A Nitric Oxide-Releasing Salbutamol Elicits Potent Relaxant and Anti-Inflammatory Activities

Vincent Lagente, Emmanuel Naline, Isabelle Guenon, Marianne Corbel, Elisabeth Boichot, Jean-Luc Burgaud, Piero Del Soldato, and Charles Advenier

Laboratoire de Pharmacodynamie et de Pharmacologie Moléculaire, Institut National de la Sante et de la Recherche Medecale, Université de Rennes 1, Rennes, France (V.L., I.G., M.C., E.B.); UPRES EA220-Pharmacologie, U.F.R. Biomédicale des Saints-Pères, Paris, France (E.N., C.A.); NicOx S.A., Sophia-Antipolis, France (J.L.B., P.D.S.)

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ABSTRACT

$\beta_2$-Adrenoceptor agonists are widely used in the treatment of pulmonary diseases. We have investigated the relaxant and anti-inflammatory activities of NCX-950 (a $\alpha$-$\beta$-[1,1-dimethylaminomethyl]-4-hydroxy-1,3-benzenedimethanol nitrate) in human isolated bronchi and on lipopolysaccharide (LPS)-induced acute airway inflammation in mice. NCX-950 (10$^{-8}$–10$^{-5}$ M) elicited a relaxation of human isolated bronchi moderately higher than salbutamol, which was reduced by a $\beta$-adrenergic blocking drug, propranolol, but not by an inhibitor of guanylate cyclase, ODQ (1H-[1,2,4]oxadiazolo[4,3-b]quinoxalin-1-one). The treatment of mice with NCX-950 (1, 10, and 100 $\mu$M aerosol) markedly inhibited the neutrophil influx induced by LPS aerosol in bronchoalveolar lavage (BAL) fluid, whereas salbutamol at equimolar doses elicited a moderate inhibition. Pre-treatment of mice with NCX-950 (100 $\mu$M) also significantly reduced tumor necrosis factor-α, interleukin-6 (IL-6), transforming growth factor-β, and matrix metalloproteinase-9 release in BAL fluid, whereas salbutamol was ineffective. Propranolol, but not ODQ, suppressed the inhibitory activity of NCX-950 on neutrophil influx and IL-6 release in BAL fluids. A nitric oxide-releasing silde- nanfi NCX-911 ([5-[2-ethoxy-5-(4-methylpiperidinylsulfonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one nitrate), but not sildenafl (100 $\mu$M) also reduced the neutrophil influx following LPS exposure in mice. This study reported that NCX-950 elicits potent relaxant and anti-inflammatory activities compared with salbutamol, and these effects may be mainly due to the activation of the $\beta_2$-adrenoceptor rather than the cGMP pathway.

$\beta_2$-Adrenoceptor agonists are widely used for the treatment of pulmonary diseases such as asthma or chronic obstructive pulmonary diseases (COPD) (Price and Clissold, 1989; Naline et al., 1994). Such compounds are well described to elicit potent relaxation of airway smooth muscle but they also inhibit bronchoconstriction in response to several spasmodens, whereas they presented none or few anti-inflammatory properties (Johnson and Coleman, 1995). Therefore, the combination of $\beta_2$-adrenoceptor agonists and glucocorticoids constitutes an established treatment for asthma and COPD as currently proposed in the International Consensus Conference (American Thoracic Society, 1987; Pauwels et al., 2001). Indeed, corticosteroids are potent and effective anti-inflammatory drugs.

Endogenous nitric oxide (NO) may play an essential role in the physiological regulation of airway function and has been implicated in the pathogenesis of airway diseases (Barnes and Liew, 1995; Nevin and Broadley, 2002). The metabolic pathway of NO generation has been recognized in various cells, where it provides a signal transduction leading to soluble guanylate cyclase stimulation, intracellular cGMP accumulation, and vasodilation (Moncada et al., 1991; Ignarro, 1999). Moreover, NO is able to elicit several functions in airways such as the modulation of airway smooth muscle tone and airway hyperresponsiveness but also anti-inflammatory activity (for review, see Nevin and Broadley, 2002; Eynott et al., 2002).

Recently, a series of new compounds have been synthesized in which a NO-releasing group has been linked to parent ABBREVIATIONS: COPD, chronic obstructive pulmonary disease; NO, nitric oxide; NOS, nitric-oxide synthase; NCX-950, $\alpha$-$\beta$-[1,1-dimethyl-ethylamino)methyl]-4-hydroxy-1,3-benzenedimethanol nitrate; NCX-911, 5-[2-ethoxy-5-(4-methylpiperidinylsulfonyl)phenyl]-1-methyl-3-n-propyl-1,6-dihydro-7H-pyrazolo[4,3-d]pyrimidin-7-one nitrate; LPS, lipopolysaccharide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-b]quinoxalin-1-one; TGF, transforming growth factor; BAL, bronchoalveolar lavage; TNF, tumor necrosis factor; IL, interleukin; TBS, Tris-buffered saline; GEA 3175, 1,2,3,4-oxatriazolium-3-(3-chloro-2-methylphenyl)-5-[[4-methylphenyl]sulfonyl][amino], hydroxide inner salt.
molecules. The strategy has been to identify novel molecules with an improved profile of pharmacological activity either in terms of enhanced therapeutic efficacy or reduced side effects. Several of these compounds (referred to as nitric oxide-donating drugs) (Keeble and Moore, 2002) have been described, including NCX-950 (NO-salbutamol) and NCX-911 (NO-sildenafil).

Recent data suggest that NCX-950 presents additional bronchodilator effects due to the release of NO (Tallet et al., 2001). Moreover, inhalation of NCX-950 showed greater bronchodilator activity than salbutamol in inhibiting histamine-induced bronchoconstriction in conscious guinea pigs (Toward et al., 2001).

The aim of this study was to compare the bronchodilatory effects of NCX-950 with the reference compound, the short-acting β2-adrenoceptor agonist salbutamol, on the human isolated bronchus. Moreover, we also investigated the possible anti-inflammatory activity of NCX-950 in lipopolysaccharide (LPS)-induced pulmonary inflammation in mice as a model of experimental COPD. The goal of this study was also to analyze the mechanism of action of this compound, namely the influence of β2-adrenoceptor stimulation on the one hand and the role of NO release on the other hand.

Materials and Methods

Materials. LPS from Escherichia coli (0.55 B5), gelatin, salbutamol [α-(l-butyramino)methyl]-4-hydroxy-m-xylene-α,α′-diol), ODQ [(R)-1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride (propranolol), 1H-[1,2,4]oxadiazolo[4,3-g][1,2,4]oxadiazolo[4,3-1-one], EDTA, theophylline (3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). May-Grinwald and Giemsa stains were purchased from RAL (Paris, France). Sodium pentobarbital was obtained from Sanofi santé animale (Libourne, France). Acrylamide was purchased from ICN Pharmaceuticals Biochemicals Division (Aurora, Ohio). Coomassie Blue was purchased from Bio-Rad (München, Germany). RPMI 1640, penicillin, streptomycin, L-glutamine, SDS, and Tris solution were obtained from Eurobio (Les Ulis, France). Anti-mouse TGβ-β monoclonal antibodies were provided by R&D Systems (Minneapolis, MN). NCX-950 (NO-salbutamol), NCX-911 (NO-sildenafil), and sildenafil were synthesized by NicOx (Sophia-Antipolis, France). Salbutamol and NCX-950 were dissolved in ethanol (0.55 B5), gelatin, salbutamol (0.55 B5), l-methionine maximal relaxation. Pretreatment with propranolol (10−8–10−6 M) (a β-adrenergic-blocking drug) and/or ODQ (10−5 M) (an inhibitor of guanylate cyclase, to inhibit NO effect) were performed 30 min before relaxation-response curves. Only one concentration-response curve to salbutamol, sildenafil, NCX-950, and NCX-911 was recorded in each ring. Experiments were performed on bronchi of 4 to 12 patients.

For the determination of duration of action (Fig. 1), human bronchi were prepared under similar conditions. After equilibration, salbutamol and NCX-950 were added to the bath at a concentration (3 × 10−3 M) giving approximately 85% of maximal response. When maximal relaxation was obtained, the bronchi were washed and allowed to return to basal tone. The time from addition to the bath to attainment of 50% of maximal relaxation by the compound (t1/2 onset) and the time to return, after washing, from maximal relaxation to 50% basal tone (t1/2 recovery) were calculated (Fig. 1).

Animals and Experimental Protocols. Ten-week-old male BALB/c mice (CERJ, Le Genest Saint Isle, France) were exposed for 30 min to an aerosol of either NCX-950 (1, 10, or 100 μM), salbutamol (1, 10, or 100 μM), NCX-911 (100 μM), or sildenafil (100 μM). In another set of experiments, the administration of NCX-950 and salbutamol was preceded by an i.p. treatment of mice with propranolol (1 mg/kg) or ODQ (2 mg/kg), 1 h before exposure to NCX-950 or salbutamol. For exposure, mice were placed into a Plexiglas chamber (20 × 30 × 20 cm) directly connected to a Shinmed SW-966 ultrasonic nebulizer (Shining World Health Care Co., Ltd., Taipei Hsien, Taiwan) that generated particles with an aerodynamic diameter that averaged 0.5 to 3 μm. After 15 min of resting time, mice were exposed for 30 min to an aerosol of LPS (100 μg/ml) in saline solution or to an aerosol of the saline solution alone (negative control group). The aerosol was administered under the same conditions as previously described for drug treatment using a Devilbiss Ultraneb 99 ultrasonic nebulizer (Devilbiss, Sommerset, PA) that generated particles with an aerodynamic diameter that averaged 0.5 to 3 μm. All experiments involving animals were approved by the local ethical committee.

Bronchoalveolar Lavage. Twenty-four hours after LPS exposure, mice were anesthetized i.p. with pentobarbital sodium (60 mg/kg). After semi-excision of the trachea, a plastic cannula was inserted and airspaces were washed using a 1-ml syringe with 0.5 ml of 0.9% NaCl containing 2.6 mM EDTA. This operation was realized four times, and the recovery of the total lavage volume (2 ml) ex-
ceed 95%. Bronchoalveolar lavage (BAL) was centrifuged (600g for 10 min, 4°C), and the fluid phase of the first milliliter of BAL fluids was aliquoted and frozen at −80°C until the mediators were assessed.

After red blood cell lysis, cell pellets were resuspended in 200 μl of saline solution (NaCl, 0.9%). Total cell count was evaluated using a hemacytometer, and viability was determined by the trypan blue exclusion method. Cells were adjusted to a concentration of 5 × 10⁵ cells/ml in saline. After cytocentrifugation (Cytoptr 7620, Wescor, Logan, UT) at 700 rpm for 10 min, cells were stained with May Grünwald-Giemsa. Differential cell counts were made on 200 cells using standard morphological criteria.

Cytokine and Metalloproteinase Measurements. Amounts of TNF-α or IL-6 were quantified in the BAL fluids by enzyme-linked immunosorbent assay method (R&D Systems). For zymography analysis, aliquots of BAL fluids were subjected to electrophoresis on a 4.5% acrylamide stacking gel/7% acrylamide separating gel containing 1 mg/ml gelatin in the presence of SDS under nonreducing conditions, as previously described (Corbel et al., 2001). After electrophoresis, gels were washed twice with 2.5% Triton X-100, rinsed with distilled water at 37°C overnight in 50 mM Tris, 5 mM CaCl₂, 2 mM ZnCl₂, pH 8. The gels were stained with Coomassie Brilliant Blue and destained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activities appeared as clear bands against a blue background. Molecular weights of gelatinolytic bands were estimated using recombinant protein molecular weight markers (10,000–225,000) (Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK). Enzyme amount was quantified by measuring the intensity of the negative bands using a densitometric analyzer with Densilab software (Bioprobe, Montreuil sous Bois, France). Results were expressed as a percentage of a band of migration of an internal standard loaded onto each gel to allow comparison between gels.

For TGF-β determination by Western blot, standardized protein quantities (2 μg) of BAL fluid were loaded onto SDS-polyacrylamide gel electrophoresis under reducing conditions and were transferred electrophoretically onto nitrocellulose membranes (Hybond-ECL, Amersham Biosciences UK Ltd., Little Chalfont, Buckinghamshire, UK). After blocking the filters with 3% bovine serum albumin in Tris-buffered saline (TBS), they were incubated with an anti-mouse TGF-β monoclonal antibody diluted in TBS containing 0.1% bovine serum albumin and 0.3% NP40. The filters were then washed three times for 10 min in TBS and incubated with peroxidase conjugate IgG. All incubations were performed at room temperature for 2 h. Antibody binding was detected by an ECL system (Amersham Biosciences UK Ltd.), and blots were exposed to X-ray films. Results were expressed as a percentage of a sample loaded onto the filter. This sample was used as an internal standard of intensity to allow comparison between filters. Under reducing conditions, this antibody detects the active TGF-β homodimer (25 kDa).

Expression of the Results and Statistical Analysis. Relaxation of human isolated bronchi was expressed as a percentage of the relaxation produced by theophylline (3 × 10⁻³ M); −log EC₅₀ values represent the negative logarithm of the concentration of drug that induces a relaxation equal to 50% of the maximal effect of theophylline (3 × 10⁻³ M). Statistical analysis of the results was performed using analysis of variance and Student’s t test (two-tailed, for paired or unpaired data). All values in the text are expressed as mean ± S.E.M.; P < 0.05 was considered to be significant.

For in vivo experiments, the results are expressed as mean ± S.E.M. Statistical analysis was performed with Statview software (SAS, Cary, NC) on an Apple computer. Analysis of treatment effects between the different groups was performed with analysis of variance. Comparison of multiple treatment interactions was realized by Newman-Keuls tests. Other comparisons were performed with the Mann and Whitney U test. For each analysis, P values less than 0.05 were considered to be statistically significant.

Results

Concentration-Response Curves of Salbutamol, NCX-950, Sildenafil, and NCX-911. On human bronchi, salbutamol and NCX-950 induced a concentration-response-dependent relaxation (Fig. 2). −log EC₅₀ and maximal effect (effect observed for 10⁻⁵ M) were 7.14 ± 0.14 and 85 ± 3% for NCX-950 and 6.88 ± 0.16 and 77 ± 4% for salbutamol (n = 1–19 experiments, 9–11 patients). Statistical analysis of concentration-response curves showed that the responses induced by NCX-950 were significantly (P < 0.05) higher than that induced by salbutamol.

Sildenafil and NCX-911 also elicited a concentration-response-dependent relaxation (Fig. 3). However, the maximum relaxation obtained with both compounds is limited with a maximum of 35 to 40% at the concentration of 10⁻⁵ M. Statistical analysis of concentration-response curves showed that the responses induced by NCX-911 were significantly (n = 14 experiments, 4 patients; P < 0.05) higher than that induced by sildenafil.

Influence of Propranolol or ODQ on Salbutamol and NCX-950 Concentration Responses Curves. Incubation of bronchi with propranolol (10⁻⁶ to 10⁻⁶ M) elicited a rightward shift of NCX-950 and salbutamol concentration-response curves (Fig. 4). However, in the presence of 10⁻⁶ M propranolol, NCX-950 (10⁻⁴ M) induced a significant greater relaxation of human bronchi than salbutamol under similar conditions (29 ± 2%, n = 8/3 versus 15 ± 5%, n = 8/3, respectively, P < 0.05).

Figure 5 shows that in the presence of the guanylate cyclase inhibitor ODQ (10⁻⁵ M), the concentration-response curves of NCX-950 and salbutamol were not significantly modified compared with the control (Fig. 2). In the presence of propranolol (10⁻⁶ M) + ODQ (10⁻⁵ M) (Fig. 4B), the effects of NCX-950 and salbutamol were similar to those obtained in the presence of propranolol (10⁻⁶ M) alone (Fig. 4A). However, in this condition the relaxant effect of NCX-950 was higher than that observed with salbutamol (Fig. 5B).

Duration of Action. Table 1 shows that the kinetics of effect, onset, and duration of action of salbutamol and NCX-950 (3 × 10⁻⁷ M), at a concentration giving 85% of maximal effect, were similar.

Effect of NCX-950 and Salbutamol on LPS-Induced Change in Cell Composition of Bronchoalveolar Lavage. Exposure of mice to LPS elicited a marked and significant increase in the number of total cells. The cell composition of the bronchoalveolar lavage fluid is mainly constituted of macrophages in the control mice, but a major influx of neutrophils was noted following LPS exposure (Table 2). NCX-950 dose dependently inhibited the increase in total BAL cells as well as macrophages and neutrophils induced by LPS aerosol. The maximum inhibitory effect was observed using the dose of 100 μM. Salbutamol was effective in reducing the increase in total cells and neutrophils only at the dose of 100 μM, but this effect was less important than that noted with NCX-950 (Table 2).

Effect of NCX-950 and Salbutamol on LPS-Induced Cytokine Release and Metalloproteinase Activity in Bronchoalveolar Lavage Fluid. A significant increase in IL-6, TNF-α, TGF-β, and MMP-9 release in the BAL.
fluids of mice exposed to LPS was observed in comparison to the release in the BAL fluids of control mice. NCX-950 dose dependently and significantly reduced the increased IL-6 level (Fig. 6). The maximum inhibition was observed with the highest concentration of NCX-950 (100 μM). In addition, the highest dose of NCX-950 (100 μM) also inhibited the increase in the release of TNF-α (Fig. 6) and TGF-β and MMP-9 activity (Fig. 7) in the BAL fluid of mice exposed to LPS. In contrast, salbutamol exposure did not show any inhibition of IL-6, TNF-α, TGF-β, and MMP-9 release in the BAL fluid of mice exposed to LPS. Moreover, a tendency to an increased cell influx (Table 2) and IL-6 release (Fig. 6) was noted in the BAL fluids of mice treated with 1 μM salbutamol and exposed to LPS.

**Influence of Propranolol or ODQ on Salbutamol and NCX-950-Induced Inhibition of Inflammatory Cell Influx and IL-6 Release in BAL Fluids.** Treatment of mice with propranolol markedly reduced the inhibition of neutrophil influx elicited by NCX-950, since no difference was noted whether or not the mice were exposed to NC-950 or were not

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**Fig. 2.** Effects of salbutamol (■) and NCX-950 (□) on the human isolated bronchus. Results are expressed as percentage of theophylline-induced relaxation. Values are mean ± S.E.M. (n = 12–19 experiments, 9–11 patients). ● vehicle.

**Fig. 3.** Effects of sildenafil (■) and NCX-911 (□) on the human isolated bronchus. Results are expressed as percentage of theophylline-induced relaxation. Values are mean ± S.E.M. (n = 14 experiments, 4 patients).
Influence of NCX-950 and salbutamol on LPS-induced changes in bronchoalveolar lavage cell composition.

**TABLE 2**
Expressed as percentage of theophylline-induced relaxation. Values are mean ± S.E.M. (n = 8–10 experiments, 3–4 patients). ○, vehicle.

Effect of Treatment with Sildenafil and NO-Sildenafil (NCX-911). To analyze the importance of the activity of NO production from this particular molecule, we have investigated the influence of sildenafil and NO-sildenafil (NCX-911) on the change of cell composition in the BAL fluid using similar protocols. Only a moderate but significant reduction of LPS-induced increase in the number of total cells and neutrophils has been noted under the treatment with NCX-911 (100 μM) (Table 4).

**Discussion**

The present study showed that a NO-releasing salbutamol compound (NCX-950), elicits potent bronchorelaxant and anti-inflammatory activities in the airways. These effects seem to be associated mainly with the stimulation of β2-adrenoceptor. However, our results suggest that the release of NO

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 4.** Influence of propranolol (○, 10^{-6}; ▲, 10^{-7}; and △, 10^{-8} M) on concentration-response curves of NCX-950 (A) and salbutamol (B). Results are expressed as ratio of theophylline-induced relaxation. Values are mean ± S.E.M. (n = 8–10 experiments, 3–4 patients). ○, vehicle.

TABLE 1
Onset and duration of action of theophylline, salbutamol and NCX950

<table>
<thead>
<tr>
<th>n</th>
<th>Onset of Action</th>
<th>Recovery of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time</td>
<td>Ratio/Theophylline</td>
</tr>
<tr>
<td>Theophylline (10^{-4} M)</td>
<td>6</td>
<td>162 ± 37</td>
</tr>
<tr>
<td>NCX-950 (3 × 10^{-7} M)</td>
<td>6</td>
<td>118 ± 17</td>
</tr>
<tr>
<td>Salbutamol (3 × 10^{-7} M)</td>
<td>6</td>
<td>142 ± 33</td>
</tr>
</tbody>
</table>

TABLE 2
Influence of NCX-950 and salbutamol on LPS-induced changes in bronchoalveolar lavage cell composition.

BALB/c mice were exposed for 30 min to an aerosol of NCX-950 (1, 10, and 100 μM) or salbutamol (1, 10, and 100 μM). After 15 min of resting time, mice were exposed for 30 min to an aerosol of LPS (100 μg/ml) in saline solution or to an aerosol of the saline solution alone (negative control group). Results are expressed in 10^4 ± S.E.M.

<table>
<thead>
<tr>
<th>Dose</th>
<th>n</th>
<th>Treatment LPS</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>–</td>
<td>16</td>
<td>–</td>
<td>7.78 ± 1.03</td>
<td>7.59 ± 1.04</td>
</tr>
<tr>
<td>–</td>
<td>23</td>
<td>+</td>
<td>51.29 ± 3.99***</td>
<td>8.79 ± 0.71*</td>
<td>42.03 ± 3.61***</td>
</tr>
<tr>
<td>NCX-950</td>
<td>100</td>
<td>6</td>
<td>–</td>
<td>5.17 ± 0.58</td>
<td>5.03 ± 0.59</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>+</td>
<td>38.57 ± 6.06</td>
<td>5.78 ± 0.89</td>
<td>32.2 ± 5.48</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>+</td>
<td>26.81 ± 4.24a</td>
<td>3.84 ± 0.51b</td>
<td>22.25 ± 3.95a</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>+</td>
<td>15.68 ± 0.97a</td>
<td>2.90 ± 0.31b</td>
<td>12.61 ± 0.69a</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>100</td>
<td>8</td>
<td>–</td>
<td>13.67 ± 1.50</td>
<td>13.48 ± 1.53</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>+</td>
<td>64.20 ± 4.48</td>
<td>8.50 ± 1.62</td>
<td>51.54 ± 3.41</td>
</tr>
<tr>
<td>10</td>
<td>8</td>
<td>+</td>
<td>40.50 ± 3.09</td>
<td>9.58 ± 1.20</td>
<td>30.24 ± 3.75</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>+</td>
<td>32.8 ± 2.26b</td>
<td>7.72 ± 1.53</td>
<td>24.67 ± 1.77b</td>
</tr>
</tbody>
</table>

n, number of mice.

* P < 0.05; *** P < 0.001 in comparison with nontreated control mice exposed to saline solution alone.

a P < 0.01; b P < 0.05 in comparison with nontreated control mice exposed to LPS aerosol.
from the NO moiety may be involved in the effects of NCX-950 since the effects of NCX-950 are slightly but significantly superior, at equimolar doses, to that of salbutamol.

β₂-Adrenoceptor agonists are widely used for the treatment of pulmonary diseases such as asthma and COPD (for review, see Price and Clissold, 1989). Indeed, salbutamol inhibits bronchoconstriction of airway smooth muscle in response to spasmogens (Cockroft et al., 1977). Since NO is also a bronchodilator (Dupuy et al., 1992), a NO-releasing salbutamol compound may therefore provide a more powerful bronchodilator effect than salbutamol alone. NCX-950 is a NO-releasing salbutamol and is a chemically stable agent, by which NO release is achieved enzymatically following exposure to biological tissues. The precise identity of the enzymes involved is not yet clear, but a lot of evidence obtained to date suggests that release of the NO moiety from an NODD occurs as a result of the activity of esterase enzymes (Cirino et al., 1995; Keeble et al., 2001; Burgaud et al., 2002).

In the present study, we showed that salbutamol and NCX-950 induced a concentration-response-dependent relaxation of human isolated airways with a similar kinetic of action. However, comparison of concentration-response curves showed that relaxation induced by NCX-950 was reasonably higher than that induced by salbutamol. These results confirm the previous data showing that NCX-950 presents additional in vitro and in vivo bronchodilator effects due to the release of NO (Tallet et al., 2001; Toward et al., 2001).

The β-adrenoceptor blocker, propranolol, induced a shift to the right of NCX-950 and salbutamol concentration-response curves. Interestingly, in the presence of propranolol, NCX-950 induced a small but significant greater relaxation of human bronchi than salbutamol under similar conditions. This indicates that NCX-950 is less sensitive to propranolol than salbutamol and suggests that the relaxant effect of NCX-950 may be due in part to a β₂-independent mechanism, which is probably due to the release of NO. In contrast, ODQ had no significant effects on both NCX-950 and salbutamol concentration-response curves, suggesting that NO is not a major component in the relaxant effect of NCX-950. However, ODQ did not cause total inhibition of the relaxation of isolated airways induced by GEA 3175 or sodium nitroprusside, two other NO donors (Hernandez et al., 1998; Hjoberg et al., 1999). These results may also support the existence of additional mechanisms, other than guanylyl cyclase activation, for NO-induced smooth muscle relaxation.

β₂-Adrenoceptor agonists such as salbutamol exert their

Fig. 5. Effects of salbutamol (□) and NCX-950 (■) on the human isolated bronchus in the presence of ODQ 10⁻⁵ M (A) or ODQ 10⁻⁵ M + propranolol 10⁻⁶ M (B). Results are expressed as percentage of theophylline-induced relaxation. Values are mean ± S.E.M. (n = 5–6 experiments, 1–4 patients). ○, vehicle.

Fig. 6. Effect of NCX-950 and salbutamol pretreatment on the level of IL-6 (A) and TNF-α (B) in bronchoalveolar lavage fluids from LPS or vehicle (saline)-exposed mice. NCX-950 and salbutamol (Sal) were used at concentrations of 1, 10, and 100 μM. **, P < 0.01; ###, P < 0.001 in comparison with vehicle-saline (veh-sal) mice; #, P < 0.05; ##, P < 0.01 in comparison with veh-LPS mice. NaCl, 0.9%. n = 6 to 23.
bronchodilating properties by stimulating cAMP production, whereas NO is thought to relax smooth muscle by increasing cGMP. Heaslip et al. (1987) demonstrated that cAMP- and cGMP-dependent mechanisms induce relaxations of the guinea pig trachea that are functionally additive. Therefore, it may be suggested that coadministration of a β2-adrenoceptor agonist and a NO donor may produce an additive bronchodilating response. Indeed, Rolla et al. (1995), in moderate asthmatics, showed that coadministration of inhaled salbutamol and inhaled nitroglycerine (0.2 mg) gave an additive bronchodilating effect over salbutamol alone. Inhalation of β2-adrenoceptor agonist immediately after NO inhalation gave a slightly greater increase in specific airway conductance over β2-agonist alone (Hogman et al., 1993). This suggests that the NO released by NO salbutamol may potentiate the bronchodilator effect of the β-adrenergic agonist. Due to the superior bronchial effects of β2-adrenoceptor agonists, the administration of NO donors alone appears unlikely. Their concomitant use with current bronchodilator therapy, however, may prove useful. NO donors with a prolonged duration of action with minimal side effects may be of particular benefit (Nevin and Broadley, 2002).

The moderate bronchodilator activity was also demonstrated by the results obtained with NCX-911, a NO-releasing sildenafil compound. Indeed, sildenafil is a weak bronchodilator compound, whereas NO-sildenafil (NCX-911) may present a slight (moderate) but significant increased relaxation of human bronchi. This may indicate that NO release elicits some bronchodilator activity.

As expected, LPS exposure of BALB/c mice was characterized by a massive recruitment of inflammatory cells in the airways, namely neutrophils, that was accompanied by an increase in cytokine level and a marked enhancement in MMP-9 activity in BAL fluid compared with saline-exposed mice (Corbel et al., 2001). These observations suggested that the model used in our study reproduced some features of acute lung injury and COPD. We showed that aerosol administration of NCX-950 inhibited neutrophil recruitment, IL-6, TNF-α, TGF-β release, and MMP-9 activity in BAL fluids from mice exposed to LPS aerosol. In contrast, salbutamol moderately inhibited neutrophil influx at high doses and failed to reduce the increase in cytokine release and MMP-9 activity in the BAL fluid of mice exposed to LPS by aerosol, showing that NCX-950 exerts anti-inflammatory effects through the NO release. The present study clearly reported that the reduction of the inflammatory process was associated with a decrease in MMP-9 activity in BAL. Although alveolar macrophages spontaneously release MMP-9 (Weglus et al., 1990), it seems also that neutrophils are mainly involved in MMP-9 secretion (Hibbs et al., 1985; Yao et al., 1997; Atkinson and Senior, 2003). In addition, activated neutrophils produce other proteases and reactive oxygen species that activate latent metalloproteases to their active forms (Palmgren et al., 1992). Therefore, it is conceivable that the

### Table 3
Interactions of propranolol and ODQ on the changes in bronchoalveolar lavage cell composition elicited by NCX-950 and salbutamol on LPS-exposed mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>LPS</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>5</td>
<td>–</td>
<td>9.39 ± 2.22</td>
<td>9.20 ± 2.19</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>NCX 950</td>
<td>8</td>
<td>+</td>
<td>41.85 ± 6.07***</td>
<td>9.20 ± 1.13</td>
<td>32.61 ± 5.41***</td>
</tr>
<tr>
<td>Propranolol</td>
<td>6</td>
<td>+</td>
<td>28.33 ± 3.46</td>
<td>12.30 ± 1.69</td>
<td>16.03 ± 3.00**</td>
</tr>
<tr>
<td>ODQ</td>
<td>6</td>
<td>+</td>
<td>7.49 ± 1.47</td>
<td>7.20 ± 1.39</td>
<td>0.29 ± 0.10</td>
</tr>
<tr>
<td>NCX 950</td>
<td>6</td>
<td>+</td>
<td>42.20 ± 6.60</td>
<td>10.03 ± 1.14</td>
<td>32.11 ± 5.69</td>
</tr>
<tr>
<td>ODQ</td>
<td>5</td>
<td>–</td>
<td>2.72 ± 1.27</td>
<td>7.20 ± 1.27</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>+ NCX 950</td>
<td>6</td>
<td>+</td>
<td>47.67 ± 8.32</td>
<td>13.40 ± 2.20</td>
<td>34.21 ± 6.79</td>
</tr>
<tr>
<td>Propranolol</td>
<td>8</td>
<td>–</td>
<td>7.87 ± 1.98</td>
<td>7.72 ± 1.96</td>
<td>0.15 ± 0.07</td>
</tr>
<tr>
<td>ODQ</td>
<td>6</td>
<td>–</td>
<td>40.57 ± 9.32</td>
<td>8.80 ± 1.13</td>
<td>31.72 ± 8.73</td>
</tr>
<tr>
<td>+ NCX 950</td>
<td>6</td>
<td>+</td>
<td>18.17 ± 1.21*</td>
<td>8.38 ± 0.72</td>
<td>9.79 ± 0.63b</td>
</tr>
</tbody>
</table>

* a, number of mice.  
*** P < 0.001 in comparison with nontreated control mice exposed to saline solution alone.  
* P < 0.05, b P < 0.01 in comparison with nontreated control mice exposed to LPS aerosol.

### Table 4
Influence of NCX-911 (NO-sildenafil) and sildenafil on LPS-induced changes in bronchoalveolar lavage cell composition

<table>
<thead>
<tr>
<th>Dose</th>
<th>n</th>
<th>Treatment LPS</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Neutrophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>16</td>
<td>–</td>
<td>8.05 ± 1.03</td>
<td>7.89 ± 1.01</td>
<td>1.63 ± 0.03</td>
</tr>
<tr>
<td>NCX 911</td>
<td>19</td>
<td>+</td>
<td>64.5 ± 5.5***</td>
<td>11.81 ± 1.43*</td>
<td>52.40 ± 4.71***</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>100</td>
<td>6</td>
<td>–</td>
<td>5.15 ± 1.16</td>
<td>5.11 ± 1.15</td>
</tr>
<tr>
<td>100</td>
<td>7</td>
<td>–</td>
<td>51.48 ± 5.28*</td>
<td>13.12 ± 2.33</td>
<td>35.53 ± 4.10</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>–</td>
<td>10.81 ± 1.59</td>
<td>10.70 ± 1.57</td>
<td>1.06 ± 0.04</td>
</tr>
<tr>
<td>100</td>
<td>16</td>
<td>+</td>
<td>54.61 ± 10.38</td>
<td>11.06 ± 1.46</td>
<td>42.86 ± 9.26</td>
</tr>
</tbody>
</table>

* a, number of mice.  
* P < 0.05; *** P < 0.001 in comparison with nontreated control mice exposed to saline solution alone.  
* P < 0.05 in comparison with nontreated control mice exposed to LPS aerosol.
reduction of MMP-9 observed in our experiments might be due to reduced airway neutrophilia caused by NCX-950 pretreatment as previously demonstrated with phosphodiesterase-4 inhibitors (Corbel et al., 2001). The reduction of MMP-9 activity is consistent with the suppression of cytokine-mediated induction of MMP-9 in airway epithelial cells by S-nitrosothiols (Okamoto et al., 2002).

In vitro, TGF-β stimulates fibroblast production of ECM proteins including collagen and fibronectin leading to fibrosis. We presently reported that NCX-950 pretreatment of LPS-exposed mice elicited a marked inhibition of TGF-β release in BAL. On the basis of the data obtained on MMP-9 and TGF-β release, these results suggest that NCX-950 may modulate airway remodeling occurring in several pathologies such as acute lung injury, acute respiratory distress syndrome, and COPD.

Similar to that reported for the relaxant effect of NCX-950, propranolol but not ODQ inhibited the anti-inflammatory activity of this compound, suggesting that the mechanism of action is mediated through an interaction by the β2-adrenoceptor rather than the guanylyl cyclase activation. Controversial experiments have been reported regarding the effect of NO on pulmonary inflammatory process. Indeed, it has been described that NO may contribute to inflammation of the airways (Xiong et al., 1999). However, other studies suggest that constitutional NOS-derived NO and not inducible NOS-derived NO may be pro-inflammatory (Nevin and Broadley, 2002). In contrast, NO may also reduce leukocyte chemotaxis (Kuo et al., 1997) and adherence of leukocytes to the vascular endothelial cell wall (Conran et al., 2001). Inducible NOS-deficient mice that were treated with endotoxin showed enhanced leukocyte accumulation in lung tissue compared with wild-type mice similarly treated with endotoxin (Hickey et al., 1997). The exogenous administration of NO has also been found to exert anti-inflammatory effects (Nevin and Broadley, 2002). Indeed, inhaled NO has been shown to attenuate neutrophil activation and cytokine release in lungs of patients with acute respiratory distress syndrome (Chol-
let-Martin et al., 1996). Inhaled NO, however, did not show anti-inflammatory properties in microvasculature of endotoxin-treated cats (Fox-Robichaud et al., 1998). The anti-inflammatory and pro-inflammatory effects of NO may also be mediated by mechanisms independent of guanylate cyclase activation (Coleman, 2001).

In the present study, we showed that NCX-911, a NO-releasing sildenafil compound, but not sildenafil, at equimolar doses, are able to moderately but significantly reduce LPS-induced neutrophil influx in BAL fluids of mice. These results suggest that endogenous NO release and the subsequent guanylyl cyclase activation are partly involved in the anti-inflammatory effect of NCX-950. It may also suggest that the NO component of NCX-950 may facilitate the activation of β2-adrenoceptor by the salbutamol structure of NCX-950. The enhancement of activity of salbutamol through incorporation of an NO-releasing moiety is consistent with similar enhancement of activity of other types of drugs such as NO-nonsteroidal anti-inflammatory drug and NO-corticosteroid (Keeble and Moore, 2002). Whether or not the mechanism underlying the enhancement of activity and reduced toxicity is the same for all of these types of derivatises is not clear, but certainly it would appear likely that NO mediates a substantial component of the improvement in therapeutic activity. A possible alternative explanation is increased potency at the β2-receptor for NCX-950 when compared with salbutamol. This possibility has been investigated for the enhanced anti-inflammatory properties of nitro-prednisolone (Paul-Clark et al., 2003).

In conclusion, we showed that the nitric oxide-releasing salbutamol, NCX-950, elicits potent relaxant and anti-inflammatory activities compared with salbutamol, and these effects may be mainly due to the activation of β2-adrenoceptor rather than cGMP pathway. These data also suggest that NCX-950 could be proposed as a very interesting compound in the treatment of lung diseases associated with airway obstruction, inflammation, and tissue remodeling such as COPD.

References
Blackwell, Cambridge, UK.
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References