated control cells, in peroxynitrite-treated cells, and in peroxynitrite- and MEG-treated cells. Data are expressed as means  $\pm$  S.E. ( $n =$  six to nine wells). \*\*, significant DNA strand breakage in response to peroxynitrite ( $p < 0.01$ ); #, significant protective effect of MEG against peroxynitrite-induced DNA single strand breakage ( $p < 0.05$ ).

bined extra- and intracellular action of MEG (see also “Discussion”).

**MEG Protects against Suppression of Vascular Contractility in Response to Peroxynitrite in Thoracic Aortic Rings**—As previously reported (27), peroxynitrite (1 mM) caused a marked delayed suppression of vascular contractility in thoracic aortic rings (Fig. 6). This effect was significantly reduced by pretreatment of the blood vessels with 300  $\mu$ M MEG (Fig. 6).

**MEG Does Not Inhibit Vascular and Cytotoxic Effects of NO**—In precontracted aortic rings, SNAP caused a dose-dependent vasodilation, which was significantly enhanced by MEG (Fig. 7A). Exposure of the J774 cells to SNAP, a NO donor compound, caused a suppression of mitochondrial respiration over 24 h (Fig. 7B). Pretreatment of the cells with MEG did not afford protection against this effect (Fig. 7B).

## DISCUSSION

Our results demonstrate that MEG and related mercaptoalkylguanidines are potent scavengers of peroxynitrite and inhibit multiple peroxynitrite-induced oxidative processes (Fig.

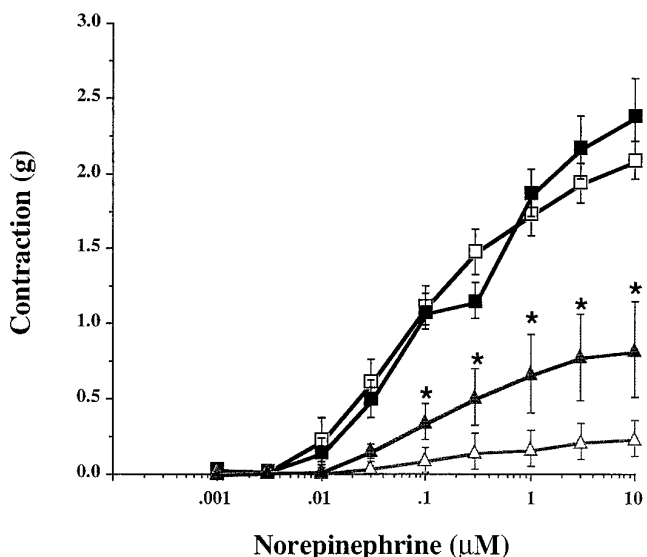


FIG. 6. Effect of peroxynitrite (1 mM) on the contractile activity of norepinephrine (1 nM to 10  $\mu$ M) in thoracic aortic rings and effect of *in vitro* pretreatment with MEG (300  $\mu$ M) on the response.  $\square$ , vehicle-treated controls;  $\blacksquare$ , MEG-treated rings without peroxynitrite treatment;  $\triangle$ , peroxynitrite-treated rings without MEG pretreatment;  $\blacktriangle$ , peroxynitrite-treated rings with MEG pretreatment. Peroxynitrite significantly decreased contractility at 0.1–10  $\mu$ M ( $p < 0.01$ ). \*, significant protection by MEG against the peroxynitrite-induced vascular hyporeactivity ( $p < 0.05$ ). Data are expressed as means  $\pm$  S.E. ( $n = 4$ –10 vascular rings).

8). The potency of MEG appears to be comparable with that of the currently known peroxynitrite scavengers glutathione, cysteine, cysteine methyl ester, and penicillamine (2, 4, 15). The structure-activity relationship of the potencies of MEG and related compounds shows that the free thiol is crucial in this inhibitory effect. In addition, replacement of the sulfur with selenium or *N*-methylation of MEG tends to cause a slight reduction in the inhibitory potency (Table I). It is conceivable that *N*-methylation of the guanidino nitrogen may change the  $pK_a$  of the thiol and thus change the bell shape of the curve determining the rate constant.

Aminoguanidine did not inhibit the oxidation of cytochrome  $c^{2+}$ , but reduced the peroxynitrite-induced hydroxylation of benzoate and the nitration of 4-HPA. Moreover, we observed no second-order reaction between peroxynitrite and aminoguanidine or GED in the stopped-flow experiments. These findings are consistent with the view that cytochrome  $c^{2+}$  oxidation detects primarily second-order oxidative processes (first-order on peroxynitrite and first-order in the target molecule), and benzoate hydroxylation detects primarily first-order processes (zero-order in the target molecule) (see also Refs. 54, 55, 60, and 61). Thus, based on our results, we propose that the free thiol group of MEG and related compounds is crucial in the inhibition of reactions depending on the ground state form of peroxynitrite, while the guanidine or hydrazine group interferes in processes depending on the activated intermediate derived from peroxynitrous acid (ONOOH\*), as indicated in Fig. 8. In the latter effect, the strong reducing properties of the hydrazine moiety ( $-\text{NH}-\text{NH}_2$ ) may play an important role. As accessory mechanism, guanidines could also react with the 1-electron reduction product of peroxynitrite, nitrogen dioxide ( $\text{NO}_2$ ).

The second-order rate constant for the reaction of MEG with peroxynitrite was determined to be  $1900 \text{ M}^{-1} \text{ s}^{-1}$  and is similar to those previously found for the reaction of peroxynitrite with cysteine, glutathione, and the single thiol group of albumin (15) (5000, 1500, and  $2700 \text{ M}^{-1} \text{ s}^{-1}$ , respectively). Interestingly, the

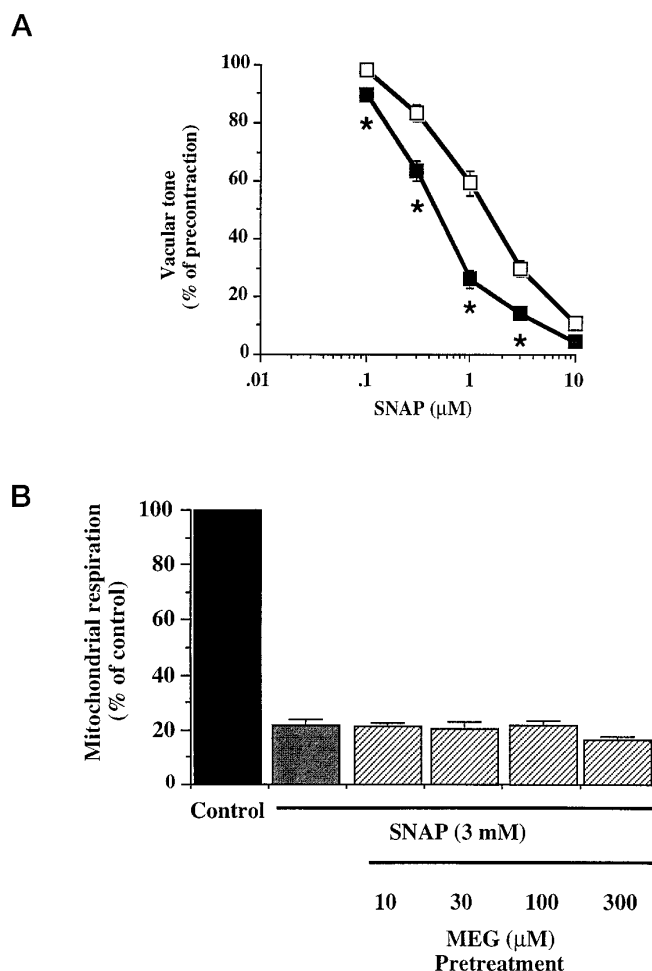


FIG. 7. A, effect of MEG (1 mM) on the relaxant activity of SNAP in thoracic aortic rings precontracted with noradrenaline.  $\square$ , response to SNAP alone;  $\blacksquare$ , response to SNAP in the presence of MEG. Data are expressed as means  $\pm$  S.E. ( $n =$  four to six vascular rings). \*, a significant enhancement by MEG of the SNAP-induced vascular relaxations ( $p < 0.05$ ). B, effect of MEG (10, 30, 100, and 300  $\mu$ M) on the SNAP-induced (3 mM) suppression of mitochondrial respiration in J774 cells. Data are expressed as percent of mitochondrial respiration in untreated (control) cells. Data are expressed as means  $\pm$  S.E. ( $n =$  six to nine wells). There was a significant ( $p < 0.01$ ) suppression of mitochondrial respiration in response to SNAP, which was not significantly altered by MEG.

corresponding disulfide of MEG, GED, did not react in a second-order process, further supporting the view that the second-order reactivity of MEG with peroxynitrite relies on the reaction with the free thiol. The data with GED are also consistent with the lack of increased peroxynitrite decomposition rate in the presence of aminoguanidine and guanidine since the guanidine moiety would be present in GED and aminoguanidine as well.

Our data demonstrate that MEG protects cells and tissues against peroxynitrite-induced cytotoxic effects. Since MEG reacts with peroxynitrite at rates comparable to that of glutathione (the intracellular concentration of which can be as high as 10 mM), while many other guanidines including aminoguanidine do not react in second-order kinetics with peroxynitrite, it is not kinetically apparent how these inhibitors inhibit peroxynitrite-dependent oxidation processes in cellular systems. A likely possibility is the extracellular trapping of peroxynitrite and/or its oxidation products. In addition, at the intracellular level, the inhibitors may well accumulate manifold to efficiently out-compete other targets of peroxynitrite such as met-

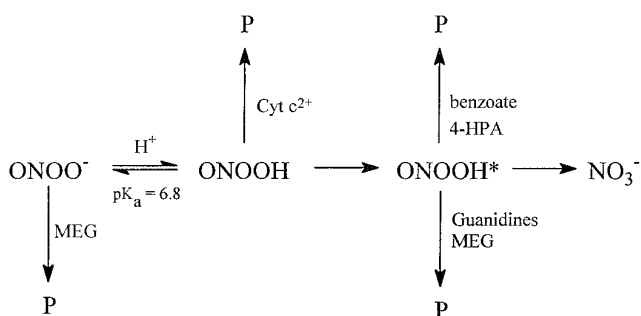


FIG. 8. Proposed mechanisms for the reactivity of peroxynitrite and the sites of actions of MEG and related compounds. Peroxynitrite anion ( $\text{ONOO}^-$ ) is produced by the rapid reaction of NO and superoxide. The reaction of the sulfhydryl group of various compounds, including MEG, occurs primarily via a direct reaction with  $\text{ONOO}^-$ . Peroxynitrite anion is in fast protonation equilibrium with peroxynitrous acid ( $\text{ONOOH}$ ). Peroxynitrous acid directly reacts with cytochrome  $c^{2+}$ , a process that will be inhibited by MEG reactions with peroxynitrite. Peroxynitrous acid will also undergo a rate-limiting transition to an activated intermediate ( $\text{ONOOH}^*$ ), which can oxidize a target molecule such as benzoate or nitrate 4-HPA. This process can be inhibited by thiol-containing compounds (69) such as MEG as well as by guanidines and hydrazines. Guanidines could also react with  $\text{NO}_2$ , which may also participate in their inhibitory effects on 4-HPA nitration (70).

alloproteins and glutathione (15, 61). In this respect, it must be emphasized that the reactions of peroxynitrite in cellular and biological systems are modified by a number of factors, such as bicarbonate, thiols, lipids, cellular proteins, and so on. As shown in this study and in a number of previous studies, in cellular systems exposed to peroxynitrite, a variety of oxidative processes occur (inhibition of mitochondrial enzymes, lipid peroxidation, protein oxidation, DNA injury, etc.). From the available data in the literature, it is not defined what is the relative contribution of peroxynitrite in triggering injury by direct reactions *versus* causing cell damage due to the formation and migration of secondary oxidants such as lipid hydroperoxides from the membrane into the interior of the cell. Current observations with intact cells indicate that peroxynitrite can diffuse to intracellular compartments by more than one mechanism and promote intracellular nitration (62).<sup>4</sup> Taken together, although the mechanism of the protection by MEG against peroxynitrite-induced damage at the cellular level is not clear at the present time, our results clearly demonstrate that 1) MEG inhibits multiple peroxynitrite-induced oxidative processes *in vitro*, and 2) MEG protects various biological systems against the injury induced by supraphysiological concentrations of peroxynitrite. Based on these observations, we suggest that peroxynitrite scavenging (neutralization of peroxynitrite-triggered oxidative processes) may be a therapeutically important pharmacological property of this compound.

Various *S*-nitroso adducts of cysteine, glutathione, and other thiols have been described (63). However, it appears that in neutral anaerobic solutions, NO does not react with thiol-containing compounds to form *S*-nitroso adducts, whereas under aerobic conditions, the formation of *S*-nitroso adducts results from a reaction with intermediates generated in the NO/O<sub>2</sub> reaction (64). In the experiments with cultured J774 macrophages, MEG failed to prevent the suppression of mitochondrial respiration in response to SNAP. Under our experimental conditions, MEG did not inhibit the vascular relaxations elicited by SNAP, but, on the contrary, we observed a slight but significant enhancement. These observations indicate that MEG is not a direct scavenger of NO. We have previously demonstrated that MEG enhances the endothelium-dependent

relaxations in response to acetylcholine (46), a finding that we were unable to explain at that point. One possibility is that MEG enhances the release of NO from SNAP. Another possibility is that MEG, by scavenging peroxynitrite (which is known to be produced when endothelial cells are stimulated with the endothelium-dependent relaxant agent; see Ref. 65), or scavenging oxyradicals (see below) may enhance the relaxations in response to SNAP.

While MEG is not a scavenger of NO, MEG may scavenge various oxyradicals (66, 67). In this respect, previous studies have demonstrated that MEG inhibits radiation-induced DNA injury and cytotoxicity and acts as a modest radioprotective agent in rodents (66, 67). However, neither the exact nature of the oxygen-derived species that react with MEG nor the chemistry of these reactions has been established in these prior studies. In any case, as previously suggested (49, 50), oxyradical scavenging by MEG may reduce the amount of peroxynitrite formed and thus can be considered an additional mode of anti-inflammatory action of these compounds.

The combined effect of MEG as a selective iNOS inhibitor (46) and peroxynitrite scavenger (this study) may be extremely useful in pathophysiological conditions associated with induction of iNOS and peroxynitrite production. In fact, there are *in vivo* data with the isothiourea AETU (which rearranges to form MEG) or with authentic MEG that show that these agents have remarkable protective effects against the vascular failure, hypotension, mortality, and hepatic failure in endotoxic shock (46, 47, 53, 55) and hemorrhagic shock (68). The relative contribution of the iNOS inhibitory *versus* peroxynitrite-scavenging effect in the protective actions remains to be further investigated, but it appears likely that MEG reduces the amounts of NO formed by inhibiting iNOS and also scavenges part of the peroxynitrite produced by residual iNOS activity or from NO produced by constitutive NOS isoforms. Since our data also demonstrate that aminoguanidine is an inhibitor of the peroxynitrite-induced oxidation of benzoate and inhibits the nitration of 4-hydroxyphenylacetic acid, it is possible that some of the previously reported anti-inflammatory effects of aminoguanidine in a variety of experimental models (see the Introduction) may be related to inhibition of peroxynitrite-induced oxidative processes, in addition to direct inhibition of the enzymatic activity of iNOS.

In conclusion, here we have demonstrated that certain guanidine-based NOS inhibitors (in particular, mercaptoalkylguanidines) are potent scavengers of peroxynitrite and protect biological systems against the cytotoxic effects of authentic peroxynitrite. We propose that a combined mode of action (iNOS inhibition, peroxynitrite scavenging, and oxyradical scavenging) is likely to explain their previously reported protective effects in various models of shock and inflammation.

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