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Reduction by N^G-nitro-L-arginine of H_2O_2-induced endothelial cell injury

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1 The effects of three analogues of N^G-nitro-L-arginine (L-NOARG) and N^G-monomethyl-L-arginine (L-NMMA), inhibitors of nitric oxide (NO) synthase, on hydrogen peroxide (H_2O_2)-induced endothelial cell injury were studied.

2 Endothelial cell injury was assessed by measuring the release of intracellular lactate dehydrogenase (LDH) and ^{51}Cr.

3 Addition of H_2O_2 (250-1,000 μM) to endothelial cells induced the release of LDH dose-dependently. The release of LDH was reduced by pretreatment with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-4}-4×10^{-3} M), L-NOARG (10^{-4}-4×10^{-3} M) and N^G-nitro-L-arginine benzyl ester (L-NABE, 10^{-4}-4×10^{-3} M), inhibitors of NO synthase.

4 L-NOARG analogues also reduced H_2O_2-induced ^{51}Cr release from endothelial cells, while L-NMMA had no effect.

5 The protective effect of L-NAME was not reversed by addition of L-arginine (L-Arg, 1-10 mM).

6 Both L-NAME and L-NMMA completely inhibited L-Arg metabolism to L-citrulline coupled with NO synthesis.

7 These findings suggest that L-NOARG analogues but not L-NMMA reduced H_2O_2-induced endothelial cell injury, and that these effects may not be related to inhibition of NO production.

Keywords: Endothelial cells; N^G-nitro-L-arginine; N^G-nitro-L-arginine methyl ester; N^G-nitro-L-arginine benzyl ester; N^G-monomethyl-L-arginine methyl ester; L-arginine; hydrogen peroxide; cytotoxicity

Introduction

Reactive oxygen species have been implicated in the development of many diseases such as ischaemia-reperfusion injury (Granger, 1988) and inflammation (Fantone & Ward, 1982; Henson & Johnston, 1987). Although the mechanism of cell death induced by reactive oxygen species has not been determined in detail, they are known to cause lipid peroxidation (Bus *et al.*, 1974), DNA strand breakage (Brawn & Fridovich, 1981; Spragg, 1991) and a variety of changes in proteins (Freeman & Crapo, 1982). It is generally accepted that hydroxyl radicals, a highly reactive form of oxygen, are responsible for the oxidant injury. It has been proposed that the hydroxyl radical is derived from superoxide anion or hydrogen peroxide (H_2O_2) in the presence of iron (iron-catalyzed HaberWeiss (Fenton) reaction). Beckman *et al.* (1990) suggested that generation of hydroxyl radicals via the Haber-Weiss pathway may be limited *in vivo* and they proposed that nitric oxide (NO) reacts with superoxide anion in pathological states to produce cytotoxic species such as peroxynitrite and hydroxyl radicals. Moreover, Noronha-Dutra *et al.* (1993) have shown that NO reacts with H_2O_2 to produce singlet oxygen, a highly reactive form of oxygen. NO may exert cytotoxic effects in the presence of reactive oxygen species. Therefore, the role of NO in the process of pathogenesis has been studied. In these studies, NO synthase inhibitors have been used widely (Dawson *et al.*, 1992; Matheis *et al.*, 1992; Patel *et al.*, 1993).

Vascular endothelial cells generate NO via a Ca^{2+}/calmodulin-dependent constitutive enzyme which catalyzes the conversion of L-arginine to L-citrulline (Mayer *et al.*, 1989; Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Schmidt *et al.*, 1992). Vascular endothelial cells are one of the major biological targets of oxygen radical species produced by activated neutrophils and macrophages. Thus, vascular endothelial cells may be exposed to NO and reactive oxygen species simultaneously. Therefore, we investigated the role of NO in H_2O_2-induced endothelial cell injury. In the present study, we found differences in effect between N^G-nitro-L-arginine analogues (L-NOARG analogues) and N^G-monomethyl-L-arginine (L-NMMA), inhibitors of NO synthase, on H_2O_2-induced endothelial cell injury. L-NOARG analogues but not L-NMMA reduce H_2O_2-induced endothelial cell injury and this protective effect may not be related to the inhibition of NO production.

Methods

Endothelial cell culture

Fresh bovine thoracic aortae obtained from an abattoir were kept in phosphate buffered saline (pH 7.4) with penicillin (200 u ml^{-1}) and streptomycin (200 $\mu g\ ml^{-1}$). Aortae were trimmed free of adhering fat and connective tissue, and washed with phosphate buffered saline. Endothelial cells were obtained by scraping the luminal surface with a razor blade (Shasby & Shasby, 1986), and were cultured in minimal essential medium (MEM) containing penicillin (100 u ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and 10% foetal calf serum (FCS). Cells were finally grown on Cytodex 3 microcarrier beads (Pharmacia).

Endothelial cells were characterized by microscopic observation and incorporation of acetylated low density lipoprotein labelled with 1,1′-dioctadecyl-1-3,3,3′3′-tetramethylindocarbocyanine perchlorate (McGuire & Orkin, 1987). Cells at 6 and 7 passages were used for the experiments.

Lactate dehydrogenase (LDH) release

Confluent endothelial cells on microcarrier beads were washed five times with Krebs solution (pH 7.4) containing (mM): NaCl 118.5, KCl 4.74, $CaCl_2 \cdot 2H_2O$ 2.5, $MgSO_4 \cdot 7H_2O$

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1.18, KH_2PO_4 1.18, $NaHCO_3$ 2.5, glucose 11 and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) 10. Cells (50 µl of microcarrier beads, approximately 1×10^6 cells) were then treated with various concentrations of H_2O_2 (250–1,000 µM) at 37°C in 0.5 ml of Krebs solution. LDH activity was determined in cell supernatants and cell fractions of endothelial cells solubilized in 2% Triton X-100 (Thies & Autor, 1991). The percentage of the total LDH activity (supernatant fraction + cell fraction) released into the supernatant fraction was then calculated.

^{51}Cr release

Confluent endothelial cells on microcarrier beads (approximately 1.4×10^7 cells) were radioactively labelled with 18.5 MBq of $Na_2{}^{51}CrO_4$ (Amersham) in 5 ml of culture medium for 16 h at 37°C. This medium was removed and cells were washed five times with Krebs solution. Cells (50 µl of microcarrier beads, approximately 1×10^6 cells) were then exposed to H_2O_2 (500 µM) at 37°C in 0.5 ml of Krebs solution for 3 h, after which time 200 µl of cell-free medium was recovered to determine ^{51}Cr radioactivity (supernatant fraction). The remaining cells were solubilized by addition of 500 µl of 2% Triton X-100, and 200 µl of medium was recovered to determine ^{51}Cr radioactivity (cell fraction). The percentage of the total ^{51}Cr radioactivity (supernatant fraction + cell fraction) released into the superatant fraction was calculated.

Measurement of L-arginine metabolism

Confluent cells on microcarrier beads were transferred to culture medium containing neither L-arginine nor FCS for 24 h prior to the experiment to deplete L-arginine (L-Arg) content. L-Arg-depleted cells were washed 5 times with Krebs solution. The washed cells (100 µl of microcarrier beads, approximately 2×10^6 cells) were incubated at 37°C in 290 µl of Krebs solution containing 37 kBq L-[^{3}H]-arginine (2.29 TBq mmol^{-1}) for 5 min, after which time 10 µl of ionomycin (final concentration of 10^{-6} M) was added and the reaction began. The reaction was terminated with 10 µl of perchloric acid (final concentration of 2%), and after 30 min the reaction mixture was sonicated (5 s) and centrifuged for 10 min at 3,000 r.p.m. [^{3}H]-citrulline formation was determined by high performance liquid chromatography (h.p.l.c.) by a modified version of the method of Rees *et al.* (1990). The supernatant was applied to h.p.l.c. using an ODS-80Ts (4.6 mm i.d. × 150 mm, Tosoh Co., Japan) with a mobile phase of 25 mM sodium acetate (pH 4.35) containing 15 mM sodium hexane sulphonate, and radioactivity in each fraction containing [^{3}H]-citrulline was determined with a liquid scintillation spectrometer.

Materials

N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME), N^G-nitro-L-arginine benzyl ester (L-NABE), N^G-monomethyl-L-arginine acetate salt (L-NMMA) and N^G-nitro-D-arginine methyl ester (D-NAME) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). H_2O_2 (30% solution) was obtained from Wako Chemicals (Japan). Minimal essential medium was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Foetal calf serum was purchased from Boehringer Mannheim. L-[2,3,4,5-^{3}H]-arginine monohydrochloride (2.29 TBq mmol^{-1}) was purchased from Amersham. All other reagents were of the highest grade commercially available.

Statistical analysis

Results are expressed as mean ± s.e.mean of *n* observations. For multiple comparisons, either Duncan's or Dunnett's test was used. A paired *t* test was used in those experiments where only two groups were being compared. In all cases, a *P* value of less than 0.05 was considered statistically significant.

Results

Effects of L-arginine and nitric oxide inhibitors on H_2O_2-induced endothelial cell injury

Endothelial cell injury was assessed by measuring the release of intracellular LDH. Addition of H_2O_2 (250–1,000 µM) to endothelial cells caused LDH release in a dose-dependent manner after a delay of approximately 1 h (Figure 1).

Figure 2 shows the effects of L-NOARG, L-NAME and L-NABE (all at 10^{-6}–4×10^{-3} M), inhibitors of NO synthase, on H_2O_2-induced endothelial cell injury. Pretreatment of cells with all L-NOARG analogues reduced H_2O_2-induced LDH release from endothelial cells. The order of protective effect was as follows: L-NABE > L-NAME = L-NOARG. However, the D-enantiomer of NAME had no effect (data not shown).

The effects of L-Arg, L-NMMA and L-NOARG analogues on H_2O_2-induced ^{51}Cr release from endothelial cells, another marker of cell injury, are shown in Figure 3. Treatment with L-Arg (2 mM) or L-NMMA (1 mM) had no effect, while L-NOARG (1 mM), L-NAME (1 mM) and L-NABE (1 mM) reduced H_2O_2-induced ^{51}Cr release.

Thus, three L-NOARG analogues, but not L-NMMA, reduced H_2O_2-induced endothelial cell injury. Further results are referred to the effect of L-NAME.

Effects of L-arginine on the protective effect of N^G-nitro-L-arginine methyl ester

L-NAME (10^{-6}–4×10^{-3} M) was added to endothelial cell cultures in the presence of various concentrations of L-Arg (1–10 mM), and H_2O_2-induced endothelial cell injury was assessed. L-Arg at no concentration investigated affected the reduction of H_2O_2-induced LDH release by L-NAME (Figure 4).

Effect of nitric oxide synthase inhibitors on nitric oxide synthesis

To determine the effects of L-NMMA and L-NAME on NO synthase activity we used L-citrulline (L-Cit) formation from L-Arg as a marker for NO synthesis. Ionomycin-stimulated L-Cit formation was inhibited by L-NAME and L-NMMA in a dose-dependent manner, with maximum inhibition at concentrations in excess of 10^{-5} and 10^{-3} M, respectively (Figure 5).

Discussion

L-NOARG analogues and L-NMMA are known to be NO synthase inhibitors. Recently, Frew *et al.* (1993) reported that L-NOARG blocks basal and agonist-stimulated production of NO, while L-NMMA blocks basal production but not agonist-stimulated production of NO in rat aortae. However, there have been reports that L-NMMA inhibits agonist-stimulated production of NO in many tissues and cells including rat thoracic aortae (Rees *et al.*, 1990), bovine aortic endothelial cells (Ishii *et al.*, 1990) and rabbit thoracic aortae (Zembowicz *et al.*, 1993). Previously, we reported that the Ca^{2+} ionophore ionomycin-stimulated NO synthesis in endothelial cells can be determined by measurement of L-citrulline (L-Cit) formation from L-Arg (Shimizu *et al.*, 1993). In the present study, both L-NAME and L-NMMA inhibited L-Cit formation from L-Arg coupled with NO synthase in endothelial cells. Thus, both L-NOARG analogues and L-NMMA acted as inhibitors of NO synthesis.

We found that L-NOARG analogues reduced H_2O_2-induced endothelial cell injury. Surprisingly, despite its inhibition of L-Arg metabolism, L-NMMA did not reduce H_2O_2-induced endothelial cell injury. Inhibition of NO synthase by L-NOARG analogues is reversible (Mayer *et al.*, 1993), and can be reversed by L-Arg (Ishii *et al.*, 1990; Mayer *et al.*, 1993; Frew *et al.*, 1993). However, the protective effect of L-NAME was neither reversed in the presence of L-Arg nor increased in the presence of L-NMMA (data not shown), and thus may not involve the inhibition of NO production.

It is generally accepted that highly reactive hydroxyl radicals (·OH) formed via the iron-catalyzed Haber-Weiss (Fenton) reaction, are responsible for oxidant-induced injury of endothelial cells (Todoki *et al.*, 1992). We have observed inhibition of H_2O_2-induced endothelial cell injury by pretreatment with N-(2-mercaptopropionyl)-glycine and 1,3-dimethyl-2-thiourea, ·OH scavengers, and dipyridyl, an iron chelator (data not shown). Therefore, ·OH was responsible for H_2O_2-induced endothelial cell injury. Recently, NO has been shown to react with superoxide anion and H_2O_2 to produce highly cytotoxic such as peroxynitrite, ·OH and singlet oxygen (Beckman *et al.*, 1990; Hogg *et al.*, 1992; Noronha-Dutra *et al.*, 1993). However, addition of L-Arg to endothelial cells did not affect H_2O_2-induced endothelial cell injury. Moreover, carboxy-PTIO, a NO scavenger (Akaike *et al.*, 1993), also showed no effect on H_2O_2-induced endothelial cell injury (data not shown). Thus, under our conditions, NO could not be responsible for H_2O_2-induced endothelial injury. Recently, it was suggested that L-NMMA functions as an alternative substrate for NO synthase, and produces NO and L-Cit (Olken & Marletta, 1993). Frew *et al.* (1993) showed that pretreatment with L-NMMA reduces the ability of L-NOARG to inhibit acetylcholine-induced relaxation in rat aortae, but that the effects of L-NAME were not affected by

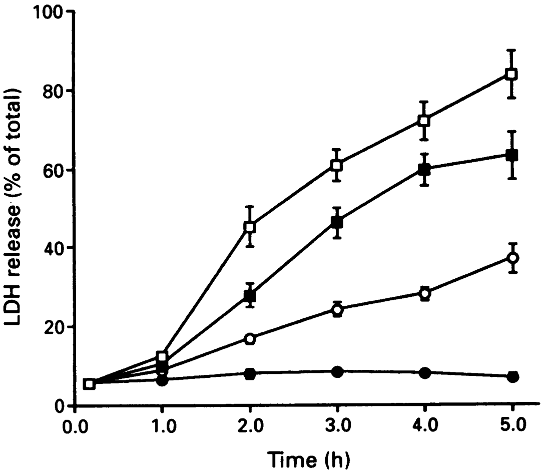

Figure 1 Time course and concentration-response curves of lactate dehydrogenase (LDH) release from endothelial cells exposed to H_2O_2. Cells were incubated in the presence of various concentrations of H_2O_2 (control, ●; 250, ○; 500 ■ and 1,000 μM □) at 37°C. At the indicated times after addition of the H_2O_2, the percentage of the total LDH released into the medium was determined. Results are the means ± s.e.mean of three different experiments performed in triplicate.

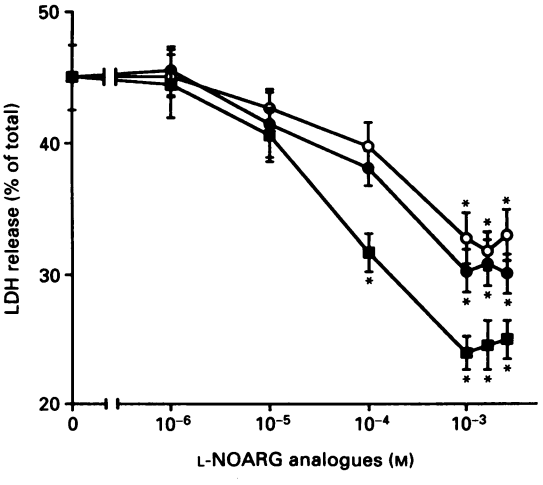

Figure 2 Effects of N^G-nitro-L-arginine analogues on H_2O_2-induced lactate dehydrogenase (LDH) release from endothelial cells. Cells were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-6}–4×10^{-3} M, ○), N^G-nitro-L-arginine (L-NOARG, 10^{-6}–4×10^{-3} M, ●) or N^G-nitro-L-arginine benzyl ester (L-NABE, 10^{-6}–4×10^{-3} M, ■) for 10 min at 37°C, followed by incubation with H_2O_2 (500 μM) for 3 h at 37°C. Results are the means ± s.e.mean of four different experiments performed in triplicate. *Significantly different from cells treated with H_2O_2 alone ($P<0.05$).

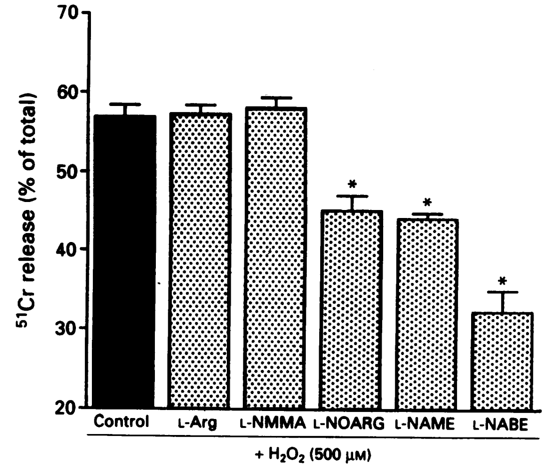

Figure 3 Effects of L-arginine (L-Arg), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NOARG), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine benzyl ester (L-NABE) on H_2O_2-induced ^{51}Cr release from endothelial cells. Cells were pretreated with Krebs solution (control), L-Arg (1 mM), L-NMMA (1 mM), L-NOARG (1 mM), L-NAME (1 mM) or L-NABE (1 mM) for 10 min at 37°C, followed by incubation with H_2O_2 (500 μM) for 3 h at 37°C. Results are the means ± s.e.mean of four different experiments performed in triplicate. *Significantly different from cells treated with H_2O_2 alone (control, $P<0.05$).

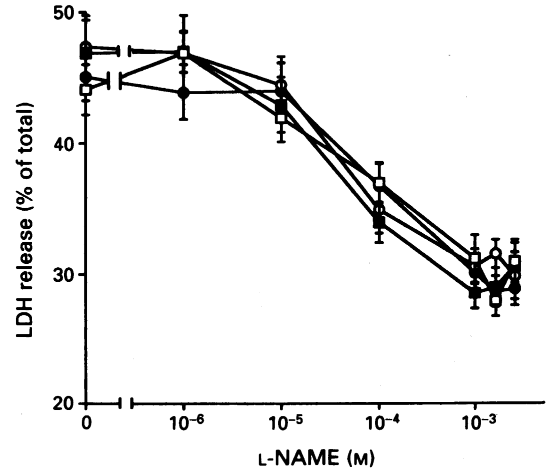

Figure 4 Effect of L-arginine (L-Arg) on the protective effect of N^G-nitro-L-arginine methyl ester. Cells were pretreated with N^G-nitro-L-arginine methyl ester (L-NAME, 10^{-6}–4×10^{-3} M) in the presence of L-Arg (0 ●; 1 ○; 5 ■; 10 mM □) for 10 min at 37°C, followed by incubation with H_2O_2 (500 μM) for 3 h at 37°C. Results are the means ± s.e.mean for four different experiments performed in triplicate.

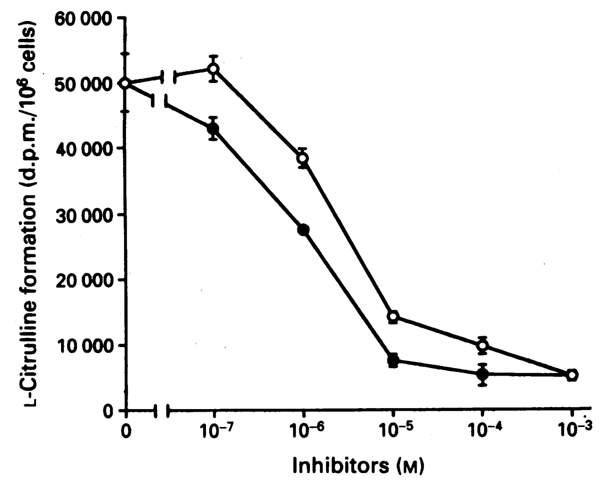

Figure 5 Effects of N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA) on L-citrulline formation from L-arginine induced by ionomycin. Cells were incubated for 4 min at 37°C in the presence of ionomycin (10^{-6} M), and L-NAME (10^{-7}–10^{-3} M, ●) or L-NMMA (10^{-7}–10^{-3} M, ○) were added 10 min before a supplement of ionomycin. Results are the means ± s.e.mean for triplicate assays.

simultaneous addition of L-NMMA (data not shown). This result also suggests that NO may not be responsible for H_2O_2-induced endothelial cell injury. From these results, L-NOARG analogues may reduce cell injury without inhibition of NO production.

NO synthase generates NO from L-Arg in the presence of Ca^{2+}/calmodulin, NADPH, and tetrahydrobiopterin (H_4 biopterin) in vascular endothelial cells and brain (Mayer *et al.*, 1989; Förstermann *et al.*, 1991; Pollock *et al.*, 1991; Schmidt *et al.*, 1992). However, NO synthase also generates H_2O_2 from molecular oxygen at low concentrations of L-Arg and H_4biopterin (Mayer *et al.*, 1991; Heinzel *et al.*, 1992). Both L-NOARG and L-NAME have been shown to block the substrate-independent generation of H_2O_2, whereas L-NMMA has no effect on this reaction. When we investigated the effects of L-NOARG analogues on H_2O_2-induced endothelial cell injury, L-Arg was not supplemented in the medium, and the H_4biopterin content of endothelial cells may be lowered by addition of H_2O_2. Moreover, we reported previously, that addition of H_2O_2 to endothelial cells increases intracellular Ca^{2+} concentration (Saito *et al.*, 1993). Therefore, addition of H_2O_2 to endothelial cells may stimulate NO synthase to produce H_2O_2. Accordingly, it is possible that L-NOARG analogues block the L-Arg-independent generation of H_2O_2 by NO synthase and consequently reduce H_2O_2-induced endothelial cell injury. However, addition of L-Arg, even at a high concentration (10 mM), did not alter the protective effects of L-NOARG analogues. Although the mechanism is not clear, we speculate that intracellular L-Arg contents were lowered by H_2O_2, probably by inhibition of the incorporation of L-Arg into cells. A detailed study will be required to test this hypothesis.

In conclusion, we have shown that despite the similar inhibition by L-NOARG analogues and L-NMMA of L-Cit formation from L-Arg coupled with NO production, L-NOARG analogues reduced H_2O_2-induced endothelial cell injury, while L-NMMA did not. The effects of L-NOARG analogues may be independent of NO production.

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