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Scavenging of hydroxyl radicals but not of peroxynitrite by inhibitors and substrates of nitric oxide synthases

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1 The nitric oxide synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) is widely used to study the role of $NO^\bullet$ in physiological and pathological processes, including its role in the generation of the cytotoxic species peroxynitrite ($ONOO^-$) and of reactive oxygen radicals such as hydroxyl ($OH^\bullet$). Often L-NAME is applied to tissues at mM concentrations. At such high concentrations, it might act as a free radical scavenger. A similar possibility might apply to the use of high levels of arginine to study the role of $NO^\bullet$ in atherogenesis.

2 We therefore examined the rate of scavenging of $OH^\bullet$ by L-NAME and found that L-NAME reacts more quickly with $OH^\bullet$ than the established '$OH^\bullet$ scavenger' mannitol and the widely used '$OH^\bullet$ trap' salicylate. However, D-NAME can scavenge $OH^\bullet$ at rates equal to L-NAME. Both L- and D-arginine were also good $OH^\bullet$ scavengers, comparable in effectiveness to mannitol.

3 Neither L-NAME, D-NAME, L-arginine nor D-arginine was able to inhibit $ONOO^-$-dependent nitration of tyrosine, suggesting that they are unlikely to be scavengers of $ONOO^-$-derived nitrating species.

4 Neither L-NAME, D-NAME, L-arginine nor D-arginine was able to inhibit the inactivation of α_1-antiproteinase by $ONOO^-$, suggesting that they cannot prevent direct oxidations by peroxynitrite.

5 We conclude that L-NAME has sufficient activity as an $OH^\bullet$ scavenger to confound certain pharmacological experiments. However, this explanation of its biological effects can be ruled out if control experiments show that D-NAME has no effect and that L-arginine (also a free radical scavenger) antagonizes the action of L-NAME.

Keywords: Nitric oxide; nitric oxide synthase; hydroxyl radical; peroxynitrite; L-NAME

Introduction

Nitric oxide, a biologically-generated free radical ($NO^\bullet$), plays an enormous number of physiological roles. By contrast, overproduction of $NO^\bullet$ has been suggested to contribute to the pathology of several diseases, including neurodegenerative disease and chronic inflammation (for reviews see Moncada *et al*., 1991; Choi, 1993; Beal, 1997). Inhibitors of nitric oxide synthase enzymes, such as N^G-nitro-L-arginine methyl ester (L-NAME), are widely used to probe the role of $NO^\bullet$ in physiological and pathological phenomena (Moncada *et al*., 1991). One of the mechanisms by which excess $NO^\bullet$ can cause tissue injury is by contributing to the generation of highly-reactive oxygen radicals, such as hydroxyl, $OH^\bullet$ or a species closely resembling $OH^\bullet$ (Beckman *et al*., 1991; 1994; Kaur *et al*., 1997). Also relevant is the reaction of $NO^\bullet$ with superoxide ($O_2^{\bullet-}$) radicals to produce the cytotoxic species peroxynitrite, $ONOO^-$ (Beckman *et al*., 1994).

Often, nitric oxide synthase (NOS) inhibitors are added to biological systems at high concentrations. For example, millimolar levels of L-NAME are often used to inhibit NOS in isolated phagocytes and 10 mM levels have been used in studies of the role of $NO^\bullet$ in hydroxyl radical formation in brain (Smith *et al*., 1994; Lancelot *et al*., 1995; Dikshit *et al*., 1996). However, a recent study published in this journal (Dikshit *et al*., 1996) raised the possibility that L-NAME could be a free radical scavenger. Only 300 μM levels of L-NAME were sufficient to decrease luminol-dependent chemiluminescence in phagocytes, an assay of free radical generation. Dikshit *et al*. (1996) did not identify the radical scavenged, except to note that it was not superoxide, or related to myeloperoxidase.

The present paper describes a rigorous study of the ability of L-NAME and related compounds to scavenge $NO^\bullet$-derived cytotoxic species, namely $OH^\bullet$ and $ONOO^-$. For hydroxyl radical, we determined the exact rate constants for the reactions, from which it is possible to assess the likely physiological significance of any $OH^\bullet$ scavenging observed.

Two assays were used to screen for the ability of L-NAME and related compounds to protect against damage by $ONOO^-$, tyrosine nitration and α_1-antiproteinase inactivation (Whiteman & Halliwell, 1996). Two different types of assay were used because reactions of $ONOO^-$ at pH 7.4 are extremely complex and different 'scavengers' can exert protection by different mechanisms (Pryor & Squadrito, 1995). Tyrosine nitration was chosen because nitration of this amino acid, apparently by the action of $ONOO^-$-derived nitrating species (not including $OH^\bullet$) has been demonstrated to occur *in vivo* (Beckman *et al*., 1994; Pryor & Squadrito, 1995). Inactivation of α_1-antiproteinase was selected because damage to this protein has been shown to occur at sites of chronic inflammation and the generation of $ONOO^-$ provides a plausible mechanism (reviewed by Halliwell, 1995a).

Methods

Measurement of hydroxyl radical scavenging

Hydroxyl radicals were generated by a mixture of ascorbate, Fe^{3+}-EDTA and H_2O_2 essentially as described by Halliwell *et al*. (1987). Reaction mixtures contained, in a final volume of 1.0 ml, the following reagents at the final concentrations stated: deoxyribose (usually 2.8 mM), KH_2PO_4-KOH buffer, pH 7.4 (10 mM), $FeCl_3$ (25 μM), EDTA (100 μM), H_2O_2 (2.8 mM), and ascorbate (100 μM). Solutions of $FeCl_3$ and ascorbate were made up immediately before use in deaerated water and $FeCl_3$ and EDTA were premixed before addition to reaction mixtures. Reaction mixtures were incubated at 37°C for 1 h and colour developed as described in Halliwell *et al*. (1987); 1 ml each of 1% (w/v) thiobarbituric acid in 50 mM

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NaOH and of 2.8% (w/v) trichloroacetic acid were added followed by heating at 80°C for 20 min. The rate of deoxyribose degradation was constant over the 1 h incubation period. Rate constants for reaction of scavengers with $OH^{\bullet}$ were calculated from the equation

$$\frac{1}{A} = \frac{1}{A^0} \left(1 + \frac{k_S[S]}{k_{DR}[DR]}\right),$$

where A is the absorbance in the presence of a scavenger S at concentration [S], [DR] is the deoxyribose concentration and A^0 is the absorbance in the absence of a scavenger. A plot of $1/A$ against [S] should give a straight line of slope $k_S/k_{DR}[DR]A^0$ with an intercept on the y-axis of $1/A^0$. The rate constant k_S for reaction of S with $OH^{\bullet}$ is obtained from the slope of the line, by use of the rate constant for deoxyribose (k_{DR}) as 3.1×10^9 M^{-1} s^{-1} (Halliwell *et al.*, 1987).

None of the compounds tested interfered with the assay procedures; the controls summarized by Halliwell (1995b) were used. Essentially, none of the compounds interfered with the measurement of deoxyribose damage products—they had no inhibitory effect when added at the end of the assay with the chromogenic reagents used to measure deoxyribose degradation. None of the compounds themselves formed a chromogen when deoxyribose was omitted from the reaction mixture.

Nitration of tyrosine by $ONOO^-$

A 10 ml stock solution (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) KOH followed by 250 μl 5% phosphoric acid with 1.5 ml water. Then 100 μl of the tyrosine solution together with 100 μl of compound to be tested was added to a plastic test tube containing 795 μl buffer (500 mM K_2HPO_4-$KHPO_4$, pH 7.4) and incubated in a water bath at 37°C for 15 min. After this time $ONOO^-$ (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.

H.p.l.c. determination of 3-nitrotyrosine (3-NO_2 Tyr)

Measurement of 3-NO_2 Tyr was performed by means of a Spherisorb 5 μm ODS2 C_{18} column (Wellington House, Cheshire, U.K.) with a guard column (Hibar from BDH, Poole, Dorset, U.K.) and C_{18} cartridge. The eluent was 500 mM K_2HPO_4-$KHPO_4$, pH 3.01, with 20% methanol (v/v) at a flow rate of 1.0 ml min^{-1} through a Polymer Laboratories pump (Essex Road, Church Stretton, U.K.) and u.v. detector set at 274 nm. The 3-NO_2 Tyr detected was confirmed by spiking with standards. Peak heights of tyrosine and 3-NO_2 Tyr were measured and concentrations calculated from a standard curve. The limit of sensitivity was 0.5 μM 3-NO_2 Tyr. Under these conditions the retention time was typically 3.6 min. None of the compounds tested co-eluted with 3-NO_2 Tyr on the high performance liquid chromatography (h.p.l.c.) chromatograms. If the compounds tested were pre-mixed with 3-NO_2 Tyr before analysis, they had no effect on the amount of 3-NO_2 Tyr detected by subsequent h.p.l.c.

Inactivation of α_1-antiproteinase by $ONOO^-$

The activity of elastase can be measured with a spectrophotometer. Hydrolysis of the synthetic substrate N-succinyl(Ala)$_3$-*p*-nitroanilide (SANA) 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.

α_1-Antiproteinase was dissolved in phosphate buffered saline, pH 7.4 (140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, 2.9 mM KH_2PO_4) to a concentration of 4.0 mg ml^{-1} and elastase in the same buffer to 5.0 mg ml^{-1}. The volume of α_1-antiproteinase required to inhibit elastase activity by 80–90% (usually 60–70 μl) was added to buffer (500 mM K_2HPO_4-$KHPO_4$, pH 7.4) with or without 100 μl of the compound to be tested, to give a volume of 945 μl, and incubated in a water bath at 37°C for 15 min. Then $ONOO^-$ (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 (SANA) was added and the rate of the reaction followed at 410 nm for 30 s. Assays of elastase alone showed that none of the compounds examined had a direct effect on this enzyme. Assays of elastase plus α_1-antiproteinase (without $ONOO^-$) showed that none of the compounds affected the ability of α_1-antiproteinase to inhibit elastase. Data are shown in Figure 3.

Reagents

All reagents were of the highest purity available from Sigma Chemical Corp., except that $ONOO^-$ was synthesized and its concentration determined essentially as described by Beckman *et al.* (1994). Briefly, 20 ml of an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with 20 ml KNO_2 (0.6 M) on ice for one second and the reaction quenched with 20 ml ice-cold NaOH (1.2 M). Residual H_2O_2 was removed by adding 10–15 mg prewashed granular MnO_2. The solution was then filtered and frozen overnight at −20°C. The top layer of the solution was collected for the experiment. Concentrations of stock $ONOO^-$ were determined before each experiment at 302 nm by use of a molar extinction coefficient of 1670 cm^{-1} M$^-$ (Beckman *et al.*, 1994). Concentrations of 250–300 mM were usually obtained.

Results

Scavenging of hydroxyl radicals

A mixture of ascorbate, H_2O_2 and Fe^{3+}−EDTA generates $OH^{\bullet}$ radicals:

$$Fe^{3+}EDTA + \text{ascorbate} \rightarrow \text{ascorbate}^{\bullet} + Fe^{2+} - EDTA$$

$$Fe^{2+} - EDTA + H_2O_2 \rightarrow Fe^{3+} - EDTA + OH^{\bullet} + OH^-$$

The $OH^{\bullet}$ is allowed to react with the sugar 2-deoxy-D-ribose at pH 7.4, and a chromogen is generated. Any added scavenger of $OH^{\bullet}$ will compete with deoxyribose for the $OH^{\bullet}$ generated and decrease chromogen formation (Halliwell *et al.*, 1987). Analysis of the concentration-dependence of the competition allows determination of the second-order rate constant for reaction of the scavenging compound with $OH^{\bullet}$, as explained in the Methods section. Figure 1a illustrates this for mannitol, a well-established $OH^{\bullet}$ scavenger frequently used in biological systems (Halliwell & Gutteridge, 1989) and employed here as a positive control. Its rate constant for reaction with $OH^{\bullet}$ is 1.9×10^9 M^{-1}s^{-1}, in agreement with previous studies (Halliwell & Gutteridge, 1989). The competition plot is linear, indicating a direct competition between mannitol and deoxyribose for the $OH^{\bullet}$ generated.

L-NAME and D-NAME were examined by the same procedure (Figure 1b,c) and both were found to be powerful $OH^{\bullet}$ scavengers, over three times better than mannitol (Table 1). L-Arginine and D-arginine were also found to be good $OH^{\bullet}$ scavengers, broadly comparable in effectiveness to mannitol (Figure 1d,e; Table 1). None of these compounds had any confounding effect on the deoxyribose assay itself,

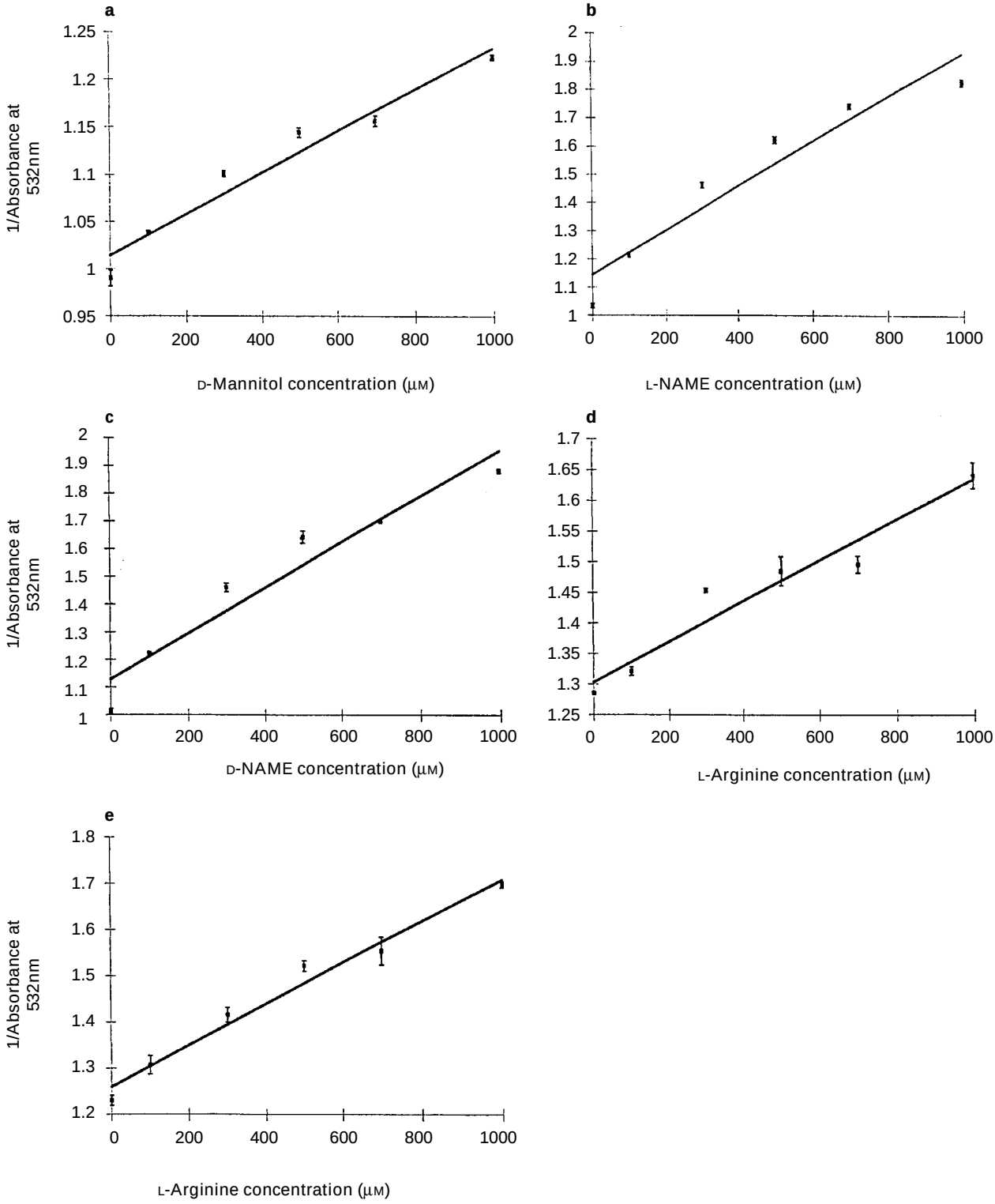

Figure 1 Competitive scavenging of hydroxyl radicals. Assays are as described in the Methods section: (a) mannitol, (b) L-NAME, (c) D-NAME, (d) L-arginine and (e) D-arginine. Compounds were included in the reaction mixtures at the final concentrations stated. None of the above compounds interfered with the deoxyribose assay. Points are mean and vertical lines show s.d., $n \geqslant 3$.

when the controls specified in the Methods section were used (Halliwell, 1995b).

Effect on nitration of tyrosine by peroxynitrite

When peroxynitrite is added to the amino acid tyrosine, 3-nitrotyrosine is generated (Beckman *et al*., 1994). The chemistry of nitration is complex, but $OH^{\bullet}$ is probably not involved (Pryor & Squadrito, 1995). Formation of nitrotyrosine is frequently observed *in vivo* and taken as a biomarker of $ONOO^-$ generation (Beckman *et al*., 1994). Scavengers of $ONOO^-$ inhibit tyrosine nitration; Figure 2 shows data for reduced glutathione (GSH) used as a positive control. GSH is well-known to scavenge $ONOO^-$ (Beckman *et al*., 1994; Pryor & Squadrito, 1995) and, as expected, was a powerful inhibitor of nitration (Figure 2). By contrast L-NAME, D-NAME, L-arginine or D-arginine had no significant effect on nitration of tyrosine by $ONOO^-$ (Figure 2).

Table 1 Rate constants for hydroxyl radical scavenging

Compound	*Rate constant* ($M^{-1} s^{-1}$)
Mannitol	$(1.9 \pm 0.1) \times 10^9$
L-Arginine	$(1.7 \pm 0.4) \times 10^9$
D-Arginine	$(2.6 \pm 0.2) \times 10^9$
L-NAME	$(7.0 \pm 0.5) \times 10^9$
D-NAME	$(7.1 \pm 0.3) \times 10^9$

Results are mean $\pm$ s.d., $n \geqslant 3$.

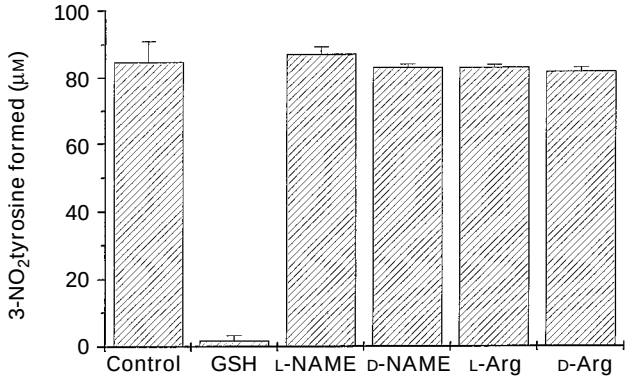

Figure 2 Inhibition of tyrosine nitration. Peroxynitrite (1.0 mM) was added to tyrosine (1 mM) and incubated for 10 min at 37°C and pH 7.4. 3-Nitrotyrosine was measured by h.p.l.c. Compounds tested for inhibitory capacity were included with the tyrosine at 1.0 mM final concentration before $ONOO^-$ was added. None of the compounds interfered with h.p.l.c. analysis of 3-nitrotyrosine (see Methods section for more details). Data are mean $\pm$ s.d., $n \geqslant 3$.

Effect on inactivation of α_1-antiproteinase by peroxynitrite

α_1-Antiproteinase, the major inhibitor of serine proteases in human body fluids, is rapidly inactivated on addition of $ONOO^-$. Inactivation occurs by direct attack of $ONOO^-$ on an essential methionine residue, and $OH^\bullet$ is not involved (Moreno & Pryor, 1992; Whiteman & Halliwell, 1996). α_1-Antiproteinase is assayed in the laboratory by its ability to inhibit the enzyme elastase. Figure 3 shows that L-NAME, D-NAME, L-arginine and D-arginine had no direct effect on elastase (stippled columns). If α_1-antiproteinase was added, elastase was inhibited (Figure 3, open columns); none of the test compounds interfered with this inhibition. If α_1-antiproteinase was first treated with $ONOO^-$, its ability to inhibit elastase was decreased (Figure 3, hatched columns). The presence of a scavenger of $ONOO^-$ decreases this inactivation, as is demonstrated with GSH in Figure 3. By contrast, L-NAME, D-NAME, L-arginine and D-arginine had no protective effect: they could not prevent the inactivation of α_1-antiproteinase by $ONOO^-$.

Discussion

Our data show, in agreement with the conclusion of Dikshit *et al.* (1996), that L-NAME has the ability to act as a free radical scavenger. In particular we showed that it is a powerful scavenger of $OH^\bullet$ radicals and should be used with caution as a 'specific' NOS inhibitor in situations where such radicals are being generated (Smith *et al.*, 1994; Lancelot *et al.*, 1995). For example, salicylate at 1–10 mM is often infused into physiological systems as a 'trap' for $OH^\bullet$, so detecting $OH^\bullet$ by the formation of salicylate hydroxylation products (Grootveld & Halliwell, 1986; Lancelot *et al.*, 1995). Yet the rate constant for reaction of salicylate with $OH^\bullet$ is 6×10^9 $M^{-1}s^{-1}$, broadly comparable with L-NAME.

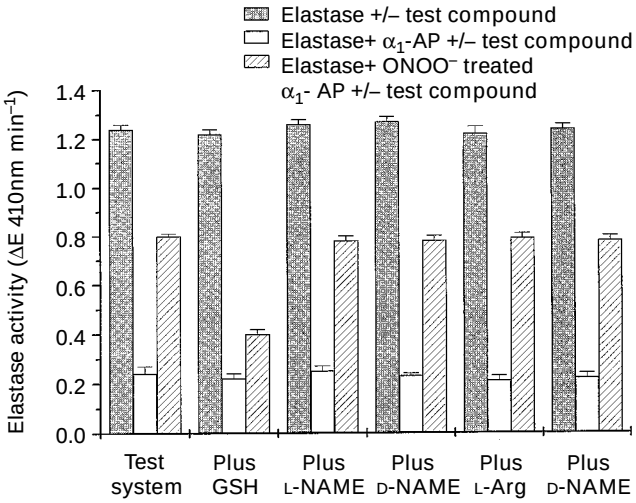

Figure 3 Prevention of inactivation of α_1-antiproteinase by $ONOO^-$. α_1-Antiproteinase (α_1-AP) was incubated with 500 μM peroxynitrite. Elastase was then added. Residual elastase activity was measured as described in the Methods section. None of the compounds tested had any direct effect on elastase or the ability of α_1-antiproteinase to inhibit elastase. GSH could decrease inactivation of α_1-antiproteinase by $ONOO^-$, but the other compounds tested could not. GSH added after the inactivation had taken place could not protect, i.e. it cannot restore activity to α_1-antiproteinase inactivated by $ONOO^-$.

Hence co-infusion of salicylate and L-NAME at comparable concentrations in studies attempting to examine the role of $NO^\bullet$ in $OH^\bullet$ generation may lead to an inhibition of salicylate hydroxylation that has nothing to do with NOS inhibition but is due to $OH^\bullet$ scavenging by L-NAME. Similarly, mannitol at mM concentrations has been shown to inhibit a wide range of physiological phenomena as evidence for the role of $OH^\bullet$ (Halliwell & Gutteridge, 1989). Yet L-NAME is a better $OH^\bullet$ scavenger than mannitol by a factor of over three.

However, our data also show that D-NAME is an equally good $OH^\bullet$ scavenger and that both L-arginine and D-arginine are fairly good $OH^\bullet$ scavengers (Table 1). It is usual (but by no means universal) in biological experiments with L-NAME to perform control experiments showing that an inhibitory action of L-NAME is not reproduced by D-NAME, and is antagonized by addition of L-arginine. Our data show that such controls are essential. If an inhibitory action of L-NAME is not reproduced by D-NAME and is antagonized by L-arginine (but not D-arginine) then it is very unlikely to be due to $OH^\bullet$ scavenging. In addition, high concentrations of L-arginine have been used in some experiments to explore the putative role of $NO^\bullet$ in atherogenesis (e.g. Drexler *et al.*, 1991); it is not impossible that free radical scavenging by L-arginine might contribute to its beneficial effects since atherosclerosis involves free radical damage (Halliwell & Gutteridge, 1989).

Our data also show that neither L-NAME, D-NAME, L-arginine or D-arginine, appear to be scavengers of $ONOO^-$, in that they cannot inhibit inactivation of α_1-antiproteinase or tyrosine nitration on addition of this species. Since our paper was submitted, Szabo *et al.* (1997) have shown that mercaptoethylguanidine and guanidine inhibitors of iNOS do scavenge $ONOO^-$ and that this can confound experiments. Our data show that this artefact does not seem to be a problem with L-NAME.

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