Chronic Lipopolysaccharide Exposure on Airway Function, Cell Infiltration, and Nitric Oxide Generation in Conscious Guinea Pigs: Effect of Rolipram and Dexamethasone

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ABSTRACT

This study investigated whether a correlation between airway hyperreactivity (AHR), leukocyte influx, and nitric oxide (NO) existed in guinea pigs chronically exposed to lipopolysaccharide (LPS). The effect of the corticosteroid, dexamethasone, or phosphodiesterase-4 (PDE4) inhibitor, rolipram, on these features was studied. Airway function was measured in conscious guinea pigs (specific airways conductance) before and after single, double, or chronic (nine) LPS (30 μg·ml⁻¹·h) exposures. Airway reactivity to inhaled histamine (1 mM, 20 s) was assessed before and at various times after LPS challenges. Leukocytes and NO metabolites were measured in bronchoalveolar lavage fluid (BALF). AHR occurred at 1 h after a single LPS challenge and was resolved by 4 h. This coincided with reduction and recovery, respectively, of BALF NO levels. The AHR and NO deficiency were extended to 4 h, after a double LPS exposure. Chronic LPS exposures, 48 h apart, initially caused persistent bronchodilations, whereas later exposures produced progressively persistent bronchoconstrictions. There was AHR 24 h after the eighth challenge. Twenty-four hours after the ninth LPS exposure, macrophages, neutrophils, eosinophils, and NO metabolites were elevated in BALF. Dexamethasone (20 mg·kg⁻¹·i.p.) or rolipram (1 mg·kg⁻¹·i.p.) prevented single and chronic LPS-induced AHR, the respective deficiency and elevation in NO metabolites, and the chronic LPS-induced leukocyte influx. Dexamethasone exacerbated, whereas rolipram reversed, the chronic LPS-induced bronchoconstrictions. This study demonstrates for the first time that chronic LPS causes persistent bronchoconstriction, neutrophilic inflammation, AHR, and NO overproduction in guinea pig airways. These rolipram-sensitive features suggest the potential of PDE4 inhibitors in airway disease.

Exaggerated constrictor responses of the airways [airway hyperreactivity (AHR)] and persistent pulmonary inflammation, driven by neutrophils or eosinophils, respectively, are characteristic features of chronic obstructive pulmonary disease (COPD) and asthma (Postma and Kerstjens, 1998). Patients with COPD (Agusti et al., 1999) and asthma (Saleh et al., 1998) exhale increased levels of nitric oxide (NO).

In normal lungs, NO is synthesized by the constitutive nitric-oxide synthase (cNOS) isoforms expressed in nonadrenergic noncholinergic neurones (nNOS), pulmonary vascular endothelium (eNOS), and airway epithelial cells (nNOS and eNOS) (Nijkamp and Folkerts, 1995). These isoforms are regulated by intracellular Ca²⁺ calmodulin concentration, are also localized in platelets, neutrophils, and mast cells, and generate picomolar levels of NO (Moncada, 1992). By contrast, the inflammatory inducible NOS (iNOS) isoform is Ca²⁺ independent and produces nanomolar levels of NO (Moncada, 1992) and can be induced in epithelial, smooth muscle, and inflammatory (e.g., macrophages and eosinophils) cells (Barnes et al., 1999). Regulated at the transcriptional level in response to proinflammatory cytokines, such as interleukin 1β, tumor necrosis factor-α (TNF-α), or lipopolysaccharide (LPS), activity of the iNOS gene is dependent on nuclear factor-κB (NF-κB) promoter binding (Barnes and Adcock, 1997).

The lipophilic free-radical NO passes readily into smooth muscle to activate soluble guanylate cyclase, resulting in increased cGMP to induce bronchodilation that can antagonize cholinergic bronchoconstriction (Dupuy et al., 1992). Basal (physiological) levels of cNOS-derived NO are anti-AHR and anti-inflammatory, maintain bronchodilatory tone, inhibit neurotransmission, and suppress both leukocyte activation and microvascular leakage (Barnes et al., 1999; Colasanti and Suzuki, 2000). Higher lung concentrations of NO, although having beneficial antibacterial and antiviral activity, promote pulmonary leukocyte influx through: increased

**ABBREVIATIONS:** AHR, airway hyperreactivity; COPD, chronic obstructive pulmonary disease; NO, nitric oxide; cNOS, constitutive nitric-oxide synthase; nNOS, neuronal nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; FEV₁, forced expiratory volume in 1 s; PDE4, phosphodiesterase isozyme 4; BAL, bronchoalveolar lavage; sGaw, specific airways conductance; DMSO, dimethyl sulfoxide; L-NAME, N°-nitro-L-arginine methyl ester.
microvascular leakage, neutrophil adhesion, and Th1 lymphocyte inhibition, thus favoring the development of Th2 responses (e.g., eosinophilia) (Fukatsu et al., 1997; Barnes et al., 1999). Under pathological conditions, excessive NO also reacts with inflammatory-derived superoxide anions, resulting in formation of cytotoxic peroxynitrite, which promotes tissue damage and AHR in guinea pigs (Beckman, 1996; Sadeghi-Hashjin et al., 1996). Thus, NO appears capable of exerting contradictory effects on airway caliber, reactivity, and inflammation.

In asthma and patients with COPD, exhaled NO correlates with AHR, iNOS up-regulation, leukocyte influx, and peroxynitrite formation and negatively correlates with lung function (FEV1) (Saleh et al., 1998; Agusti et al., 1999; Ichinose et al., 2000). Although anti-inflammatory steroids are the mainstay of asthma treatment (British Thoracic Society, 1993), by ameliorating these inflammatory parameters (Saleh et al., 1998), there is little indication of their benefit on disease progression in COPD (Burge, 1999). Recent attention has focused on the inhibition of phosphodiesterase isoenzyme-4 (PDE4), as a target for COPD and asthma (Torphy et al., 1999). Evidence suggests that the subsequent intracellular elevation of cAMP induces airway smooth muscle relaxation, inhibits acute allergen-induced AHR, and suppresses immunocompetent cell activation and migration (Danahay and Broadley, 1997; Torphy et al., 1999; Ikemura et al., 2000).

Many acute animal models have been developed to study features of asthma and COPD (Pauwels et al., 1991). However, few in vivo models emulate the chronic inflammatory nature of these multifactorial diseases, examine progressive lung function over many days (without anesthesia influencing vagal tone or sensory reflexes), and stimulate AHR associated with neutrophilia, as in COPD. Previously, we have demonstrated in conscious guinea pigs that a single aerosolized LPS inhalation caused the early development of AHR to histamine, persisting for 2 h, and influx of neutrophils into the airways that peaked 24 h later (Toward and Broadley, 2000). During the period of LPS-induced AHR, the stable NO metabolites (nitrite and nitrate) in the bronchoalveolar lavage (BAL) fluid were reduced. The resolution in AHR coincided with a recovery in NO generation, 4 h later. The NO deficiency during LPS-induced AHR is in contrast to the elevated expired levels associated with AHR in asthmatics (Saleh et al., 1998) and patients with COPD (Postma and Kerstjens, 1998; Agusti et al., 1999). It is also contrary to the anticipated TNF-α-mediated induction of iNOS-derived NO (Barnes and Adcock, 1997). Kips et al. (1992) have shown that inhibition of TNF-α attenuates neutrophil influx and AHR in rats exposed to LPS.

This study aimed to characterize the effects of double and chronic (nine exposures, 48 h apart) LPS exposures on lung function, AHR, cell infiltration, and NO production as a more clinically germane model of the chronic pulmonary inflammatory diseases, COPD, and asthma. The second aim was to examine the effects of the corticosteroid, dexamethasone, and PDE4 inhibitor, rolipram, on the acute and chronic LPS-induced inflammation.1

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treated animals after the ninth exposure to LPS or saline, each time in a separate group of animals. This dose of spasmoden caused a small threshold bronchoconstriction in naive animals. To examine if the effects of dexamethasone or rolipram on LPS-induced AHR were due to bronchodilator activity exerting functional antagonism of the histamine-induced bronchoconstriction, a higher dose of histamine (nose-only, 3 mM, 20 s) causing a significant bronchoconstriction was used before and after saline (LPS vehicle) exposures. Measurements of sGaw were taken before and at 0, 5, and 10 min after exposure to the spasmoden.

**Administration of Anti-inflammatory Compounds**

In single and chronic exposure studies, dexamethasone (20 mg·kg⁻¹) or rolipram (1 mg·kg⁻¹) were administered (i.p.) 24 and 0.5 h before exposure to LPS or saline. In chronic exposure studies, dexamethasone or rolipram was further administered at 24 and 47 h after each subsequent exposure. The last dose of dexamethasone was administered 47 h after the eighth exposure. Animals treated with rolipram during the chronic LPS study developed a persistent bronchodilation, which was projected to have prevented an accurate measurement of airway reactivity to histamine 24 h after the eighth or ninth exposure. Consequently, the last dose of rolipram was given 24 h after the eighth exposure, which allowed sGaw to recover to baseline values, at 24 h after the ninth LPS exposure. The histamine challenge was therefore made 24 h after the ninth LPS exposure in both rolipram-treated groups (chronic LPS and chronic saline). Doses were selected based upon data from other studies using models of inflammation and that were without adverse effects (dexamethasone: Whelan et al., 1995; Toward and Broadley, 1999) (rolipram: Danahay and Broadley, 1997). No animal appeared to be in respiratory distress or to exhibit other signs of discomfort during the exposure regimes or during any of the other procedures described.

**Bronchoalveolar Lavage**

In single and double exposure studies, with and without dexamethasone or rolipram treatment, the animals underwent a BAL within 20 min of assessing post-exposure airway reactivity to histamine, or 24 h after exposure to LPS or saline. In chronic studies, the animals were lavaged 24 h after the ninth exposure to LPS or saline. The guinea pigs were overdose with pentobarbitone sodium (400 mg·kg⁻¹ i.p., Euthatal) and the trachea cannulated. A 1% solution of EDTA (1 mL·100 g⁻¹ of 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 hemocytometer (Neubauer). A Cytospin smear (Shandon Centrifuge: 1000 rpm, 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) was counted.

**Measurement of NO Production**

The remaining BAL sample was then centrifuged (1200 rpm, 6 min), and the supernatant was removed and frozen (−20°C). A spectrophotometric assay was used to determine stable decomposition products of NO (nitrite and nitrate) based on the Griess reaction, as described by Grisham and coworkers (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 the conversion of nitrate 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 oxidized by incubating (25°C, 10 min) with potassium ferricyanide (1 mM). The samples were then incubated (25°C) with 1 mL of the Griess reagent [naphthylenediamine: 0.2% (w/v), sulfanilamide: 2% (w/v), and solubilized in double distilled water: 95% and phosphoric acid: 5% (w/v)] 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 maximum linear limit of detection for the assay was 1 mM.

**Drugs and Solutions**

Dexamethasone-21-phosphate disodium salt, dimethyl sulfoxide (DMSO), EDTA disodium salt, FAD disodium salt, histamine diphosphate salt, LPS (Escherichia coli O26:B6), HEPES free acid, NADPH (reduced form), naphthylenediamine, phosphoric acid, potassium ferricyanide, rolipram, and sulfanilamide were purchased from Sigma (Poole, Dorset, UK), Euthatal from Rhone Merieux (Harlow, Essex, UK), and Aspergillus nitrate reductase (NADPH: nitrate oxidoreductase, EC 1.6.6.2) from Boehringer Mannheim (Indianapolis, IN). Rolipram and dexamethasone were dissolved in 50% DMSO, 50% sterile saline (0.9% NaCl; Baxter Healthcare, Thetford, UK) and introduced as 0.1-mL volumes made up with saline. Unless otherwise indicated, all other solutions were made up in sterile saline.

**Data Analysis**

To reduce intersubject variability, changes in sGaw from the baseline sGaw values taken before a procedure are presented as a percentage of the mean baseline value preceding the first LPS challenge. Absolute values of baseline sGaw are stated in the figure legends. BAL fluid cell counts and the concentration of NO metabolites were compared using analysis of variance, followed by Scheffe’s post hoc analysis. Changes in airway function 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**

**Lung Function**

**Effect of Multiple Exposures to LPS on Airway Function.** Exposure of guinea pigs to LPS or the LPS vehicle (saline) caused falls in sGaw, indicating bronchoconstriction, whether it was a single exposure or the first of the double or multiple exposures. The first exposure to LPS in the chronic exposure study caused an immediate bronchoconstriction (−12.4 ± 10.7% decrease from baseline sGaw values) lasting 30 min, which was not significantly different (p > 0.05) from the response to saline (−20.5 ± 3.6%) (Fig. 1). This profile of transient bronchoconstriction followed by recovery at 30 min was essentially repeated after each subsequent saline exposure. After the second LPS exposure, there was a significant bronchodilation compared with baseline sGaw values (+27.4 ± 10.7%) and the saline group (15 min after exposure: p < 0.05 and p < 0.001, respectively). At 47 h, the baseline was still significantly (p < 0.01) raised, compared with the saline-exposed group. After the third exposure to LPS, there was still a significant bronchodilation, compared with the saline group, but recovery to baseline values 24 h later. The fourth, fifth, and sixth LPS exposures elicited responses comparable with the saline group. The seventh, eighth, and ninth exposures to LPS caused a decline in sGaw (−16.9 ± 5.9, −20.6 ± 2.8, and −23.1 ± 4.7 peak percent decrease from baseline sGaw values), with a progressive increase in duration of bronchoconstriction. At the eighth exposure, there was still significant (p < 0.05) bronchoconstriction at 30 min, whereas after the ninth challenge, the bronchoconstriction remained at 19 h after exposure.

**Effect of Dexamethasone or Rolipram Treatment on Airway Function Responses to Multiple Exposures to LPS.** The profile of airway function in animals chronically
exposed to LPS with and without dexamethasone treatment was not significantly different, except for a period of persistent bronchodilation (p < 0.05) during the intermediate exposures (3, 4, and 5) and an exaggerated duration of bronchoconstriction during the later exposures, compared with the LPS only group (Fig. 2). In contrast, rolipram treatment revealed bronchoconstriction (p < 0.05) during the early LPS exposures and a persistent baseline bronchodilation during the later exposures. These later bronchodilations returned to baseline sGaw values when rolipram was withdrawn 24 h after the eighth exposure to LPS. Lung function during single and chronic saline exposure regimes was not significantly different in the absence or presence of dexamethasone or rolipram treatment (data not shown).

Airway Responsiveness to Inhaled Spasmogen after Single, Double, and Chronic Exposure to LPS. Inhalation of histamine (1 mM, 20 s) before or 1 h after a single saline exposure failed to produce a significant bronchoconstriction (Fig. 3a and Fig. 4a). However, 1 h after a single exposure to LPS, there was a significant (p < 0.02) bronchoconstriction to histamine, compared with before exposure, indicating AHR (Figs. 3b and 4a). Airway reactivity to histamine was recovered 4 h later (p > 0.05, Fig. 4a). Further, challenging animals with double exposures to LPS, 48 h apart, prolonged the duration of AHR to at least 4 h, whereas chronic (nine) exposures prolonged the AHR to at least 24 h after the eighth exposure (Fig. 4, a and b).

Effect of Dexamethasone or Rolipram on Airway Responsiveness to Inhaled Spasmogen after Exposure to LPS. In animals treated with rolipram or dexamethasone, there was no significant difference (p < 0.05) between inhaled histamine (1 mM, 20 s) responses before and 1 h after a single LPS exposure, indicating an inhibition of the acute LPS-induced AHR (Figs. 3c and 4a). A higher dose of inhaled histamine (3 mM, 20 s), before saline exposure, caused a significant (p < 0.01) bronchoconstriction (−22.6 ± 6.7 and

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Fig. 1. Effect of chronic exposure (nine, 60-min exposures, 48 h apart) to nebulized LPS (30 µg · ml⁻¹) or LPS vehicle (pathogen-free saline) on the airway function of conscious guinea pigs. The sGaw was measured at 0, 0.25, 0.5, 24, and 47 h after each exposure, except after the ninth exposure when sGaw was recorded at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 19, and 24 h. Each point represents the mean ± S.E.M (n = 6) change in sGaw expressed as a percentage of the baseline (B) sGaw values [sGaw (nl⁻¹ cm of H₂O−¹): saline = 0.45 ± 0.01; LPS = 0.46 ± 0.3]. Negative values represent bronchoconstriction. Significance of differences from baseline values (∗p < 0.05, **p < 0.01, and ***p < 0.001) or between LPS and saline exposure (‘p < 0.05, ‘‘p < 0.01, and ‘‘‘p < 0.001) was determined by analysis of variance (single factor), followed by Student’s t test. Lung function during single exposure to saline with dexamethasone (D; rolipram: R). The last rolipram dose was given 24 h after the eighth exposure to LPS. Lung function during chronic exposure (nine, 60-min exposures, 48 h apart) to nebulized LPS (30 µg · ml⁻¹) or LPS vehicle (pathogen-free saline) on the airway function of conscious guinea pigs. Treatment was administered (i.p.) 24 and 0.5 h before the first exposure and at 24 and 47 h after each subsequent exposure (dexamethasone: D; rolipram: R). The last rolipram dose was given 24 h after the eighth exposure to LPS to allow lung function recovery to baseline values for an accurate assessment of airway reactivity to histamine at 24 h after the ninth exposure. The sGaw was measured at 0, 0.25, 0.5, 24, and 47 h after each exposure, except after the ninth exposure when sGaw was recorded at 0, 0.25, 0.5, 1, 1.5, 2, 3, 4, 5, 19, and 24 h. Each point represents the mean ± S.E.M (n = 6) change in sGaw expressed as a percentage of the baseline (B) sGaw values [sGaw (nl⁻¹ cm of H₂O−¹): LPS = 0.46 ± 0.3; LPS and dexamethasone = 0.40 ± 0.01; LPS and rolipram = 0.32 ± 0.01]. Negative values represent bronchoconstriction. Significance of differences between LPS exposure with and without dexamethasone treatment (∗p < 0.05 and ‘‘‘p < 0.001) or LPS exposure with and without rolipram treatment (∗p < 0.05, **p < 0.02, ***p < 0.01, and ****p < 0.001) was determined by analysis of variance (single factor), followed by Student’s unpaired t test. Lung function during chronic exposure to saline with dexamethasone or rolipram treatment (data not shown) was not significantly different (p > 0.05) from saline alone (Fig. 1).
Baseline sGaw values [sGaw (s^-1 cm of H_2O)] before exposure, i.p.) in conscious guinea pigs. Each point represents the mean ± S.E.M. (n = 6) change in sGaw expressed as a percentage of the baseline sGaw values [sGaw (s^-1 cm of H_2O)]; a, before = 0.44 ± 0.02 and after = 0.36 ± 0.02; b, before = 0.30 ± 0.02 and after = 0.31 ± 0.02; c, before = 0.36 ± 0.01 and after = 0.36 ± 0.01; and d, before = 0.38 ± 0.01 and after = 0.38 ± 0.01. Negative values represent bronchoconstriction. Significance from baseline sGaw (*p < 0.02 and **p < 0.01) and between responses to histamine before and after exposure (’p < 0.02) was determined by analysis of variance (single factor), followed by Student’s paired t test. Airway reactivity to: histamine (1 mM, 20 s) before and at 1 h after a single exposure, i.p.; d, histamine (3 mM, 20 s) before and 1 h after a single exposure to nebulized saline and rolipram treatment (1 mg · kg^-1, 24 and 0.5 h before exposure, i.p.); d, before

-28.9 ± 8.9% decrease from baseline sGaw values, respectively. After treating the animals with rolipram or dexamethasone, the same histamine dose, 1 h after a single saline exposure, caused a bronchoconstriction (−20.2 ± 4.6 and −31.6 ± 6.2% decrease from baseline sGaw values, respectively) that was not significantly (p > 0.05) different (Figs. 3a and 4a). The chronic LPS-induced AHR to histamine (1 mM, 20s), in guinea pigs treated with dexamethasone or rolipram, was also inhibited at 24 h after the eighth and ninth exposures, respectively (Fig. 4b). However, the airway responsiveness to the higher dose of histamine (3 mM, 20 s) 24 h after the eighth and ninth saline exposures, in dexamethasone or rolipram-treated animals, respectively, was not different (p > 0.05) from the pre-exposure response (Fig. 4b). There was no statistically significant difference between starting baseline values of sGaw for any of the groups studied.

Leukocyte Infiltration

**Effect of Single, Double, and Chronic Exposure to LPS on Leukocytes Recovered from Bronchoalveolar Lavage Fluid.** There was a tangible increase in the number of macrophages, neutrophils (p < 0.001 and p < 0.001, respectively), and small increase in eosinophils retrieved from the BAL fluid at 24 h after a single LPS exposure, compared with that removed from naive animals or at 24 h after saline exposure (Table 1). A double exposure to LPS, 48 h apart, caused a 2-fold increase in macrophage infiltration into the airways, compared with the single exposure, although eosinophil and neutrophil populations were not significantly different from those recovered after a single exposure to LPS. Compared with naive animals or those subjected to a single exposure to LPS, guinea pigs exposed to chronic LPS had significantly elevated levels of BAL fluid macrophages (p < 0.001 and p < 0.001, respectively), eosinophils (p < 0.001 and p < 0.001, respectively), and neutrophils (p < 0.001 and p < 0.05, respectively). Chronic LPS exposure also caused a 58% increase in the total cellular influx into the airways, compared with a double exposure. There was no significant difference (p > 0.05) between the recovered cell populations from naive animals, or those after a single or chronic saline exposure.

**Effect of Dexamethasone or Rolipram on Leukocytes Recovered from BAL Fluid after Exposure to LPS.** Treatment with dexamethasone attenuated the airway influx of neutrophils by over one-third, 24 h after a single LPS exposure (Table 1). In chronic studies, dosing with dexamethasone or rolipram reduced the number of macrophages (21 and 45% decrease, respectively), eosinophils (92 and 86% decrease, respectively), and neutrophils (88 and 63% decrease, respectively) in the BAL fluid, at 24 h after the ninth LPS exposure. Interestingly, both dexamethasone and rolipram treatment increased the BAL fluid macrophage population 24 h after an acute saline or LPS challenge and in the chronic saline studies.

**Nitric Oxide.**

The variations in concentrations of the individual NO metabolites (nitrate and nitrite) in BAL fluid were synchronous.
chronic exposure (60 min, 48 h apart) to nebulized LPS (30 

before and after subsequent exposure, i.p.). Each point represents the mean 
treatment (24 and 0.5 h before exposure and 24 and 47 h after each 
absence (before 
6 
5 
0.02) and double (before 
0.36 
0.01, before 
0.38 
0.01, and after 
0.38 
0.01, respectively) treatment and at 4 h after a single (before 
0.33 
0.01 and 
0.30 
0.02) and double (before 
0.46 
0.03 and after 
0.41 
0.02) exposure to LPS; b, 24 h after chronic exposure to LPS or saline in the absence (before 
0.44 
0.02 and after 
0.46 
0.02, before 
0.47 
0.01 and after 
0.42 
0.01, respectively) and presence of dexamethasone (before 
0.42 
0.0 and after 
0.38 
0.02, before 
0.37 
0.01 and after 
0.43 
0.02, respectively) or rolipram (before 
0.36 
0.01 and after 
0.36 
0.01, before 
0.38 
0.01 and after 
0.38 
0.01, respectively) treatment and at 4 h after a single (before 
0.33 
0.01 and 
0.30 
0.02) and double (before 
0.46 
0.03 and after 
0.41 
0.02) exposure to LPS; b, 24 h after chronic exposure to LPS or saline in the absence (before 
0.44 
0.02 and after 
0.46 
0.02, before 
0.47 
0.01 and after 
0.42 
0.01, respectively) and presence of dexamethasone (before 
0.42 
0.0 and after 
0.38 
0.02, before 
0.37 
0.01 and after 
0.43 
0.02, respectively) or rolipram (before 
0.36 
0.01 and after 
0.36 
0.02, before 
0.38 
0.02 and after 
0.37 
0.03, respectively) treatment. Negative values represent broncho- 
Discussion

We have previously demonstrated that, 1 h after a single 
-fluids recovered from bronchoalveolar lavage fluid after exposure to LPS. 
metabolites in 
BAL fluid were significantly greater (p < 0.05) than after LPS alone, but 
significantly different (p > 0.05) from saline-exposed animals treated with 
dexamethasone or rolipram (Fig. 5). Thus, dexamethasone and rolipram prevented 
reduction in NO metabolites observed during the acute LPS-induced AHR.
In chronic LPS-exposed animals, dosed with dexamethasone (p < 0.001) or rolipram (p < 0.01), the increased production of NO metabolites was significantly suppressed to naive levels (Fig. 6). The NO metabolites were not significantly different (p > 0.05) in BAL fluid from naive guinea pigs, or those exposed to chronic saline with and without dexamethasone or rolipram treatment.

However, lower levels of nitrate were analyzed, due to the rate-limiting nitrate reductase conversion to measurable nitrite (Fig. 5 and Fig. 6). Therefore, to describe changes in the NO metabolites assayed, reference is made to the combined nitrate and nitrite values.

Effects of Single, Double, and Chronic Exposure to LPS on Nitric Oxide Metabolites Recovered from BAL

Fluid. At 1 h after a single exposure to saline, the concentration of NO metabolites was significantly increased (41%) in the BAL fluid, compared with that found in naive animals (Fig. 5). However, during the period of AHR induced by a single exposure to LPS (1 and 2 h), the concentration of NO metabolites was significantly reduced, compared with that found in BAL fluid from naive animals (p < 0.01) and at 1 h after saline exposure (p < 0.001). At 4 h after a single LPS exposure, NO metabolite levels were not significantly different (p > 0.05) from those observed at 1 h after saline exposure. However, at 4 h after a double exposure regime, during the period of prolonged AHR, the NO metabolites were significantly reduced to levels observed during the acute LPS-induced AHR. Chronic exposure to LPS caused a significant elevation in the concentration of BAL fluid NO metabolites at 24 h after the last exposure, compared with naive animals (p < 0.001), or at 24 h after a single LPS (p < 0.001) or chronic saline (p < 0.01) exposure (145, 130, and 48% increase in the combined nitrate and nitrite levels, respectively) (Fig. 6).

Effect of Dexamethasone or Rolipram on Nitric Oxide Metabolites Recovered from Bronchoalveolar Lavage Fluid after Exposure to LPS. NO metabolites in BAL fluid removed from animals treated with dexamethasone or rolipram at 1 h after a single exposure to LPS were significantly greater (p < 0.05) than after LPS alone, but not significantly different (p > 0.05) from saline-exposed animals treated with dexamethasone or rolipram (Fig. 5). Thus, dexamethasone and rolipram prevented the reduction in NO metabolites observed during the acute LPS-induced AHR.

variance (single factor), followed by Scheffe’s post hoc analysis. Compared differences in the NO metabolites were determined by analysis of variance (single factor), followed by Scheffe’s post hoc analysis indicated as **p < 0.001, *p < 0.001, and **p < 0.001.

**Fig. 5.** NO production, determined as the individual and combined metabolites (NO, NOx, and NOy), recovered from guinea pig BAL fluid before (naive) and after a single exposure (60 min) to nebulized LPS (30 μg·ml⁻¹) or vehicle (saline), in the absence (1, 2, 4 h and 1 h, respectively) and presence of dexamethasone (20 μg·kg⁻¹, 1 h) and rolipram (1 μg·kg⁻¹, 1 h) treatment (24 and 0.5 h before exposure, i.p.), or 4 h after a double exposure (60 min) to LPS (30 μg·ml⁻¹, 48 h apart). Each point represents the mean ± S.E.M. (n = 6) of the NO metabolite concentration. Significant differences in the NO metabolites were determined by analysis of variance (single factor), followed by Scheffe’s post hoc analysis. Compared with: 1 h after saline exposure (*p < 0.02, **p < 0.01, and ***p < 0.001) and naive animals (p < 0.05 and ***p < 0.001). The conditions associated with AHR are indicated.

NO-generating epithelium (Nijkamp and Folkerts, 1995). NO is produced from the enzymatic conversion of L-arginine to L-citrulline by NOS (Schulz and Triggle, 1994), and three possible mechanisms may explain a dysfunction in cNOS-derived NO generation during acute LPS-induced AHR. First, a reduced substrate (L-arginine) concentration could arise from inhibition of cellular L-arginine uptake by inflammatory cell-derived polyatomic proteins (De Boer et al., 1999) or elevated arginine degradation via arginase (Nijkamp and Folkerts, 1995). Intracellular L-arginine levels are, however, normally in excess of demand (McCall and Vallance, 1992). Second, LPS could suppress NOS activity by phosphorylation of nNOS or eNOS tyrosine residues (Colasanti and Suzuki, 2000). Third, reduced NO could arise from its increased sequestration by rapid interaction with proteins and lipids (Nijkamp and Folkerts, 1995) or with superoxide (O²⁻) to yield the powerful oxidant peroxynitrite (ONOO⁻) (Beckman, 1996), which may induce AHR (Sadeghi-Hashjin et al., 1996) through epithelial damage (Barnes, 1996) or impairment of β-adrenoceptors (Kanazawa et al., 1999). Peroxynitrite or the peroxynitrite-induced nitration product, nitrotyrosine, were not determined and their roles could not be assessed.

This phenomenon of NO deficiency followed by recovery, during AHR and its resolution, has been described by others in comparable guinea pig models of lung inflammation (Nijkamp and Folkerts, 1995: parainfluenza virus; Schuiling et al., 1998: allergen). In the current study, dexamethasone or rolipram on airway influx of inflammatory cells after single, double and chronic LPS or vehicle exposure.

### Table 1

<table>
<thead>
<tr>
<th>Cells per Bronchoalveolar Lavage Sample (x10⁶)</th>
<th>Total Cell Count</th>
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</thead>
<tbody>
<tr>
<td><strong>Macrophages</strong></td>
<td><strong>Eosinophils</strong></td>
</tr>
<tr>
<td>Naive</td>
<td>6.7 ± 0.1**</td>
</tr>
<tr>
<td>Single saline (24 h)</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>Single LPS (24 h)</td>
<td>27.5 ± 3.1***</td>
</tr>
<tr>
<td>Single saline and dex (24 h)</td>
<td>17.8 ± 1.8</td>
</tr>
<tr>
<td>Single LPS and dex (24 h)</td>
<td>39.1 ± 5.4</td>
</tr>
<tr>
<td>Single saline and rolipram (24 h)</td>
<td>15.8 ± 1.7</td>
</tr>
<tr>
<td>Single LPS and rolipram (24 h)</td>
<td>105.3 ± 11.4</td>
</tr>
<tr>
<td>Double LPS (24 h)</td>
<td>57.3 ± 4.8***</td>
</tr>
<tr>
<td>Chronic saline (24 h)</td>
<td>2.2 ± 0.3***</td>
</tr>
<tr>
<td>Chronic LPS (24 h)</td>
<td>92.9 ± 14.2</td>
</tr>
<tr>
<td>Chronic saline and dex (24 h)</td>
<td>15.6 ± 1.6</td>
</tr>
<tr>
<td>Chronic LPS and dex (24 h)</td>
<td>73.7 ± 4.4***</td>
</tr>
<tr>
<td>Chronic saline and rolipram (24 h)</td>
<td>15.9 ± 1.4</td>
</tr>
<tr>
<td>Chronic LPS and rolipram (24 h)</td>
<td>51.1 ± 4.9***</td>
</tr>
</tbody>
</table>

Significance of differences in the cells retrieved were compared with cells from naïve animals and after a single LPS or chronic LPS exposure and determined by analysis of variance (single factor), followed by Scheffe’s post hoc analysis indicated as **p < 0.01, *p < 0.001, and ***p < 0.001.
rolipram inhibited the AHR after a single LPS exposure and interestingly, prevented the deficiency in NO. In other studies, dexamethasone and rolipram inhibited the AHR associated with allergen-induced (Danahay and Broadley, 1997, 1998) and viral-induced (Mehta et al., 1997; Iekumura et al., 2000) inflammation, but effects on NO synthesis were not examined. In this study, steroid (Barnes and Adcock, 1997) and PDE4 inhibitor (Torphy et al., 1999) protection from LPS-induced NO deficiency may be explained by common mechanisms suppressing proinflammatory cell activity and AHR. Saline exposure initially increased NO release, possibly from the “shear stress” to epithelial cells. The NO would cause cGMP-dependent smooth muscle relaxation (Schulz and Triggle, 1994) and aid recovery from the transient bronchoconstrictions. The saline-induced bronchoconstriction may be due to saline condensing in the airways, causing a reflex bronchoconstriction or obstructed airway conductance.

The effects of chronic LPS inhalation on airway function have not been studied before. In this study, chronic LPS had a biphasic effect on lung function. Initial exposures caused persistent bronchodilation, whereas later exposures caused progressively increased periods of bronchoconstriction. The inflammatory cell population and duration of AHR after LPS were dependent on the exposure number. AHR was prolonged from 2 h after a single exposure to at least 24 h after the eighth exposure. Thus, as in asthmatic and COPD patients, where AHR correlates with disease progression (reduced FEV1 and airways leukocytes) (Postma and Kerstjens, 1998), in this model AHR is prognostic of disease progression.

The mechanism for neutrophilic inflammation is likely to be orchestrated from chemotactic factors, such as TNF-α and interleukin-8, released into the airways by macrophages (Kips et al., 1992). Both dexamethasone and rolipram inhibit TNF-α (Barnes et al., 1999; Torphy et al., 1999) and were equipotent in attenuating the total airway infiltration of inflammatory cells and AHR after chronic LPS exposure. However, although the anti-inflammatory compounds inhibited AHR after a single LPS exposure, they did not significantly attenuate cell influx. This discrepancy in the efficacy of dexamethasone and rolipram appears to depend on the time of administration. In this study, animals were dosed 24 and 0.5 h before exposure and in chronic studies, 24 and 47 h after each successive exposure. Whelan et al. (1995) demonstrated that the inhibitory effect of dexamethasone (50 mg · kg⁻¹ i.p.) on LPS-induced neutrophilia was lost when given 23 h before LPS dosing. Danahay and Broadley (1997) showed that PDE4 inhibitors were effective when administered 30 min before and essentially 6 h after the inflammatory stimuli. Banner and Page (1995) showed that chronic administration, for several days, was required for PDE4 inhibitor efficacy. This infers that the efficacy of these anti-inflammatory compounds may have deteriorated at the time of BAL, 24 h after an acute LPS exposure, and that dexamethasone and rolipram have improved efficacy when allowed to accumulate in the body. It was of interest that macrophage numbers rose after dexamethasone or rolipram treatment, the reason for which was not examined further.

Contrary to the acute LPS-induced AHR coinciding with NO deficiency, chronic LPS inhalation caused AHR associated with NO overproduction, presumably derived from iNOS (Kristof et al., 1998). Previously, we described an elevated NO production coinciding with airway hyporeactivity, 48 h after a single exposure to LPS (Toward and Broadley, 2000). This elevated NO appears to be a rebound response to attenuate inflammation and exaggerated bronchoconstriction (Barnes et al., 1999). Elevated NO levels would also explain the raised baseline sGaw during the second and third LPS exposures, in the current study. Recent evidence indicates that basal levels of NO suppress NF-κB dissociation from the inhibitory transcription factor IκB complex and also prevent NF-κB binding to the promoter region of the iNOS gene (Colasanti and Suzuki, 2000). Thus, in the present study, the initial deficiency in NO during the early LPS exposures would facilitate activation of the machinery to induce iNOS expression. However, an overproduction of iNOS-derived NO and underlying inflammation would cause peroxy nitrite formation, which exacerbates airway damage and edema (Kristof et al., 1998). We have performed airway histology after chronic LPS exposure (manuscript in preparation) and found evidence of edema, epithelial disruption, extensive leukocyte infiltration, and goblet cell metaplasia. Contrary to findings of Stolk et al. (1992) in hamsters after chronic intratracheal LPS installations, however, there was no irreversible lung tissue destruction (emphysema). The epithelial damage would impair epithelial-derived relaxing factor generation (e.g., prostaglandin E2 and NO), expose sensory nerves and smooth muscle (Nijkamp and Folkerts, 1995), and narrow airway caliber (Pare and Hogg, 1989), which would explain the prolonged AHR and persistent bronchoconstriction associated with later LPS exposures.

Both, dexamethasone and rolipram prevented the elevated NO and protracted AHR. Dexamethasone inhibits NF-κB activation and expression of iNOS through increased transcription and expression of IκB (Barnes and Adcock, 1997). Consequently, a dexamethasone-induced suppression of iNOS-derived NO would explain the normalized NO metabolites 24 h after the last exposure. The exacerbated bronchoconstrictions after dexamethasone treatment appear to be independent of the steroid-sensitive NO overproduction, AHR, and leukocyte infiltration, and requires further histological investigation, which will be addressed in our follow-up histological studies.

Elevated intracellular cAMP levels, after rolipram treatment, suppresses cell activation and inflammatory processes (Torphy et al., 1999), thus attenuating iNOS induction and subsequent NO overproduction 24 h after the last exposure. The intriguing development of persistent bronchodilation after intermediate-late LPS exposures was not seen in saline-exposed animals and subsided when rolipram dosing was stopped. Therefore, this potentially clinically favorable cAMP-dependent bronchodilation appeared to be LPS-dependent. A NO component to the bronchodilation cannot be ruled out, because NO metabolites were assessed when bronchodilation had subsided. Induction by LPS of the cyclooxygenase-2-derived bronchodilator prostaglandin E2 (Uhlig et al., 1997), further augmenting rolipram-elevated cAMP levels, may also be involved. Attenuation of the AHR by rolipram was not due to the persistent bronchodilation through functional antagonism, since lung function had returned to baseline levels when assessing AHR. Furthermore, there was no functional antagonism from the PDE inhibition by rolipram alone, since the bronchoconstriction to a larger dose of histamine was not attenuated in chronic saline-exposed animals.

In summary, this study demonstrates that, in conscious
guinea pigs, both dexamethasone and rolipram inhibit chronic LPS-induced AHR and inflammatory exacerbation, suppressing inflammatory cell influx and NO overproduction. As with asthma and COPD, NO production after chronic LPS correlated with airway hyperreactivity and inflammation. In common with COPD, dexamethasone exacerbated disease progression, whereas rolipram improved lung function. These results support the development of PDE4 inhibitors for the treatment of COPD or severe asthma.

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References


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