Nerve Growth Factor Inhibits Na\textsuperscript{+}/H\textsuperscript{+} Exchange and HCO\textsubscript{3}\textsuperscript{−} Absorption through Parallel Phosphatidylinositol 3-Kinase-m\textsuperscript{TOR} and ERK Pathways in Thick Ascending Limb*  

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In the medullary thick ascending limb, inhibiting the basolateral NHE1 Na\textsuperscript{+}/H\textsuperscript{+} exchanger with nerve growth factor (NGF) induces actin cytoskeleton remodeling that secondarily inhibits apical NHE3 and transepithelial HCO\textsubscript{3}\textsuperscript{−} absorption. The inhibition by NGF is mediated 50% through activation of extracellular signal-regulated kinase (ERK). Here we examined the signaling pathway responsible for the remainder of the NGF-induced inhibition. Inhibition of HCO\textsubscript{3}\textsuperscript{−} absorption was reduced 45% by the phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin or LY294002 and 50% by rapamycin, a specific inhibitor of mammalian target of rapamycin (mTOR), a downstream effector of PI3K. The combination of a PI3K inhibitor plus rapamycin did not cause a further reduction in the inhibition by NGF. In contrast, the combination of a PI3K inhibitor plus the MEK/ERK inhibitor U0126 completely eliminated inhibition by NGF. Rapamycin decreased NGF-induced inhibition of basolateral NHE1 by 45%. NGF induced a 2-fold increase in phosphorylation of Akt, a PI3K target linked to mTOR activation, and a 2.2-fold increase in the activity of p70 S6 kinase, a downstream effector of mTOR. p70 S6 kinase activation was blocked by wortmannin and rapamycin, consistent with PI3K, mTOR, and p70 S6 kinase in a linear pathway. Rapamycin-sensitive inhibition of NHE1 by NGF was associated with an increased level of phosphorylated mTOR in the basolateral membrane domain. These findings indicate that NGF inhibits HCO\textsubscript{3}\textsuperscript{−} absorption in the medullary thick ascending limb through the parallel activation of PI3K-mTOR and ERK signaling pathways, which converge to inhibit NHE1. The results identify a role for mTOR in the regulation of Na\textsuperscript{+}/H\textsuperscript{+} exchange activity and implicate NHE1 as a possible downstream effector contributing to mTOR’s effects on cell growth, proliferation, survival, and tumorigenesis.

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger isoform NHE1\textsuperscript{2} is expressed ubiquitously in the plasma membrane of nonpolarized cells and in the basolateral membrane of epithelial cells, where it plays essential roles in basic cell functions such as the maintenance of intracellular pH (pHi) and cell volume (1–3). NHE1 is involved in other important cellular processes, including proliferation, survival, adhesion, migration, and tumor formation (2–7). These specialized functions involve regulation of NHE1 by a variety of receptor-mediated signaling networks as well as physical interactions of NHE1 with the actin cytoskeleton (2, 3, 5). By comparison, the role of NHE1 in epithelial function remains poorly understood. In particular, the contributions of NHE1 to transepithelial acid-base transport and the possible mechanisms involved are largely undefined.

The medullary thick ascending limb (MTAL) of the mammalian kidney participates in acid-base regulation by reabsorbing most of the filtered HCO\textsubscript{3}\textsuperscript{−} not reabsorbed by the proximal tubule (8, 9). Absorption of HCO\textsubscript{3}\textsuperscript{−} by the MTAL depends on H\textsuperscript{+} secretion mediated by the apical membrane NHE3 Na\textsuperscript{+}/H\textsuperscript{+} exchanger (8, 10–13) and basolateral HCO\textsubscript{3}\textsuperscript{−} efflux, which involves Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchange (14). The MTAL also expresses basolateral NHE1, and we have recently identified a novel role for this exchanger in transepithelial HCO\textsubscript{3}\textsuperscript{−} absorption. Inhibition of NHE1 with amiloride or nerve growth factor (NGF) or by NHE1 knock-out results secondarily in inhibition of apical NHE3, thereby decreasing HCO\textsubscript{3}\textsuperscript{−} absorption (15–17). NHE1 modulates NHE3 activity by regulating the organization of the actin cytoskeleton (18). The rate of luminal H\textsuperscript{+} secretion and transepithelial HCO\textsubscript{3}\textsuperscript{−} absorption in the MTAL thus depends on a regulatory interaction between the basolateral and apical membrane Na\textsuperscript{+}/H\textsuperscript{+} exchangers, whereby basolateral NHE1 enhances the activity of apical NHE3 (15–18).

Based on the above findings, a key to understanding the role of NHE1 in epithelial function lies in identifying cell signals that modify transepithelial acid transport through effects on basolateral NHE1 activity. Recently we demonstrated that NGF inhibits HCO\textsubscript{3}\textsuperscript{−} absorption in the MTAL by inhibiting NHE1 through activation of the extracellular signal-regulated kinase (ERK) signaling pathway (19). However, blocking ERK activation eliminated only ~50% of the NGF-induced transport regulation (19). Thus, an additional signaling pathway must play a role in mediating the inhibition of NHE1 by NGF.

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2 The abbreviations used are: NHE, Na\textsuperscript{+}/H\textsuperscript{+} exchanger; pH, intracellular pH; MTAL, medullary thick ascending limb; NGF, nerve growth factor; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol 3-kinase; mTOR, mammalian target of rapamycin; S6K, p70 ribosomal S6 kinase; MEK, mitogen-activated protein kinase/ERK kinase; TSC, tuberous sclerosis complex; ΔHCO\textsubscript{3}\textsuperscript{−}, absolute rate of HCO\textsubscript{3}\textsuperscript{−} absorption; MOPS, 4-morpholinepropanesulfonic acid.
The present study was designed to identify the signaling pathway responsible for the remainder of NGF-induced inhibition of NHE1 and HCO₃⁻ absorption in the MTAL. We show that NGF decreases HCO₃⁻ absorption through a phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin (mTOR)-dependent signaling pathway that functions in parallel with ERK to inhibit NHE1. These studies identify a previously unrecognized role for mTOR in regulating Na⁺/H⁺ exchange activity and the absorptive function of renal tubules. The results implicate NHE1 as a possible downstream effector contributing to mTOR’s effects on cell growth, proliferation, and tumorigenesis.

**EXPERIMENTAL PROCEDURES**

Tubule Perfusion and Measurement of Net HCO₃⁻ Absorption—MTALs from male Sprague-Dawley rats (50–100 g body wt; Taconic, Germantown, NY) were isolated and perfused in vitro as previously described (16, 20). Tubules were dissected from the inner stripe of the outer medulla at 10 °C in control bath solution (see below), transferred to a bath chamber on the stage of an inverted microscope, and mounted on concentric glass pipettes for perfusion at 37 °C. For HCO₃⁻ transport experiments, the tubules were perfused and bathed in control solution that contained 146 mM Na⁺, 4 mM K⁺, 122 mM Cl⁻, 25 mM HCO₃⁻, 2.0 mM Ca²⁺, 1.5 mM Mg²⁺, 2.0 mM phosphate, 1.2 mM SO₄²⁻, 1.0 mM citrate, 2.0 mM lactate, 5.5 mM glucose, and 5 mM HEPES (equilibrated with 100% O₂; titrated to pH 7.4). Basolateral Na⁺/H⁺ exchange activity was determined by measurement of the initial rate of pH recovery increase after the addition of 145 mM Na⁺ to the bath solution (Na⁺ replaced N-methyl-d-glucammonium), the intracellular buffering power, and cell volume as described (16, 19). Interruption of pH recovery at various points along the recovery curve permits determination of the Na⁺/H⁺ exchange rate over a range of pH values, with appropriate corrections for a variable background acid loading rate (16, 22). The Na⁺-dependent pH recovery rate was inhibited ≥90% by bath ethylisopropyl amiloride (50 μM) under all experimental conditions.

**Inner Stripe Tissue Preparation and Immunoblotting**—The inner stripe tissue preparation used to study signaling proteins has been previously described (19, 21, 23). In brief, thin strips of tissue were microdissected at 10 °C from the inner stripe of the outer medulla, the region of the kidney highly enriched in MTALs. The tissue strips were then divided into four samples of equal amount and incubated in vitro at 37 °C in the same solutions used for HCO₃⁻ transport experiments (19, 21, 23). The specific protocols used for incubations are given under “Results” (Figs. 7 and 9D). After incubation, the tissue was resuspended in ice-cold modified radioimmune precipitation assay lysis buffer, pH 7.5, plus 1 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (1:400; Sigma), homogenized, and lysed for 4 h at 4 °C. Lysates were cleared by centrifugation (4000 × g for 10 min), and supernatants were separated into aliquots and stored at −80 °C. Samples of equal protein content were separated by SDS-PAGE on 8 or 10% gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline + 0.1% Tween 20 + 1–5% bovine serum albumin at 4 °C and incubated overnight at 4 °C with antiphospho-akt-Ser-473 (1:1000) or anti-Akt (1:2500) antibodies (Cell Signaling Technology), or with antiphospho-mTOR-Ser-2448 (1:500; BIOSOURCE) or antimTOR (1:250; Santa Cruz Biotechnology) antibodies. After washing in Tris-buffered saline + 0.1% Tween 20, horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was applied, and immunoreactive bands were detected by chemiluminescence (Luminol Reagent, Santa Cruz Biotechnology). Band intensities were quantified by densitometry.

*p70 S6 Kinase (S6K) Assay*—Inner stripe tissue was incubated in vitro at 37 °C in the absence and presence of NGF and various inhibitors, and cell lysates were prepared as described above. The specific incubation conditions are indicated under “Results” (Fig. 8). For immunoprecipitation, equal amounts of sample protein (500 μg) were incubated for 2 h at 4 °C with 4 μg of rabbit anti-p70 S6K antibody (Upstate Biotechnology) coupled to protein A/G-agarose beads (Santa Cruz). The immune complexes were then washed 3 times in Triton X-100 lysis buffer and one time in assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM sodium β-glycerophosphate, 5 mM EGTA, 1 mM Na₃VO₄, and 1 mM dithiothreitol). Kinase activity in immunoprecipitates was measured using a p70 S6K assay kit (Upstate). In brief, immunoprecipitates were incubated for 15 min at 30 °C in 50 μl final volume of assay dilution buffer containing 5 μM S6K substrate peptide 2 (KKRNRTLTK), inhibitor mixture containing protein kinase C and A and CdK inhibitors, magne-
tubule, which provides a cross-sectional view of cells in the lateral tubule walls (18). For individual experiments, two to four tubules from the same kidney for each experimental condition were fixed and stained identically and imaged in a single session at identical settings of illumination, gain, and exposure time. Two-dimensional image analysis was performed using MetaMorph software in which boxes (4.5 × 1.2 μm) were positioned on linear regions of basolateral and apical membrane domains, and pixel intensity per unit area was determined for each region. Two to four cells were analyzed in each tubule, and the values were averaged. Fluorescence intensity for NGF-treated tubules was expressed as a percentage of the control value measured in the same experiment.

RESULTS

PI3K Inhibitors Reduce Inhibition of HCO₃⁻ Absorption by NGF—Under control conditions, adding 0.7 nM NGF to the bath decreased HCO₃⁻ absorption in isolated MTALs by 39%, from 13.7 ± 0.6 to 8.4 ± 0.6 pmol/min/mm (Fig. 1A). In MTALs bathed with the PI3K inhibitor wortmannin (100 nM) or LY294002 (20 μM), NGF decreased HCO₃⁻ absorption only by 21%, from 13.5 ± 0.8 to 10.6 ± 0.7 pmol/min/mm (Fig. 1B). The net decrease in HCO₃⁻ absorption induced by NGF was reduced 45% by the PI3K inhibitors (5.3 ± 0.3 pmol/min/mm without inhibitors versus 2.9 ± 0.3 pmol/min/mm with inhibitors; p < 0.001). In previous studies, inhibition of HCO₃⁻ absorption by NGF was reduced 50–60% by inhibitors of ERK activation (19). As shown in Fig. 1C, the combination of a PI3K inhibitor plus a MEK/ERK inhibitor (U0126) completely eliminated the inhibition by NGF, indicating that the inhibitory effects of PI3K and ERK are additive. These results support the view that NGF inhibits HCO₃⁻ absorption through the parallel activation of PI3K- and ERK-dependent signaling pathways.

Rapamycin Reduces Inhibition of HCO₃⁻ Absorption by NGF—An important downstream effector of PI3K in growth factor signaling is mTOR, which is specifically inhibited by the immunosuppressive drug rapamycin (25). Similar to the preceding results with PI3K inhibitors, NGF decreased HCO₃⁻ absorption by 38% under control conditions (from 14.8 ± 0.4 to 9.2 ± 0.5 pmol/min/mm) but only by 19% (from 14.3 ± 0.4 to 11.6 ± 0.6 pmol/min/mm) in MTALs bathed with 20 nM rapamycin (Fig. 2, A and B). The net decrease in HCO₃⁻ absorption induced by NGF was reduced 50% by rapamycin (p < 0.001). The combination of rapamycin plus U0126 again completely eliminated the inhibition by NGF (Fig. 2C). These results support a role for mTOR in mediating the inhibition of HCO₃⁻ absorption by NGF and show that inhibition via the rapamycin-sensitive pathway is additive to inhibition mediated through ERK.

FIGURE 1. Inhibition of HCO₃⁻ absorption by NGF is reduced by PI3K inhibitors. Rat MTALs were isolated and perfused in vitro in control solution (A), bathed with 100 nM wortmannin or 20 μM LY294002 (B), or bathed with a PI3K inhibitor plus the MEK/ERK inhibitor U0126 (15 μM) (C). NGF (0.7 nM) was then added to and removed from the bath solution. Data points are average values for single tubules. Lines connect paired measurements made in the same tubule. p values are for paired t tests. NS, not significant. HCO₃⁻, absolute rate of HCO₃⁻ absorption. Mean values are given under "Results."
Because PI3K inhibitors and rapamycin reduced the inhibition from 13.4 to 1.04 pmol/min/mm (Fig. 3), a decrease similar to that observed with either inhibitor alone (Figs. 1B and 2B). Thus, combining a PI3K inhibitor with rapamycin does not cause a further reduction in the inhibition by NGF, consistent with these agents blocking a common regulatory pathway. These results support the view that PI3K and mTOR are components of a common signaling pathway that inhibits HCO₃⁻ absorption.

**Specificity of Inhibitors in Regulation of HCO₃⁻ Absorption**—To assess the specificity of rapamycin actions on HCO₃⁻ absorption, we examined factors that inhibit HCO₃⁻ absorption through signaling pathways not involving PI3K. Angiotensin II inhibits HCO₃⁻ absorption in the MTAL through cytochrome P450 (26). In MTALs bathed with rapamycin, angiotensin II decreased HCO₃⁻ absorption by 32 ± 1% (Fig. 4A), an effect similar to that observed under identical conditions in the absence of the inhibitor (26). In previous studies we found that rapamycin also has no effect on inhibition of HCO₃⁻ absorption by aldosterone, which is mediated through ERK (24). Thus, rapamycin selectively reduces inhibition of HCO₃⁻ absorption by NGF that depends on PI3K. Consistent with these findings, the inhibition of HCO₃⁻ absorption by NGF is additive to inhibition by angiotensin II (Fig. 4B) and aldosterone (27), further confirming that these factors act through distinct signaling pathways. Additional experiments evaluated the specificity of the PI3K inhibitor + ERK inhibitor combination by examining arginine vasopressin, which inhibits HCO₃⁻ absorption via cAMP (20). In MTALs bathed with LY294002 plus U0126, vasopressin decreased HCO₃⁻ absorption by 45 ± 5% (Fig. 4C), an effect similar to that observed in the absence of the inhibitors (20). Thus, the effect of the PI3K-ERK inhibitor combination to eliminate inhibition of HCO₃⁻ absorption is selective for NGF (Fig. 1C) and is not the result of nonspecific metabolic or cytotoxic effects on the tubule cells.

**Rapamycin Reduces Inhibition of Basolateral Na⁺/H⁺ Exchange by NGF**—In the MTAL, NGF decreases HCO₃⁻ absorption through primary inhibition of the basolateral NHE1 Na⁺/H⁺ exchanger (16, 17). To determine whether the PI3K-mTOR pathway is involved in mediating inhibition of basolateral Na⁺/H⁺ exchange, we examined the effects of NGF in the absence and presence of rapamycin. Under control conditions, NGF decreased basolateral Na⁺/H⁺ exchange activity at all pH values studied (control versus NGF; Fig. 5A). In MTALs bathed with rapamycin, the inhibition by NGF was significantly reduced (rapamycin + NGF, Fig. 5A). Overall, the net decrease in basolateral Na⁺/H⁺ exchange activity induced by NGF was reduced 45% by rapamycin (p < 0.05; Fig. 5B). Rapamycin alone did not affect basolateral Na⁺/H⁺ exchange activity (Fig. 5B). These results demonstrate that NGF inhibits basolateral Na⁺/H⁺ exchange via a rapamycin-sensitive
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FIGURE 4. Specificity of inhibitors in regulation of HCO₃⁻ absorption. A, MTALs were bathed with rapamycin (Rap, 20 nm), and then angiotensin II (Ang II, 10 nm) was added to and removed from the bath solution. B, MTALs were bathed with angiotensin II (10 nm), and then NGF (0.7 nm) was added to and removed from the bath solution. C, MTALs were bathed with LY294002 (20 μM) and then arginine vasopressin (AVP) (0.1 μM) was added to and removed from the bath solution. JHCO₃⁻, data points, lines, and p values are as in Fig. 1. Mean values are given under "Results."

FIGURE 5. Inhibition of basolateral Na⁺/H⁺ exchange by NGF is reduced by rapamycin. A, MTALs were studied under control conditions and with NGF (0.7 nm) or NGF + rapamycin (Rap, 20 nm) in the bath solution. Basolateral Na⁺/H⁺ exchange rates (JNa⁺/H⁺) were determined at various pH values from initial rates of pH increase after the addition of Na⁺ to the bath solution (see "Experimental Procedures"). Data points are from 11 control tubules, 11 tubules with NGF, and 7 tubules with rapamycin + NGF. B, mean basolateral Na⁺/H⁺ exchange rates for the three conditions in panel A plus additional data from seven tubules studied with rapamycin alone. *, p < 0.05 versus control or rapamycin; #, p < 0.05 versus NGF (analysis of variance).

pathway and are consistent with mTOR acting downstream of PI3K to mediate NGF-induced inhibition of NHE1 and HCO₃⁻ absorption.

PI3K Inhibitors and Rapamycin Do Not Affect Inhibition By Bath Amiloride—Inhibiting basolateral Na⁺/H⁺ exchange decreases HCO₃⁻ absorption in the MTAL by inducing actin cytoskeleton remodeling that secondarily inhibits apical Na⁺/H⁺ exchange (18). Thus, an additional mechanism through which PI3K-mTOR signaling could affect HCO₃⁻ absorption is by modifying the regulatory interaction between basolateral and apical Na⁺/H⁺ exchangers. To test this, we took advantage of our previous finding that the interaction between exchangers and the resulting inhibition of HCO₃⁻ absorption can be induced directly by inhibiting basolateral NHE1 with bath amiloride (16–18). Under control conditions, the addition of 10 μM amiloride to the bath decreased HCO₃⁻ absorption by 31%, from 14.1 ± 0.6 to 9.7 ± 0.7 pmol/min/mm S6K is an important downstream effector of mTOR (25). To test the effect of NGF on S6K, inner stripe tissue was exposed to NGF in vitro for 15 min, and S6K activity was measured by immune complex assay. As shown in Fig. 8, NGF increased S6K activity 2.2-fold. This activation was blocked by wortmannin or rapamycin, consistent with PI3K, mTOR, and S6K in a linear signaling pathway. In contrast, S6K activation was not blocked by the MEK/ERK inhibitor U0126, confirming further that activation of the PI3K-mTOR-S6K pathway by NGF occurs independently of the activation of ERK. These results indicate that NGF increases S6K activity via a PI3K- and mTOR-dependent pathway and suggest that S6K may be a downstream effector of PI3K and mTOR in mediating NGF-induced inhibition of NHE1 and HCO₃⁻ absorption (see "Discussion").

NGF Increases Phosphorylated mTOR Level in the Basolateral Membrane Domain—The sensitivity to rapamycin (Figs. 2 and 5) suggests that the inhibition of NHE1 and HCO₃⁻ absorp-
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**DISCUSSION**

Previously we identified a novel role for the basolateral NHE1 Na\(^+\)/H\(^+\) exchanger in transepithelial HCO\(_3\)\(^-\) absorption in the renal MTAL (15–17). In the present study we examined the effects of NGF to identify signal transduction pathways that regulate transepacellular H\(^+\) secretion through effects on NHE1 activity. The results reveal a new role for PI3K-mTOR signaling in the acute regulation of NHE1 and provide the first evidence that mTOR is involved in regulating the absorptive function of renal tubules.

A model for NGF-induced regulation in the MTAL based on our current and previous (16–19) findings is presented in Fig. 10. Binding of NGF to its basolateral cell surface receptor (TrkA) induces parallel activation of PI3K and ERK signaling cascades. Activation of PI3K leads to the downstream activation of Akt, mTOR, and S6K. The PI3K-mTOR and ERK pathways converge to inhibit basolateral NHE1. This in turn induces actin cytoskeleton remodeling that secondarily inhibits apical NHE3, resulting in decreased luminal H\(^+\) secretion and transepithelial HCO\(_3\)\(^-\) absorption (16, 18). The PI3K-mTOR and ERK pathways function independently to inhibit NHE1, with each pathway accounting for ~50% of the NGF-induced transport inhibition.

The role of NHE1 in processes such as proliferation and survival, adhesion and migration, and the motile and invasive properties of tumor cells (1–7) has led to extensive investigation of cell signaling pathways that regulate NHE1 activity. NHE1 is regulated by diverse stimuli acting through receptor tyrosine kinases, G protein-coupled receptors, and integrin...
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**FIGURE 8. NGF increases S6K activity.** Inner stripe tissue was incubated in vitro at 37 °C in the absence (Cont) and presence of 100 nm wortmannin (Wort), 20 nm rapamycin (Rap), or 15 µM U0126 for 15 min, then treated with 0.7 nm NGF for 15 min. S6K was immunoprecipitated, and in vitro kinase activity determined by phosphorylation of S6 peptide substrate (see "Experimental Procedures"). Phosphorylated products were spotted onto phosphocellulose paper and quantified by scintillation counting. Phosphotransferase activity of S6K is presented as a percentage of control activity measured in the same experiment. Data are means ± S.E. for five independent experiments.

**FIGURE 9. NGF increases p-mTOR labeling in the basolateral membrane domain.** A–C, MTALs were incubated in vitro in control solution (A), 0.7 nm NGF (B), or 100 nm wortmannin + NGF (C) for 15 min, then fixed, permeabilized, and stained with antiphospho-mTOR-Ser-2448 antibody (p-mTOR). Tubules were analyzed by confocal immunofluorescence as described under "Experimental Procedures." Images are z-axis sections (<0.4 µm) taken through a plane at the center of the tubule showing a cross-sectional view of cells in the lateral tubule walls (18). NGF increased p-mTOR labeling in the basolateral membrane domain (arrowheads). Quantification of membrane fluorescence intensity is given under "Results." Images are representative of five independent experiments. Bar, 5 µm. D, inner stripe tissue was incubated in vitro at 37 °C in the absence (Cont) and presence of 0.7 nm NGF for 15 min. Cell lysates were immunoblotted with antiphospho-mTOR-Ser-2448 (p-mTOR) and anti-mTOR (mTOR) antibodies. Blots are representative of three separate experiments. Bars show densitometric analysis of mTOR phosphorylation, presented as a percentage of the control level measured in the same experiment. Data are means ± S.E. (n = 3), *p < 0.05 versus control.

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- **Activation of NHE1 in response to growth factors and other stimuli involves phosphorylation of the exchanger by various kinases, including the ERK-regulated kinase p90\(^{ROCK}\) (29), the RhoA target p160 Rho-associated kinase 1 (p160\(^{ROCK}\)) (30), and the serine/threonine kinase Nck-interacting kinase (NIK) (31). NHE1 also is modulated through physical interactions with other regulatory proteins such as Ca\(^{2+}\)-calmodulin and calcineurin B homologous proteins (CHIPs) (32–34). A signaling molecule importantly involved in the control of cell growth, proliferation, motility, and transformation is PI3K (25, 35–37). Although PI3K has been implicated in regulation of Na\(^+\)/H\(^+\) exchange activity in mammary epithelial cells and breast tumor cells (38, 39), PI3K signaling generally has not been ascribed a significant role in regulating NHE1 and its physiological functions (1–3, 5). In the present study we demonstrate that PI3K plays a major role in NGF-induced inhibition of HCO\(_3\)\(^-\) absorption, mediated through inhibition of NHE1. Thus, PI3K signaling is importantly involved in regulating the epithelial function of NHE1 to control transcellular H\(^+\) secretion in MTAL cells. Of significance, we found that PI3K signaling inhibits NHE1 activity, contrary to the stimulation of NHE1 by other growth-related signaling pathways (1–3). It remains to be determined whether PI3K inhibits NHE1 in other epithelial or nonepithelial cells or if this represents a specialized response of the MTAL. It is conceivable that PI3K could function in a negative feedback or compensatory pathway that serves to modulate the stimulation of NHE1 by other mitogenic pathways. Such a system would be analogous to the role of PI3K-Akt signaling in innate immune regulation, where it serves as a negative regulator of immune receptor signaling to limit the magnitude of proinflammatory responses that can lead to organ damage (40, 41). Consistent with this possibility, inhibiting PI3K potentiates activation of Na\(^+\)/H\(^+\) exchange that was responsible for increased motility and invasion of breast epithelial tumor cells during serum deprivation (39). These results support a role for PI3K in suppressing NHE1 stimulation that leads to tumor cell transformation and metastasis.

- mTOR plays a central role in the control of cell growth, survival, and proliferation, with abnormal elevation of PI3K-mTOR signaling a contributing factor in tumorigenesis (25, 37, 42). To our knowledge, no previous studies have reported a role for mTOR in the regulation of Na\(^+\)/H\(^+\) exchange activity. Results
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TSC2 gene lead to the development of renal tumors that are sensitive to rapamycin, indicating an important role for TSC suppression of mTOR signaling in renal cells (25, 47, 48). The results of the present study raise the possibility that mTOR-mediated regulation of NHE1 could play a role in mTOR-dependent renal cell proliferation and tumor development.

Rapamycin specifically inhibits mTOR by forming an inhibitory complex with FKBP12, the intracellular rapamycin receptor. The rapamycin-FKBP12 complex binds directly to mTOR, which destabilizes multiprotein mTOR signaling complexes and inhibits the ability of mTOR to activate S6K and other downstream signals (25, 46). The efficacy of rapamycin in suppressing cell proliferation and the mammalian immune system has led to its use in multiple clinical settings, including as an immunosuppressant in kidney transplants, to prevent restenosis in cardiovascular stents, and as an antitumor agent (25, 37, 42, 49, 50). Beneficial effects of rapamycin to prevent progression of chronic kidney disease also have been reported in experimental models (51). Our study provides new evidence that rapamycin at therapeutic concentrations influences the regulation of NHE1 and its cell functions through inhibition of PI3K-mTOR signaling. Rapamycin had no significant effect on NHE1 activity or HCO<sub>3</sub><sup>-</sup> absorption under basal conditions but impaired their regulation by NGF. In view of the defined roles of NHE1 in cell growth, proliferation, and migration, it will be important in future studies to determine whether the ability of rapamycin to modify NHE1 regulation via mTOR may contribute to the antiproliferative, immunosuppressive, or antitumor properties of this drug. Our findings in the MTAL also raise the possibility that rapamycin could impair the regulation of renal tubule functions such as Na<sup>+</sup> absorption and H<sup>+</sup> secretion that depend on Na<sup>+</sup>/H<sup>+</sup> exchange activity.

The two major downstream effectors of mTOR are S6K1, which enhances translational efficiency, and eukaryotic initiation factor 4E-binding protein (4E-BP1), a translational repressor protein (25, 46). We propose that S6K1 functions downstream of PI3K-mTOR to mediate NGF-induced inhibition of NHE1 in the MTAL based on the following observations. First, NGF increased S6K activity under conditions similar to those used in HCO<sub>3</sub> absorption experiments. Second, the stimulation of S6K was blocked by wortmannin and rapamycin but not by the MEK/ERK inhibitor U0126. Thus, the inhibitor sensitivity of S6K activation correlates directly with that observed for PI3K-dependent inhibition of HCO<sub>3</sub><sup>-</sup> absorption. Third, the PI3K-mTOR-dependent transport regulation occurs within a time frame (<15 min) that correlates with increased S6K activity but is unlikely to be mediated through mTOR-dependent up-regulation of transcriptional factors and protein synthesis. Fourth, S6K is a member of the AGC family of serine/threonine kinases that includes several well defined regulators of epithelial transport proteins, including protein kinases A, C, and G, serum and glucocorticoid-induced protein kinase, and p90 ribosomal S6 kinase (52, 53). Our study is the first to implicate a role for S6K in regulating Na<sup>+</sup>/H<sup>+</sup> exchange activity and the transport function of renal tubules. Because of the lack of selective S6K inhibitors, we were unable to evaluate the functional significance of S6K activation for Na<sup>+</sup>/H<sup>+</sup> exchange regulation in the perfused MTAL. Future studies using cell systems that enable

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**FIGURE 10. Model of signaling pathways mediating inhibition of NHE1 and HCO<sub>3</sub> absorption by NGF in the MTAL.** NGF induces parallel activation of PI3K-mTOR and ERK signaling pathways, which function independently to inhibit basolateral NHE1. Inhibition of NHE1 induces actin cytoskeleton remodeling that secondarily inhibits apical NHE3, resulting in decreased luminal H<sup>+</sup> secretion and transepithelial HCO<sub>3</sub><sup>-</sup> absorption. This conclusion is supported by several lines of evidence. 1) Inhibition of HCO<sub>3</sub> absorption by NGF was reduced by wortmannin or LY294002, two chemically unrelated PI3K inhibitors with different mechanisms of action shown previously to block PI3K-dependent transport regulation in the MTAL (21). Wortmannin was studied at a concentration that selectively inhibits PI3K, with no significant effects against mTOR, other PI3K-related kinases, and a broad range of protein kinases (43–45). 2) NGF increased wortmannin-sensitive phosphorylation of the PI3K substrate Akt, confirming PI3K activation. 3) Inhibition of NHE1 and HCO<sub>3</sub><sup>-</sup> absorption were reduced by rapamycin, a specific inhibitor of mTOR signaling (see below). 4) The effects of PI3K inhibitors and rapamycin to reduce inhibition by NGF were similar in magnitude and not additive, consistent with PI3K and mTOR as components of a common inhibitory pathway. 5) Rapamycin selectively blocked PI3K-dependent inhibition by NGF but had no effect on inhibition of HCO<sub>3</sub> absorption by stimuli that act through pathways not involving PI3K (angiotensin II and aldosterone). 6) Inhibition of NHE1 by rapamycin was associated with an increased level of phosphorylated mTOR in the basolateral membrane domain. 7) NGF-induced stimulation of the mTOR target S6K was blocked by both PI3K inhibitors and rapamycin. Taken together, these results support PI3K and mTOR as components of a common signaling pathway that inhibits NHE1 and HCO<sub>3</sub><sup>-</sup> absorption. In mammalian cells, the biochemical link between PI3K and mTOR involves Akt-induced phosphorylation and inactivation of the protein tuberous sclerosis 2 (TSC2), which negatively regulates mTOR through the small G protein Rheb (25, 42, 46). Whether TSC2 and Rheb play a role in mediating PI3K-mTOR-dependent regulation of NHE1 in the MTAL remains to be determined. Inactivating mutations in the
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constitutive activation and/or knockdown of S6K will be required to establish directly a role for S6K in NHE1 regulation. Additional mTOR-associated proteins, such as protein phosphatase 2 (25, 54, 55), also could be involved in mTOR regulation of NHE1 activity.

Regulatory interactions between the PI3K-mTOR and ERK signaling pathways have been described in other systems. For example, ERK1/2 can induce p90\(^{RSK}\)-catalyzed phosphorylation and inactivation of TSC2, resulting in increased mTOR signaling and S6K activation independent of PI3K (46, 56, 57). Conversely, rapamycin has been shown to diminish growth factor-induced ERK activation in some cells, an effect that may involve mTOR modulating ERK phosphorylation through PP2A (55). Several findings suggest that these interactions are minimal or absent in the MTAL, at least with respect to NGF-induced inhibition of NHE1 and HCO\(_3\)\(^-\) absorption. First, the effect of ERK inhibitors to diminish inhibition by NGF is quantitatively similar in the absence or presence of a functional PI3K-mTOR pathway (Figs. 1 and 2) (Ref. 19), arguing against a contribution of mTOR to ERK activation. Second, PI3K inhibitors reduce NGF-induced inhibition by an amount virtually identical to that observed with rapamycin (Figs. 1-3), arguing against an effect of ERK to activate mTOR-dependent regulation independently of PI3K. Third, activation of S6K by NGF was similar in the absence and presence of a MEK/ERK inhibitor (Fig. 8). Thus, our results provide no evidence for significant cross-talk between the PI3K-mTOR and ERK pathways and suggest that these pathways function independently to inhibit NHE1 and HCO\(_3\)\(^-\) absorption. Previous studies also have identified an internal feedback loop within the PI3K-mTOR-S6K pathway involving phosphorylation of mTOR by S6K. In this mechanism, S6K is primarily activated downstream of PI3K via the Akt-TSC1/2-Rheb-mTOR pathway. Activated S6K in turn directly phosphorylates mTOR at Ser-2448 (28, 58). It is presently unclear whether this feedback loop functions as a positive or negative regulator of mTOR signaling (28, 58). In the MTAL we found that NGF activates S6K via a PI3K- and mTOR-dependent pathway and that NGF stimulation results in increased Ser-2448 phosphorylation of mTOR in the basolateral membrane domain, consistent with feedback regulation of mTOR through S6K. Further studies will be required to confirm this and to evaluate the possibility that phosphorylation of mTOR by S6K may be involved in basolateral mTOR targeting and/or in mTOR-dependent regulation of NHE1 activity.

PI3K has been shown to play a role in acute regulation of the epithelial Na\(^+\)/H\(^+\) exchanger NHE3 by growth factors and other stimuli (59, 60). In the MTAL, hyposmolality increases NHE3 activity and HCO\(_3\)\(^-\) absorption through activation of PI3K (13, 21). These effects are blocked by rapamycin, indicating that mTOR functions downstream of PI3K to stimulate NHE3 and HCO\(_3\)\(^-\) absorption in MTAL cells (61). These findings can be contrasted directly with the regulatory effects of NGF; activation of PI3K-mTOR signaling by NGF results in inhibition of HCO\(_3\)\(^-\) absorption through primary inhibition of NHE1, with no direct coupling to NHE3 (Fig. 5) (Ref. 16). Thus, in the MTAL the PI3K-mTOR pathway can be targeted specifically to inhibit basolateral NHE1 or stimulate apical NHE3 depending on the physiological stimulus. The exact mechanisms that target PI3K-mTOR signals to regulate different Na\(^+\)/H\(^+\) exchangers in different epithelial membrane domains will be important to identify. The NGF-induced inhibition of NHE1 is associated with an increase in phosphorylated mTOR in the basolateral membrane. Thus, targeted activation of mTOR may be a component of PI3K-mTOR signal specificity in MTAL cells.

Neurotrophins and their receptors are highly expressed in the kidney, but their roles in kidney function are not understood (16). Our studies establish directly that NGF can influence the transport function of renal tubules. The change in HCO\(_3\)\(^-\) absorption induced by NGF in the MTAL is comparable in magnitude to that observed with other regulatory factors, including angiotensin II, aldosterone, chronic metabolic acidosis and alkalosis, and dietary sodium intake (8, 26, 27). The results of the present study show that the inhibition of HCO\(_3\)\(^-\) absorption by NGF is mediated through a rapamycin-sensitive PI3K-mTOR pathway that is distinct from the signaling pathways involved in inhibition by angiotensin II, aldosterone, and vasopressin, thereby enabling NGF to regulate MTAL HCO\(_3\)\(^-\) absorption in the presence of these other hormones (Fig. 4) (Refs. 20, 24, 26, 27, and 62). These findings support a significant role for NGF in the physiological control of MTAL function.

In summary, NGF inhibits NHE1 in the MTAL through the parallel activation of PI3K-mTOR and ERK signaling pathways. The decrease in NHE1 activity results secondarily in inhibition of apical NHE3 and transepithelial HCO\(_3\)\(^-\) absorption (16–18). These studies provide the first evidence that mTOR regulates Na\(^+\)/H\(^+\) exchange activity and identify an important role for PI3K-mTOR signaling in regulating the epithelial function of NHE1 to enhance tranacellular H\(^+\) secretion (Fig. 10). Our findings raise the possibility that NHE1 could function as a downstream target of mTOR that plays a role in mediating mTOR’s effects on cell growth, survival, proliferation, and tumorigenesis.

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