British Journal of Pharmacology (1998) 125, 711–716
http://www.stockton-press.co.uk/bjp

Lipopolysaccharide enhances neurogenic plasma exudation in guinea-pig airways

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1 Lipopolysaccharide (LPS) is implicated in many pulmonary and airway inflammatory diseases. Tachykinins released from nerve endings increase vascular permeability. In this study, we have assessed the enhancement by LPS of tachykinin-mediated plasma exudation in guinea-pig airways, and examined the role of oxidants as well as leukocyte adherence.

2 LPS (100 μg kg^{-1}, i.v.) was administered 0–3 h before bilateral electrical stimulation of the cervical vagus nerves in animals anaesthetized with urethane and ventilated. Vagal stimulation increased vascular permeability in the airways. LPS enhanced the vagally-mediated plasma exudation with the peak effect at 1 h after LPS administration. LPS alone induced no significant plasma exudation. LPS also enhanced exogenous substance P (10^{-8} mol kg^{-1}, i.v.)-induced plasma exudation.

3 The NK-1 receptor antagonist L-732,138 abolished vagally-induced plasma exudation and significantly inhibited the enhancement by LPS. Pretreatment with superoxide dismutase (SOD, 5000 U kg^{-1}, i.p.) did not affect the vagally-induced plasma exudation, but inhibited the LPS-enhanced neurogenic plasma leakage. The LPS-enhanced vagally-induced plasma exudation was not completely inhibited by either L-732,138 or SOD pretreatment alone, but was blocked by the combination of both pretreatments.

4 Neutrophil depletion by cyclophosphamide alone did not influence vagally-induced plasma exudation, but significantly inhibited the LPS-enhanced response.

5 In conclusion, we have demonstrated LPS enhanced neurogenic plasma exudation by augmenting the response to tachykinins, partly through NK-1 receptors, to directly increase vascular permeability or to enhance leukocyte adhesion-mediated endothelial cell injury. Tachykinins released from nerve endings may contribute to endotoxin-related airway inflammatory responses.

Keywords: Tachykinin; lipopolysaccharide; oxidants; neutrophils; vascular permeability; respiratory system

Introduction

Lipopolysaccharide (LPS), a major constituent of the cell walls of gram-negative bacteria, can interact with inflammatory cells and endothelial cells. LPS is present in a wide variety of environments and is implicated in several inflammatory airway diseases. Inhalation of airborne LPS has been shown to cause bronchoconstriction in man (Jamison & Lowry, 1986) and bronchial hyperresponsiveness in sheep (Hutchinson *et al*., 1983). The administration of LPS into airways can result in an inflammatory response, including leukocyte infiltration, oedema and increased vascular permeability (Hudson *et al*., 1997; Pauwels *et al*., 1990; Esbenshade *et al*., 1982), thereby playing a role in the aggravation of asthma (Hutchinson *et al*., 1983) and in the pathogenesis of pulmonary oedema and lung injury *in vitro* when a large dose of LPS is administered (Harlar *et al*., 1983).

Tachykinins released from sensory C-fibre endings induce an inflammatory response known as neurogenic inflammation (Jancso *et al*., 1968), characterized by vasodilatation (Piedimonte *et al*., 1993), increased post-capillary venule permeability (Katayama *et al*., 1993) and increased neutrophil adherence to blood vessels (Katayama *et al*., 1993; Umeno *et al*., 1990). Several mediators released in endotoxaemia, such as bradykinin, histamine, and prostaglandins have been shown to enhance the chemosensitivity of sensory nerve endings (Kaufman *et al*., 1980; Lee & Morton, 1993; 1995). Therefore, it is possible that endotoxin may interact with tachykinins to potentiate neurogenic inflammation in the airways. The purpose of this study was to determine the effect of a low dose of LPS, administered intravenously, on neurogenic plasma exudation in anaesthetized guinea-pigs and explore the possible mechanisms underlying the exudative response.

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Methods

Animal preparation

Male Dunkin-Hartley guinea-pigs (350–500 g body weight) were obtained in pathogen-free containers and were maintained in closed-circulation cubicles. The animals were anaesthetized by an intraperitoneal injection of urethane (2 g kg^{-1}) and put in the supine position. The trachea and left external jugular artery were cannulated. The animals were ventilated with a small animal constant volume respiratory pump (Harvard Apparatus, Ltd., Edenbridge, U.K.) operating at 60 strokes min^{-1} of 1 ml of laboratory air per 100 g body weight. Drugs were injected *via* the jugular veins. The cervical vagus nerves were carefully dissected free bilaterally and sectioned at the level of the fourth to fifth tracheal cartilage rings. Their caudal ends were placed across bipolar platinum electrodes and were stimulated (10 Hz, 5 V, 5 ms for 3 min) by square-wave pulses (model SD9 stimulator; Grass Instruments, Quincy, MA, U.S.A.) (Kuo *et al*., 1992). The effect of dissection and nerve manipulation on pulmonary vascular leakage was assessed by repeating the process described above

without electrical stimulation (sham stimulation). The responses to vagus nerve stimulation were assessed by monitoring the overflow pressure with a differential pressure transducer (Farnell Electronic Components Ltd., Leeds, U.K.) and blood pressure *via* an indwelling cannula filled with heparin-saline (10 u ml^{-1}) in the left carotid artery.

Experimental procedures

Animals were treated with intravenous administration of either LPS (100 $\mu g\ kg^{-1}$) or the same volume of 0.9% saline (1 ml kg^{-1}) immediately, or 1, 2, or 3 h before vilateral vagal stimulation (5 V, 5 ms, 10 Hz for 3 min). Five min before nerve or sham stimulation, ^{125}I-human serum albumin (3 $\mu Ci\ kg^{-1}$) was given intravenously. In one set of animals, an NK-1 receptor antagonist (N-acetyl-L-Tryptophan, 3,5-bistrifluoromethyl benzyl ester, L-732,138) or its vehicle, 0.9% saline was given intravenously 15 min before nerve stimulation in the presence or absence of LPS pretreatment.

Three minutes after vagus nerve stimulation, the chest was opened and 10 ml blood was sampled *via* cardiac puncture. A blunt-end needle connected to a tube installed in a roller pump was inserted into the main pulmonary artery through a right ventriculotomy. The heart was clamped and the left atrium incised to allow the outflow of perfusate. Any ^{125}I-albumin remaining in the pulmonary vascular bed was flushed out by perfusion of the pulmonary vascular bed with warmed 0.9% saline. Perfusion was continued until the outflow was clear and the lungs became completely white. The remaining intravascular ^{125}I-albumin in the tracheobronchial vascular bed was also flushed out, as described previously (Kuo *et al*., 1992). The lower trachea and main bronchi were dissected, cleaned of surface blood, blotted dry of surface liquid, weighed, and dried in a vacuum freezer (at $-60^{\circ}C$ and 4mBar) to a constant weight.

Assessment of microvascular permeability

Plasma albumin leakage was assessed using an ^{125}I-albumin leakage index as described previously (Kuo *et al*., 1992). Radioactivity was measured in dry tracheobronchi with their corresponding plasma (500 μl), in a gamma counter (Packard Instruments, U.K.). The albumin leakage index was calculated from the tissue radioactivity (c.p.m. g^{-1}) divided by the plasma radioactivity (c.p.m. μl^{-1}) and expressed as tissue plasma content (ml g^{-1} dry tissue).

Pretreatment with superoxide dismutase (SOD)

SOD (300,000 u of specific activity: 4166 u mg^{-1} protein) was dissolved in 0.9% saline (5000 u ml^{-1}) and stored at $-20^{\circ}C$. In one group of animals, the animals were pretreated with SOD (5000 u kg^{-1}) 5 days and 1 day before experiment. On the day of experimentation, another dose of SOD was given 30 min before LPS administration. The control animals were pretreated as above with heat-inactivated SOD.

Neutrophil depletion

To evaluate possible involvement of neutrophils in the enhancement effect of LPS on neurogenic plasma exudation, the animals were depleted of circulating neutrophils by a method described previously (Kuo *et al*., 1997). Cyclophosphamide (50 mg kg^{-1}, i.p.) was injected 5 days and 1 day before the experiment. On the day before LPS pretreatment, another cyclphosphamide injection was given. Pretreatment with cyclophosphamide caused a $91.9 \pm 2.5\%$ ($n=5$) decrease in leukocytes in the circulation.

Specificity of an NK-1 receptor antagonist L-732,138

L-732,138 is a specific competitive tachykinin NK-1 receptor antagonist (Cascieri *et al*., 1994). To determine the specificity of L-732,138 at a dose relevant to this study, L-732,138 (10^{-2} mol kg^{-1} i.v., $n=4$) was administered 15 min before histamine administration (300 $\mu g\ kg^{-1}$, i.v.). Animals were prepared as above and microvascular permeability was assessed 30 min after histamine administration. The inhibitory effect of L-732,138 on acute exudative responses induced by histamine were compared with animals receiving histamine alone with 0.9% saline pretreatment ($n=4$).

Reagents

LPS from *Escherichia coli* (serotype 026:B6); cyclophosphamide, SOD, NK-1 receptor antagonist, L-732,138 (N-acetyl-L-Tryptophan 3,5-bistrifluoromethyl benzyle ester), histamine, SP and urethane were purchased from Sigma (St. Louis, MO, U.S.A.). ^{125}I-human serum albumin was obtained from Amersham Life Science Inc. (Illinois, U.S.A.). All drugs were prepared freshly on the day of experimentation.

Studies

Standard formulae were used for the analysis. Data did not approximate a Gaussian distribution, whereby the mean value did not approximate the median value. Nonparametric statistical analyses was therefore employed, and the probability of differences between groups was initially assessed by Kruskal-Wallace analysis. The number of animals in a group was too small to allow for a strict median test between groups, and subsequent analysis was performed using the Mann-Whitney U test (two-tailed) to assess the significance of differences between groups. To minimize the possibility of obtaining chance significance as a result of multiple comparisons, preplanned comparisons between specific groups were made and the significant values confirmed using Newman-Keuls analysis. Data in Results are means $\pm$ s.e.mean. The null hypothesis was rejected at $P<0.05$.

Results

Effect of LPS on vagally-induced plasma exudation

Vagal stimulation caused a significant increase in plasma leakage (0.33 ± 0.05 ml g^{-1} tissue, $n=10$, $P<0.01$) in tracheobronchial tissues compared with sham stimulation (0.05 ± 0.02 ml g^{-1} tissue, $n=8$) (Figure 1). Intravenous administration of low dose LPS (100 $\mu g\ kg^{-1}$) alone did not induce any significant change in plasma leakage in the tracheobronchial tissues (0.04 ± 0.02 ml g^{-1} tissue, $n=4$) (Figure 1), but markedly enhanced the vagally-induced plasma leakage (0.68 ± 0.11 ml g^{-1} tissue, $n=6$, $P<0.02$) compared with the vagal-stimulation alone group (Figure 1). The enhancement effect of LPS pretreatment waned with time and was significant only at 1 h before nerve stimulation (Figure 1).

Inhibitory effect of the NK-1 receptor antagonist

Pretreatment with an NK-1 receptor antagonist (L-732,138, 10^{-3} mol kg^{-1}, i.v., $n=5$), abolished the vagally-induced

plasma leakage in tracheobronchial tissues (0.08 ± 0.02 ml g^{-1} tissue) (Figure 2), and dose-dependently inhibited the LPS-enhanced vagally-induced plasma exudation in traceobronchial tissues (0.42 ± 0.03 ml g^{-1} tissue at 10^{-4} mol kg^{-1}, $n=4$; 0.26 ± 0.03 ml g^{-1} tissue at 10^{-3} mol kg^{-1}, $n=5$; and 0.27 ± 0.05 ml g^{-1} tissue at 10^{-2} mol kg^{-1}, $n=5$) (Figure 2).

Specificity of L-732,138

Histamine administration (300 μg kg^{-1}, i.v.) induced a significant increase in plasma exudation (0.48 ± 0.10 ml g^{-1} tissue, $n=4$, $P<0.05$) in tracheobronchial tissues compared with vehicle (0.9% saline) stimulation (0.04 ± 0.02 ml g^{-1} tissue, $n=5$). Pretreatment with L-732,138 (10^{-2} mol kg^{-1} i.v., $n=4$) failed to modify the response to histamine (0.43 ± 0.01 ml g^{-1} tissue, $n=4$).

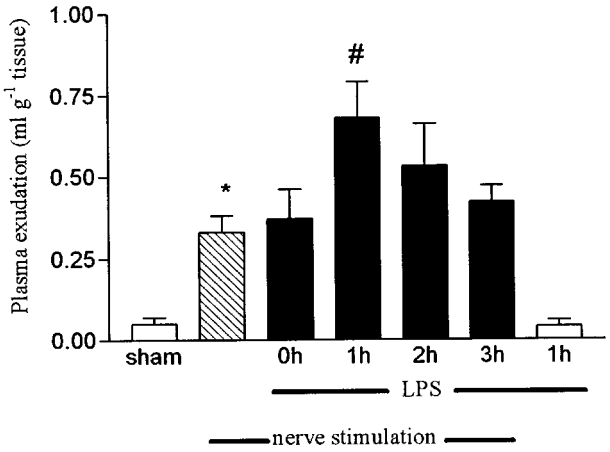

Figure 1 Time course for the effect of lipopolysaccharide (LPS) on vagally-induced plasma exudation in guinea-pig airways. LPS was administered 0, 1, 2 and 3 h before nerve stimulation. Data are mean ± s.e.mean. $*P<0.01$ compared with sham stimulation (sham). $\#P<0.02$ compared with nerve stimulation alone.

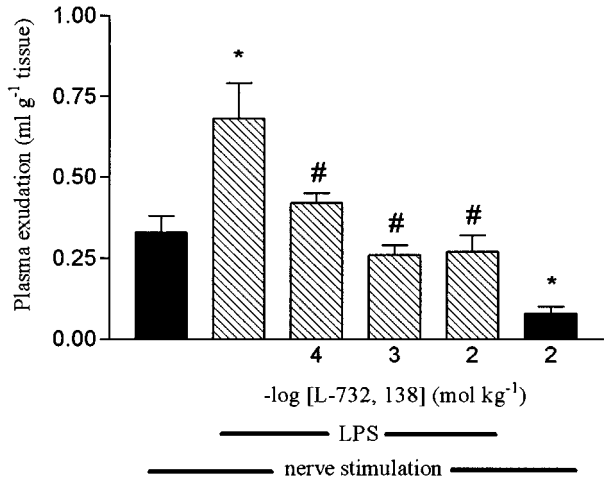

Figure 2 Dose-dependent inhibitory effect of an NK-1 receptor antagonist, L-732,138, (10^{-4}, 10^{-3}, and 10^{-2} mol kg^{-1}) on nerve stimulation-induced or lipopolysaccharide (LPS)-enhanced neurogenic plasma exudation. Data are mean ± s.e.mean. $*P<0.01$ compared with nerve stimulation alone; $\#P<0.05$ compared with LPS-enhanced neurogenic plasma exudation alone.

Effect of LPS on substance P-induced plasma exudation

Administration of substance P (10^{-8} mol kg^{-1}, i.v.) induced a similar magnitude of plasma leakage in tracheobronchial tissues (0.34 ± 0.04 ml g^{-1} tissue, $n=6$) as those induced by electrical stimulation of the vagus nerves (Figure 3). LPS pretreatment significantly enhanced SP-induced vascular leakage of plasma in tracheobronchial tissues (0.51 ± 0.04 ml g^{-1} tissue, $n=5$, $P<0.02$) (Figure 3).

SOD pretreatment

Pretreatment with SOD did not significantly inhibit the vagally induced tracheobronchial vascular leakage of plasma (0.28 ± 0.09 ml g^{-1} tissue, $n=5$) (Figure 4), but markedly inhibited the LPS-enhancement of vagally-induced plasma leakage (0.39 ± 0.08 ml g^{-1} tissue, $n=5$, $P<0.04$) when compared with the control group which received pretreatment with heat-inactivated SOD (0.62 ± 0.09 ml g^{-1} tissue, $n=5$) (Figure 4). Combination of SOD with L-732,138 (10^{-2} mol kg^{-1}, i.v.) pretreatment abolished the LPS-enhanced plasma exudation (0.09 ± 0.02 ml g^{-1} tissue, $n=5$) (Figure 4).

Depletion of leukocytes by cyclophosphamide

Cyclophosphamide pretreatment did not significantly affect vagally-induced plasma leakage (0.41 ± 0.11 ml g^{-1} tissue, $n=4$), but significantly inhibited the LPS-enhanced response (0.33 ± 0.04 ml g^{-1} tissue, $n=6$, $P<0.02$) (Figure 5).

Discussion

Our results demonstrate that low dose LPS alone did not induce plasma exudation, but significantly enhanced vagally-induced vascular leakage in tracheobronchial tissues. The enhancement by LPS was significantly inhibited by an NK-1 receptor antagonist, suggesting the enhanced vascular permeability was mediated *via* NK-1 receptors by an increased response to tachykinins.

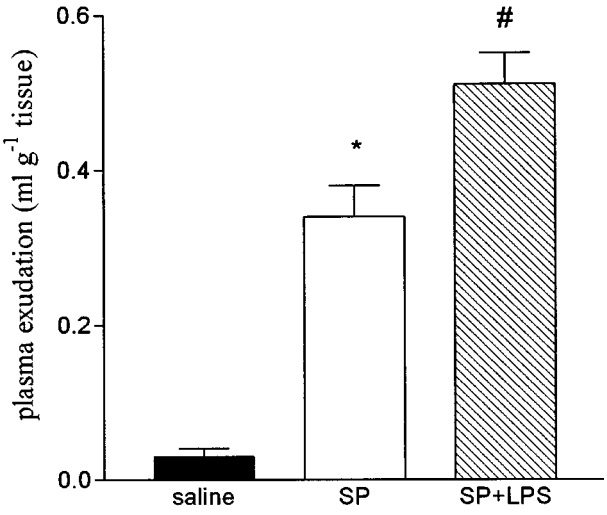

Figure 3 Effect of lipopolysaccharide (LPS) on exogenous administration of substance P (SP)-induced plasma exudation. Data are mean ± s.e.mean. $*P<0.01$ compared with normal saline control (saline); $\#P<0.05$ compared with SP alone.

LPS induces a release of several inflammatory mediators. Some of these, such as histamine, bradykinin, and prostaglandins, have been shown to enhance chemosensitivity of bronchopulmonary C-fibers (Kaufman *et al*., 1980; Lee & Morton, 1993; 1995). In addition, LPS is able to directly stimulate tachykinins release from spinal cord (Bret-Dibat *et al*., 1994) and sympathetic ganglia (Shadiack *et al*., 1994). The increased release of tachykinins from sensory C-fibers induced by LPS has been demonstrated to induce airway hyperresponsiveness in guinea-pigs (Jarreau *et al*., 1994; Loeffler *et al*., 1997). LPS induces maximal release of tachykinins 1 h after stimulation (Bret-Dibat *et al*., 1994), compatible with the maximal enhancement by LPS shown in this study. Thus, it is possible that the mechanism involved by LPS-enhanced vagally-induced plasma leakage is mediated *via* sensitizing or stimulatory effects on the C-fibre sensory nerve endings to release an increased amount of tachykinins. However, our results also showed that LPS enhanced SP-induced plasma exudation, indicating the enhancement by LPS also may be *via* a post-synaptic response. LPS has been reported to upregulate the expression of mRNA encoding for SP receptors in rat macrophages and neuronal cells (Bost *et al*., 1992). However, LPS does not increase the number or affinity of SP receptors, although LPS enhances SP-induced tumor necrosis-α secretion from neuroglial cells (Luber-Narod *et al*., 1994). Thus, it is not known whether the LPS-enhanced SP-mediated plasma leakage shown herein is *via* an upregulation of SP receptors.

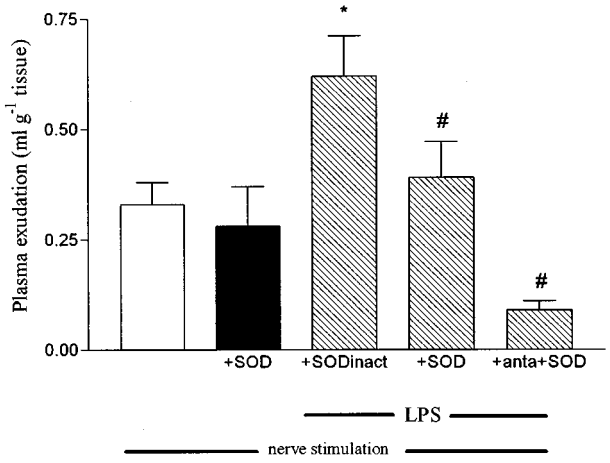

Figure 4 Effect of superoxide dismutase (SOD) and an NK-1 receptor antagonist, L-732,138, (anta) on neurogenic plasma exudation in the absence or presence of lipopolysaccharide (LPS) pretreatment. Data are mean$\pm$s.e.mean. *$P<0.01$ compared with nerve stimulation alone. #$P<0.05$ compared with animals pretreated with inactivated SOD (SODinact).

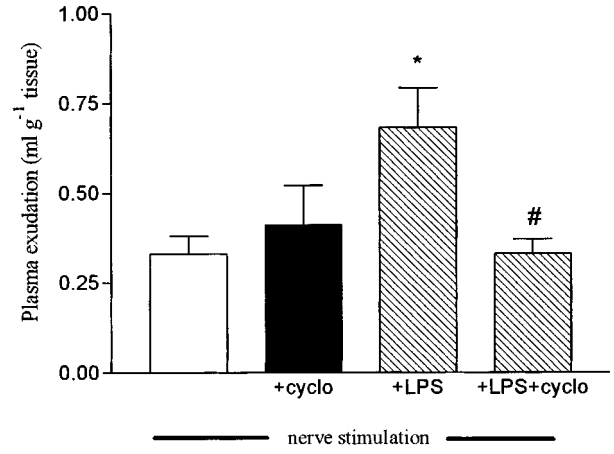

Figure 5 The inhibitory effect of cyclophosphamide pretreatment (cyclo) on neurogenic plasma exudation with or without lipopolysaccharide (LPS) pretreatment. Data are mean$\pm$s.e.mean. *$P<0.01$ compared with nerve stimulation alone; #$P<0.05$ compared with LPS-enhanced neurogenic plasma exudation alone.

NK-1 receptors mediate leukocyte adhesion to airway endothelial cells by upregulation of P-selectin (Baluk *et al*., 1995). Substance P also induces the expression of intercellular adhesion molecule-1 (ICAM-1) on vascular endothelial cells (Nakagawa *et al*., 1995). Neutrophil adherence to vascular endothelium is associated with increased vascular permeability in airway neurogenic inflammation (Umeno *et al*., 1990; Baluk *et al*., 1995), although the number of neutrophils do not correspond to the magnitude of vascular permeability (Baluk *et al*., 1995). Nevertheless, the sequestrated neutrophils under the stimulation with trace amount of LPS may induce neutrophil-mediated vascular permeability (Smedly *et al*., 1986; Worthen *et al*., 1987; Uchiba *et al*., 1995). LPS induces pulmonary accumulation of neutrophil reaching maximum levels 30 min to 2 h after administration, with decay thereafter (Uchiba *et al*., 1995). The latter time course for LPS-induced neutrophil sequestration is compatible with that for LPS-enhanced neurogenic plasma exudation in our results. Furthermore, in the present study, depletion of circulating neutrophils by cyclophosphamide greatly attenuated the LPS-enhanced neurogenic plasma exudation, suggesting neutrophils were implicated in this response. Thus, LPS may augment NK-1 receptor-mediated neutrophil adhesion to endothelium to enhance neutrophil-mediated vascular permeability.

LPS increases the release of reactive oxygen intermediates which are capable of injuring pulmonary endothelial cells (Shiki *et al*., 1987; Brigham & Meyrick, 1986) and involved in the development of tachykinin-mediated neurogenic plasma exudation (Lei *et al*., 1996) as well as bronchoconstriction in the airways (Lai *et al*., 1993). Our results demonstrated that pretreatment with SOD failed to inhibit the vagally-induced microvascular permeability, but significantly inhibited the LPS enhanced response, indicating that the generation of oxygen radicals, especially superoxide anion, were implicated in the LPS-enhanced neurogenic plasma exudation in the airways. The LPS-enhanced neurogenic plasma exudation was not completely inhibited by either SOD or L-732,138 pretreatment alone, but was abolished by both pretreatments in combination, indicating that superoxide anions contribute to the responses other than augmenting the NK-1 receptor-mediated plasma exudation. Superoxide anions inactivate airway neutral endopeptidase (Dusser *et al*., 1989) which is a peptidase involved in the degradation of several proinflammatory peptides, such as tachykinins and kinins. An inhibition of neutral endopeptidase in the airways enhances SP or other tachykinin-mediated airway responses, including plasma exudation (Borson *et al*., 1989). Thus, LPS may increase the production of superoxide anions to inactivate neutral endopeptidase and then increase SP and other tachykinins activities through NK-1 receptors to induce plasma exudation in guinea-pig airways.

Previous studies have failed to detect obvious oedema in the airways mucosa, even in the face of marked plasma exudation responses (Persson, 1991). The lack of oedema is attributable to a swift luminal entry of extravasated plasma (Erjefalt & Persson, 1991). Thus, although we did not measure extravasated plasma that entered the airway lumen, the increased

vascular permeability to plasma albumin demonstrated in the present study is not necessarily accompanied by oedema in trachobronchial tissues.

There is always a problem with extrapolating findings in animal airways to the human condition, considering the different neural control in airways among species. In comparison with rodent airways, SP-immunoreactive nerves are sparse in human airways (Bowden & Gibbins, 1992). However, SP concentrations are increased in pulmonary oedema fluid in patients with adult respiratory distress syndrome (Espiritu *et al*., 1992) and in the bronchoalveolar lavage of normal subjects exposed to ozone (Hazbun *et al*., 1993), indicating that SP is involved in the pathophysiology of human inflammatory pulmonary diseases. Whether SP and NK-1 receptors also mediate human endotoxin-induced respiratory dysfunction deserves future study.

In conclusion, we have demonstrated that LPS enhanced neurogenic plasma exudation by augmenting the responses to tachykinins partly through NK-1 receptors to directly increase vascular permeability or to enhance leukocyte adhesion-mediated endothelial cell injury. Tachykinins released from nerve endings may contribute to endotoxin-related airway inflammatory responses.

This project was supported by NSC87-2314-B182-041.

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(Received April 15, 1998
Revised July 22, 1998
Accepted July 24, 1998)