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## Effect of nitric oxide on epithelial ion transports in noncystic fibrosis and cystic fibrosis human proximal and distal airways

Sabine Blouquit-Laye,<sup>1</sup> Luc Dannhoffer,<sup>1</sup> Camille Braun,<sup>1</sup> Anh-Tuan Dinh-Xuan,<sup>2</sup> Edouard Sage,<sup>3</sup> and Thierry Chinet<sup>1</sup>

<sup>1</sup>UPRES EA220, UFR Paris Ile de France Ouest, Université de Versailles Saint Quentin en Yvelines, Boulogne, France;

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**Blouquit-Laye S, Dannhoffer L, Braun C, Dinh-Xuan A, Sage E, Chinet T.** Effect of nitric oxide on epithelial ion transports in noncystic fibrosis and cystic fibrosis human proximal and distal airways. *Am J Physiol Lung Cell Mol Physiol* 303: L617–L625, 2012. First published July 6, 2012; doi:10.1152/ajplung.00368.2011.—The airways of patients with cystic fibrosis (CF) exhibit decreased nitric oxide (NO) concentrations, which might affect airway function. The aim of this study was to determine the effects of NO on ion transport in human airway epithelia. Primary cultures of non-CF and CF bronchial and bronchiolar epithelial cells were exposed to the NO donor sodium nitroprusside (SNP), and bioelectric variables were measured in Ussing chambers. Amiloride was added to inhibit the Na<sup>+</sup> channel ENaC, and forskolin and ATP were added successively to stimulate cAMP- and Ca<sup>2+</sup>-dependent Cl<sup>−</sup> secretions, respectively. The involvement of cGMP was assessed by measuring the intracellular cGMP concentration in bronchial cells exposed to SNP and the ion transports in cultures exposed to 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one, an inhibitor of the soluble guanylate cyclase (ODQ), or to 8Z, a cocktail of 8-bromo-cGMP and zaprinast (phosphodiesterase 5 inhibitor). SNP decreased the baseline short-circuit current (*I*<sub>sc</sub>) and the changes in *I*<sub>sc</sub> induced by amiloride, forskolin, and ATP in non-CF bronchial and bronchiolar cultures. The mechanism of this inhibition was studied in bronchial cells. SNP increased the intracellular cGMP concentration ([cGMP]<sub>i</sub>). The inhibitory effect of SNP was abolished by 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, an NO scavenger (PTIO) and ODQ and was partly mimicked by increasing [cGMP]<sub>i</sub>. In CF cultures, SNP did not significantly modify ion transport; in CF bronchial cells, 8Z had no effect; however, SNP increased the [cGMP]<sub>i</sub>. In conclusion, exogenous NO may reduce transepithelial Na<sup>+</sup> absorption and Cl<sup>−</sup> secretion in human non-CF airway epithelia through a cGMP-dependent pathway. In CF airways, the NO/cGMP pathway appears to exert no effect on transepithelial ion transport.

sodium nitroprusside; primary cell culture; bronchi; bronchioles

NITRIC OXIDE (NO) is an important physiological regulator in the lungs that is involved in vasodilatation, neurotransmission, and inflammatory responses (6, 19, 63). NO production is dependent on NO synthases and occurs in the pulmonary macrophages, neutrophils, and endothelial and epithelial cells of the airways (3, 46, 63). The three NO synthase isoforms (neuronal, inducible, and endothelial) are present in the lung; however, the inducible isoform is considered to be the main source of NO in airway epithelial cells (28, 46). NO production in airway epithelial cells can be stimulated by mediators such as histamine (55, 61, 62) or by contact with neutrophils (46).

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NO can signal through several pathways, depending on the environment, the quantity of NO produced, and the kinetics of production (32). It is currently thought that oxidative reactions occur at “high” NO concentrations (above 1 μM) and lead to auto-oxidation of NO and the production of dinitrogen trioxide, which is responsible for the nitration or nitrosylation of proteins such as p21, ras, and prostacyclin synthase. However, the main target of NO is soluble guanylate cyclase, which produces cGMP and is responsible for most of the downstream effects of NO. In human airway epithelial cells, NO and cGMP are involved in the regulation of numerous functions, including cilia beat frequency (54), ion permeability (21, 23), secretion of interleukin-8 (58), and promotion of airway wound repair (13).

The concentration of NO in exhaled breath is used as a marker of airway inflammation in several airway diseases. It is elevated in asthma exacerbations, allergic rhinitis, and acute respiratory distress syndrome (7, 31, 39) and decreased in asthmatic infants treated with inhaled corticosteroids and in patients with community-acquired pneumonia after treatment and recovery (17, 27). The NO concentration in exhaled breath condensate (EBC) is higher in cystic fibrosis (CF) patients than controls, whereas the fractional exhaled NO concentration is usually lower in CF patients. Zetterquist et al. (67) suggested that the nitrite (NO<sub>2</sub><sup>−</sup>) in the EBC mainly originates in the pharyngo-oral tract as a result of bacterial activity, thereby masking the decreased NO production characteristic of lower airways in CF patients. Other studies revealed lower levels of inducible NO synthase in the bronchial epithelium of CF patients relative to normal subjects (20, 38, 66, 67). In CF cells, the expression of cystic fibrosis transmembrane conductance regulator (CFTR) is decreased or absent. In vivo and in vitro experiments have shown positive correlation between CFTR activity, NO production, and NO synthase expression (60), suggesting that the absence of normal CFTR may explain the low NO concentration in exhaled air from CF patients.

Because NO has antibacterial properties, low NO concentrations may aggravate the susceptibility of CF airways to bacterial infections. Low NO level may also influence epithelial ion transport, which is a major determinant of mucociliary clearance in airways (18, 22, 24, 29, 35, 37, 41, 62). The airways are lined by a thin layer of airway surface liquid (ASL), which is finely adjusted to the length of the outstretched epithelial cell cilia by ion and fluid movements. The ASL facilitates cilia beating and mucociliary clearance. In CF, the defect in CFTR disturbs transepithelial ion transport and leads to decreased ASL height and impaired mucociliary clearance. These deficiencies lead to bacterial infection and inflammation with progressive deterioration of pulmonary function (12). In

murine nasal and alveolar epithelia, constitutive NO decreases the amiloride-sensitive  $\text{Na}^+$  absorption and stimulates  $\text{Cl}^-$  secretion (18, 24). Tamaoki et al. (62) found that NO production by epithelial cells may be involved in tachykinin-induced  $\text{Cl}^-$  secretion in rabbit tracheal mucosa. Duszyk (22) observed that NO activates transepithelial anion secretion via a cGMP-dependent pathway in Calu-3 cells. Moreover, endogenous NO and cGMP stimulate cilia beat frequency in airway epithelial cells (26, 34, 43, 53). These studies suggest that, in human airways, NO likely plays a role in the control of transepithelial electrolytes, mucus secretion, and therefore mucociliary clearance.

Because the decreased concentration of NO observed in CF airways might be partially responsible for the ion transport defect associated with CF, we hypothesized that providing exogenous NO to CF airway epithelial cells could improve the transepithelial ion transport. We therefore exposed primary cultures of human non-CF and CF bronchial and bronchiolar epithelial cells to the NO donor sodium nitroprusside (SNP).

## MATERIALS AND METHODS

**Cell isolation and culture.** Non-CF lung fragments were obtained from 40 patients [ $65.7 \pm 2.6$  (SE) yr] who underwent surgery for lung cancer. CF samples were obtained from 15 patients ( $25.5 \pm 2.1$  yr) who underwent lung transplantation; 9 patients were homozygous for the F508del mutation in CFTR, the others were heterozygous, and all of them, except for one, possessed the F508del allele. Tissues were collected and processed in compliance with the current French public health legislation (articles L.1235–2 and L.1245–2, code de la santé publique, www.legifrance.gouv.fr). Each participating institution informed the patients and made sure that they were not opposed to the use of surgical samples, removed during a medical act, for research purpose. Samples and cell cultures were discarded after use. Human bronchi were identified on the basis of an outer diameter of 3–12 mm and the presence of cartilage. Bronchioles were identified by the absence of cartilage and an outer diameter of  $\leq 2$  mm. Bronchial and bronchiolar epithelial cells were isolated and cultured on collagen matrix supports (CMS) at the air-liquid interface in six-well plates as previously described (10).

**Measurement of nitrate/ $\text{NO}_2^-$  concentrations released by SNP in the culture medium.** SNP ( $10^{-4}$  M) was dissolved in six-well plates filled with 1.7 ml culture medium/well. Some wells contained a CMS with a confluent culture of non-CF bronchial cells. The plates were incubated at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and 100% humidity (Binder, Tuttlingen, Germany). Samples of culture medium were taken at time ( $T$ ) 2 min,  $T_{5\text{min}}$ ,  $T_{15\text{min}}$ , and  $T_{30\text{min}}$  and immediately processed for total nitrate ( $\text{NO}_3^-$ )/ $\text{NO}_2^-$  production measurement.

NO transforms to  $\text{NO}_2^-$  and then to  $\text{NO}_3^-$ . The  $\text{NO}_2^- + \text{NO}_3^-$  level was measured by the Griess reaction after the conversion of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  using nitrate reductase (40). Fifty-microliter samples were incubated with 40  $\mu\text{l}$  of a phosphate buffer containing 20 mU nitrate reductase and 0.9 mM NADPH for 15 min at  $37^\circ\text{C}$ . After this step, the samples contain only  $\text{NO}_2^-$ . Samples were then incubated with 25  $\mu\text{l}$  Griess solution for 15 min at room temperature. The Griess solution, which reacts with  $\text{NO}_2^-$ , was composed of a mixture of equal proportions of 0.5% naphthylethylenediamine dihydrochloride in 2 N HCl and 5% sulfanilamide in 2 N HCl. Absorbances were read at 550 nm using a spectrophotometer (Biochrom, Cambridge, UK).  $\text{NO}_2^-$  concentrations were compared with results obtained with a series of known  $\text{NO}_2^-$  concentrations.

**Measurement of SNP toxicity.** After enzymatic dissociation, suspended non-CF bronchial cells were immediately exposed to  $10^{-4}$  M SNP or vehicle (distilled water) for 30 min in an incubator at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$ , and 100% humidity. Toxicity was assessed using the 0.1%

trypan blue exclusion test, and the number of dead cells (stained blue) was counted on a hemocytometer at  $T_0$  and  $T_{30\text{min}}$ .

**Ussing chamber studies.** Confluent cultures were mounted in Ussing chambers. The short-circuit current ( $I_{\text{sc}}$ ), the potential difference (PD), and the transepithelial resistance ( $R$ ) were measured under baseline conditions. The effect of apical exposure to  $10^{-5}$  M amiloride, an inhibitor of the major epithelial  $\text{Na}^+$  channel ENaC, on  $I_{\text{sc}}$  was recorded. We also measured the effect of successive addition of  $10^{-5}$  M forskolin, an agonist of adenylyl cyclase, and  $10^{-4}$  M ATP, an agonist of  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretion, on  $I_{\text{sc}}$  in the presence of amiloride, as previously described (11).

To study the effects of NO on the bioelectric variables of airway epithelial cells, the cultures were apically and basolaterally preincubated with  $10^{-4}$  M SNP or vehicle for 30 min in the incubator. The cultures were immediately mounted in Ussing chambers after the preincubation, and their baseline bioelectric properties and the changes in  $I_{\text{sc}}$  induced by the successive addition of amiloride, forskolin, and ATP were measured (the entire Ussing chamber protocol lasted 30–40 min). During Ussing experiments, the cultures were still exposed to SNP at the same concentration.

**Effect of the NO scavenger PTIO on SNP-induced  $I_{\text{sc}}$  decreases in non-CF bronchial cultures.** Cultures were exposed to the membrane-impermeable NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO). The stock solution of PTIO (0.1 M) was protected from light and kept no longer than 1 day. Cultures were exposed 30 min in the incubator to  $10^{-4}$  M SNP and PTIO vehicle (PBS) or to  $10^{-4}$  M SNP and  $10^{-3}$  M PTIO. Cultures were mounted immediately in Ussing chambers after preincubation and were still exposed to SNP or SNP + PTIO.

**Measurement of the intracellular concentration of cGMP in bronchial cells exposed to SNP.** The intracellular concentration of cGMP was measured using a competitive enzyme immunoassay (cGMP EIA kit; Cayman Chemical, Ann Arbor, MI). The non-CF and CF bronchial cultures exposed to  $10^{-4}$  M SNP or vehicle for 30 min in the incubator were processed according to the manufacturer's recommendations.

**Effect of the modulation of the intracellular concentration of cGMP on the bioelectric properties of non-CF bronchial epithelial cell cultures.** Because the effects of NO may be mediated by an increase in the intracellular concentration of cGMP, we tested the effect on epithelial ion transport of an inhibition of cGMP production. Non-CF bronchial cultures were apically and basolaterally preincubated for 20 min with  $10^{-5}$  M 1H-[1, 2, 4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ), an inhibitor of the soluble guanylate cyclase, or vehicle [0.0001% dimethyl sulfoxide (DMSO)] before SNP exposure. This experiment was performed at  $37^\circ\text{C}$  in the incubator, and cultures were still exposed to ODQ during SNP exposure. The cultures were then mounted in Ussing chambers and were continuously exposed to ODQ and SNP.

We also tested the effect of increased intracellular cGMP on ion transport in non-CF and CF bronchial cultures. Cultures were mounted in Ussing chambers, baseline bioelectric variables were measured, and cultures were apically exposed to a cocktail of  $10^{-4}$  M 8-bromo-cGMP (a permeant analog of cGMP) and  $10^{-4}$  M zaprinast (an inhibitor of the phosphodiesterase 5, responsible for the cGMP degradation) or vehicles (distilled water and 0.1% DMSO, respectively). After 20 min, the sequence amiloride-forskolin-ATP was applied.

**Chemicals and statistical analysis.** Unless otherwise indicated, chemicals and drugs were purchased from Sigma Aldrich (Saint Quentin Fallavier, France).

Values are expressed as means  $\pm$  SE. Comparisons between treated and control cultures were performed using the unpaired Student's  $t$ -test. A  $P$  value  $< 0.05$  was considered significant.

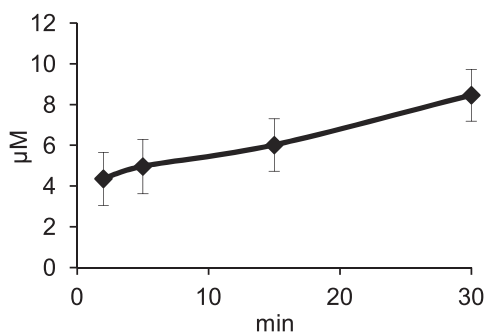


Fig. 1. Measurement of nitrate/nitrite production in the culture medium after sodium nitroprusside (SNP) dissolution. SNP ( $10^{-4}$  M) was dissolved in culture medium. The plate was kept in the incubator, and nitrate/nitrite concentration was measured in samples taken at 2, 5, 15, and 30 min (5 different experiments).

## RESULTS

**Measurement of  $NO_3^-/NO_2^-$  concentrations produced by SNP.** Figure 1 displays the  $NO_3^-/NO_2^-$  measurements in samples of culture medium taken at various times after SNP dissolution. After 2 min, the accumulation was linear over time ( $0.14 \mu\text{M}/\text{min}$ ,  $n = 5$ ), indicating continuous release of NO from SNP. Similar results were obtained in samples of culture medium from wells containing a confluent non-CF bronchial culture ( $n = 6$ , data not shown).

**Measurement of SNP toxicity.** Samples of control cells and cells exposed to  $10^{-4}$  M SNP had a nonsignificantly different percentage of dead cells at  $T_0$  ( $3.6 \pm 2.3$  and  $3.3 \pm 2\%$ , respectively) and  $T_{30\text{min}}$  ( $1.8 \pm 0.9$  and  $3.8 \pm 0.8\%$ , respectively, mean of 3 different experiments, data not shown).

**Effects of SNP on the bioelectric properties of epithelial cell cultures.** Non-CF bronchial and bronchiolar cultures exposed to SNP showed a significant decrease in baseline  $I_{sc}$  compared with cultures exposed to the vehicle. SNP significantly inhibited the changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP in bronchial and bronchiolar cultures (Table 1 and Figs. 2–4), with the exception of ATP in bronchiolar cultures.

Table 1. Effect of SNP on the baseline bioelectric properties of cultured human airway epithelial cells

	PD, mV	$I_{sc}$ , $\mu\text{A}/\text{cm}^2$	$R$ , $\Omega \cdot \text{cm}^2$
<i>Non-CF Bronchial Cultures</i>			
Vehicle ( $n = 28$ )	$-5.1 \pm 0.4$	$48.8 \pm 5.0$	$117.6 \pm 7.5$
SNP ( $n = 30$ )	$-3.3 \pm 0.4^*$	$32.5 \pm 4.4^*$	$101.6 \pm 5.4$
<i>CF Bronchial Cultures</i>			
Vehicle ( $n = 24$ )	$-8.3 \pm 1.5$	$133.5 \pm 31.3$	$96.1 \pm 12.3$
SNP ( $n = 21$ )	$-7.4 \pm 1.3$	$97.5 \pm 27.1$	$111.6 \pm 13.0$
<i>Non-CF Bronchiolar Cultures</i>			
Vehicle ( $n = 28$ )	$-3.7 \pm 0.4$	$86.8 \pm 10.9$	$55.7 \pm 7.1$
SNP ( $n = 26$ )	$-2.4 \pm 0.2^*$	$59.1 \pm 8.2^*$	$58.7 \pm 8.9$
<i>CF Bronchiolar Cultures</i>			
Vehicle ( $n = 23$ )	$-5.5 \pm 0.8$	$48.2 \pm 6.6$	$123.5 \pm 15.6$
SNP ( $n = 22$ )	$-5.2 \pm 0.6$	$38.1 \pm 8.7$	$163.8 \pm 17.7$

Values are means  $\pm$  SE;  $n$ , no. of patients. PD, potential difference;  $I_{sc}$ , short-circuit current;  $R$ , resistance.

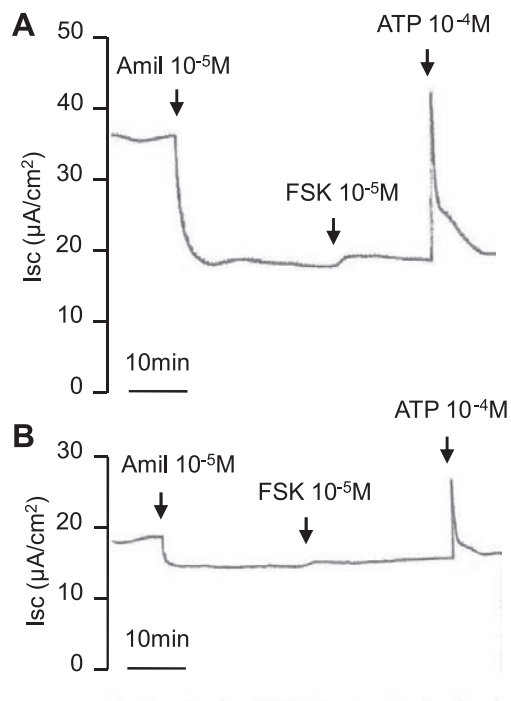


Fig. 2. Representative short-circuit current ( $I_{sc}$ ) tracings from noncystic fibrosis (CF) bronchial cultures exposed to SNP. Confluent cultures were exposed 30 min in an incubator to vehicle (A) or  $10^{-4}$  M SNP (B). In the Ussing chambers, the cultures were still exposed to SNP or vehicle. Amiloride (Amil,  $10^{-5}$  M), forskolin (FSK,  $10^{-5}$  M), and ATP ( $10^{-4}$  M) were added sequentially.

SNP tended to inhibit baseline bioelectric properties and the changes in  $I_{sc}$  induced by amiloride, forskolin, or ATP in CF bronchial and bronchiolar cultures (Table 1 and Figs. 3 and 4); however, this inhibition was not significant. There was no difference between F508del homozygous and heterozygous cell cultures (data not shown).

**Effects of SNP on the intracellular concentration of cGMP in non-CF and CF bronchial epithelial cell cultures.** The intracellular cGMP concentration in non-CF bronchial cells exposed to  $10^{-4}$  M SNP was significantly higher than that of control cells ( $0.5 \pm 0.2$  vs.  $0.02 \pm 0.02$  pmol/ $10^6$  cells,  $n = 4$ ). CF bronchial cells exposed to SNP displayed a similar increase in the intracellular concentration of cGMP ( $0.72 \pm 0.08$  vs.  $0.17 \pm 0.03$  pmol/ $10^6$  cells,  $n = 4$ ).

**Effect of the NO scavenger PTIO on SNP-induced  $I_{sc}$  decreases in non-CF bronchial cultures.** Compared with the control group (vehicle), non-CF bronchial cultures exposed to SNP + PTIO displayed nonsignificantly different baseline  $I_{sc}$  and changes in  $I_{sc}$  in response to amiloride, forskolin, and ATP (Fig. 5). This result suggests that scavenging NO abolishes the inhibitory effect of SNP on ion transport.

Cultures exposed to SNP + PTIO displayed significantly higher changes in forskolin and ATP response than cultures exposed to SNP. Baseline  $I_{sc}$  and change in amiloride response tended to be higher in the SNP + PTIO group; however, the differences were nonsignificant.

**Effect of ODQ on the inhibition of ion transport induced by SNP in non-CF bronchial epithelial cell cultures.** Non-CF bronchial cultures exposed to ODQ and SNP displayed similar PD and significantly lower basal  $R$  than cultures exposed to the vehicle of ODQ and SNP ( $-3.2 \pm 0.3$  vs.  $-3.6 \pm 0.3$  mV,

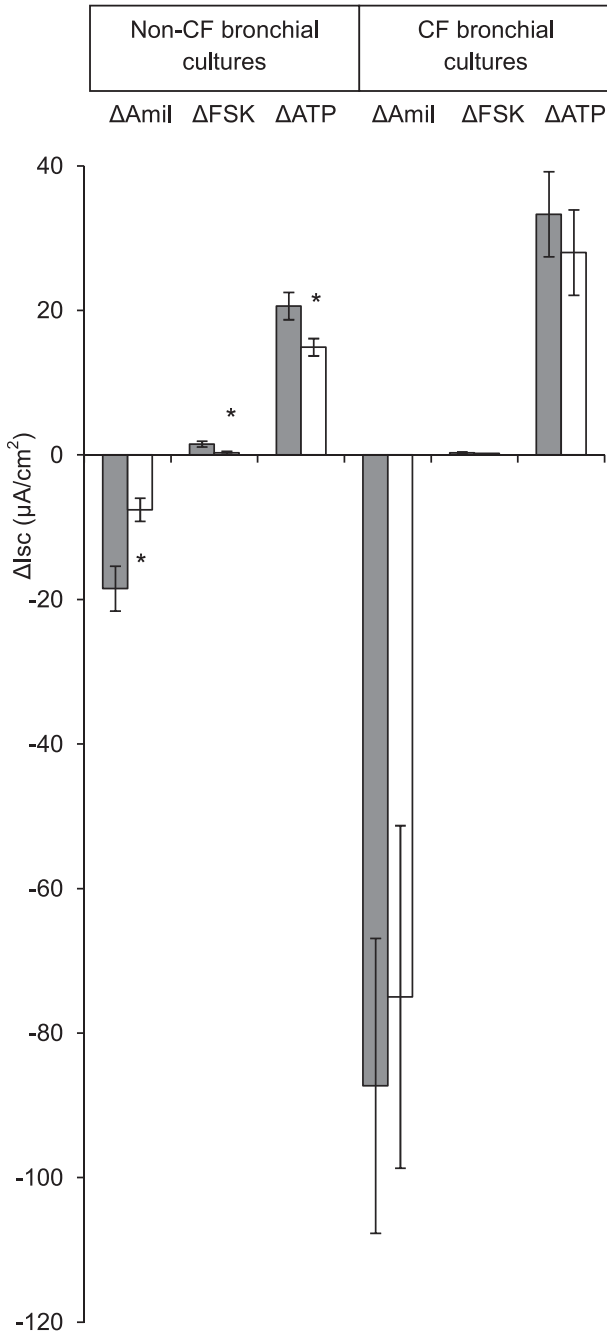


Fig. 3. Effect of  $10^{-4}$  M SNP on the changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP in non-CF bronchial and CF bronchial epithelial cell cultures. Amiloride ( $10^{-5}$  M), forskolin ( $10^{-5}$  M), and ATP ( $10^{-4}$  M) were added sequentially, and changes in  $I_{sc}$  were measured after the addition of each drug. \*Significant difference between vehicle (gray bars) and SNP (white bars) ( $P < 0.05$ ).

respectively, and  $57.4 \pm 4.1$  vs.  $80.2 \pm 8.4 \Omega\cdot\text{cm}^2$ , respectively,  $P < 0.05$ ). The baseline  $I_{sc}$  and the changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP were significantly higher in the cultures exposed to ODQ and SNP compared with cultures exposed to the vehicle of ODQ and SNP (Fig. 6). These results suggest that ODQ abolished the inhibitory effect of SNP on the bioelectric properties of non-CF bronchial cultures.

*Effect of increasing the intracellular concentration of cGMP on the bioelectric properties in non-CF and CF bronchial epithelial cell cultures.* In non-CF bronchial cells, the cocktail of 8-bromo-cGMP and zaprinast induced a similar decrease of the baseline  $I_{sc}$  than the vehicles ( $-14.1 \pm 3.9$  vs.  $-13.4 \pm 4.4 \mu\text{A}/\text{cm}^2$ , respectively). In non-CF cultures exposed to both 8-bromo-cGMP and zaprinast, the changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP were lower than in cultures exposed to vehicles, with a significant difference observed for forskolin and ATP (Fig. 7). These results suggest that increasing the intracellular cGMP concentration partly mimics the effect of SNP in non-CF bronchial cultures.

In contrast, increasing the intracellular cGMP concentration had no significant effect on changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP in CF bronchial cells (Fig. 7).

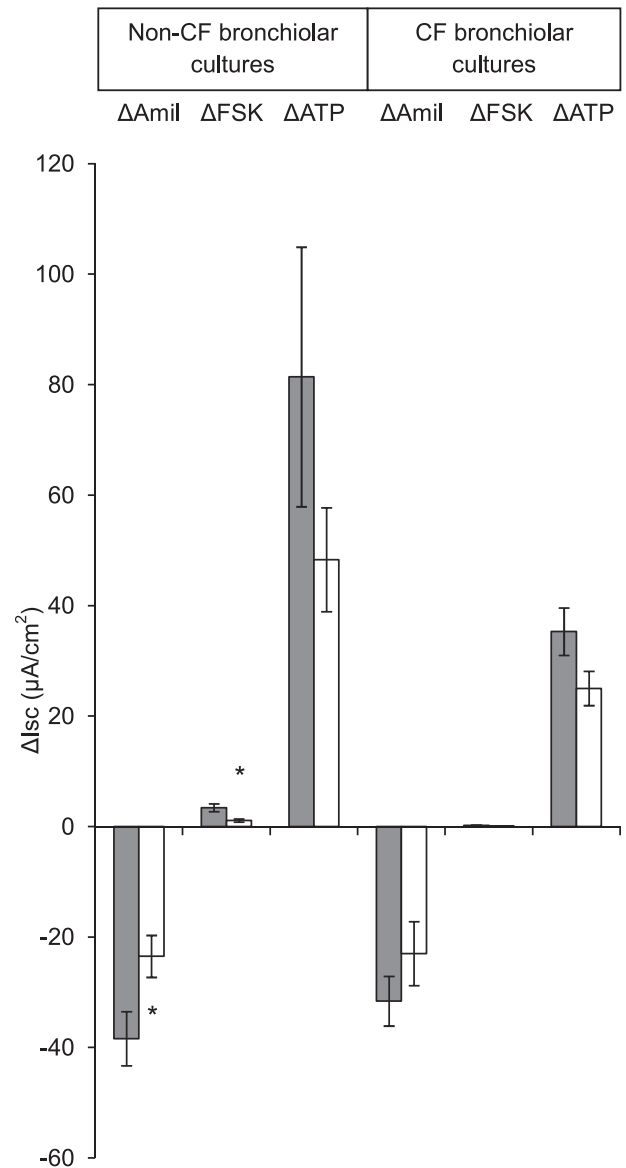


Fig. 4. Effect of  $10^{-4}$  M SNP on  $I_{sc}$  and the changes in  $I_{sc}$  induced by amiloride, forskolin, and ATP in non-CF bronchiolar and CF bronchiolar epithelial cell cultures. Amiloride ( $10^{-5}$  M), forskolin ( $10^{-5}$  M), and ATP ( $10^{-4}$  M) were added sequentially, and changes in  $I_{sc}$  were measured after the addition of each drug. \*Significant difference between vehicle (gray bars) and SNP (white bars) ( $P < 0.05$ ).

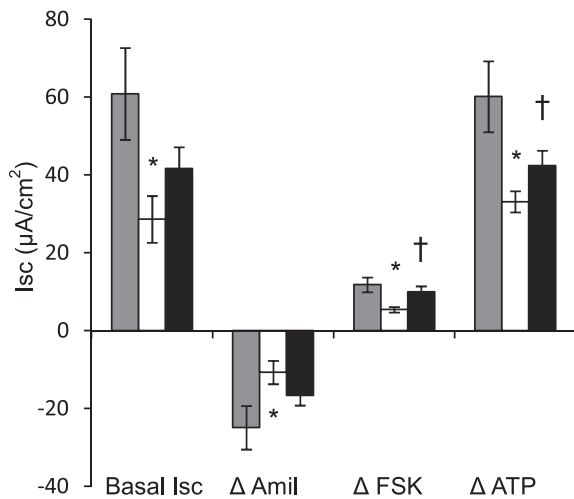


Fig. 5. Effect of 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) on the inhibition of ion transport induced by SNP in non-CF bronchial epithelial cell cultures. Cultures were simultaneously preincubated with  $10^{-3}$  M PTIO (or vehicle) and with  $10^{-4}$  M SNP before insertion in the Ussing chambers. After the measurement of baseline  $I_{sc}$ , amiloride ( $10^{-5}$  M), forskolin ( $10^{-5}$  M), and ATP ( $10^{-4}$  M) were added sequentially, and changes in  $I_{sc}$  were measured after the addition of each drug. Gray bars, control (vehicles,  $n = 10$ ); white bars, the SNP group ( $n = 19$ ); black bars, SNP + PTIO ( $n = 23$ ). \*Significantly different from the control group ( $P < 0.05$ ). †Significantly different from the SNP group ( $P < 0.05$ ).

## DISCUSSION

The aim of this study was to assess the role of NO in the regulation of airway transepithelial ion transport. The NO donor SNP at  $10^{-4}$  M inhibited  $\text{Na}^+$  absorption and  $\text{Cl}^-$  secretion in human non-CF bronchial and bronchiolar epithelial cells, at least in part through NO release and an increase in

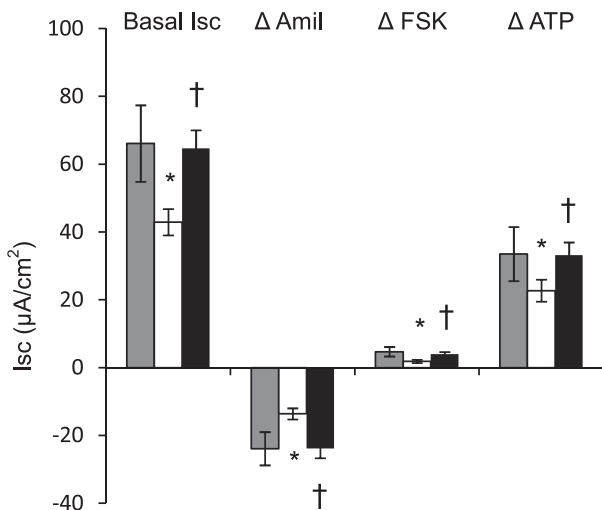


Fig. 6. Effect of 1H-[1,2,4]oxadiazolo-[4,3-a]quinoxalin-1-one (ODQ) on the inhibition of ion transport induced by SNP in non-CF bronchial epithelial cell cultures. Cultures were preincubated with  $10^{-5}$  M ODQ (or vehicle) and with  $10^{-4}$  M SNP before insertion in the Ussing chambers. After the measurement of baseline  $I_{sc}$ , amiloride ( $10^{-5}$  M), forskolin ( $10^{-5}$  M), and ATP ( $10^{-4}$  M) were added sequentially, and changes in  $I_{sc}$  were measured after the addition of each drug. Gray bars, the control group (vehicles,  $n = 13$ ); white bars, the SNP group ( $n = 24$ ); black bars, the SNP + ODQ group ( $n = 23$ ). \*Significantly different from the control group ( $P < 0.05$ ). †Significantly different from the SNP group ( $P < 0.05$ ).

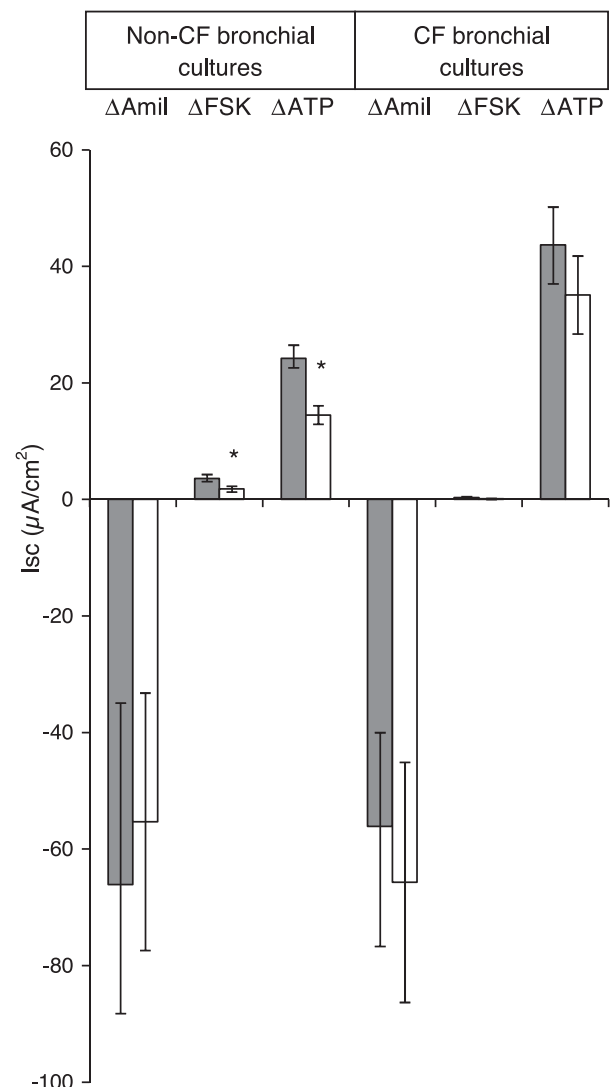


Fig. 7. Effect of increased intracellular concentration of cGMP on the bioelectric properties in non-CF and CF bronchial epithelial cell cultures. Cultures were simultaneously exposed to  $10^{-4}$  M 8-bromo-cGMP and  $10^{-4}$  M zaprinast ( $n = 11$  for non-CF and 10 for CF, white bars) or vehicles ( $n = 10$  for non-CF and for CF, gray bars) after stabilization of the bioelectric variables in the Ussing chambers. Changes in  $I_{sc}$  were then measured after the subsequent addition of amiloride ( $10^{-5}$  M), forskolin ( $10^{-5}$  M), and ATP ( $10^{-4}$  M). \*Significantly different from the control group ( $P < 0.05$ ).

the intracellular cGMP level. Ion transport in CF bronchial and bronchiolar cultures was unaffected by SNP, despite the fact that SNP increased the cGMP level in CF bronchial cells. Increasing cGMP level in CF bronchial cultures had no effect on ion transport, suggesting a failure of CF cells to respond to NO and cGMP.

Measurements of  $\text{NO}_3^-/\text{NO}_2^-$  concentrations revealed that, from 2 min, SNP regularly produced NO in our model, suggesting that, during the time of the experiment, the cultures were exposed to a regular NO level. Similarly, Chen et al. (14) found that, after an immediate higher NO formation ( $1 \mu\text{M}$ ),  $10 \mu\text{M}$  of the NO donor *S*-nitrosoglutathione provided a steady-state lower value ( $0.3 \mu\text{M}$ ), lasting over 45 min. The kinetic of accumulation of  $\text{NO}_3^-/\text{NO}_2^-$  was similar in our culture medium alone and in wells containing a confluent cell culture. SNP

requires the presence of tissue or reducing agents such as thiol groups to produce NO (8, 45). Our culture medium contains an adequate concentration of thiol groups [0.26 vs. 0.2 mM for Bates et al. (8)]. Living cells metabolize NO. The cell culture may therefore modify the NO concentration in the culture medium; however, our cell inserts are very small (diameter 2 and 3.5 mm) compared with the volume of culture medium (1.7 ml). The impact of cellular NO clearance on  $\text{NO}_3^-/\text{NO}_2^-$  level during 30 min was probably negligible. Rolla et al. (51) found  $\text{NO}_3^-/\text{NO}_2^-$  concentrations of 12.3  $\mu\text{M}$  (range 11.1–41.9) and 9.4  $\mu\text{M}$  (range 4.6–10.9) in the EBC of normal and end-stage renal disease patients, respectively. Likewise, Ratnawati et al. (50) found a  $\text{NO}_3^-/\text{NO}_2^-$  concentration range of 4.8–8.4  $\mu\text{M}$  in EBC, with higher values in asthmatic and atopic subjects. Ojoo et al. (48) found a  $\text{NO}_3^-/\text{NO}_2^-$  concentration of 29.9  $\mu\text{M}$  in the EBC of CF patients, which was higher than that of control subjects (11.4  $\mu\text{M}$ ). These observations indicate that the maximal  $\text{NO}_3^-/\text{NO}_2^-$  concentrations produced by SNP in the present study did not exceed concentrations measured in samples of human EBC. Bates et al. (8) found that  $10^{-3}$  M SNP provided 50 nM NO/s in vitro. The lifetime of NO is 6–10 s, especially in well-oxygenized media used in vitro; the NO level probably did not exceed 0.5  $\mu\text{M}$  in the experiments of Bates et al. SNP ( $10^{-4}$  M) probably did not exceed this value either. It is difficult to assess in vivo NO levels. The generally admitted physiological NO concentration is  $<1$   $\mu\text{M}$ . Hall and Garthwaite (30) suggested that a lower physiological NO level range is obtained with recent and more reliable measurement techniques: from 100 pM to 5 nM. However, almost all of the cited studies were performed in brain tissues, liver, and aorta (endothelium and smooth muscle cells). The NO concentration delivered by SNP in the present study is probably  $<1$   $\mu\text{M}$  and is likely to be present in normal and pathological human airways.

SNP may exert a toxic effect that could explain the decreases in baseline  $I_{sc}$  and changes in the  $I_{sc}$  induced by all of the tested drugs (amiloride, forskolin, and ATP). SNP releases cyanide during NO production. Cyanide and NO are toxic compounds for respiration (8). It has been shown that NO inhibits the cytochrome *c* oxidase (30). Mitochondrial damage induced by a 24-h SNP exposure ( $10^{-4}$  M) leads to 20% cell death in PC-12 cells (49) and to 14% human synovial cell death ( $5 \times 10^{-4}$  M SNP) (16). NO may also reduce ionic gradients and amplitude of the changes in  $I_{sc}$  induced by the drugs by inhibiting the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity (2). We found no increase in the percentage of dead cells 30 min after SNP exposure; the global time of exposure is probably insufficient to induce significant mitochondrial damage. Rückes-Nilges et al. (52) worked with SNP concentrations 10 and 20 times higher than the concentration used in the present study and did not find a tendency to a general decrease in bioelectric variables that could suggest a toxic effect. NO can induce an oxidative stress that may impact the junctional integrity of epithelial cells (4, 21); however, the *R* in our cultures did not change in response to SNP, suggesting that tight junctions were not affected in our model. Overall, these results suggest that SNP did not exert significant toxic effects in our cultures.

The NO scavenger PTIO inhibited the effect of SNP on ion transports, in a significant manner for forskolin- and ATP-dependent  $\text{Cl}^-$  secretion, suggesting that NO is responsible for most of the inhibitory effect of SNP on ion transport. We found

that PTIO did not totally inhibit the effect of SNP, possibly because PTIO imperfectly scavenges NO. It has been shown that PTIOs such as carboxy-PTIO are less effective NO scavengers than hemoglobin (42). However, we cannot exclude that a part of the SNP effect may be independent on its NO release. Despite the fact that NO may have pro-oxidative effect, the main target of NO is soluble guanylate cyclase, and NO donors are known to increase intracellular cGMP levels (47, 56). We used several protocols to confirm the role of cGMP in the inhibitory effect of SNP on ion transport in our cultures and found that SNP acts principally via an increase in intracellular cGMP concentration. The main part of the inhibitory effect of SNP on ion transport in our cultures is transmitted through the NO/cGMP pathway.

Scavenging NO or inhibiting the soluble guanylate cyclase abolished the inhibitory effect of SNP on the baseline  $I_{sc}$  and the amiloride response, suggesting that NO inhibits transepithelial  $\text{Na}^+$  transport in non-CF airway epithelium. PTIO imperfectly inhibited the SNP effect. The remaining NO concentration may be sufficient to partly decrease the baseline  $I_{sc}$  and the amiloride response. At last, increasing intracellular cGMP level only partly mimicked the inhibitory effect of SNP on  $\text{Na}^+$  transport. The basal intracellular cGMP level was probably sufficient to partly inhibit  $\text{Na}^+$  transport. An inhibitory effect of NO on the transepithelial  $\text{Na}^+$  transport has already been described in animal airways (29, 33, 35). Guo et al. (29) found that NO inhibited 60% of the amiloride-sensitive  $I_{sc}$  in cultured rat alveolar type II cells after permeabilization of the basolateral membrane. NO also inhibited 60% of the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity in apically permeabilized cultures. The reduction of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activity leads to a decreased driving force for  $\text{Na}^+$  entry through the apical membrane (2). A patch-clamp study in A549 cells showed that the amiloride-sensitive  $I_{sc}$  is inhibited by exogenous NO and by increasing the 8-bromo-cGMP level (41). Similar results were observed in cultured H441 cells, a cell line with morphologic features of Clara cells, exposed to two different NO donors (1, 57) and in freshly isolated human nasal cells exposed to  $3 \times 10^{-4}$  M SNP (5). In contrast, Rückes-Nilges et al. (52) did not find an effect of SNP (1 mM) on amiloride-sensitive  $\text{Na}^+$  transport in primary cultured human nasal epithelium. These observations suggest that NO inhibits transepithelial  $\text{Na}^+$  absorption in human airways, except for the nasal level. Similarly, in the rodent kidney, NO inhibits the  $\text{Na}^+\text{-K}^+\text{-ATPase}$  in the proximal tubule but not in the collecting duct (2).

In our model, the forskolin-induced increase in  $I_{sc}$  is supported by a sustained increase in intracellular cAMP concentration leading to CFTR-dependent  $\text{Cl}^-$  secretion. ATP induces an  $I_{sc}$  increase via  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels, leading to a fast and large  $\text{Cl}^-$  secretion. Scavenging NO completely abolished the inhibitory effect of SNP, and an increase in cGMP mimicked the effect of SNP on cAMP- and  $\text{Ca}^{2+}$ -dependent  $\text{Cl}^-$  secretions in bronchial cultures. These results are in contradiction with previous studies in airway epithelial models that concluded that NO has either no effect or a stimulatory effect on  $\text{Cl}^-$  secretion. Endogenous NO stimulated  $\text{Cl}^-$  secretion in nasal murine cells (24) and A549 cells (37). Exogenous NO had a stimulatory effect in Calu-3 cells (15, 22), A549 cells (37), the murine nose in vivo (15), and in human nasal cells impaled with a microelectrode (5). In contrast, exogenous NO did not have any effect on  $\text{Cl}^-$  secretion in

fresh rat alveolar type II cells (15) or in primary human nasal epithelial cell cultures (26, 52). In the studies on fresh human nasal cells and primary human nasal cell cultures, NO was provided by SNP at 300  $\mu\text{M}$  (5, 26) or 1 mM (52). The effect or the absence of effect on  $\text{Cl}^-$  secretion was confirmed by the use of another NO donor (spermine NONOate) or the exposure to 8-bromo-cGMP (5, 26, 52). These differences are not due to different culture conditions, since Geary et al. (26) and Rückes-Nilges et al. (52) used the same culture medium as our medium. To our knowledge, no other reports in the literature have discussed the effects of SNP or NO donors on primary human bronchial and bronchiolar epithelial cells. The inhibitory effect of the NO/cGMP system on epithelial  $\text{Cl}^-$  secretion may be limited to the bronchial and bronchiolar area. Each airway level, as characterized by typical epithelial morphology, may have specific functions. We have previously shown that there are significant differences in transepithelial ion transport between the bronchial and bronchiolar levels (11). Upon longer exposure to exogenous NO (24–96 h), an inhibitory effect on CFTR and cAMP-mediated  $\text{Cl}^-$  secretion was observed in human airway and kidney epithelial cell lines (9, 36). Stimulation of endogenous NO production also inhibited adenylyl cyclase and cAMP-dependent  $\text{Cl}^-$  secretion in human cholangiocytes (59) and in rodent colonic epithelia (25, 44). Overall, these studies suggest that inhibition of cAMP-dependent  $\text{Cl}^-$  secretion by NO is possible in epithelia, which is in accordance with our results. To our knowledge, the only available reference about the effect of NO donors on ATP-dependent  $\text{Cl}^-$  secretion in airway epithelia is the work of Rückes-Nilges et al. (52). They showed that SNP and spermine NONOate have no effect on  $\text{Cl}^-$  conductance in human nasal epithelia. It was also observed that NO mediates hyporesponsiveness to cAMP, but not to  $\text{Ca}^{2+}$ -dependent secretagogues, in the rodent colon (25). The SNP-induced decrease in ATP-dependent  $\text{Cl}^-$  secretion in human bronchial and bronchiolar cultures described in our study seems specific to these airway levels.

Human bronchial and bronchiolar epithelia absorb NaCl and fluid under basal conditions and are able to secrete NaCl and fluid in response to the stimulation of  $\text{Cl}^-$  channels by agonists or by tidal breath-induced shear stress (12, 64). Our study suggests that exogenous NO limits baseline NaCl absorption and selective NaCl secretion, potentially contributing to reduced amplitude of transepithelial fluid movements.

SNP tended to decrease baseline  $I_{\text{sc}}$  and drug-induced changes in  $I_{\text{sc}}$  in CF cultures; however, cGMP did not have a significant effect on ion transport. This suggests that SNP may have a small inhibitory effect independent on its NO release, but the activation of the NO/cGMP pathway has no effect on ion transport in CF cultures. This pathway seems to be functional in CF cells, since the cGMP level in CF bronchial cultures was increased by SNP exposure. Baker et al. (5) also observed that NO has different effect in control and pathological tissue. They observed that the ion permeability of the nasal epithelium of asthmatic patients did not change in response to NO or cGMP, contrary to healthy epithelium. The use of channel inhibitors suggests that either the basolateral  $\text{Na}^+\text{-K}^+\text{-2Cl}^-$  cotransporter is not functioning or that the defect lies in the pathways that regulate secretion. It has been shown in vivo that CF patients with higher nasal PDs also have lower levels of exhaled NO, suggesting that the reduced NO concentration is correlated with  $\text{Na}^+$  hyperabsorption (65). Therefore, expos-

ing CF airways to NO would counterbalance  $\text{Na}^+$  hyperabsorption and potentiate ATP-induced  $\text{Cl}^-$  secretion. However, Rückes-Nilges et al. (52) found that NO has no beneficial effect on ion transport in cultured human CF nasal epithelial cells, which is in accordance with our results in bronchi and bronchioles. Although the lack of CFTR inhibits inducible NO synthase (60), this cannot explain why exogenous NO does not affect transepithelial ion transport. The failure of CF cultures to respond to the activation of the NO/cGMP system remains unexplained.

In conclusion, NO is an inhibitor of the amiloride-sensitive  $\text{Na}^+$  absorption and the cAMP- and ATP-dependent  $\text{Cl}^-$  secretions in human non-CF bronchial and bronchiolar epithelia. In contrast, NO has no effect on these ion transports in CF counterparts. Potential therapeutic interventions aimed at correcting the NO deficiency in CF probably will not significantly improve transepithelial ion transport.

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#### DISCLOSURES

The authors declare that they have no competing interests.

#### AUTHOR CONTRIBUTIONS

Author contributions: S.B.-L., A.-T.D.-X., and T.C. conception and design of research; S.B.-L., L.D., and C.B. performed experiments; S.B.-L., L.D., C.B., and T.C. analyzed data; S.B.-L., L.D., and T.C. interpreted results of experiments; S.B.-L. prepared figures; S.B.-L., L.D., A.-T.D.-X., and T.C. drafted manuscript; S.B.-L. and E.S. edited and revised manuscript; S.B.-L., L.D., C.B., A.-T.D.-X., E.S., and T.C. approved final version of manuscript.

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