Role of SGK1 in nitric oxide inhibition of ENaC in Na\(^{+}\)-transporting epithelia

My N. Helms, Ling Yu, Bela Malik, Dean J. Kleinhenz, C. Michael Hart, and Douglas C. Eaton

1Department of Physiology, Emory University School of Medicine; and 2Department of Medicine, Atlanta Veterans Affairs Medical Center and Emory University Medical Center, Atlanta, Georgia

Submitted 6 January 2005; accepted in final form 12 April 2005

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

http://www.ajpcell.org

C717
motif with the ubiquitin ligase Nedd4-2, which would lead to the eventual degradation of surface ENaC proteins (9, 39). However, because SGK1 is expressed in a wide range of tissues and several pathways may lead to the regulation of amiloride-sensitive Na⁺ transport, additional SGK1 effectors may be involved in SGK1’s signal transduction cascade, leading to the upregulation of ENaC activity. Therefore, our current study examined the physiological interaction between SGK1 and iNOS to determine whether the NO inhibition of amiloride-sensitive Na⁺ channel activity and corticosteroid inhibition of NOS were mediated by SGK1.

METHODS

Cell culture and type II pneumocyte isolation. Low-passage X. laevis kidney distal nephron A6 and M1 CCD cells were purchased from the American Type Culture Collection (Manassas, VA). M1 cells were maintained in plastic flasks (Corning, Corning, NY) at 37°C in a humidified incubator with 5% CO₂ in air. The M1 culture medium consisted of DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and 1.5 μM aldosterone, and 1% streptomycin, and 0.6% penicillin. A6 cells were cultured in a mixture of Coon’s F-12 medium (3 parts) and Leibovitz’s L-15 medium (7 parts) (Irving Scientific, Santa Ana, CA) in a final concentration of 104 mM NaCl, 25 mM NaHCO₃, 0.6% penicillin, 1% streptomycin, 10% (vol/vol) fetal bovine serum, and 1.5 μM aldosterone at 4°C and 26°C.

Primary mouse alveolar type II (ATII) cells were isolated and maintained as described previously (24). All procedures involving animals were reviewed and approved by our Institutional Animal Care and Use Committee. Briefly, 3–mo-old BALB/c mice were anesthetized with pentobarbital sodium and then killed after the lungs were perfused with PBS. Lungs were removed from the animal, and subsequently enzyme digested with dispase and 0.1 mg/ml DNAse in DMEM. Dispersed cells were then passed through a 20-μm nylon mesh and purified using the differential adherence technique (10).

Electrophysiological measurements. With the use of patch-clamp techniques, cell-attached recordings were established on the apical membrane of A6 cells and grown on permeable supports. Polished micropipettes were pulled from filaments of borosilicate glass capillaries (TW-150; World Precision Instruments) with a two-stage vertical puller (Narishige, Tokyo, Japan). The resistance of fire-polished pipettes were between 5 and 10 MΩ when filled with pipette solution containing (in mM) 96 NaCl, 3.4 KCl, 0.8 CaCl₂, 0.8 MgCl₂, and 10 HEPES, pH 7.4. Under the above culture conditions, a high-resistance seal (>20 GΩ) was usually formed after slight negative pressure was applied to the patch membrane. Channel currents were sampled at 5 kHz with a patch-clamp amplifier (Axopatch 200B; Molecular Devices, Union City, CA) and filtered at 1 kHz with a low-pass Bessel filter. Data were recorded using a computer equipped with AxoScope8 software (Molecular Devices). The open probability (Pₒ) of the channels was calculated using FETCHAN in pCLAMP6 software. Experiments were conducted at 22–23°C.

A6 and M1 cells were grown to confluence on Transwell-permeable supports (Corning, Acton, MA). After ~20 days in culture, the potential difference (PD) and transepithelial resistance (Rₜₑₚ) across cell monolayers were measured using an epithelial voltmeter equipped with stick electrodes (World Precision Instruments, Sarasota, FL). The equivalent short-circuit current (Iₑₛₜ) was calculated according to Ohm’s law (Iₑₛₜ = PD/Rₜₑₚ) and then corrected for the surface area of the Transwell insert.

Immunoprecipitation of iNOS and Western blot analysis. A6, M1, and ATII cells were rinsed three times with PBS before being lysed with 600 μl of lysis buffer (150 mM NaCl, 10 mM NaPO₄, pH 7.4, 0.1% SDS, 1% Nonidet P-40, 0.25% Na⁺-deoxycholate, and freshly prepared 1× protease inhibitor cocktail), and protein concentration was determined using bicinchoninic acid protein assay reagent (Pierce Chemical, Rockford, IL). iNOS immunoprecipitations were performed with rabbit polyclonal iNOS antibody (Upstate, Lake Placid, NY). To coimmunoprecipitate iNOS with SGK1, 3 μl of rabbit polyclonal anti-SGK1 antibody were incubated as described previously (46) with the cell lysate overnight at 4°C. The next day, immunoprecipitated protein complexes were immobilized with ImmunoPure protein A beads (Pierce Chemical), electrophoresed on a 7.5% acrylamide gel under denaturing conditions, and then transferred to Protran nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membrane was blocked in TBST buffer (10 mM Tris, pH 7.5, 70 mM NaCl, and 0.1% Tween) with 5% dry milk and then incubated with 1 μg/ml mouse monoclonal anti-iNOS antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. IgG-hors eradish peroxidase (HRP)-labeled secondary antibody (KPL, Gaithersburg, MD) was added at a concentration of 1 μg/ml TBST and incubated for another 1 h at room temperature. After being washed thoroughly, HRP signal was detected using the enhanced chemiluminescence substrate and Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunocytochemistry. Primary mouse ATII cells were allowed to adhere overnight on 1-mm round poly-l-lysine-coated coverslips (Becton Dickinson Labware, Bedford, MA) in growth medium and then were fixed with 4% paraformaldehyde for 10 min at room temperature. A 1:1,000 dilution of polyclonal rabbit anti-SGK1 and mouse monoclonal iNOS antibody (see above) was diluted in Ca²⁺/Mg²⁺- and MgCl₂-free PBS with 3% horse serum and 1% BSA and then applied to the cells for 1 h at room temperature. The cells were then washed three times with blocking buffer, followed by application to the cells of a 1:10,000 mix of Alexa Fluor 488-labeled goat anti-rabbit and Alexa Fluor 568-labeled goat anti-mouse antibodies (Molecular Probes, Eugene, OR) for an additional 1 h at room temperature. Cells were washed and fixed again and then mounted onto a slide with antifade reagent (Molecular Probes). Subcellular localization of SGK1 and iNOS was analyzed using standard confocal microscopy, and 1-μm-thick sections throughout the cell were sequentially imaged. The emission data for SGK1 and iNOS were collected separately and subsequently superimposed using LSM 5 Image Browser software (Carl Zeiss, Thornwood, NY).

In vitro SGK1 kinase assay. Active SGK1 enzyme (Δ1–60, S422D) was purchased from Upstate, and the kinase assay procedures were performed as recommended by the manufacturer. Briefly, in a 50-μl reaction volume, 25 ng of SGK1, 10 μCi [γ-³²P]ATP, 10 μM cold ATP, and PKA/PKC inhibitors were incubated at 30°C for 10 min with either hiNOS enzyme (Alexis Biochemicals, San Diego, CA) or iNOS oligopeptides custom produced by Sigma Genosys (The Woodlands, TX) as described in the text. Subsequent to incubation, a 35-μl aliquot of the reaction was transferred to phosducinulose squares, washed, and read in scintillation liquid. Data were recorded as counts per minute (cpm), which indicate γ-³²P incorporation into iNOS substrate. Assay buffer was substituted for hiNOS or oligopeptides in negative background control groups.

³²P labeling. Confuent M1 cells were serum and hormone deprived for 72 h and then rinsed twice with sodium phosphate-free DMEM (Invitrogen). The cells were then labeled with 0.5 mCi ³²P (Amersham Biosciences) for 6 h and then treated with 1.5 μg of aldosterone (or remained in serum- and hormone-free medium as control) for an additional 4 h. Radioactively labeled lysates were then immunoprecipitated using anti-iNOS or SGK1 antibody, separated on 7.5% denaturing gel, and transferred to nitrocellulose using the procedures described above. Quantification of ³²P-labeled iNOS protein was enhanced with the use of Molecular Dynamics PhosphorScreen (Sunnyvale, CA) and quantified using ImageQuant software obtained from Amersham Biosciences.

Measurement of NO release. Measurement of NO release was performed on freshly isolated ATII cells. Immediately after isolation, ATII cells were seeded onto Costar Transwell 12-mm inserts (Corn-
ing) at confluent densities. NO release was determined by measuring NO and its oxidation products, NO2 and NO3, from the culture medium as described in (27). Briefly, after incubation with or without aldosterone overnight, culture medium was collected and injected into a vessel containing 0.8% NaCl in 1 N HCl at 95°C. NO was detected using a chemiluminescence NO analyzer (model 280; Sievers, Boulder, CO), and standard curves were generated using 0.1–10 μM NaNO3 in serum- and hormone-free DMEM.

Statistical evaluation. Statistical analyses were performed using Student’s t-test, with statistical significance defined as P < 0.05. Data are expressed as means ± SE. ANOVA among multiple parameters was performed using the Holm t-test, which allows sequential comparison of unadjusted P values.

RESULTS

PAPA-NONOate release of NO decreases transepithelial Na+ transport in A6 and M1 cells. Because of conflicting reports in the literature, we decided to examine the effect of NO and the role of SGK1 in regulating iNOS in three different cell systems. One type of cell studied was primary cultures of ATII cells because of the potential importance of NO in lung inflammation and because inhaled NO is often used to improve blood flow in neonates. A second type of cell studied was mouse M1 cells, a renal cell line derived from CCD, because of reports that NO can alter Na+ transport in the distal nephron. The third type of cells studied was A6 cells, a cell line originally derived from Xenopus kidney that shares some properties with both mammalian lung cells and mammalian distal nephron cells.

Several previous studies have shown opposing data regarding the effect of NO on Na+ transport. For example, infusion of substances such as acetylcholine (which causes NO release into the renal artery) increases urinary volume and decreases Na+ absorption in in vivo animal models (35). Application of acetylcholine and NO donors such as spermine NONOate and nitroglycerin to M1 cells also has shown that NO directly decreases net Na+ flux in mouse CCD cell lines (40). However, other studies have reported that sodium nitroprusside release of NO fails to change net Na+ flux (19) or that NO can even stimulate amiloride-sensitive Na+ channels in rat CCD cells (32). Because such differences could be attributed to differences in the properties of the cell types tested or even the specific NO donor used, we tested the effect of PAPA-NONOate on both the A6 Xenopus distal renal cells and in the mouse M1 CCD cell lines to better understand NO’s role in regulating ENaC. Both cell lines are model systems for studying amiloride-sensitive Na+ transport. The NO donor PAPA-NONOate is a zwitterion capable of rapidly releasing 2 M NO per mole of parent compound. The half-life of PAPA-NONOate at room temperature is ~76.6 min in PBS and culture medium (20). We found that very low (50 nM-3 μM) concentrations of PAPA-NONOate were effective in decreasing Isc values in both A6 and M1 cell lines within 1 min, and persisted for at least 10 min without significant change. Figure 1 shows the effect of on Isc 5 min after applying 1.5 μM PAPA-NONOate to the apical membrane of both A6 and M1 cell lines. As a control for the NO donor compound, we allowed the same concentration of PAPA-NONOate to expire at room temperature overnight and then applied the expired PAPA-NONOate to similarly maintained A6 and M1 cells. This inactivated compound is not capable of donating NO. In this way, we could determine whether an effect on Na+ transport was due to the release of NO or to the metabolites of the parent compound. The data shown in Fig. 1, A and B, left, are expressed as %Isc decrease (from pretreatment Isc measurements) after 1.5 μM active PAPA-NONOate or inactivated compound was added to the apical membrane. Active PAPA-NONOate significantly decreased the Isc in A6 cells from an average value of 8.23 ± 0.68 μA/cm2 to 5.91 ± 0.62 μA/cm2, an ~29% decrease in Isc, n = 20 (Fig. 1A, left). Application of the inactivated PAPA-NONOate did not substantially affect Isc values of A6 cells. The ~3% decrease (n = 12) after applying the control compound did not cause a significant decrease in Na+ current compared with pretreatment Isc values (Fig. 1A, right). However, in A6 cells, the percent current decrease in 1.5 μM PAPA-NONOate vs.

![Figure 1](https://example.com/fig1.png)

**A** Nitric oxide (NO) donor drug L-propanamine, 3,2-hydroxy-2-nitroso-1-propylhidrazino (PAPA-NONOate) release of NO decreases amiloride-sensitive, equivalent short-circuit current (Isc) in A6 and M1 epithelial cell monolayers. A and B: 1.5 μM active PAPA-NONOate decreased Isc by 29% in A6 cells (n = 20; A) and by 44% in M1 cells (n = 12; B). This %Isc decrease from initial untreated values differed significantly from the lower 3% Isc decrease in A6 cells (n = 12) and 19% in M1 cells (n = 12) caused by inactivated NO donor compound in A6 cells (right; control). C and D: amiloride substantially decreased Isc in untreated (no NONOate) A6 cells 67% (n = 12; C) and 76% in M1 cells (n = 36; D). Subsequent application of 1.5 μM PAPA-NONOate to amiloride-inhibited A6 and M1 cells did not further decrease Isc values. *P < 0.0005 (A–D).
inactive compound was statistically significant ($P < 0.0005$). Similarly, 1.5 $\mu$M PAPA-NONOate significantly decreased amiloride-sensitive $I_{sc}$ of M1 cells by 44%, from $10.63 \pm 0.60 \mu$A/cm$^2$ to $6.0 \pm 0.34 \mu$A/cm$^2$, $n = 12$ (Fig. 1B, left). The $I_{sc}$ values of M1 cells declined by 19% in the control studies, $n = 12$ (Fig. 1B, right). Again, statistical comparison between the 1.5 $\mu$M NONOate-treated M1 group vs. control compound showed a significant decrease in the changes in current ($P < 0.0005$).

Figure 1, C and D, right, shows that the PAPA-NONOate-inhibited current consists largely of an amiloride-sensitive component. Application of amiloride significantly decreased A6 $I_{sc}$ values from $9.25 \pm 1.09 \mu$A/cm$^2$ to $3.08 \pm 0.04 \mu$A/cm$^2$ in A6 cells and from $6.1 \pm 0.34 \mu$A/cm$^2$ to $1.46 \pm 0.10 \mu$A/cm$^2$ in M1 cells ($P < 0.0005$). Furthermore, application of 1.5 $\mu$M PAPA-NONOate to amiloride-inhibited A6 and M1 cells did not further decrease $I_{sc}$, also shown in Fig. 1, C and D. Together, these data suggest that NO release from PAPA-NONOate compound specifically inhibits ENaC activity as measured by transepithelial $I_{sc}$ recordings.

**NO decreases the $P_o$ of ENaC in A6 cells.** When applied to the apical surface of A6 cells, 1.5 $\mu$M PAPA-NONOate decreased the $P_o$ of ENaC from $0.186 \pm 0.043$ to $0.045 \pm 0.009$ ($P < 0.05$) without significantly changing the unitary current of the channel (Fig. 2A and B). The top trace in Fig. 2A is a representative cell-attached single-channel recording from a renal A6 cell before application of PAPA-NONOate showing typical ENaC activity. The bottom trace shown in Fig. 2A shows the same single-channel recording 3 min after application of NO donor, with an apparent decrease in ENaC activity. The $P_o$ was calculated from seven independently performed patch-clamp studies, and average values are shown in Fig. 2B. In a separate study, similar to the transepithelial $I_{sc}$ studies described above, we also added 1.5 $\mu$M inactivated PAPA-NONOate after an initial control recording period. Again, we found that the parent NO donor and its metabolites were not responsible for the NO-induced decrease in ENaC $P_o$. Inactivated PAPA-NONOate did not substantially decrease $P_o$ values in A6 cells (Fig. 2C).

Our present findings in M1 and A6 renal epithelial cells are consistent with those described in our previous report that NO inhibits lung Na$^+$ transport (24) and provides further evidence for NO’s inhibitory role on ENaC activity in kidney epithelial cells.

**SGK1 associates with iNOS in communoprecipitation studies and in vivo.** The lung and kidney express high levels of iNOS mRNA and protein in response to cell injury (25). Consequently, under some circumstances, very high levels of NO can be produced. Even in unstimulated conditions, however, measurable quantities of NO are produced from iNOS (16). This implies that in the absence of any NOS inhibitor, ENaC $P_o$ will always be reduced to a greater or lesser extent by NO. Often, when it is necessary to increase Na$^+$ transport, new channels are inserted in the apical membrane of Na$^+$-transporting epithelial cells. If the $P_o$ of the newly inserted channels is reduced by endogenous NO, then Na$^+$ transport will be limited even though there are new apical transporters. Aldosterone increases Na$^+$ transport, and one mechanism by which it produces this increase is by increasing the number of apical channels. The increase is produced by an activation of SGK1. After promoting the insertion of new channels, however, it makes sense that the $P_o$ of the new channels would not be inhibited by NO. Therefore, we examined whether SGK1 might also increase $P_o$ by inhibiting NOS and NO production while promoting an increase in the number of channels. To test this hypothesis, we first studied whether SGK1, an important

---

Fig. 2. NO decreases the open probability ($P_o$) of epithelial Na$^+$ channel (ENaC) in A6 cells. A: after a control recording period (top trace), 1.5 $\mu$M NONOate was added to the apical surface of A6 cells and recording continued for an additional 5 min (bottom trace). Arrows indicate closed state, and downward deflections represent channel activity. B: results of seven independent patch-clamp studies show that NO significantly decreased $P_o$ in A6 cells. *$P < 0.02$. C: inactivated PAPA-NONOate did not substantially decrease $P_o$ values in A6 single-channel measurements ($n = 4$).
regulator of Na\(^+\) transport, is associated with iNOS in Na\(^+\)-transporting epithelia. Our model for SGK1 regulation of ENaC activity presumed that SGK1 decreases iNOS activity via direct phosphorylation of iNOS to maintain low levels of NO. Inhibition of iNOS is a plausible mechanism for normal ENaC function because pharmacologically inhibiting iNOS with the use of \(\text{N}^\text{G}-\text{nitro-L-arginine methyl ester (37)}\) and iNOS\(^{-/-}\) mice (22) renders these mice iNOS deficient and hypertensive.

We first showed that iNOS is easily detectable in Na\(^+\)-transporting epithelia and coimmunoprecipitates with SGK1. In each 7.5% PAGE Western blot analysis assay, the left bands in Fig. 3A show iNOS immunoprecipitated from A6 cell lysate (Fig. 3A, 1), M1 lysate (Fig. 3A, 2), and primary ATII cell lysate (Fig. 3A, 3) using anti-iNOS antibody. These immunoreactive bands serve as the positive signal control for the coimmunoprecipitation of iNOS with SGK1 in the right lanes of each respective blot in Fig. 3A. With the use of the same experimental protocol used for 1–3 in Fig. 3A, iNOS protein did not coimmunoprecipitate with rabbit polyclonal anti-GAPDH antibody from A6, M1, and ATII cell lysate (Fig. 3A, negative control).

The coimmunoprecipitation of SGK1 and iNOS in lung ATII primary cells and renal epithelia strongly suggests that these proteins are associated and exist in proximity to each other in the cells. Therefore, using immunofluorescence microscopy, we next investigated the subcellular localization of SGK1 and iNOS in ATII cells. ATII cells were costained with Alexa 488-conjugated anti-rabbit antibodies subsequent to polyclonal SGK1 antibody labeling (green in Fig. 3B), as well as Alexa 568-conjugated anti-mouse antibody after mouse monoclonal iNOS binding (red in Fig. 3B). The left and middle columns in Fig. 3B show that SGK1 and iNOS, respectively, are predominantly cytoplasmic or at the cell membrane in lung epithelium.

![Fig. 3. Serum- and glucocorticoid-inducible kinase (SGK1) and inducible nitric oxide synthase (iNOS) are closely associated in alveolar Na\(^+\)-transporting epithelium. A: 7.5% SDS-PAGE gel showing iNOS immunoprecipitated with anti-iNOS antibody (positive control) and coimmunoprecipitated with anti-SGK1 antibody in A6 cells (1), M1 cells (2), and primary cultures of alveolar type II (ATII) epithelial cells (3). iNOS protein did not coimmunoprecipitate with rabbit polyclonal anti-GAPDH antibody (Chemicon, Temecula, CA) in the negative control (right column). All immunoblot analysis was performed with mouse monoclonal anti-iNOS antibody. C: immunofluorescent staining of paraformaldehyde-fixed ATII cells showing colocalization of endogenous SGK1 and iNOS. In the left and middle columns, confocal images show the same field of cells grown in the presence of serum and steroid hormone, costained for SGK1 (green) and iNOS (red), respectively. The images in the right column show the composite images of SGK1 and iNOS and demonstrate that the two proteins colocalize in ATII cells. Columns show 1-μm-thick sections through the cells.](http://ajpcell.physiology.org/)
cells. The composite image of SGK1 and iNOS staining patterns (Fig. 3B, right column) shows an overlap of the spectral signals and strongly suggests that SGK1 and iNOS are closely associated in ATII cells. In control studies, no red or green emission was detected in ATII cells stained with Alexa 568 and Alexa 488 secondary antibodies only (data not shown). Our data are in agreement with findings in previous reports of cytoplasmic SGK1 (2, 3) and iNOS (7) expression in mammary and gastric tumor cells, respectively, and demonstrate that SGK1 and iNOS are present together in ATII cells.

**SGK1 phosphorylates iNOS in vitro**

To demonstrate that SGK1 is capable of phosphorylating iNOS, we performed in vitro kinase assays in which active SGK1 enzyme was incubated with iNOS protein in the presence of radioactively labeled \[^{32}\text{P}ATP\]. Figure 4A shows that iNOS is phosphorylated by SGK1 at high levels similar to those of the positive SGK-tide control (28, 36). Because SGK1 is a Ser/Thr kinase, we next identified which amino acid residues on iNOS are specifically phosphorylated by SGK1.

**Figure 4B** shows the amino acids surrounding miNOS serine residues S733 and S903 (which share high sequence identity with the human isoform of iNOS) that were predicted by NetPhos software (Technical University of Denmark) to be potential sites of phosphorylation. Therefore, we generated the oligopeptides HAKNVFTMRLKSQQNLQSEK (peptide 1; S733) and KPRYYSISSSQDH (peptide 2; S903) and performed additional kinase assays to test these peptides as SGK1-phosphorylated substrates. Figure 4C shows that 6.4 ± 2.8% and 12.88 ± 5.28% of 80 μM peptides 1 and 2, respectively, had incorporated \[^{32}\text{P}ATP\] compared with 18.30 ± 8.34% of 80 μM SGK-tide (data not shown). SGK1 did not phosphorylate the control synthetic peptide CFVRSVSGFQLPED, which contained a Ser and a −3 Arg residue in the same context as peptides 1 and 2 above background levels (data not shown). Compared with this finding, the percent \[^{32}\text{P}ATP\] incorporation into peptides 1 and 2, respectively, in Fig. 4 is significant.

Our current data show that SGK1 is capable of phosphorylating iNOS and that the two proteins are closely associated in ATII cells freshly obtained from mouse alveolar epithelium. Below, we describe a novel aldosterone-dependent, posttranslational modification of iNOS in M1 CCD cells.

**Aldosterone alters in vivo phosphorylation of iNOS and decreases NO production in Na\(^{+}\)-transporting epithelia.** First, we metabolically labeled M1 cells with \[^{32}\text{P}i\] in the presence or absence of 1.5 μM aldosterone. Subsequently, we immunoprecipitated iNOS from \[^{32}\text{P}\]-labeled cells and subjected the immunoprecipitate to SDS-PAGE using a 7.5% gel. The outlined region in Fig. 5A highlights \[^{32}\text{P}\]-labeled iNOS protein (left) and its corresponding pixel intensity profile (middle). Figure 5A, right, shows the results of Western blot analysis performed to confirm that the \[^{32}\text{P}\]-labeled band in the left column was indeed immunoreactive with anti-iNOS antibody. The bands in the autoradiogram and Western blot analysis overlap precisely when the images are superimposed. Similarly, Fig. 5B shows that iNOS protein, which coimmunoprecipitated with anti-SGK1 antibody, from cells grown without aldosterone (left) exhibited less phosphorylated iNOS protein compared with M1 cells grown with aldosterone (right) aldosterone. The average pixel intensities of phosphorylated iNOS from the autoradiogram are expressed as percent control in Fig. 5C. Aldosterone increased the level of \[^{32}\text{P}\]-labeled iNOS in M1

---

**Fig. 4. SGK1 phosphorylates iNOS in vitro.** A: SGK1 phosphorylates hiNOS at levels comparable to the synthetic SGK-tide control. B and C: amino acids surrounding miNOS S733 and S903 are highly conserved in human and murine isoforms. Oligopeptides HAKNVFTMRLKSQQNLQSEK and KPRYYSISSSQDH are both phosphorylated in vitro by the SGK1 enzyme. CPM, counts per minute.
cells ~300% above control levels, regardless of whether iNOS protein was directly immunoprecipitated using anti-iNOS antibody (Fig. 5A) or coimmunoprecipitated with polyclonal SGK1 antibody. In both A and B, phosphorimaging shows an increase in the intensity of 32P-labeled iNOS proteins in response to 1.5 μM aldosterone (left). The same 32P-labeled membrane was examined using Western blot analysis and probed with mouse monoclonal anti-iNOS antibody to confirm that the phosphorylated band in A and B was immunoreactive with iNOS antibody. C: %control of [32P]ATP incorporation was quantified on the basis of the band intensity profiles above background levels in the intensity profiles. Aldosterone increased the level of phosphorylated iNOS in M1 cells ~3-fold. Values are shown as %control, with samples without aldosterone treatment (open bars) set as 100%, the closed bar representing immunoprecipitated iNOS using anti-iNOS antibody, and the shaded bar representing iNOS that coimmunoprecipitated with SGK1.

DISCUSSION
Several types of NO-releasing compounds, such as S-nitrosothiols, organic nitrates, and secondary amine/NO complexes, are commercially available and reportedly have different...
effects on Na\(^{+}\) channel activity. Our present studies show that NO released from 1.5 μM PAPA-NONOate donor is very effective in decreasing amiloride-sensitive Na\(^{+}\) current in M1 and A6 cells within minutes. NO generated from PAPA-NONOate is spontaneously released, and 2 M NO per mol of parent compound is effectively produced. The high amounts of NO released from PAPA-NONOate drug are similar to the micromolar amounts produced in response to iNOS activation and may account for the immediate effect of PAPA-NONOate on inhibiting ENaC activity.

Our findings in the M1 CCD and A6 distal nephron cell lines are similar to findings in two other independent groups. Stoos and colleagues (40, 41) showed that acetylcholine-induced NO release from endothelial cells, as well as addition of the NO donor spermine NONOate, inhibited Na\(^{+}\) reabsorption in CCD cells. Rückes-Nilges et al. (38) reported that 1 mM of sodium nitroprusside clearly inhibited amiloride-sensitive Na\(^{+}\) reabsorption in Xenopus kidney A6 distal nephron cell lines. However, in their study, the NO donors sodium nitroprusside and spermine NONOate did not alter either the amiloride-sensitive or the amiloride-insensitive portions of \(I_{\text{Na}}\) in primary cultures of human nasal epithelial cells. It is becoming apparent that perhaps the efficiency of NO release from different NO donors may effect ion transport differently. The effect of NO also may depend on the cell type examined. For example, our donors may effect ion transport differently. The effect of NO ent that perhaps the efficiency of NO release from different NO cultures of human nasal epithelial cells. It is becoming appar-

SGK1, which is important in mediating both early and late phases of aldosterone activity (1, 6, 33), also may be the key regulator of NO production and iNOS activity after corticoste-
roid stimulation. Our in vitro kinase assays identified iNOS as a novel SGK1 substrate. Specifically, miNOS oligopeptides that were 20 (peptide 1; S733) and 13 (peptide 2; S903) amino acid residues long were phosphorylated by SGK1. Shorter, nine-amino acid peptide sequences surrounding hiNOS S114, S749, S909, S917, and S965 were not phosphorylated effect-

Figure 7 summarizes and illustrates our model of aldoste-
rone-regulated Na\(^{+}\) transport, involving the SGK1, iNOS, and NO components. In the absence of aldosterone or after direct application of PAPA-NONOate, NO immediately inhibits ENaC by reducing ENaC \(P_{o}\), without altering the unitary current or apparent channel density. However, aldosterone-induced increases in the expression of SGK1 may lead to the phosphorylation and hence inactivation of iNOS protein. In-
deed, our studies have shown that local production of NO decreases after aldosterone treatment, which may be an important mechanism involved in controlling ENaC activity.
Although the specific mechanism behind NO inhibition of Na\(^+\) channel activity is not identified in this study and remains unclear, we found that NO modification of ENaCs, or ENaC-regulatory proteins, is not immediately reversible by sequestrering endogenous NO levels. We performed single-channel patch-clamp studies in the presence of carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl (cPTIO; Wako Chemicals, Richmond, VA), a specific NO scavenger. Application of 300 nM cPTIO to A6 cells did not acutely increase ENaC activity (data not shown). Other studies have similarly demonstrated that NO inhibits amiloride-sensitive current in lung cells. These studies also put forth convincing evidence that NO may regulate Na\(^+\) transport via both cGMP-dependent and cGMP-independent pathways. First, Jain et al. (24) demonstrated that NO donors GSNO and SNAP acted via a cGMP pathway. Pretreating cells with methylene blue, an inhibitor of guanylyl cyclase, blocked the inhibitory effects of the NO donors. Jain et al. further implicated cGMP in the NO-regulatory pathway by demonstrating that the permeable analog of cGMP, 8-bromoguanosine 3',5'-cyclic monophosphate (8-BrcGMP), decreased the \(I_{sc}\) of Na\(^+\) channels in alveolar lung cells. Next, by pretreating cells with the PKG inhibitor, KT-5823, and blocking the inhibitory effects of GSNO, Jain et al. demonstrated that PKG may be positioned between cGMP and the effector. Lakrak and colleagues (24, 31) also showed that PAPA-NONOate and spermine NONOate decreased 8.6-pS amiloride-sensitive ENaC via cGMP-dependent mechanisms using both whole cell and single-cell patch-clamp techniques in A549 human alveolar cell lines. Similarly to the aforementioned study by Jain et al., Lakrak and colleagues observed a decrease in inward Na\(^+\) current when A549 cells were perfused with 8-BrcGMP.

Contrary to these findings, Guo et al. (15) reported a cGMP-independent mechanism of ENaC regulation by NO donors. Although they, too, reported that NO generated by PAPA-NONOate inhibited 60% of the amiloride-sensitive \(I_{sc}\) in cultured ATII monolayers, they also reported that the NO-induced decrease in alveolar \(I_{sc}\) was not accompanied by an increase in intracellular cGMP levels. Alternatively, the inhibitory effect of NO on ENaC may occur through direct interaction of NO with the channel or with other ENaC-regulatory proteins. DuVall et al. (11) recently suggested that direct nitration or nitrOSylation of key Tyr residues on the outer borders of the transmembrane domain (TM) of \(\alpha\)-ENaC subunit (Y134 and Y137 in TM1; Y482, Y484, and Y485 in TM2) may alter ENaC activity.

Overall, the present data support an inhibitory effect of NO on ENaC activity in both M1 CCD and A6 epithelial cell lines. In addition, we have shown that the mechanisms by which aldosterone regulates ENaC function include phosphorylation of iNOS and decreased synthesis of NO, possibly through the SGK1 signaling pathway.

ACKNOWLEDGMENTS

We thank B. J. Duke for maintaining M1 and A6 cell cultures. Meral Ciblak and Julie Self provided excellent technical assistance in isolating primary cultures of ATII cells.

GRANTS

This research was supported by National Institutes of Health Grants R01-HL-071621 and R37-DK-37963.

REFERENCES

SGK1 REGULATION OF ENaC BY NO

C726


