DETANO and Nitrated Lipids Increase Chloride Secretion across Lung Airway Cells

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We investigated the cellular mechanisms by which nitric oxide (NO) increases chloride (Cl⁻) secretion across lung epithelial cells *in vitro* and *in vivo*. Addition of (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino] diazen-1-ium-1, 2-diolate (DETANONOate [DETANO]; 1–1,000 μ M) into apical compartments of Ussing chambers containing Calu-3 cells increased short-circuit currents (I_{sc}) from 5.2 ± 0.8 to $15.0 \pm 2.1 \mu\text{A}/\text{cm}^2$ ($X \pm 1 \text{ SE}$; $n = 7$; $P < 0.001$). NO generated from two nitrated lipids (nitrolinoleic and nitrooleic acids; 1–10 μ M) also increased I_{sc} by about 100%. Similar effects were noted across basolaterally, but not apically, permeabilized Calu-3 cells. None of these NO donors increased I_{sc} in Calu-3 cells pretreated with 10 μ M 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (an inhibitor of soluble guanylyl cyclase). Scavenging of NO either prevented or reversed the increase of I_{sc} . These data indicate that NO stimulation of soluble guanylyl cyclase was sufficient and necessary for the increase of I_{sc} via stimulation of the apical cystic fibrosis transmembrane regulator (CFTR). Both Calu-3 and alveolar type II (ATII) cells contained CFTR, as demonstrated by *in vitro* phosphorylation of immunoprecipitated CFTR by protein kinase (PK) A. PKGII (but not PKGI) phosphorylated CFTR immunoprecipitated from Calu-3 cells. Corresponding values in ATII cells were below the threshold of detection. Furthermore, DETANO, 8-Br-cGMP, or 8-(4-chlorophenylthio)-cGMP (up to 2 mM each) did not increase Cl⁻ secretion across amiloride-treated ATII cells *in vitro*. Measurements of nasal potential differences in anesthetized mice showed that perfusion of the nares with DETANO activated glybenclamide-sensitive Cl⁻ secretion. These findings suggest that small concentrations of NO donors may prove beneficial in stimulating Cl⁻ secretion across airway cells without promoting alveolar edema.

Keywords: Calu-3 cells; cystic fibrosis transmembrane conductance regulator; nasal potential difference; protein kinase G type II; alveolar type II cells

Previous studies have shown that airway cells absorb sodium (Na⁺) and secrete chloride (Cl⁻) and bicarbonate ions via active transport mechanisms (1). Na⁺ ions enter the apical membranes of lung epithelial cells via both cation- and amiloride-sensitive Na⁺ channels, and exit across the basolateral membranes by the ouabain-sensitive Na-K-ATPase (2). Cl⁻ ions enter the basolateral membranes via a number of pathways, including the Na⁺-K⁺-2Cl⁻ cotransporter, and exit across the apical membrane down their electrochemical gradient via the cystic fibrosis transmembrane conductance regulator (CFTR) or other types of Cl⁻

CLINICAL RELEVANCE

This research attempts to understand the basic mechanisms by which nitric oxide alters ion transport across lung epithelial cells. This may form the rational basis for the use of nitric oxide as a therapeutic tool in cystic fibrosis.

channels (1). The coordinated transport of these ions creates osmotic forces responsible for the movement of fluid across airways. Fluid and solute transport play vital roles in proper hydration of airway mucus, mucociliary clearance, and innate immunity.

The CFTR, a 1,480-amino acid protein, is a member of the traffic ATPase family, functions as a cAMP-regulated Cl⁻ channel, and controls other ion-conductive pathways, including Na⁺ channels, as well as ATP transport (1, 3). Although an earlier study did not identify cAMP-stimulated Cl⁻ conductance in freshly isolated rabbit alveolar type II (ATII) cells (4), more recent studies have provided convincing evidence of the presence of both immunoreactive and functional CFTR in both fetal (5) and adult ATII cells (6). For example, Nielsen and colleagues (7) showed that agents that increased cAMP increased Cl⁻ secretion across the alveolar epithelium of rabbits *in vivo*, whereas the studies of Fang and colleagues (8) demonstrated that functional CFTR is necessary for increased Na⁺ reabsorption across alveolar epithelial cells by agents that increase intracellular cAMP. These studies helped to establish the importance of CFTR in fluid homeostasis across both airway and alveolar epithelia.

Because of their location, airway and alveolar cells are continuously exposed to nitric oxide (NO), present in cigarette smoke, environmental pollutants, oxidant gases, or generated endogenously by nitric oxide synthases (NOS) that are known to be present in both airway and inflammatory cells of murine and human lungs (9–11). Increased inducible NOS levels have been found in airway cells and human lung tissue obtained from patients with acute respiratory distress syndrome (ARDS) (11, 12) and other inflammatory lung diseases. Furthermore, inhaled NO has been shown to be beneficial in mitigating both idiopathic pulmonary hypertension of the newborn (13) and systemic ischemia–reperfusion injury (14).

The biological effects of NO and its congeners depend on their concentration, the biochemical composition of the targets, and the presence of other reactive intermediates. At low concentrations, the physiological effects of NO are dominated by its rapid and reversible reactions with heme proteins, especially the NO-mediated activation of soluble guanylyl cyclase (sGC), resulting in an increase in cellular cGMP (15). At higher concentrations of NO, or in the presence of transition metals and/or superoxide, NO will exert its effects through oxidative and covalent modification of biomolecules via formation of higher nitrogen oxides (12, 16–19).

Regulation of CFTR by NO and its congeners is complex; whereas S-nitrosation (i.e., addition of a nitrosonium to a re-

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Figure 1. Nitrolinoleic acid (LNO₂) and (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate (DETANO) increase short-circuit current (*I*_{sc}) in Calu-3 cells. Confluent monolayers of Calu-3 cells were mounted in Ussing chambers. LNO₂ (0.01–10 μM; left panels) or DETANO (1–1,000 μM; right panels) were added into apical compartments of the Ussing chambers. In paired experiments, equivalent amounts of the parent compound (linoleic acid [LA]) or vehicle (NaOH) were added in similar fashion. (A and C) Typical recordings of *I*_{sc} in individual experiments. Mean values ± 1 SE are shown in Table 1. (B and D) Percent of maximum *I*_{sc} versus concentration of LNO₂ or DETANO; percent of maximum *I*_{sc} = $((I_x - I_0) / (I_{max} - I_0)) \times 100$, where *I*₀ is the *I*_{sc} obtained in the absence of LNO₂ or DETANO, *I*_x is the steady-state current at a given concentration (μM) of LNO₂ or DETANO, and *I*_{max} is the steady-state value of *I*_{sc} after addition of either 10 μM LNO₂ or 1,000 μM DETANO. Data were fitted by spline curves using Slide Write for Windows 6. Values for 50% stimulation (~0.06 μM for LNO₂ and 12 μM for DETANO) were calculated by extrapolation from these figures.

duced cysteine, resulting in nitrosothiol) of CFTR increases maturation and function of both wild-type and ΔF508 CFTR (20, 21), glutathionylation, oxidation, or nitration of CFTR are associated with loss of function and decreased levels of apical

CFTR in polarized cells (19, 22, 23). In addition, activation of cGMP-dependent protein kinase (PK) type II (PKGII) is associated with phosphorylation of CFTR and increased Cl⁻ secretion across intestinal and airway cells (24, 25). However,

TABLE 1. STEADY-STATE EFFECTS OF NITROLINOLEIC OR LINOLEIC ACID, DETANO OR NaOH, AND THEIR CORRESPONDING VEHICLE CONTROLS, ON SHORT-CIRCUIT CURRENT AND TRANSEPITHELIAL RESISTANCE OF CALU-3 MOUNTED IN USSING CHAMBERS

Variable	Baseline	Concentration (μM)					
		0.01	0.1	1	10	100	1,000
<i>I</i> _{sc} : LA (6)	5.8 ± 0.5	5.9 ± 0.6	6.0 ± 0.6	6.5 ± 0.4	5.5 ± 0.8	—	—
Δ <i>I</i> _{sc} : LA	—	0.2 ± 0.2	0.3 ± 0.2	0.5 ± 0.5	0.5 ± 0.9	—	—
<i>I</i> _{sc} : LNO ₂ (7)	6.4 ± 0.7	6.6 ± 0.7	7.9 ± 0.7*	9.2 ± 0.4*	12.4 ± 2.2*	—	—
Δ <i>I</i> _{sc} : LNO ₂	—	0.2 ± 0.1	1.5 ± 0.3†	2.3 ± 0.4†	5.3 ± 2.1†	—	—
<i>R</i> _t : LA (6)	351 ± 43	332 ± 39	298 ± 38	285 ± 41	264 ± 42	—	—
Δ <i>R</i> _t : LA	—	-19 ± 9	-52 ± 17	-66 ± 10	-87 ± 15	—	—
<i>R</i> _t : LNO ₂ (7)	364 ± 39	342 ± 35	325 ± 35	296 ± 37	234 ± 30*	—	—
Δ <i>R</i> _t : LNO ₂	—	-22 ± 7	-38 ± 10	-67 ± 18	-144 ± 20†	—	—
<i>I</i> _{sc} : NaOH (4)	5.1 ± 0.7	—	—	5.3 ± 0.6	5.6 ± 0.5	6.3 ± 0.5	6.8 ± 0.5
Δ <i>I</i> _{sc} : NaOH	—	—	—	0.3 ± 0.2	0.5 ± 0.3	1.2 ± 0.6	1.7 ± 0.7
<i>I</i> _{sc} : DETANO (7)	5.2 ± 0.8	—	—	7.1 ± 1.1*	9.8 ± 1.5*	13.7 ± 2.1*	15.0 ± 2.1*
Δ <i>I</i> _{sc} : DETANO	—	—	—	1.9 ± 0.6	4.5 ± 1.2†	8.5 ± 1.7†	9.7 ± 1.8†
<i>R</i> _t : NaOH (4)	324 ± 14	—	—	321 ± 11	313 ± 12	286 ± 10	268 ± 8
Δ <i>R</i> _t : NaOH	—	—	—	-1.9 ± 0.6	-4.5 ± 1.2†	-8.5 ± 1.7†	-9.7 ± 1.8†
<i>R</i> _t : DETANO (7)	334 ± 21	—	—	330 ± 19	326 ± 20	294 ± 18	262 ± 23
Δ <i>R</i> _t : DETANO	—	—	—	-4 ± 2	-7 ± 4	-40 ± 12	-72 ± 23

Definition of abbreviations: DETANO, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate; *I*_{sc}, short-circuit current; LA, linoleic acid; LNO₂, nitrolinoleic acid; *R*_t, transepithelial resistance.

Δ*I*_{sc} (μA/cm²) or Δ*R*_t (Ω × cm²) were calculated by subtracting the steady-state value at the indicated variable for the indicated concentration from the corresponding baseline values of the same filter. Values are means ± 1 SE; numbers in parentheses indicate number of monolayers. Positive Δ*I*_{sc} or Δ*R*_t indicate that the variable was increased, whereas negative numbers indicate that it was decreased.

* *P* < 0.05 compared to the corresponding baseline value.

† *P* < 0.05 compared to the corresponding vehicle control group.

TABLE 2. STEADY-STATE EFFECTS OF 8-pCPTcAMP OR 8-pCPTcGMP ON SHORT-CIRCUIT CURRENT AND TRANSEPITHELIAL RESISTANCE OF CALU-3 CELLS MOUNTED IN USSING CHAMBERS

	<i>n</i>	<i>I_{sc}</i>		ΔI_{sc}	<i>R_t</i>		ΔR_t
		Baseline	1 mM	1 mM	Baseline	1 mM	1 mM
Veh	7	8.9 ± 0.9	9.6 ± 1.1	0.8 ± 0.6	459 ± 54	425 ± 57	-33 ± 8
cAMP	9	14.9 ± 1.5	29.4 ± 2.8*	14.8 ± 1.7†	255 ± 10	167 ± 22*	-89 ± 21†
cGMP	6	10.4 ± 1.0	25.3 ± 2.1*	14.2 ± 3.5†	360 ± 109	230 ± 81*	-130 ± 17†

Definition of abbreviation: Veh, vehicle for cAMP, cGMP (H₂O).

ΔI_{sc} ($\mu A/cm^2$) and ΔR_t ($\Omega \times cm^2$) calculated by subtracting the steady-state values at the indicated concentrations from the corresponding baseline values of the same filter. All reagents were added into the apical compartments of Ussing chambers and *I_{sc}* and *R_t* were measured continuously until they reached steady-state values. Positive ΔI_{sc} or ΔR_t values indicate that the variable was increased, whereas negative numbers indicate that it was decreased. Values are means ± 1 SE; *n* = number of monolayers.

* *P* < 0.05 compared to the corresponding baseline value.

† *P* < 0.05 compared to the corresponding vehicle control group.

other studies have shown that cGMP-dependent phosphorylation of CFTR in airway cells was not associated with increased activity as a Cl⁻ channel (26).

Presently, a number of important questions remain unanswered. First, the mechanisms by which physiological concentrations of NO regulate Cl⁻ secretion across airway have not been elucidated. Here, we demonstrate that submicromolar concentrations of NO, released either by the synthetic NO donor, (Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl) amino] diazen-1-ium-1, 2-diolate (DETANONOate [DETANO]) or by two nitrated lipids (nitrolinoleic acid [LNO₂] and nitrooleic acid [ONO₂]), known to be present in both lipoproteins and red blood cell membranes of normal humans, and capable of acting as NO donors *in vivo* (27), stimulate Cl⁻ secretion across airway cells both *in vitro* and *in vivo* by stimulating sCG, which, in turn, activates apical CFTR, but not basolateral transporters. Furthermore, PKGII (but not PKGI) phosphorylates CFTR immunoprecipitated from Calu-3 cells *in vitro*. Second, it is unclear whether NO regulates Cl⁻ secretion across ATII cells. Here, we demonstrate that ATII cells grown to confluent monolayers contain CFTR in their apical membranes and PKGII. Agents that increase intracellular cAMP increase basal levels of Cl⁻ secretion. However, even when present in very large concentrations, neither DETANO nor cell-permeable forms of cGMP (8-(4-chlorophenylthio)-[CPT]-cGMP or Br-cGMP) were able to activate Cl⁻ secretion across these monolayers or across the alveolar epithelium *in vivo*, most likely due to the low levels of basal CFTR in these cells.

MATERIALS AND METHODS

Experiments using animals were approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of the University of Alabama at Birmingham.

Cell Culture

Human airway mucosal gland Calu-3 cells (HTB-55; ATCC, Manassas, VA) were plated at a density of 10⁶ cells/cm² onto type 3470 Costar Transwell inserts (0.45- μm pore size, 0.33-cm² surface area; Corning Inc., Corning, NY) and grown using Eagle's minimal essential medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg /ml each), and incubated at 37°C in humidified 21% O₂ and 5% CO₂ mixture. They were cultured for 2 days under a liquid (apical)-liquid (basolateral) interface, and for an additional 3 to 4 days using an air (apical)-liquid interface, as previously described (23, 28). The medium was replaced every 48 hours. Alveolar type II (ATII) cells were isolated from adult male Sprague-Dawley rats (175–225 g), as previously described (29). Briefly, the rats were anesthetized and killed by an intraperitoneal pentobarbital injection (260 mg/kg body weight). The lungs were perfused with buffered saline, removed from the thoracic cavity, and lavaged five times with solution I, containing 140 mM NaCl, 5 mM KCl, 2.5 mM Na₂HPO₄, 6 mM glucose, 0.2 mM EGTA, and 10 mM HEPES, pH 7.4; then lavaged five times with solution II, containing 140 mM NaCl, 5 mM KCl, 2.5 mM Na₂HPO₄, 1.3 mM MgSO₄, and 2 mM CaCl₂, to remove macrophages. Subsequently, the lungs were filled with solution II (37°C) containing elastase (2 U/ml; Worthington, Lakewood, NJ). Proteolytic digestion was stopped after 30 minutes by addition of cold (4°C) PBS containing 10% (vol/vol) heat-inactivated FBS and DNAase. The lungs were minced with dissecting scissors into small

TABLE 3. STEADY-STATE VALUES OF CALU-3 CELLS SHORT-CIRCUIT CURRENT BEFORE TREATMENT (BASELINE), AFTER PRETREATMENT WITH 1H-[1,2,4]OXADIAZOLO[4,3-A]QUINOXALIN-1-ONE OR OXYMYOGLOBIN, AND AFTER SUBSEQUENT ADDITION OF NITROLINOLEIC ACID, ONO₂ AND DETANO INTO THE APICAL COMPARTMENT OF THE USSING CHAMBERS

<i>n</i>	Baseline	Veh	ODQ (10 μM)	oxyMb (300 μM)	LNO ₂ (10 μM)	ONO ₂ (7.5 μM)	DETANO (50 μM)
8	5.4 ± 0.6	5.4 ± 0.6	—	—	11.6 ± 1.5*†	—	—
7	3.5 ± 0.9	—	3.8 ± 0.9	—	4.2 ± 0.9	—	—
3	9.1 ± 1.0	—	—	9.2 ± 2.0	9.9 ± 2.0	—	—
7	7.8 ± 2.4	—	—	—	—	11.9 ± 2.6*†	—
3	6.1 ± 0.4	—	—	6.0 ± 0.6	—	5.9 ± 0.8	—
3	8.7 ± 1.7	8.7 ± 1.7	—	—	—	—	18.3 ± 2.8*†
5	6.2 ± 1.1	—	6.2 ± 1.1	—	—	—	7.2 ± 1.4
4	8.4 ± 1.8	—	—	8.8 ± 2.0	—	—	10.3 ± 2.6

Definition of Abbreviations: ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; ONO₂, nitrooleic acid; oxyMb, oxymyoglobin; Veh, Vehicle solution for the indicated variable.

Values are means ± 1 SE; *n* = number of monolayers. Values shown were obtained in a different set of monolayers than those in Table 1.

* *P* < 0.05 compared to the corresponding baseline value in the same row.

† *P* < 0.005 compared to either corresponding ODQ or oxyMb values in the same column.

Figure 2. 1H-(1, 2, 4)-oxadiazolo (4, 3-a) guinoxalin-1-one (ODQ) prevents the effect of nitric oxide (NO)-induced I_{sc} in the Calu-3 cells. Addition of either 10 μM LNO₂ or 50 μM DETANO increased I_{sc} , which was reversed by oxymyoglobin (oxyMb; 400 μM). Pretreatment of Calu-3 cells with ODQ (10 μM) twice a day for 3 days prevented the increased of I_{sc} by either LNO₂ or DETANO (A and B). However, addition of CPT-cAMP (0.05 mM) into the apical compartment after ODQ increased I_{sc} , indicating that ODQ did not cause nonspecific cellular damage. Results of typical experiments are shown; for mean values $\pm$ 1 SE see Table 3.

pieces, resuspended into the medium, and stirred for 20 minutes at 4°C. The suspension was filtered through nylon gauze of decreasing porosity of 150 and 41 μm (Tetko, Briarcliff Manor, NY) to remove debris. ATII cells were layered on sterile, disposable Petri dishes (FALCON; Becton Dickinson Labware, Franklin Lakes, NJ) percolated with rat IgG, brought to a final concentration of 0.5 mg/ml in 50 mM Tris (pH 9.5), incubated overnight at 4°C, rinsed with PBS several times before using, and incubated at 37°C in 5% CO₂/21% O₂ for 1 hour. The cells ($\sim 1 \times 10^7$ cells/rat) were removed by carefully panning the plates, pelleted by centrifugation, and resuspended in Dulbecco's modified Eagle's medium (GIBCO-Invitrogen, Carlsbad, CA) containing 10% FBS, 2 mM L-glutamine, and penicillin and streptomycin (100 $\mu\text{g}/\text{ml}$ each). Viability, quantified by trypan blue exclusion, was higher than 95%. Subsequently, ATII cells were seeded onto permeable polycarbonate cell culture filters (type 3413; Corning Inc.) with a 0.4- μm pore size at a density 5×10^6 cm² at 37°C in a humidified mixture of 21% O₂ and 5% CO₂ for 3 to 5 days. At that time, the apical solution was removed, and cells were maintained in 400 nM dexamethasone (Sigma, St. Louis, MO) culture medium for at least 2 days from the time that they were able to exclude fluid. Total time in culture was between 5 and 7 days.

Measurements of Short-Circuit Current and Electrical Resistance

Short-circuit currents (I_{sc}) and transepithelial resistance (R_t) were measured, as previously described (5, 28). Briefly, filters containing monolayers of either Calu-3 or ATII cells were mounted in Ussing chambers (Jim's Instruments, Iowa City, IA) and bathed on both sides with solutions containing: 120 mM NaCl, 25 mM NaHCO₃, 3.3 mM KH₂PO₄, 0.83 mM K₂HPO₄, 1.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES (Na⁺ free), 10 mM mannitol (apical compartment), and 10 mM glucose (basolateral compartment). Osmolarity of all solutions, as measured by a freezing depression osmometer (Wescor, Inc., Logan, UT), was between 290 and 300 mOsm. Bath solutions were stirred vigorously by bubbling continuously with 95% O₂, 5% CO₂ at 37°C (pH

7.4). Monolayers were short circuited to 0 mV and I_{sc} were measured with an epithelial voltage clamp (VCC-600; Physiologic Instruments, San Diego, CA). A 10-mV pulse of 1-second duration was imposed every 10 seconds to monitor R_t , which was calculated with Ohm's law. Data were collected using the Acquire and Analyze program, version 1.45 (Physiologic Instruments).

After the establishment of steady-state values of I_{sc} and R_t (usually within 10–20 min from mounting the filters to the Ussing chambers), nitrated lipids (1–10 μM , LNO₂ or ONO₂), or DETANO (1–1,000 μM), as well as various inhibitors, were added into the apical compartments, and I_{sc} and R_t were measured continuously until a new steady state was reached. To evaluate the effect of LNO₂ and DETANO on apical membrane Cl^- pathways, confluent monolayers of Calu-3 were mounted in Ussing chambers containing nonsymmetrical Cl^- solutions (apical, 5 mM; basolateral, 125 mM). Subsequently, the basolateral membranes were permeabilized by the addition of the pore-forming antibiotic, amphotericin B (10 μM), into the basolateral compartments. Complete permeabilization was verified by the fact that addition of ouabain (1–3 mM) into the basolateral compartment had no effect on I_{sc} (in contrast to rapidly decreasing I_{sc} to zero in intact monolayers). Nitrated lipids and DETANO were then added into the apical compartments of the Ussing chambers, as described above.

NO Donors

LNO₂ was provided by Dr. B.A. Freeman (Department of Pharmacology, University of Pittsburgh), and was synthesized and purified as described previously (30, 31). Diethylenetriamine NONOate (DETA-NO) was purchased from Cayman Chemicals (Ann Arbor, MI); its concentration was determined with a Beckman DU 7400 spectrophotometer (Beckman Coulter, Fullerton, CA) at 252 nm ($\epsilon_{252\text{nm}} = 7640 \text{ M}^{-1} \times \text{cm}^{-1}$). Oxymyoglobin (oxyMb) concentrations were determined by using the extinction coefficient per heme group $\epsilon_{582\text{nm}}$ of 14.4 $\text{M}^{-1} \text{cm}^{-1}$. ONO₂ was purchased from Cayman Chemicals.

Detection of NO

Concentrations of NO released by DETANO were determined by using an NO-specific electrode (ISO NOP), and data were acquired with an Apollo 4000 radical analyzer (both from World Precision Instruments, Sarasota, FL). The electrodes were placed in a glass reaction vessel maintained at 37°C with a water jacket, and calibrated by adding known concentrations of NO via a fast-releasing NO donor (ProliNONOate; Alexis Biochemicals, San Diego, CA). Filters with and without Calu-3 and ATII monolayers were placed into the reaction chamber, and NO was monitored after the addition of DETANO and oxyMb.

Levels of NO released by ONO₂ and LNO₂ were undetectable by our electrode, in part due to the very large increase in background current caused by the ethanol vehicle. Instead, we measured the rate of NO release from nitrated lipids according to the method of Schopfer and colleagues (30) using oxyMb, which is oxidized to metmyoglobin (metMb) by NO. Nitrated lipids (10 μM) were incubated with 25 μM oxyMb in Normal Ringer's solution at 37°C, and the oxidation of oxyMb to metMb was monitored by the characteristic decrease in the α and β absorption peaks at 582 and 544 nm, respectively, by using a Shimadzu UV-2501PC spectrophotometer. NO release was quantified with an ϵ_{582} of 0.0121 $\mu\text{M}^{-1} \text{cm}^{-1}$, and assuming a 1:1 stoichiometry of NO release and oxyMb oxidation. To account for baseline drift, the spectra were normalized by setting the absorbance at the isosbestic wavelength (525 nm) to zero. Auto-oxidation of oxy-Mb was subtracted from the signal by including 25 μM oxyMb in the reference cuvette.

Detection of CFTR by Immunofluorescence

After the completion of Ussing chamber studies, confluent monolayers of Calu3 cells were washed three times with cold Dulbecco's Phosphate Buffered Saline (DPBS) containing calcium and magnesium, fixed with 3% formaldehyde for 45 minutes at room temperature, and permeabilized by the addition of a 3:1 mixture of methanol:acetic acid at –20°C for 30 minutes. The fixed cells were treated with 1:20 goat serum in PBS (blocking buffer) for 1 hour at room temperature, and incubated with the primary antibody (mouse monoclonal anti-CFTR antibody [24-1; Chemicon, Temecula, CA]) at 1:100 dilutions with blocking buffer. In control studies, cells were incubated with equal amounts of nonimmune mouse

Figure 3. Cystic fibrosis transmembrane conductance regulator (CFTR) inhibitors reversed the NO increase of I_{sc} in Calu-3 cells. Glybenclamide (0.3 mM) or CFTR inhibitor-172 (CFTR_{inh172}; 1–100 μ M) added into the apical compartments of Calu-3 cells rapidly reversed the increase of I_{sc} by either LNO₂ (10 μ M; left panel) or DETANO (50 μ M; right panel). Results of typical experiments are shown; for mean values $\pm$ 1 SE see Table 4.

IgG (10 μ g/ml; Sigma) in blocking buffer for 2 hours at room temperature. All cells were then incubated with the secondary antibody (goat anti-mouse IgG-Alexa, 488 nm, 1:500; Molecular Probes, Eugene, OR) for 2 hours at room temperature in the dark. To visualize nuclei, cells were incubated with Hoechst 33258 (20 μ g/ml in PBS; Hoechst, Sigma, St. Louis, MO) for 5 minutes at room temperature. The samples were mounted on the stage of a Leitz Orthoplan epifluorescence microscope with epifluorescence and Hoffman modulation contrast optics (Leitz, Buntown Instrument Co., Mt. Airy, MD). Images were obtained with either a Photometric CH250 liquid-cooled charge-coupled device, high-resolution, monochromatic camera, or a Photometric Coolsnap HQ charge-coupled device, high-resolution, monochromatic camera (Roper Scientific, Tucson, AZ). Images were processed and analyzed with Image acquisition software (IPLab Spectrum; Scanalytics, Fairfax, VA). For imaging side views of the cell monolayers, the filters were folded sharply, and the cells at the folded edge were photographed as previously described (23).

SDS-PAGE and Immunoblotting for PKGI and PKGII

Calu-3 and ATII cells were washed three times with PBS and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate) with complete mini, EDTA-free protease inhibitor (Roche, Mannheim, Germany). The lysates were cleared at 16,000 $\times$ g at 4°C for 10 minutes. A total of 50 μ g of protein were boiled in SDS sampling buffer for 5 minutes, subjected to 10% SDS-PAGE, and transferred to polyvinylidene fluoride membrane. The membranes were

blocked with 5% nonfat dry milk in PBS, and probed with rabbit anti-PKGI antibody (1:2,000, PAS-PK005; Stresses, Victoria, and BC Canada) or goat anti-PKGII antibody (1:1,000, sc-10346; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. After washing off nonspecific binding for three times with PBS, the membranes were blotted with goat anti-rabbit horseradish peroxidase (Pierce, Rockford, IL) or donkey anti-goat horseradish peroxidase (Santa Cruz Biotechnology) at 1:5,000 for 1 hour at room temperature. The signal was detected by Super Signal West Dura chemiluminescence substrates (Pierce) and exposed to Fuji medical X-ray films (Fujifilm Medical Systems, Stamford, CT) (32).

Immunoprecipitation and *In Vitro* Phosphorylation of CFTR by PKA, PKGI, and PKGII

Calu-3 and murine ATII cells expressing wild-type CFTR were grown on semipermeable supports as for Ussing chamber studies. In some cases, Calu-3 cells were also grown on plastic dishes with similar results. Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.05% Na-deoxycholate, 0.01% SDS, and 50mM TRIS-Cl) in the presence of protease inhibitor (Complete Mini; Roche Diagnostics, Indianapolis, IN). Equal amounts of the lysates were immunoprecipitated for CFTR using the 24-1 antibody and protein A agarose (Roche Diagnostics) overnight. CFTR was phosphorylated with [γ -³²P] ATP (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the cAMP-dependent PKA catalytic subunit (Promega, Madison, WI), cGMP-dependent PKGI (Invitrogen) and PKGII (Invitrogen and Sigma),

TABLE 4. STEADY-STATE VALUES OF CALU-3 SHORT-CIRCUIT CURRENT MOUNTED IN USSING CHAMBER AFTER ADDITION OF EITHER NITROLINOLEIC ACID OR DETANO, FOLLOWED BY OXYMB, GLYBENCLAMIDE, CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR INHIBITOR-172, OR SIMILAR VOLUME OF VEHICLE (EITHER H₂O OR DMSO)

n	Baseline	LNO ₂ (10 μ M)	DETANO (50 μ M)	Plus Veh	Plus oxyMb (0.3 mM)	Plus Glyb (0.3 mM)	Plus CFTR _{inh} (0.1 mM)
12	5.9 $\pm$ 0.5	11.6 $\pm$ 1.0*	—	10.2 $\pm$ 1.0	—	—	—
9	6.0 $\pm$ 0.7	11.6 $\pm$ 1.3*	—	—	6.4 $\pm$ 1.8†	—	—
3	7.0 $\pm$ 0.9	16.2 $\pm$ 1.6*	—	—	—	3.9 $\pm$ 1.7†	—
4	3.7 $\pm$ 2.0	13.2 $\pm$ 1.8*	—	—	—	—	9.9 $\pm$ 0.8†
12	10.2 $\pm$ 1.0	—	17.3 $\pm$ 2.3*	17.0 $\pm$ 2.8	—	—	—
8	11.5 $\pm$ 1.6	—	19.1 $\pm$ 3.2*	—	14.0 $\pm$ 1.9†	—	—
5	7.7 $\pm$ 0.4	—	15.0 $\pm$ 1.4*	—	—	5.0 $\pm$ 1.3†	—
4	5.5 $\pm$ 1.9	—	9.9 $\pm$ 3.4*	—	—	—	5.0 $\pm$ 1.5†

Definition of abbreviations: CFTR_{inh}, cystic fibrosis transmembrane conductance regulator inhibitor-172; Glyb, glybenclamide.

Measurements in a given row were conducted in the same monolayer. Values are means $\pm$ 1 SE; n = number of monolayers.

* P < 0.05 compared to the corresponding baseline value in the same row.

† P < 0.05 compared to either the corresponding LNO₂ or DETANO values in the same row.

Figure 4. LNO₂ and DETANO increase *I*_{sc} across basolaterally permeabilized Calu-3 monolayers. Confluent monolayers of Calu-3 cells were mounted in Ussing chamber containing nonsymmetrical chloride (Cl⁻) solutions (apical, 5 mM; basolateral, 125 mM). Amphotericin B (10 μM) and ouabain (1 mM) were added into the basolateral compartment, followed 10 to 20 minutes later by LNO₂ (10 μM; A–C) or DETANO (50 μM; D). Addition of oxyMb (0.4 mM; A, C, and D) or glybenclamide (0.3 mM; B) into the apical compartments rapidly decreased *I*_{sc}. Pretreatment of Calu-3 cells with oxyMb (0.4 mM; A) or glybenclamide (0.3 mM; B) and ODQ (10 μM) twice a day for 3 days prevented the increased of *I*_{sc} by either LNO₂ or DETANO (C and D). Results of typical experiments are shown. Mean values ± 1 SE are shown in Tables 5 and 6.

according to the manufacturers recommendations. Labeled CFTR was analyzed by SDS-PAGE, autoradiography, and phosphor imaging analysis, as described previously (33).

Nasal Potential Differences

Nasal potential differences (NPDs) were measured with the technique of Knowles and colleagues (34) and Barker and colleagues (35). Balb/c mice were anesthetized with isoflurane (2–3% [vol/vol] in O₂) or ketamine/xylazine (4 ml/g body weight). The nasal cannula was inserted to a distance of 3 mm from the nasal orifice. A reference bridge was placed in the subcutaneous space of the hind leg. In mice, the perfusion rate was constant at 10 μl/minute to the nasal surface, with low (6 mM) Cl⁻ solutions. The following solutions were perfused in succession: (1) low Cl⁻ solution, containing 0.1 mM amiloride (low Cl⁻); (2) low Cl⁻ with DETANO (either 0.1 or 0.5 mM) or low Cl⁻ with 0.1mM oxyMb plus 0.1 mM DETANO; and (3) low Cl⁻ with glybenclamide (0.3 mM). In some cases, we also perfused the nares with low Cl⁻ containing forskolin (20 μM; Calbiochem, La Jolla, CA).

Statistical Analysis

All values are expressed as means ± 1 SE. The Δ*I*_{sc} was calculated from the difference between the initial value (baseline) and the value after adding LNO₂ or DETANO. Data were analyzed by one-way ANOVA with Bonferroni’s test or Student’s paired *t* test, as appropriate. *P* values of less than 0.05 were considered significant.

TABLE 5. STEADY-STATE EFFECTS OF APICALLY ADDED NITROLINOLEIC ACID OR DETANO ON SHORT-CIRCUIT CURRENT (μA/cm²) OF BASOLATERALLY PERMEABILIZED CALU-3 MONOLAYERS MOUNTED IN USSING CHAMBERS

<i>n</i>	Baseline	LNO ₂ (10 μM)	DETANO (50 μM)	oxyMb (0.3 mM)	CFTR _{inh} (0.1 mM)	Glyb (0.3 mM)
6	-3.1 ± 0.9	21.6 ± 6.0*†		3.3 ± 1.6	—	—
3	-8.0 ± 0.9	13.1 ± 7.3*†		—	—	4.1 ± 7.9
7	-9.5 ± 3.2		18.9 ± 5.5*†	4.4 ± 0.8	—	—
4	-2.4 ± 1.2		10.3 ± 2.9*†	—	3.0 ± 2.2	—

Oxy-Mb, CFTR_{inh}, or glybenclamide were added into the apical compartments after achieving steady-state values. Values are mean *I*_{sc} ± 1 SE; *n* = number of monolayers.
* *P* < 0.05 compared to each corresponding baseline.
† *P* < 0.05 compared to inhibitor.

RESULTS

DETANO, LNO₂, and ONO₂ Increase Cl⁻ Secretion across Calu-3 Cells by Activating sGC

Addition of DETANO (1–1,000 μM) into the apical compartments of Ussing chambers containing Calu-3 cells increased *I*_{sc} by a maximum of about 300% within 30 seconds (Figure 1 and Table 1). Higher levels of DETANO or subsequent addition of either 8-Br-cGMP or 8-CPT-cGMP into the apical compartment did not increase *I*_{sc} further (data not shown). Based on these data, we calculated that 50% of the maximum stimulation of *I*_{sc} by DETANO occurred at about 12 μM (Figure 1). LNO₂ (0.01–10 μM), added into the apical compartment, also increased *I*_{sc} in a dose–response fashion (Figure 1), with a 50% maximum stimulation occurring at 0.06 μM; apical addition of 10 μM LNO₂ decreased *R*_t to a larger extent than did DETANO (Table 1). For this reason, higher concentrations of LNO₂ were not tested. Cell-permeable analogs of cGMP (CPT-cGMP) increased *I*_{sc} to the same extent as DETANO or CPT-cAMP (about 300%; Tables 1 and 2). ONO₂ (7.5 μM) increased *I*_{sc} by 50% (Table 3) and decreased *R*_t in a manner similar to that of LNO₂.

To understand whether activation of sGC was responsible for the increase of *I*_{sc}, we pretreated Calu-3 cells with the sGC inhibitor, 10 μM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) for 3 days before mounting them in Ussing chambers. Previously published results (and our current measurements) show that this treatment does not alter *R*_t, and totally prevents formation of cGMP after exposure to NO donors (36). As seen

TABLE 6. STEADY-STATE EFFECTS OF APICALLY ADDED LNO₂ OR DETANO IN THE APICAL COMPARTMENTS OF USSING CHAMBERS CONTAINING BASOLATERALLY PERMEABILIZED CALU-3 CELLS PRETREATED WITH EITHER OXYMB OR ODQ

<i>n</i>	Baseline	oxyMb (300 μM)	ODQ (10 μM)	LNO ₂ (10 μM)	DETANO (50 μM)
4	1.1 ± 2.0	1.8 ± 1.7	—	1.6 ± 1.3	—
3	4.2 ± 1.0	—	2.6 ± 1.0	2.4 ± 0.9	—
5	6.2 ± 1.1	6.2 ± 1.2	—	—	7.2 ± 1.4
5	-2.4 ± 0.9	—	-2.3 ± 0.9	—	-2.4 ± 0.9

LNO₂ or DETANO did not increase *I*_{sc} across basolaterally permeabilized monolayers pretreated with ODQ or oxyMb. Values are mean *I*_{sc} ± 1 SE; *n* = number of monolayers.

TABLE 7. STEADY-STATE EFFECTS OF THE VARIOUS AGENTS ON SHORT-CIRCUIT CURRENT OF RAT ALVEOLAR TYPE II CELLS MOUNTED IN USSING CHAMBERS

Baseline	Amiloride (100 μ M)	Agent Added (n)	Steady-State Value	cAMP	CFTR _{inh} (100 μ M)
7.5 $\pm$ 1.1	-0.6 $\pm$ 1.0*	Veh (5)	-0.6 $\pm$ 1.2	2.7 $\pm$ 0.7 [†]	-0.3 $\pm$ 1.0 [†]
6.3 $\pm$ 2.3	1.0 $\pm$ 0.6*	DETANO (4)	0.2 $\pm$ 1.1	2.5 $\pm$ 1.3 [†]	1.4 $\pm$ 1.5 [†]
6.8 $\pm$ 0.8	1.1 $\pm$ 0.5*	Bay41-2272 (9)	1.3 $\pm$ 0.6	2.4 $\pm$ 0.5 [†]	0.5 $\pm$ 0.9 [†]
6.5 $\pm$ 1.6	0.7 $\pm$ 1.3*	Br-cGMP (3)	1.0 $\pm$ 1.6	3.0 $\pm$ 2.0 [†]	0.9 $\pm$ 2.7 [†]
3.4 $\pm$ 0.4	1.2 $\pm$ 0.3*	8-pCPT-cGMP (3)	0.1 $\pm$ 0.3	3.5 $\pm$ 0.7 [†]	2.2 $\pm$ 0.7 [†]

After stable I_{sc} values (μ A/cm²) were obtained, amiloride was added into the apical compartment to inhibit apical Na⁺ transporters, followed by either vehicle (0.01 mM NaOH), 1 mM DETANO, Bay 41-2272 (100 μ M), 8pCPT-cGMP (2 mM), Br-cGMP (2 mM). After I_{sc} reached a stable baseline, we added a mixture of agents to stimulate cAMP (0.1 mM; 8-pCPT-cAMP, 0.1 mM IBMX, 10 μ M forskolin), followed by CFTR_{inh}. Values are means $\pm$ 1 SE; n = number of monolayers.

* $P < 0.05$ compared to each corresponding baseline value.

[†] $P < 0.05$ compared to the corresponding value to the column in the immediate left.

in Figure 2 and Table 3, pretreatment of Calu-3 cells with ODO totally prevented both the LNO₂ and DETANO increase of I_{sc} . However, I_{sc} increased significantly after addition of CPT-cAMP (Figure 2) in ODO-treated cells (ΔI_{sc} for ODO + 50 μ M CPT-cAMP = 3.3 $\pm$ 1.2; n = 6; ΔI_{sc} for DMSO + 50 μ M CPT-cAMP = 4.8 $\pm$ 1.4; n = 3; P = 0.5), indicating that the action of ODO was specific, and not due to generalized cellular injury. Furthermore, data shown in Figure 2 show that ODO treatment did not alter I_{sc} values. R_t values in vehicle versus ODO-treated Calu-3 cells were: 360 $\pm$ 24 (n = 3) versus 383 $\pm$ 15 (n = 10) (means $\pm$ 1 SE; n = number of monolayers; P = 0.5).

LNO₂ and DETANO Increase I_{sc} by Activating Apical Cl⁻ Pathways

Evidence that DETANO and LNO₂ stimulation of anion secretion involves CFTR was provided by two different types of experiments. First, as shown in Figure 3 and Table 4, addition of either glybenclamide (which blocks both CFTR and K_{ATP} channels) or the specific CFTR inhibitor-172 (CFTR_{inh172}) (37) into the apical compartments quickly reversed the LNO₂ or DETANO increase of I_{sc} . Second, addition of either LNO₂ or DETANO into the apical compartments containing basolaterally permeabilized Calu-3 monolayers increased Cl⁻ secretion, which was either reversed by oxyMb or prevented by pretreatment of Calu-3 cells with either 0.3 mM glybenclamide or 10 μ M ODO (Figure 4 and Table 6). None of these agents (i.e., DETANO, LNO₂, or ONO₂) increased I_{sc} when added into the apical compartments of Ussing chambers housing apically permeabilized Calu-3 cell monolayers (data not shown).

Measurements of NO Release

Measurements of NO concentration with an ISO-NO meter, placed in a reaction chamber containing Calu-3 cells, showed that 50 μ M DETANO released about 300 nM NO (Figure 5A). Addition of oxyMb (0.1–1 μ M) into the apical compartments decreased NO until it was converted to metMb. Higher levels of oxyMb (10 μ M) decreased NO to nondetectable levels for more than 60 minutes. As shown in Figure 5B, scavenging of NO was accompanied by a decrease of I_{sc} (Figure 5B).

Neither LNO₂ nor ONO₂, at the concentrations used in our studies (10 and 1 μ M, respectively), generated sufficient NO to be detected with the ISO-NO meter. Thus, NO levels were determined from difference spectra for the oxidation of oxyMb to metMb taken at 10-minute intervals, showing the character-

Figure 5. Measurements of NO release by DETANO. DETANO (50 μ M) was added into a reaction chamber containing a confluent monolayer of Calu-3 cells. OxyMb was added at times indicated by arrows. (A) Time course of oxyMb inhibition of NO levels (measured by the ISO-NO meter). (B) oxyMb inhibition of I_{sc} after addition of DETANO. Results are shown of typical experiments that were repeated five different times.

istic decreases in the 582- and 544-nm maxima, as previously described (30) (Figures 6 and 7). These measurements show that 10 μ M of either LNO₂ or ONO₂ generated less than 1 μ M NO (Figures 6 and 7).

Identification of CFTR in Calu-3 Cells and ATII Cells by Immunofluorescence and *In Vitro* Phosphorylation

Confluent monolayers of either Calu-3 or ATII cells were immunostained with CFTR antibodies and secondary fluorescent antibodies, as described in MATERIALS AND METHODS section. Staining was seen both at the level of apical membranes (in folded filters) and in the cytoplasm by confocal microscopy (Figure 8).

CFTR was also detected by *in vitro* phosphorylation of immunoprecipitated protein by PKA. As can be seen in Figure 9, phosphorylated CFTR was detected in both Calu-3 and ATII cells. However, CFTR levels in ATII cells were less than 5% of those in Calu-3 cells.

In Vitro Phosphorylation of CFTR by PKGII

We immunoprecipitated CFTR from confluent Calu-3 monolayers and investigated whether it could be phosphorylated by the PKG/cGMP system. As shown in Figure 10B, CFTR was readily phosphorylated by the catalytic subunit of PKA, and by PKGII in the presence of Br-cGMP. Because of differences in the specific activities of the PKA and PKGII, as well as differences in the number of phosphorylation sites for PKA and PKG, conclusions regarding relative phosphorylation efficiencies of CFTR by these kinases is not possible. However, PKGI failed to phosphorylate CFTR. Western blotting studies showed that both Calu-3 and ATII cells contain PKGII, detected as a single band of approximately 77 kD. This band was not detected when extracted cellular proteins from Calu-3

Figure 6. Measurements of NO release by LNO_2 . (A) Difference spectra for the oxidation of oxyMb (25 μM) to metMb by LNO_2 (10 μM) taken at 10-minute intervals, showing the characteristic decreases in the 582- and 544-nm maxima (arrows). The absorbance at the isosbestic wavelength for the oxyMb-metMb conversion (525 nm) was set to zero. (B) Time course of NO generation, as determined by the decrease in absorbance at 582 nm, is shown. Data represent means $\pm$ SE for three different experiments. (C) Time course of I_{sc} increase after addition of LNO_2 (1 μM) into the apical chamber of an Ussing chamber containing Calu-3 cells. Addition of oxyMb (1–100 μM) rapidly reversed the increase of I_{sc} in a different set of experiments, addition of oxyMb (100 μM) into the apical chambers prevented the increase of I_{sc} by 10 μM LNO_2 (data not shown). (D) ΔI_{sc} calculated by subtracting the plateau value of I_{sc} after addition of the indicated amount of oxyMb (x axis) from the I_{sc} after addition of 1 μM LNO_2 (see C). Negative values indicate a decrease of I_{sc} . Mean values $\pm$ 1 SE; $n = 3$; $*P < 0.05$ compared to the absence of oxyMb (using one- and two-tailed t tests, respectively).

cells were immunostained with the PKGII antibody in the presence of the immunizing peptide (Figure 10A). Furthermore, extracted proteins from rat brain tissue and ATII, but not from Calu-3 cells, showed specific immunostaining for PKGI (Figure 10A).

DETANO Increases Cl^- Secretion across Murine Nasal Airways *In Vivo*

NPDs were measured in six Balb/c mice during perfusion with a low- Cl^- Ringers solution containing 100 μM amiloride (38, 39). Baseline NPD values were about -11 mV. As shown in Figure 11, addition of 100 μM DETANO in the perfusate

hyperpolarized the NPD by 5 mV. Subsequent perfusion with 0.3 mM glybenclamide inhibited about 50% of this response. Addition of 100 μM oxyMb in the perfusate before 100 μM DETANO completely prevented the increase in NPD, demonstrating that it was mediated through the release of NO. As shown in Figure 5, the steady-state concentration of NO in the medium after addition of 50 μM DETANO equaled 300 nM, well below the range reported in inflamed airways (40).

NO Donors Do Not Stimulate Cl^- Secretion across ATII Cells

When they form confluent monolayers, rat ATII cells exhibit vectorial Na^+ and Cl^- transport (7, 8, 29, 41). As seen in Figure

Figure 7. Measurements of NO release by nitrooleate (ONO_2). (A) Difference spectra for the oxidation of oxyMb (25 μM) to metMb by ONO_2 (10 μM) taken at 10-minute intervals, showing the characteristic decreases in the 582- and 544-nm maxima (arrows). The absorbance at the isosbestic wavelength for the MbO_2 -metMb conversion (525 nm) was set to zero. (B) Time course of NO generation, as determined by the decrease in absorbance at 582 nm. Data represent means $\pm$ SE for three different experiments. (C) Black line, time course of I_{sc} increase after addition of ONO_2 (1 μM) into the apical chamber of an Ussing chamber containing Calu-3 cells; grey line, same as above, except that oxyMb (100 μM) was added into the apical compartments before ONO_2 . (D) ΔI_{sc} calculated by subtracting the plateau value of I_{sc} after addition of the indicated amount of oxyMb (x axis) from the I_{sc} after addition of 1 μM ONO_2 (see C). Negative values indicate a decrease of I_{sc} . Mean values $\pm$ 1 SE; $n = 3$; $*P < 0.05$ compared to the corresponding value in the absence of oxyMb (using one- and two-tailed t tests, respectively).

Figure 8. Immunocytochemical detection of CFTR in Calu-3 (left panels) and rat alveolar type II (ATII) cells (right panels). Calu-3 (A–D) and rat ATII (E–H) cells were immunostained with either a mouse monoclonal anti-CFTR antibody (B, D, F, and H) or with an equivalent amount of nonimmune mouse IgG (A, C, E, and G) followed by a secondary antibody, goat anti-mouse IgG Alexa 488 (green fluorescence). Nuclei were stained with Hoechst 33258 dye. In some cases (A, B, E, and F), the filters were folded to visualize the apical membranes. Characteristic figures were chosen from either 4 (folded) or 10 (unfolded) different records.

12, addition of 100 μ M amiloride into the apical compartments of Ussing chambers containing confluent monolayers of rat ATII cells rapidly decreased I_{sc} . Subsequent addition of DETANO (up to 1 mM, which generated more than 1 μ M NO), BAY 41-2272 (a compound known to stimulate sGC via non-cGMP-dependent pathways) (42), or Br-cGMP into the same compartments did not increase I_{sc} . On the other hand, addition of 8-CPT-cAMP or 8-Br-cAMP, forskolin, and isobutylmethylxanthine (IBMX, a non-specific inhibitor of phosphodiesterases) increased glybenclamide-sensitive I_{sc} , indicating the presence of functional CFTR (Figure 12 and Table 7) (complementing our findings that ATII cells contain CFTR, both by indirect immunofluorescence and *in vitro* phosphorylation by PKA). Finally, addition of the specific CFTR_{inh172} into the apical compartment also decreased I_{sc} to

zero, indicating that functional CFTR is crucial to Na^+ transport in these cells. In contrast to ATII cells, DETANO increased I_{sc} across amiloride-treated 16-human bronchial epithelial (HBE) cells, an airway epithelial cell line containing both epithelial Na^+ channel and CFTR (23), with 50% of maximum stimulation occurring at 460 μ M DETANO (data not shown).

DISCUSSION

Here, we demonstrate that: (1) both Calu-3 and murine type II cells contain CFTR and PKGII; (2) physiological concentrations of NO, released by either DETANO or nitrated lipids added in the apical medium, stimulate Cl^- secretion across Calu-3 cells *in vitro* by stimulating CFTR activity; (3) NO released by DETANO stimulates Cl^- secretion across murine airways *in vivo*; (4) the presence of NO *per se* in the apical medium was sufficient and necessary for the observed increase of Cl^- secretion; (5) both PKA and PKGII (but not PKGI) phosphorylate CFTR immunoprecipitated from Calu-3 *in vitro*; and (6) very large concentrations of NO, cell-permeable analogs of cGMP, or BAY (which stimulates sGC directly) fail to stimulate Cl^- secretion across confluent monolayers of rat ATII cells *in vitro*.

Previous studies have documented the presence of CFTR in alveolar epithelial cells by immunofluorescence, Western blotting, and patch clamp studies in rat ATII cells (6). Furthermore, others have shown that functional CFTR is necessary for increased Na^+ transport by β -agonists across the mouse alveolar epithelium. CFTR^(-/-) mice fail to increase their Na^+ -dependent fluid clearance in response to cAMP, indicating that CFTR plays an important role in lung fluid balance (8, 41). We demonstrated the presence of CFTR in ATII cells, grown to confluency using an air-liquid interface, by a variety of techniques, including: immunocytochemical staining at the level of their apical membranes; *in vitro* phosphorylation of immuno-

Figure 9. *In vitro* phosphorylation of immunoprecipitated CFTR from Calu-3 and ATII cells by protein kinase (PK). A. Calu-3 and ATII cells were grown on permeable supports. When confluent, CFTR was immunoprecipitated with anti-(M3A7) monoclonal antibody and *in vitro*-phosphorylated using $\gamma^{32}P$ ATP and PKA, separated by SDS-PAGE, and detected with autoradiography as described in the text. To enhance detection, we used about twice as much protein from ATII than from Calu-3 cells for immunoprecipitation. Results presented are from a typical experiment, repeated twice with similar results.

using $\gamma^{32}P$ ATP and PKA, separated by SDS-PAGE, and detected with autoradiography as described in the text. To enhance detection, we used about twice as much protein from ATII than from Calu-3 cells for immunoprecipitation. Results presented are from a typical experiment, repeated twice with similar results.

Figure 10. Phosphorylation of CFTR by PKGII. (A) Cellular proteins from either Calu-3 or ATII cells were separated with SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunostained with PKGII antibodies (lanes 1–3), PKGII antibodies along in the presence of the immunizing peptide (lane 4) or PKGI antibodies (lanes 6–7); positive control (rat brain extract; lane 5). Results are shown of a typical experiment that was repeated three times. (B) Calu-3 cells expressing wild-type CFTR were grown on plastic dishes. Cells were lysed in RIPA buffer and CFTR was immunoprecipitated from equal amounts of cells using 24-1 and protein A Agarose. CFTR was *in vitro* phosphorylated with PKA (lane 1), PKGI (lane 3), PKGII (lanes 4 and 6) and PKGI plus PKGII (lane 5) according to the manufacturer’s recommendations. Results are those of a typical experiment that was repeated three times. Grey arrow above the 75-kD MW marker shows the location of PKGII; white arrow (right) shows the location of PKGI.

precipitated CFTR by PKA; and the fact that addition of forskolin or Br-cAMP after apical application of amiloride increased I_{sc} significantly. Similar results were obtained with Na⁺-free solutions, indicating that the increase of I_{sc} was not due to the movement of Na⁺ ions through amiloride-insensitive pathways (data not shown). These results are in agreement with our previous findings showing activation of Cl⁻ secretion across the rabbit alveolar epithelium and confluent monolayers of rabbit ATII cells by agents that increased cAMP *in vivo* (7). Most importantly, in spite of the fact that more than 90% of baseline I_{sc} in rat ATII was amiloride sensitive (in contrast to about 50% in ATII cells harvested from newborn rats [5]), addition of glybenclamide (an agent that inhibits both CFTR and K⁺ activated ATP channels) or CFTR_{inh172} (a specific inhibitor of CFTR [37]) decreased I_{sc} significantly. Similarly, addition of CFTR_{inh172} in the alveolar lumen of mice decreased alveolar fluid clearance to about the same extent (data not shown). These findings indicate that the Cl⁻ moves across the alveolar epithelium mainly through CFTR channels.

In spite of the fact that both Calu-3 and ATII cells contain CFTR, NO and agents that increase cGMP increased Cl⁻ secretion across the former, but not the latter. Our Western blotting studies indicate that both Calu-3 and ATII cells contain significant amounts of PKGII, and PKGII (but not PKGI), phosphorylated CFTR immunoprecipitated from Calu-3 cells *in vitro*. These results are in agreement with previous findings showing PKGII, but not PKGI, phosphorylated CFTR and increased Cl⁻ secretion across intestinal and airway cells (24, 25). The most likely reason that NO did not stimulate Cl⁻ secretion across ATII cells is that these cells contain very low levels of CFTR as compared with Calu-3 cells. Indeed, considerably higher levels of NO were needed to stimulate Cl⁻ secretion across 16HBE cells (half maximum stimulation occurring at 460 nM as compared with 11 nM for Calu-3 cells). The 16HBE cells are an airway epithelial cell line that contains much less CFTR as compared with Calu-3 cells (23). The lack of stimulation of Cl⁻ secretion by NO across ATII cells agrees with previously reported *in vivo* data, showing that intratracheal instillation of DETANO (up to 3 mM) decreased amiloride-sensitive, Na⁺-driven alveolar fluid reabsorption, but did not stimulate Cl⁻-driven fluid secretion across the alveolar epithelium of rabbits (43).

There is significant evidence that NO down-regulates the activity of both renal and alveolar epithelial cell cation channels via cGMP-dependent and cGMP-independent mechanisms. For example, Guo and colleagues (29) demonstrated that addition of NO donors decreased amiloride-sensitive I_{sc} across confluent monolayers of rat ATII cells with a concentration producing 50% inhibition of about 500 nM. Jain and colleagues (44) reported that cGMP and S-nitrosoglutathione (GSNO) significantly decreased single-channel activity in rat ATII cells. However, the effects of NO and cGMP on Cl⁻ secretion has not been assessed until recently. Kaestle and colleagues (45) reported that elevation of pulmonary artery pressure of isolated, perfused lungs leads to an up-regulation of endothelial NOS, which results in pulmonary edema by decreasing Na⁺ reabsorption across the alveolar space. In the same study, these investigators also showed that increased levels of NO had no effect on Cl⁻ secretion across the alveolar epithelium, in agreement with our findings in rat ATII cells. Furthermore, the lack of response was not due to the presence of epithelial Na⁺ channel, as DETANO increased glybenclamide-sensitive I_{sc} across 16HBE140 cells after inhibition of vectorial Na⁺ transport with apical amiloride (data not shown).

Recent studies report that interactions between reactive nitrogen intermediates, unsaturated fatty acids, and lipid oxidation intermediates lead to the formation of oxidation and nitration products (46). LNO₂ has been found in human plasma lipoproteins and plasma membranes at concentrations of about 500 nM (46), making it one of the most abundant biological sources of NO. Cole and colleagues (47) reported a 38-fold increase in the concentration of nitroalkenes in the edema fluid of patients with ARDS. Thus, levels of nitrated lipids used in our studies ($\leq 10 \mu\text{M}$) are well within the concentrations reported *in vivo* in patients with ARDS ($\sim 15 \mu\text{M}$). Nitrated lipids exert their effects *in vivo* and *in vitro* by a variety of mechanisms, including activation of cAMP (48), cGMP (30), or cGMP-independent mechanisms (49).

Here, we were able to demonstrate that both LNO₂ and ONO₂ generate significant amounts of NO. Furthermore, incubation of Calu-3 cells with ODQ totally prevented the LNO₂ increase of Cl⁻ secretion. These data indicate that the nitrated lipid activation of CFTR occurred exclusively via NO release and stimulation of guanylyl cyclase. In contrast, earlier experiments from this laboratory have shown that GSNO, another

Figure 11. DETANO increases Cl^- secretion across nasal airways *in vivo*. Recordings of nasal potential differences (NPDs) in Balb/c mice perfused with 6 mM Cl^- solution containing 100 μM amiloride to block Na^+ absorption and enhance Cl^- secretion. (A) Addition of 0.1 mM DETANO (or 0.5 mM; data not shown) resulted in significant hyperpolarization (indicative of enhanced Cl^- secretion), which was blocked partially by glybenclamide (0.3 mM). (B) Addition of equal concentrations of oxyMb and DETANO (0.1 mM) into the perfusate had no effect on NPD. (C) Mean values of NPD changes (± 1 SE; $n = 3$); * $P < 0.05$ compared to vehicle; # $P < 0.05$ compared to DETANO. Dotted lines indicate baseline levels before addition of DETANO

physiological carrier of NO, can activate CFTR via both cGMP-dependent and independent mechanisms (28). Similar to GSNO, nitrated lipids are capable of covalent modification of nucleophilic residues (50). The different cGMP-independent profiles of CFTR activation by LNO_2 and GSNO may therefore indicate specificity in the ability to post-translationally modify CFTR. Alternatively, CFTR might exhibit specificity in the functional response to different covalent modifications—for

example, increased activity after thiol nitrosation by GSNO, but not after alkylation by nitrated lipids.

In summary, our results indicate that nanomolar concentrations of NO released by either DETANO or nitrated lipids stimulate Cl^- secretion across glandular and airway epithelial cells *in vitro* and *in vivo* via cGMP-dependent mechanisms. Specifically, NO stimulates sGC, resulting in increased cGMP levels and activation of PKGII, leading to phosphorylation and

Figure 12. cAMP but not cGMP increased Cl^- I_{sc} across rat A10 cells. Confluent monolayers of rat A10 cells were mounted in Ussing chambers as described in MATERIALS AND METHODS. (A) Addition of amiloride (100 μM ; an inhibitor of epithelial Na^+ channels) into the apical compartment rapidly decreased I_{sc} , indicating that the majority of I_{sc} is due to the movement of Na^+ ions. cAMP (100 μM CPT-cAMP, 10 μM forskolin, and 100 μM IBMX), added into the apical compartments, increased I_{sc} , which was either reversed (*reversed*) or prevented (*grey line*) by the CFTR_{inh}172 (20 μM). (A–D) DETANO (1–1,000 μM ; B), Bay 41-2272 (1–100 μM ; C), Br-cGMP (1–2,000 μM ; D), or CPT-cGMP (1–2,000 μM ; data not shown) did not increase I_{sc} after addition of amiloride. Addition of CFTR_{inh}172 (1–200 μM ; *grey line* in D) into the apical compartment decreased I_{sc} , indicating that the majority of I_{sc} is also due to the movement of Cl^- ions. Results are those of typical experiments that were repeated at least three times each. Mean values ± 1 SE are shown in Table 7.

activation of CFTR. On the other hand, even at very high concentrations, NO does not increase Cl⁻ secretion across AII cells, even though these cells contain both CFTR (albeit, at much lower levels) and PKGII. The reason for the lack of response of AII cells has not been elucidated. However, because alveolar fluid balance depends on both Na⁺ reabsorption and Cl⁻ secretion, an acute increase of NO concentrations (as seen in hydrostatic edema [51], bacterial infections [52], or administration of inhaled NO) will not enhance fluid accumulation in the alveolar space secondary to Cl⁻ secretion.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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