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Airway reactivity, inflammatory cell influx and nitric oxide in guinea-pig airways after lipopolysaccharide inhalation

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1 The aim of this study was to investigate the relationship between airway reactivity, leukocyte influx and nitric oxide (NO), in conscious guinea-pigs after aerosolized lipopolysaccharide (LPS) exposure.

2 Inhaled histamine (1 mM, 20 s), causing no bronchoconstriction before LPS exposure (30 μg ml^{-1}, 1 h), caused bronchoconstriction at 0.5 and 1 h ($P<0.02$) after LPS exposure. This airway hyperreactivity (AHR) recovered by 2 h. In contrast, 48 h after LPS exposure, the response from a previously bronchoconstrictor dose of histamine (3 mM, 20 s) was attenuated ($P<0.01$) i.e. airway hyporeactivity (AHOR).

3 Investigation of the cellular content of bronchoalveolar lavage fluid (BALF) from these animals revealed a rapid (0.5 h: 691 fold increase) and progressive neutrophil influx after LPS exposure (24 h: $36.3\pm2.3\times10^6$ cells per sample), that subsided 48 h later. Macrophages and eosinophils also time-dependently increased (0.5 h: 4.6 ± 0.4 and 0.1 ± 0.05; 48 h: 31.0 ± 6.0 and $1.8\pm0.3\times10^6$ cells per sample, respectively) after LPS, compared to vehicle exposure (24 h: neutrophils, eosinophils and macrophages: 0.28 ± 0.19, 0.31 ± 0.04 and $4.96\pm0.43\times10^6$ cells per sample, respectively).

4 The combined NO metabolites in BALF, after vehicle (1 h), or LPS (1 h: AHR and 48 h: AHOR) exposure, were respectively increased (41%, $P<0.01$), decreased (47%, $P<0.01$) and further increased (80%, $P<0.001$), compared with naïve animals.

5 Inhaled N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME: 1.2 and 12 mM, 15 min), reduced BALF NO metabolites 2 h later, but did not cause AHR to histamine ($P>0.05$). When L-NAME inhalation followed LPS, AHR was prolonged from 1 h to at least 4 h ($P<0.01$).

6 In summary, aerosolized LPS inhalation caused neutrophil and macrophage airways infiltration, and an early development of AHR followed 48 h later by AHOR to histamine. AHR and AHOR coincided with a respective reduction and elevation in airways NO (metabolites). Thus, NO may aid recovery from AHR, as inhibition of its production prolongs AHR. However, NO deficiency alone is not responsible for LPS-induced AHR.

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Abbreviations: AHOR, airway hyporeactivity; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; cyclic AMP, adenosine 3′,5′ cyclic monophosphate; cyclic GMP, guanosine 3′,5′ cyclic monophosphate; cNOS, constitutive nitric oxide synthase; COPD, chronic obstructive pulmonary disease; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]; iNOS, inducible nitric oxide synthase; L-NAME, N$^{\omega}$-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; NADPH, nicotinamide adenine dinucleotide phosphate; NED, naphthyleneethylenediamine; NO, nitric oxide; NOS, nitric oxide synthase; sGaw, specific airways conductance; TNF-α, tumour necrosis factor-α

Introduction

Exposure of asthmatic subjects (Alving *et al*., 1993) or atopic guinea-pigs (Iijima *et al*., 1998) to inhaled antigen, and exacerbations in patients with chronic obstructive pulmonary disease (COPD) (Bhowmik *et al*., 1998; Corradi *et al*., 1999), lead to an increase in levels of exhaled nitric oxide (NO). Lipopolysaccharide (LPS) induces widespread tissue expression of inducible nitric oxide synthase mRNA (Liu *et al*., 1993). Aerosolized LPS inhalation also produces airways infiltration of predominantly neutrophils and macrophages and airway hyperreactivity (AHR) (Folkerts *et al*., 1988; Vrugt & Aalberes, 1993; Toward & Broadley, 1999).

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NO is a second messenger molecule, synthesized from the conversion of L-arginine to L-citrulline by a group of haemoproteins known as nitric oxide synthases (NOS) (Schini-Kerth & Vanhoutte, 1995). In normal lungs, NO is synthesized by two constitutive NOS (cNOS) isoforms expressed in nonadrenergic noncholinergic neurones (nNOS), vascular endothelial cells (eNOS) and airway epithelial cells (nNOS and eNOS) (Gaston *et al*., 1994). These isoforms, regulated by intracellular Ca^{2+} calmodulin concentration, are also localized in platelets, neutrophils and mast cells (Nathan, 1992) and generate picomolar levels of NO (Moncada, 1992). By contrast, a third inflammatory and inducible NOS (iNOS) isoform is Ca^{2+}-independent and produces nanomolar levels of NO (Moncada, 1992). Regulated at the transcriptional level in response to pro-inflammatory cytokines, e.g.

interleukin 1-β (IL-1β) and tumour necrosis factor-α (TNF-α) or LPS, activity of the iNOS gene is dependent on nuclear factor-κB (NF-κB) promoter binding (Lowenstein *et al.*, 1993; Xie *et al.*, 1994). Consequently, the iNOS isoform can be induced in a variety of cells, including epithelial, smooth muscle and inflammatory (e.g. macrophages and eosinophils) cells (Barnes & Belvisi, 1993).

The lipophilic free radical NO passes readily into smooth muscle to activate soluble guanylate cyclase, resulting in increased guanosine 3′,5′ cyclic monophosphate (cyclic GMP) to induce bronchodilation (Schulz & Triggle, 1994). NO may also have a role in leukocyte adhesion to endothelial cells and vascular permeability (Kubes *et al.*, 1991). Schuiling *et al.*, (1998) suggested that an over-production of NO in atopic guinea-pigs, presumably iNOS mediated, benefits recovery from AHR. However, in inflammatory conditions NO can quickly react with superoxide anion, resulting in the cytotoxic formation of peroxynitrite which promotes tissue damage and AHR (Nijkamp & Folkerts, 1995; Giaid *et al.*, 1998). Thus, NO appears to be capable of exerting contradictory effects on airway reactivity.

Since LPS can induce expression of NOS mRNA and has the ability to cause cell infiltration and AHR, the aim of this study was to investigate the relationships between airway reactivity, cell influx and NO production in the conscious guinea-pig after an acute LPS challenge. The stable NO decomposition products, nitrate (NO_3) and nitrite (NO_2), were assayed from lavage fluid taken from the lung to allow a surrogate indication of NO synthesis (Kanazawa *et al.*, 1998). The competitive analogue of L-arginine, L-NAME (N^{ω}-nitro-L-arginine methyl ester) was also used as a non-selective NOS inhibitor of NO synthesis. The LPS- and L-NAME -induced variations in NO production were correlated with changes in airway reactivity and leukocyte influx into the airways by methods previously used in this laboratory (Danahay & Broadley, 1997). Preliminary findings from these studies have been presented to the British Pharmacological Society (Toward & Broadley, 1999; 2000).

Method

Animals

Groups of six male Dunkin-Hartley guinea-pigs (Halls, Staffs., U.K.), weighing 300–400 g were used throughout. Animals received food and water *ad libitum*, and lighting was maintained in the room (22±2°C) on a 12 h cycle. This work complied with the guidelines for the care and use of laboratory animals according to the Animals (Scientific Procedures) Act 1986 and Glaxo-Wellcome policy.

Measurement of respiratory function

Whole body plethysmography of the conscious guinea-pig was used to monitor airway function, recorded as specific airways conductance (sGaw). The method was as described by Griffiths-Johnson and co-workers (1988), although a computerised data acquisition system replaced the original oscilloscope and angle resolver (Danahay & Broadley, 1997).

Animals were fitted with a face mask and placed in a restrainer which was then slid into the plethysmography chamber. As the animal breathed, a computer with a Biopack data acquisition system, ran AcqKnowledge software which acquired and stored data referring to the airflow across a pneumotachograph (Mercury FIL). The resulting change in box volume (pressure) was simultaneously measured. Changes in air flow and box pressure were measured by two pressure transducers, UP1 and UP2, respectively. The resultant waveforms could then be rapidly analysed by comparing the gradients of the flow and the box pressure waves at a point where flow tended towards zero, i.e. in the first 30 ms of expiration. A function of these parameters, correcting for ambient pressure and the weight of the animal, determines a value for sGaw. A minimum of five breaths were analysed for each animal at each timepoint. Before each experiment the animals were handled and familiarized with the equipment to reduce stress.

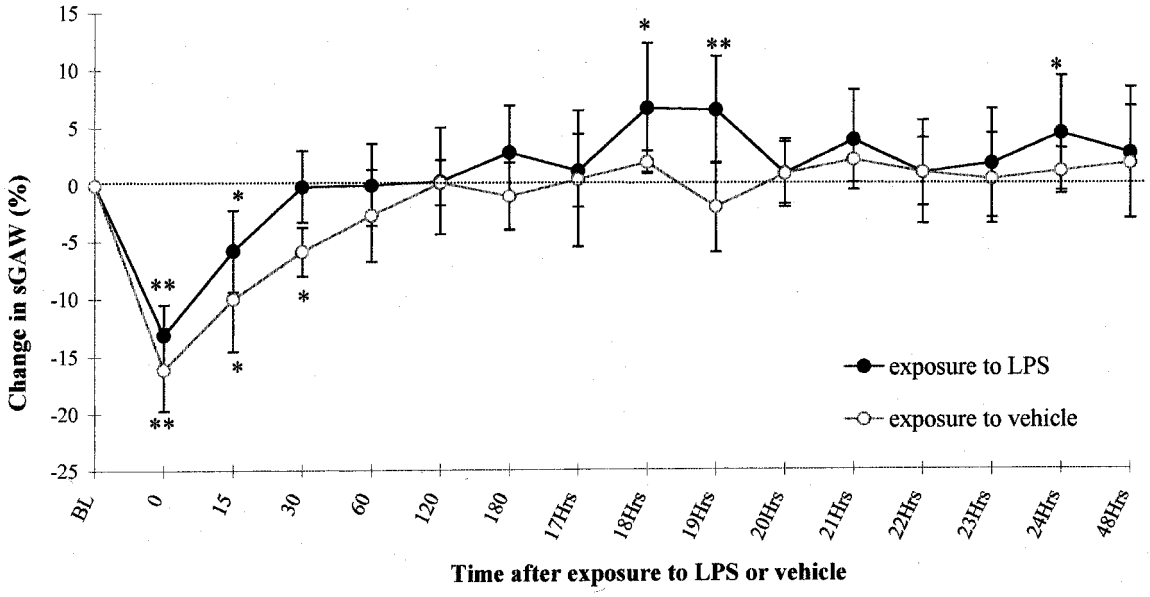

Figure 1 The effect of a single exposure (60 min) to nebulized lipopolysaccharide (LPS: 30 μg ml^{-1}), or the LPS vehicle (pathogen-free saline) on the airway function of conscious guinea-pigs. Each point represents the mean±s.e.mean (n=6–36) change in sGaw expressed as a percentage of the baseline sGaw values (sGaw (sec^{-1} cmH_2O^{-1}): saline=0.40±0.02; LPS=0.38±0.02). Negative values represent bronchoconstriction. Significance of differences from baseline values were determined by analysis of variance (single factor), followed by a Student's paired t-test indicated as *$P<0.05$ or **$P=0.02$.

Inhalation exposures

LPS exposures Animals were exposed for 1 h to a nebulized solution of LPS (30 μg ml^{-1}) or the LPS vehicle (0.9% pathogen-free saline) in a sealed chamber ($620 \times 300 \times 420$ mm). A Wright nebulizer, supplied with air at a pressure of 20 lb. p.s.i., was used to deliver the aerosol at a rate of 0.5 ml min^{-1}. sGaw was measured twice prior to exposure (baseline) and then at regular intervals (0, 15, 30 min and hourly) after the exposure.

Spasmogen exposures Airway reactivity to nebulized histamine (1 mM, 20 s) was assessed 24 h before and at various times after exposure to LPS or the LPS vehicle, each time in a separate group of animals. This dose of spasmogen, delivered as a nose-only exposure, caused a small threshold bronchoconstriction. To examine airway hyporeactivity, or any residual bronchodilator effect at 48 h after exposure to LPS, a higher dose of histamine (nose-only, 3 mM, 20 s) was used, which produced a significant bronchoconstriction. Measurements of sGaw were taken before and at 0, 5 and 10 min after exposure to spasmogen.

L-NAME exposures In other animals, unchallenged, or 2 h after exposure to LPS or vehicle, nebulised L-NAME (1.2 or 12 mM, 15 min) was delivered as a whole body exposure (chamber: $300 \times 180 \times 180$ mm). These inhaled doses of L-NAME were based upon those used by others to inhibit nitric oxide production without any significant effect on cardiovascular parameters (Nijkamp *et al*., 1993; Schuiling *et al*., 1998), however, the aerosol characteristics (particle size, flow rates) may not be identical. Measurements of sGaw were taken throughout the experiment and airway reactivity to histamine (nose-only, 1 mM, 20 s) was assessed 24 h before and at 105 min after exposure to L-NAME (4 h after exposure to LPS or vehicle). No animal appeared to be in any respiratory distress during this exposure regime or any of the other procedures described.

Bronchoalveolar lavage

Within 20 min of assessing post-exposure airway reactivity to histamine, the animals underwent a bronchoalveolar lavage (BAL). The guinea-pigs were overdosed with pentobarbitone sodium (400 mg kg^{-1}, i.p., Euthatal) and the trachea cannulated. A 1% solution of EDTA (1 ml 100 g^{-1} body weight) was flushed through the cannula into the lungs, and recovered 3 min later. This was repeated and a total cell count (cells ml^{-1}) of the pooled BAL fluid was determined using a haemocytometer (Neubauer). A Cytospin smear (Shandon Centrifuge: 1000 r.p.m., 7 min) of the sample was differentially stained (Leishman's: 1.5% in methanol, 6 min), and a minimum of 500 cells (macrophages, eosinophils and neutrophils) were counted.

Measurement of nitric oxide (NO) production

The remaining BAL sample was then centrifuged (1200 r.p.m., 6 min), the supernatant removed and frozen (-20°C). A spectrophotometric assay was used to determine the decomposition products of NO (nitrite and nitrate) based on the Griess reaction, as described by Grisham *et al*. (1996). Briefly, 100 μl samples of the BAL fluid were incubated (37°C) for 30 min with HEPES buffer (50 mM, pH 7.4), FAD (5 μM), NADPH (0.1 mM), distilled water (290 μl) and nitrate reductase (0.2 U ml^{-1}) for nitrate conversion to nitrite. In an identical set of tubes, nitrate reductase was omitted for determining nitrite only. Any unreacted NADPH in the solution (500 μl) was then oxidised by incubating (25°C) for 10 min with potassium ferricyanide (1 mM). The samples were then incubated (25°C) with 1 ml of the Griess reagent (NED: 0.2% (w v^{-1}), sulfanilamide: 2% (w v^{-1}), solubilized in double distilled water: 95% and phosphoric acid: 5% (v v^{-1})), for 10 min and the absorbance measured at 543 nm. To maintain standard conditions, all the samples to be compared were assayed at the same time. The linear limit of detection for the assay was 1 mM.

Drugs and solutions

Lipopolysaccharide (LPS: *Escherichia coli* O26:B6), histamine diphosphate salt, N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME)

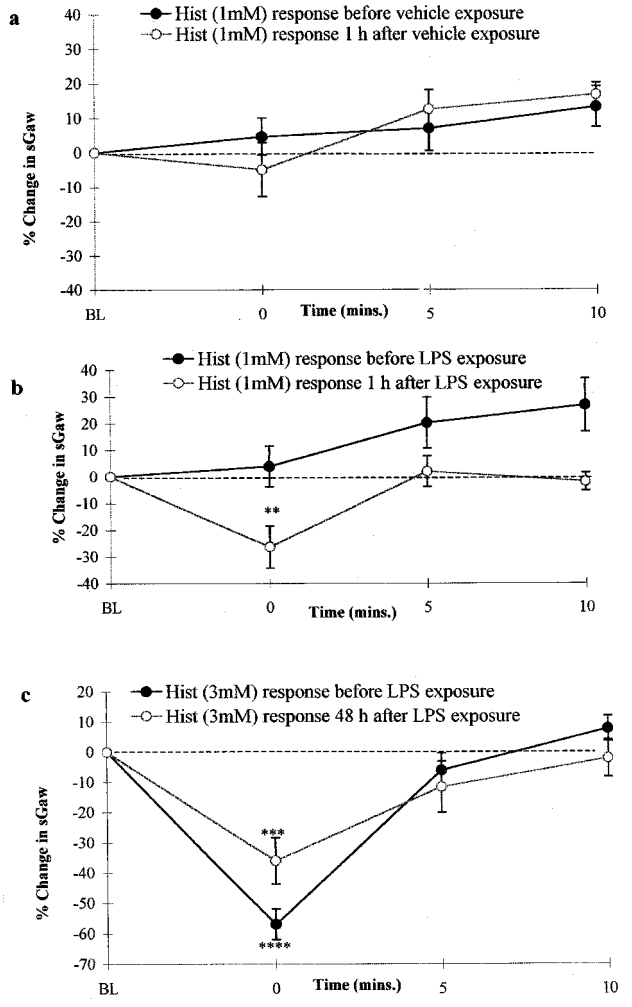

Figure 2 The responsiveness of the airways to a nose-only exposure to histamine (Hist); (a: Hist, 1 mM, 20 s) before and 1 h after exposure (60 min) to nebulized vehicle (saline), (b: Hist, 1 mM, 20 s) before and 1 h after exposure (60 min) to nebulized LPS, (c: Hist, 3 mM, 20 s) before and 48 h after exposure (60 min) to nebulized LPS, in conscious guinea-pigs. Each point represents the mean$\pm$ s.e.mean ($n=6$) change in sGaw expressed as a percentage of the baseline sGaw values (sGaw (sec^{-1} cmH_2O^{-1}): (a) before$=$ 0.44 ± 0.02 and after$=0.36\pm0.02$; (b) before$=0.30\pm0.02$ and after$=0.31\pm0.02$; (b) before$=0.32\pm0.01$ and after$=0.35\pm0.01$). Negative values represent bronchoconstriction. Significance from baseline sGaw was determined by analysis of variance (single factor), followed by a Student's paired *t*-test denoted as $^{*}P<0.05$, $^{**}P=0.02$, $^{***}P<0.01$ and $^{****}P<0.001$. Airway reactivity to Hist (3 mM), before and after vehicle exposure (saline) was not significantly different ($P>0.05$, data not shown).

hydrochloride, flavin adenine dinucleotide (FAD) disodium salt, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES) free acid, nicotinamide adenine dinucleotide phosphate (NADPH: reduced form), potassium ferricyanide, ethylenediaminetetraacetic acid (EDTA) disodium salt, naphthyleneethylenediamine (NED), sulphanilamide and phosphoric acid were purchased from Sigma (Poole, Dorset, U.K.), Euthatal from Rhone Meriux (Harlow, Essex, U.K.) and *Aspergillus* nitrate reductase (NADPH: nitrate oxidoreductase, EC 1.6.6.2) from Boehringer Mannheim (Indianapolis, U.S.A.). Unless otherwise indicated, all solutions were made up in sterile saline (0.9% NaCl: Baxter Healthcare, U.K.).

Data analysis

To reduce inter-subject variability, changes in sGaw from the baseline sGaw values taken before a procedure, are presented as a percentage of the baseline value. Absolute values of baseline sGaw are stated in the figure legends. Changes in airway function, BAL fluid cell count and the concentration of NO metabolites were compared using analysis of variance, followed by the appropriate paired or unpaired Student's *t*-test. Differences were considered statistically significant when $P<0.05$.

Results

Effect of exposure to LPS on airway function

Exposure to nebulized LPS (30 μg ml^{-1}, 60 min) caused an immediate bronchoconstriction lasting 30 min (Figure 1). However, this was no different from the response after an exposure to the LPS vehicle (pathogen-free saline, 60 min). There was a small but significant bronchodilation with the LPS exposed group at 18, 19 and 24 h after challenge, which was not seen with the saline exposed group.

Airway responsiveness to inhaled spasmogen after exposure to LPS

Inhalation of histamine (1 mM, 20 s) prior to LPS exposure failed to produce a significant bronchoconstriction. However, 0.5 h after exposure to LPS, there was a significant ($P<0.05$) bronchoconstriction to histamine, indicating AHR (Figures 2b and 3). There was also AHR to inhaled histamine (1 mM, 20 s) at 1 h after LPS, a lack of bronchoconstriction before exposure being converted to a significant ($P<0.05$) bronchoconstriction afterwards (Figure 3). At 2 h, there was a non-significant ($P>0.05$) bronchoconstriction, but at 4 and 24 h the AHR had subsided. Exposing guinea-pigs to a higher dose of inhaled histamine (3 mM, 20 s) before LPS exposure

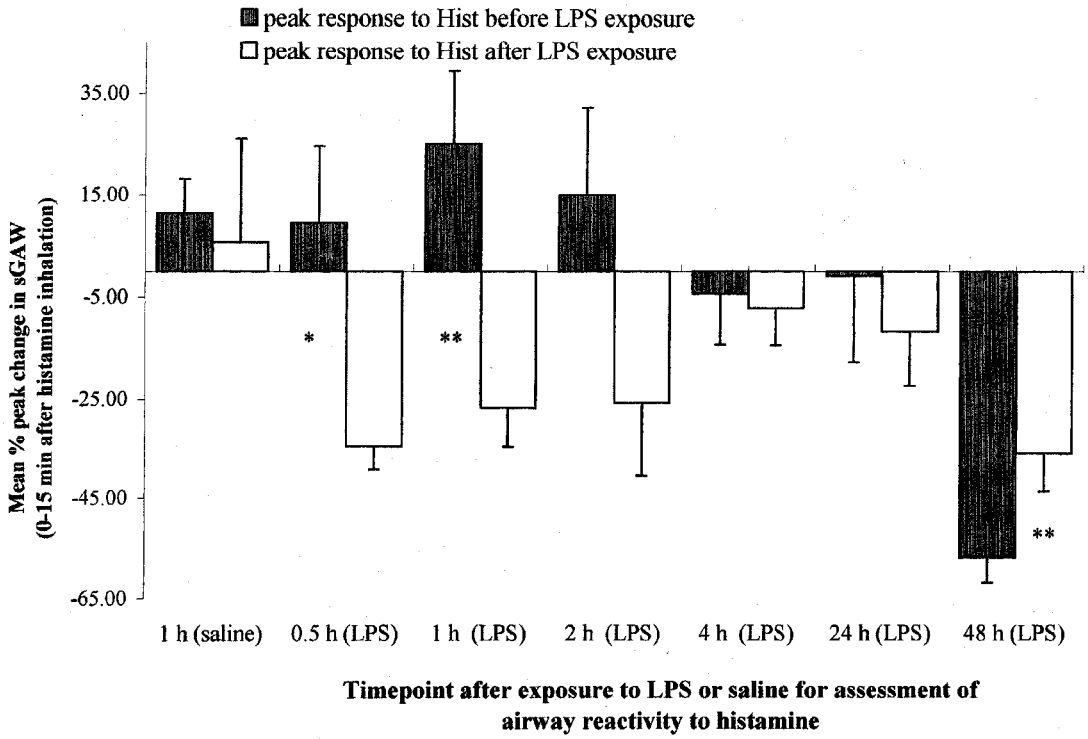

Figure 3 The peak responses of the airways to nose only exposures to histamine (Hist: 1 and 3 (48 h only) mM, 20 s) before and after exposure to nebulized LPS (30 μg ml^{-1}) or vehicle in conscious guinea-pigs. Each point represents the mean ± s.e.mean ($n=6$) of the individual peak change in sGaw from baseline (BL) sGaw values expressed as a percentage of the BL, after a nose-only exposure to Hist. Airway reactivity to Hist was assessed at 0.5 (BL sGaw (sec^{-1} cmH_2O^{-1}): before = 0.34 ± 0.01 and after = 0.39 ± 0.03), 1 (BL sGaw: before = 0.31 ± 0.03 and after = 0.31 ± 0.02), 2 (BL sGaw: before = 0.27 ± 0.01 and after = 0.26 ± 0.02), 4 (BL sGaw: before = 0.33 ± 0.01 and after = 0.30 ± 0.02), 24 (BL sGaw: before = 0.44 ± 0.04 and after = 0.48 ± 0.04), 48 (BL sGaw: before = 0.32 ± 0.01 and after = 0.35 ± 0.01) hours after exposure to LPS and at 1 h (BL sGaw: before = 0.46 ± 0.04 and after = 0.36 ± 0.02) after exposure to saline. Negative values represent bronchoconstriction. Significance of differences between the before and after exposure response to Hist were determined by analysis of variance (single factor), followed by a Student's paired *t*-test denoted as $*P<0.05$, $**P<0.01$. Airway reactivity to Hist, before and after vehicle exposure, or 4 and 24 h after LPS exposure was not significantly different from baseline sGaw values ($P>0.05$).

caused a significant bronchoconstriction ($-56.9\pm5.0\%$ decrease from baseline sGaw values). The same dose, 48 h after LPS, produced a significantly ($P<0.01$) reduced bronchoconstriction ($-35.9\pm6.7\%$ decrease from baseline sGaw values) compared to the pre-exposure response to histamine, indicating airway hyporeactivity (Figure 2c). Exposure to the LPS vehicle did not alter the airway responsiveness to histamine at 1 h after challenge (Figure 2a).

Effect of exposure to L-NAME on airway function

Exposure to nebulised L-NAME (1.2 or 12 mM, 15 min), in unchallenged animals, or 2 h after an exposure to LPS caused an immediate bronchoconstriction (unchallenged: 12 mM L-NAME $=-20.6\pm8.4\%$; LPS: 1.2 mM L-NAME $=-20.3\pm2.2\%$, 12 mM L-NAME $=-23.7\pm7.3\%$ decrease from BL sGaw) which recovered to baseline sGaw values 30 min later (data not shown). This immediate bronchoconstriction was not significantly ($P>0.05$) different, however, from that observed after saline exposure alone (Figure 1). There were no other changes in airway function that significantly deviated from the baseline sGaw values.

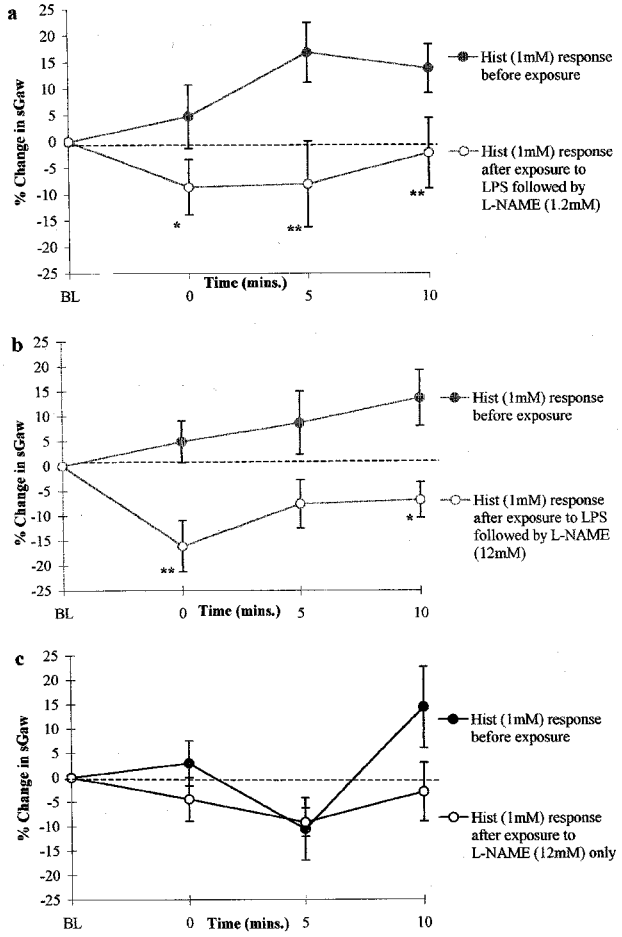

Figure 4 The effect of L-NAME (a: 1.2 mM, b & c: 12 mM) on the responsiveness of the airways to a nose-only exposure to histamine (Hist; 1 mM, 20 s): (a and b) before and 4 h after exposure to nebulized LPS, 105 min after nebulized L-NAME; (c) before and 105 min after exposure to nebulized L-NAME only, in conscious guinea-pigs. L-NAME was administered by inhalation (15 min), 2 h after the LPS exposure (60 min, a and b), or to naïve animals (c). Each point represents the mean $\pm$ s.e.mean ($n=6$) change in sGaw expressed as a percentage of the baseline (BL) sGaw values (BL sGaw ($sec^{-1}\,cmH_2O^{-1}$): (a) before $=0.40\pm0.02$ and after $=0.38\pm0.02$; (b) before $=0.43\pm0.04$ and after $=0.39\pm0.01$; (c) before $=0.34\pm0.01$ and after $=0.32\pm0.01$). Negative values represent bronchoconstriction. Airway reactivity to Hist, before and after nebulized vehicle exposure (saline) and L-NAME (1.2 or 12 mM, 15 min) was not significantly different ($P<0.05$, data not shown). Significance of differences between the before and after L-NAME exposure response to Hist was determined by analysis of variance (single factor), followed by a Student's paired *t*-test denoted as $^*P=0.02$, $^{**}P<0.01$.

Airway responsiveness to inhaled spasmogen after exposure to LPS followed by L-NAME

When exposure to nebulized LPS was followed 2 h later by L-NAME, a dose-dependent increase in reactivity to inhaled histamine was observed at 4 h after the LPS (L-NAME: 1.2 mM $=-8.6\pm5.2$, 12 mM $=-16.1\pm5.1$ peak per cent change from BL sGaw: Figure 4a,b, respectively). This contrasts with the lack of AHR to histamine, 4 h after exposure to LPS alone (Figure 3). The airway response to inhaled histamine was not significantly ($P>0.05$) different before or 105 min after a 15 min exposure to L-NAME with (data not shown), or without (Figure 4c) prior exposure to vehicle 2 h earlier. There was no statistically significant difference between starting baseline values of sGaw for any of the groups of animals studied (Figures 2, 3 and 4).

Leukocyte infiltration

There was a substantial increase in the number of neutrophils retrieved from the BAL fluid 30 min after the LPS exposure, with a progressive further increase up to 24 h. At 48 h after LPS exposure the recoverable neutrophil population subsided. Macrophages increased progressively up to 48 h after the LPS challenge, which was accompanied by a small increase in the number of eosinophils (Figure 5).

Compared with naïve animals, there was a significantly increased macrophage and eosinophil population in the BAL fluid, 105 min after exposure to nebulized L-NAME only (Figure 6). In animals exposed to vehicle followed by L-NAME, there was a significantly elevated number of macrophages and eosinophils recovered in the BAL fluid at 4 h after vehicle exposure, compared to vehicle alone. Exposure to nebulized L-NAME after LPS further increased the number of macrophages recovered in the BAL fluid at 4 h after LPS exposure, compared with the effect of LPS alone. However, the neutrophil influx into the lungs was not increased by L-NAME and L-NAME did not cause a significant ($P>0.05$) change to the LPS-induced increases in either neutrophil or eosinophil populations at 4 h.

Effect of exposure to LPS on nitric oxide metabolites recovered from BAL fluid

The changes in the spectrophotometrically determined concentrations of the individual NO metabolites (nitrate and nitrite) were synchronous. However, lower levels of nitrate were analysed, due to the rate limiting nitrate reductase conversion to measurable nitrite (Figure 7). Therefore, to describe changes in the assayed NO metabolites, the combined nitrate and nitrite levels are referred to.

After the LPS exposure, the concentration of NO metabolites significantly decreased in the BAL fluid during the period of AHR (1 and 2 h) (Figure 7). However, 1 h after vehicle exposure the concentration of NO metabolites significantly increased, compared with naïve animals. The NO metabolites concentration in BAL fluid at 4 h after the LPS exposure, was not significantly different ($P>0.05$) to that

recovered at 1 h after the vehicle exposure. At 24 h, there was no significant difference ($P>0.05$) in NO metabolite concentration between naïve, LPS or vehicle exposed groups. During the period of hyporeactivity (48 h after LPS exposure), the concentration of NO metabolites significantly increased ($P<0.001$) above the vehicle control.

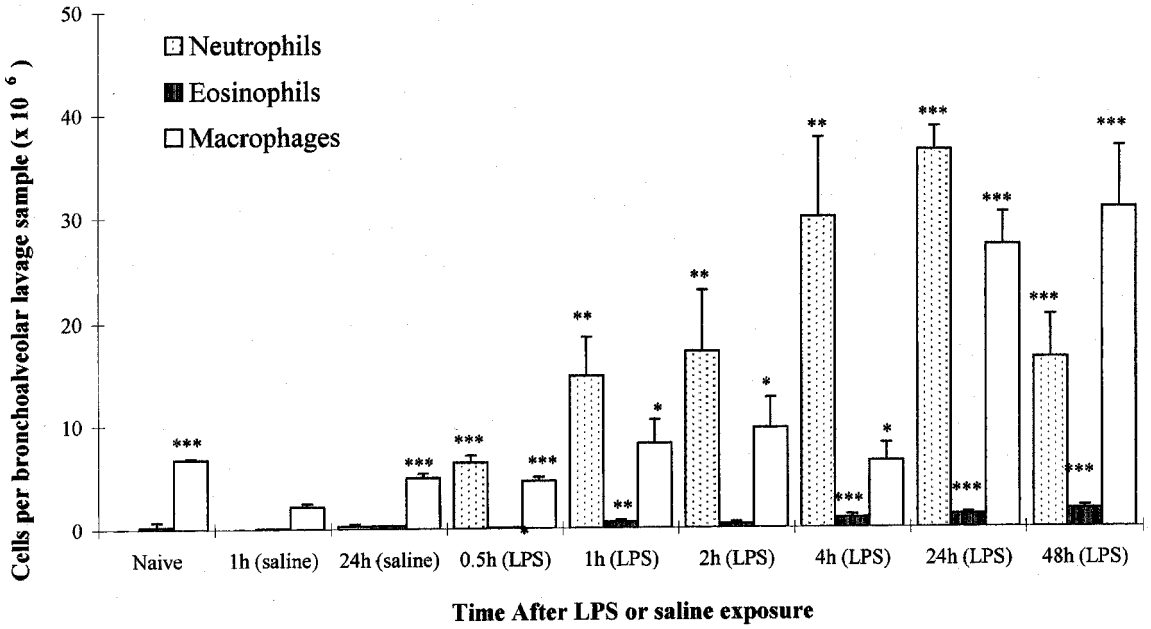

Figure 5 Differential cell (macrophage, eosinophils, neutrophils) counts of bronchoalveolar lavage (BAL) fluid removed from guinea-pigs before (naïve) and after exposure (60 min) to nebulized LPS (30 μg ml^{-1}: 0.5, 1, 2, 4, 24 and 48 h), or vehicle (saline: 1 and 24 h). Each point represents the mean$\pm$s.e.mean ($n=6$) of the differential cells per sample ($\times 10^6$). Significance of differences in the cells retrieved after LPS exposure were compared with cells at 1 h after vehicle exposure and determined by analysis of variance (single factor), followed by a Student's unpaired t-test indicated as $^*P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

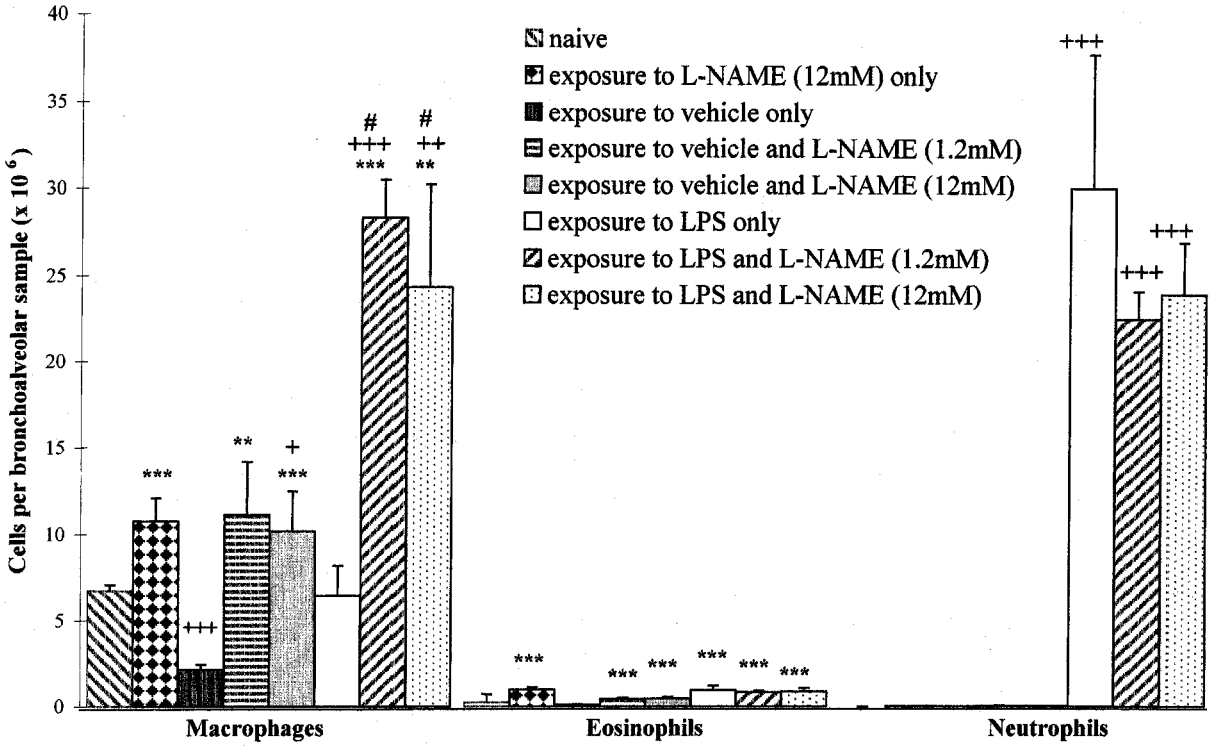

Figure 6 Differential cell counts in bronchoalveolar lavage (BAL) fluid removed from naïve guinea-pigs and after exposure to: nebulized (15 min) L-NAME only (12 mM, 105 min after exposure); nebulized (60 min) LPS only (30 μg ml^{-1}, 4 h after exposure); LPS and nebulized (15 min, 2 h after LPS exposure) L-NAME (1.2 mM and 12 mM, 4 h after LPS exposure); nebulized (60 min) vehicle only (saline, 1 h after exposure); or vehicle and nebulized (15 min, 2 h after vehicle exposure) L-NAME (1.2 mM and 12 mM, 4 h after vehicle exposure). Each point represents the mean$\pm$s.e.mean ($n=6$) of the differential cells per sample ($\times 10^6$). Significance of differences from naïve ($^+P<0.05$, $^{++}P<0.01$ and $^{+++}P<0.001$), from 1 h after vehicle control ($^{**}P<0.01$ and $^{***}P<0.001$), or from 4 h after LPS exposure ($^{\#}P<0.001$) was determined by analysis of variance (single factor), followed by a Student's unpaired t-test.

Exposure to nebulized L-NAME (1.2 or 12 mM, 15 min) in unchallenged animals, or 2 h after an exposure to vehicle, caused a significant reduction in the BAL fluid NO metabolites 105 min later, compared to concentrations in naïve animals, or at 1 h after vehicle (Figure 8). Similarly, L-NAME exposure after LPS significantly decreased the concentration of NO metabolites in the BAL fluid at 4 h, compared to concentrations at 4 h after LPS exposure alone, or in naïve animals. With or without prior exposure to LPS or vehicle 2 h earlier, L-NAME exposure attenuated the production of NO metabolites in animals 105 min later, to levels below that recovered from the BAL fluid during the

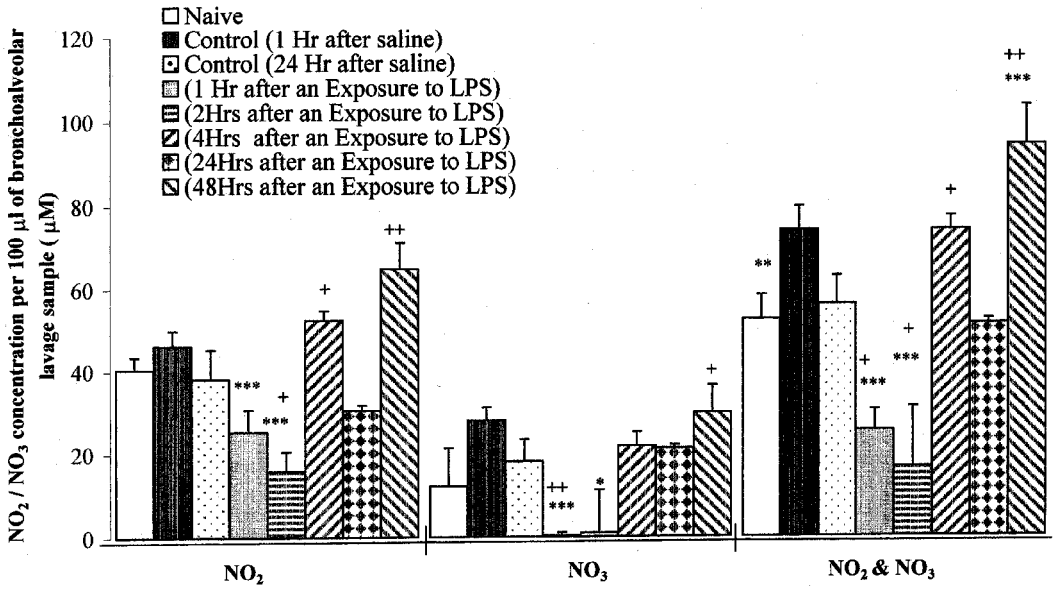

Figure 7 Nitric oxide (NO) production, determined as the individual and combined metabolites (NO_2 and NO_3) recovered from guinea-pig bronchoalveolar lavage (BAL) fluid before (naïve) and after exposure (60 min) to nebulized LPS (30 μg ml^{-1}: 1, 2, 4, 24, 48 h), or the vehicle (saline: 1 and 24 h). Each point represents the mean$\pm$s.e.mean ($n=6$) of the NO metabolite concentration. Significant differences in the NO metabolites were determined by analysis of variance (single factor), followed by a Student's unpaired t-test. Compared with: 1 h after saline exposure ($^{*}P=0.02$, $^{**}P<0.01$ and $^{***}P<0.001$) and naïve animals ($^{+}P<0.01$ and $^{++}P<0.001$).

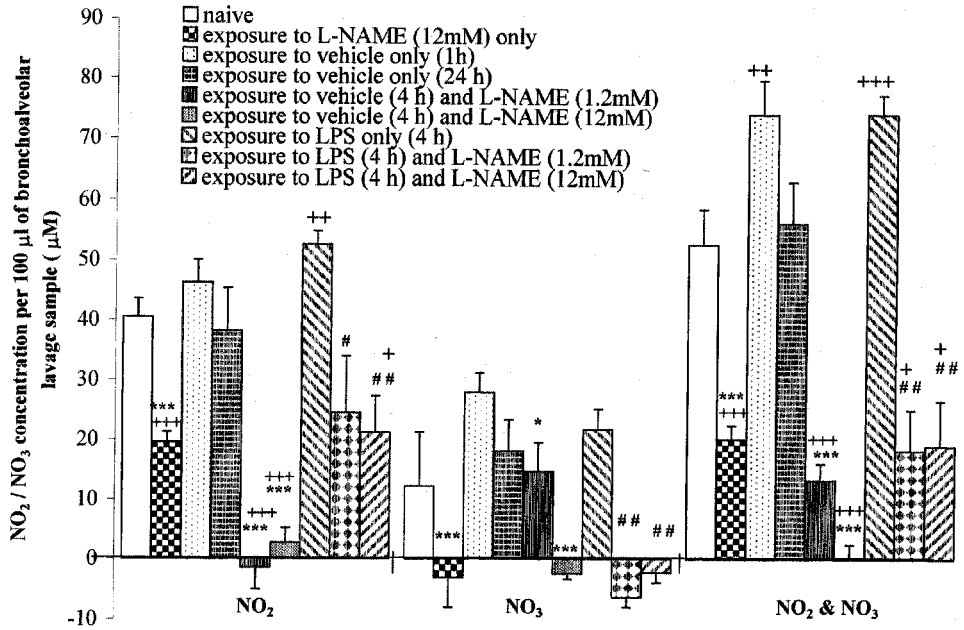

Figure 8 Nitric oxide (NO) production, determined as the individual and combined metabolites (NO_2 and NO_3) recovered from guinea-pig bronchoalveolar lavage (BAL) fluid before (naïve) and after exposure to: nebulized (60 min) LPS only (30 μg ml^{-1}, BAL: 4 h after exposure); nebulized (15 min) L-NAME only (12 mM, BAL: 105 min after exposure); LPS and nebulized L-NAME (15 min, 2 h after LPS exposure, 1.2 mM and 12 mM, BAL: 4 h after LPS exposure); nebulized vehicle only (saline, BAL: 1 and 24 h after exposure); or vehicle and nebulized L-NAME (15 min, 2 h after vehicle exposure, 1.2 mM and 12 mM, BAL: 4 h after vehicle exposure). Each point represents the mean$\pm$s.e.mean ($n=6$) of the NO metabolites concentration. Significance of differences in the NO metabolites between naïve animals ($^{+}P<0.05$, $^{++}P<0.01$ and $^{+++}P<0.001$), 1 h after vehicle control ($^{*}P<0.05$ and $^{***}P<0.001$) and 4 h after LPS ($^{\#}P<0.01$, $^{\#\#}P<0.001$) exposed animals were determined by analysis of variance (single factor), followed by a Student's unpaired t-test.

period of LPS induced-AHR (1–2 h after LPS alone) (compare Figures 7 and 8).

Discussion

Epidemiological studies have shown that the degree of AHR, an increased responsiveness of the airways to non-specific stimuli, correlates with the severity of COPD and asthma (Rijcken *et al*., 1997). These respective neutrophilic and eosinophilic inflammatory diseases are also associated with an elevated pulmonary production of NO, measured in exhaled air and as NO metabolites in BAL fluid (Kanazawa *et al*., 1998). Animal models have been developed to study antigen-induced airway hyperreactivity (Sanjar & Morley, 1990; Johnson & Broadley, 1999). However, there are few *in vivo* models that simulate AHR associated with a neutrophilic condition, as in COPD. Most studies have utilized anaesthetized animals to determine lung function, which may influence vagal tone or sensory reflexes. In the present study, lung function was examined in conscious animals. Using a similar method, Iijima *et al*. (1998) have demonstrated in antigen-exposed atopic guinea-pigs, an increase in exhaled NO during the early and late airway response. Schuiling *et al*. (1998), in contrast, proposed that a *deficiency* in NO contributes to the AHR after the early asthmatic response and a recovery in NO production aids the reversal of AHR after the late asthmatic response. However, Alving *et al*., (1993) compared the production of NO in air exhaled through the nose or the mouth and suggested that in normal human airways, NO production is restricted to the nasal mucosa. In asthmatics, the level of exhaled NO produced during oral breathing was increased 2–3 fold, indicating lower airway involvement. Since the guinea-pig is an obligatory nose-breather (Finney & Firsberg, 1994), airway-produced NO in expired air may be masked by a nasal production. Consequently, NO (metabolites) determination in BAL fluid in the current study, although invasive and restricted to a single time point, allows a more accurate indication of airway NO synthesis (Kanazawa, *et al*., 1998). The aqueous environment in the lung with an acidic pH can influence the spontaneous autoxidation product of NO (Grisham *et al*., 1996). Consequently, we assayed for the two prominent oxidized metabolites formed from NO (NO_2 and NO_3), although the physiological pathway for the autoxidation of NO to these derivatives is unclear (Moncada, 1992).

In the present study, inhalation of LPS caused an early development of AHR to histamine. From 2–24 h after the exposure, airway reactivity was restored. However, 48 h after LPS exposure, the airways expressed a decreased responsiveness, or hyporeactivity, to histamine. Young & Nicholls (1997) have documented a similar biphasic change in airway reactivity to methacholine after exposure to LPS. Isolated tracheal spirals from guinea-pigs exposed to LPS have also been shown to exhibit hyperreactivity (Young & Nicholls, 1996) and hyporeactivity (Folkerts *et al*., 1988) to histamine and carbachol. This suggests that the LPS-induced changes in airway reactivity are not specific to histamine and in common with asthmatics and COPD patients, non-specific stimuli (histamine, methacholine or cold air) can be used to assess AHR (Postma & Kerstjens, 1998).

Many studies have documented the prominence of the neutrophil after an administration of LPS, either *via* systemic or inhaled routes (Snella & Rylander, 1985; Brigham & Meyrick, 1986; Lefort *et al*., 1998). However, few have documented the time-course of leukocyte infiltration into the lungs and correlated this with changes in airway reactivity after LPS inhalation. In the present study, there was a rapid influx of neutrophils into the airways 30 min after LPS exposure, which persisted for 24 h. This finding is in agreement with Brigham & Meyrick (1986), who noted a 3 fold increase in the number of neutrophils in the airways, 15 min after the start of an LPS infusion. Although delayed in comparison, there was also a time-dependent increase in the macrophage and eosinophil population recovered from the BAL fluid. The difference in cell population recovered in BAL fluid from naïve animals and at 1 or 24 h after saline was probably due to a slight bronchoconstriction at 1 h after saline exposure, which would reduce airway calibre and reduce lavage reclamation. The mechanism for the LPS-induced neutrophilic inflammation is likely to be initially orchestrated from chemotactic factors, such as TNF-α and interlukin-8 (IL-8), released into the airways by resident macrophages (Snella & Rylander, 1985), epithelial cells and lymphocytes (Brigham & Meyrick, 1986; Kuo *et al*., 1997). Kips *et al*. (1992) showed in rats exposed to LPS, that inhibition of TNF-α reduces the neutrophil influx and AHR. The early synthesis of TNF-α in response to LPS (Goncalves de Moraes *et al*., 1996) activates other pro-inflammatory mediators including deleterious cytotoxins (proteases and superoxides), arachidonic acid metabolites, nuclear transcription factors (e.g. NF-κB) and cell adhesion molecules (Brigham & Meyrick, 1986; Albelda *et al*., 1994; Barnes & Adcock, 1997). Bronchial biopsies from patients with COPD show similar inflammatory processes and sputum samples have elevated TNF-α, IL-8 and proteolytic enzyme levels (Barnes, 1998). In the present study, the LPS-induced AHR resolved at 4 h, well before the peak cell influx occurred (neutrophils at 24 h, eosinophils and macrophages at least 48 h). This infers that either: cell influx into the airways and AHR are discrete symptoms; or that protective mechanism(s) mask the underlying AHR that persists with the cell inflammation. AHR to histamine did not occur at 1 h after a saline challenge, although it caused a similar direct bronchoconstriction to the LPS challenge. Thus, the AHR is not related to this common transient bronchoconstriction and it was not considered necessary to examine for AHR at subsequent times after the saline challenge.

Exposure to saline initially increased NO release, possibly activated from the 'shear stress' to epithelial cells, which would oppose the transient saline-induced bronchoconstriction with a cyclic GMP-dependent smooth muscle relaxation (Schulz & Triggle, 1994). By contrast, exposure to LPS initially inhibited NO synthesis and the reduced NO levels coincided with the period of AHR. This is opposite to the observation of an elevated level of expired NO following antigen exposure (Iijima *et al*., 1998). During the recovery from AHR there was an increase in NO production, followed by a return to levels found in naïve animals. NO metabolites in BAL fluid were then further enhanced during the period of hyporeactivity. This evidence suggests that an impaired cNOS ability to produce NO contributes to the AHR immediately after LPS exposure. Using a perfused airway preparation, De Boer *et al*. (1996) also concluded that hyperreactivity after the allergen-induced early asthmatic reaction was associated with a NO deficiency. De Boer *et al*. (1999) also demonstrated that a limitation of the NOS substrate (L-arginine) may underlie the reduced cNOS activity and subsequent AHR after the early asthmatic reaction. The current study also suggests that the period of airway hyporeactivity coincides with an over-production of NO,

possibly from NF-κB activation of the iNOS gene (Liu *et al*., 1993). This is in agreement with Kips *et al*. (1995), who also concluded that the LPS-induced hyporesponsiveness is NO-mediated, the NO being synthesized from iNOS. At 105 min after L-NAME inhalation the NO metabolites in BAL fluid were reduced to levels equivalent to those observed during the period of AHR (1–2 h after LPS). A similar inhaled dose of L-NAME was used by Nijkamp *et al*. (1993) who demonstrated a significant AHR to histamine in anaesthetized guinea-pigs. However, in our model exposure to L-NAME with or without prior exposure to saline 2 h earlier did not induce a significant AHR to inhaled histamine. This was probably because we used a shorter period of exposure to L-NAME and assessed AHR 105 min later than Nijkamp *et al*. (1993). Although the levels of NO (metabolites) were significantly reduced at the time of assessing airway reactivity after L-NAME, it is possible that other anti-AHR mechanism(s) compensate for the deficiency of endogenous NO and prevent AHR from occurring. Exposure to LPS followed by L-NAME caused a significantly increased bronchoconstriction to histamine, prolonging the duration of AHR to 4 h. This data suggests that a deficiency of NO alone is not responsible for the LPS-induced AHR and for AHR to occur, other LPS generated AHR mediators are required. However, the initial recovery from LPS-induced AHR is NO-dependent. Whether the NO production is from a cNOS or iNOS isoform is unknown, but implies that NO acts as a functional antagonist to the exaggerated histamine-induced bronchoconstriction.

Different mechanisms may account for the LPS-induced NO deficiency promoting AHR. L-NAME increases mucus production and inhibits mucociliary clearance (Barnes, 1999), which could reduce airway calibre and thus potentiate the airway resistance from a given bronchoconstrictor (Pare & Hogg, 1989; Postma & Kersjens, 1998). However, iNOS 'knock-out' mice produce reduced oedema after LPS exposure, which would increase airway calibre and thus reduce airway resistance from a given bronchoconstrictor (Kristof, *et al*., 1998). Also, histological examination of the lungs from the present study showed no morphological features to support a geometric reduction in airway calibre (unpublished observations) and the rapid onset of AHR after LPS exposure suggests a biochemical mechanism. Interestingly, Folkerts *et al*. (1989) have demonstrated in isolated tracheal spirals from guinea-pigs pre-treated with LPS, a decreased prostaglandin E_2 (PGE_2) production by the epithelial layer. The PGE_2-induced second messenger, adenosine 3′,5′ cyclic monophosphate (cyclic AMP), like the NO-induced second messenger cyclic GMP, causes airway smooth muscle relaxation (Hakonarson & Grunstein, 1998). These two second messengers can act synergistically to enhance their activity *via* a cyclic GMP inhibition of cyclic AMP phosphodiesterase (Maurice & Haslam, 1990). Consequently, a reduced NO and PGE_2 production and respective cyclic GMP and cyclic AMP formation after exposure to LPS, could augment airway smooth muscle contractility to histamine. This could also explain the antagonistic effects of phosphodiesterase inhibitors on LPS-induced AHR (Uno *et al*., 1998). The reaction of NO and superoxide products from activated cells to form the toxic metabolite peroxynitrite is unlikely to cause the AHR observed in the present study, as NO synthesis is reduced. However, after sustained LPS exposure, peroxynitrite may be detrimental.

The degree of cellular infiltration into the airways is determined by three main stages: rolling and firm adhesion to the bronchial microvasculature, trans-endothelial and -epithelial penetration and chemotactic migration (Hellewell, 1999). In the current study, L-NAME inhalation enhanced macrophage and eosinophil influx into the airways of unchallenged and LPS, or saline challenged animals, but slightly reduced the LPS-induced neutrophil infiltration. NO has been shown to inhibit leukocyte adherence to endothelial cells and migration (Kubes *et al*., 1991; Bloomfield *et al*., 1997), which would explain the L-NAME-induced augmentation in eosinophil and macrophage influx. Also, Kips *et al*. (1995) found that L-NAME did not influence the LPS-induced increase of neutrophils in BAL fluid. However, contrary to these findings, in an IL-8-dependent mechanism, the LPS-induced TNF-α production acts as a potent neutrophil chemotractant, which is potentiated by pre-treatment with L-NAME (Kuo *et al*., 1997). Similarly, the role of NO in vascular permeability is controversial and conflicting results have been reported. NOS inhibitors applied to the airway surface increase plasma exudation, suggesting that basal release of NO has an inhibitory effect on microvascular leakage (Erjefalt *et al*., 1994). However, endogenous NO may increase the exudation of plasma by increasing blood flow to leaky post-capillary venules (Kuo, *et al*., 1992). Li *et al*. (1998) have also demonstrated that L-NAME inhibited an LPS-induced increase in epithelial permeability. The role of NO in inflammatory cell infiltration into the airways is complex as NO appears capable of contrarily influencing the three main stages by which inflammatory cells may infiltrate the airways. The inflammatory condition and the degree of NO synthesis are likely to affect the regulation of these processes, which may explain the discrepancies in the reported role of NO in cell influx into the airways.

In summary, this study demonstrates that aerosolized LPS inhalation causes an acute AHR to histamine in conscious guinea-pigs, which coincides with a deficiency in cNOS synthesised NO. The recovery of airway reactivity appears to be associated with increased NO levels, although the NOS isoform involved is unclear. The LPS-induced airway hyporeactivity observed at 48 h was associated with over-production of NO, most likely from an iNOS source. Reduced NO production after LPS exposure may also enhance the influx of eosinophils and macrophages, but not the predominant neutrophil infiltration.

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References

ALBELDA, S.M., SMITH, C.W. & WARD, P.A. (1994). Adhesion molecules and inflammatory injury. *FASEB. J.,* **8,** 504–512.

ALVING, K., WEITZBERG, E. & LUNDBERG, J.M. (1993). Increased amount of nitric oxide in exhaled air of asthmatics. *Eur. Respir. J.,* **6,** 1368–1370.

BARNES, P.J. (1998). Chronic obstructive pulmonary disease: new opportunities for drug development. *Trends Pharmacol. Sci.,* **19,** 415–423.

BARNES, P.J. & ADCOCK, I.M. (1997). NF-κB: a pivotal role in asthma and a new target for therapy. *Trends Pharmacol. Sci.,* **18,** 46 – 51.

BARNES, P.J. & BELVISI, M.G. (1993). Nitric oxide and lung disease. *Thorax,* **48,** 1034 – 1043.

BARNES, P.J., CHUNG, K.F. & PAGE, C.P. (1999). Inflammatory mediators of asthma: an update. *Pharmacol. Rev.,* **50,** 515 – 575.

BHOWMIK, A., SEEUNGAL, T., ROLAND, M. & WEDZICHA, J.A. (1998). Exhaled nitric oxide in patients with COPD. Proceedings from the British Thoracic Society winter meeeting. *Thorax,* **53,** 158 P.

BLOOMFIELD, G.L., HOLLOWAY, S., RIDINGS, P.C., FISCHER, B.J., BLOCHER, C.R., SHOLLEY, M., BUNCH, T., SUGERMAN, H.J. & FOWLER, A.A. (1997). Pretreatment with inhaled nitric oxide inhibits neutrophil migration and oxidative activity resulting in attenuated sepsis-induced acute lung injury. *Crit. Care Med.,* **25,** 584 – 593.

BRIGHAM, K.L. & MEYRICK, B. (1986). State of art: endotoxin and lung injury. *Am. Rev. Respir. Dis.,* **133,** 913 – 927.

CORRADI, M., MAGATI, M., CACCIANI, G.C., CONSIGLI, G.F., DE MUNARI, E. & PESCI, A. (1999). Increased exhaled nitric oxide in patients with stable chronic obstructive pulmonary disease. *Thorax,* **54,** 572 – 575.

DANAHAY, H. & BROADLEY, K.J. (1997). Effects of inhibitors of phosphodiesterase, on antigen-induced bronchial hyperreactivity in conscious sensitised guinea-pigs and airway leukocyte infiltration. *Br. J. Pharmacol.,* **120,** 289 – 297.

DE BOER, J., DUYVENDAK, M., SCHUURMAN, F.E., POUW, F.M.H., ZAAGSMA, J. & MEURS, H. (1999). Role of L-arginine in the deficiency of nitric oxide and airway hyperreactivity after the allergen-induced early asthmatic reaction in guinea-pigs. *Br. J. Pharmacol.,* **128,** 1114 – 1120.

DE BOER, J., MEURS, H., COERS, W., KOOPAL, BOTTONE, A.E., VISSER, A.C., TIMENS, W. & ZAAGSMA, J. (1996). Deficiency of nitric oxide in allergen-induced airway hyperreactivity to contractile agonists after the early asthmatic reaction: an ex-vivo study. *Br. J. Pharmacol.,* **119,** 1109 – 1116.

ERJEFALT, J.S., ERJEFALT, I., SUNDLER, F. & PERSSON, C.G. (1994). Mucosal nitric oxide may tonically suppress airway plasma exudation. *Am. J. Crit. Care Med.,* **150,** 227 – 232.

FINNEY, M.J. & FIRSBERG, K.J. (1994). Quantification of nasal involvement in a guinea-pig plethysmograph. *J. Appl. Physiol.,* **76,** 1432 – 1438.

FOLKERTS, G., ENGELS, F. & NIJKAMP, F.P. (1989). Endotoxin-induced hyperreactivity of the guinea-pig isolated trachea coincides with decreased prostaglandin E_2 production by the epithealial layer. *Br. J. Pharmacol.,* **96,** 388 – 394.

FOLKERTS, G., HENRICKS, P.A.J., SLOOTWEG, P.J. & NIJKAMP, F.P. (1988). Endotoxin-induced inflammation and injury of the guinea-pig respiratory airways cause hyporeactivity. *Am Rev. Respir. Dis.,* **137,** 1441 – 1448.

FOLKERTS, G. & NIJKAMP, F.P. (1998). Airway epithelium: more than just a barrier!. *Trends Pharmacol. Sci.,* **19,** 334 – 341.

GASTON, B., DRAZEN, J.M., LOSCALZO, J. & STAMLER, J.S. (1994). The biology of nitrogen oxides in the airways. *Am. J. Respir. Crit. Care Med.,* **149,** 538 – 551.

GIAID, A., SALEH, D., LIM, S., BARNES, P.J. &ERNST, P. (1998). Formation of peroxynitrite in asthmatic airways. *FASEB. J.,* **12,** 929 – 937.

GONCALVES DE MORAES, V.L., VARGAFTIG, B.B., LEFORT, J., MEAGER, A. & CHIGNARD, M. (1996). Effect of cyclo-oxygenase inhibitors and modulators of cyclic AMP formation on lipopolysaccharide-induced neutrophil infiltration in mouse lung. *Br. J. Pharmacol.,* **117,** 1792 – 1796.

GRIFFITHS-JOHNSON, D.A., NICHOLLS, P.J. & McDERMOTT, M. (1988). Measurement of specific airways conductance in guinea-pigs. A non-invasive method. *J. Pharmacol. Meth.,* **19,** 233 – 342.

GRISHAM, M.B., JOHNSON, G.G. & LANCASTER. J.R. (1996). Quantification of nitrate and nitrite in extracellular fluids. *Meth. Enzymology,* **268,** 237 – 246.

HAKONARSON, H. & GRUNSTEIN, M.M. (1998). Regulation of second messengers associated with airway smooth muscle contraction and relaxation. *Am J. Respir. Crit. Care Med.,* **158,** S115 – S122.

HELLEWELL, P.G. (1999). Adhesion molecule strategies. *Pulmonary Pharmacol. & Ther.,* **12,** 137 – 141.

IIJIMA, H., UCHIDA, Y., ENDO, T., XIANG, A., SHIRATO, M., NOMURA, A. & HASEGAWA, S. (1998). Role of endogenous nitric oxide in allergen-induced airway responses in guinea-pigs. *Br. J. Pharmacol.,* **124,** 1019 – 1028.

JOHNSON, A. & BROADLEY, K.J. (1999). Airway hyperresponsiveness in anaesthetised guinea-pigs 18 – 24 hours after antigen inhalation dose not occur with all intravenously administered spasmogens. *Pharmacol. & Toxicol.,* **84,** 281 – 287.

KANAZAWA, H., SHOJI, S., YOSHIKAWA, T., HIRATA, K. & YOSHIKAWA, J. (1998). Increased production of endogenous nitric oxide in patients with bronchial asthma and chronic obstructive pulmonary disease. *Clin. Exp. Allergy,* **10,** 1244 – 1250.

KIPS, J.C., LEFEBVRE, R.A., PELEMAN, R.A. & JOOS, G.F. (1995). The effect of a nitric oxide synthase inhibitor on the modulation of airway responsiveness in rats. *Am. J. Respir. Crit. Care Med.,* **151,** 1165 – 1169.

KIPS, J.C., TAVERNIER, J. & PAUWELS, R.A. (1992). Tumour necrosis factor causes bronchial hyperresponsiveness in rats. *Am. Rev. Respir. Dis.,* **145,** 332 – 336.

KRISTOF, A.S., GOLDBERG, P., LAUBACH, V. & HUSSAIN, S.N.A. (1998). Role of inducible nitric oxide synthase in endotoxin-induced acute lung injury. *Am. J. Respir. Crit. Care Med.,* **158,** 1883 – 1889.

KUBES, P., SUZUKI, M. & GRANGER, D.N. (1991). Nitric oxide: an endogenous modulator of leukocyte adhesion. *Proc. Natl. Acad. Sci. USA,* **88,** 4651 – 4655.

KUO, H.-P., HWANG, K.-H., LIN, H.-C., WANG, C.-H. & LU, L.-C. (1997). Effect of endogenous nitric oxide on tumour necrosis factor-α-induced leukosequestration and IL-8 release in guinea-pigs airways *in vivo*. *Br. J. Pharmacol.,* **122,** 103 – 111.

KUO, H.-P. LIU, S. & BARNES, P.J. (1992). The effect of endogenous nitric oxide on neurogenic plasma exudation in guinea-pig airways. *Eur. J. Pharmacol.,* **221,** 385 – 388.

LEFORT, J., SINGER, M. & LEDUC, D. (1998). Systemic administration of endotoxin induces bronchopulmonary hyperreactivity dissociated from TNF-alpha formation and neutrophil sequestration into the murine lungs. *J. Immunol.,* **161,** 474 – 480.

LI, X.Y., DONALDSON, K. & MACNEE, W. (1998). Lipopolysaccharide-induced alveolar epithelial permeability: the role of nitric oxide. *Am. J. Respir. Crit. Care Med.,* **157,** 1027 – 1033.

LIU, S., ADCOCK, I.M., OLD, R.W., BARNES, P.J. & EVANS, T.W. (1993). Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem. Biophys. Res. Commun.,* **196,** 1208 – 1213.

LOWENSTEIN, C.J., ALLEY, E.W., RAVAL, P., SNOWMAN, A.M., SNYDER, S.H., RUSSELL, S.W. & MURPHY, W.J. (1993). Macrophage nitric oxide synthase gene: two upstream regions mediate induction by interferon-γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA,* **90,** 9730 – 9734.

MAURICE, D.H. & HASLAM, R.J. (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol. Pharmacol.,* **37,** 671 – 681.

MONCADA, S. (1992). The L-arginine: nitric oxide pathway. *Acta Physiol. Scand.,* **145,** 201 – 227.

NATHAN, C.F. (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J.,* **6,** 3051 – 3064.

NIJKAMP, F.P. & FOLKERTS, G. (1995). Nitric oxide and bronchial hyperresponsiveness. *Arch. Int. Pharmacodyn.,* **329,** 97 – 110.

NIJKAMP, F.P., VAN DER LINDE, H.J. & FOLKERTS, G. (1993). Nitric oxide synthesis inhibitors induce airway hyperresponsiveness in the guinea-pig in vivo and in vitro: Role of the epithelium. *Am. Rev. Respir. Dis.,* **148,** 727 – 734.

PARE, P.D. & HOGG, J.C. (1989). Mechanics of airway narrowing. *Am. Rev. Respir. Dis.,* **139,** 242 – 246.

POSTMA, D.S. & KERSTJENS, H.A.M. (1998). Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. *Am J. Respir. Crit. Care Med.,* **158,** S187 – S192.

RIJCKEN, A., SCHOUTEN, J.P. & WEISS, S.T. (1997). Airways responsiveness and development and remission of chronic respiratory disease in adults. *Lancet,* **350,** 1431 – 1434.

SANJAR, S. & MORLEY, J. (1990). Antigen-challenge induces pulmonary eosinophil accumulation and airway hyperreactivity in sensitised guinea-pigs: the effect of anti-asthma drugs. *Br. J. Pharmacol.,* **99,** 679 – 686.

SCHINI-KERTH, V.B. & VANHOUTTE, P.M. (1995). Review article: nitric oxide synthases in vascular cells. *Exp. Physiol.,* **80,** 885 – 905.

SCHUILING, M., ZUIDHOF, A.B., BONOUVRIE, M.A.A., VENEMA, N., ZAAGSMA, J. & MEURS, H. (1998). Role of nitric oxide in the development and partial reversal of allergen-induced airway hyperreactivity in conscious, unrestrained guinea-pigs. *Br. J. Pharmacol.,* **123,** 1450 – 1456.

SCHULTZ, R. & TRIGGLE, C.R. (1994). Role of NO in vascular smooth muscle and cardiac muscle function. *Trends Pharmacol. Sci.,* **15,** 255 – 259.

SNELLA, M.C. & RYLANDER, R. (1985). Endotoxin inhalation induces neutrophil chemotaxis by alveolar macrophages. *Agents Actions.,* **16,** 521 – 526.

TOWARD, T.J. & BROADLEY, K.J. (1999). Characteristics of airway hyperreactivity, cell influx and steroid sensitivity after inhaled LPS in conscious guinea-pigs. *Br. J. Pharmacol.,* **128,** 275P.

TOWARD, T.J. & BROADLEY, K.J. (2000). The role of NO on changes in airway reactivity induced by exposure of guinea-pigs to inhaled LPS. *Br. J. Pharmacol.,* **129,** 228P.

UNO, T., TANAKA, H. & NAGAI, H. (1998). Effects of isoenzyme selective phosphodiesterase inhibitors on bacterial lipopolysaccharide-induced bronchial hyperreactivity in guinea-pigs. *Gen. Pharmacol.,* **30,** 167 – 173.

VRUGT, B. & AALBERS, R. (1993). Inflammation and bronchial hyperresponsiveness in allergic asthma and chronic obstructive pulmonary disease. *Respir. Med.,* **87,** B3 – 7.

XIE, Q.-W., KASHIWABARA, Y. & NATHAN, C. (1994). Role of transcription factor NF-κB/ Rel in induction of nitric oxide synthase. *J. Biol. Chem.,* **269,** 4705 – 4708.

YOUNG, R.S. & NICHOLLS, P.J. (1996). A comparison of the effects of LPS and detoxified LPS on reactivity changes induced in the guinea-pig airways. *Proceedings from the 20th Cotton Dust Research Conference; Nashville Tennessee, U.S.A.* 333 – 336.

YOUNG, R.S. & NICHOLLS, P.J. (1997). A comparison of the pulmonary effects of lipopolysaccharide, Lipid A and detoxified lipopolysaccharide in the guinea-pig. *Proceedings from the Beltwide Cotton Dust Research Conference; New Orleans, Louisiana, U.S.A.* 208 – 210.

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