Nitric oxide inhibits Na\(^+\) absorption across cultured alveolar type II monolayers

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Nitric oxide inhibits Na\(^+\) absorption across cultured alveolar type II monolayers. Am. J. Physiol. 274 (Lung Cell, Mol. Physiol. 18): L369–L377, 1998.—We examined the mechanisms by which nitric oxide (NO) decreased vectorial Na\(^+\) transport across confluent monolayers of rat alveolar type II (ATII) cells grown on permeable supports. Amiloride (10 μM) applied to the apical side of monolayers inhibited ~90% of the equivalent (I\(_{sc}\)) and the short-circuit (I\(_{sc}\)) current, with an half-maximal inhibitory concentration (IC\(_{50}\)) of 0.85 μM, indicating that Na\(^+\) entry into ATII cells occurred through amiloride-sensitive Na\(^+\) channels. NO generated by spermine NONOate and papa NONOate added to both sides of the monolayers decreased I\(_{sc}\), and increased transepithelial resistance in a concentration-dependent fashion (IC\(_{50}\) = 0.4 μM · NO). These changes were prevented or reversed by addition of oxyhemoglobin (50 μM). Incubation of ATII monolayers with 8-bromo-guanosine 3',5'-cyclic monophosphate (400 μM) had no effect on transepithelial Na\(^+\) transport. When the basolateral membranes of ATII cells were permeabilized with amphotericin B (10 μM) in the presence of a mucosal-to-serosal Na\(^+\) gradient (145:25 mM), NO (generated by 100 μM papa NONOate) inhibited ~60% of the amiloride-sensitive I\(_{sc}\). In addition, after permeabilization of the apical membranes, NO inhibited the I\(_{sc}\) [a measure of Na\(^+\)-K\(^+\)-adenosinetriphosphatase (ATPase) activity] by ~60%. We concluded that NO at nontoxic concentrations decreased Na\(^+\) absorption across cultured ATII monolayers by inhibiting both the amiloride-sensitive Na\(^+\) channels and Na\(^+\)-K\(^+\)-ATPase through guanosine 3',5'-cyclic monophosphate-dependent mechanisms.

Recent evidence indicates that the adult alveolar epithelium is capable of actively transporting Na\(^+\). Several groups have demonstrated the reabsorption of intratracheally instilled isotonic fluid or plasma from the alveolar into the interstitial space both in anesthetized animals and isolated perfused lungs (33, 36). Amiloride or N-ethyl-N-isopropyl amiloride (EIPA), added into the alveolar space, and ouabain, injected into the vascular compartment, decreased the rate of fluid reabsorption by ~40–50 and 70–80%, respectively (2, 14, 32, 49). In addition, 23Na\(^+\) uptake and bioelectrical measurements across either freshly isolated or cultured alveolar type II (ATII) cells are consistent with the existence of vectorial Na\(^+\) transport; Na\(^+\) diffuses passively across the ATII apical membranes, mainly through cation channels, down a favorable electrochemical gradient maintained by Na\(^+\)-K\(^+\)-adenosinetriphosphatase (ATPase) and is then actively transported across the basolateral membrane by ouabain-sensitive Na\(^+\)-K\(^+\)-ATPase (8, 19, 38). K\(^+\) leaves ATII cells, driven by a favorable electrochemical gradient, through K\(^+\) channels located in the basolateral membrane. The active Na\(^+\) transport across the alveolar epithelium creates an osmotic gradient for fluid movement from the alveolar to the interstitial space, thus helping to maintain the alveoli free of fluid, especially after oxidant injury to the alveolar epithelium (34, 49).

Because of their location, alveolar epithelial cells are often exposed to increased intracellular and extracellular concentrations of reactive oxygen and nitrogen species generated by oxidant gases, inflammatory cells, or enzymatic generators such as xanthine oxidase. These agents may cause significant damage to alveolar epithelial cells, resulting in functional and structural abnormalities. Recently, interest has been focused on the potential contribution of nitric oxide (NO) to this injury. Pertinent sources of pulmonary NO include activated macrophages (1, 20) and ATII cells (22, 37). Thus, in a number of pathological conditions, significant amounts of NO may be present in the epithelial lining fluid, in close proximity to the alveolar epithelial cells.

NO has complex biological reactivity, and its physiological effects depend on its concentration and redox state, on the nature of the target molecules, and on the presence of other free radicals (42). There is ample evidence to indicate that NO modulates both ion channel and Na\(^+\)-K\(^+\)-ATPase activity in a variety of epithelial cells by altering guanosine 3',5'-cyclic monophosphate (cGMP) and ATP levels or by modifying key amino acids of these proteins (4, 15, 26, 43–45). Similarly, Compeau et al. (9) demonstrated that reactive nitrogen species, released by activated alveolar macrophages, inhibited the Ca\(^2+\)-activated cation channel density of fetal epithelial cells. However, the mechanisms by which NO alters the Na\(^+\) transport across cultured adult alveolar epithelial cells have not been studied in detail. This is an important question to address, since NO inhalation has been advocated as a means of selectively reducing pulmonary hypertension and improving systemic oxygenation in patients with a variety of clinical situations (3).

We therefore designed a series of experiments to examine the mechanisms by which NO modulates Na\(^+\) transport across rat ATII cells cultured on permeable supports until they formed resistive monolayers. Our results indicate that NO at nontoxic concentrations (~10 μM) likely to be encountered in vivo during acute and chronic inflammation (12, 28) inhibits both apical amiloride-sensitive Na\(^+\) channels and basolat-
eral Na\(^+\)-K\(^+\)-ATPase by cGMP- and ATP-independent mechanisms and decreases vectorial Na\(^+\) transport across cultured ATII cells.

**METHODS**

ATII cell isolation and culture. ATII cells were isolated from the lungs of young adult male Sprague-Dawley rats (−150 g) as previously described (19). Briefly, the rats were anesthetized and killed by an intraperitoneal pentobarbital sodium injection (260 mg/kg body wt). The lungs were perfused with buffered saline, removed en bloc from the thoracic cavity, and lavaged with a balanced salt solution and buffered saline alternately to remove macrophages. Subsequently, the lungs were filled with Joklik’s modified minimum essential medium (MEM; 37°C) containing elastase (2 U/ml in MEM; Worthington, Lakewood, NJ) and deoxyribonuclease (DNase). Proteolytic digestion was stopped after 30 min by addition of cold (4°C) MEM containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) and DNase. The lungs were minced and stirred for 20 min at 4°C. The suspension was filtered through nylon gauze of decreasing porosity (Tetko, Briarcliff Manor, NY) to remove debris. ATII cells were filtered through nylon gauze of decreasing porosity (Tetko, Briarcliff Manor, NY) to remove debris. ATII cells were seeded onto 0.4-µm pore, 3.5-cm² Transwell tissue culture filters at a density of 1.5 × 10⁶ cells/cm² and were maintained in MEM containing 10% FBS and DNase. The lungs were minced with dissecting scissors into small pieces, resuspended in the medium, and stirred for 20 min at 4°C. The suspension was filtered through nylon gauze of decreasing porosity (Tetko, Briarcliff Manor, NY) to remove debris. ATII cells were layered on sterile disposable petri dishes (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) precoated with rat immunoglobulin G and incubated at 37°C in 5% CO₂–95% air for 1 h (10). Nonadhering cells (−1 × 10⁷ cells/rat) were removed by carefully panning the plates, pelleted by centrifugation, and resuspended in minimal essential medium (MEM) containing 10% FBS and 0.1 µM dexamethasone. More than 85% of these cells were identified as ATII cells by the modified Papanicolaou stain or by staining positive for alkaline phosphatase. Viability, quantified by trypan blue exclusion, was >95%.

Subsequently, ATII cells were seeded onto 0.4-µm pore, 33-cm² Transwell tissue culture filters at a density of 1.5 × 10⁶ cells/cm² and were maintained in MEM containing 10% FBS, 0.1 µM dexamethasone, 100 µg/ml penicillin, and 100 µg/ml streptomycin at 37°C with 95% air-5% CO₂. The medium was changed every other day after plating.

Generation and measurement of ·NO. ·NO was generated by the spontaneous decomposition of either spermine NONOate or papa NONOate (Cayman Chemical, Ann Arbor, MI). NONOate stocks were prepared by dissolving it in 100 µM phosphate buffer (pH 8.5) just before use. Evolution of ·NO in the culture medium (pH 7.4, 37°C) was measured with an ISO-NO electrochemical probe (World Precision Instruments, Sarasota, FL) connected to an IBM-compatible computer via an analog-to-digital converter. Mean ·NO concentration values were calculated as previously described (17).

Measurement of biocurrents of ATII cells. Spontaneous potential difference (PD) and transepithelial resistance (Rₑₑₑ) across ATII cell monolayers were measured daily, beginning after 2 days in culture, using an Epithelial Voltohmeter equipped with chopstick-style electrodes (World Precision Instruments). Equivalent short-circuit current (Iₑₑₑ) was calculated by Ohm’s law (Iₑₑₑ = PD/Rₑₑₑ). Only monolayers achieving Rₑₑₑ values > 800 Ω·cm² within 4–6 days after seeding were used in our studies.

In the initial set of experiments, we added spermine NONOate or papa NONOate into both the apical and basolateral bathing solutions of the monolayers and measured PD and Rₑₑₑ for a 60-min period. In a number of cases, measurements were repeated in the presence of oxyhemoglobin (50 µM), a scavenger of ·NO, or vehicle alone (100 mM phosphate buffer, pH 8.5).

In subsequent studies designed to offer insight into the mechanisms involved, ATII cell monolayers were mounted in modified Ussing chambers. The monolayers were bathed by a solution containing (in mM) 145 Na⁺, 5 K⁺, 125 Cl⁻, 1.2 Ca²⁺, 1.2 Mg²⁺, 25 HCO₃⁻, 3.3 H₂PO₄⁻, and 0.8 HPO₄²⁻ (pH 7.4) at 37°C gassed with 95% O₂–5% CO₂ (solution 1). Glucose (10 mM) and mannitol (10 mM) were added to the basolateral and apical solutions, respectively. We substituted glucose with mannitol in the apical solution to minimize the contribution of a putative Na⁺–glucose cotransporter to Na⁺ influx in ATII cells. A transepithelial voltage clamp (EC-825; Warner Instruments, Hamden, CT) was used for the continuous monitoring of PD and Iₑₑₑ. After mounting the filters containing ATII cells into the Ussing chambers, the short-circuit current (Iₑₑₑ) was allowed to stabilize before beginning each experiment (−10 min). Iₑₑₑ was determined using Ohm’s law from the observed change in Iₑₑₑ resulting from 5-s square-voltage pulses (1 or 2 mV) imposed across the monolayer.

In some cases, amiloride (0.01–100 µM) or EIPA (0.1–200 µM) was added into the apical bathing solution, and Iₑₑₑ was recorded until a new baseline value was reached. In other cases, spermine NONOate or papa NONOate was added to both the apical and basolateral bathing solutions, and Iₑₑₑ and Rₑₑₑ were monitored for −20 min. At this point, amiloride (10 µM) was added to the apical chamber, and Iₑₑₑ was recorded for an additional 15 min. All measurements were repeated in the presence of oxyhemoglobin (50 µM) or after addition of the appropriate vehicle.

Effects of ·NO on apical Na⁺ conductance. To further understand the mechanisms by which ·NO decreased Iₑₑₑ, we quantified the effects of ·NO on the apical and basolateral ATII cell transporters by selectively permeabilizing each compartment as described (21). ATII cell monolayers were mounted in Ussing chambers. The composition of the apical compartment solution was as described above (solution 1). The basolateral compartment contained (in mM) 25 Na⁺, 5 K⁺, 125 Cl⁻, 1.2 Ca²⁺, 1.2 Mg²⁺, 25 HCO₃⁻, 3.3 H₂PO₄⁻, 0.8 HPO₄²⁻ (pH 7.4), and 120 N-methyl-D-glucamine (an impermeant cation) at 37°C gassed with 95% O₂–5% CO₂ (mucosal-to-serosal Na⁺ gradient 145:25 mM). After the Iₑₑₑ reached steady state, papa NONOate (100 µM) or an equivalent amount of vehicle control was added into both the apical and basolateral bathing fluids for 20 min, at which time the basolateral membrane was permeabilized by the addition of amphotericin B (10 µM), a monovalent ionophore, to the basolateral bathing solution. Once the Iₑₑₑ reached a new steady state, amiloride (10 µM) was added to the mucosal bathing solution, and the difference current, representing the amiloride-sensitive component of the Na⁺ current across the apical membrane, was calculated.

Effects of ·NO on Na⁺-K⁺-ATPase activity. ATII cell monolayers were mounted in Ussing chambers under short-circuit conditions and bathed with solution 1 (145 mM Na⁺). After the Iₑₑₑ reached steady state, amiloride (10 µM) was added to the apical bathing fluid, which decreased Iₑₑₑ to nondetectable levels. When a new steady state was reached, papa NONOate (100 µM) or an equivalent amount of vehicle control was added into both the apical and basolateral bathing fluids for 20 min, and at this time, the apical membrane was permeabilized by addition of amphotericin B (10 µM) to the mucosal bathing solution. Under these conditions, the Iₑₑₑ reflects the movement of Na⁺ across the basolateral membrane by Na⁺-K⁺-ATPase.

cGMP radioimmunoassay. cGMP concentrations in ATII cells were determined by radioimmunoassay as previously described (18). ATII monolayers on filters were treated with vehicle or with spermine NONOate (200 µM) added to both
bathing solutions for either 5 or 45 min, the medium was aspirated, and the cells were washed three times in ice-cold phosphate-buffered saline and extracted with a 50% methanol-0.1 N HCl mixture. The acidified methanol extract was lyophilized and processed for cGMP radioimmunoassay. The data are expressed in picomoles per milligram cell protein, measured by the bioluminescent method (41).

ATP measurement. ATP levels of cultured ATII cells were measured by a bioluminescent assay using luciferin as a substrate in the presence of firefly luciferase (Sigma, St. Louis, MO). Emitted light was measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). ATII cell ATP values were calculated by comparison with the measured light emission of an ATP standard solution.

Statistical analysis. Results are expressed as means ± SE. Student’s t-test or paired t-test, as indicated in the legends, was used for statistical analysis between two group means. Statistical differences among multiple group means were determined using one-way analysis of variance and the Bonferroni modification of the t-test.

RESULTS

In the present study, -50% of ATII cell monolayers had $R_{te} > 800 \Omega \cdot cm^2$ between 4 and 6 days in culture. Under basal conditions, transepithelial currents measured either under open-circuit ($I_{eq}$) or short-circuit ($I_{sc}$) conditions were 4–8 µA/cm². Mean values (±SE) for the various bioelectrical variables for these monolayers are shown in Table 1. Addition of amiloride (10 µM) to the apical bath rapidly decreased both $I_{eq}$ and $I_{sc}$ by ~90%, with a half-maximal inhibitory concentration ($IC_{50}$) of 0.85 µM (Table 1 and Fig. 1). In contrast, the $IC_{50}$ for 4-aminopyridine was 64 µM. Addition of ouabain (0.5–1 mM) into the basolateral bath completely abolished both $I_{eq}$ and $I_{sc}$ (data not shown).

Addition of 200 µM of spermine NONOate, which generates a steady-state concentration of ~1.5 µM NO (Fig. 2), to the apical and basolateral bathing solutions decreased $I_{eq}$ and increased $R_{te}$ by ~80 and 30% respectively, 45 min postinstillation (Fig. 3). To avoid damage to the filters by the electrodes, in subsequent studies, bioelectrical properties were measured just before the addition of spermine NONOate and 45 min later, by which time a new steady state was reached. These values are summarized in Fig. 4. Addition of oxyhemoglobin (50 µM) to both compartments just before the NO donors decreased NO concentration to baseline levels and prevented the decrease in $I_{eq}$ (Figs. 4 and 5). Moreover, addition of oxyhemoglobin 45 min after spermine NONOate reversed the inhibition of $I_{eq}$ (Fig. 6). Oxyhemoglobin alone had no effect on the bioelectrical properties of ATII cell monolayers.

The inhibitory effects of NO on $I_{eq}$ were concentration dependent, with an $IC_{50}$ of 0.4 µM (Fig. 7) irrespective of which NO donor was used. Papa NONOate (100 µM) released similar amounts of NO as 200 µM of spermine NONOate (Fig. 2) and inhibited $I_{eq}$ to the same extent (75–80% of control) as 200 µM of spermine NONOate (Fig. 7).

ATII cell cGMP levels measured either just before or 5 and 45 min after addition of 200 µM spermine NONOate into the medium were 0.66 ± 0.22 (n = 4), 0.51 ± 0.25 (n = 3), and 0.44 ± 0.25 (n = 3) pmol/mg protein, respectively (mean ± SE; n = number of filters). Addition of 8-bromo-cGMP (400 µM) to both bathing solutions did not alter either $I_{eq}$ or $I_{sc}$ within a 45-min period.

When the apical and basolateral compartments were bathed with symmetrical Na⁺ solutions (145 mM NaCl), permeabilization of the basolateral membrane with amphotericin B (10 µM) resulted in a rapid decrease in the $I_{sc}$ to nondetectable levels (data not shown). In contrast, in the presence of a Na⁺ concentration gradient across the monolayers (mucosal to serosal = 145:25 mM), permeabilization of the basolateral membrane increased $I_{sc}$ from 8.6 ± 0.4 to 13.8 ± 0.7 µA/cm² (n = 4). Subsequent addition of amiloride (10 µM) into the apical compartment decreased the $I_{sc}$ to 3.8 ± 0.9 µA/cm². Thus the amiloride-sensitive fraction of the amphotericin B-induced current ($ΔI_{sc,amil}$; the difference between these two currents) was 10.1 ± 0.7 µA/cm² (Fig. 8). Addition of 100 µM papa NONOate in both the apical and basolateral compartments decreased $ΔI_{sc,amil}$ to 3.8 ± 0.8 µA/cm² (n = 4; Fig. 8). These data suggest that NO inhibits Na⁺ influx through amiloride-sensitive channels located in the apical membranes of cultured ATII cells.

In the presence of symmetrical Na⁺ solutions (145 mM NaCl), addition of amiloride (10 µM) into the apical bath decreased $I_{sc}$ to 0.5 ± 0.2 µA/cm². Subsequent permeabilization of the apical membrane with amphotericin B (10 µM) increased the $I_{sc}$ from 0.5 to 0.2 to 3.8 ± 0.3 µA/cm². The difference between these two currents ($ΔI_{sc,pump}$ = 3.3 ± 0.3 µA/cm²; n = 6; Fig. 9) represents the current generated by Na⁺-K⁺-ATPase. Addition of 100 µM papa NONOate to both bathing solutions for ~20 min decreased $ΔI_{sc,pump}$ from 3.8 ± 0.3 to 1.4 ± 0.3 µA/cm² (Fig. 9). The $ΔI_{sc,pump}$ was completely inhibited by the addition of ouabain (1 mM) into the basolateral bathing solution (Fig. 9).

ATII cell ATP values under control conditions were 0.14 ± 0.004 nmol/µg protein (n = 4). After exposure of

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<th>Table 1. Bioelectric measurements on rat ATII cell monolayers</th>
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Data are means ± SE; n = 7 filters. PD, potential difference (apical membrane negative); $R_{te}$, transepithelial resistance; $I_{eq}$, equivalent short-circuit current; $I_{sc}$, short-circuit current; N/A, not recorded. * Significantly different (P < 0.05) from control values (paired t-test).
ATII cells to 100 µM papa NONOate for 20 min in the manner described, their ATP value decreased to $0.083 \pm 0.009$ ($n = 3$). To investigate whether this 45% decrease in ATP contributed to the $\zeta$NO-induced decline in ATII cell diffusion, we measured $D_{\text{Isc,pump}}$ after addition of 2-deoxy-D-glucose (1 mM) into the basolateral medium of permeabilized ATII cell monolayers in the absence of $\zeta$NO donors. All conditions were as described above except that we substituted glucose with mannitol in the apical compartment. Twenty minutes post-2-deoxy-D-glucose addition, ATII ATP values also decreased by 45% ($0.086 \pm 0.003$; $n = 3$), but $D_{\text{Isc,pump}}$ remained unchanged from control levels. These data indicate that the $\zeta$NO-induced decrease in $D_{\text{Isc,pump}}$ was not the result of decreased ATP availability.

**DISCUSSION**

Data presented herein indicate that acute exposure of ATII monolayers to $\zeta$NO generated by either spermine or papa NONOate decreased their basal rate of Na⁺ absorption by inhibiting both the apically located amiloride-sensitive Na⁺ channels and the basolaterally located Na⁺ channels. The inhibition of Na⁺ absorption by $\zeta$NO was reversible, as shown by the time course of spermine NONOate effects on equivalent short-circuit current ($I_{\text{eq}}$) and transepithelial resistance ($R_{\text{te}}$). Addition of spermine NONOate (200 µM) to both sides of culture medium significantly decreased $I_{\text{eq}}$ and increased $R_{\text{te}}$ within 30–60 min (A; $n = 6$). $I_{\text{eq}}$ and $R_{\text{te}}$ were not affected by vehicle treatments (B; $n = 3$).

45% ($0.086 \pm 0.003$; $n = 3$), but $\Delta I_{\text{sc,pump}}$ remained unchanged from control levels. These data indicate that the $\zeta$NO-induced decrease in $\Delta I_{\text{sc,pump}}$ was not the result of decreased ATP availability.

**Fig. 1.** Effects of amiloride and N-ethyl-N-isopropyl amiloride (EIPA) on short-circuit current ($I_{\text{sc}}$) across alveolar type II (ATII) monolayers. A: representative traces showing that $I_{\text{sc}}$ was inhibited by ~90% (from 4.8 ± 0.2 to 0.5 ± 0.1 µA/cm², $n = 7$) by amiloride (amil; 10 µM; top trace) but only ~15% (from 5.3 ± 0.4 to 4.5 ± 0.4 µA/cm², $n = 7$) by EIPA (10 µM; bottom trace). B: sensitivity of $I_{\text{sc}}$ to amiloride and EIPA. From the concentration-response curves, half-maximal inhibitory concentration ($IC_{50}$) was calculated, by linear regression using the linear portion of data, to be 0.85 µM for amiloride and 64 µM for EIPA ($n = 3$ for each data point). [Inhibitor], inhibitor concentration.

**Fig. 2.** Representative traces demonstrating the amount of nitric oxide (NO) released from NO donors. Similar amounts of NO (~1.5 µM at peak levels) were released from papa NONOate (100 µM) and spermine NONOate (200 µM) in the medium ($n \geq 3$). [NO], NO concentration.

**Fig. 3.** Time course of spermine NONOate effects on equivalent short-circuit current ($I_{\text{eq}}$) and transepithelial resistance ($R_{\text{te}}$). Addition of spermine NONOate (200 µM) to both sides of culture medium significantly decreased $I_{\text{eq}}$ and increased $R_{\text{te}}$ within 30–60 min (A; $n = 6$). $I_{\text{eq}}$ and $R_{\text{te}}$ were not affected by vehicle treatments (B; $n = 3$).

**Fig. 4.** Protection of oxyhemoglobin on the inhibitory effects of $\zeta$NO on $I_{\text{eq}}$. Oxyhemoglobin (HB; 50 µM) had no effect on $I_{\text{eq}}$ when added to the medium on both sides of filters. When added before spermine NONOate (SP; 200 µM), the inhibitory effects of spermine NONOate on $I_{\text{eq}}$ were completely blocked. *Significantly different from vehicle control. Initial untreated $I_{\text{eq}}$ values for each experimental group are as follows: control, 7.4 ± 0.8 µA/cm² ($n = 7$); SP, 7.2 ± 1.0 µA/cm² ($n = 8$); HB, 4.6 ± 0.3 µA/cm² ($n = 3$); SP + HB, 4.6 ± 0.2 µA/cm² ($n = 4$).
The observed decreases in transepithelial Na$^{+}$-selective (I$_{sc}$) paracellular resistance. Because Na$^{+}$ could not be attributed to cell death or decreased cell number, these findings provide insight into the mechanisms responsible for alveolar edema formation in pathological situations, these findings provide insight into the limiting of alveolar fluid in pathological settings. For example, Ischiropoulos et al. (20) reported that activated rat alveolar macrophages and alveolar epithelial cells release large amounts of NO in the epithelial lining fluid and have not been measured directly. However, various studies indicated that alveolar macrophages may produce 0.1 nmol · NO·min$^{-1}$·10$^{6}$ cells$^{-1}$, which may generate micromolar concentrations in the epithelial lining fluid. In other studies, Malinski et al. (28) measured 2–4 µM NO in ischemic brain during cerebral ischemia, and Gaston et al. (12) reported the presence of 4 µM nitrosothiols in the distal airway fluid of patients with pneumonia. Thus it seems reasonable to assume that concentrations of NO used in this study are likely to be encountered in vivo.

Amiloride-sensitive epithelial Na$^{+}$ channels (ENaC) are classified as either high (H-type) or low (L-type) affinity channels depending on their binding affinities for amiloride and its structural analogs. H-type channels display the following structure/inhibitory pattern relationship: phenamil, benzamil >>> amiloride >>> EIPA. In contrast, L-type channels are inhibited to the same extent by EIPA and amiloride (29). Patch-clamp and $^{22}$Na$^{+}$ flux measurements in either freshly isolated ATII cells or those in primary culture (18–24 h of culture), as well as measurements of single channels of a putative Na$^{+}$ channel protein purified from freshly isolated rabbit ATII cells, which was reconstituted in lipid bilayers, are consistent with the presence of L-type (EIPA-inhibitable) Na$^{+}$-selective channels (30, 40, 48, 50, 51). Finally, intratracheal instillation of EIPA inhibited Na$^{+}$ reabsorption across the rat alveolar epithelium in vivo to the same degree as amiloride (49). In contrast, data presented herein indicate that amiloride added to the apical compartments was about two orders of magnitude more effective than EIPA in limiting the extent of alveolar fluid in pathological situations (37, 39, 47). For example, Ischiropoulos et al. (20) reported that activated rat alveolar macrophages may produce 0.1 nmol · NO·min$^{-1}$·10$^{6}$ cells$^{-1}$, which may generate micromolar concentrations in the epithelial lining fluid. In other studies, Malinski et al. (28) measured 2–4 µM NO in ischemic brain during cerebral ischemia, and Gaston et al. (12) reported the presence of 4 µM nitrosothiols in the distal airway fluid of patients with pneumonia. Thus it seems reasonable to assume that concentrations of NO used in this study are likely to be encountered in the alveolar space in a number of pathological situations.

An important question that needs to be addressed is whether the concentrations of NO used in these experiments are likely to be encountered in vivo. Data shown in Fig. 7 indicate that the IC$_{50}$ for I$_{sc}$ inhibition was 0.4 µM. The concentration of NO in the alveolar epithelial lining fluid has not been measured directly. However, various studies indicated that alveolar macrophages and alveolar epithelial cells release large amounts of NO in the epithelial lining fluid in a variety of pathological situations (37, 39, 47).

Addition of spermine NONOate (SP; 200 µM) to both sides of culture medium significantly decreased I$_{sc}$ ($p < 0.05$). Oxyhemoglobin alone had no effect on I$_{sc}$ (data not shown).

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inhibiting \( I_{sc} \) across ATII cell monolayers. These results are consistent with the presence of high amiloride affinity (H-type) channels in cultured ATII cells. However, it should be kept in mind that, with increasing time in culture, ATII cells undergo a number of important phenotypic changes, including decreased ability to secrete surfactant lipids (11), decreased levels of both the amount and mRNA for surfactant apoproteins (25), and internalization of Na\(^+\) channel proteins (48). It is unclear whether these alterations represent degenerative changes or transformation of ATII to ATI cells. Taken as a whole, these data coupled with our previous findings indicate that culture conditions will influence the expression of Na\(^+\) channels in ATII cells.

Our results indicate that vectorial Na\(^+\) transport across ATII cells was inhibited by \( \cdot \)NO in a dose-response and reversible fashion. Transepithelial Na\(^+\) absorption requires the coordinated functions of both the apical Na\(^+\) channels and the basolateral Na\(^+\)-K\(^+\)-ATPase. The results of studies in which the apical or basolateral membranes of ATII cells were selectively permeabilized by amphotericin B clearly indicate that \( \cdot \)NO inhibited the entry of Na\(^+\) across the apical membranes and their exit across the basolateral membranes. These biological effects of \( \cdot \)NO may be exerted by 1) increasing cGMP or activating cGMP kinase, 2) decreasing cellular ATP levels, and 3) direct interaction with critical amino acids of these transport proteins.

There is evidence to indicate that \( \cdot \)NO modulates cation channel activity by increasing cGMP levels. Light et al. (24) demonstrated the presence of a 28-pS cation channel in rat renal innermedullary collecting duct cells, the activity of which was decreased both by cGMP per se and via cGMP kinase-induced phosphorylation. \( \cdot \)NO released from bradykinin stimulated endothelial cells, or spermine NONOate decreased net \(^{22}\)Na\(^+\) flux across isolated perfused cortical collecting ducts (45) and decreased Na\(^+\) \( I_{sc} \) across a cortical...

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**Fig. 8.** Effects of \( \cdot \)NO on amiloride-sensitive apical membrane Na\(^+\) channels. Amphotericin B (ampho; 10 \( \mu \)M) increased the \( I_{sc} \), which was inhibited by amiloride (10 \( \mu \)M; A). Application of papa NONOate (100 \( \mu \)M) decreased the amiloride-sensitive current (B). Mean values of amiloride-sensitive Na\(^+\) currents (\( \Delta I_{sc,amil} \)) across apical membranes in the presence or absence of \( \cdot \)NO are shown in C. *Significantly different from control (Student's t-test; \( n = 4 \) for each group).

**Fig. 9.** Effects of \( \cdot \)NO on basolateral membrane Na\(^+\)-K\(^+\)-ATPase activity. Amiloride (10 \( \mu \)M) decreased the \( I_{sc} \), which was increased by amphotericin B (A). Addition of ouabain (1 mM) in the basolateral compartment totally abolished the amphotericin B-induced increase in \( I_{sc} \) (\( \Delta I_{sc,pump} \)). Application of papa NONOate (100 \( \mu \)M) also decreased \( \Delta I_{sc,pump} \). Mean values of these variables are shown in C. *Significantly different from control (Student's t-test; \( n = 4 \) for each group).
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collecting duct (CCD) cell line while increasing their cGMP content (43, 44). Selective permeabilization of the apical membranes of the CCD cells with nystatin reversed the inhibition of Isc. Based on these findings, it was concluded that ·NO inhibited CCD apical Na⁺ channels by increasing their cGMP content (44). In contrast to these studies, we found no change in ATII cell cGMP levels after exposure of ATII cells to ·NO donors. Furthermore, addition of 8-bromo-cGMP, the cell-permeable form of cGMP, to the bathing solutions of ATII cells did not alter Isc. Taken as a whole, these findings indicate that, contrary to what was observed in other cell types, the ·NO-induced decrease in Isc across cultured ATII cells was not due to an increase in intracellular cGMP. In accordance with these results, the 22Na⁺ permeability-surface area product in isolated perfused lungs was not decreased after addition of 8-bromo-cGMP in the perfusate (13).

·NO has also been shown to cause cellular energy depletion by activating the nuclear enzyme poly-ADP-riboseyl transferase (52) or by inhibiting cytochrome-c oxidase (46). Data shown here indicate that, in agreement with these studies and our previous measurements in freshly isolated ATII cells (17), exposure of cultured ATII cells to 100 µM papa NONOate decreased their ATP levels by 45%. However, the fact that a similar decrease in ATP levels caused by 2-deoxy-o-glucose did not inhibit the Na⁺-K⁺-ATPase current indicates that the ·NO decrease in Isc and pump currents was not the result of decreased ATP availability.

Other potential mechanisms by which ·NO may modify vectorial Na⁺ transport across ATII cell monolayers is by oxidizing and/or nitrosylating key thiols in either the transport proteins themselves (Na⁺ channels and Na⁺-K⁺-ATPase) or other proteins such as actin, which are important in their functional regulation (7). S-nitrosylation of critical thiols at the N-methyl-D-aspartate-receptor redox modulatory site downregulated channel activity (26). ·NO generated from various donors decreased single-channel activity of a Ca²⁺-activated, 25-pS cation channel in rat brown fat cells (23). This blockade was reversible by dithiothreitol, suggesting that ·NO decreased the activity of the channels by oxidizing a critical sulfhydryl group known to be present on their cystolic side. Finally, ·NO increased the activity of Ca²⁺-activated K⁺ channels in excised membrane patches from rabbit aortic smooth muscle cells in the absence of Mg²⁺, guanine nucleotides, or kinases that are required for cGMP-dependent channel activation (4). Covalent modification of the sulfhydryl groups of these K⁺ and Ca²⁺ channels with N-ethylmaleimide prevented the ·NO-induced activation, suggesting that it resulted from the modification of sulfhydryl groups present in the channel or other membrane constituents.

A cDNA encoding an amiloride-sensitive Na⁺ channel (α-rat (r) ENaC) was cloned from the colon of salt-deprived rats using functional RNA expression (5, 6). Two additional subunits named β-rENaC and γ-rENaC with molecular masses of 72 and 75 kDa, respectively, were subsequently cloned (6). Expression of α-, β-, and γ-rENaC mRNA in adult ATII cells has been demonstrated by both in situ hybridization and Northern blot analysis (31, 35, 50). The external loop of α-rENaC, the pore-forming unit of the Na⁺ channel, contains 14 cysteines, any or all of which may be subject to modification by reactive oxygen-nitrogen species (6). Thiol groups have been reported to be important for the activity and possibly subunit assembly of Na⁺-K⁺-ATPase (27). cGMP-independent regulatory effects of ·NO on Na⁺-K⁺-ATPase have been reported in isolated rabbit aorta (15) and cultured human corpus cavernosum smooth muscle cells (16). We have been unable to demonstrate reversibility of ·NO-induced inhibition of vectorial Na⁺ transport in our studies, since both dithiothreitol and N-ethylmaleimide decrease the basal Na⁺ currents (data not shown). Thus the exact mechanisms for the inhibitory effects of ·NO on Na⁺ channels and Na⁺-K⁺-ATPase in cultured ATII cells remain to be determined.

In summary, our results indicate that ·NO at nontoxic concentrations (~1.0 µM) likely to be encountered in vivo during acute and chronic inflammation inhibits both apical amiloride-sensitive cation channels and the basolateral Na⁺-K⁺-ATPase by cGMP and ATP-independent mechanisms and decreases vectorial Na⁺ transport across cultured ATII cells. Our previous data also indicate that peroxynitrite, an agent formed by the reaction of ·NO with superoxide, decreases amiloride-sensitive 22Na⁺ uptake in freshly isolated rabbit ATII cells (19). Decreased transepithelial Na⁺ absorption across alveolar epithelial cells, brought about by reactive nitrogen and oxygen species, may reduce the rate of alveolar fluid absorption and thus enhance alveolar edema formation in acute lung injury.

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