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Cigarette smoke-inhibition of neurogenic bronchoconstriction in guinea-pigs *in vivo*: involvement of exogenous and endogenous nitric oxide

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1 We investigated the effect of acute inhalation of cigarette smoke on subsequent non-adrenergic, non-cholinergic (NANC) neural bronchoconstriction in anaesthetized guinea-pigs *in vivo* by use of pulmonary insufflation pressure (PIP) as an index of airway tone. The contribution of endogenous nitric oxide (NO) was investigated with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). The contribution of plasma exudation to the response was investigated with Evans blue dye as a plasma marker.

2 Inhalation of 50 tidal volumes of cigarette smoke or air had no significant effect on baseline PIP. In the presence of propranolol and atropine (1 mg kg^{-1} each), electrical stimulation of the vagus nerves in animals given air 30 min previously induced a frequency-dependent increase in PIP above sham stimulated controls (16 fold increase at 2.5 Hz, 24 fold increase at 10 Hz). In contrast, in smoke-exposed animals, the increase in subsequent vagally-induced PIP was markedly less than in the air controls (90% less at 2.5 Hz, 76% less at 10 Hz).

3 L-NAME (10 mg kg^{-1}), given 10 min before air or smoke, potentiated subsequent vagally-induced (2.5 Hz) NANC bronchoconstriction by 338% in smoke-exposed animals, but had no significant effect in air-exposed animals. The inactive enantiomer D-NAME (10 mg kg^{-1}) had no effect, and the potentiation by L-NAME was partially reversed by the NO-precursor L-arginine (100 mg kg^{-1}). Vagal stimulation did not affect the magnitude of vagally-induced bronchoconstriction 30 min later.

4 Cigarette smoke exposure reduced the magnitude of subsequent bronchoconstriction induced by neurokinin A (NKA) by 37% compared with the effect of NKA in air-exposed animals. L-NAME had no significant effect on the smoke-induced inhibition of NKA-induced bronchoconstriction.

5 Vagally-induced plasma exudation in the main bronchi was greater in smoke-exposed animals compared with air-exposed animals (120% greater at 2.5 Hz, 82% greater at 10 Hz).

6 We conclude that cigarette smoke-induced inhibition of subsequent NANC neurogenic bronchoconstriction is not associated with inhibition of airway plasma exudation and is mediated in part via exogenous smoke-derived NO, or another bronchoprotective molecule, and by endogenous NO.

Keywords: Cigarette smoke; nitric oxide; NO; nitric oxide synthase; NOS; airway tone; bronchoconstriction; plasma exudation

Introduction

Cigarette smoke is a powerful irritant of the respiratory tract. Acute inhalation triggers a number of protective neural responses including coughing (Widdicombe, 1995), mucus secretion (Peatfield *et al*., 1986; Kuo *et al*., 1992), plasma exudation (Lundberg & Saria, 1983) and bronchoconstriction (Hartiala *et al*., 1984). The neural pathways involved in the responses include direct stimulation of the central nervous system (Hartiala *et al*., 1984), direct activation of autonomic ganglia (Peatfield *et al*., 1986), reflex activation of sensory nerve endings in upper and lower airways (Lee *et al*., 1989), and local (axonal) reflex release of sensory neuropeptides from capsaicin-sensitive sensory nerves (Lundberg & Saria, 1983). With such a battery of excitatory neuronal pathways involved, it is surprising that in the anaesthetized pig, inhalation of cigarette smoke induces bronchodilatation (Alving *et al*., 1993). The latter authors concluded that nitric oxide (NO), derived from the smoke (Norman & Keith, 1965), was the most likely mediator of the bronchodilatation (Dupuy *et al*., 1992). We have found that in the anaesthetized guinea-pig inhalation of cigarette smoke induced marked plasma exudation but had no effect on airway tone (Hirayama *et al*., 1993; Lei *et al*., 1993). The lack of bronchoconstriction in response to cigarette smoke could be due to functional antagonism by smoke-derived NO of neuronally-induced bronchoconstriction. It could also be due to tissue-derived NO because we have shown that endogenous NO regulates the magnitude of neurogenic bronchoconstriction *in vivo* in guinea-pigs (Lei *et al*., 1993).

Cigarette smoke has other activity in the airways. For example, it induces bronchoconstrictor hyperresponsiveness to substance P in guinea-pigs by inactivating neutral endopeptidase, an endogenous enzyme which degrades sensory neuropeptides (Dusser *et al*., 1989). From this observation, it would appear that NO derived from the smoke does not play a primary bronchodilator or bronchoprotective role, at least to an exogenously administered spasmogen. However, it would be interesting to determine whether or not cigarette smoke has any effect on subsequent neurally-induced bronchoconstriction, either via exogenous NO or endogenous NO, or both.

The aims of the present study were to determine in guinea pigs *in vivo*: (1) whether inhalation of cigarette smoke would modulate subsequent neurogenic bronchoconstriction, and (2) to determine the mechanisms of any modulatory activity. To simplify interpretation of the data, only one neural pathway was investigated. Animals were pretreated with propranolol and atropine to eliminate adrenergic and cholinergic neural influences. Excitatory airway responses to the remaining non-adrenergic, non-cholinergic (NANC) neural pathway are due to stimulation of a population of capsaicin-sensitive C-fibres which subserve a motor function, and may be termed 'sensory-efferent' nerves (Maggi & Meli, 1988). The neurotransmitters of these nerves are the sensory neuropeptides which include

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calcitonin gene-related peptide and the tachykinins substance P and neurokinin A (NKA) (Barnes *et al*., 1991). The contribution of endogenously-released NO to the response was investigated by use of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (Rees *et al*., 1990), and the NO precursor L-arginine (Moncada *et al*., 1989). Pulmonary insufflation presure (PIP) was used as an index of airway patency (an increase in PIP indicates bronchoconstriction). Bronchial neurogenic plasma exudation was determined with the plasma marker Evans blue dye (Rogers *et al*., 1989). Exudation acted as an 'internal control' to assess, (1) whether any effects of smoke were specific for bronchoconstriction, and (2) whether simple tachyphylaxis or refractoriness to smoke accounted for any change in subsequent neurogenic airway response.

Methods

Animal preparation

Male Dunkin-Hartley outbred guinea-pigs (Charles River, Margate, Kent; 350–400 g body weight) were housed in a temperature controlled room (20°C) with food and water freely available. They were anaesthetized with urethane (2 g kg^{-1}, i.p.; 25% w/v in 0.9% w/v saline), laid supine on a heated blanket (37°C rectal temperature; Homeothermic System, Harvard Apparatus Ltd., Edenbridge, Kent), and the cervical jugular veins were exposed for the injection of drugs (i.v.). The dose of urethane was chosen because, from previous studies in our laboratory, stable traces for blood pressure (Belvisi *et al*., 1990) and PIP (Lei *et al*., 1993) are maintained without the need for paralysing or over-anaesthetizing the animal. To monitor the physiological condition of the animals and to establish drug activity, blood pressure was recorded from the left carotid artery via an indwelling Portex cannula filled with heparin-saline (2 u ml^{-1}). Animals were mechanically ventilated at constant volume and positive pressure via a tracheostomy cannula at a tidal volume of 10 ml kg^{-1} and a rate of 60 breaths min^{-1} which maintained blood gases at (mean ± s.e.mean) PaO_2 114 ± 2 mmHg, $PaCO_2$ 37 ± 1, SaO_2 98 ± 1 (pH 7.4). A side arm in the expiratory limb of the ventilatory circuit was used to measure PIP. Both cervical vagus nerves were exposed, sectioned below the nodose ganglia (to avoid stimulating the central nervous system during vagal stimulation) and their cut ends looped over bipolar platinum electrodes (Subminiature Electrode, Harvard Apparatus Ltd., Edenbridge, Kent). A double channel square wave stimulator (Model S88, Grass Instruments, Quincy, U.S.A.) was used to stimulate the nerves at either 2.5 Hz or 10 Hz with other parameters held at 5 V, 5 ms for 3 min: 2.5 Hz is threshold and 10 Hz just submaximal for activation of sensory-efferent nerves (Belvisi *et al*., 1990).

Preparation and administration of cigarette smoke

Cigarettes used were commercially available, unfiltered, and in the U.K. Government category 'middle tar' (nicotine 1.2, CO 11 mg per cigarette). Cigarettes were lit in a fume cupboard where a constant laminar flow prevented smoke accumulation in the room. After the cigarette had burned down approximately 0.5 cm, smoke was drawn into a 60 ml polypropylene syringe over an interval of 2 s. The first four syringefuls of smoke were discarded, and the fifth syringeful was introduced in a series of tidal volumes into the trachea and lungs via a three-way stopcock just rostral to the endotracheal tube, according to the following protocol. At the end of expiration, noted from the recorder trace, the stopcock tap to the lungs was turned to 'off'. This registered maximal deflections on the pressure trace (see Figure 1). A tidal volume of smoke was blown immediately into the cannula and the tap turned back to the lungs. Smoke was introduced at the end of expiration to avoid increasing the effective tidal volume and, therefore, any impact on resting PIP which lung hyperinflation may have. Fifty tidal volumes of smoke were used. Thus, for a 400 g guinea-pig, at a tidal volume of 10 ml kg^{-1}, a total of 200 ml smoke was delivered from 3–4 cigarettes (3–4 60 ml syringes), depending upon burning rate. This 'dose' has been shown to be near maximal for induction of airway neurogenic plasma exudation in a similar preparation and, as such, demonstrates that it effectively stimulates nerves (Lei *et al*., 1995). Air drawn through unlit cigarettes was used as a control procedure. Each tidal volume of air or smoke was separated from the next by 5–6 tidal volumes of room air. Smoke or air administration took ~12 min.

Protocols

Fifteen min after the end of administration of cigarette smoke or air, propranolol and atropine (1 mg kg^{-1} each) were injected i.v. to eliminate the effects on bronchoconstriction and plasma exudation of stimulation of adrenergic and cholinergic nerves, respectively. The dose of each drug has been shown previously to block effectively adrenergic and cholinergic-mediated responses (Lei *et al*., 1993). Fifteen minutes later, the vagus nerves were stimulated (i.e. 30 min after the end of air or smoke inhalation). Sham stimulated animals underwent comparable surgery and vagus nerve preparation but the stimulator was not activated. In certain animals, neurokinin A (NKA) was administered at the vagal stimulation time point. The dose of NKA used was 3.6 nmol kg^{-1} which, in pilot studies, gave an increase in PIP which was submaximal and comparable to that of vagal stimulation without prior air or cigarette smoke administration.

A similar protocol to the above was used to determine the effect of vagus nerve stimulation on subsequent NANC neurogenic bronchoconstriction. Both vagus nerves were stimulated electrically at 2.5 Hz (5 V, 5 ms for 3 min). This frequency was chosen because it produces a bronchoconstriction, due to activation of cholinergic and sensory-efferent nerves, which is similar to the NANC neural bronchoconstriction induced by 10 Hz (Lei *et al*., 1993). Vagal stimulation was followed 15 min later by administration of propranolol and atropine, and 15 min later by a second vagal stimulation at either 2.5 Hz or 10 Hz.

In animals for determination of plasma exudation, Evans blue dye (25 mg kg^{-1} in a volume of 1 ml kg^{-1}) was injected i.v. 1 min before vagal stimulation (Rogers *et al*., 1989; Lei *et al*., 1993). Five min after cessation of vagal stimulation, intravascular dye was expelled from the systemic circulation (including the bronchial circulation) at 100 mmHg pressure and the Evans blue remaining in the main bronchi was extracted in formamide for 16 h. The concentration of extractable dye was determined by spectrophotometry at 620 nm and the tissue content expressed as ng dye mg^{-1} wet weight main bronchi.

The effective doses and timing of administration of L-NAME, D-NAME (H-D-Arg(NO_2)oMe. HCl) and L-arginine have been determined by us previously (Lei *et al*., 1993), but had to be established for the present experimental protocols. Consequently, L-NAME or D-NAME (10 mg kg^{-1} for each: submaximal dose for L-NAME potentiation of vagally-induced bronchoconstriction) were given 10 min before smoke or air (i.e. ~52 min before vagal stimulation), or 10 min before vagal stimulation. In order to investigate the specificity of any response to L-NAME,L-arginine (100 mg kg^{-1}) was administered 9 min after L-NAME (i.e. 1 min before cigarette smoke, air or vagal stimulation).

Drugs

The following drugs were used: L-NAME, NKA, L-arginine (Sigma Chemical Co. Ltd., Poole, Dorset); D-NAME (Bachem Feinchemikalen AG, Bubendorf, Switzerland); atropine sulphate (Pharma Hameln, G.m.b.H., Germany); propranolol

hydrochloride (Imperial Chemical Industries PLC, Macclesfield, Cheshire); heparin sodium (CP Pharmaceuticals Ltd., Wrexham, North Wales). L-NAME and L-arginine were dissolved in distilled water on each day of experimentation. NKA was dissolved in 0.9% (w/v) saline at a concentration of 0.1 μmol ml^{-1}, and was aliquoted and stored frozen at -20°C. On the days of experimentation, aliquots were thawed and dissolved in saline to the final concentration before use. All drugs were injected in a volume of 0.1 ml 100g^{-1} body weight.

Data analysis

Data were analysed by use of Minitab software (The Pennsylvania State University, University Park, U.S.A.). Changes in PIP were calculated from the original traces in cmH_2O and are expressed in Results as mean and one s.e.mean. Data for PIP and plasma exudation did not approximate a normal distribution and the significance of differences between predetermined experimental groups was assessed by the Mann-Whitney U-test (two tailed). Mean arterial blood pressure was calculated from original traces as: diastolic pressure + 0.33 (systolic pressure − diastolic pressure). Data for blood pressure were assessed for group differences by one-way analysis of variance. Subsequent analysis of differences between predetermined groups was made by Student's t test for unpaired observations (two-tailed). Spearman's rank correlation coefficient was used to determine the relationship between baseline PIP and increase in PIP with vagal stimulation. For all tests, the null hypothesis of no difference between groups was rejected at $P<0.05$.

Results

Effect of air or cigarette smoke on vagally-induced bronchoconstriction

Mean baseline PIP (measured just before any treatment) was 7.1 cmH_2O ($\pm$0.1, $n=83$). Inhalation of 50 tidal volumes of cigarette smoke or air had no significant effect on PIP (mean increase of 0.3$\pm$0.3 cmH_2O for both) (Figure 1). Similarly, there was no significant change in PIP in smoke-exposed animals in response to sham stimulation 30 min after cigarette smoke (Figure 2). In the air-exposed, sham-stimulated group, three animals demonstrated no increase in PIP and one demonstrated an increase of 0.9 cmH_2O (11%) above baseline (group mean of 0.2$\pm$0.3 cmH_2O, 3%, above baseline; not significant: Figure 2).

Vagal stimulation of animals having air inhalation 30 min previously induced a marked increase in PIP above the air-administered, sham-stimulated controls (Figure 1). The increase showed a frequency-related trend with a 3.0 cmH_2O (16 fold) increase above the air controls at 2.5 Hz and a 4.6 cmH_2O (24 fold) increase above the air controls at 10 Hz (Figure 2). Vagal stimulation of animals which had previously inhaled smoke also increased PIP (Figure 1), with a frequency-related trend (Figure 2). However, the increase in PIP after smoke was markedly less than in the air controls at both frequencies of stimulation used: 90% less at 2.5 Hz and 76% less at 10 Hz (Figure 2). The inhibitory effect of cigarette smoke on vagally-induced increase in PIP was not related to PIP before vagal stimulation ($r_s=0.1$, $n=11$).

In the following experiments in which the mechanisms underlying the inhibitory effect of cigarette smoke were investigated, all vagal stimulations were at 2.5 Hz to allow for any potentiation due to drug intervention.

Effect of alterations in NO metabolism on neurogenic bronchoconstriction

When L-NAME was given 10 min before inhalation of air, subsequent vagally-induced increases in PIP were not significantly changed compared with the values without L-NAME (Figure 3a). In contrast, when given 10 min before smoke, L-NAME potentiated vagally-induced increases in PIP by 2.7 cmH_2O (338%) (Figure 3a).

D-NAME (10 mg kg^{-1}, 10 min before inhalation), did not significantly affect vagally-induced PIP in smoke-exposed animals (mean increase in PIP of 1.2$\pm$0.5 cmH_2O, 19%, $n=7$, with D-NAME compared with an increase of 0.8$\pm$0.4 cmH_2O, 13%, $n=17$, without D-NAME). L-Arginine (100 mg kg^{-1}, 1 min before inhalation) had no significant efffect on the lack of potentiation by L-NAME of neurogenic bronchoconstriction in air-exposed animals, but inhibited the potentiation in smoke-exposed animals by 93% (Figure 3a).

Figure 1 Original tracings showing examples of the effect of inhalation of 50 tidal volumes of cigarette smoke or air on subsequent non-adrenergic, non-cholinergic neural bronchoconstriction in anaesthetized guinea-pigs. (a) Cigarette smoke exposed animal, blood pressure, (b) simultaneous trace of pulmonary insufflation pressure (PIP, an index of airway tone), (c) air exposed animal, blood pressure, (d) simultaneous PIP trace. Prior cigarette smoke exposure protected against bronchoconstriction induced by electrical stimulation of the vagus nerves (VS) in the presence of propranolol and atropine. Note change of chart speed from 1 min markers to 5 min markers.

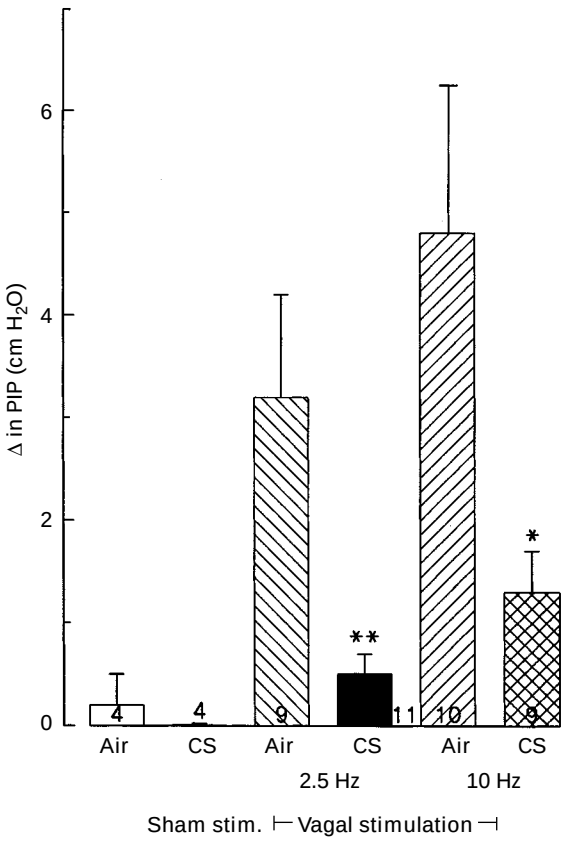


Figure 2 Cigarette smoke (CS) inhibition of neurogenic bronchoconstriction in anaesthetized guinea-pigs *in vivo*. Fifty tidal volumes of air or CS were inhaled followed 30 min later by electrical stimulation of both vagus nerves at one of two frequencies (5 V, 5 ms, 3 min), or sham stimulation. Data are mean increase above baseline in pulmonary insufflation pressure (PIP: an index of airway patency), vertical lines show s.e.mean, for the number of animals indicated in the columns. $*P<0.05$, $**P<0.01$ compared with corresponding value with air.

In contrast to the differential effect of L-NAME in air-exposed and smoke-exposed animals when given before the inhalation, L-NAME potentiated vagally-induced increases in PIP in both groups of animals when given 10 min before vagal stimulation (Figure 3b): by 5.0 cmH_2O (139%) in the air-exposed animals, and by 3.1 cmH_2O (443%) in the smoke-exposed animals. L-Arginine (100 mg kg^{-1}, 1 min before vagal stimulation) inhibited the potentiation by L-NAME by 74% in smoke-exposed animals and by 77% in air-exposed animals (Figure 3b).

Effect of vagal stimulation on subsequent vagally-induced bronchoconstriction

Vagal stimulation at 2.5 Hz increased PIP by 131% from a baseline of 6.3 ± 0.4 cmH_2O ($n=10$) to 14.6 ± 1.4 cmH_2O. Sham stimulation had no effect on PIP (i.e. 0% change, $n=10$). Neither sham stimulation nor stimulation at 2.5 Hz significantly affected subsequent NANC neurogenic bronchoconstriction: at 2.5 Hz, previous sham = 58 ± 8% increase above baseline, previous 2.5 Hz = 63 ± 9% increase; at 10 Hz, previous sham = 82 ± 13% increase above baseline, previous 2.5 Hz = 84 ± 11% increase.

Effect of air or CS on NKA-induced bronchoconstriction and the effect of L-NAME

In air-exposed animals, NKA (3.6 nmol kg^{-1}) induced an increase in PIP of 3.6 ± 0.5 cmH_2O (52%) ($n=6$) above baseline. In cigarette smoke-exposed animals, the NKA-induced increase in PIP was significantly ($P<0.05$) less (by 37%) than in air-exposed animals: increase of 2.3 ± 0.4 cmH_2O (33%) ($n=6$) above baseline. In contrast to its potentiating effect on neurogenic bronchoconstriction in smoke-exposed animals (see above), L-NAME (10 mg kg^{-1}), given 10 min before smoke inhalation, had no potentiating effect on NKA-induced bronchoconstriction: increase in PIP of 2.4 ± 0.3 cmH_2O (35%) ($n=7$) above baseline (not significantly different to the increase of 2.3 cmH_2O above).

Effect of air or CS on vagally-induced plasma exudation

The Evans blue dye content of the main bronchi was 126% greater in smoke-exposed, sham-stimulated animals compared with the air-exposed, sham-stimulated animals (Figure 4). Vagal stimulation increased the tissue dye content of the main bronchi, with a frequency-related trend, in both air-exposed and smoke-exposed animals. At both frequencies of stimulation used, Evans blue dye content was greater in smoke-exposed than air-exposed animals: 120% greater at 2.5 Hz and 82% greater at 10 Hz.

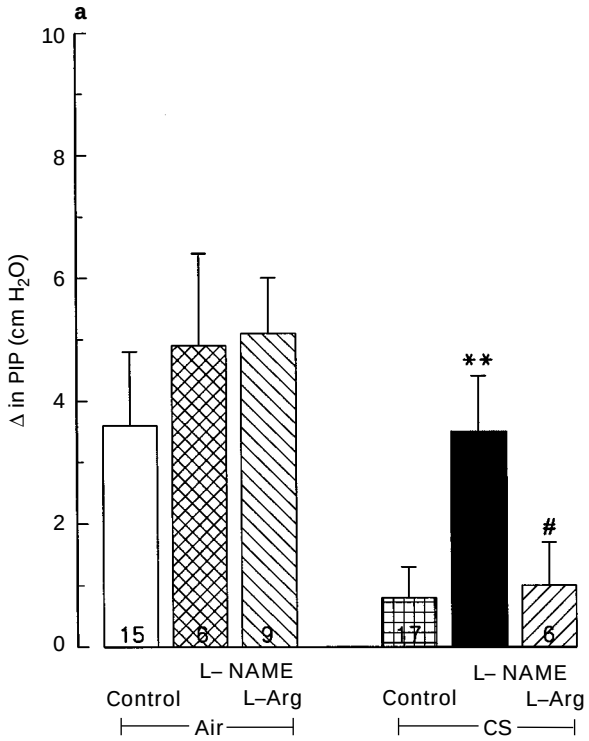
Blood pressure

Mean baseline carotid artery pressure (measured just before any treatment) was 41.2 mmHg (±0.7, $n=83$). Neither administration of air nor cigarette smoke had any significant effect on blood pressure (Figure 1). L-NAME (10 mg kg^{-1}) significantly ($P<0.01$) increased blood pressure whether given before or after cigarette smoke or air administration: overall increase above baseline of 27.5 ± 3.8 mmHg (~67%).

Discussion

The present study found that, in guinea-pigs *in vivo*, prior inhalation of fifty volumes of cigarette smoke markedly reduced the magnitude of subsequent vagally-induced non-cholinergic bronchoconstriction. Non-cholinergic bronchoconstriction *in vivo* is due to stimulation of sensory-efferent nerves with release of sensory neuropeptides, of which NKA and, to a lesser extent, substance P, interacting with tachykinin NK_2 receptors, are the principal contractors of airway smooth muscle (Barnes *et al*., 1991; Hirayama *et al*., 1993). Cigarette smoke itself did not have any significant effect on airway tone, which is consistent with previous observations (Hirayama *et al*., 1993; Lei *et al*., 1995), and the magnitude of baseline PIP was not related to the initial PIP. In addition, we found herein that vagal stimulation 30 min before subsequent vagal stimulation did not inhibit neurogenic bronchoconstriction. Thus, the inhibition by cigarette smoke of neurogenic bronchoconstriction is due to the delayed action(s) of an inhibitory factor(s), either in the smoke or released endogenously by the smoke. The following discussion considers possibilities based on the present data.

The inhibitory effect could be merely due to tachyphylaxis or refractoriness of the later response after the initial administration of cigarette smoke. However, in the present study, stimulation of the vagus nerves, in the presence of propranolol and atropine, induced not only bronchoconstriction but also an increase in the tissue content of Evans blue dye in the main bronchi. The dye binds to albumin, and its exudation into the airways correlates with that of [^{125}I]-serum albumin (Rogers *et al*., 1989), which indicates that the increase in bronchial dye content in the present study was due to plasma exudation. In contrast to its inhibitory effect on neurogenic bronchoconstriction herein, prior inhalation of cigarette smoke did not inhibit vagally-induced plasma exudation. NANC neurogenic plasma exudation is due exclusively to stimulation of sensory-efferent nerves (Lundberg & Saria, 1983; Belvisi *et al*., 1990). In contrast to non-cholinergic bronchoconstriction, the principal mechanism of neurogenic plasma exudation is substance P, and to a lesser extent NKA, interaction with tachykinin NK_1 receptors (Hirayama *et al*., 1993). Thus, the increase in neu-



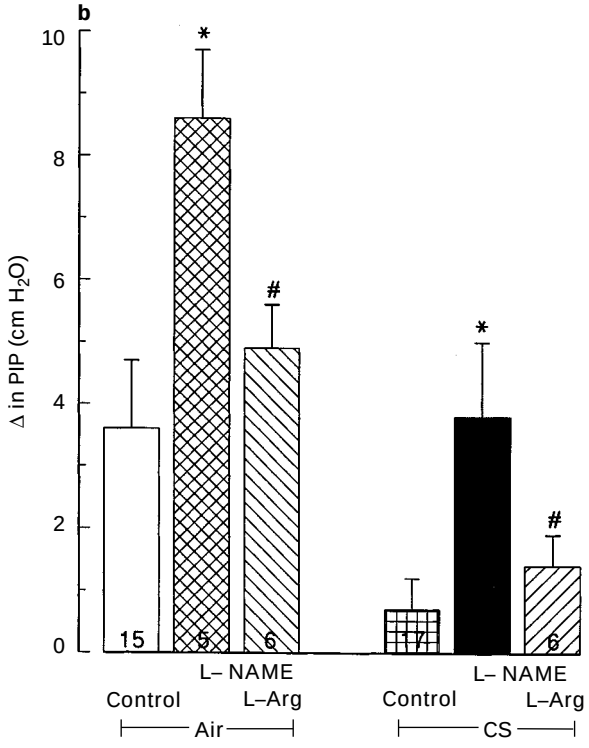


Figure 3 Effect of modifications in metabolism of endogenous nitric oxide (NO) on neurogenic bronchoconstriction in anaesthetized guinea-pigs *in vivo*. (a) The NO synthase inhibitor L-NAME (10 mg kg^{-1}), alone or in combination with the NO precursor L-arginine (L-Arg: 100 mg kg^{-1}), was injected i.v. followed 10 min later by inhalation of 50 tidal volumes of air or cigarette smoke (CS). Thirty min later both vagus nerves were stimulated electrically (5 V, 5 ms, 3 min). (b) Fifty tidal volumes of air or cigarette smoke (CS) were inhaled followed 20 min later by injection of the NO synthase inhibitor L-NAME, alone or in combination with the NO precursor L-arginine (L-Arg). Ten min later both vagus nerves were stimulated electrically. Data are mean increase above baseline in pulmonary insufflation pressure (PIP: an index of airway patency), vertical lines show s.e.mean, for the number of animals indicated in the columns. $*P<0.05$, $**P<0.01$ compared with corresponding control; $^{\#}P<0.05$ compared with L-NAME.

rogenic plasma exudation demonstrates that the airway sensory-efferent neural pathway does not exhibit tachyphylaxis or refractoriness to cigarette smoke under the present experimental circumstances. The lack of tachyphylaxis to sequential vagal stimulations (see above) is further evidence that neurogenic bronchoconstriction does not demonstrate reduced sensitivity under the present experimental conditions. The reduced neurogenic bronchoconstrictor response seen after prior administration of smoke must, therefore, be associated with an effect of the smoke. The plasma exudation data show also that, at least for the two parameters measured in the present study, cigarette smoke inhibition of subsequent NANC neurogenic airway responses is selective for bronchoconstriction rather than plasma exudation. Consequently, the inhibitory factor(s) must be selective for inhibition of bronchoconstriction rather than plasma exudation. Nitric oxide is a bronchodilator and inhibits bronchoconstriction (see below) whilst increasing airway plasma exudation (Kuo *et al.*, 1992) and is, therefore, a candidate for the inhibitory factor.

Cigarette smoke contains a number of gaseous components, including NO which is present at concentrations of 400–1,000 p.p.m. per puff of smoke (Norman & Keith, 1965). In guinea-pigs, inhalation of 300 p.p.m. NO has only a mild bronchodilatory effect, as measured by pulmonary resistance (R_L), but gives considerable protection against a bronchoconstrictor challenge (Dupuy *et al.*, 1992). Thus, in the present study, the inhibition by cigarette smoke of neurogenic bronchoconstriction could be due to the bronchoprotective

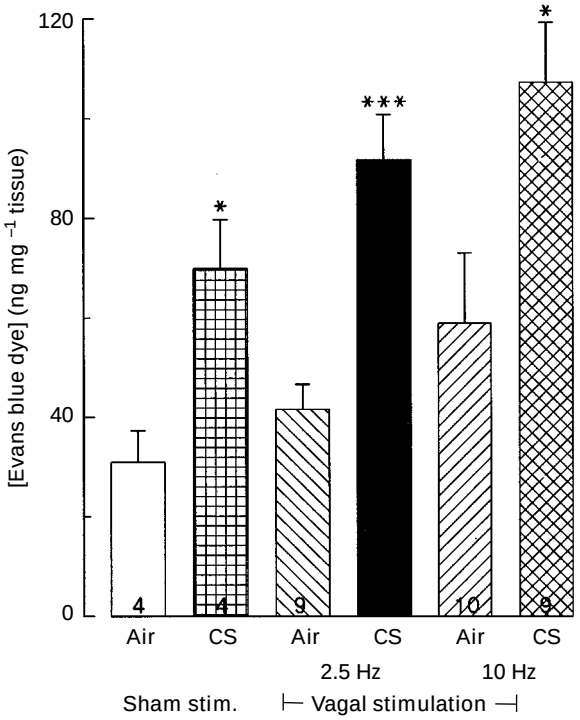


Figure 4 Effect of cigarette smoke (CS) or air inhalation on neurogenic plasma exudation in the main bronchi of anaesthetized guinea-pigs *in vivo*. Fifty tidal volumes of air or CS were inhaled followed 30 min later by electrical stimulation of both vagus nerves at one of two frequencies (5 V , 5 ms, 3 min), or sham stimulation. Data are mean tissue content of the plasma marker Evans blue dye, vertical lines show s.e.mean, for number of animals indicated in the columns. $*P<0.05$, $***P<0.001$ compared with corresponding value with air.

action of smoke-derived NO. The bronchodilator and bronchoprotective actions of inhaled NO are short-lived (Dupuy *et al*., 1995), and would be expected to have expired by the time of the vagal stimulation in the present study. However, NO degradation in cigarette smoke has different kinetics to that of inhaled NO, with a calculated half-life of 83 min when diluted in the lung (Norman & Keith, 1965). Thus, NO in the smoke is at a concentration and duration of action consistent with a bronchoprotective role against neurogenic bronchoconstriction in the present study. In addition, there may be other bronchoprotective substances in the smoke. For example, carbon monoxide is an important constituent of cigarette smoke (Guerin *et al*., 1979) and relaxes smooth muscle (Marks *et al*., 1991). The present study supports the idea of bronchoprotective agents in the smoke because cigarette smoke inhibited bronchoconstriction induced by NKA (given at the same time point as vagal stimulation). The inhibition was not altered by the NO synthase inhibitor L-NAME, which indicates either that exogenous rather than endogenous NO was the mediator of the inhibitory effect, or that another endogenous bronchoprotective molecule, not susceptible to NO synthase inhibition, was involved. Whatever the bronchoprotective mediator, inhibition of NKA-induced bronchoconstriction was only partial ($\sim$37%), compared with the almost complete inhibition by cigarette smoke of vagally-induced bronchoconstriction. Thus, cigarette smoke-derived NO, or another bronchoprotective molecule, accounts for approximately a third of the inhibitory actions of cigarette smoke on neurogenic bronchoconstriction. The remainder appears to be due primarily to endogenous NO.

We have shown previously that endogenous NO regulates the magnitude of NANC neural bronchoconstriction in guinea-pigs *in vivo* (Lei *et al*., 1993). In the present study L-NAME, given 10 min previously potentiated vagally-induced bronchoconstriction in both cigarette smoke-exposed and air-exposed animals. This observation is consistent with the concept of inhibition of NO generation in association with nerve activation leading to reduced bronchodilator activity and increased bronchoconstriction. The reversal of the potentiating effect of L-NAME by the NO precursor L-arginine indicates that the effect of L-NAME was selective for NO synthase. The lack of potentiation of bronchoconstriction by the inactive enantiomer D-NAME argues against a non-selective effect of L-NAME. The above observations demonstrate that the NO-modulating drugs used herein are appropriately active at the doses used.

In contrast to the potentiating effect of L-NAME in both air-exposed and smoke-exposed animals when given 10 min before vagal stimulation, L-NAME given 10 min before inhalation (i.e. at least 50 min before vagal stimulation) potentiated neurogenic bronchoconstriction only in smoke-exposed animals. The potentiation was partially reversed by L-arginine, demonstrating a selective effect on NO metabolism. Our interpretation of the differential effect of L-NAME is that under normal circumstances the *in vivo* activity of L-NAME is not maintained over 50 min. This suggestion would be consistent with administration of L-NAME *in vivo* 10–15 min before administration of vasodilators (Rees *et al*., 1990) or stimulation of the vagus nerves (Lefebre *et al*., 1992). Thus, in the air-exposed animals, the NO-inhibitory action of L-NAME was not present during vagal stimulation and consequently there was no enhancement of the neurogenic bronchoconstrictor response. In contrast, generation of NO during cigarette smoke exposure was inhibited by L-NAME, and this had a carry-over effect during subsequent vagal stimulation. The nature of the carry-over effect is unknown, but may be associated with the comparatively long lasting action of cigarette smoke. In contrast to most stimuli which induce a rapid increase in airway plasma exudation which resolves in a few minutes (Rogers & Evans, 1992), cigarette smoke-induced plasma exudation can be maintained for up to two hours, depending upon airway level (Lei *et al*., 1995). The mechanisms underlying the long-lasting exudation are unknown. However, it is possible that smoke-induced generation of endogenous NO, either from nerves or another source, may contribute to airway plasma exudation (Kuo *et al*., 1992), and to the bronchoprotection seen herein. Specific studies are required to test this hypothesis.

We found previously that endogenous vasoactive intestinal polypeptide (VIP) contributes to regulation of NANC neurogenic bronchoconstriction *in vivo* in guinea-pigs (Lei *et al*., 1993). VIP, released by cigarette smoke-activation of nerves, may, therefore, have contributed to the cigarette smoke-inhibition of neurogenic bronchoconstriction observed herein. In the absence of potent and selective VIP receptor antagonists (Watson & Girdlestone, 1996), the principal approach in determining a role for VIP in a given response is to use α-chymotrypsin, which degrades VIP (Altiere & Diamond, 1984; 1985), and which has been used to investigate the role of endogenous VIP in guinea-pig airways *in vitro* (Ellis & Farmer, 1989; Li & Rand, 1991) and *in vivo* (Lei *et al*., 1993). However, to degrade VIP effectively, α-chymotrypsin is administered typically 30–60 min before any investigation of the effects of VIP, and its activity is comparatively long-lasting (Ellis & Farmer, 1989; Li & Rand, 1991; Lei *et al*., 1993). Thus, the protocol of the present study precluded investigation of the contribution of VIP to the cigarette smoke inhibition because we would have been uncertain as to whether the α-chymotrypsin was acting directly on the vagally-induced bronchoconstriction rather than influencing the preceding cigarette smoke administration.

In conclusion, cigarette smoke-inhibition of NANC neurogenic bronchoconstriction is mediated by exogenous NO, or another bronchodilator molecule derived from the smoke, and by endogenous NO, and is unrelated to changes in airway plasma exudation. Endogenous VIP is not excluded as an additional mediator of the inhibition.

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