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Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence

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1 The objective of this study was to investigate the ability of aminoguanidine, methylguanidine and guanidine to inhibit free radicals or metabolites generated by either stimulated human leucocytes or cell-free systems using luminol-enhanced chemiluminescence (CL).

2 Aminoguanidine (0.1 μM – 10 mM), methylguanidine (10 μM – 10 mM) and guanidine (10 μM – 10 mM) produced concentration-dependent inhibition (96 ± 0.1%, $n=7$, 59 ± 1.3%, $n=6$, and 62 ± 3%, $n=6$, $P<0.05$ at 10 mM, respectively) in FMLP-stimulated leucocytes CL.

3 In cell-free experiments, hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hydroxyl radical and peroxynitrite-induced CL responses were initiated by hydrogen peroxide (3.5 mM), NaOCl (50 μM), $FeSO_4$ (40 nM) and peroxynitrite (20 nM), respectively. Aminoguanidine, methylguanidine and guanidine produced concentration-dependent inhibition in H_2O_2-(69 ± 0.7%, $n=7$, 26 ± 1%, $n=6$, and 15 ± 0.5%, $n=6$, at 1 mM, respectively) and HOCl-(84 ± 0.3%, $n=6$, 50 ± 1%, $n=6$, and 29 ± 1%, $n=7$, at 1 mM, respectively) induced luminol CL. Peroxynitrite-induced CL was markedly attenuated in a concentration-dependent manner by aminoguanidine (99 ± 0.1%, $n=6$, at 10 mM), methylguanidine (5 ± 0.2%, $n=6$, at 10 mM) and guanidine (27 ± 0.4%, $n=7$, at 10 mM). However, inhibition with aminoguanidine was found to be more marked than with methylguanidine and guanidine. Aminoguanidine (95 ± 0.5%, $n=6$, at 1 mM) and methylguanidine (25 ± 1%, $n=6$, at 1 mM), but not guanidine (2 ± 1%, $n=6$, at 1 mM), significantly decreased ferrous iron-induced CL.

4 Collectively, these data suggest that aminoguanidine and a high concentration ($\geqslant$0.1 mM) of methylguanidine have direct scavenging activities against H_2O_2, HOCl, hydroxyl radical and peroxynitrite. Guanidine, at a high concentration ($\geqslant$0.1 mM), scavenges H_2O_2, HOCl and peroxynitrite, but not the hydroxyl radical. These direct scavenging properties may contribute to inhibitory effects of these compounds on human leucocyte CL.

Keywords: Chemiluminescence; aminoguanidine; methylguanidine; guanidine; free radicals; peroxynitrite; antioxidant activity

Introduction

The formation of reactive oxygen species and metabolites appear to play a significant role in many pathological states including diabetes, ischaemia-reperfusion injury and endotoxaemia (Halliwell & Gutteridge, 1991). Stimulated inflammatory cells represent a major source of oxygen radicals and metabolites. Leucocytes, which are activated by a variety of stimuli, produce a series of reactive oxygen species. The initial product of oxygen reduction is superoxide, generated during a respiratory burst. Following this superoxide generation, other oxygen metabolites may then be formed including H_2O_2, singlet oxygen, hydroxyl radical and HOCl, the latter being formed in a reaction catalyzed by myeloperoxidase (Reilly *et al*., 1991). By a biosynthetically distinct pathway, another free radical, nitric oxide, has been demonstrated to be released from leucocytes on activation by different stimuli (Wright *et al*., 1989). Nitric oxide is derived from the guanidino nitrogen of L-arginine by the action of nitric oxide synthase (NOS) enzymes. In L-arginine-depleted macrophages, inducible NOS is capable of generating superoxide radical in addition to nitric oxide (Xia & Zweier, 1997). It has been shown that superoxide radical can react with nitric oxide to form the potent oxidant peroxynitrite (Beckman *et al*., 1990). The peroxynitrite anion is rapidly protonated to peroxynitrous acid which decomposes with a half-life of about one second at pH 7.4 (Beckman *et al*., 1990; Pryor & Squadrito, 1995). Peroxynitrous acid decomposes rapidly to nitrate and can serve as a precursor for other potent reactive species, including nitrogen dioxide, nitronium ion and an intermediate with hydroxyl radical-like reactivity towards a variety of biological molecules (Pryor & Squadrito, 1995). Recent data have provided evidence for *in vivo* formation of peroxynitrite in several pathological conditions. Interaction of guanidine and derivatives with peroxynitrite have not been fully characterized, although it has been suggested that aminoguanidine inhibits lipid peroxidation (Philis-Tsimikas *et al*., 1995) and reacts with an active form of peroxynitrous acid or nitrogen dioxide (Szabo *et al*., 1997). Although aminoguanidine, methylguanidine and guanidine are known to inhibit NOS (Griffiths *et al*., 1993; Sorrentino *et al*., 1997), there is no current evidence that these substances, which are structurally related to the guanidino group of L-arginine, have free radical scavenging activity.

CL has been widely used as a sensitive assay for monitoring free radicals and reactive metabolites from enzyme, cell or organ systems (Archer *et al*., 1989; Demiryürek *et al*., 1994). Generation of reactive oxygen species and metabolites emits light which can be monitored by a variety of luminometers (Van Dyke *et al*., 1986). Light emission can be markedly amplified by luminol which measures a mixture of oxygen-derived species (Betts, 1986; Van Dyke *et al*., 1986). In the present study, we used the chemiluminescence method for assessment of the scavenging activity of aminoguanidine,

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methylguanidine and guanidine against free radicals or metabolites generated by either stimulated human leucocytes or cell-free systems.

Methods

CL generated from human leucocytes

Isolation and separation of leucocytes Human venous blood was obtained from healthy volunteers and leucocytes were isolated according to methods described previously (Demiryürek *et al*., 1998). Blood (9 ml) was taken into tubes containing 3.8% sodium citrate (1 ml). Dextran was added and allowed to sediment at room temperature for 60 min. The leucocyte rich supernatant was removed and centrifuged at 900 r.p.m. for 20 min. Erythrocyte lysis was performed by washing cells with 0.2% NaCl for 30 s and mixing immediately with double volume of 1.6% NaCl. Then, leucocytes were centrifuged at 900 r.p.m. for 15 min and the pellet was resuspended in Hank's buffered salt solution (HBSS) containing 1 mM calcium (pH 7.4). Leucocytes were washed with HBSS three times. After leucocyte count in a cell counter (Contraves, Digicell 300, Zurich, Switzerland), cell yield was adjusted to 10^7 cells ml^{-1} (stock cell suspension) by adding HBSS. Cell viability was assessed by a trypan blue exclusion test and more than 98% of the cells were found to be viable leucocytes ($n=6$). The stock cell suspension was stored at room temperature until use and found to contain 69 ± 7% neutrophils, 35 ± 6% lymphocytes and 4 ± 2% monocytes ($n=3$).

FMLP-induced CL from human leucocytes Stock leucocyte cell suspension (0.1 ml) was diluted with HBSS in a cuvette (total volume of 1 ml) and 20 μl luminol (50 μM; final cuvette concentration) was added, producing a final cell yield of 10^6 cells ml^{-1}. Then a stimulant, N-formyl-methionyl-leucyl-phenylalanine (FMLP), was added to yield final cuvette concentrations of 2 μM. Luminol-CL was measured at 37°C using a chemiluminometer (Bio-Orbit 1250 Luminometer, Turku, Finland). The CL produced was measured continuously and recorded on a computer by using the Luminometer 1250 programme (version 1.12, BioOrbit) for 10 min (Demiryürek *et al*., 1998).

CL generated in cell-free systems

H_2O_2-induced CL H_2O_2 (3.5 mM) was injected to phosphate buffered saline (PBS, 10 mM KH_2PO_4 and 150 mM NaCl, pH 7.4) and luminol (250 μM, prepared daily in 2 M NaOH and diluted with PBS) mixture and generated CL at 37°C was measured continuously for 10 min.

HOCl-induced CL HOCl was prepared as previously described by Vissers *et al*. (1994). NaOCl was diluted with PBS and the pH of the solution readjusted to 7.4 immediately before the addition to the CL cuvette. At this pH, the solution will contain approximately 1:1 HOCl and OCl^- and is subsequently referred to as HOCl. HOCl (50 μM) was injected in to the PBS and luminol (250 μM) mixture to induce CL which was measured continuously for 3 min (Demiryürek *et al*., 1998).

Ferrous iron-induced CL Hydroxyl radical was generated by addition of ferrous iron to the buffer solution as described previously (Green *et al*., 1994; Demiryürek *et al*., 1998). Ferrous iron reduces molecular oxygen to superoxide radical, which in turn dismutates to hydrogen peroxide. Further reduction of H_2O_2 by Fe^{2+} produces the highly reactive hydroxyl radical (Murphy *et al*., 1993). Freshly prepared $FeSO_4$ (40 nM) was added to the PBS plus luminol (250 μM) mixture and CL was recorded continuously for 3 min.

Peroxynitrite synthesis and CL Peroxynitrite was prepared using a quenched flow reaction as described previously (Beckman *et al*., 1994). Briefly, an aqueous solution of 0.6 M sodium nitrite was rapidly mixed with an equal volume of 0.6 M H_2O_2 containing 0.7 M HCl and immediately quenched with the same volume of 1.2 M NaOH. All reactions were performed on ice. Excess H_2O_2 was removed by addition of manganese dioxide (MnO_2) powder to the peroxynitrite solution. The mixture was shaken for 5 min and then MnO_2 was removed by passage over a cellulose acetate disposable filter. The solution was used freshly or frozen at -20°C for as long as a week. The final concentration of peroxynitrite was determined spectrophotometrically in 1.2 M NaOH ($\varepsilon_{302}=1670\ M^{-1}\ cm^{-1}$). Dilutions of this peroxynitrite stock solution were made in 1.2 M NaOH with the final dilution in 0.1 M NaOH before use.

Luminol-enhanced CL were measured at 37°C as described previously (Kahraman & Demiryürek, 1997). PBS was mixed with luminol (250 μM) in a cuvette. After adding catalase (50 u ml^{-1}) to the cuvette to remove H_2O_2 left after MnO_2 treatment, peroxynitrite at 20 nM was injected and the CL produced was measured continuously and recorded for 3 min.

Experimental protocol and statistics

The effects of various concentrations of aminoguanidine, methylguanidine and guanidine were examined by addition to the mixture before the stimulant. Duplicate assays were performed in all experiments. Results were calculated as peak CL or a % of the peak CL and expressed as mean $\pm$ s.e.mean; n refers to the number of individual volunteers (for leucocyte experiment) and number of experiments (for cell free assays). Analysis of variance (ANOVA) was used to assess the observed differences in CL between concentrations. If significant differences were detected by ANOVA, individual means were compared with control by using a Student-Newman-Keuls test. Differences were considered to be statistically significant when the P value was less than 0.05.

Materials

Aminoguanidine hemisulphate, methylguanidine hydrochloride, guanidine hydrochloride, luminol sodium, ferrous sulphate heptahydrate, catalase (from bovine liver), sodium hypochlorite, FMLP were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). H_2O_2 was purchased from Merck (Darmstadt, Germany).

Results

Effects of aminoguanidine, methylguanidine and guanidine on luminol-CL in leucocytes

FMLP (2 μM) produced 4055 ± 91 mV ($n=43$) CL signal in the leucocyte assay (Figure 1a). FMLP-induced response was depressed by either aminoguanidine, methylguanidine or guanidine in a concentration-dependent manner. The most marked reduction of the FMLP-induced luminol CL was

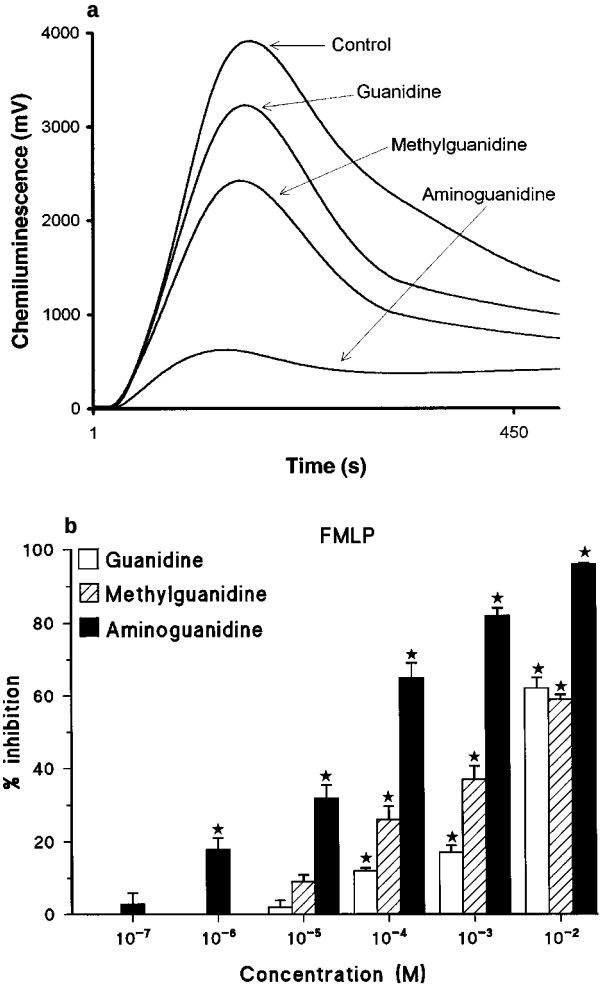

Figure 1 Typical traces representing the effects of guanidine, methylguanidine, and aminoguanidine at 1 mM on FMLP (2 μM)-induced luminol-enhanced chemiluminescence in human isolated leukocytes (a). Concentration-dependent effects of guanidine ($n=6$), methylguanidine ($n=6-7$) and aminoguanidine ($n=6-7$) on FMLP-induced luminol chemiluminescence are shown in (b). Data are shown as mean$\pm$s.e.mean. *$P<0.05$ significantly less than its control value.

observed with aminoguanidine (Figure 1a and b). A significant decrease in the presence of aminoguanidine in FMLP-induced luminol CL was recorded at 1 μM (18$\pm$3%, $n=6$), whereas marked reductions with methylguanidine and guanidine were observed at 0.1 mM. At the concentrations tested, the maximum reduction with aminoguanidine was observed at 10 mM (96$\pm$0.1%, $n=7$). At the same concentration, methylguanidine and guanidine generated only 59$\pm$1.3% ($n=6$) and 62$\pm$3% ($n=6$) inhibitions, respectively.

Effects of aminoguanidine, methylguanidine and guanidine on luminol-CL in the cell free systems

The concentrations of 3.5 mM, 50 μM, 40 nM and 20 nM were selected for H_2O_2, HOCl, $FeSO_4$ and peroxynitrite, respectively, and generated CL peaks (4153$\pm$32 mV, $n=54$, H_2O_2, 4168$\pm$40 mV, $n=48$, HOCl, 4015$\pm$34 mV, $n=49$, $FeSO_4$ and 4179$\pm$66 mV, $n=46$, peroxynitrite) were found to be comparable with that produced by FMLP stimulation in leucocyte experiments.

In luminol CL, all substances inhibited the H_2O_2-induced response in a concentration-dependent manner (Figure 2a). At the concentrations tested, maximum inhibition was recorded at 1 mM (69$\pm$0.7%, $n=7$) in the presence of aminoguanidine. Similar to leucocyte experiments, reductions in H_2O_2-induced luminol CL were less marked with methylguanidine (26$\pm$1%, $n=6$, 1 mM) and guanidine (15$\pm$0.5%, $n=6$, 1 mM).

HOCl-induced luminol CL was also inhibited by these guanidine derivatives and guanidine itself in a concentration-dependent manner. Significant inhibition with aminoguanidine was observed at 1 μM (14$\pm$0.6%, $n=6$) and there was a pronounced inhibition at 1 mM (84$\pm$0.3%, $n=6$). Methylguanidine and guanidine had significant effects at 0.1 and 1 mM. Guanidine was about three times less potent than aminoguanidine at 1 mM inducing only 29$\pm$1% ($n=7$) inhibition (Figure 2b). The maximum inhibition with methylguanidine at 1 mM was 50$\pm$1% ($n=6$).

Aminoguanidine and methylguanidine produced concentration-dependent inhibition of $FeSO_4$-induced luminol CL. Inhibition with aminoguanidine was apparent at 1 μM (11$\pm$2%, $n=7$) and there was almost a complete inhibition at 1 mM (95$\pm$0.5%, $n=6$). The maximum suppression by 1 mM methylguanidine was only 25$\pm$1% ($n=6$). However, guanidine had no effect on ferrous iron-induced CL at any concentration tested (2$\pm$1%, $n=6$, 1 mM) (Figure 2c).

Aminoguanidine generated the most potent inhibition of peroxynitrite-induced luminol CL, almost totally depressing it at 10 mM (99$\pm$0.1%, $n=6$) (Figure 2d). Aminoguanidine had an inhibitory effect at a concentration as low as 0.1 μM (7$\pm$1%, $n=6$). Methylguanidine was almost ineffective and caused only 5$\pm$0.2% ($n=6$) reduction at 10 mM. Guanidine produced significant inhibitions between 0.1 (8$\pm$1%, $n=6$) and 10 mM (27$\pm$0.4%, $n=7$).

Discussion

The binding of FMLP to its specific membrane receptors on phagocytes stimulates the NADPH-oxidase, the enzyme responsible for the production of superoxide radical, mainly by activation via phospholipase C and an increase in Ca^{2+} influx from the medium (Thelen *et al*., 1993). In the present study, guanidine and derivatives, aminoguanidine being the most potent, reduced FMLP-induced leucocyte CL. Several studies have suggested that nitric oxide interacts with the respiratory burst of activated inflammatory cells (Wang *et al*., 1991; Demiryürek *et al*., 1997). Simultaneous production of nitric oxide and superoxide and formation of peroxynitrite have been shown with activated alveolar macrophages (Ischiropoulos *et al*., 1992). Wang *et al*. (1991) demonstrated that luminol-enhanced CL was dependent on the simultaneous production of nitric oxide and superoxide from phorbol ester stimulated rat Kupffer cells. Endogenous nitric oxide may play an important role in the measurement of free radicals released from porcine leucocytes, assessed by luminol-enhanced CL, and compounds with nitric oxide-releasing properties decrease CL, possibly by interfering with free radical generation (Demiryürek *et al*., 1997). These compounds can also decrease FMLP-stimulated leucocyte CL by inhibiting NOS, since aminoguanidine (Griffiths *et al*., 1993; Laszlo *et al*., 1995) as well as methylguanidine (Sorrentino *et al*., 1997) and guanidine (Sorrentino *et al*., 1997) are shown to be NOS inhibitors and inhibition of nitric oxide formation will inhibit the peroxynitrite production.

An enhanced formation of nitric oxide contributes to the acute and delayed hypotension and vascular hyporeactivity to contractile agents in endotoxic shock and aminoguanidine inhibits inducible NOS activity and attenuates the delayed

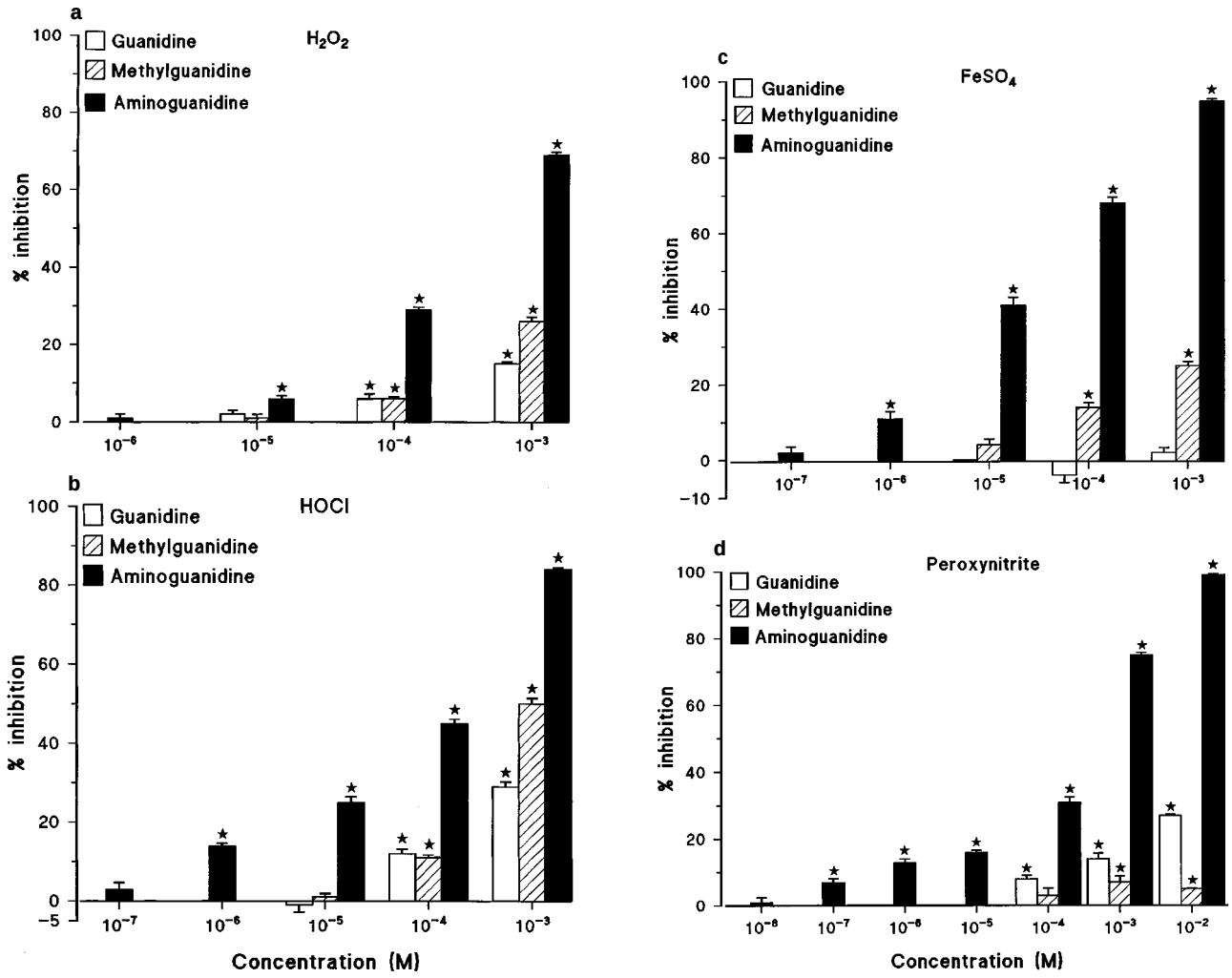

Figure 2 Concentration-dependent effects of guanidine, methylguanidine and aminoguanidine on H_2O_2 (3.5 mM) (a, $n=6-7$, $n=6$ and $n=6-8$, respectively), HOCl (50 μM) (b, $n=7-10$, $n=6-7$ and $n=6-9$, respectively), $FeSO_4$ (40 nM) (c, $n=6$, $n=6$ and $n=6-7$, respectively) and peroxynitrite (20 nM)-induced luminol-enhanced chemiluminescence (d, $n=6-7$, $n=6$ and $n=6-7$, respectively). Data are shown as mean $\pm$ s.e.mean $^*P<0.05$ significantly less than its control value.

circulatory failure and improves survival (Wu *et al.*, 1995). In previous study (Szabo *et al.*, 1997), aminoguanidine has been shown to inhibit the peroxynitrite-induced benzoate hydroxylation and 4-hydroxyphenylacetic acid nitration, indicating that it reacts with the active form of peroxynitrous acid or nitrogen dioxide radical. In this study, we have shown that peroxynitrite reacts with luminol to yield chemiluminescence. This finding agrees with previous results showing that luminol can detect peroxynitrite generated by chemical synthesis (Radi *et al.*, 1993). Aminoguanidine displays pro-oxidant activity at low concentrations (0.01 mM) and antioxidant activity at high concentrations (0.05 to 1 mM) against low density lipoprotein (Philis-Tsimikas *et al.*, 1995). Lipid oxidation leads to the formation of reactive aldehydes and aminoguanidine was found to be an efficient scavenger of α,β-unsaturated aldehydes (Al-Abed & Bucala, 1997). In the present study, aminoguanidine was shown to be a potent scavenger of peroxynitrite in addition to oxygen free radicals or metabolites. Peroxynitrite is one of the most potent reactive metabolites for the initiation of lipid peroxidation (Radi *et al.*, 1991). Therefore, aminoguanidine appears to inhibit lipid peroxidation by scavenging peroxynitrite, as shown in this study, and these results suggest that one of the mechanisms of action of aminoguanidine *in vivo* is to protect tissue components from the damaging effects of oxidative stress. In our experiments, although guanidine was found to have the ability to act as a peroxynitrite scavenger at high concentrations, there were no concentration-dependent effects in the presence of methylguanidine, suggesting that the ability of a compound to act as a peroxynitrite scavenger does not involve its antioxidant activity. It also appears that methyl substitution of a guanidine molecule depresses the peroxynitrite scavenging activity of a compound.

In a present study, we have shown, for the first time, that all the guanidine substances tested were effective as hypochlorous acid scavengers. Although production of the superoxide is one of the major mechanisms by which phagocytes destroy microorganisms, the major toxicity of superoxide is due to the secondary generation of HOCl from H_2O_2 and chloride ions, catalyzed by the enzyme myeloperoxidase, which is present in neutrophils (Reilly *et al.*, 1991).

Although aminoguanidine irreversibly inhibits catalase at high concentrations and produces hydrogen peroxide in the presence of a transition metal-catalyzed process (Ou & Wolff, 1993), it is unlikely that these effects are involved in our experiment since these reactions have been shown to occur over extended periods of time ($\geqslant$1 h) *in vitro* (Ou & Wolff,

1993). Furthermore, in our study, aminoguanidine and, less potently, methylguanidine and guanidine were found to be scavengers of H_2O_2.

Our data also show that aminoguanidine and methylguanidine are hydroxyl radical scavengers. However, guanidine has no effect on hydroxyl radical generating system, suggesting that amino or methyl substitution of a guanidine molecule is necessary for the hydroxyl radical scavenging activity.

The ability of guanidine and its analogues to scavenge free radicals and metabolites may have significant implications in the pathogenesis of certain diseases such as uraemia and chronic renal failure. These pathological conditions are generally characterized by reduced urine production, decreased excretion of methylguanidine and guanidine (Orita *et al*., 1978), their accumulation in both plasma and tissues of patients (Orita *et al*., 1981; Kikuchi *et al*., 1981) and peroxynitrite production (Fukuyama *et al*., 1997). Although methylguanidine and guanidine produced marked inhibition at concentrations more than 100 μM in all the present experiments and these concentrations are higher than levels measured in the plasma of patients with chronic renal failure (3 μM for guanidine and 27 μM for methylguanidine) (Kikuchi *et al*., 1981; Orita *et al*., 1981), it should be pointed out that guanidine and methylguanidine can accumulate intracellularly at concentrations to 5 to 7 times higher than in plasma (Orita *et al*., 1981) and furthermore, they can act synergistically with other compounds. Aminoguanidine has been shown to reduce the peroxynitrite-induced hydroxylation of benzoate with an EC_{50} value of 10 mM (Szabo *et al*., 1997). Moreover, when the reported IC_{50} values of guanidine, methylguanidine and aminoguanidine for neuronal constitutive NOS (2310, 290 and 779 μM, respectively) and for inducible NOS (1140, 370 and 43 μM respectively) (Sorrentino *et al*., 1997) are taken into consideration, our results suggest that guanidine and its derivatives exert free radical scavenging activity at their effective concentrations. Inhibition of diabetic vascular functional changes by aminoguanidine or methylguanidine (Tilton *et al*., 1993) may also involve their free radical and metabolite scavenging activity.

In conclusion, we have demonstrated that aminoguanidine, methylguanidine and guanidine are scavengers of peroxynitrite and oxygen free radicals and protect biological systems against the cytotoxic effects of these reactive species and metabolites. It is likely that a combined mode of action of guanidine and its derivatives (NOS inhibition, peroxynitrite scavenging, and oxygen free radical scavenging) is involved in this action. There is no evidence that aminoguanidine, methylguanidine and guanidine interfere with free radical production of leucocytes, inhibit NADPH oxidase or myeloperoxidase enzyme. However, effects of these guanidine compounds on leucocyte CL may be due to direct scavenging activities against H_2O_2, HOCl, hydroxyl radical or peroxynitrite.

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