

Induction of nitric oxide synthase by lipopolysaccharide inhalation enhances substance P-induced microvascular leakage in guinea-pigs

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ABSTRACT: Inducible nitric oxide (NO) synthase (iNOS)-mediated hyperproduction of NO in airways has been reported in asthmatic patients. However, the role of NO in the pathogenesis of asthma has not yet been fully elucidated. The aim of this study was to examine whether the iNOS-derived NO affects airway microvascular leakage, one of the characteristic features of asthmatic airway inflammation.

Guinea-pigs were exposed to lipopolysaccharide (LPS) (1 mg·mL⁻¹) by inhalation in order to induce iNOS in the airways, and the histochemical staining of reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase activity was determined 5 h after the inhalation to confirm the iNOS induction. Airway microvascular leakage to subthreshold doses of substance P (0.3 µg·kg⁻¹, *i.v.*) was also examined in the absence and presence of an iNOS inhibitor (aminoguanidine) in LPS- or saline-exposed (control) animals using Evans blue dye and Monastral blue dye.

In the LPS-exposed animals, increased NADPH-diaphorase activity was observed in the airway microvasculature compared with the control animals. Substance P caused significant airway microvascular leakage assessed by Evans blue dye in all airway levels in the LPS-exposed animals but not in the control group. This was also confirmed by Monastral blue dye extravasation. Aminoguanidine abolished this LPS-induced enhancement of plasma leakage to substance P without changing the systemic blood pressure.

These results may suggest that inducible nitric oxide synthase-derived nitric oxide is capable of potentiating neurogenic plasma leakage in airways.

Eur Respir J 1998; 12: 831–836.

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Keywords: Evans blue
microvascular leakage
Monastral blue
nitric oxide

Received: February 19 1997
Accepted after revision May 10 1998

In asthmatic airways, excessive production of nitric oxide (NO) gas has been demonstrated by analysis of the expired gas [1, 2]. Since steroid inhalation suppresses this hyperproduction of NO in asthmatic airways [2], inducible nitric oxide synthase (iNOS) appears to be responsible for the production of excessive NO. The expression of iNOS, regulated at the level of transcription [3], is closely related to a variety of inflammatory processes, since inflammatory cytokines such as interferon- γ , interleukin (IL)-1 β and tumour necrosis factor (TNF)- α induce the expression of messenger ribonucleic acid (mRNA) for iNOS [3]. In human airways, immunostaining for iNOS has revealed the presence of this enzyme in airway epithelium and vascular endothelium as well as in macrophages after inflammatory stimulation using lipopolysaccharide (LPS) [4]. In addition, it has been reported that tracheal epithelium taken from asthmatic patients expresses iNOS activity more abundantly than that from normal individuals [5]. Therefore, it is likely that the inflammatory nature of asthmatic airways [6] is related to the expression of iNOS in this disease. However, the role of excessively produced NO in asthmatic airways has not been well elucidated. Since NO has bronchodilatory activity [7], it is possible

that it plays a role in counterbalancing the excessive bronchoconstriction in asthmatic airways. In contrast, NO could promote airway inflammation through the formation of a potent oxygen radical, peroxynitrite, *via* interaction with superoxide [8]. Recently, it has been reported that peroxynitrite can contribute to airway hyperreactivity [9]. In this case, NO may exacerbate asthmatic airway inflammation.

Airway microvascular leakage is one of the common features observed in asthmatic airway inflammation [6]. NO has also been reported to have various and sometimes opposite effects on the airway microvascular leakage. Inhibition of NO synthesis has been reported to increase basal airway microvascular permeability [10]. In contrast, sensory neuropeptide-mediated airway plasma leakage has been demonstrated to involve NO production in guinea-pig airways [11]. It has also been reported that antigen-induced airway plasma leakage is potently inhibited by a NOS inhibitor [12]. These controversial reports give rise to the speculation that NO acts protectively against airway microvascular leakage in basal conditions, but not after inflammatory stimulation. However, the way in which airway microvascular leakage is affected by the induction of iNOS has never been reported. The present study, aimed

to elucidate whether iNOS-derived NO affects substance P-induced airway microvascular leakage using an iNOS inhibitor, aminoguanidine [13]. Substance P was chosen as an inducer of microvascular leakage because the effect of substance P in airways mimics asthmatic airway inflammation by itself [14] and has been reported to be elevated in the induced sputum of asthmatics [15]. For this purpose, LPS was used to induce iNOS in guinea-pig airways and airway microvascular leakage was assessed by Evans blue dye and Monastral blue dye.

Methods

Animal preparation

Male Dunkin-Hartley guinea-pigs (Funabashi Farm, Sendai, Japan, total $n=66$) weighing 300–350 g were used. The animals were divided into two groups and exposed to saline aerosol (control group) or LPS aerosol (1 mg·mL⁻¹, LPS group) delivered by an ultrasonic nebulizer (Omron, NE-U12, Tokyo, Japan; output 0.8 mL·min⁻¹) for 3 min in a plexiglas exposure chamber (24.5 × 40.5 × 15.0 cm) under spontaneous breathing. Five hours after the inhalation, reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase staining was performed. Further, quantification of airway microvascular permeability to substance P was studied in each group as follows. Guinea-pigs were anaesthetized with urethane (2 g·kg⁻¹, *i.p.*) and placed on a thermostat operation table (Model SN-662; Shinano Seisakusho, Tokyo, Japan) which kept the animal's body temperature at 37°C. The trachea was cannulated and the lungs were ventilated artificially with a small animal constant-volume ventilator (Model SN-480-7; Shinano Seisakusho) at a frequency of 60 strokes·min⁻¹ and a tidal volume of 1 mL·100 g body weight⁻¹. A polyethylene catheter was cannulated into the carotid artery to monitor systemic blood pressure. The jugular veins were prepared for direct injection of intravenous drugs. All studies were performed in the presence of atropine (1 mg·kg⁻¹, *i.v.*) and propranolol (1 mg·kg⁻¹, *i.v.*) to avoid muscarinic and β -adrenergic neural modifications, respectively. All of the experiments performed in this study were conducted with the consent of the Ethics Committee for the Use of Experimental Animals of the Tohoku University School of Medicine.

NADPH-diaphorase histochemical staining study

Animals were divided into two groups: group 1, saline inhalation, saline (1 mL·kg⁻¹, *i.p.*) pretreated and saline (1 mL·kg⁻¹, *i.v.*) ($n=5$); group 2, LPS inhalation, saline pretreated and saline ($n=5$). The thorax was opened and the systemic circulation was perfused with 50 mL of ice-cold saline, following 350 mL of 2% paraformaldehyde in phosphate buffer (pH 7.4, 0.1 M) solution. After removal of the trachea and the lungs, the trachea was immersed in the same fixative solution for 12 h at 4°C, and further immersed for 24 h at 4°C in 0.1 M phosphate buffer containing 10% sucrose. The tracheal tissues were then sectioned at a thickness of 12 μ m with a cryostat, cutting along the longitudinal axis of the airways. All cryosections were

mounted on chrom-alum, gelatin-coated glass slides and prepared for the later processing for histological examination by haematoxylin-eosin stain and histochemical examination for NADPH-diaphorase activity.

NADPH-diaphorase histochemical staining was performed by incubating slide-mounted tissue sections with 1 mM β -NADPH/0.2 mM nitroblue tetrazolium/0.1 M Tris-HCl, pH 8.0/0.2% Triton X-100 for 30–60 min at 37°C [16]. The reaction was stopped by rinsing the sections in phosphate-buffered saline (pH 7.2, 0.01 M). The tissue sections were photographed with Fuji Neopan F-film (Iso 32, Fuji Photo Film Co., Tokyo, Japan).

Airway permeability study

Evans blue dye study. Evans blue dye was filtered using 0.22 μ m Millipore filters (MILLEX-GV; Millipore Products Division, Bedford, MA, USA) before use. Animals were divided into six groups: group 1, saline inhalation, saline (1 mL·kg⁻¹, *i.p.*) pretreated and saline (1 mL·kg⁻¹, *i.v.*) ($n=5$); group 2, LPS inhalation, saline pretreated and saline ($n=5$); group 3, LPS inhalation, aminoguanidine (100 μ mol·kg⁻¹, *i.p.*) pretreated and saline ($n=5$); group 4, saline inhalation, saline pretreated and substance P (0.3 μ g·kg⁻¹, *i.v.*) ($n=5$); group 5, LPS inhalation, saline pretreated and substance P ($n=6$); and group 6, LPS inhalation, aminoguanidine pretreated and substance P ($n=5$). Five minutes after the pretreatment with saline (vehicle for aminoguanidine) or aminoguanidine, Evans blue dye (30 mg·kg⁻¹, *i.v.*) was administered. One minute after the dye injection, saline (vehicle for substance P) or substance P was administered.

The details of the quantification of extravasated Evans blue dye have previously been described [12, 17]. In brief, 5 min after the induction of leakage by substance P, the thorax was opened and the systemic circulation was perfused with saline at 100 mmHg using a needle passed through a left ventriculotomy into the aorta to remove intravascular dye. The removed lungs with trachea were divided into three components: lower part of the trachea, main bronchi and intrapulmonary airways. The tissue dye content extracted in formamide at 37°C for 16 h was quantified from light absorbance at 620 nm using a Labsystem multiskan bichromatic (Labsystem, Helsinki, Finland).

Monastral blue dye study. In another set of studies, baseline and substance P-induced endothelial gap formation was evaluated using Monastral blue dye, which is known to be extravasated from leaky vessels and trapped at the endothelial gaps of these vessels [18]. Thus, Monastral blue dye (particle size 50–300 nm), sonicated in an ultrasonic cleaner for 5 min and filtrated by a 5 μ m Millipore filter, was used as a marker of endothelial gap formation. Animals were divided into five groups: group 1, saline inhalation, saline (1 mL·kg⁻¹, *i.p.*) pretreated and saline (1 mL·kg⁻¹, *i.v.*) ($n=5$); group 2, LPS inhalation, saline pretreated and saline ($n=5$); group 3, saline inhalation, saline pretreated and substance P (0.3 μ g·kg⁻¹, *i.v.*) ($n=5$); group 4, LPS inhalation, saline pretreated and substance P ($n=5$); and group 5, LPS inhalation, aminoguanidine (100 μ mol·kg⁻¹, *i.p.*) pretreated and substance P ($n=5$). Administration of Monastral blue dye (30 mg·kg⁻¹, *i.v.*) was performed 1 min before the saline or substance P (0.3 μ g·kg⁻¹) injection.

One minute after the saline or substance P injection, the animals were perfused with 1% paraformaldehyde in phosphate-buffered saline, as in the Evans blue dye study. In order to quantify the extravasated Monastral blue dye, tracheal whole mounts were prepared. The tissues were immersed in 1% paraformaldehyde for 2 h at 4°C and washed overnight in distilled water. Then, the trachea was soaked in glycerol for 20 h at room temperature, followed by dehydration in 100% ethanol. The rehydrated trachea was flattened between two glass slides held tightly by clips for 24 h in 100% ethanol, cleared in toluene for 15 min and mounted on glass slides. Five images of intercartilaginous regions from each tracheal whole-mount preparation were viewed with image-analysing software (MacScope; Mitani Co., Fukui, Japan) using an Apple Macintosh computer connected to the microscope. The dye extravasation was quantified as area densities, *i.e.* the percentages of tracheal mucosa occupied by Monastral blue-labelled blood vessels.

Drugs

The following drugs were used: LPS, Monastral blue dye and aminoguanidine (Sigma Chemical Co., St Louis, MO, USA), substance P (Peptide Institute, Osaka, Japan), Evans blue dye (Aldrich Chemical Co., Milwaukee, WI, USA), atropine sulphate (Tanabe Pharmaceutical, Osaka, Japan), propranolol hydroxide, nitroblue tetrazolium, paraformaldehyde and Triton-X (Wako Pure Chemical Industries, Osaka, Japan), β -NADPH (Oriental Yeast Co., Tokyo, Japan) and saline (Ohtsuka Chemical Co., Tokyo, Japan).

Statistical analysis

Data are expressed as means \pm SEM. Multiple comparisons of mean data of dye extravasation among the groups were performed by one-way analysis of variance followed by Scheffé's test as a *post hoc* test. Probability values <0.05 were considered significant.

Results

NADPH-diaphorase staining

In the tracheal sections taken from both control and LPS-exposed animals, NADPH-diaphorase activity was observed to the same degree in basal cells of the airway epithelium, the endothelium of intra-airway large vessels and nerve fibres surrounding airway smooth muscle bundles. In contrast, NADPH-diaphorase staining in submucosal microvasculature was enhanced in LPS-exposed compared with control guinea-pigs (fig. 1). Haematoxylin-eosin staining of the tracheal tissues demonstrated massive infiltration of polynuclear and mononuclear cells in the LPS-exposed group but not in the control group. NADPH-diaphorase staining was also observed in the relatively large infiltrated cells.

Effect of LPS inhalation on substance P-induced airway Evans blue leakage

LPS inhalation by itself produced no significant extravasation of Evans blue dye compared with saline inhala-

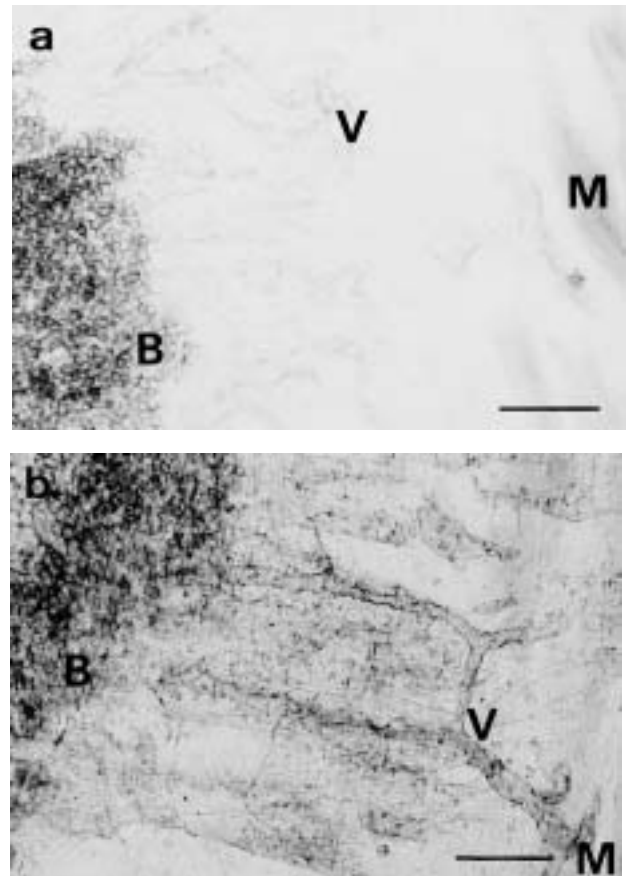


Fig. 1. – Representative histochemical demonstration of reduced nicotinamide-adenine dinucleotide phosphate (NADPH)-diaphorase activity in the submucosal layer of guinea-pig trachea. Strong staining of NADPH-diaphorase activity is observed only in the basal cells of airway epithelium in the saline-treated animal (a), whereas the submucosal microvasculature is also strongly stained in the lipopolysaccharide-treated animal (b). B: basal cells of airway epithelium; V: submucosal microvasculature; M: smooth muscle. (Internal scale bars = 80 μ m.)

tion in any airway portion (fig. 2). A low dose of substance P (0.3 μ g·kg⁻¹, *i.v.*) did not cause significant Evans blue dye leakage in control animals, but it caused significant leakage at all airway levels in the LPS inhalation group (fig. 2). Aminoguanidine pretreatment significantly inhibited the substance P-induced leakage observed in the LPS inhalation group (fig. 2).

Effect of LPS inhalation on substance P-induced endothelial gap formation

LPS inhalation itself did not affect the airway microvascular gap formation assessed by Monastral blue dye (figs. 3 and 4).

Substance P (0.3 μ g·kg⁻¹, *i.v.*) caused significant endothelial gap formation in both saline and LPS inhalation animals (figs 4 and 5). The degree of Monastral blue staining was significantly enhanced in the LPS exposure group compared with that in control animals (figs. 4 and 5). This enhancement was completely reversed by aminoguanidine pretreatment (figs. 4 and 5).

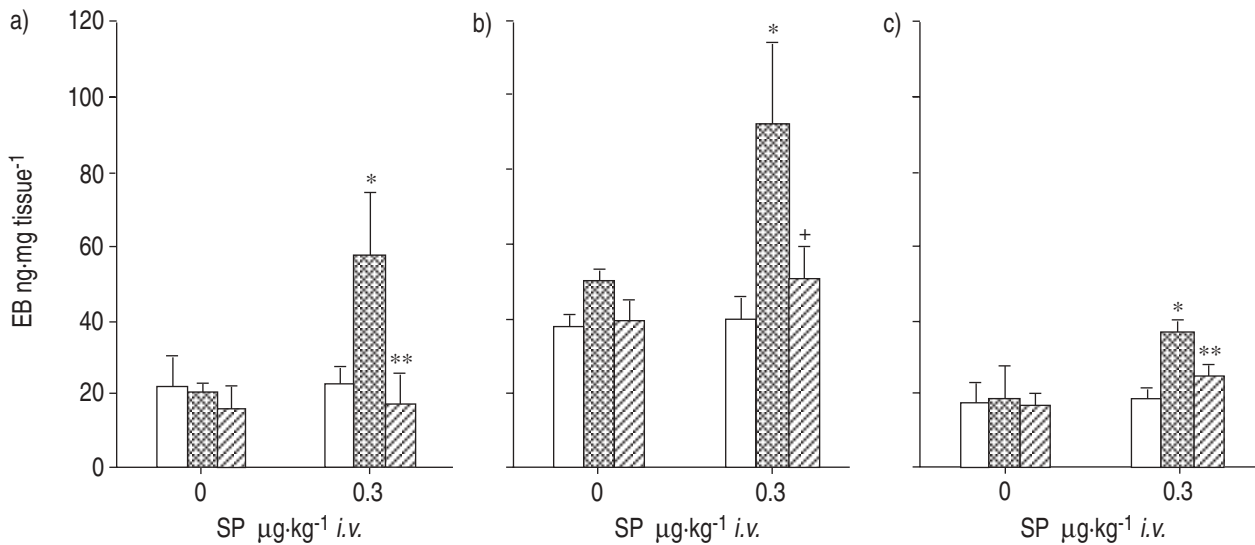


Fig. 2. – Histograms illustrating the effect of lipopolysaccharide (LPS) inhalation on Evans blue (EB) dye leakage in a) trachea, b) main bronchi, and c) intrapulmonary airways. □ : saline inhalation + saline pretreatment; ▨ : LPS inhalation + saline pretreatment; ▩ : LPS inhalation + aminoguanidine pretreatment. All values are mean \pm SEM of five to seven animals. *: $p < 0.05$ compared with the values of LPS inhalation, saline (1 mL.kg⁻¹, *i.p.*) pretreated and saline (1 mL.kg⁻¹, *i.v.*). †: $p < 0.05$; **: $p < 0.01$ compared with the values of LPS inhalation, saline pretreated and substance P (SP; 0.3 µg.kg⁻¹, *i.v.*).

Discussion

It was demonstrated in this study that LPS inhalation enhanced NADPH-diaphorase staining of airway microvasculature and infiltrated mononuclear cells, indicating that iNOS induction occurred at these sites. Further, the airway vascular permeability induced by a subthreshold dose of substance P was enhanced by LPS inhalation and this enhancement was suppressed by pretreatment with the iNOS inhibitor aminoguanidine. These results suggest the possibility that the induction of iNOS at the site of plasma leakage is capable of potentiating airway microvascular permeability.

LPS is a multicytokine inducer which has been found to induce iNOS activity in a variety of organs including airways [3, 19]. In the present study, LPS inhalation enhanced the staining of NADPH-diaphorase activity in the

mucosal microvasculature and induced positive staining in some of the infiltrated mononuclear cells. The staining of NADPH-diaphorase activity by chemical reaction has been used for the screening of NOS activity [20]. In human and rat airways, the localization of NADPH-diaphorase activity has been demonstrated to include all of the immunolocalization of NOS antigen [4]. Therefore, the difference in NADPH-diaphorase activity between the saline and the LPS-exposed animals suggests that NOS was induced by LPS inhalation at these sites. It has been demonstrated that cardiac microvascular endothelial cells express a cytokine-inducible form of NOS [21]. Therefore, the enhanced NADPH-diaphorase activity in the submucosal microvasculature seems to reflect the expression of iNOS at the submucosal microvascular endothelium. The site of airway microvascular leakage has been demonstrated to be the postcapillary venules [22]. In order to identify the site

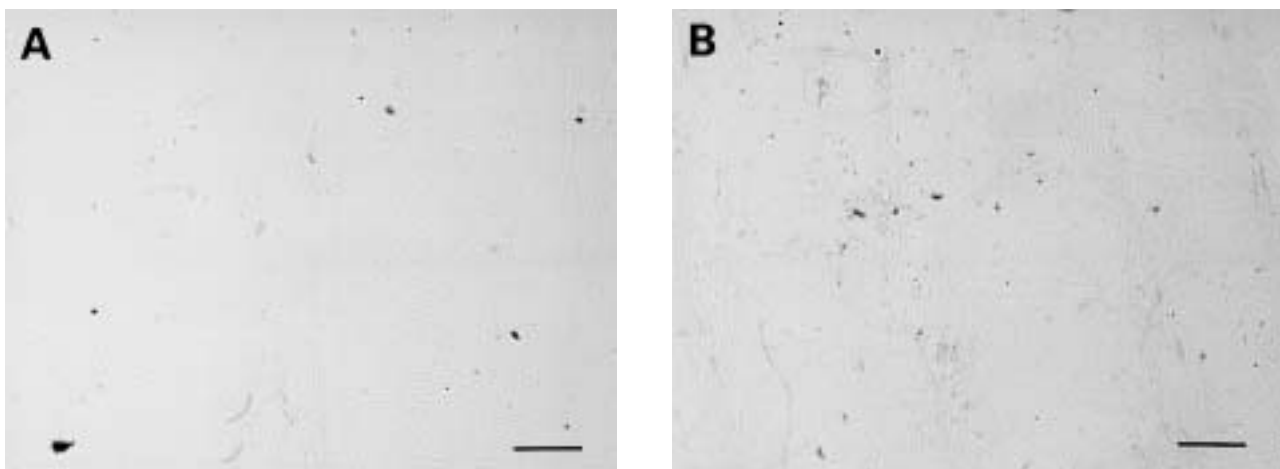


Fig. 3. – Light micrographs of tracheal whole preparations showing basal endothelial gap formation assessed by Monastral blue dye trapping. Only a few venules labelled by Monastral blue were seen in tracheal mucosa overlying the cartilaginous rings of the saline inhalation (A) and lipopolysaccharide inhalation groups (B). (Internal scale bar = 400 µm.)

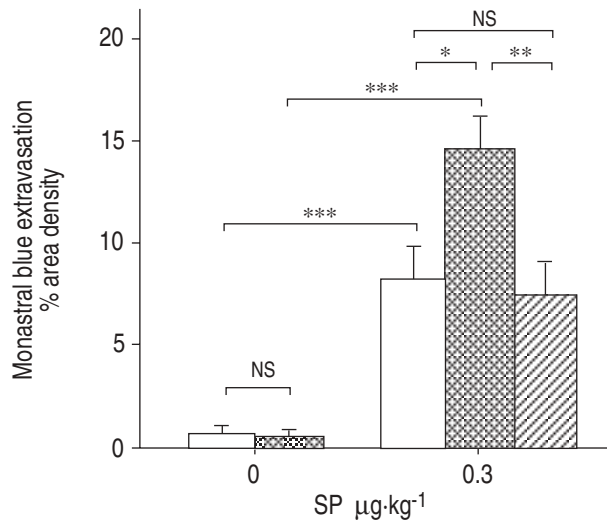


Fig. 4. – Quantification of airway endothelial gap formation evaluated by Monastral blue dye extravasation in guinea-pig trachea. □ : saline inhalation + saline pretreatment; ▨ : LPS inhalation + saline pretreatment; ▩ : LPS inhalation + aminoguanidine pretreatment. All values are mean ± SEM. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$. SP: substance P.

of plasma leakage in this model, Monastral blue dye was used to label the leaky sites. Monastral blue dye has been shown to extravasate only in abnormally permeable endothelium and then to be trapped at the basal lamina of the leaky vessels [23]. In LPS-exposed animals, the submucosal microvasculature, which possessed strong NADPH-diaphorase activity, was labelled by Monastral blue dye after injection of substance P (data not shown). This suggests that enhanced NOS activity occurred at the site of microvascular leakage. It has also been well established that macrophages express iNOS, as reported in rat airways [4]. Thus, the mononuclear cells possessing NADPH-diaphorase-positive staining observed in the present study could be macrophages, which infiltrated into airway tissues after LPS inhalation.

In the LPS-exposed animals, subthreshold doses of substance P provoked significant Monastral blue dye and Evans blue dye extravasation and this enhancement was suppressed by the pretreatment with aminoguanidine ($100 \mu\text{mol}\cdot\text{kg}^{-1}$). The selectivity of aminoguanidine as an iNOS inhibitor has been reported to be relatively weak [24]. However, the dose of aminoguanidine chosen here was reported to be selective for iNOS in an *in vivo* study [24] and did not affect systemic blood pressure by intravenous injection in the present study, indicating that this dose of aminoguanidine had no effect on iNOS in the arterial endothelium in the present model. Aminoguanidine also has the ability to inhibit another enzyme, histamine deaminase [12]. However, this action is unlikely to explain the inhibitory effect against the enhancement of plasma leakage by LPS, since the inhibition of histamine degradation appears to potentiate rather than suppress microvascular leakage.

The mechanisms by which the induction of iNOS potentiates substance P-induced plasma leakage are still uncertain. It has previously been reported that NOS inhibitor pretreatment reduces substance P-induced airway plasma leakage in guinea-pigs [17], suggesting that endogenous NO is involved in the process of plasma leakage, followed

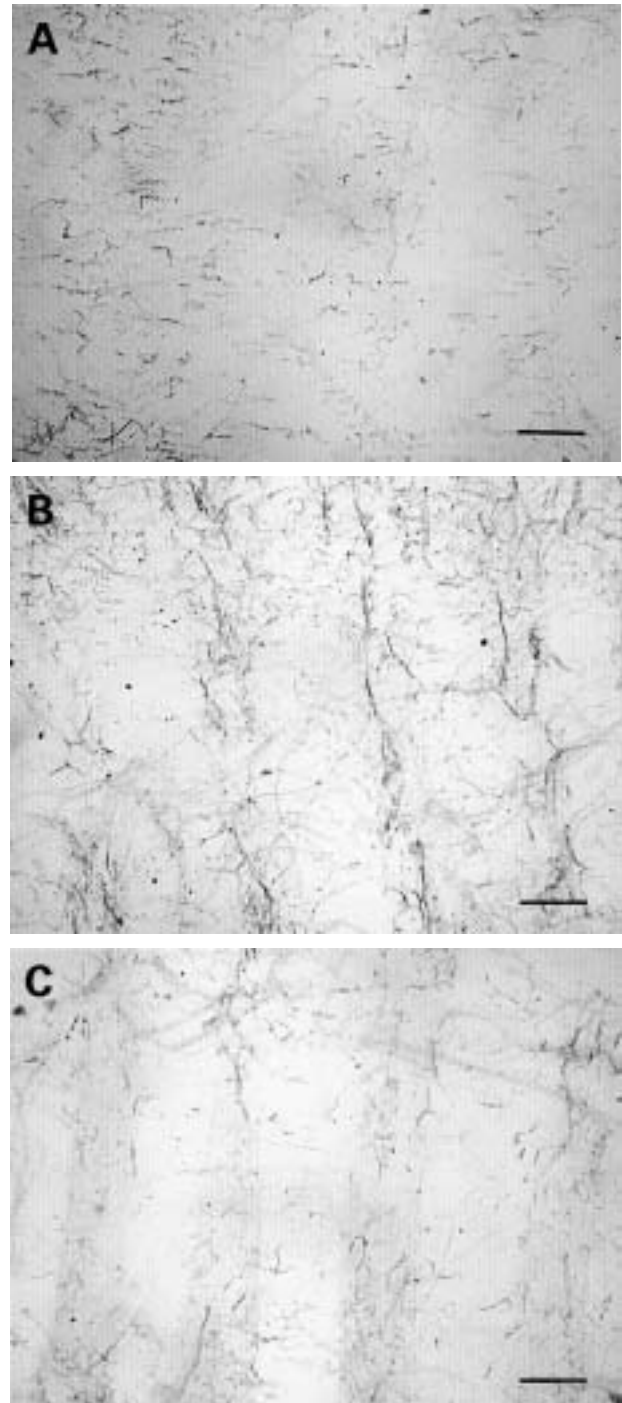


Fig. 5. – Monastral blue dye trapping between the endothelial cells in trachea: a) saline inhalation, saline pretreated and substance P (SP; $0.3 \mu\text{g}\cdot\text{kg}^{-1}$, *i.v.*); b) lipopolysaccharide (LPS) inhalation, saline pretreated and SP; and c) LPS inhalation, aminoguanidine pretreated and SP. (Internal scale bars = $400 \mu\text{m}$).

by neurokinin 1 (NK_1) receptor activation in this species. Thus, the hyperproduction of NO by iNOS at the sites of microvascular leakage may modify the signal transduction after NK_1 receptor stimulation. An alternative explanation is the involvement of a potent oxygen radical, peroxynitrite, in the LPS-induced potentiation of substance P-mediated plasma leakage. Besides NOS induction, LPS is

reported to prime neutrophils for increased superoxide anion production in lungs [25]. NO reacts very rapidly with superoxide anion by a diffusion-limited reaction, resulting in the formation of peroxynitrite [8, 26] which has a potent vascular injury effect [27]. Thus, it is possible that peroxynitrite-mediated vascular injury at the microvascular leaky site has a synergistic action with substance P and results in vascular hyperpermeability.

In summary, this study demonstrated that the induction of inducible nitric oxide synthase is capable of potentiating airway microvascular leakage to substance P, suggesting the possibility that the excessive production of nitric oxide worsens asthmatic airway inflammation. Although the role of excessively expired nitric oxide from asthmatic patients [1, 2] in the pathogenesis still needs to be elucidated, the pro-inflammatory action of nitric oxide through inducible nitric oxide synthase formation, such as the enhancement of airway microvascular leakage, should be taken into consideration.

Acknowledgement: The authors thank B. Bell for reading the manuscript.

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