British Journal of Pharmacology (1999) 126, 1646–1652
http://www.stockton-press.co.uk/bjp

Modulation of peroxynitrite- and hypochlorous acid-induced inactivation of α_1-antiproteinase by mercaptoethylguanidine

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1 Peroxynitrite is a cytotoxic species that can be formed, among other mechanisms, by the rapid reaction of superoxide with nitric oxide. Peroxynitrite formation has been implicated in a wide range of neurodegenerative and chronic inflammatory diseases, as has the formation of hypochlorous acid by myeloperoxidase.

2 There is considerable interest in the development of peroxynitrite scavengers as therapeutic agents. The thiol compound mercaptoethylguanidine has been suggested to fulfil this role since it has recently been shown to be not only a potent inhibitor of inducible nitric oxide synthase but also a scavenger of peroxynitrite. Indeed, it has been shown to be protective in some experimental models of circulatory shock and inflammation at plasma levels in the approximate range 100–300 μM.

3 One protein inactivated by peroxynitrite is the major inhibitor of serine proteinases in human body fluids, α_1-antiproteinase. At high (250–1000 μM) concentrations, mercaptoethylguanidine was found to be effective in preventing peroxynitrite-mediated tyrosine nitration and α_1-AP inactivation.

4 By contrast, lower concentrations of mercaptoethylguanidine (1–60 μM) enhanced the inactivation of α_1-antiproteinase by peroxynitrite.

5 At all concentrations tested (1–1000 μM), mercaptoethylguanidine decreased the inactivation of α_1-antiproteinase by hypochlorous acid.

6 We suggest that products of reaction of mercaptoethylguanidine with peroxynitrite or peroxynitrite-derived products could cause damage to α_1-antiproteinase, and possibly other proteins *in vivo*, whereas scavenging of hypochlorous acid by mercaptoethylguanidine could contribute to its anti-inflammatory action *in vivo*.

Keywords: Peroxynitrite; nitric oxide; inflammation; mercaptoethylguanidine; iNOS; hypochlorous acid

Abbreviations: α_1-AP, α_1-antiproteinase; DNA, deoxyribonucleic acid; K_2HPO_4, dipotassium hydrogen phosphate; Na_2HPO_4, disodium hydrogen phosphate; HPLC, high performance liquid chromatography; HOCl, hypochlorous acid; MEG, mercaptoethylguanidine; L-NAME, N_ω-nitro-L-arginine methyl ester; NO, nitric oxide; $ONOO^-$, peroxynitrite; KCl, potassium chloride; K_2HPO_4, potassium dihydrogen phosphate; NaCl, sodium chloride; SANA, N-succinyl-(ala)$_3$-*p*-nitroanilide; $O_2^{\bullet -}$, superoxide anion

Introduction

The free radical gas nitric oxide ($NO^\bullet$) has many important physiological functions, but its overproduction may contribute to the pathology of several diseases including rheumatoid arthritis (Kaur & Halliwell, 1994; Jang & Murrell, 1998), gastrointestinal inflammation (Miller *et al*., 1994; Miampamba & Sharkey, 1997), circulatory shock (Fukayama *et al*., 1997; Zingarelli *et al*., 1997a) and various neurodegenerative diseases (Abe *et al*., 1997; Beal *et al*., 1997; Good *et al*., 1996; 1998). Part of the toxicity associated with nitric oxide involves its very fast reaction with superoxide radical ($O_2^{\bullet -}$) to give peroxynitrite, $ONOO^-$. The rate constant for this formation has been determined to be 6.7 (± 0.9) $\times 10^9$ M^{-1} s^{-1} (Huie & Padmaja, 1993).

$$NO^\bullet + O_2^{\bullet -} \longrightarrow ONOO^-$$

At physiological pH, peroxynitrite protonates to form peroxynitrous acid (ONOOH) and decomposes by a series of pathways to generate multiple toxic products with the reactivities of the nitryl cation (NO_2^+), nitrogen dioxide radical ($^\bullet NO_2$) and hydroxyl radical ($^\bullet OH$) (Pryor & Squadrito, 1995; Merényi & Lind, 1997; Kaur *et al*., 1997). The addition of peroxynitrite to biological systems can result in peroxidation of lipids, oxidation of methionine and -SH residues in proteins, depletion of antioxidants, displacement of metals from metalloproteins, deoxyribonucleic acid (DNA) oxidation and nitration, and nitration of protein tyrosine residues (reviewed in: Beckman *et al*., 1994; Pryor & Squadrito, 1995; Halliwell, 1997a).

The thiol compound mercaptoethylguanidine, has been developed as an inhibitor of inducible nitric oxide synthase (iNOS) (Southan *et al*., 1996) and is a potent scavenger of peroxynitrite with a second order rate constant of $1.9 \times 10^3 \pm 64$ M^{-1} s^{-1} (Szabó *et al*., 1997). It also has some inhibitory effect on cyclo-oxygenase-1 and -2 (Zingarelli *et al*., 1997b). By contrast, N_ω-nitro-L-arginine methyl ester (L-NAME) does not scavenge peroxynitrite at a significant rate (Rehman *et al*., 1997). Mercaptoethylguanidine has been claimed to exert beneficial effects in experimental animal models of shock (Zingarelli *et al*., 1997b), inflammation (Cuzzocrea *et al*., 1998; Lohinai *et al*., 1998) and arthritis (Brahn *et al*., 1998), presumably by acting as an inducible nitric oxide synthase inhibitor and/or a peroxynitrite scavenger. However, we have recently shown that at concentrations lower than 60 μM, certain thiols and disulphides increase the toxicity

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of peroxynitrite (Whiteman & Halliwell, 1997) and it is important to know if this could apply to mercaptoethylguanidine before advocating its therapeutic use. As a model system for such effects we use α_1-antiproteinase (α_1-AP) (Whiteman & Halliwell, 1997; Halliwell & Whiteman, 1998) the major inhibitor of serine proteases (such as elastase) in human body fluids. α_1-AP is inactivated at sites of inflammation (Chidwick *et al*., 1991) and has been shown to be inactivated by peroxynitrite *in vitro* (Moreno & Pyror, 1992). It can also be inactivated by hypochlorous acid (HOCl), generated by the enzyme myeloperoxidase from neutrophils at sites of inflammation (Weiss, 1989). The action of mercaptoethylguanidine on hypochlorous acid has never been reported.

In this paper the interactions of mercaptoethylguanidine with peroxynitrite and hypochlorous acid are described.

Methods

Synthesis of mercaptoethylguanidine

Mercaptoethylguanidine hydrochloride was synthesised by Dr Garry Southan at Inotek Corporation (Cincinnati, Ohio, U.S.A.). The following procedure was used to generate free (basic) mercaptoethylguanidine in solution: 160 ml of Amberlite IRA 402 (OH-) in 100 ml purified water was degassed thoroughly and saturated with nitrogen. Aminoethyl-isothiourea (Sigma, 20 g) was added with stirring and continually stirred at room temperature under nitrogen for 12–15 min. The solution was then filtered to remove the resin. The freshly prepared solution of mercaptoethylguanidine (free base, pH ~ 11) was brought to pH 7.4 with careful addition of a solution containing succinic acid and sodium hydroxide (both approximately 3 M). The mixture was concentrated by rotary evaporation followed by lyophilization. The white solid product dissolves readily in water or saline to give a solution of pH 7.4. The formula of the salt is close to the expected stoichiometry of 1:1:1 with formula weight of 259, 46% of which is mercaptoethylguanidine by mass. Purity of the preparation (>98%) was confirmed by high performance liquid chromatography (HPLC) analysis.

Synthesis of peroxynitrite

Synthesis of peroxynitrite was essentially as described in Beckman *et al*. (1994). Briefly, an acidic solution (0.6 M hydrochloric acid) of hydrogen peroxide (0.7 M) was mixed with potassium nitrite (0.6 M) on ice for 1 s and the reaction quenched with ice cold sodium hydroxide (1.2 M). Residual hydrogen peroxide was removed by mixing with granular manganese dioxide prewashed with sodium hydroxide (1.2 M). The stock solution was filtered and then frozen overnight (−20°C) and the top layer of the solution collected for the experiment. Concentrations of stock peroxynitrite were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1} (Hughes & Nicklin, 1968). Concentrations of 200–250 mM were usually obtained. Once thawed, peroxynitrite solutions were kept on ice for no longer than 30 min before use (Beckman *et al*., 1994).

Preparation of hypochlorous acid

Hypochlorite (OCl^-) concentrations were determined immediately prior to experimentation at 290 nm using a molar extinction coefficient of 350 M^{-1} cm^{-1} (Morris, 1966). Hypochlorous acid (HOCl) has a pKa of 7.46, thus the term hypochlorous acid is used here to represent a mixture of hypochlorous acid (HOCl) and hypochlorite (OCl^-).

Effect of mercaptoethylguanidine on the nitration of tyrosine by peroxynitrite

This was conducted essentially as described in Whiteman & Halliwell, (1996a). Addition of peroxynitrite to tyrosine at physiological pH leads to the rapid nitration of tyrosine to give 3-nitrotyrosine.

A 10 ml stock concentration (10 mM) of D,L-tyrosine was prepared by dissolving the required amount of tyrosine in 8 ml water with 250 μl (10% w/v) potassium hydroxide followed by 250 μl 5% (v/v) phosphoric acid with 1.5 ml water. Mercaptoethylguanidine was dissolved in phosphate buffer (500 mM $K_2HPO_4-KH_2PO_4$, pH 7.4) fresh before use to give a stock concentration of 10 mM. One hundred microlitres of the tyrosine solution together with 100 μl of phosphate buffer or test compound (mercaptoethylguanidine or reduced glutathione) solution was added to a plastic test tube containing 795 μl buffer (500 mM, pH 7.4) to give final concentrations of 1, 3, 6, 15, 30, 60, 125, 250, 500 and 1000 μM. The samples were then incubated in a water bath at 37°C for 15 min. After this time peroxynitrite (typically 5 μl) was added to give a final concentration of 1 mM, the tubes were vortexed for 15 s and incubated for a further 15 min. The pH of the reaction mixture was measured after every experiment and found to be 7.4–7.5. Reduced glutathione, dissolved in buffer as above, was used at the same concentrations instead of mercaptoethylguanidine, as a positive control (Whiteman & Halliwell, 1996a). Control experiments containing 1 mM succinic acid (residual from mercaptoethylguanidine synthesis) were also performed.

High performance liquid chromatography measurement of 3-nitrotyrosine

This was conducted essentially as described in Whiteman & Halliwell (1996a). Briefly, high performance liquid chromatography separation was carried out on a Spherisorb 5 μm ODS2 C_{18} column (Wellington House, Cheshire, England) with a guard column (Hibar from BDH, Poole, England) and C_{18} cartridge. The eluant was 500 mM KH_2PO_4-H_3PO_4, pH 3.01, with 20% methanol (v/v) at a flow rate of 1.0 ml m^{-1} through a Polymer Laboratories pump (Essex Road, Church Stretton, England) and a UV detector set at 274 nm. Peak heights of 3-nitrotyrosine were measured and concentrations calculated from a standard curve. The limit of sensitivity was 0.5 μM 3-nitrotyrosine: typical retention time under our experimental conditions was 3.6 min.

Effect of mercaptoethylguanidine on the inactivation of α_1-antiproteinase by peroxynitrite

α_1-antiproteinase was dissolved in phosphate buffered saline, pH 7.4 (in mM): NaCl 140, KCl 2.7, Na_2HPO_4 16, KH_2PO_4 2.9 to a concentration of 4.0 mg ml^{-1} and elastase was also dissolved in the same buffer to 5.0 mg ml^{-1} (Whiteman & Halliwell, 1996b). The volume of α_1-antiproteinase required to inhibit elastase activity 80–90% (usually 60–70 μl) was added to buffer (K_2HPO_4-KH_2PO_4 500 mM, pH 7.4) with or without 100 μl of the compound to be tested (mercaptoethylguanidine or reduced glutathione) to give a volume of 945 μl. The final concentrations of test compounds used were 1, 3, 6, 15, 30, 60, 125, 250, 500 and 1000 μM. The samples were then incubated in a water bath at 37°C for 15 min. Then peroxynitrite (typically 5 μl) was added to give a final concentration of

500 μM. The sample was vortexed for 15 s and incubated for 5 min, elastase (usually 50 μl) was added and the sample further incubated at 37°C for 15 min followed by addition of 2.0 ml buffer. Then after 15 min, 100 μl of substrate (*N*-succinyl(Ala)$_3$-*p*-nitroanilide) was added and the rate of reaction followed at 410 nm for 30 s as a measure of elastase activity. Hydrolysis of the synthetic substrate *N*-succinyl(Ala)$_3$-*p*-nitroanilide by elastase liberates the chromogen *p*-nitroaniline at a rate proportional to elastase activity. The addition of α_1-antiproteinase decreases elastase activity and measurement of the extent of inhibition allows assessment of α_1-antiproteinase activity. These times were selected from our previous study as being sufficient for the elastase inhibitory capacity of α_1-antiproteinase damaged by peroxynitrite to be exerted (Whiteman & Halliwell, 1996a).

Controls without peroxynitrite addition, were performed to assess any direct action of mercaptoethylguanidine or reduced glutathione on the activity of elastase or α_1-antiproteinase. Additional control experiments containing 1 mM succinic acid were also performed.

Effect of mercaptoethylguanidine on peroxynitrite-treated α_1-antiproteinase

α_1-antiproteinase was prepared as described above and incubated for 15 min in 500 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.4), peroxynitrite (500 μM) added and the samples vortexed for 15 s. After this time 1 mM mercaptoethylguanidine was added. The samples were then incubated at 37°C for 1 h, elastase (typically 50 μl) added and residual elastase activity measured after the addition of 2.0 ml K_2HPO_4-KH_2PO_4 buffer (pH 7.4) and 100 μl *N*-succinyl(Ala)$_3$-*p*-nitroanilide as described above.

Effect of peroxynitrite concentration on aggravated α_1-antiproteinase inactivation by mercaptoethylguanidine

α_1-antiproteinase was prepared as described above and incubated for 15 min in 500 mM K_2HPO_4-KH_2PO_4 buffer (pH 7.4) containing 30 μM of mercaptoethylguanidine or reduced glutathione. After this time, increasing concentrations of peroxynitrite (15–1000 μM) were added and the samples incubated for a further 5 min and elastase (typically 50 μl) added for 15 min. Residual elastase activity was then measured as described above.

Effect of mercaptoethylguanidine on the inactivation of α_1-antiproteinase by hypochlorous acid

α_1-antiproteinase was prepared as described above and incubated in 500 mM phosphate buffer (K_2HPO_4-KH_2PO_4, pH 7.4) for 15 min in the presence of varied concentrations (1–1000 μM) of mercaptoethylguanidine or reduced glutathione. After this time 5 μM hypochlorous acid was added, the samples vortexed for 15 s and further incubation at 37°C for 1 h was carried out to ensure that residual elastase-inhibitory capacity of α_1-antiproteinase damaged by hypochlorous acid could be exerted. Residual elastase activity was then measured as described above. The effect of 1 mM succinic acid, residual from mercaptoethylguanidine synthesis, at protecting α_1-AP from HOCl-dependent inactivation was also examined.

Data analysis

All graphs are plotted with mean ± standard error of the mean (s.e.mean). In all cases the mean values were calculated from data taken from at least four separate experiments performed on separate days using freshly prepared reagents. Where significance testing was performed, an independent *t*-test (Students; two populations) was used.

Materials

N-succinyl-(ala)$_3$-*p*-nitroanilide (SANA), elastase (E0258), α_1-antiproteinase (A9024), D,L-tyrosine, reduced glutathione, 3-nitrotyrosine and all other reagents were from Sigma Chemical Corp., (Poole, Dorset, England).

Results

Effect of mercaptoethylguanidine on inactivation of α_1-antiproteinase by peroxynitrite

Addition of peroxynitrite to α_1-antiproteinase caused inactivation of its elastase-inhibitory capacity (Figures 1A and B). A peroxynitrite concentration of 500 μM was chosen for most experiments to achieve substantial but not complete inactivation, so that both decreases and increases in the extent of inactivation could be detected (Whiteman & Halliwell, 1996a). Figure 1A also shows that addition of 1 mM mercaptoethylguanidine substantially protected α_1-antiproteinase from peroxynitrite-mediated inactivation, although mercaptoethylguanidine was not as effective as reduced glutathione, used as a positive control since it is known to be a good scavenger of peroxynitrite (Beckman *et al*., 1994). By contrast, the addition of low concentrations of mercaptoethylguanidine (1–125 μM) reproducibly aggravated α_1-antiproteinase inactivation by peroxynitrite (Figure 1A). However, at concentrations greater than 250 μM (e.g. 1 mM, Figure 1), mercaptoethylguanidine protected α_1-antiproteinase from inactivation. Reduced glutathione did not aggravate α_1-antiproteinase inactivation at any concentration as expected from previous results (Whiteman & Halliwell, 1996a). Figure 1B shows the effect of peroxynitrite concentration on the activation of α_1-antiproteinase by peroxynitrite in the presence of 30 μM mercaptoethylguanidine. Mercaptoethylguanidine reproducibly aggravated α_1-antiproteinase inactivation at all peroxynitrite concentrations tested (1–1000 μM). Even when low (1–60 μM) peroxynitrite concentrations were added, significant aggravation was observed. Reduced glutathione, by contrast did not aggravate inactivation.

Figure 2 shows control experiments. For example, mercaptoethylguanidine had no direct effect on elastase or the ability of α_1-antiproteinase to inhibit elastase (Figure 2A). If mercaptoethylguanidine was added to the reaction mixture 5 min after addition or peroxynitrite to α_1-antiproteinase, it had no effect, neither stimulating nor decreasing inactivation (Figure 2B). Since inactivation of α_1-antiproteinase by peroxynitrite is complete within 5 min (Whiteman & Halliwell, 1996a), it follows that mercaptoethylguanidine is not acting by altering the residual activity of α_1-antiproteinase after damage by peroxynitrite. Similarly, incubation of mercaptoethylguanidine with peroxynitrite for 5 min before adding α_1-antiproteinase did not give any observable inactivation of α_1-antiproteinase subsequently added (Figure 2C). Therefore, the products of reaction of mercaptoethylguanidine with peroxynitrite that cause the inactivation of α_1-antiproteinase do not persist in the reaction mixture.

Additional control experiments showed that succinic acid (residual from mercaptoethylguanidine synthesis) did not significantly scavenge peroxynitrite or aggravate peroxyni-

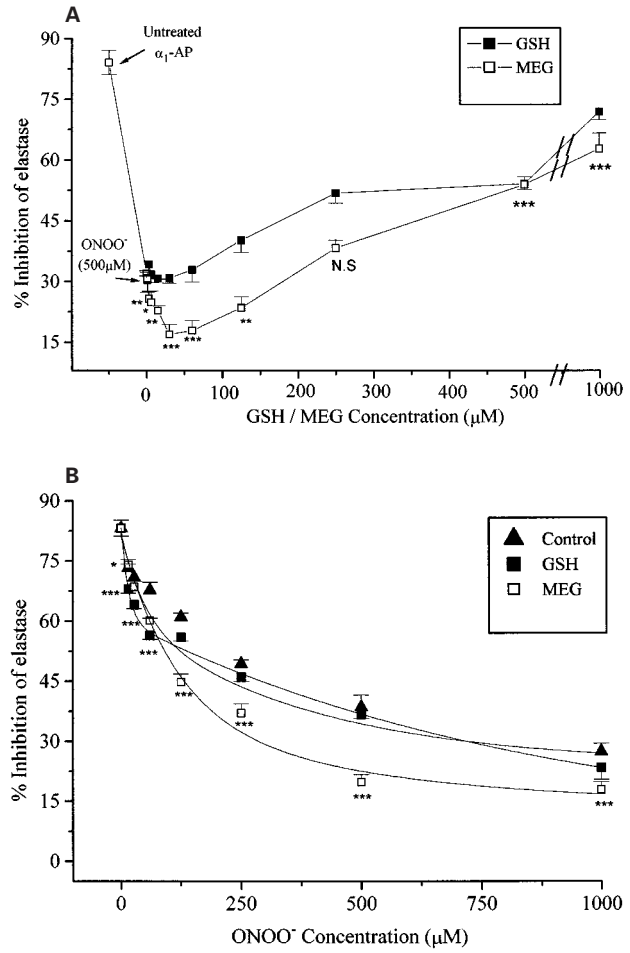

Figure 1 Aggravation of peroxynitrite ($ONOO^-$)-mediated α_1-antiproteinase (α_1-AP) inactivation by mercaptoethylguanidine (MEG): a comparison with reduced glutathione (GSH). (A) $ONOO^-$ (500 μM) was added to α_1-AP in the presence of increasing concentrations of MEG. The ranges of mercaptoethylguanidine and GSH used were 1–1000 μM. (B) Increasing concentrations of $ONOO^-$ were added to α_1-AP in the presence of 30 μM MEG. All experiments were conducted at pH 7.4 and residual elastase activity was measured as described in the Methods section. Results are expressed as mean ± s.e.mean of four or more separate experiments. Statistical significance was evaluated using Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$. $^{***}P<0.001$, N.S, not statistically significant compared to $ONOO^-$ treated α_1-AP.

trite-mediated inactivation of α_1-antiproteinase (data not shown).

Effect of mercaptoethylguanidine on nitration of tyrosine by peroxynitrite

Addition of peroxynitrite to the amino acid tyrosine leads to its nitration and the formation of 3-nitrotyrosine (Beckman *et al*., 1994). A 1 mM concentration of tyrosine was used to obtain significant amounts of 3-nitrotyrosine for ease of measurement, but similar data were obtained at lower peroxynitrite concentrations. Mercaptoethylguanidine inhibited under all reaction conditions examined. Unlike its action with α_1-antiproteinase, mercaptoethylguanidine did not stimulate tyrosine nitration at any concentration tested (Figure 3). Even at low concentrations (60 μM) compared to 1 mM peroxynitrite, mercaptoethylguanidine decreased peroxynitrite-mediated tyrosine nitration to an extent not significantly different to that of the positive control, reduced glutathione, known to be a powerful scavenger of peroxynitrite (Beckman

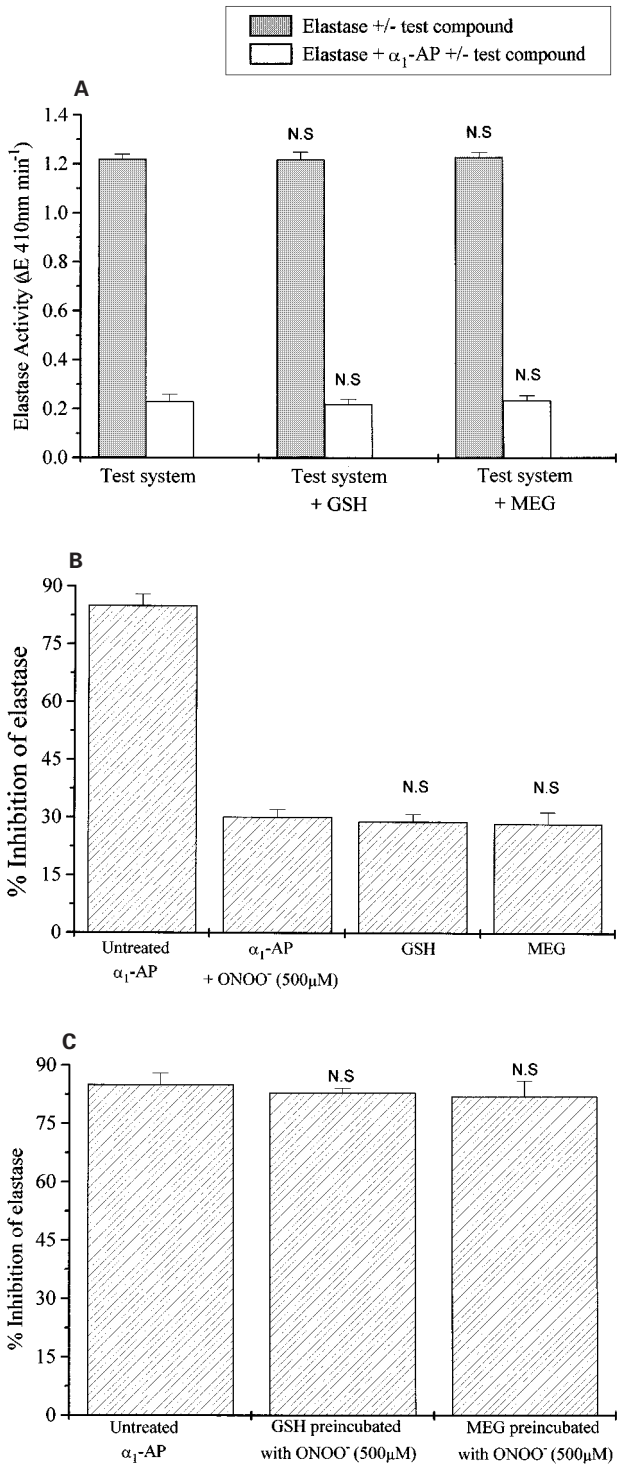

Figure 2 Control experiments. (A) α_1-Antiproteinase (α_1-AP) inactivation by peroxynitrite ($ONOO^-$); the effect of mercaptoethylguanidine (MEG) and reduced glutathione (GSH). The test system consisted of vehicle (500 mM KH_2PO_4-K_2HPO_4 buffer pH 7.4) with either elastase or elastase plus α_1-AP. (B) α_1-AP was incubated with $ONOO^-$ (500 μM) and buffer for 5 min, then MEG or GSH (1 mM) added followed by a further 60 min incubation. Statistical significance was evaluated using Student's *t*-test. N.S; not statistically significant compared to $ONOO^-$ treated α_1-AP. (C) $ONOO^-$ (500 μM) was incubated with mercaptoethylguanidine (MEG) or reduced glutathione (GSH; 30 μM) for 5 min, then α_1-antiproteinase (α_1-AP) was added followed by a further 30 min incubation. All experiments were conducted at pH 7.4 as described in the Methods section. Results are expressed as mean ± s.e.mean of four or more separate experiments. Statistical significance was evaluated using Student's *t*-test. N.S; not statistically significant compared to vehicle treated α_1-AP.

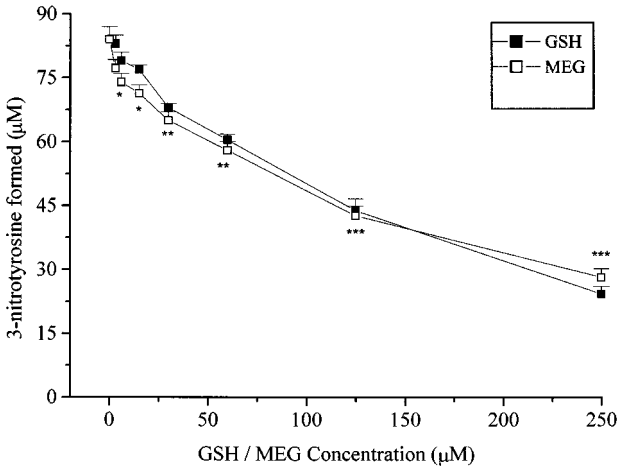

Figure 3 Effect of mercaptoethylguanidine (MEG) on the nitration of tyrosine by peroxynitrite. Peroxynitrite (1 mM) was added to tyrosine (1 mM) at pH 7.4 in the presence of increasing concentrations of MEG or reduced glutathione (GSH). The formation of 3-nitrotyrosine was measured by high performance liquid chromatography as described in the Methods section. Results are expressed as Mean ± s.e.mean of six or more separate experiments. Statistical significance was evaluated using Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to tyrosine with peroxynitrite (1 mM) add vehicle. There was no statistically significant difference between MEG and GSH at any of the concentrations tested.

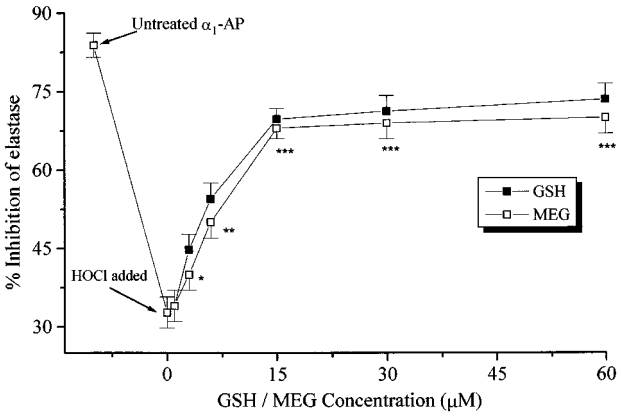

Figure 4 Effect of mercaptoethylguanidine (MEG) on hypochlorous acid-mediated inactivation of α_1-antiproteinase at pH 7.4. Experiments were conducted as described in the Methods section. Results are expressed as mean ± s.e.mean of four or more separate experiments. Statistical significance was evaluated using Student's *t*-test. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared to hypochlorous acid-treated α_1-antiproteinase. There was no statistically significant difference between MEG and GSH at any of the concentrations tested.

et al., 1994; Whiteman & Halliwell, 1996a). Succinic acid (residual from mercaptoethylguanidine synthesis) when tested up to 1 mM, did not significantly inhibit tyrosine nitration (data not shown).

Effect of mercaptoethylguanidine on inactivation of α_1-antiproteinase by hypochlorous acid

Another agent known to inhibit α_1-antiproteinase *in vitro* and *in vivo* is hypochlorous acid (Weiss *et al*., 1989). Concentrations of mercaptoethylguanidine that aggravated α_1-antiproteinase inactivation by peroxynitrite did not promote its inactivation by hypochlorous acid. Indeed, all concentrations tested protected α_1-antiproteinase from hypochlorous acid-mediated inactivation to an extent not significantly different from the positive control, reduced glutathione (Figure 4). Succinic acid tested up to 1 mM concentrations did not significantly protect α_1-antiproteinase from HOCl-mediated inactivation (data not shown).

Discussion

Previously (Whiteman & Halliwell, 1997), our laboratory has shown that certain thiols and disulphides at low concentrations can aggravate the inactivation of α_1-antiproteinase upon addition of peroxynitrite, whereas at high concentrations they are excellent peroxynitrite scavengers (Whiteman & Halliwell, 1996b). These properties should be considered before advocating the use of thiols as antioxidants (Halliwell, 1997b), and so we investigated the behaviour of mercaptoethylguanidine, which has been suggested for use in the therapy of chronic inflammation and septic shock, as an inhibitor of inducible nitric oxide synthase and powerful scavenger of peroxynitrite (Zingarelli *et al*., 1997b; Cuzzocrea *et al*., 1998; Lohinai *et al*., 1998; Brahn *et al*., 1998). We found that mercaptoethylguanidine at low concentrations (1–125 μM) aggravated peroxynitrite-mediated damage to α_1-antiproteinase but at higher concentrations (>250 μM) it was able to protect α_1-antiproteinase.

The usual doses of mercaptoethylguanidine given to animals have been 3–10 mg kg^{-1} body weight. By using a rough estimation (10% of body weight considered as blood volume), this corresponds to initial plasma levels of approximately 100–300 μM, which overlaps into the range at which stimulatory effects of damage by peroxynitrite would be feasible. These peak concentrations presumably decline rapidly as mercaptoethylguanidine is excreted/metabolized, bringing them further into the 'pro-oxidant' range.

Our data also raise the possibility of another important action of mercaptoethylguanidine, the scavenging of hypochlorous acid, which occurs over a range of mercaptoethylguanidine concentrations. Hypochlorous acid is cytotoxic and oxidises many important biomolecules including DNA repair enzymes, collagen, ascorbate and sulphydryls (Halliwell *et al*., 1987; Schraufstatter *et al*., 1990; Van Rensberg *et al*., 1991; Prutz, 1996). It also chlorinates fatty acids and cholesterol in cell membranes (Carr *et al*., 1997) as well as oxidizing and chlorinating deoxyribonucleic acid (Whiteman *et al*., 1997) and chlorinating proteins (Domigan *et al*., 1995; Kettle, 1996). The protection of these biomolecules by scavenging hypochlorous acid could be a beneficial effect of mercaptoethylguanidine, perhaps even outweighing its ability to exacerbate damage to α_1-antiproteinase by peroxynitrite.

The mechanism for the aggravation of peroxynitrite-dependent α_1-antiproteinase inactivation may involve the one electron oxidation of the thiol moiety of mercaptoethylguanidine by peroxynitrite since aggravated α_1-antiproteinase inactivation was not observed with the poor one electron oxidant but powerful two electron oxidant hypochlorous acid. The two electron oxidation of mercaptoethylguanidine by hypochlorous acid would lead to the formation of mercaptoethylguanidine-disulphide. Indeed, the reaction of hypochlorous acid with thiols (presumably including mercaptoethylguanidine) does not produce free radical species (Folkes *et al*., 1995). A one electron oxidation of the thiol moiety by peroxynitrite would produce thiyl radicals (RS$^\bullet$), which in oxygenated solutions can form RSOO$^\bullet$, RSO$^\bullet$, and reactive oxysulphur radicals are thought to be capable of inactivating α_1-antiproteinase (Aruoma *et al*., 1989). Indeed,

thiols are known to react with peroxynitrite to generate thiyl and thiyl-derived radicals (Karoui *et al*., 1996; Gatti *et al*., 1994) that can inactivate several enzymes (Bremner *et al*., 1987). The species responsible for mercaptoethylguanidine aggravation of peroxynitrite inactivation of α_1-antiproteinase were shown to be short lived (Figure 2C) consistent with a free radical mechanism. Identification of such species deserves further attention since quenching them could limit potential toxic effects of thiol compounds such as mercaptoethylguanidine, for treating conditions in which peroxynitrite generation is implicated such as rheumatoid arthritis (Kaur & Halliwell, 1994), gastrointestinal inflammation (Miller *et al*., 1994; Miampamba & Sharkey, 1997), circulatory shock (Fukayama *et al*., 1997) and neurodegenerative diseases (Abe *et al*., 1997; Beal *et al*., 1997; Good *et al*., 1996;1998).

In summary, it is possible that while scavenging hypochlorous acid and peroxynitrite (Szabó *et al*., 1997), inhibiting inducible nitric oxide synthase and cyclo-oxygenases (Southan *et al*., 1996; Zingarelli *et al*., 1997b) and suppressing the symptoms of inflammation (Brahn *et al*., 1998; Lohinai *et al*., 1998) and shock (Zingarelli *et al*., 1997a), mercaptoethylguanidine might exacerbate oxidative damage to some other biomolecules *in vivo* and give rise to side effects. Therefore in screening thiols and thiols-related compounds for peroxynitrite scavenging activities, the concentration ranges to be used must be carefully considered and several assays of scavenging activity performed (Halliwell, 1997b; Halliwell & Whiteman, 1998). Given the growing interest in the therapeutic development of peroxynitrite scavengers (Hooper *et al*., 1998; Brahn *et al*., 1998) it is most important that their effects be thoroughly characterized.

We are grateful to the Arthritis and Rheumatism Campaign (ARC) for their research support.

References

ABE, K., PAN, L.H., WATANABE, M., KONNO, H., KATO, T. & ITOYAMA, Y. (1997). Upregulation of protein-tyrosine nitration in the anterior horn cells of amyotrophic lateral sclerosis. *Neurol. Res.,* **19,** 124 – 128.

ARUOMA, O.I., HALLIWELL, B., BUTLER, J. & HOEY, B.M. (1989). Apparent inactivation of α_1-antiproteinase by sulphur-containing radicals derived from penicillamine. *Biochem. Pharmacol.,* **38,** 4353 – 4357.

BEAL, M.F., FERRANTE, R.J., BROWNE, S.E., MATTHEWS, R.T., KOWALL, N.W. & BROWN, R.H. (1997). Increased 3-nitrotyrosine in both sporadic and familial amyotrophic lateral sclerosis. *Ann. Neurol.,* **42,** 644 – 654.

BECKMAN, J.S., CHEN, J., ISCHIROPOLOUS, H. & CROW, J.P. (1994). Oxidative chemistry of peroxynitrite. *Methods Enzymol.,* **233,** 229 – 240.

BRAHN, E., BANQUERIGO, M.L., FIRESTEIN, G.S., BOYLE, D.L., SALZMAN, A.L. & SZABÓ, C. (1998). Collagen-induced arthritis: Reversal by mercaptoethylguanidine, a novel anti-inflammatory agent with a combined mechanism of action. *J. Rheumatol.,* **25,** 1785 – 1793.

BREMNER, J.C.M., SIMONE, G. & TAMBA, M. (1987). Sulphur peroxy radicals in enzyme inactivation. *Radiat. Res.* (Proc. 8th Int. Congr. Radiat. Res., Edinburgh) **1,** A42 – 8.

CARR, A., VANDERBERG, J.J.M. & WINTERBOURN, C.C. (1997). Chlorination of cholesterol in cell membranes by hypochlorous acid. *Arch. Biochem. Biophys.,* **332,** 63 – 69.

CHIDWICK, K., ZHANG, Z., WINYARD, P.G., FARRELL, A.J. & BLAKE, D.R. (1991). Inactivation of the elastase inhibitory activity of α_1-antitrypsin in fresh samples of synovial fluid from patients with rheumatoid arthritis. *Annals. Rheum. Dis.,* **50,** 78 – 79.

CUZZOCREA, S., ZINGARELLI, B., HAKE, P., SALZMAN, A.L. & SZABÓ, C. (1998). Anti-inflammatory effects of mercaptoethylguanidine, a novel combined inhibitor of nitric oxide synthase and peroxynitrite scavenger, in carrageenan-induced models of inflammation. *Free Rad. Biol. Med.,* **24,** 450 – 459.

DOMIGAN, N.M., CHARLTON, T.S., DUNCAN, M.W., WINTERBOURN, C.C. & KETTLE, A.J. (1995). Chlorination of tyrosyl residues in peptides by myeloperoxidase and human neutrophils. *J. Biol. Chem.,* **270,** 16542 – 16548.

FOLKES, L.K., CANDEIAS, L.P. & WARDMAN, P. (1995). Kinetics and mechanisms of hypochlorous acid reactions. *Arch. Biochem. Biophys.,* **323,** 120 – 126.

FUKAYAMA, N., TAKEBAYASHI, Y., HIDA, M., ISHIDA, H., ICHIMORI, K. & NAKAZAWA, H. (1997). Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Rad. Biol. Med.,* **22,** 771 – 774.

GATTI, R.M., RADI, R. & AUGUSTO, O. (1994). Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical. *FEBS Letts.,* **348,** 287 – 290.

GOOD, P.F., HSU, A., PERL, D.P. & OLANOW, C.W. (1998). Protein nitration in Parkinson's disease. *J. Neuropath. Exptl. Neurol.,* **57,** 338 – 342.

GOOD, P.F., WERNER, P., HSU, A., OLANOW, C.W. & PERL, D.P. (1996). Evidence for neuronal oxidative damage in Alzheimer's disease. *Am. J. Path.,* **149,** 21 – 28.

HALLIWELL, B. (1997a). What nitrates tyrosine? *FEBS Letts.,* **411,** 157 – 160.

HALLIWELL, B. (1997b). Antioxidants! The basics-what they are and how to evaluate them. *Adv. Pharmacol.,* **38,** 3 – 20.

HALLIWELL, B., WASIL, M. & GROOTVELD, M. (1987). Biologically significant scavenging of the myeloperoxidase-derived oxidant hypochlorous acid by ascorbic acid: Implications for antioxidant protection in the inflamed rheumatoid joint. *FEBS Letts.,* **231,** 15 – 18.

HALLIWELL, B. & WHITEMAN, M. (1998). Measurement of peroxynitrite scavengers. *Meth. Enzymol.,* **301,** 333 – 342.

HOOPER, D.C., SPITSIN, S., KEAN, R.B., CHAMPION, J.M., DICKSON, G.M., CHAUDHRY, J. & KOPROWSKI, H. (1998). Uric acid: A natural scavenger of peroxynitrite in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 675 – 680.

HUGHES, M.N. & NICKLIN, H.G. (1968). The chemistry of pernitrites. Part I. Kinetics and decomposition of pernitrous acid. *J. Chem. Soc. (A).* 450 – 452.

HUIE, R.E. & PADMAJA, S. (1993). The reaction of superoxide with nitric oxide. *Free Rad. Res.,* **18,** 195 – 199.

JANG, D. & MURRELL, G.A.C. (1998). Nitric oxide in arthritis. *Free Radic. Biol. Med.,* **24,** 1511 – 1519.

KAROUI, H., HOGG, N., FREJAVILLE, T. & KALYANARAMAN, B. (1996). Characteristics of sulfur-centred radical intermediates formed during the oxidation of thiols and sulfite by peroxynitrite. *J. Biol. Chem.,* **271,** 6000 – 6009.

KAUR, H. & HALLIWELL, B. (1994). Evidence for nitric oxide-mediated oxidative damage in chronic inflammation: nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Letts.,* **350,** 9 – 12.

KAUR, H., WHITEMAN, M. & HALLIWELL, B. (1997). Peroxynitrite-dependent aromatic hydroxylation and nitration of salicylate and phenylalanine. Is hydroxyl radical involved? *Free Rad. Res.,* **26,** 71 – 82.

KETTLE, A.J. (1996). Neutrophils convert tyrosine residues in albumin to chlorotyrosine. *FEBS Letts.,* **379,** 103 – 106.

LOHINAI, Z., BENDEKM, P., FEHER, E., GYORFI, A., ROSIVELL, C., FAZEKAS, A., SALZMAN, A.L. & SZABÓ, C. (1998). Protective effects of mercaptoethylguanidine, a selective inhibitor of inducible nitric oxide synthase in ligature-induced peridontitis in the rat. *Br. J. Pharmacol.,* **123,** 353 – 360.

MERÉNYI, G. & LIND, J. (1997). Thermodynamics of peroxynitrite and its CO_2 adduct. *Chem. Res. Toxicol.,* **10,** 1216 – 1220.

MIAMPAMBA, M.A. & SHARKEY, K.A. (1997). Distribution of inducible nitric oxide synthase and nitrotyrosine in the rat colon in experimental colitis. *Gastroenterol.,* **112,** SS A1041 (Abstract).

MILLER, M.J.S., ZHANG, X.J., SADOWSKAKROWICKA, H. & CLARK, D.A. (1994). A potential role for peroxynitrite in guinea-pig ileitis induced by trinitrobenzensulfonic acid. *Gastroenterol.,* **106,** A735.

MORENO, J.J. & PRYOR, W.A. (1992). Inactivation of α_1-proteinase inhibitor by peroxynitrite. *Chem. Res. Toxicol.,* **5,** 425–431.

MORRIS, J.C. (1966). The acid ionization constant of hypochlorous acid from 5 to 35 degrees. *J. Phys. Chem.,* **70,** 3798–3805.

PRUTZ, W.A. (1996). Hypochlorous acid interactions with thiols, nucleotides, DNA and other biological substrates. *Arch. Biochem. Biophys.,* **332,** 110–120.

PRYOR, W.A. & SQUADRITO, G.L. (1995). The chemistry of peroxynitrite: A product from the reaction of nitric oxide with superoxide. *Am. J. Physiol.,* **12,** L699–L722.

REHMAN, A., WHITEMAN, M. & HALLIWELL, B. (1997). Scavenging of hydroxyl radicals but not of peroxynitrite by inhibitors and substrates of nitric oxide synthase. *Br. J. Pharmacol.,* **122,** 1702–1706.

SCHRAUFSTATTER, I.U., BROWNE, K., HARRIS, A., HYSLOP, P.A., JACKSON, P.A., QUEHENBERGER, O. & COCHRANE, C.G. (1990). Mechanisms of hypochlorite injury of target cells. *J. Clin. Invest.,* **85,** 554–562.

SOUTHAN, G.J., ZINGARELLI, B., O'CONNOR, M., SALZMAN, A.L. & SZABÓ, C. (1996). Spontaneous rearrangement of aminoalkyliothioureas into mercaptoalkylguanidines, a novel class of nitric oxide synthase inhibitors with selectivity towards the inducible form. *Br. J. Pharmacol.,* **117,** 619–632.

SZABÓ, C., FERER-SUETA, G., ZINGARELLI, B., SOUTHAN, G.J., SALZMAN, A.L. & RADI, R. (1997). Mercaptoethylguanidine and guanidine inhibitors of nitric oxide synthase react with peroxynitrite and protect against peroxynitrite-induce oxidative damage. *J. Biol. Chem.,* **272,** 9030–9036.

VAN RENSBERG, C.E.J., VAN STADEN, A.M. & ANDERSON, R. (1991). Inactivation of Poly(ADP-ribose) polymerase by hypochlorous acid. *Free Rad. Biol. Med.,* **1,** 285–291.

WEISS, S.J. (1989). Tissue destruction by neutrophils. *New Eng. J. Med.,* **320,** 365–376.

WHITEMAN, M. & HALLIWELL, B. (1996a). Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. *Free Rad. Res.,* **25,** 275–283.

WHITEMAN, M. & HALLIWELL, B. (1996b). Protection against peroxynitrite-dependent tyrosine nitration and α_1-antiproteinase inactivation by oxidized and reduced lipoic acid. *FEBS Letts.,* **379,** 74–76.

WHITEMAN, M. & HALLIWELL, B. (1997) Thiols and disulphides can aggravate peroxynitrite-dependent inactivation of α_1-antiproteinase. *FEBS Letts.,* **414,** 497–500.

WHITEMAN, M., JENNER, A.M. & HALLIWELL, B. (1997). Hypochlorous acid-induced base modifications in isolated calf thymus DNA. *Chem. Res. Toxicol.,* **10,** 1240–1246.

ZINGARELLI, B., ISCHIROPOLOUS, H., SALZMAN, A.L. & SZABÓ, C. (1997a). Amelioration by mercaptoethylguanidine of the vascular and energetic failure in haemorrhagic shock in the anaesthetised rat. *Eur. J. Pharmacol.,* **338,** 55–65.

ZINGARELLI, B., SOUTHAN, G.J., GILAD, E., O'CONNOR, M., SALZMAN, A.L. & SZABÓ, C. (1997b). The inhibitory effects of mercaptoethylguanidines on cyclo-oxygenase activity. *Br. J. Pharmacol.,* **120,** 357–366.

(Received September 22, 1998
Accepted January 13, 1999)