Concomitant administration of nitric oxide and glucocorticoids improves protection against bronchoconstriction in a murine model of asthma

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Concomitant administration of nitric oxide and glucocorticoids improves protection against bronchoconstriction in a murine model of asthma. J Appl Physiol 109: 521–531, 2010. First published June 10, 2010; doi:10.1152/japplphysiol.01317.2009.— Glucocorticoids (GC) remain the first choice of treatment in asthma, but GC therapy is not always effective and is associated with side effects. In a porcine study in our laboratory, simultaneous administration of GC and nitric oxide (NO) attenuated the endotoxin-induced inflammatory response and made GC treatment more effective than inhaled NO or steroids alone. In the present study, we aimed to further investigate the interactions between NO and GC treatment in two murine models of asthma. Inflammation was induced by endotoxin, ovalbumin, or a combination of both. With an animal ventilator and a forced oscillation method (FlexiVent), lung mechanics and airway reactivity to methacholine in response to various treatments were assessed. We also describe histology and glucocorticoid receptor (GR) protein expression in response to inhaled NO treatment [40 ppm NO gas or NO donors sodium nitroprusside (SNP) or diethylamine NONOate (DEA/NO)]. SNP and GC provided protection against bronchoconstriction to a similar degree in the model of severe asthma. When GC-treated mice were given SNP, maximum airway reactivity was further reduced. Similar effects were seen after DEA/NO delivery to GC-treated animals. Using 1-[1,2,4]-oxadiazolo-[4,3-c]-quinoxalin-1-one (ODQ), a soluble guanylate cyclase inhibitor, we found this effect of NO donors to be mediated through a cGMP-independent mechanism. In the severe model, prolonged NO treatment restored or even increased the nuclear levels of GR. In conclusion, in our murine model of severe asthma GC treatment provided protection to only a limited degree against bronchoconstriction, while concomitant treatment with a NO donor was markedly more potent than the use of either NO or GC alone.

forced oscillation; glucocorticoid sensitivity; lung mechanics; airway inflammation

ALLERGIC ASTHMA is an inflammatory lung disorder responsible for significant morbidity and mortality worldwide (3). Glucocorticoids (GC) remain the first choice of treatment (12), but GC therapy is not always completely effective and is associated with side effects and steroid resistance (4). In some steroid-resistant patients, GC has no effect at all (29).

GC are lipophilic molecules that interact with the ubiquitous cytoplasmic and nuclear glucocorticoid receptors (GR), through which they exert their effects (11). Previous studies have shown that there are points of interaction between the effector pathways of GC and the gaseous molecule nitric oxide (NO) (13, 16, 19, 31, 44, 50, 55). NO, endogenously produced by several different types of cells (17), is involved in airway disease (42), and NO content is increased in the exhaled air of asthmatic patients (1). NO is a weak bronchodilator and shows bronchoprotective effects against bronchoconstriction and airway reactivity (25, 42, 49). It is clear that NO is not merely a marker of airway inflammation but can also act in an anti-inflammatory manner by inhibiting the expression of many genes thought to be involved in inflammatory diseases (41, 48, 51, 57).

In a recent porcine study in our laboratory (13), infusion of lipopolysaccharide (LPS) induced acute general inflammation, and LPS downregulated GR while inhalation of NO upregulated the expression of GR. Simultaneous administration of GC and NO blunted the inflammatory response and almost preserved the normal histology of the lung (13). The blunting effect on the inflammatory response was striking compared with that of inhaled NO or steroids alone. When a receptor antagonist was used to block the GR, neither NO alone nor NO together with a steroid had any effect on the inflammatory response.

On the basis of these results, we have developed murine models for a further in-depth assessment of the interactions between NO and GC and the effects on GC treatment. To investigate different methods of inflammatory induction and various inflammatory patterns, we studied these interactions in three different groups of animals: 1) the first group of mice was given ovalbumin (OVA)-induced airway inflammation in accordance with an established model (32, 33); 2) a second group was given LPS-induced airway inflammation by exposure to inhaled aerosolized LPS; and 3) in the third group these two models were combined as modified from the model of Murakami et al. (40). OVA induces primarily an eosinophilic inflammation, with many similarities to the changes seen in asthmatic patients (8, 27), while LPS exposure induces an infiltration of neutrophils (6, 45). LPS has been considered to contribute to a worsening of asthma symptoms, e.g., airway inflammation (14, 21).

In this study, we used a murine ventilator and a forced oscillation technique (FOT) (FlexiVent, Scireq, Montreal, PQ, Canada) to evaluate lung mechanics and airway reactivity in response to various treatments, and together with the physiology we also describe the histology and GR protein expression. We address the role of combining GC treatment and exogenous NO treatment, the latter delivered by inhalation of 40 ppm NO or by inhalation of the aerosolized NO donors sodium nitroprusside (SNP) or diethylamine NONOate (DEA/NO). We also used a soluble guanylate cyclase (sGC) inhibitor, 1-H-[1,2,4]oxadiazolo-[4,3-c]-quinoxalin-1-one (ODQ), to investigate whether NO is mediated through a guanosine 3’,5’-cyclic monophosphate (cGMP) mechanism.

MATERIALS AND METHODS

Animals

Female BALB/c mice (Taconic M&B) were used in this study. They were housed in plastic cages with absorbent bedding material and were maintained on a 12-h daylight cycle. Food and water were

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provided ad libitum. Animal care and the experimental protocols were approved by the Regional Ethics Committee on Animal Experiments in Uppsala (C86/5 and C64/8). Mice were 6–7 wk of age when the airway inflammation protocol started and 9–10 wk when their airway physiology was assessed (n = 6–12 mice/group).

Experimental Protocols

Airway inflammation protocol. Acute airway inflammation was induced by intraperitoneal (IP) injections of 10 μg of OVA (Sigma-Aldrich) emulsified in Al(OH)₃ (Sigma-Aldrich) on day 0 and day 7. One group of mice was then challenged with 1% OVA diluted in phosphate-buffered saline (PBS, Sigma-Aldrich) (Fig. 1). Animals were subjected to inhaled aerosolized OVA or PBS for 30 min on days 14–16. Aerosol exposure was performed in a chamber coupled to a nebulizer (Devilbiss UltraNeb, Sunrise Medical). The chamber was divided into pie-shaped compartments with individual boxes for each animal, providing equal and simultaneous exposure to the allergen. A second group of mice were exposed to inhaled aerosolized LPS (Escherichia coli serotype 0111:B4, Sigma-Aldrich; dissolved in H₂O) diluted in PBS without OVA (Fig. 1). Aerosol exposure was performed as described above. The concentration of LPS in the nebulizer was 0.005% (wt/vol). A third group of animals was also subjected to inhaled aerosolized LPS diluted in PBS simultaneous with OVA (Fig. 1). Aerosol exposure was performed as described above. The concentration of LPS in the nebulizer was 0.005% (wt/vol). A third group of animals was also subjected to inhaled aerosolized LPS diluted in PBS simultaneous with OVA (Fig. 1). Aerosol exposure was performed as described above. The concentration of OVA in the nebulizer was 1% (wt/vol), and the concentration of LPS in the nebulizer was 0.005% (wt/vol). Another group of mice was challenged with OVA and LPS on days 14–16 and 21–23.

Glucocorticoid treatment. Mice receiving GC received an injection of GC (hydrocortisone sodium succinate, Pfizer, 0.375 g/kg IP). Control groups were given vehicle (PBS). GC or vehicle was given as an IP injection on days 14–16 immediately before OVA challenge and 1 h before the assessment of lung mechanics on day 17 (Fig. 1 and Table 1).

Preparation of Animals

On the day of assessment of lung mechanics, the animals were weighed and anesthetized with pentobarbital sodium (90 mg/kg IP, from local suppliers). Mice were tracheostomized with an 18-gauge cannula and mechanically ventilated in a quasi-sinusoidal fashion with a small-animal ventilator (FlexiVent, Scireq) at a frequency of 2.5 Hz and a tidal volume (VT) of 12 ml/kg body wt. Once ventilation was established, bilateral holes were cut in the chest wall so that the pleural pressure would equal the body surface pressure and the rib cage would not interfere with lung movement. This made strict lung mechanics measurement possible. A positive end-expiratory pressure (PEEP) of 3 cmH₂O was applied by submerging the expiratory line in water. A warming pad prevented cooling of the animal. Four sigh maneuvers at three times VT were performed at the beginning of the experiment to establish stable baseline lung mechanics and to ensure a similar volume history before the experiments. The mice were then allowed a 5-min resting period before the experiment began.

Analysis of Lung Mechanics

Dynamic lung mechanics were measured by applying a sinusoidal standardized breath and analyzed with FOT. During the forced oscillatory maneuver, the ventilator piston delivers 13 superimposed sinusoidal frequencies, ranging from 1.0 to 20.5 Hz, during 2 s (Prime 2), at the mouse’s airway opening. Harmonic distortion in the system was avoided by using mutually prime frequencies (24). With the dynamic calibration signal characteristics, the Fourier transformations of the recordings of pressure and volume displacement within the ventilator cylinder (Pcyl and Vcyl) can be used to calculate the respiratory system input impedance (Zrs) (23). Fitting the Zrs to an advanced model of respiratory mechanics, the constant phase model (24), allows the lung mechanics to be divided into central and peripheral components. The primary parameters obtained are the Newtonian resistance (Rₙ), a close approximation of resistance in the central airways; tissue damping (G), which is closely related to tissue resistance and reflects energy dissipation in the lung tissues; and tissue elastance (H), which characterizes tissue stiffness and reflects energy storage in the tissues (7, 28). To investigate the maximum response of each dose of methacholine (MCh) for Rₙ, G, and H, Prime 2 was continuously measured over a period of 2 min with a standardized script.

Assessment of Airway Responsiveness

To assess airway responsiveness, mice were given incremental doses of MCh intravenously in the lateral tail vein [0 (PBS), 0.03, 0.1, 0.3, 1, and 3 mg/kg] at 3-min intervals. MCh (acetyl-β-methylcholine chloride, Sigma-Aldrich) was diluted in PBS (Sigma-Aldrich) with 10 U/ml of heparin, and a volume of 2,000 μl/kg was injected at each injection.

Table 1. Groups and experimental protocols

<table>
<thead>
<tr>
<th>Groups and Protocols</th>
<th>Treatment</th>
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<tbody>
<tr>
<td>A (OVA)</td>
<td>With vehicle None SNP With GC (0.375 g/kg) DEA/NO None SNP</td>
</tr>
<tr>
<td>B (LPS)</td>
<td>With vehicle None SNP With GC (0.375 g/kg) None SNP</td>
</tr>
<tr>
<td>C (OVA/LPS)</td>
<td>With vehicle None SNP With GC (0.375 g/kg) DEA/NO NO gas None SNP DEA/NO NO gas</td>
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Mice were randomly allocated into the listed groups and experimental protocols. OVA, ovalbumin; LPS, lipopolysaccharide; GC, glucocorticoids; SNP, sodium nitroprusside; DEA/NO, diethylamine NONOate; NO, nitric oxide.

![Fig. 1. Schematic overview of the airway inflammation protocol and treatment. Group A: ovalbumin (OVA); group B: lipopolysaccharide (LPS); group C: OVA/LPS. NO, nitric oxide; BAL, bronchoalveolar lavage; GC, glucocorticoid.](http://jap.physiology.org/Downloaded from http://jap.physiology.org/ on October 30, 2017)
Nitric Oxide Donor Delivery

SNP (sodium nitroprusside salt, 5 mg/kg) or DEA/NO (2.6 mg/kg) was diluted in PBS, and a volume of 10 μl was given over 10 s with an aerosol (Aeroneb, Aerogen) into the trachea before each dose of PBS and MCh (Fig. 1 and Table 1). These procedures were performed without interference with the ventilation pattern. Solutions were kept cool in the dark between loadings of the aerosol.

Nitric Oxide Delivery

NO gas exposure (40 ppm for 3 or 6 h) was performed in a chamber coupled to an INOvent delivery system for NO therapy (Datex-Ohmeda). The chamber was divided into pie-shaped compartments with individual boxes for each animal, providing equal and simultaneous exposure to NO. There were no detectable levels of NO₂ in the system. Mice receiving NO gas underwent a longer OVA/LPS challenge protocol for 2 wk (6 challenges).

ODQ Treatment

Mice received an injection of ODQ (20 mg/kg IP, Calbiochem) similar to that described by Zingarelli et al. (58). ODQ was dissolved in DMSO (25 mg/ml) and then diluted in PBS. Control groups were given vehicle. Mice were given IP injections of ODQ or vehicle 1 h before assessment of the airway mechanics on day 17. To assess the inhibitory effects of sGC this ODQ dose was first evaluated in naive mice.

Bronchoalveolar Lavage

After completion of the lung mechanics experiment, the mice were exsanguinated and subjected to bronchoalveolar lavage (BAL). The lungs were lavaged three times via the tracheal tube with a total volume of 1 ml of PBS containing 0.6 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich). The BAL fluid was then immediately centrifuged (10 min, 4°C, 1,200 rpm). After the supernatant was removed, the cell pellet was resuspended for 2 min at room temperature in 100 μl of erythrocyte lysis buffer containing 0.15 M NH₄Cl, 1.0 mM KHCO₃, and 0.1 mM EDTA. The suspension was then diluted with 1 ml of PBS containing 0.05% bovine serum albumin (Sigma-Aldrich) and recentrifuged (10 min, 4°C, 1,200 rpm). Leukocytes were counted manually in a hemocytometer so that 50,000 cells were suspended into a pressure of 20 cmH₂O. The trachea was tied off, and the tissue was immediately removed, the cell pellet was resuspended for 2 min at room temperature in 100 μl of erythrocyte lysis buffer containing 0.15 M NH₄Cl, 1.0 mM KHCO₃, and 0.1 mM EDTA. The suspension was then diluted with 1 ml of PBS containing 0.05% bovine serum albumin (Sigma-Aldrich) and recentrifuged (10 min, 4°C, 1,200 rpm). Leukocytes were counted manually in a hemocytometer so that 50,000 cells could be loaded and centrifuged with a Cytospin centrifuge (Shandon Cytospin 3 cytocentrifuge, cell preparation system). Cytocentrifuged preparations were stained with May-Grünwald-Giemsa reagent, and differential cell counts of pulmonary inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were made with standard morphological criteria and counting 300 cells per cytospin preparation.

Histological Evaluation

After BAL, the lungs were inflated with 4% paraformaldehyde solution to a pressure of 20 cmH₂O. The trachea was tied off, and the lungs were removed, stored overnight in 4% paraformaldehyde, and then stored in 70% ethanol at room temperature until the time for embedding. After being embedded in paraffin, the tissue was cut into 4-μm thick sections and mounted on positively charged slides. To assess inflammatory cell infiltration, the sections were deparaffinized, dehydrated, and stained with hematoxylin and eosin (H & E). H & E-stained sections were examined by bright field microscopy (Axioskop 40 microscope, Carl Zeiss, Göttingen, Germany), and images were captured with a high-resolution digital camera system (AxioCam, Axiovision 2007, Carl Zeiss Imaging Systems, Göttingen, Germany). Repeated blind histopathological analyses were performed, and representative images have been selected.

Immunohistochemistry: Glucocorticoid Receptor

After being embedded in paraffin, the tissue was cut into 4-μm sections and mounted on positively charged slides. Tissue sections were deparaffinized in xylene and alcohol. All sections were heated in a microwave (750 W) in citrate buffer [citric acid and distilled H₂O (dH₂O), pH 6.0] for 10 min. After cooling, tissue sections were incubated in 3% H₂O₂ for 30 min to block endogenous peroxidase activity. This was followed by incubation (in a humidified box at room temperature) with 1.5% normal blocking goat serum (X0907 DAKO) in PBS for ~1 h. Thereafter, the sections were incubated overnight at 4°C with primary antibody rabbit anti-GR (GRα, P-20, 1:400, Santa Cruz Biotechnology, Santa Cruz, CA). The overnight incubation was followed by incubation for 45 min with a secondary antibody, goat anti-rabbit in PBS + 1.5% normal goat serum (1:100, DAKO). This was followed by incubation for 30 min with streptavidin-biotin complex (ABC technique) by a standard procedure (VECTORSTAIN Elite ABC Kit). The staining was made visible with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector SK4200) for 5 min. Sections were lightly stained in hematoxylin and dehydrated with ethanol and xylene. Stained sections were examined with the same equipment as for histology evaluation. All these procedures were also carried out on sections of lung tissue that had been incubated in the absence of primary antibody (negative control). After each staining step, the tissue sections were carefully washed with PBS.

Protein Assays

After completion of the lung mechanics experiment, the mice were exsanguinated and the lungs were resected. The tissue was immediately frozen in liquid nitrogen and stored at −70°C. Protein extracts were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents for the preparation of cytoplasmic and nuclear protein extracts from tissue in accordance with the manufacturer’s instructions (Thermo Scientific, Nordic Biolabs). All samples were stored at −70°C. To determine protein concentrations, absorbance was measured with the Quanti-FL Protein Assay Kit for use with the Qubit fluorometer (Invitrogen). Protein samples were diluted with dH₂O to make even loading and boiled for 5 min together with sample buffer [0.5 M Tris pH 6.8, 20% SDS, glycerol, and mercaptoethanol (Sigma-Aldrich)]. Samples were then separated by 8% SDS-polyacrylamide gel electrophoresis. After separation, proteins were transferred to a nitrocellulose membrane (Trans-Blot, Bio-Rad). The membranes were soaked in Lumi-LightPLUS (Roche Diagnostics) reagents for 1 h. Thereafter, the sections were incubated overnight at 4°C with primary antibody rabbit anti-GR (GR α, P-20, 1:400, Santa Cruz Biotechnology, Santa Cruz, CA). The overnight incubation was followed by incubation for 30 min with streptavidin-biotin complex (ABC technique) by a standard procedure (VECTORSTAIN Elite ABC Kit). The staining was made visible with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Vector SK4200) for 5 min. Sections were lightly stained in hematoxylin and dehydrated with ethanol and xylene. Stained sections were examined with the same equipment as for histology evaluation. All these procedures were also carried out on sections of lung tissue that had been incubated in the absence of primary antibody (negative control). After each staining step, the tissue sections were carefully washed with PBS.

Statistical Analysis

Results are presented as means ± SE. Statistical significance was assessed by parametric methods using a two-way analysis of variance (ANOVA) to determine differences between groups, followed by a Bonferroni post hoc test. When appropriate, a one-way ANOVA or Student’s unpaired t-test was used. A statistical result with P < 0.05 was considered significant. The statistical analyses were carried out by 10.220.33.1 on October 30, 2017 http://jap.physiology.org/ Downloaded from
RESULTS

Lung Mechanics

OVA. In OVA-inflamed airways, inhaled aerosolized SNP reduced the maximum MCh-induced bronchoconstriction ($R_N$) by 43% from $5.3 \pm 0.4$ to $3.0 \pm 0.6$ cm$H_2O \cdot s \cdot ml^{-1}$ ($P < 0.01$). Similar effects were seen in animals receiving another NO donor, inhaled DEA/NO (by 56% from $4.6 \pm 0.3$ to $2.0 \pm 0.3$ cm$H_2O \cdot s \cdot ml^{-1}$, $P < 0.001$). Inhaled aerosolized vehicle (PBS) did not affect the MCh-induced bronchoconstriction. Treatment with GC significantly decreased the maximum $R_N$ by 64% from $5.3 \pm 0.4$ to $1.9 \pm 0.3$ cm$H_2O \cdot s \cdot ml^{-1}$ ($P < 0.001$), and cotreatment with SNP did not further reduce the MCh-induced bronchoconstriction. In LPS-challenged mice, the increase in $G$ was similar to that in OVA-challenged mice ($P > 0.05$). As shown in Fig. 2, there were significant increases in $H$ compared with the OVA groups ($P < 0.001$). GC had no protective effect on either maximum $G$ or $H$ ($P > 0.05$).

OVA/LPS. The combination challenge consisting of OVA/LPS produced a maximum airway reactivity similar to that of the OVA challenge alone (OVA/LPS: $5.0 \pm 0.4$ cm$H_2O \cdot s \cdot ml^{-1}$, OVA: $5.3 \pm 0.4$ cm$H_2O \cdot s \cdot ml^{-1}$, $P > 0.05$; Fig. 3A).

In OVA/LPS groups, $G$ and $H$ were higher than in the OVA-challenged mice. In OVA/LPS-challenged mice, the increase in $G$ was significantly higher than in LPS-challenged mice ($P < 0.05$). There were no significant differences between the LPS and OVA/LPS groups. In OVA/LPS mice, GC treatment had protective effects on both $G$ and $H$ (both $P > 0.01$; Fig. 2).

LPS. In animals subjected to LPS challenge, inhaled aerosolized SNP had no effect on maximum MCh-induced bronchoconstriction. Pretreating LPS-challenged animals with GC reduced maximum $R_N$ by 51% (from $3.5 \pm 0.5$ to $1.7 \pm 0.3$ cm$H_2O \cdot s \cdot ml^{-1}$, $P < 0.0001$), and cotreatment with SNP did not further reduce the MCh-induced bronchoconstriction. In LPS-challenged mice, the increase in $G$ was similar to that in OVA-challenged mice ($P > 0.05$). As shown in Fig. 2, there were significant increases in $H$ compared with the OVA groups ($P < 0.001$). GC had no protective effect on either maximum $G$ or $H$ ($P > 0.05$).

and graphs were prepared with GraphPad Prism (version 4.0 GraphPad Software, San Diego, CA).

Fig. 3. Measurements of MCh-induced Newtonian resistance ($R_N$) were performed with FOT (Zrs measurements, Prime 2). OVA/LPS-challenged groups received inhaled NO donors [sodium nitroprusside (SNP; A), diethylamine NONOate (DEA/NO; B)], and all treated groups received GC treatment and concomitant treatment with both NO and GC. Values are means ± SE. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

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Inhaled SNP reduced the maximum \( R_N \) by 42\% (from 5.0 ± 0.4 to 3.0 ± 0.3 cmH\(_2\)O·s·ml\(^{-1}\), \( P < 0.0001 \)), and treatment with GC had a similar effect, with a reduction of the maximum \( R_N \) by 45\% (from 5.0 ± 0.4 to 2.8 ± 0.3 cmH\(_2\)O·s·ml\(^{-1}\), \( P < 0.0001 \)); Fig. 3A). When GC-pretreated mice also received SNP, a potentiating effect was seen, and the maximum \( R_N \) was reduced by a further 50\% (from 2.8 ± 0.3 to 1.4 ± 0.2 cmH\(_2\)O·s·ml\(^{-1}\), \( P = 0.001 \), Fig. 3A). Repetition of the experiment with another NO donor, inhaled DEA/NO, showed similar results. DEA/NO reduced \( R_N \) by 50\% (from 5.0 ± 0.4 to 2.5 ± 0.6 cmH\(_2\)O·s·ml\(^{-1}\), \( P < 0.001 \)), and combination treatment with NO donor and GC reduced \( R_N \) by a further 45\% (from 2.5 ± 0.6 to 1.5 ± 0.3 cmH\(_2\)O·s·ml\(^{-1}\), \( P = 0.001 \); Fig. 3B).

To investigate whether this potentiating effect was due to a cGMP-dependent mechanism we evaluated airway responsiveness after ODQ pretreatment. The effectiveness of ODQ to inhibit sGC, and thereby subsequent cGMP formation, was first evaluated in naive mice, in which ODQ abolished the effect of inhaled aerosolized SNP and had no further effects on airway reactivity [maximum MCh-induced bronchoconstriction (\( R_N \))]: vehicle+PBS 2.9 ± 0.3, vehicle+SNP 1.9 ± 0.2, and ODQ+SNP 2.8 ± 0.3 cmH\(_2\)O·s·ml\(^{-1}\) (vehicle+SNP vs. ODQ+SNP, \( P < 0.0004 \); vehicle+SNP vs. SNP, \( P < 0.0001 \); vehicle+PBS vs. ODQ+SNP, \( P > 0.05 \)); PBS was administered as a vehicle for SNP]. Despite ODQ pretreatment, simultaneous administration of GC and SNP in OVA/LPS-treated mice still resulted in a marked drop in \( R_N \) (1.9 ± 0.1 cmH\(_2\)O·s·ml\(^{-1}\)). This suggests that the potentiating mechanism of concomitant treatment with NO and steroid is mediated through a cGMP-independent pathway.

**Bronchoalveolar Lavage**

**OVA.** OVA-challenged mice had 431,500 ± 88,800 cells/ml in BAL. GC treatment decreased the total BAL cell count to 131,900 ± 22,000 (\( P < 0.0001 \)) and reduced the number of eosinophils from 49.9 ± 6.7\% to 3.1 ± 1.9\% (\( P < 0.001 \)) (Table 2 and Fig. 4A).

**LPS.** The total BAL cell count in LPS-challenged animals was significantly lower than in OVA-challenged animals, and GC treatment had no effect on the total cell numbers (169,200 ± 39,500 cells/ml with and 153,000 ± 42,700 cells/ml without GC, \( P > 0.05 \)). LPS challenge induced a significant increase of neutrophils from 49.9 ± 6.7\% (\( P < 0.016 \)) (Table 2 and Fig. 4B).

**Histological Evaluation**

In all three models of inflammation, the lungs exhibited circulatory changes and cellular inflammatory infiltrates of varying degrees of severity. Circulatory changes may be considered a feature of the inflammation and were characterized by edema, congestion, and hemorrhages. Edema, together with extravasated leukocytes, was most obvious in dilated perivascular spaces, indicating leakage of fluid from the vessels as a result of increased permeability associated with the inflammatory reaction. The severity of the changes varied not only between the groups but also between the individual mice in each group. The OVA/LPS group showed the most severe lesions, closely followed by the OVA group. The LPS group had the mildest lesions.

**OVA.** Lung histological evaluation of H & E sections by light microscopy revealed that OVA-challenged animals had signs of mild to moderate airway inflammation with a distribution of inflammatory cells surrounding the airways and within the alveolar spaces. The perivascular space was the site most commonly infiltrated by inflammatory cells, exhibiting polymorphs, monocytes, and macrophages. The peribronchial areas were generally infiltrated as a spill-off effect from adjacent perivascular spaces and exhibited mixed leukocytes, but with more mononuclear cells than the other sites. OVA-challenged mice exhibited a very mild edema in the parenchyma. Animals treated with GC showed fewer signs of inflammation, and the number of inflammatory cells was markedly less.

**LPS.** LPS-challenged mice had signs of mild airway inflammation in the tissue and around the airway tree, in contrast to the OVA- and OVA/LPS-treated groups. In the peripheral airways of the LPS mice, the alveoli appeared somewhat dilated, and it has been reported previously that LPS may induce emphysema-like changes in murine lungs (9). The mice displayed occasional alveolar macrophages and inflammatory cells, but their numbers were too low to be regarded as a significant change. The signs of inflammation were reduced after GC treatment.

**OVA/LPS.** OVA/LPS challenge enhanced the airway inflammation, and there were signs of moderate to severe airway inflammation in the tissue and around the airway tree. The alveoli were affected in severely inflamed lungs, often in a patchy distribution. The cells most commonly noted in alveoli were macrophages and occasional multinucleate giant cells. The giant cells displayed up to ~20 nuclei and originated by...
fusion of macrophages in the alveolar lumen (54). Giant cells occurred in those lungs that exhibited extensive changes. Mice with severe lesions exhibited leukocytes migrating through the wall of small- and medium-sized arterioles. These leukocytes were found in the vascular lumen, adhering to the endothelium and between endothelial cells, and also in the tunica intima, the tunica media, the adventitia, and the perivascular tissues. Heavily infiltrated vessels exhibited changes of the endothelium. OVA/LPS-treated mice also showed a moderate edema/emphysema in the parenchyma. GC treatment reduced the signs of inflammation.

**GRα Immunoreactivity**

GRα immunoreactivity was present in endothelial/epithelial cells of pulmonary arteries, veins, bronchioles, and alveolus in mice with airway inflammation (Fig. 5). GRα was very intensely expressed in the tissue in OVA-challenged mice. There was less intense staining of GRα in LPS-challenged mice than OVA-challenged mice. There was no further immunoreactivity of GRα in OVA/LPS-challenged mice than in OVA-challenged mice. After GC treatment the immunoreactivity of GRα was decreased in all groups.

**GR Protein Expression**

In animals without any inflammation, the PBS-challenged control mice, GR protein levels were slightly higher in the nuclear fraction than in the cytoplasmic fraction isolated from whole lung homogenates. Subsequent to both OVA and OVA/LPS challenges, GR protein expression was reduced in the nuclear fraction (Fig. 6A). In OVA/LPS-challenged animals, GC treatment restored the normal nuclear-to-cytosolic ratio seen in animals without any inflammation (Fig. 6B) and increased the GR protein levels in both nuclear and cytosolic fractions. SNP administration abolished the effect of GC in
OVA/LPS-challenged animals, while DEA/NO had an intermediate reductive effect on the GC-induced increase in GR protein levels (Fig. 6B). To evaluate whether these effects of SNP and DEA/NO were due to effects of NO rather than to possible alternative effects of the NO donor compounds, the protein expression analysis was repeated after administration of 40 ppm NO gas (Fig. 6C). In the OVA/LPS-challenged animals treated with GC, NO administration for 3 h reduced the nuclear GR expression and modestly increased the GR in the cytosol. After NO administration for 6 h, the nuclear levels of GR were restored or even increased above the levels seen in the group without NO, and the cytosolic GR levels were also restored.

**DISCUSSION**

The aim of this study was to characterize the interplay between GC and NO and their effects on airway function in murine models of airway inflammation. Lung function has been thoroughly characterized in mice suffering from airway inflammation induced by OVA, by LPS, or by a combination of OVA and LPS. We have evaluated the lung mechanics, airway reactivity, lung histology, and GR protein expression after GC and/or NO treatment in these different inflammatory models. Our main finding is that in a model of severe asthma cotreatment with GC and NO provides a better protection against MCh-induced bronchoconstriction than either NO or GC alone. We also conclude that the combined OVA/LPS model resembles severe human asthma better than the more conventional OVA model. The OVA model is in many respects similar to acute asthmatic inflammation with, e.g., eosinophil and airway hyperreactiveness, whereas the OVA/LPS model also showed a strong increase in neutrophils together with eosinophils, airway hyperreactiveness, and a poor response to GC treatment, which are some of the characteristics of severe asthma (35, 56).

Interactions between NO and GR have been reported in a porcine LPS inflammatory model (13) in which a combination treatment with GC and NO reduced the inflammatory response and prevented many of the pathological changes seen in lung histology. The combined treatment was strikingly more effective than treatment with either NO or GC alone. In the present study of mice with airway inflammation, it was also found that the combination treatment with NO donors and GC was more bronchoprotective than either treatment alone. This effect was seen when mice were challenged with OVA/LPS to induce airway inflammation, but there was no enhanced effect of GC+NO combination treatment in mice that had received either a pure OVA- or a pure LPS-induced airway inflammation.

In a recent study on human subjects, Hållström et al. (26) employed low-dose LPS challenges in combination with 30 ppm inhaled NO. They saw no changes in the plasma inflammatory response or any other advantageous effects of the combination treatment. Lung function was not measured in...
their study, but they report that the LPS challenge did not cause coughing, wheezing, or any change in the respiratory rate, which implies that the challenge was mild and did not cause any bronchoconstriction. It is conceivable that the beneficial effects on airway inflammation and airway function of a combined NO and GC treatment might be species dependent, but it is more probable that the dose of endotoxin and the timing of the NO administration may be important. The timing of the LPS exposure is also relevant since the complexity of the effects on the allergic responses may depend on the time of exposure (53).

It is interesting that the combined treatment with NO donors and GC was effective in the OVA/LPS model but not in either the OVA or the LPS single-challenge models. Mice challenged with OVA/LPS exhibited no further increase in airway reactivity to MCh than the OVA-challenged animals, and the effect of NO donors (SNP) on airway resistance was similar in the different groups, but GC had a lesser effect in the OVA/LPS group. Thus challenging mice with OVA/LPS according to this protocol led to a model that reduced their GC sensitivity. While speculating on the mechanism, we may report that this reduction in GC sensitivity is counteracted by a concomitant treatment with NO.

Further investigations of the effects of NO on these three different inflammatory models also showed other differences among the groups. The NO donor SNP was much less potent in LPS-challenged mice than in the OVA and OVA/LPS groups. One reason for this may of course be that LPS induced less airway reactivity to MCh and that there was therefore less constriction to counteract. This is supported by the fact that SNP treatment induced a similar airway resistance in all three groups studied, so that the maximum bronchoprotective effect may also have been reached in the LPS group. This is not, however, supported by the results that both GC and SNP+GC treatments markedly reduced airway constriction in the LPS-challenged animals, leaving room for further improvement.

With regard to peripheral lung mechanics, the LPS group differed from the OVA and OVA/LPS groups also in its response to GC treatment. Although a good effect against bronchoconstriction, measured as MCh-induced increase in airway resistance, was noted, subjects in the LPS-challenged group showed no improvement whatsoever in either G or H after GC treatment. Since both OVA- and OVA/LPS-challenged subjects showed a strong improvement in both G and H after GC treatment, we speculate that OVA may in fact provide protection against this lack of LPS-induced peripheral improvement in airway function. Several studies have reported changes in airway and immune responses after combining LPS and OVA. In studies on rats, Tulic’ and colleagues (52, 53) have shown that exposure to LPS increases airway reactivity as does exposure to OVA, whereas combining LPS and OVA leads to a reduced airway hyperreactivity (AHR) and reduced cellular influx. These authors report that both OVA and LPS challenges lead to an increase in inducible nitric oxide synthase (iNOS) and a decrease in constitutive NOS (cNOS), although these changes are inhibited by a combined OVA/LPS challenge. In the present study, we did not measure endogenous NOS levels, but we saw no diminished AHR after the combined OVA/LPS treatment. Tulic’ and colleagues also describe a reduced cellular influx with OVA/LPS compared with a single OVA treatment. In contrast, we saw a markedly higher total cell count in OVA/LPS animals than in both OVA- and LPS-challenged mice, although the combination challenge led to a markedly reduced fraction of eosinophils, which provides support for Tulic’ and colleagues’ proposal that immunomodulatory factors are released in the combination model. They suggest that one of these factors may be interleukin (IL)-10, and others have suggested that IL-12 is the factor responsible for the LPS-induced reduction of OVA eosinophilia (22). In the present study, we have examined the effect of coadministering LPS together with an OVA challenge, which is worth noting since the time at which LPS is administered during OVA sensitization and/or OVA challenge is extremely important, the patterns of AHR and cellular and immune response being completely different depending on whether LPS is given before or during the OVA sensitization and challenge (14, 15). Despite a protocol that was slightly different from ours, LPS being nasally instilled 24 h before the start of OVA challenges, it is interesting to note that Komlósi and colleagues (36) found that murine AHR became largely steroid resistant after LPS priming. Their results are in line with our present results, which show a reduced effectiveness of GC treatment in OVA/LPS mice. Thus it is intriguing to speculate that clinical steroid resistance may be due to the concomitant exposure of inflamed and allergen-challenged airways to LPS. The authors could not explain the mechanism of steroid resistance in OVA/LPS mice, but they also showed that the selective iNOS inhibitor 1400W effectively reduced AHR in OVA-challenged mice but failed to do so in LPS+OVA-challenged mice. This is an intriguing clue since both GC (5) and 1400W suppress/inhibit iNOS, which further suggests that there may be changes in the NO system in OVA/LPS mice that are responsible for a reduction in steroid responsiveness. In the present study the effects of LPS, NO, and GC on the various NOS isoforms were not addressed, but assuming that the findings by Tulic’ and col-
leagues are also true in our model, i.e., that OVA/LPS combina-
nation treatment does not increase iNOS or decrease eNOS, we
speculate that these can be important clues to the poor response
to GC in our combination model and also a major possibility
for why exogenous NO may still work since it overrides the
iNOS/cNOS stage and directly increases cellular NO levels. In
our present study we have further investigated the interplay
between the NO system and GC effector pathways. To evaluate
whether the effects of NO donors seen in this study were due
to cGMP-dependent or cGMP-independent mechanisms, we
used a potent and selective inhibitor of sGC, ODQ. When
OVA/LPS-challenged mice were pretreated with ODQ, no
reduction was seen in the effect of a combined treatment with
SNP and GC. This suggests that the potentiating effect
achieved by combining NO donors and steroid is due to a
cGMP-independent mechanism of NO. Until quite recently, it
was generally believed that the main target of NO is guanylate
cyclase, where activation of this enzyme leads to the produc-
tion of cGMP and the subsequent relaxation of smooth muscle
in airways and pulmonary vessels. With a rapidly increasing
number of publications, a new perspective is now emerging
where the main target of NO is instead believed to be cysteine
residues in proteins, an event called S-nitrosylation (20). S-
nitrosylation would, however, not exclude activation of guan-
ylate cyclase (39). Chemically, S-nitrosylation occurs via trans-
fer of NO\(^+\) from a donor S-nitrosothiol (SNO) to an acceptor
cysteine thiol, or via an oxidative reaction of NO and cysteine
thiol, and is thus an indirect event often requiring cofactors
such as enzymes or transition metals (18). Since all major
classes of proteins carry cysteine residues that could potentially
be targeted for S-nitrosylation, it can be concluded that the
biological effects of NO may be even more diverse and more
extensive than was formerly believed. It has been suggested
that the increased levels of NO in asthmatic airways are
beneficial to airway function only if NO retains its S-nitrosy-
lation ability (47). We believe that there is a strong potential
for developing new therapeutic strategies if it is understood
how the nitrosylation system can be kept in balance. It has also
been suggested that S-nitrosylation is one mechanism by which
NO may interact indirectly with the intracellular GC effector
pathway (20). NO has been found to affect ligand binding (16,
19), dissociation from molecular chaperones (55), translocation
to the nucleus (31, 55), DNA binding (31), and histone
decacylation (30).

In the present study, we have investigated the effect of NO
donors and NO gas on GR protein expression and GR trans-
location to the nucleus. GR is widely distributed in the airways
and expressed on inflammatory and structural cells. The high-
est amount of GR has been found in endothelial and epithelial
cells (2). OVA/LPS challenge decreased nuclear GR protein
expression, and GC treatment restored this back to normal.
Cotreatment with GC and NO donors prevented some or all of
this restorative effect. To evaluate a period of NO treatment
longer than that associated merely with the acute administra-
tion of NO donors, we repeated this experiment, using inhala-
tion of 40 ppm NO for up to 6 h. Three hours of NO
administration decreased nuclear GR expression, but after 6 h
the nuclear levels were restored and even higher than those in
the group without NO. These results taken together suggest
that NO indeed affects nuclear translocation of the GR. This
may be one possible explanation for the increase in potency by
GC after concomitant treatment with NO. There may well be
several other explanations for this, and the mechanisms behind
this require further investigation. Manipulating the NO system,
whether it is by inhibition of NO by NO inhibitors or by giving
exogenous NO as in the present study, is likely to produce
different biological actions depending on the timing of the
interaction. In this study we administered NO close to the final
physiological assessment, and we expected to see primarily
direct physiological and biochemical effects of NO. Interacting
with the NO system at earlier stages, perhaps already at
sensitization or during allergen challenge, would most likely
also have complex effects on gene and protein expression that
we have not investigated in the present study.

In 2000, Paul-Clark and coworkers (43) proposed a novel
class of anti-inflammatory drugs, the nitrosteroids. They de-
scribed a derivate of the GC prednisolone with a chemical
moiety able to release NO, NCX-1015, with a higher anti-
flammatory potency than prednisolone itself. They also pre-
sented data suggesting that one mechanism by which this may
be mediated is through an enhanced activation of the GR. Later
they proposed that a specific nitration of tyrosine residues in
the GR led to the enhanced effect of this steroid derivate (44).
In their cellular system, using human peripheral blood mono-
nuclear cells (PBMCs), prednisolone, and the NO donor SNP
(nor NOC-18), concomitant treatment with prednisolone and
NO did not reproduce the effects of the nitrosteroid, nor did
treatment with a nitrosteroid with the NO-donating moiety in
a different position. They speculate that this may be due to the
high degree of stoichiometry around the target tyrosine resi-
dues. In the present study, we have investigated the nuclear
translocation of GR, which is one of several events affected by
increased nitration of the GR. On the basis of our findings, we
speculate that in our OVA/LPS model, contrary to what has
been proposed by Paul-Clark and coworkers, it is not unlikely
that there is nitration of the GR by NO. We base this hypo-
thesis on the greater efficiency after GC+NO treatment than
after either GC or NO alone, the fact that this effect is cGMP
independent, and the fact that we see an increased nuclear
translocation of GR after prolonged NO treatment.

Sensitivity to GC varies considerably among individuals,
and even within the same individual responsiveness to GC
differs among tissues (10). GC sensitivity could indirectly be
due to differences in cofactor recruitment, and the sensitivity
could be directly determined by the level and molecular nature
of GR (38). One of the possible mechanisms behind insensi-
tivity to GC treatment could be a feedback protector mecha-
nism (for review see Ref. 46). Steroids are known to modulate
GR expression in vivo (34, 37), and there is a downregulation
of GR\(\alpha\) expression in vivo and in vitro systems after GC
treatment (46). In line with this, we detected in the present
study a downregulation of GR\(\alpha\) in lung tissue from GC-treated
animals with airway inflammation.

As always, it is important to bear in mind that the effects we
see of GC and NO treatment may not be general but could of
course be limited to the specific strain of mice we are using
and/or to the specific protocols we are using to induce airway
inflammation. For this study, we chose the BALB/c mouse
because it is a strain that is highly susceptible to OVA-induced
inflammation and also responds with increased airway reactiv-
ity after induction of inflammation with most OVA protocols.
We (32) and others have previously described important strain-
specific differences in response to OVA protocols; therefore to fully validate the findings described in the present study we would like to point out that our results with the OVA/LPS protocol should best be confirmed in another strain, and preferably also in another species.

In conclusion, we see a potential for finding new strategies to increase the therapeutic effect in poor responders or patients resistant to GC treatment. To find these new pathways, combinations of well-established methods and the development of new versatile and more specific experimental models of airway inflammation are required. In this study, we have presented a murine model of severe asthma suitable for studying GC sensitivity. In this model, GC treatment provides protection to only a limited degree against MCh-induced bronchoconstriction, whereas concomitant treatment with a NO donor is only a limited degree against MCh-induced bronchoconstriction, whereas concomitant treatment with a NO donor is markedly more potent than either NO or GC alone. This suggests that cotreatment with NO and GC may be a new way of treating steroid-insensitive asthmatic patients.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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