

Endogenous nitric oxide modifies antigen-induced microvascular leakage in sensitized guinea pig airways

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To examine the role of endogenous nitric oxide in allergic airway inflammation, we investigated the effect of a nitric oxide synthase inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME), on antigen-induced airway microvascular leakage in actively sensitized guinea pigs by using Evans blue dye. Three weeks after sensitization with ovalbumin (10 μ g), the tracheas were cannulated, and lungs were artificially ventilated. Animals were pretreated with atropine and propranolol (both 1 mg/kg, intravenously) to avoid neural modification. Ovalbumin inhalation (3 mg/ml, 1 minute) challenge caused significant microvascular leakage in all airway portions, which was significantly suppressed in a dose-dependent manner by pretreatment with intravenous injection of L-NAME (1 and 10 mg/kg) but not with the inactive enantiomer D-NAME (10 mg/kg). This inhibition by L-NAME was significantly reversed by co-administration of L-arginine (100 mg/kg, intravenously). Pretreatment with a vasoconstrictor, phenylephrine (20 μ g/kg, intravenously), had no inhibitory effects on antigen-induced airway microvascular leakage despite increasing systemic blood pressure. Inhalation of representative mast cell-derived mediators, histamine (2 mg/ml, 1 minute) or leukotriene D₄ (5 μ g/ml, 1 minute), produced significant microvascular leakage in all airways. L-NAME (10 mg/kg, intravenously) partially but significantly inhibited leukotriene D₄-induced leakage, whereas histamine-induced leakage was not affected. These results suggest that endogenous nitric oxide acts to increase airway microvascular leakage after airway allergic reaction. (J Allergy Clin Immunol 1996;98:144-51.)

Key words: L-NAME, L-arginine, ovalbumin, histamine, leukotriene D₄, mast cell degranulation

Nitric oxide (NO), recognized as an air pollutant and recently as a physiologic regulator substance responsible for the endothelium-derived relaxing factor,^{1,2} has been implicated in inflammation. An increasing number of reports have suggested that NO plays a role in a variety of inflammatory processes.³⁻⁵ NO has been shown to inhibit neutrophil adherence to venular endothelium,⁶ the increase in vascular permeability in intestines,⁷ and

Abbreviations used

D-NAME:	N ω -nitro-D-arginine methyl ester
EB:	Evans blue
IV:	Intravenously
L-NAME:	N ω -nitro-L-arginine methyl ester
LT:	Leukotriene
NO:	Nitric oxide
NOS:	Nitric oxide synthase

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mast cell degranulation by scavenging oxygen radicals.⁸ These findings appear to reflect the antiinflammatory nature of NO. On the other hand, NO has also been demonstrated to have proinflammatory effects. Administration of NO synthase (NOS) inhibitors was reported to block vascular injury in the lungs⁹ and to block the edema induced by

agonists, such as bradykinin and substance P in skin¹⁰ and hamster cheek pouch,¹¹ indicating that endogenous NO exacerbates inflammation.

In relation to inflammatory diseases, hyperproduction of NO has been demonstrated in ulcerative colitis¹² and rheumatoid arthritis.¹³ In animal models of colitis¹⁴ and arthritis,¹⁵ inhibition of NO synthesis reduced inflammation, suggesting that NO acts as an inflammatory mediator. Because bronchial asthma includes the characteristics of inflammation in its pathogenesis, NO may play a role in the pathogenesis of asthma. Indeed, the expression of an inducible type of NOS has been demonstrated in epithelium and infiltrated cells in tissues taken from patients with asthma, but it is rarely seen in normal airways.¹⁶ Direct evidence for hyperproduction of NO in asthma has been obtained from the measurement of the NO concentration in expired gas.¹⁷ However, it is still unknown whether NO that is excessively produced in bronchial asthma acts as an inflammatory mediator. To study this issue in an animal model, we examined the role of endogenous NO in antigen-induced airway microvascular leakage, a component of airway inflammation, in actively sensitized guinea pigs by using a NOS inhibitor, N ω -nitro-L-arginine methyl ester (L-NAME). Airway microvascular leakage was evaluated by plasma extravasation of Evans blue (EB) dye into airway walls.

METHODS

Animal preparation

Dunkin-Hartley guinea pigs (all male, weighing approximately 200 gm each) were actively sensitized by intradermal injection of 0.5 ml of saline solution containing ovalbumin (10 μ g) and aluminum hydroxide (100 mg) on 2 consecutive days. Three weeks after the sensitization procedure, each animal was anesthetized by intraperitoneal injection of urethane (2 gm/kg) and placed on a heat pad, which kept body temperature at around 37°C. The carotid artery and jugular vein were cannulated to monitor systemic blood pressure and inject drugs, respectively. The trachea was cannulated through a tracheostoma, and the lungs were ventilated artificially with a small-animal constant volume ventilator (Model SN-480-7; Shinano Seisakusho, Tokyo, Japan) at a frequency of 60 strokes/min and a tidal volume of 1 ml/100 gm body weight. All experimental animals were pretreated with atropine and propranolol (both 1 mg/kg, intravenously [IV]) 30 minutes before experimentation in order to avoid cholinergic and β -adrenergic neural modification of plasma leakage.

Protocol

In one set of experiments, the role of endogenous NO in antigen-induced airway microvascular leakage was

examined by using a NOS inhibitor. Sensitized animals were divided into eight groups: group 1 ($n = 5$), saline inhalation pretreated with saline; group 2 ($n = 8$), ovalbumin inhalation pretreated with saline (1 ml/kg, intravenously); group 3 ($n = 3$), ovalbumin inhalation pretreated with L-NAME (1 mg/kg, intravenously); group 4 ($n = 5$), ovalbumin inhalation pretreated with L-NAME (10 mg/kg, intravenously); group 5 ($n = 5$), ovalbumin inhalation pretreated with D-NAME (10 mg/kg, intravenously); group 6 ($n = 5$), ovalbumin inhalation pretreated with L-NAME (10 mg/kg, intravenously) and L-arginine (100 mg/kg, intravenously); group 7 ($n = 5$), ovalbumin inhalation pretreated with L-NAME (10 mg/kg, intravenously) and D-arginine (100 mg/kg, intravenously); group 8 ($n = 5$), ovalbumin inhalation pretreated with L-arginine (100 mg/kg, intravenously). Then, L-NAME, D-NAME, L-arginine, or saline solution was injected 5 minutes before the administration of EB dye (30 mg/kg, intravenously), followed by inhalation of ovalbumin (3 mg/ml for 1 minute) or saline solution (for 1 minute), 1 minute after dye injection. In groups 6 and 7, L-arginine or D-arginine was intravenously administered after the systemic blood pressure increased by L-NAME reached a plateau. In the next set of experiments, we investigated the effect of a vasoconstrictor, phenylephrine (20 μ g/kg, intravenously), on antigen-induced airway microvascular leakage. In this group of studies, phenylephrine ($n = 4$) or its vehicle (saline solution, $n = 4$) was injected 1 minute before the dye injection, and ovalbumin inhalation was performed similarly. All inhalation procedures were performed with an ultrasonic nebulizer (mean particle size, 5.0 μ m, per manufacturer's specification) (NE-U11B; Omron, Tateishi Co., Tokyo, Japan). The output of this nebulizer was measured as 0.02 ml/min at 4 ml tidal volume when attached to the ventilatory system.

In another set of experiments, inhalation challenge of mast cell-derived mediators, histamine (2 mg/ml for 1 minute, $n = 5$ for each) or leukotriene (LT) D_4 (5 μ g/ml for 1 minute, $n = 5$ for each), was performed in the presence and absence of pretreatment with L-NAME (10 mg/kg, intravenously) as in the ovalbumin inhalation challenge.

These experiments were approved by the animal experiment review committee of Tohoku University School of Medicine.

Measurement of airway microvascular leakage

Vascular permeability was quantified by the extravasation of EB dye, which correlates well with the extravasation of radiolabeled albumin in guinea pig airways.¹⁸ The tissue content of EB dye after experimental intervention was determined by perfusing the systemic blood circulation with saline solution to remove intravascular dye. After the induction of the leakage (5-minute period after inhalation challenge), the thorax was opened, and a blunt-ended 13-gauge

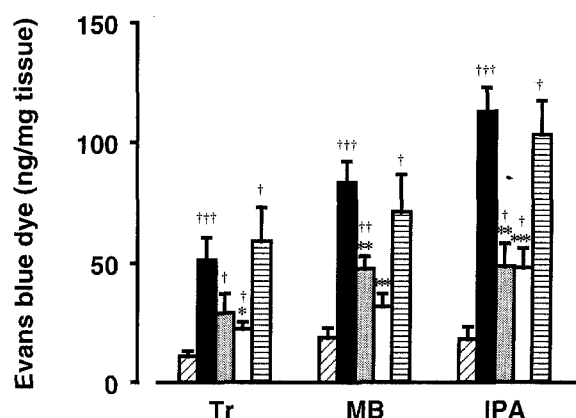


FIG. 1. Effect of intravenous injection of L-NAME on ovalbumin-induced airway microvascular leakage in sensitized guinea pigs. Ovalbumin inhalation (1 mg/ml for 1 minute, $n = 8$, filled columns) produced significant extravasation of EB dye in trachea (Tr), main bronchi (MB), and intrapulmonary airways (IPA), compared with saline inhalation (for 1 minute) ($n = 5$, obliquely-striped columns). Pretreatment with intravenous L-NAME, 1 mg/kg, ($n = 3$, dotted columns) and 10 mg/kg ($n = 5$, open columns) dose-dependently suppressed ovalbumin-induced leakage in all airway portions, whereas pretreatment with D-NAME (10 mg/kg, intravenously, $n = 5$, horizontally-striped columns) was without effect. All values are means $\pm$ SEM. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$, and $\dagger\dagger\dagger p < 0.001$ compared with the group that had saline pretreatment and saline inhalation. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ compared with the group that had saline pretreatment and ovalbumin inhalation.

needle was passed through a left ventriculotomy into the aorta. The ventricles were cross-clamped, and blood was expelled through an incision in the right atrium at a pressure of 100 mm Hg with about 100 ml of saline solution until the perfusate was clear. The lungs were then removed. The connective tissues, vasculature, and parenchyma were gently scraped; and the remaining airways were divided into three portions: the lower part of the trachea, main bronchi, and intrapulmonary airways. The tissues were blotted dry, placed in preweighed tubes, and reweighed to determine the tissue weight. The extravasated dye was extracted in formamide at 37°C for 16 hours. The dye concentration was quantified by light absorbance at 620 nm (220A spectrophotometer; Hitachi Ltd., Tokyo, Japan), and its tissue content (nanograms per milligram of tissue) was calculated from a standard curve of dye concentration in the range of 0.5 to 10 $\mu\text{g/ml}$.

Drugs

The following drugs and reagents were used: EB dye and urethane (Aldrich Chemical Co. Inc., Milwaukee, Wis.); atropine sulfate (Tanabe Chemical Co.,

Osaka, Japan); propranolol hydrochloride (Imperial Chemical Industries Plc., Macclesfield, U.K.); L-NAME, D-NAME, ovalbumin, L-arginine, D-arginine, phenylephrine hydrochloride, and LTD₄ (Sigma Chemical Co., St Louis, Mo.); and histamine hydrochloride (Wako Chemicals, Sendai, Japan).

Statistical analysis

All data were expressed as means $\pm$ SEM. Comparisons between data were performed by one-way analysis of variance, and Scheffé's F test was used as a post hoc test. Probability (p) values of less than 0.05 were considered significant.

RESULTS

Effect of L-NAME on ovalbumin-induced plasma leakage

Ovalbumin inhalation (3 mg/ml for 1 minute) caused significant EB dye extravasation in all airway portions, compared with saline inhalation (Fig. 1). Pretreatment of guinea pigs with intravenous injection of L-NAME (1 and 10 mg/kg) significantly inhibited ovalbumin-induced plasma leakage in a dose-dependent manner, whereas D-NAME (10 mg/kg, intravenously) was without effect in any airway portion (Fig. 1). Intravenous injection of L-NAME also dose-dependently increased systemic blood pressure (Fig. 2), when plateau increases were obtained within 2 to 3 minutes after the injection. D-NAME showed no significant effect on systemic blood pressure. Ovalbumin inhalation transiently decreased systemic blood pressure in each group to a similar degree (Fig. 2).

The administration of L-arginine (100 mg/kg, intravenously) after L-NAME (10 mg/kg, intravenously) completely reversed the systemic blood pressure increase by L-NAME injection and significantly reversed the inhibition of ovalbumin-induced microvascular leakage in all airway components (Fig. 3). In contrast, the inactive enantiomer of L-arginine, D-arginine (100 mg/kg, intravenously), had no reversal effect on either the increase in blood pressure or the inhibition of ovalbumin-induced plasma leakage by L-NAME (Fig. 3). Single pretreatment with L-arginine (100 mg/kg, intravenously) was without effect on systemic blood pressure by itself and had no significant effect on ovalbumin-induced plasma leakage, although plasma leakage tended to be decreased; mean EB dye contents after ovalbumin inhalation with L-arginine pretreatment were 42.0 ± 7.2 ng/mg tissue in trachea, 59.3 ± 10.9 ng/mg tissue in main bronchi, and 89.0 ± 10.4 ng/mg tissue in intrapulmonary airways, respectively.

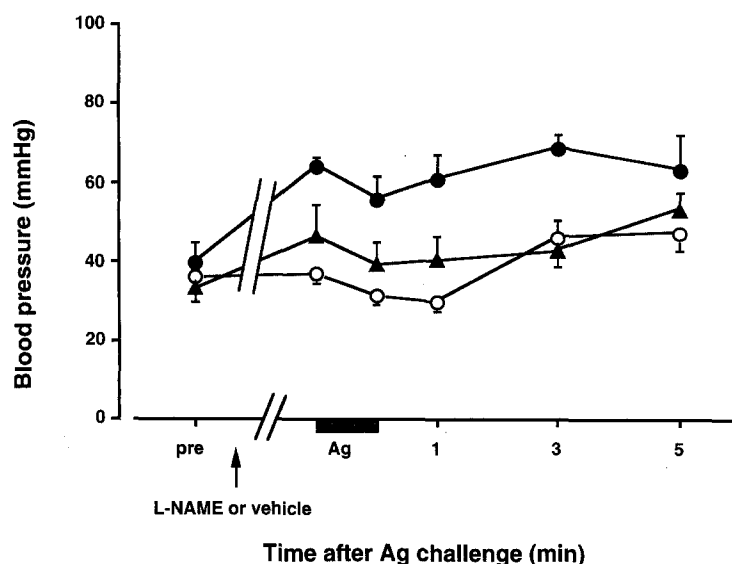


FIG. 2. Changes in systemic blood pressure during experimental procedure. All animals were pretreated with atropine and propranolol (both 1 mg/kg, IV) 30 minutes before the experiment. Vehicle pretreatment (*open circles*) did not change baseline blood pressure (*pre*), whereas intravenous pretreatment with L-NAME, 1 mg/kg, (*n* = 3, *filled triangles*) and 10 mg/kg (*n* = 5, *filled circles*) dose-dependently increased baseline blood pressure. Ovalbumin inhalation (1 mg/ml for 1 minute) transiently decreased systemic blood pressure in all groups. Ag, Antigen.

Effect of a vasoconstrictor on ovalbumin-induced plasma leakage

Intravenous injection of phenylephrine (20 μ g/kg, intravenously) increased systemic blood pressure by 23.0 ± 4.3 mm Hg at the beginning of ovalbumin inhalation. In this condition, phenylephrine was without effect on ovalbumin-induced dye extravasation in all airways (Fig. 4).

Effect of L-NAME on exogenous mediator-induced plasma leakage

Inhalation of histamine (2 mg/ml for 1 minute) or LTD₄ (5 μ g/ml for 1 minute) caused substantial plasma leakage in all airway portions (Fig. 5). L-NAME partially but significantly reduced LTD₄-induced EB dye extravasation in trachea ($p < 0.05$) and in intrapulmonary airways ($p < 0.05$) but not in main bronchi, whereas the histamine-induced change was not affected in any airway portion (Fig. 5). Mean increases in systemic blood pressure by L-NAME treatment (10 mg/kg, intravenously) were not significantly different between histamine-challenged and LTD₄-challenged groups (23 ± 5 and 20 ± 3 mm Hg, respectively).

DISCUSSION

We have demonstrated that an NOS inhibitor, L-NAME, but not its inactive enantiomer D-NAME, suppresses antigen-induced airway mi-

crovascular leakage in actively sensitized guinea pigs. Because this inhibition was partially but significantly reversed by the administration of an excessive amount of NO substrate (L-arginine) but not by D-arginine, endogenously generated NO is likely to be involved in this response.

In this *in vivo* study, we used L-NAME at doses of 1 and 10 mg/kg to inhibit endogenous NO production. Rees et al.¹⁹ have reported that L-NAME causes a dose-dependent increase in mean systemic blood pressure by inhibiting endothelial NOS activity, with a submaximal effect at 10 mg/kg in anesthetized rats. Because the effects of L-NAME on systemic blood pressure obtained in this study were comparable with this report, the doses of L-NAME appear to be appropriate for inhibiting endothelial NOS activity in guinea pigs *in vivo*.

As stated earlier, intravenously administered L-NAME (both 1 and 10 mg/kg) increased systemic blood pressure, which means that it is possible that decreased blood flow to leaky sites reduced the microvascular leakage. Indeed, epinephrine has been reported to inhibit platelet activating factor-induced plasma leakage through its vasoconstricting effect in guinea pig airways.²⁰ The protective effect of L-NAME against agonist-induced plasma leakage in guinea pig skin is also thought to be due to a local decrease in basal blood flow.²¹ Thus the decrease in local blood flow by L-NAME-induced

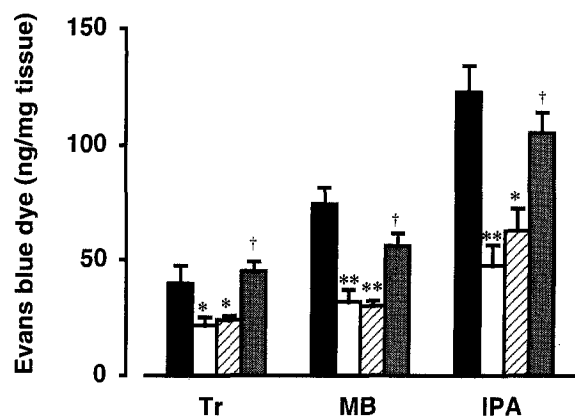


FIG. 3. Reversal effect of L-arginine on L-NAME-induced suppression of ovalbumin-induced airway microvascular leakage. Intravenous co-injection of L-arginine (100 mg/kg IV, $n = 5$, dotted columns), but not D-arginine (100 mg/kg IV, $n = 5$, obliquely-striped columns), with L-NAME significantly reversed the inhibitory effect of L-NAME (10 mg/kg IV, $n = 5$ open columns) on ovalbumin-induced EB dye extravasation ($n = 8$, closed columns) in trachea (Tr), main bronchi (MB), and intrapulmonary airways (IPA). All values are means $\pm$ SEM. * $p < 0.05$ and ** $p < 0.01$ compared with the group that had saline pretreatment and ovalbumin inhalation. † $p < 0.05$ compared with the group that had L-NAME pretreatment and ovalbumin inhalation.

vasoconstriction may appear to be responsible for this result. However, a vasoconstrictor, phenylephrine, which significantly increased systemic blood pressure, had no inhibitory effect on antigen-induced plasma leakage in this animal model. Although it is not clear that the effective sites of phenylephrine and the NOS inhibitor in the bronchial vasculatures are exactly the same, the constriction of resistance vessels alone is unlikely to explain the effect of L-NAME. In addition, it has been reported that another NOS inhibitor, N ω -monomethyl-L-arginine, causes a reduction in the diameter of arterioles but not in the diameter of venules in the hamster cheek pouch.²² Because venules are the sites of microvascular leakage in the airway wall,²³ it is possible that the inhibition of NO production does not change the blood volume at the leaky sites. Accordingly, mechanisms other than vasoconstriction are also assumed to be involved in the strong inhibition of antigen-induced plasma leakage by L-NAME.

One of the other possible mechanisms that may explain the inhibition of antigen-induced plasma leakage by L-NAME is the direct involvement of endogenous NO at the leaky sites. There are controversial reports about the role of NO in vascular permeability. In the intestines⁸ and pulmonary circulation,²⁴ the administration of

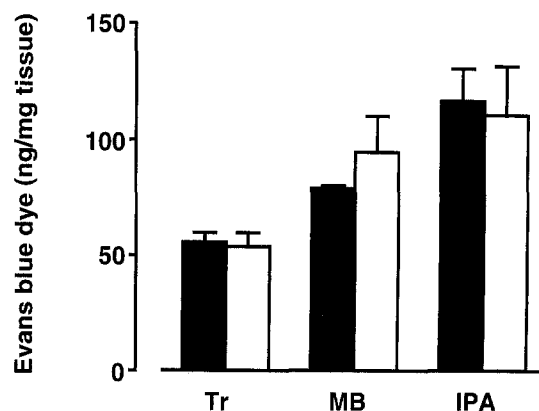


FIG. 4. Effect of phenylephrine on ovalbumin-induced airway microvascular leakage in sensitized guinea pigs. Ovalbumin inhalation with saline pretreatment ($n = 4$, filled columns) produced significant extravasation of EB dye in trachea (Tr), main bronchi (MB), and intrapulmonary airways (IPA). Pretreatment with phenylephrine (20 μ g/ml IV, $n = 4$, open columns) had no significant effect on ovalbumin-induced leakage in all airways. All values are means $\pm$ SEM.

L-NAME has been reported to increase vascular permeability. These reports suggest that endogenous NO has regulatory and protective effects on vascular permeability in unstimulated basal conditions. In contrast, neurogenic plasma exudation mediated by endogenous substance P in guinea pig bronchial circulation was reported to be inhibited by L-NAME, and this inhibition was reversed by L-arginine.²⁵ We have also reported that exogenous substance P-induced airway plasma leakage is inhibited by a NOS inhibitor in nonsensitized guinea pigs.²⁶ In hamster cheek pouch,¹¹ substance P- and bradykinin-induced plasma exudation were reduced by L-NMMA. The site of microvascular plasma leakage is thought to be postcapillary venules, and these mediators cause leakage by means of endothelial contraction of the leaky sites,²⁷ presumably by the stimulation of endothelial cell surface receptors for each mediator. Therefore these observations indicate that endogenous NO induces plasma extravasation by participating in the signal transduction after receptor activation of the postcapillary endothelial cells. This possibility may be supported by the direct evidence that substance P produces NO in bovine coronary postcapillary venular endothelial cells.²⁸ Another possible source of endogenous NO is the nervous system. Nitric oxidergic nerves are known to be distributed in guinea pig airways, including areas surrounding the vessels,²⁹ and antigen infusion has been shown to evoke neural reflex.³⁰ Thus

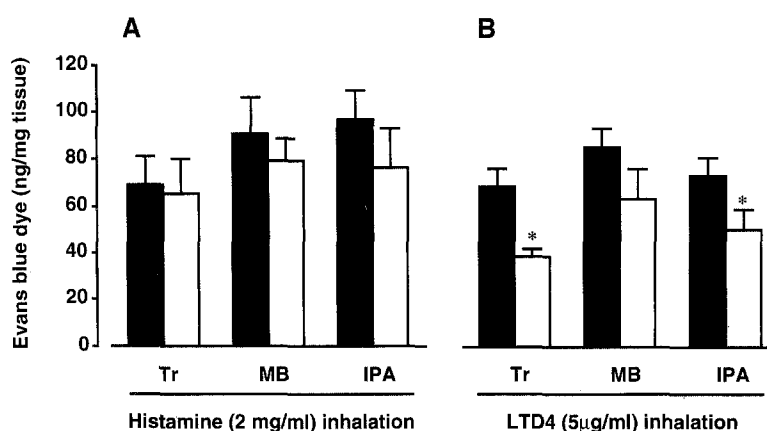


FIG. 5. Effect of intravenous injection of L-NAME on histamine-induced (**A**) and LTD₄-induced (**B**) airway microvascular leakage in sensitized guinea pigs. Pretreatment with intravenous L-NAME, 10 mg/kg, ($n = 5$ in both **A** and **B**, open columns) had no effect on histamine inhalation-induced (2 mg/ml for 1 minute, $n = 5$, filled columns) extravasation of EB dye in trachea (Tr), main bronchi (MB), and intrapulmonary airways (IPA), but significantly inhibited LTD₄-induced (5 mg/ml for 1 minute, $n = 5$, filled columns) extravasation of EB dye in trachea and IPA. All values are mean $\pm$ SEM. * $p < 0.05$ compared with the group of saline pretreatment and LTD₄ inhalation.

it is also possible that NO of neural origin modifies antigen-induced plasma leakage.

The mediators responsible for antigen-induced airway microvascular leakage in guinea pigs are thought to be mainly LTs and partly histamine.³¹ In this study LTD₄, administered exogenously by inhalation, induced microvascular leakage, which was significantly reduced by L-NAME, indicating that NO generated by the stimulation of LT receptors on postcapillary venules promotes antigen-induced plasma leakage. In contrast, histamine-mediated plasma leakage was unexpectedly unaffected by L-NAME pretreatment. Histamine causes plasma extravasation through histamine H₁ receptor on postcapillary venular endothelium.³² Thus it seems that H₁-receptor stimulation at this site does not involve NO production in the plasma leakage of guinea pig airways. The inhibition of LTD₄-induced plasma leakage by L-NAME was relatively small compared with the inhibitory effect of L-NAME on the antigen-induced response. The concentration of inhaled LTD₄ in this study was chosen to be comparable with antigen-induced leakage. As described previously, in this species the major but not only components of antigen-induced plasma leakage are LTs. Thus the tissue LT concentration after LTD₄ inhalation seems higher than that caused by endogenous release of LTs after antigen challenge in this study. The plasma leakage induced by higher concentrations of LTD₄ may include mechanisms other than NO production.

Mast cells have been reported to have NOS,³³ and this cell-derived NO may act as a negative feedback mechanism in the degranulation process.⁵ In this respect, NO seems to inhibit the allergic response. The result that single pretreatment with L-arginine tended to decrease the ovalbumin-induced plasma leakage seems to reflect this effect of NO. Nevertheless, in this study the inhibition of NO by L-NAME pretreatment reduced rather than enhanced the allergic response. During allergic airway inflammation, a variety of oxygen radicals, including superoxide, are produced from inflammatory cells, such as eosinophils³⁴ and mast cells.³⁵ NO is known to react with superoxide to produce a potent cytotoxic oxygen radical, peroxynitrite.³⁶ Thus it is possible that cytotoxic oxygen radicals produced in the presence of NO during airway allergic responses worsen airway inflammation.

In bronchial asthma, hyperproduction of NO has been demonstrated by the increased NO concentration in expired gas,¹⁷ although the exact sites of production and the role of NO in asthmatic airways remain unclear. Because NO is a potent bronchodilator,³⁷ excessively produced NO is likely to counteract narrowing of asthmatic airways, as reported in experimental conditions.³⁸ However, NO has dual functions, namely proinflammatory and antiinflammatory effects, in airway inflammation.^{35, 39} This study suggests that NO has a proinflammatory effect in airway microvascular leakage during allergic airway inflammation.

In summary, this study indicates that inhibition of endogenous NO production suppresses antigen-induced airway microvascular leakage. Although the mechanisms involved in these results are not fully known, several possibilities can be considered, such as: changes in local blood flow by inhibiting constitutive NO production in arterial endothelial cells, the involvement of NO in endogenously released LT-induced plasma leakage at postcapillary venular endothelial cells, and the interaction of NO with other oxygen radicals to worsen the allergic inflammation. If this is the case in asthmatic airways, NO has a role as a proinflammatory substance, as well as a protective role. Selective inhibition of the inflammatory nature of NO may resolve the dilemma.

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