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Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis

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1 The ability of analogues of L-arginine (N-iminoethyl-L-ornithine (L-NIO), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro-L-arginine (L-NNA)) to protect against inflammatory injury induced by activated neutrophils was investigated in rats following intradermal or intrapulmonary deposition of immune complexes.

2 The descending order of potency for protective effects of these analogues was: L-NIO > L-NMMA > L-NNA = L-NAME. The approximate IC_{50} value for L-NIO in the dermal vasculitis model was 65 μM. For all other compounds, the IC_{50} values were > 5 mM.

3 The protective effect of L-NIO in the skin was reversed in a dose-dependent manner by the presence of L-arginine, but not by D-arginine. L-Arginine also reversed the protective effects of L-NIO in immune complex-induced lung injury.

4 The protective effects of L-NIO were not associated with reductions in neutrophil accumulation, as measured by extraction from tissues of myeloperoxidase.

5 These data demonstrate that L-NIO has the most potent protective effects against immune complex-induced vascular injury induced by activated macrophages. Furthermore, they indicate that this injury is dependent upon the generation of nitric oxide.

Keywords: Nitric oxide synthase inhibitors; immune complex-induced vasculitis; lung injury

Introduction

Phagocytic cells, including neutrophils as well as macrophages, generate nitric oxide (NO) via an NO synthase that is inducible by immunological stimuli such as endotoxin (LPS) and various cytokines (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; McCall *et al.*, 1989; Schmidt *et al.*, 1989; Stuehr & Nathan, 1989; Wright *et al.*, 1989; Billiar *et al.*, 1990; Curran *et al.*, 1990). Generation of NO by macrophages has been showed to kill tumour cells due to the inactivation of iron-sulphur centres of mitochondrial enzymes (Drapier & Hibbs, 1986; Hibbs *et al.*, 1987; 1988). The broader role of NO in the inflammatory response is not well established, although the reactivity of NO or its potential conversion product, peroxynitrite anion, with sulphydryl groups indicates the possibility of cellular biochemical targets whose alteration would put tissue at risk of injury.

Recently, it has been demonstrated that IgG immune complex-initiated injury of rat lung is greatly attenuated by the presence of the L-arginine analogue, N^G-monomethyl-L-arginine (L-NMMA) (Mulligan *et al.*, 1991). The protective effects of L-NMMA were not associated with a reduced influx of neutrophils into lungs, suggesting that toxic metabolites of L-arginine may be responsible for the inflammatory injury, which is known to depend upon the recruitment and participation of neutrophils (Johnson & Ward, 1979). Since N-iminoethyl-L-ornithine (L-NIO) has recently been described as a highly potent inhibitor of phagocytic cell NO synthase (McCall *et al.*, 1991), this compound was tested for its ability to protect against immune complex-induced vascular injury in rats. In addition, the effects of L-NIO were compared with those of other L-arginine analogues, namely, L-NMMA, N^G-nitro-L-arginine (L-NNA) and its methyl ester (L-NAME).

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Methods

Models of immune complex-induced alveolitis and dermal vasculitis

With adult male (300 g) specific pathogen-free Long-Evans rats (Charles River Breeding Laboratories), immune complex reactions were induced in lung and skin by the intratracheal (2.5 mg of antibody in 300 μl) or intradermal (0.42 mg of antibody in 50 μl) injection of polyclonal rabbit IgG antibody to bovine serum albumen (anti-BSA) followed by the intravenous injection of 10 mg BSA containing trace amounts of [^{125}I]-BSA. Tissue injury was assessed at 4 h by the increase in vascular permeability. Permeability values were defined as the ratio of ^{125}I-labelled BSA in lung or skin sites to the amount present in 1.0 ml blood obtained from the inferior vena cava at the time of death. Previously, we reported that BSA-anti-BSA immune complexes produced in untreated positive control rats lung and dermal vascular permeability values of approximately 0.46 (Mulligan *et al.*, 1991). In the current studies a new batch of anti-BSA was employed, giving the indicated permeability values which were approximately double those recorded early. Accordingly, computed percentages of protein were different from those observed earlier with the different batch of antibody which was less injurious.

Tissue myeloperoxidase (MPO) content

A standard reference curve was first established by measuring MPO in lungs and skin sites that had been injected with known numbers of neutrophils. Lung and skin sites were extracted by homogenization and sonication procedures that have been previously described (Mulligan *et al.*, 1991). MPO activity in supernatant fluids was measured by the change in optical density (at 460 nm) resulting from decomposition of H_2O_2 in the presence of *o*-dianisidine.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA). Additional comparisons were made between individual groups by the use of paired or unpaired Student's t tests. All values were expressed as mean ± s.e.mean unless otherwise indicated. Statistical significance was defined as $P<0.05$.

Materials

L-NIO and L-NNA were provided by Wellcome Research Laboratories (Beckenham, Kent) while L-NAME and L-NMMA were from Cal Biochem (La Jolla, California, U.S.A.). Rabbit polyclonal IgG antibody to bovine serum albumin (anti-BSA) was from Organon Teknicka (Westchester, PA, U.S.A.).

Results

Protective effects of L-arginine analogues in dermal vascular injury

Immune complex deposition was induced by the intra-dermal injection of anti-BSA and the intravenous injection of BSA. The resulting vascular injury was measured 4 h later by leakage of [^{125}I]-albumen into skin sites. In the negative controls (omission of intravenously injected BSA) and positive controls, the permeability values were 0.07 ± 0.01 and 0.81 ± 0.03, respectively. When used, the L-arginine analogues were mixed with the anti-BSA preparations immediately prior to intradermal injection. Concentrations of the analogues employed were: 0.01, 0.1, 0.5, 1.0 and 5.0 mM. The data in Figure 1 show the comparative abilities of the four analogues of L-arginine to reduce immune complex-induced vascular injury, as measured by changes in vascular permeability. The most effective compound was L-NIO, which caused a marked reduction in the increase in vascular permeability. L-NIO had an estimated IC_{50} of 65 μM. At 1.0 mM, L-NMMA reduced the permeability change by 32%, but this was not further reduced with 5 mM L-NMMA. L-NNA and L-NAME were much less potent; at 5 mM, the protective effects were only 19% and 16%, respectively. Thus, the rank order of protective effects for these L-arginine analogues in immune complex-induced dermal vascular injury is: L-NIO > L-NMMA > L-NNA = L-NAME.

Ability of L-arginine to reverse protective effects of L-NIO

In the same model of immune complex-induced dermal vascular damage, the presence of 1.0 mM L-NIO in the anti-BSA preparation resulted in a fall in the permeability index from the positive control (absence of L-NIO), from 0.81 ± 0.03 to 0.46 ± 0.02, a 47% reduction ($P<0.001$) in the permeability index. As L-arginine was added in increasing concentrations to anti-BSA preparations which contained 1.0 mM L-NIO, the reductions (reversals) in vascular permeability reflecting the protective effects of L-NIO were correspondingly reversed: with 0.1, 0.5, 1.0, 5.0 and 10 mM L-arginine, the permeability values were: 0.55 ± 0.03 (35% reduction, $P=0.002$); 0.66 ± 0.04 (20% reduction, $P=0.049$); 0.74 ± 0.02 (9% reduction, P = NS); 0.81 ± 0.02 (0% reduction, P = NS), and 0.93 ± 0.02 (16% increase in permeability compared to the positive control, P = NS), respectively. The presence of 1.0 mM D-arginine with 1.0 mM L-NIO did not reverse the protective effects of the latter (data not shown).

Protective effects of L-NIO in immune complex induced-dermal vascular injury: effects on tissue MPO content

These experiments were carried out with positive and negative (omission of intravenously injected BSA) controls and extraction of skin sites at 4 h for MPO following deposition of immune complexes. In a comparison series of skin sites injected with anti-BSA containing 1.0 mM L-NIO alone or in combination with 1 mM D-arginine, or with 0.1, 0.5, 1.0, 5.0 or 10 mM L-arginine, a protocol similar to that described in Figure 2 was employed. The negative and positive controls showed an MPO content of 0.08 ± 0.01 and 0.39 ± 0.02, respectively. The range of MPO content in all L-NIO-injected skin sites was 0.37 ± 0.02 to 0.41 ± 0.02, none of which was statistically significantly different from the positive reference control value (data not shown). Thus, the protective effects of L-NIO in immune complex-induced vasculitis are *not* associated with a blocking of the recruitment of neutrophils into the sites containing the immune complexes.

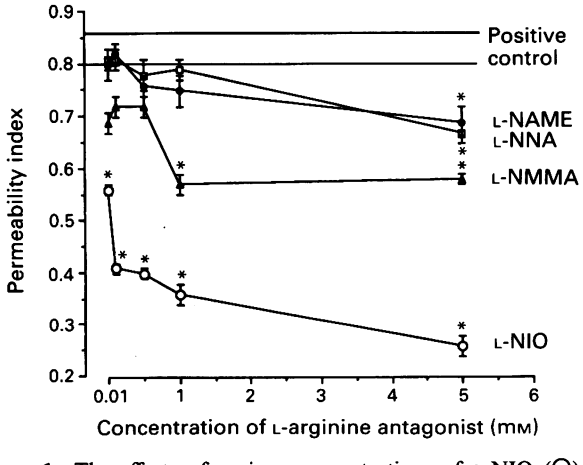

Figure 1 The effects of various concentrations of L-NIO (○), L-NMMA (▲), L-NNA (□), and L-NAME (●) on vascular injury (measured by leakage of [^{125}I]-albumen) in dermal skin sites containing IgG immune complexes. Compounds were added to the anti-BSA immediately prior to intradermal injection. All reactions were assessed 4 h after initiation of immune complex deposition. For each data point, $n=8$. For statistical purposes, comparisons were made with skin sites containing immune complexes in the absence of an L-arginine analogue. Vertical bars represent s.e.mean. For abbreviations, see text.

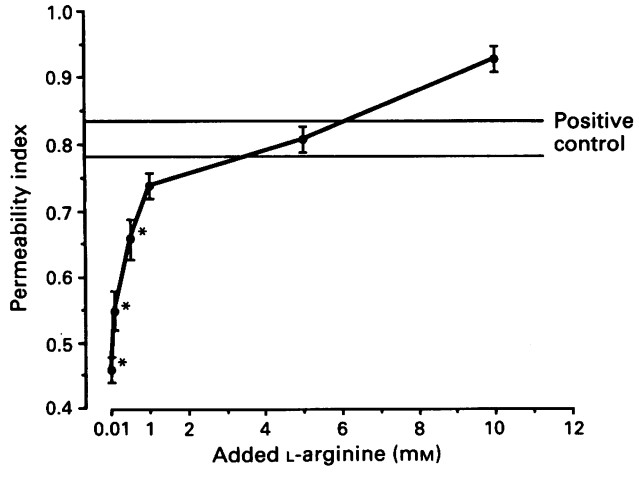

Figure 2 Reversal of protective effects of 1.0 nM L-NIO by copresence of L-arginine (●) which was added in a range of concentrations (0.01–10 nM) to the anti-BSA prior to intradermal injection. The reference positive value (for vascular permeability) was 0.81 ± 0.03 while the negative control (absence of intravenously injected BSA) was 0.07 ± 0.01. For each data point, $n=4$. For abbreviations, see text.

Protective effects of L-NIO in immune complex-induced alveolitis

IgG immune complex deposition was also induced in rat lung by the intratracheal instillation of anti-BSA (1.5 mg in a total volume of 300 μl) in the presence or absence of 5.0 mM L-NMMA or 5.0 mM L-NIO and the effects on vascular injury assessed according to the increase in lung vascular permeability. This lower dose of antibody was employed in order to accentuate differences in the protective effects of the L-arginine antagonists. For the experiments shown in Figure 3, the values for the negative (omission of intravenously injected BSA) and positive control groups were 0.16 ± 0.01 and 0.52 ± 0.02, respectively. When the negative control value was subtracted from the values of the positive control groups (treated or untreated with L-NMMA or L-NIO) and the ratios computed, 5.0 mM L-NMMA caused a 50% reduction ($P = 0.009$) in intensity of lung injury as reflected by change in vascular permeability while L-NIO caused an 82% reduction ($P = 0.002$) in lung injury (a fall in the permeability to 0.22 ± 0.01). L-NIO had statistically greater protective effects when compared to L-NMMA ($P = 0.021$), in which case the permeability value was 0.33 ± 0.01.

Additional studies on the effects of L-arginine analogues on the intensity of lung injury (as measured by permeability changes) were performed, as shown in Figure 4. For these experiments a concentration of 2.5 mg anti-BSA was employed. The negative and positive control values were 0.16 ± 0.01 and 0.75 ± 0.03, respectively. All results were compared to the positive control values (anti-BSA in the absence of any analogue of L-arginine). The presence of 5.0 mM L-NMMA, L-NAME, L-NIO, L-NIO + D-arginine, and L-NIO + L-arginine was associated with permeability values of 0.46 ± 0.02 (reduction of 49%, $P = 0.002$); 0.60 ± 0.03 (reduction of 25%, $P = 0.027$); 0.43 ± 0.02 (reduction of 54%, $P = 0.001$); 0.44 ± 0.01 (reduction of 53%, $P = 0.001$); and 0.84 ± 0.02 (intensification of injury by 15%, $P =$ NS), respectively. Thus, L-NIO is a potent inhibitor of vascular injury developing in lung following deposition of IgG immune complexes and its protective effects are reversed by L-arginine but not by D-arginine. In this experiment there are not sufficient data to compare the protective effects of L-NIO with the other analogues of L-arginine.

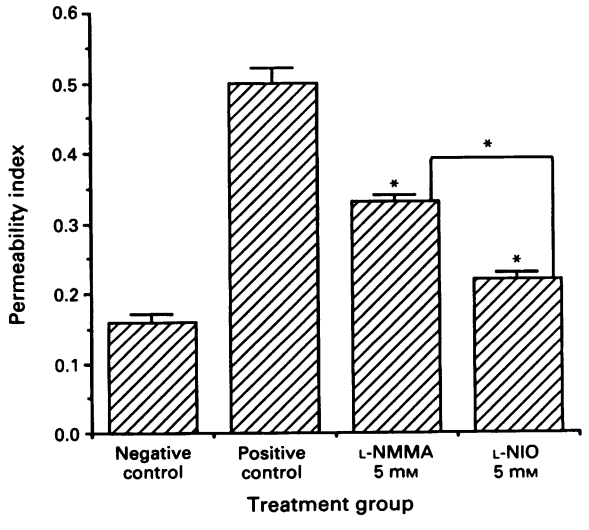

Figure 3 Protective effects (assessed by changes in vascular permeability) of L-NIO and L-NMMA on immune complex-induced pulmonary vascular injury. When employed, 5 mM L-NMMA or L-NIO was added to the anti-BSA preparation prior to its intratracheal administration. Reactions were measured 4 h after deposition of IgG immune complexes. All comparisons were to the positive control group (for each vertical column, $n = 4$). For abbreviations, see text.

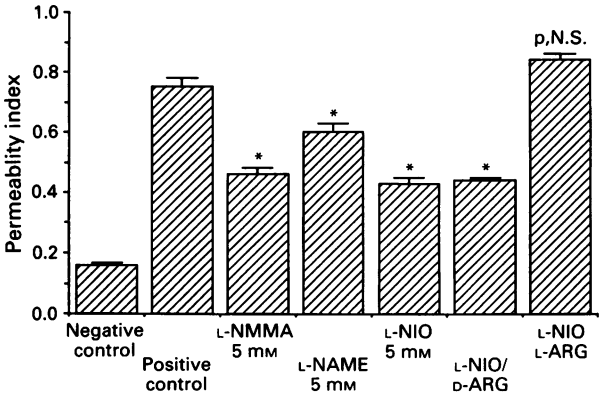

Figure 4 Ability of various analogues of L-arginine to reduce lung vascular injury (as assessed by increased vascular permeability) following intrapulmonary deposition of IgG immune complexes. All compounds employed were used at 5 nM concentrations. Statistical comparisions were made with the positive control group and represent significance of reductions in vascular permeability for each vertical column, $n = 4$. For abbreviations, see text.

When lungs from animals undergoing immune complex deposition were compared for MPO content to those lungs in which anti-BSA also contained L-NIO or L-NMMA, no difference was found in the lung content of MPO (data not shown), confirming the pattern found for the protective effects of analogues of L-arginine in the skin (described above).

Discussion

The ability of L-arginine analogues to reduce evidence of IgG immune complex-induced dermal vascular injury in rats reveals the rank order of potency: L-NIO > L-NMMA > L-NNA = L-NAME, which is in remarkable agreement with the *in vitro* effects of these compounds on their ability to block NO synthase in rat peritoneal neutrophils or in J774 cells, whether measured by interference with platelet aggregation or by direct measurement of NO formation in cytosolic fractions of cells (McCall *et al.*, 1991). The estimated IC_{50} value (65 μM) for the *in vivo* protective effects of L-NIO in skin is much higher than the value obtained *in vitro* (0.8 μM), but this is readily explained by the fact that the total volume of intradermally injected material is 50 μl, which becomes rapidly diluted as the permeability changes begin to occur in the developing acute inflammatory reaction. Since the minimal volume increase in the positive control sites is nearly 16 fold (to 800 μl) above the negative control value and the outflow, chiefly through efferent lymphatic channels, has not been measured, the effective local *in vivo* concentration cannot be accurately computed but would probably be reduced many fold below the initial concentration, perhaps to < 3 μM. A key factor in the efficacy of L-NIO when compared to the other analogues of L-arginine appears to be the more rapid onset in its inhibitory effects when added to the phagocytic cells (10 min versus 20–60 min) and the irreversible effect of L-NIO as compared to the other analogues (McCall *et al.*, 1991).

These data indicate that L-arginine analogues such as L-NIO may be useful in preventing tissue damage which is generated by toxic products of activated neutrophils (and macrophages). In the rat dermal and lung models of immune complex-induced vascular injury, neutrophils have long been shown to be key participants in the events leading to injury (Johnson & Ward, 1979; Warren *et al.*, 1990). The protective effects of antioxidants such as superoxide dismutase (SOD) and catalase have led to the suggestion that toxic metabolites

of oxygen may be key initiators of injury (Johnson & Ward, 1981). The protective effects of SOD are complicated by the fact that this compound has time-limited protective effects (2 h) in the immune complex models of injury (Johnson & Ward, 1981), which may be related to the generation of O_2-dependent chemotactic lipids (Von Zabern *et al.*, 1987; Vogt *et al.*, 1989). The recent finding that L-NMMA has significant protective effects in immune complex-induced injury in rat lung and skin (Mulligan *et al.*, 1991) has caused a re-evaluation of the pathways leading to injury and has emphasized that L-arginine, or presumably its metabolic products, are injurious, perhaps through the generation of peroxynitrite anion or the hydroxyl radical (Beckman *et al.*, 1990). The most remarkable finding in the earlier study (Mulligan *et al.*, 1991) and in the current one is that the protective effects are *not* associated with a reduced tissue accumulation of neutrophils, implying that the protective effects of L-NIO and L-NMMA are not attributable to an interference with neutrophil emigration from the vasculature. The current studies indicate that compounds capable of blocking NO formation may have significant protective effects against injury in a variety of human inflammatory diseases.

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