Published in final edited form as:

Am J Physiol Renal Physiol. 2007 February ; 292(2): F577–F585. doi:10.1152/ajprenal.00240.2006.

Characterization of the regulation of renal Na⁺/H⁺ exchanger NHE3 by insulin

Daniel G. Fuster¹, I. Alexandru Bobulescu^{1,2}, Jianning Zhang¹, James Wade³, and Orson W. Moe^{1,2}

¹ Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas ² Charles and Jane Pak Center of Mineral Metabolism and Clinical Research, University of Texas Southwestern Medical Center, Dallas, Texas ³ Department of Physiology, University of Maryland, Baltimore, Maryland

Abstract

Insulin receptors are widely distributed in the kidney and affect multiple aspects of renal function. In the proximal tubule, insulin regulates volume and acid-base regulation through stimulation of the Na⁺/H⁺ exchanger NHE3. This paper characterizes the signaling pathway by which insulin stimulates NHE3 in a cell culture model [opossum kidney (OK) cell]. Insulin has two distinct phases of action on NHE3. Chronic insulin (24 h) activates NHE3 through the classic phosphatidylinositol 3-kinase-serum- and glucocorticoid-dependent kinase 1 (PI3K-SGK1) pathway as insulin stimulates SGK1 phosphorylation and the insulin effect can be blocked by the PI3K inhibitor wortmannin or a dominant-negative SGK1. We showed that SGK1 transcript and protein are expressed in rat proximal tubule and OK cells. We previously showed that glucocorticoids augment the effect of insulin on NHE3 (Klisc J, Hu MC, Nief V, Reyes L, Fuster D, Moe OW, Ambuhl PM. *Am J Physiol Renal Physiol* 283: F532–F539, 2002). Part of this can be mediated via induction of SGK1 by glucocorticoids, and indeed the insulin effect on NHE3 can also be amplified by overexpression of SGK1. We next addressed the acute effect of insulin (1–2 h) on NHE3 by systematically examining the candidate signaling cascades and activation mechanisms of NHE3. We ruled out the PI3K-SGK1-Akt and TC10 pathways, increased surface NHE3, NHE3 phosphorylation, NHE3 association with calcineurin homologous protein 1 or megalin as mechanisms of acute activation of NHE3 by insulin. In summary, insulin stimulates NHE3 acutely via yet undefined pathways and mechanisms. The chronic effect of insulin is mediated by the classic PI3K-SGK1 route.

Keywords

Na⁺/H⁺ exchanger isoform 3; serum- and glucocorticoid-dependent kinase 1

Insulin has myriad effects on multiple organs and cell types. Although insulin receptors are believed to be widely expressed in the kidney (9,44,45,53), the renal actions of insulin are not well understood. One classic insulin effect on the kidney concerns glucose homeostasis. While the antigluconeogenic action of insulin on the kidney appears to be well established (28,39), the quantitative role of the kidney in whole-body gluconeogenesis is still a matter of some controversy (14,22). In addition to gluconeogenesis, insulin is known to alter

glomerular filtration rate, sodium excretion, phosphate excretion, and ammonium excretion, although the data supporting some of these effects are not always uniform (17,32,33,52).

In the renal proximal tubule, insulin has been described to stimulate fluid volume absorption (4) and ammonia synthesis and excretion (17,32) in addition to its antigluconeogenic effects. One protein poised along the pathways of Na^+ as well as acid-base homeostasis is the proximal tubule apical membrane Na^+/H^+ exchanger isoform 3 (NHE3). In addition to NaCl (49) and NaHCO_3 (48) absorption, apical Na^+/H^+ exchange also mediates the secretion of ammonium into the tubular lumen (43). Insulin has been shown to stimulate ammoniagenic enzymes and proximal tubule Na^+/H^+ exchange in suspended tubules (4) and in cultured proximal tubule-like cells (31).

Na^+/H^+ exchangers are transmembrane proteins ubiquitous in the animal and plant kingdom (46). In mammals, they mediate electroneutral exchange of Na^+ for H^+ . Nine mammalian isoforms have been identified which represent nine paralogs (11). Although more than one NHE isoform may mediate Na^+ transport in the proximal tubule (23), NHE3 is the predominant and best studied isoform responsible for proximal Na^+ absorption based on both antigenic and functional data (3,18,55,56,59). The importance of NHE3 in Na^+ and acid-base homeostasis is exemplified by the hypovolemic hypotension and metabolic acidosis seen in $\text{NHE3}^{-/-}$ mice despite compensation by the distal nephron (51). NHE3 can be regulated through a wide array of mechanisms, ranging from transcription, translation, protein trafficking, protein phosphorylation, and association with regulatory proteins.

The insulin receptor signals through a variety of intracellular cascades; one of the best studied pathways involves the insulin receptor substrate (IRS)-phosphatidylinositol 3-kinase (PI3K)-PI3K-dependent kinase (PKD)-serum- and glucocorticoid-dependent kinase 1 (SGK1) axis (36,47). There is evidence to suggest that the nongenomic effect of glucocorticoids may act via SGK1 (61). How insulin signals NHE3 stimulation is not known. We previously showed that the stimulatory effect of insulin on NHE3 is amplified by glucocorticoids and associated with changes in NHE3 protein and transcript levels with a complex multiphasic profile (31). In the early phase of stimulation, insulin increases NHE3 activity without changes in NHE3 protein and mRNA. At 24 h, enhanced NHE3 activity is accompanied by an increase in NHE3 protein and mRNA abundance (31). It is therefore likely that the acute and chronic stimulation of NHE3 by insulin occurs via the activation of distinct signaling pathways. In this paper, we used the marsupial epithelial cell line [opossum kidney (OK) cells], which possesses numerous characteristics of the mammalian proximal tubule, to define the mechanism of stimulation of NHE3 activity, dependence on glucocorticoids, and insulin signaling pathways.

EXPERIMENTAL PROCEDURES

Cell culture, transfection, and reagents

OK cells (19) cultured at 37°C in 95% air/5% CO_2 atmosphere were passed in high-glucose (4.5 g/l) DMEM supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were rendered quiescent postconfluence by serum removal for 48 h before the experiments. Transient transfections were performed with Lipofectamine Plus (Invitrogen, Carlsbad, CA), and efficiency was assessed by cotransfection with enhanced green fluorescent protein (eGFP) and monitoring of cells with fluorescent microscopy (typically >80% positive). Mammalian expression plasmids and inserts included COOH-terminal c-myc- and hexahistidine (His_6)-tagged opossum NHE3 (NHE3/myc/6H) in pcDNA3.1 (Invitrogen); COOH-terminal human influenza hemagglutinin (HA)-tagged mouse calcineurin homologous protein 1 (CHP1) in pMH (Roche, Indianapolis, IN); untagged wild-type mouse SGK1 and kinase-dead dominant-

negative (K127M) mouse SGK1 in pcDNA3.1; triple NH₂-terminal HA-tagged constitutively active (Q67L) and dominant-negative (T23N) TC10 in pKH3 (16); and untagged wild-type and dominant-negative (K179M) Akt in pcDNA3.1. The SGK1 constructs were a gift from D. Pearce, UCSF (San Francisco, CA). The TC10 constructs were a gift from A. Saltiel (University of Michigan, Ann Arbor, MI). The rat receptor-associated protein (RAP)-glutathione *S*-transferase (GST) fusion construct in the plasmid pGEX-DGRAP was a gift from J. Herz (University of Texas Southwestern Medical Center, Dallas, TX) (26). Rabbit anti-human SGK1 antibody was obtained from Genex Bioscience (Hayward, CA), and anti-human phosphoserine 78 SGK1 and rabbit anti-mouse phosphoserine 473 Akt antibodies were purchased from Cell Signaling (Beverly, MA). Streptavidin beads were obtained from Pierce (Rockford, IL), and protein G-agarose from Calbiochem (San Diego, CA). Unless otherwise specified, all reagents were from Sigma (St. Louis, MO).

Cloning of opossum SGK1

Total OK cell RNA was isolated using guanidium thiocyanate and acid phenol extraction. OK cell cDNA was prepared by oligo-dT-primed reverse transcription using the Thermoscript method (Invitrogen). Opossum (*Didelphis virginiana*) SGK1 ORF was cloned by PCR from OK cell cDNA using degenerate primers (forward 5'-ATGACSGTSAAARCYGAGGCT-3'; reverse 5'-TCAGAGGAARGARTCCRYRGG-3') and verified by sequencing. For RNA blotting, total OK cell RNA was size-fractionated by formaldehyde gel electrophoresis, transferred to nitrocellulose membranes, and probed with random-primed ³²P-uniformly labeled opossum SGK1 cDNA under high-stringency conditions. 18S cDNA was used in sequential probing to control for loading.

Single-tubule microdissection and RT-PCR

Rat proximal straight tubules (PST), proximal convoluted tubules (PCT), and cortical collecting ducts (CCD) were dissected as described previously (5). Total RNA from microdissected tubules was prepared using a Microprep kit from Stratagene (La Jolla, CA), and cDNA was obtained by oligo-dT-primed reverse transcription using the Thermoscript method. The following primers were used for PCR: SGK1 forward 5'-ATGACCGTCAAAACCGAGGCT-3'; reverse 5'-TCAGAGGAAGGAGTCCATAGG-3' and NHE3 forward 5'-ATGACCGTCAAAACCGAGGCT-3'; reverse 5'-TCAGAGGAAGGAGTCCATAGG-3'.

Immunocytochemistry and immunohistochemistry

For immunocytochemistry, OK cells were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized in 0.1% Triton X-100 in PBS for 3 min, and blocked by 5% bovine serum albumin in PBS at 37°C for 1 h. Specimens were incubated with rabbit anti-human SGK1 polyclonal antibody (1:200 dilution), followed by FITC-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine-conjugated phalloidin (1:50 dilution, Invitrogen). Confocal fluorescent images were visualized through a Zeiss ×100 objective lens using a Zeiss LSM-410 laser-scanning confocal microscope. Rhodamine and FITC fluorescence were detected using excitation laser wavelengths (in nm) of 568 and 488 and emission filters of 590 long-pass, 510–560 band-pass, and 670–810 band-pass, respectively.

For immunohistochemistry, anesthetized Sprague-Dawley rats were fixed by retrograde perfusion through the abdominal aorta with a solution of 2% paraformaldehyde in PBS. All animal protocols were approved by the Institutional Animal Care and Use Committee. Kidneys were sliced and further fixed for 1 h in 2% paraformaldehyde followed by 1 h in a cryoprotectant of 10% EDTA in 0.1 M Tris and frozen as previously described (54).

Cryostat sections 8- μ m thick were picked up on coverslips coated with HistoGrip (Zymed, San Francisco, CA). Sections were then treated with 0.5% SDS for 10 min to unmask antigenic sites (12). Sections washed three times with high-salt buffer (50 ml PBS, 0.5 g BSA, 1.13 g NaCl) and incubated in blocking agent (50 ml PBS, 0.5 g BSA, 0.188 g glycine, pH 7.2) for 20 min, followed by incubation with a primary antibody overnight at 4°C. Primary antibodies (anti-SGK1, Upstate Biotechnology) were diluted to 10 μ g/ml with incubation medium (50 ml PBS, 0.05 g BSA, 200 μ l 5% NaN₃). Specificity of labeling was demonstrated by inclusion of an immunogenic peptide (amino acids 399–412 of human SGK1, GKSPDSVLVTASVK) at 5 μ g/ml. After this incubation, sections were rinsed five times with high-salt buffer over the course of 1 h. Appropriate species-specific secondary antibodies coupled to Alexa 488 or 568 dyes (Molecular Probes, Eugene, OR) were diluted 1:200 with incubation medium and then incubated with the tissue sections for 2 h at 4°C, washed as above, and mounted for confocal microscopy. Confocal microscopy was performed as described above.

NHE3 activity assay

NHE3 activity was measured fluorometrically using the intracellularly trapped pH-sensitive dye BCECF as described previously (42). Cells grown on glass coverslips were loaded with 10 μ M BCECF-AM (30 min at 37°C) and intracellular pH (pH_i) was estimated from the ratio of fluorescence (λ excitation: 500 and 450 nm, λ emission: 530 nm) in a computer-controlled spectrofluorometer (QM-8/2003, Photon Technology International, London, Ontario). The 500/450-nm fluorescence ratio was calibrated to pH_i using K⁺/nigericin as described (27). Na⁺/H⁺ exchange activity was assayed as the initial rate of the Na⁺-dependent pH_i increase after an acid load using nigericin in the absence of CO₂/HCO₃⁻ (dpH_i/dt). Comparisons were always made between cells of the same passage studied on the same day. Intracellular buffer capacity (β) was measured by pulsing cells with 20 mM NH₄Cl, according to the formula $\beta = [\text{NH}_4\text{Cl}]/\Delta\text{pH}_i$. β values for control and treated cells were not significantly different (not shown).

Coimmunoprecipitation experiments in OK cells

Confluent quiescent OK cells were treated with either vehicle or agonist. After being washed with PBS, cells were lysed with ice-cold RIPA buffer [in mM: 150 NaCl, 50 Tris-HCl, pH 8.0, 5 EDTA, and 1 EGTA as well as 1% Triton X-100 (vol/vol) and protease inhibitor cocktail from Roche]. The slurry was cleared by centrifugation (109,000 g_{max} at 50,000 rpm; 30 min; 4°C; Beckman TLA 100.3 rotor), and NHE3 was precipitated with primary antiserum (NHE3 5683) at 1:200 dilution and protein G-agarose. The polyclonal NHE3 antibody (5683) has been described previously (58). After being washed with RIPA buffer, the antibody-antigen complex was eluted in SDS buffer [5 mM Tris-HCl, pH 6.8, 10% glycerol (vol/vol), 1% β -mercaptoethanol (wt/vol), 0.1% SDS (wt/vol), 0.01% bromophenol blue (wt/vol)], resolved by SDS-PAGE, and transferred to a polyvinylidene fluoride (PVDF) membrane. Immunoblotting was performed with the appropriate antisera at a 1:1,000 dilution and developed using enhanced chemiluminescence (Amersham, Pittsburgh, PA).

NHE3 phosphorylation and tryptic phosphopeptide mapping

NHE3 phosphorylation and tryptic phosphopeptide mapping were performed in intact cells as described previously (62). After incubation in phosphate-free DMEM, cells were loaded with [³²P]orthophosphate (200–330 μ Ci/ml; 120 min), and insulin, hydrocortisone, or vehicle was added for 120 min. After being washed with ice-cold TBS, cells were lysed with ice-cold phospho-RIPA buffer [in mM: 150 NaCl, 80 NaF, 50 Tris-HCl, pH 8.0, 5 EDTA, 1 EGTA, 25 Na-pyrophosphate, and 1 Na-orthovanadate as well as 1% Nonidet P-40 (vol/

vol), 0.5% deoxycholate (wt/vol), 0.1% SDS (wt/vol), and protease inhibitor cocktail]. The slurry was cleared by centrifugation at 109,000 $g_{\max}$. NHE3 immunoprecipitation and immunoblotting were performed as described above. ^{32}P content was visualized by autoradiography.

For tryptic phosphopeptide mapping, acrylamide membrane pieces containing NHE3 protein were localized by autoradiography, excised, and incubated in 100 mM acetic acid containing 0.5% polyvinylpyrrolidone (wt/vol) at 37°C for 45 min. After being washed with deionized water and 0.05% NH_4HCO_3 solution, membranes were incubated in 15 μg *N*-tyrosyl-L-phenylalanine chloromethyl-ketone-treated trypsin (Worthington, Lakewood, NJ) in 300 μl of 0.05% NH_4HCO_3 solution in a 37°C shaking water bath. Fifteen micrograms of *N*-tyrosyl-L-phenylalanine chloromethyl-ketone-treated trypsin were added after 2 and 12 h. After repeated deionized water washes and lyophilization, the dried samples were resuspended in 20 μl of electrophoresis buffer [per liter: 25 ml of 88% formic acid (wt/vol), 78 ml of glacial acetic acid, 897 ml of deionized water, pH 1.95], spotted on a cellulose TLC plate (Merck, Darmstadt, Germany), and electrophoresis was performed on a Hunter thin-layer electrophoresis apparatus with the above buffer (30 min; 1.0 kV). Separation of the peptides in the second dimension was achieved by ascending chromatography [375:250:75:300 *n*-butanol, pyridine, glacial acetic acid, deionized water (wt/vol)]. The phosphopeptides were visualized by autoradiography.

Determination of surface free and megalin-associated NHE3 fractions

Total cell surface NHE3 and cell surface megalin-associated NHE3 were determined simultaneously on the same culture dishes after treatment with insulin or vehicle for the stated time. Total cell surface NHE3 was determined by a conventional biotinylation assay as described earlier (10). To quantify cell surface megalin-associated NHE3, we used glutathione-*S*-transferase-receptor-associated protein (GST-RAP) pulldown of cell surface megalin. RAP binds megalin with high affinity, and GST-RAP has been previously used to purify and quantify megalin from both cultured cells and kidney tissue (13,20,63). We employed GST-RAP to coprecipitate megalin-associated NHE3. GST-RAP was prepared as described previously (26). Cells were incubated for 1 h with GST-RAP at 4°C to minimize internalization. GST-RAP-megalin complexes were precipitated using glutathionine beads, yielding the megalin-associated surface NHE3 fraction. The remaining supernatant was used for precipitation with streptavidin beads, constituting the non-megalin-associated surface NHE3 fraction. GST-RAP-megalin-NHE3 and streptavidin-NHE3 were eluted in SDS buffer, resolved by SDS-PAGE, and transferred to PVDF membranes as described above. Immunoblotting was performed as described above.

RESULTS

Signaling pathway for the chronic effect of insulin on NHE3

We previously showed that chronic treatment (24 h) with insulin increases NHE3 activity and NHE3 protein (31). A well-established insulin-signaling cascade is the PI3K-SGK1 pathway (36,47). Inhibition of PI3K by wortmannin did not affect baseline NHE3 activity but blocked the stimulation of NHE3 by insulin (Fig. 1A). Transfection of a kinase-dead dominant-negative SGK1 reduced the insulin stimulation of NHE3 activity, while transfection of wild-type SGK1 amplified the insulin stimulation (Fig. 1B). Neither wild-type nor dominant-negative SGK1 affected baseline NHE3 activity. Unfortunately, several attempts for small-interference RNA knockdown of endogenous SGK1 in OK cells were unsuccessful and yielded only minimal (<30%) reduction in SGK1 protein levels (data not shown). Akt is another possible pathway downstream from PI3K. Dominant-negative Akt had no effect on the chronic stimulation of NHE3 by insulin (data not shown).

Expression of SGK1 in renal proximal tubule and proximal tubule-like cell line

We detected SGK1 protein in kidney sections under baseline conditions without exogenous steroid administration (Fig. 2A). SGK1 colocalized with NHE3 in the proximal tubule, and excess immunizing SGK1 peptide blocked the signal (Fig. 2A). OK cells, which possess numerous characteristics of the mammalian proximal tubule, also express endogenous SGK1 (Fig. 2B). In addition, the SGK1 transcript was present in microdissected proximal tubules by RT-PCR (Fig. 2C). Microdissection precision was assured by coamplification of NHE3 in PST and PCT and its absence in the CCD. Opossum SGK1 cDNA shares a high similarity to mouse and human SGK1 (Fig. 3A). SGK1 transcript and protein were detected by RNA blotting (Fig. 3B) and immunoblotting (Fig. 3C), respectively, with a polyclonal antibody recognizing the 13 most COOH-terminal amino acids of human SGK1, which are highly conserved in opossum SGK1 (Fig. 3A). Glucocorticoids enhance the stimulation of NHE3 by insulin (2). This effect may be due to induction of SGK1 by glucocorticoids. Figure 3B shows induction of the SGK1 transcript by glucocorticoids but not by insulin or hypertonicity in OK cells. The glucocorticoid-induced increase in the SGK1 transcript also leads to an increase in SGK1 protein, although less prominently than the SGK1 transcript (Fig. 3C). Insulin alone has no effect on SGK1 transcript or protein abundance (data not shown). Serine 78 in SGK1 has been shown to become phosphorylated in response to growth factors (25). Both acute (2 h) and chronic (24 h) treatment with insulin lead to increased phosphorylation of SGK1 on serine 78 (Fig. 3D).

Effect of insulin on NHE3 phosphorylation

We previously showed that insulin activates NHE3 transport activity within 2 h of application with mechanisms that are distinct from the more chronic effects (31). We explored several potential mechanisms by which this could occur. Changes in NHE3 activity independent of alterations in cell surface NHE3 have been described with several agonists, where changes in NHE3 phosphorylation were postulated to modulate the intrinsic activity of the transporter (60). Insulin has no effect on whole protein phosphorylation of NHE3 (Fig. 4A). Since NHE3 is a multiphosphorylated protein and changes in single phosphorylated sites may not be evident in a whole phosphoprotein assay, we proceeded to examine individual phosphoaminoacid residues with tryptic phosphopeptide mapping. Figure 4B shows that there is no discernible difference in individual NHE3 phosphorylation sites with acute insulin treatment (10^{-6} M, 2 h) or when glucocorticoid in conjunction with insulin was applied (10^{-6} M each, 2 h). The two-dimensional profile of the tryptic phosphopeptide map is identical to that previously described for native NHE3 in OK cells (62). In clear contradistinction to hormones coupled to protein kinase A, insulin and glucocorticoids therefore acutely alter NHE3 activity without changes in surface NHE3 protein (31) or NHE3 phosphorylation.

Signal transduction pathways of acute (2 h) effect of insulin on NHE3

Insulin appears to regulate NHE3 via a biphasic mechanism (31). While the chronic effect of insulin on NHE3 appears to proceed through the PI3K-SGK1 pathway, the signaling cascade for the acute effect is not known. We explored several possibilities. First, the PI3K-SGK1 pathway that mediates the chronic effect of insulin does not appear to be involved as neither PI3K nor SGK1 blockade affects the insulin effect (Fig. 5, A and B). An alternative, but not mutually exclusive, pathway involves the cbl/CAP/TC10 complex that assembles in caveolae (15,16,57). There is some evidence that NHE3 may be present and acutely regulated in these structures (37). The dominant-negative form of the Rho-GTPase TC10 is effective in blocking this pathway (16). We overexpressed dominant-negative and constitutively active TC10 to disrupt or activate this pathway, respectively. However, neither did affect the acute effect of insulin stimulation on NHE3 (Fig. 5C). Akt was

activated in OK cells after 2 h of treatment with insulin, but dominant-negative Akt did not prevent insulin from stimulating NHE3 (Fig. 5D).

Effect of acute insulin stimulation (2 h) on NHE3-associated proteins

Another potential mechanism by which NHE3 can be regulated independently of changes in surface NHE3 protein or phosphorylation is by association with binding proteins.

Biemesderfer and co-workers (7,8) have proposed that association of brush-border NHE3 with megalin can provide a mechanism to regulate the resident pool of brush-border NHE3 transporters, and there are data from pathophysiological animal models compatible with this hypothesis (6). We therefore tested whether insulin can alter the assembly between NHE3 and megalin. Figure 6A shows that insulin does not alter total surface NHE3 or the association of NHE3 with megalin at 2 h.

Type I adenosine receptor agonists can acutely change NHE3 activity without changes in cell surface NHE3 antigen, and this alteration is associated with and dependent on NHE3 binding with CHP (21). We next tested this possible mechanism in the acute insulin model. Figure 6B shows that insulin does not alter the amount of CHP in the NHE3 immune complex nor does it alter NHE3 or CHP phosphorylation.

DISCUSSION

The role of insulin in proximal tubule function is important for Na⁺ and acid-base homeostasis. A pathogenic role has been proposed for chronic insulin-stimulated Na⁺ absorption causing volume expansion and hypertension in type II diabetes mellitus and the metabolic syndrome (50), but this conjecture has been countered by others (24). The direct antinatriuretic action of insulin has been shown in the proximal tubule (4) and thick ascending limb (30). In addition to Na⁺ absorption, insulin also stimulates ammoniagenic enzymes in the proximal tubule. Since NHE3 is the key mediator for ammonium excretion into the proximal tubule lumen (43), the physiological significance of stimulation of NHE3 activity by insulin may be germane to the provision of a urinary buffer for H⁺ excretion. Impairment of the ammoniagenic response to insulin has been proposed to cause uric acid stone formation in subjects with insulin resistance (40). Understanding the signaling pathway between insulin and NHE3 is therefore of great pathophysiological importance.

There are four key findings in the present study. First, the chronic effect of insulin on NHE3 appears to depend on the classic PI3K-SGK1 cascade. Second, the enhancement of the insulin effect by glucocorticoids can be partially explained by increased SGK1 expression. Third, neither insulin nor glucocorticoids alters NHE3 activity by a change in its phosphorylation status. Fourth, the acute stimulation of NHE3 by insulin does not involve any of the known mechanisms of insulin signaling or NHE3 regulation.

While SGK1 transcript was found in the proximal tubule (38), expression of SGK1 protein in the proximal tubule is less clear from the literature. We definitively show expression of SGK1 transcript and protein in the proximal tubule and a proximal tubule-like cell line. For our cell culture studies using OK cells, we cloned and sequenced opossum (*D. virginiana*) SGK1. Pharmacological inhibition of PI3K and dominant-negative inhibition of SGK1 blocked the effect of insulin on NHE3 activity. The small residual effect of insulin on NHE3 activity with transfection-based techniques can be accounted for by the <100% transfection efficiency. Note that overexpression of SGK1 per se did not increase baseline NHE3 activity in the absence of other agonists. This suggests that SGK1 overexpression or activation is necessary but not sufficient to stimulate NHE3 activity. Insulin possibly activates parallel cascades in addition to PI3K-SGK1 activation to effect the stimulation of NHE3.

Of note, basal NHE3 activity was reduced by the PI3K inhibitor wortmannin in the acute (2 h) experiments but was not changed in the chronic (24 h) experiments. The insulin-stimulated NHE3 activity was significantly reduced by wortmannin after 24 h, indicating that wortmannin was still active at that time point. The short-term effect of wortmannin on basal NHE3 activity is in accordance with previous studies in OK cells (1) and in AP-1 cells transfected with NHE3 (34). The lack of effect with the longer incubation time is difficult to speculate on, but this could be due at least in part to an “escape” mechanism of the baseline endosomal recycling machinery.

Glucocorticoids have been shown to exert a permissive effect on the adaptation of NHE3 to metabolic acidosis in both animals (29) and cell culture (2). We have shown that glucocorticoids enhance the insulin effect on NHE3 even in concentrations where glucocorticoids alone have no effect (31). We showed that glucocorticoids increase SGK1 transcript and protein in a proximal tubule-like cell line. The current body of data does not prove that SGK1 activation mediates the enhancing effect of glucocorticoids. However, the fact that overexpression of SGK1 by transfection also enhances the insulin effect is in keeping with this model.

Regulation of NHE3 activity is extremely complex, with an ever-expanding list of mechanisms involving transcription, protein synthesis, trafficking, and yet unidentified mechanisms of altering transporter activity. Changes in NHE3 activity without changes in plasma membrane NHE3 have been described with acute activation of protein kinase A by permeant cAMP analogs or Gs-coupled peptide hormones (41). In all these instances, NHE3 was phosphorylated on multiple serine residues by protein kinase A (35,62). Since insulin signaling involves multiple kinases, we examined for this possibility but did not find any changes in NHE3 phosphorylation in any sites associated with treatment with insulin or glucocorticoids. Acute activation of the adenosine A₁ receptor also changes NHE3 activity independently of NHE3 phosphorylation or changes in NHE3 surface protein (21). This effect was associated with and required interaction of NHE3 with CHP (21). Acute treatment with insulin, however, did not alter NHE3-CHP association in the NHE3 immune complex nor did it change CHP phosphorylation.

The classic IRS-PI3K-PDK-Akt pathway is not unique. Saltiel and others (15,16,57) have described a parallel pathway that involves the cbl/CAP/TC10 complex. We explored the contribution of the TC10 pathway but could not block the insulin effect with overexpression of the dominant-negative TC10. This may indicate that TC10 is not involved in the signaling between insulin and NHE3, or that another member of the Rho-GTPase family that is not affected by dominant-negative TC10 is present in OK cells.

In summary, we characterized the effect of insulin on proximal tubule NHE3 regulation and showed that the chronic effect is via the conventional PI3K-SGK1 pathway. We attempted to study the acute effect of insulin and managed to exclude the known mechanisms and signaling pathways.

Acknowledgments

We are grateful to Alan Saltiel for the TC10, Joachim Herz for the RAP, and David Pearce for the SGK1 constructs.

References

1. Akhter S, Kovbasnjuk O, Li X, Cavet M, Noel J, Arpin M, Hubbard AL, Donowitz M. Na⁺/H⁺ exchanger 3 is in large complexes in the center of the apical surface of proximal tubule-derived OK cells. *Am J Physiol Cell Physiol*. 2002; 283:C927–C940. [PubMed: 12176749]

2. Ambuhl PM, Yang X, Peng Y, Preisig PA, Moe OW, Alpern RJ. Glucocorticoids enhance acid activation of the Na⁺/H⁺ exchanger 3 (NHE3). *J Clin Invest.* 1999; 103:429–435. [PubMed: 9927505]
3. Amemiya M, Loffing J, Lotscher M, Kaissling B, Alpern RJ, Moe OW. Expression of NHE-3 in the apical membrane of rat renal proximal tubule and thick ascending limb. *Kidney Int.* 1995; 48:1206–1215. [PubMed: 8569082]
4. Baum M. Insulin stimulates volume absorption in the rabbit proximal convoluted tubule. *J Clin Invest.* 1987; 79:1104–1109. [PubMed: 3549779]
5. Baum M, Quigley R. Maturation of rat proximal tubule chloride permeability. *Am J Physiol Regul Integr Comp Physiol.* 2005; 289:R1659–R1664. [PubMed: 16051720]
6. Besse-Eschmann V, Klisic J, Nief V, Le Hir M, Kaissling B, Ambuhl PM. Regulation of the proximal tubular sodium/proton exchanger NHE3 in rats with puromycin aminonucleoside (PAN)-induced nephrotic syndrome. *J Am Soc Nephrol.* 2002; 13:2199–2206. [PubMed: 12191963]
7. Biemesderfer D, DeGray B, Aronson PS. Active (9.6 S) and inactive (21 S) oligomers of NHE3 in microdomains of the renal brush border. *J Biol Chem.* 2001; 276:10161–10167. [PubMed: 11120742]
8. Biemesderfer D, Nagy T, DeGray B, Aronson PS. Specific association of megalin and the Na⁺/H⁺ exchanger isoform NHE3 in the proximal tubule. *J Biol Chem.* 1999; 274:17518–17524. [PubMed: 10364184]
9. Blanchard RF, Davis PJ, Blas SD. Physical characteristics of insulin receptors on renal cell membranes. *Diabetes.* 1978; 27:88–95. [PubMed: 624445]
10. Bobulescu IA, Dwarakanath V, Zou L, Zhang J, Baum M, Moe OW. Glucocorticoids acutely increase cell surface Na⁺/H⁺ exchanger-3 (NHE3) by activation of NHE3 exocytosis. *Am J Physiol Renal Physiol.* 2005; 289:F685–F691. [PubMed: 15942046]
11. Brett CL, Donowitz M, Rao R. Evolutionary origins of eukaryotic sodium/proton exchangers. *Am J Physiol Cell Physiol.* 2005; 288:C223–C239. [PubMed: 15643048]
12. Brown D, Lydon J, McLaughlin M, Stuart-Tilley A, Tyszkowski R, Alper S. Antigen retrieval in cryostat tissue sections and cultured cells by treatment with sodium dodecyl sulfate (SDS). *Histochem Cell Biol.* 1996; 105:261–267. [PubMed: 9072183]
13. Bu G, Geuze HJ, Strous GJ, Schwartz AL. 39 kDa receptor-associated protein is an ER resident protein and molecular chaperone for LDL receptor-related protein. *EMBO J.* 1995; 14:2269–2280. [PubMed: 7774585]
14. Cano N. Bench-to-bedside review: glucose production from the kidney. *Crit Care.* 2002; 6:317–321. [PubMed: 12225606]
15. Chiang SH, Baumann CA, Kanzaki M, Thurmond DC, Watson RT, Neudauer CL, Macara IG, Pessin JE, Saltiel AR. Insulin-stimulated GLUT4 translocation requires the CAP-dependent activation of TC10. *Nature.* 2001; 410:944–948. [PubMed: 11309621]
16. Chiang SH, Hou JC, Hwang J, Pessin JE, Saltiel AR. Cloning and functional characterization of related TC10 isoforms, a subfamily of Rho proteins involved in insulin-stimulated glucose transport. *J Biol Chem.* 2002; 277:13067–13073. [PubMed: 11821390]
17. Chobanian MC, Hammerman MR. Insulin stimulates ammoniogenesis in canine renal proximal tubular segments. *Am J Physiol Renal Fluid Electrolyte Physiol.* 1987; 253:F1171–F1177.
18. Choi JY, Shah M, Lee MG, Schultheis PJ, Shull GE, Muallem S, Baum M. Novel amiloride-sensitive sodium-dependent proton secretion in the mouse proximal convoluted tubule. *J Clin Invest.* 2000; 105:1141–1146. [PubMed: 10772659]
19. Cole JA, Forte LR, Krause WJ, Thorne PK. Clonal sublines that are morphologically and functionally distinct from parental OK cells. *Am J Physiol Renal Fluid Electrolyte Physiol.* 1989; 256:F672–F679.
20. Czekay RP, Orlando RA, Woodward L, Lundstrom M, Farquhar MG. Endocytic trafficking of megalin/RAP complexes: dissociation of the complexes in late endosomes. *Mol Biol Cell.* 1997; 8:517–532. [PubMed: 9188102]
21. Di Sole F, Cerull R, Babich V, Quinones H, Gisler SM, Biber J, Murer H, Burckhardt G, Helmle-Kolb C, Moe OW. Acute regulation of Na/H exchanger NHE3 by adenosine A1 receptors is

- mediated by calcineurin homologous protein (CHP). *J Biol Chem*. 2004; 279:2962–2974. [PubMed: 14570899]
22. Gerich JE, Meyer C, Woerle HJ, Stumvoll M. Renal gluconeogenesis: its importance in human glucose homeostasis. *Diabetes Care*. 2001; 24:382–391. [PubMed: 11213896]
 23. Goyal S, Vanden Heuvel G, Aronson PS. Renal expression of novel Na^+/H^+ exchanger isoform NHE8. *Am J Physiol Renal Physiol*. 2003; 284:F467–F473. [PubMed: 12409279]
 24. Hall JE. The kidney, hypertension, and obesity. *Hypertension*. 2003; 41:625–633. [PubMed: 12623970]
 25. Hayashi M, Tapping RI, Chao TH, Lo JF, King CC, Yang Y, Lee JD. BMK1 mediates growth factor-induced cell proliferation through direct cellular activation of serum and glucocorticoid-inducible kinase. *J Biol Chem*. 2001; 276:8631–8634. [PubMed: 11254654]
 26. Herz J, Goldstein JL, Strickland DK, Ho YK, Brown MS. 39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor. *J Biol Chem*. 1991; 266:21232–21238. [PubMed: 1718973]
 27. Howlin KJ, Alpern RJ, Rector FC Jr. Amiloride inhibition of proximal tubular acidification. *Am J Physiol Renal Fluid Electrolyte Physiol*. 1985; 248:F773–F778.
 28. Joseph PK, Subrahmanyam K. Effect of growth hormone, insulin, thyroxine and cortisone on renal gluconeogenesis. *Arch Biochem Biophys*. 1968; 127:288–291. [PubMed: 5697986]
 29. Kinsella J, Cujdik T, Sacktor B. Na^+/H^+ exchange activity in renal brush border membrane vesicles in response to metabolic acidosis: the role of glucocorticoids. *Proc Natl Acad Sci USA*. 1984; 81:630–634. [PubMed: 6320201]
 30. Kirchner KA. Insulin increases loop segment chloride reabsorption in the euglycemic rat. *Am J Physiol Renal Fluid Electrolyte Physiol*. 1988; 255:F1206–F1213.
 31. Klisic J, Hu MC, Nief V, Reyes L, Fuster D, Moe OW, Ambuhl PM. Insulin activates Na^+/H^+ exchanger 3: biphasic response and glucocorticoid dependence. *Am J Physiol Renal Physiol*. 2002; 283:F532–F539. [PubMed: 12167605]
 32. Krivosikova Z, Spustova V, Dzurik R. Participation of P-dependent and P-independent glutaminases in rat kidney ammoniogenesis and their modulation by metabolic acidosis, hippurate and insulin. *Physiol Res*. 1998; 47:177–183. [PubMed: 9803482]
 33. Kubota T, Hagiwara N, Inoue A, Fujimoto M. The effect of ouabain, insulin, and cyclic AMP on the acidification of luminal fluid in the proximal tubule of bullfrog kidney. *Jpn J Physiol*. 1988; 38:549–556. [PubMed: 2853241]
 34. Kurashima K, Szabo EZ, Lukacs G, Orlowski J, Grinstein S. Endosomal recycling of the Na^+/H^+ exchanger NHE3 isoform is regulated by the phosphatidylinositol 3-kinase pathway. *J Biol Chem*. 1998; 273:20828–20836. [PubMed: 9694828]
 35. Kurashima K, Yu FH, Cabado AG, Szabo EZ, Grinstein S, Orlowski J. Identification of sites required for down-regulation of Na^+/H^+ exchanger NHE3 activity by cAMP-dependent protein kinase. phosphorylation-dependent and -independent mechanisms. *J Biol Chem*. 1997; 272:28672–28679. [PubMed: 9353335]
 36. Lang F, Cohen P. Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. *Sci STKE* 2001. 2001:RE17.
 37. Li X, Galli T, Leu S, Wade JB, Weinman EJ, Leung G, Cheong A, Louvard D, Donowitz M. Na^+/H^+ exchanger 3 (NHE3) is present in lipid rafts in the rabbit ileal brush border: a role for rafts in trafficking and rapid stimulation of NHE3. *J Physiol*. 2001; 537:537–552. [PubMed: 11731584]
 38. Loffing J, Zecevic M, Feraille E, Kaissling B, Asher C, Rossier BC, Firestone GL, Pearce D, Verrey F. Aldosterone induces rapid apical translocation of ENaC in early portion of renal collecting system: possible role of SGK. *Am J Physiol Renal Physiol*. 2001; 280:F675–F682. [PubMed: 11249859]
 39. Lupianez JA, Dileepan KN, Wagle SR. Interrelationship of somatostatin, insulin, and calcium in the control of gluconeogenesis in kidney cortex slices. *Biochem Biophys Res Commun*. 1979; 90:1153–1158. [PubMed: 518589]
 40. Maalouf NM, Cameron MA, Moe OW, Sakhaee K. Novel insights into the pathogenesis of uric acid nephrolithiasis. *Curr Opin Nephrol Hypertens*. 2004; 13:181–189. [PubMed: 15202612]

41. Moe OW. Acute regulation of proximal tubule apical membrane Na/H exchanger NHE-3: role of phosphorylation, protein trafficking, and regulatory factors. *J Am Soc Nephrol.* 1999; 10:2412–2425. [PubMed: 10541303]
42. Moe OW, Miller RT, Horie S, Cano A, Preisig PA, Alpern RJ. Differential regulation of Na/H antiporter by acid in renal epithelial cells and fibroblasts. *J Clin Invest.* 1991; 88:1703–1708. [PubMed: 1658050]
43. Nagami GT. Luminal secretion of ammonia in the mouse proximal tubule perfused in vitro. *J Clin Invest.* 1988; 81:159–164. [PubMed: 3121674]
44. Nakamura R, Emmanouel DS, Katz AI. Insulin binding sites in various segments of the rabbit nephron. *J Clin Invest.* 1983; 72:388–392. [PubMed: 6348088]
45. Nakamura R, Hayashi M, Emmanouel DS, Katz AI. Sites of insulin and glucagon metabolism in the rabbit nephron. *Am J Physiol Renal Fluid Electrolyte Physiol.* 1986; 250:F144–F150.
46. Orlowski J, Grinstein S. Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflügers Arch.* 2004; 447:549–565.
47. Pearce D. SGK1 regulation of epithelial sodium transport. *Cell Physiol Biochem.* 2003; 13:13–20. [PubMed: 12649598]
48. Preisig PA, Ives HE, Cragoe EJ Jr, Alpern RJ, Rector FC Jr. Role of the Na⁺/H⁺ antiporter in rat proximal tubule bicarbonate absorption. *J Clin Invest.* 1987; 80:970–978. [PubMed: 2888788]
49. Preisig PA, Rector FC Jr. Role of Na⁺-H⁺ antiport in rat proximal tubule NaCl absorption. *Am J Physiol Renal Fluid Electrolyte Physiol.* 1988; 255:F461–F465.
50. Reaven GM. Insulin resistance/compensatory hyperinsulinemia, essential hypertension, and cardiovascular disease. *J Clin Endocrinol Metab.* 2003; 88:2399–2403. [PubMed: 12788834]
51. Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, Giebisch G, Aronson PS, Lorenz JN, Shull GE. Renal and intestinal absorptive defects in mice lacking the NHE3 Na⁺/H⁺ exchanger. *Nat Genet.* 1998; 19:282–285. [PubMed: 9662405]
52. Takahashi N, Ito O, Abe K. Tubular effects of insulin. *Hypertens Res.* 1996; 19(Suppl 1):S41–S45. [PubMed: 9240763]
53. Talor Z, Emmanouel DS, Katz AI. Insulin binding and degradation by luminal and basolateral tubular membranes from rabbit kidney. *J Clin Invest.* 1982; 69:1136–1146. [PubMed: 7040474]
54. Wade JB, Welling PA, Donowitz M, Shenolikar S, Weinman EJ. Differential renal distribution of NHERF isoforms and their colocalization with NHE3, ezrin, and ROMK. *Am J Physiol Cell Physiol.* 2001; 280:C192–C198. [PubMed: 11121391]
55. Wang T, Yang CL, Abbiati T, Schultheis PJ, Shull GE, Giebisch G, Aronson PS. Mechanism of proximal tubule bicarbonate absorption in NHE3 null mice. *Am J Physiol Renal Physiol.* 1999; 277:F298–F302.
56. Wang T, Yang CL, Abbiati T, Shull GE, Giebisch G, Aronson PS. Essential role of NHE3 in facilitating formate-dependent NaCl absorption in the proximal tubule. *Am J Physiol Renal Physiol.* 2001; 281:F288–F292. [PubMed: 11457720]
57. Watson RT, Shigematsu S, Chiang SH, Mora S, Kanzaki M, Macara IG, Saltiel AR, Pessin JE. Lipid raft microdomain compartmentalization of TC10 is required for insulin signaling and GLUT4 translocation. *J Cell Biol.* 2001; 154:829–840. [PubMed: 11502760]
58. Wiederkehr MR, Di Sole F, Collazo R, Quinones H, Fan L, Murer H, Helmle-Kolb C, Moe OW. Characterization of acute inhibition of Na/H exchanger NHE-3 by dopamine in opossum kidney cells. *Kidney Int.* 2001; 59:197–209. [PubMed: 11135072]
59. Wu MS, Biemesderfer D, Giebisch G, Aronson PS. Role of NHE3 in mediating renal brush border Na⁺-H⁺ exchange. Adaptation to metabolic acidosis. *J Biol Chem.* 1996; 271:32749–32752. [PubMed: 8955109]
60. Yip JW, Ko WH, Viberti G, Haganir RL, Donowitz M, Tse CM. Regulation of the epithelial brush border Na⁺/H⁺ exchanger isoform 3 stably expressed in fibroblasts by fibroblast growth factor and phorbol esters is not through changes in phosphorylation of the exchanger. *J Biol Chem.* 1997; 272:18473–18480. [PubMed: 9218492]
61. Yun CC, Chen Y, Lang F. Glucocorticoid activation of Na⁺/H⁺ exchanger isoform 3 revisited. The roles of SGK1 and NHERF2. *J Biol Chem.* 2002; 277:7676–7683. [PubMed: 11751930]

62. Zhao H, Wiederkehr MR, Fan L, Collazo RL, Crowder LA, Moe OW. Acute inhibition of Na/H exchanger NHE-3 by cAMP. Role of protein kinase A and NHE-3 phosphoserines 552 and 605. *J Biol Chem.* 1999; 274:3978–3987. [PubMed: 9933588]
63. Ziak M, Kerjaschki D, Farquhar MG, Roth J. Identification of megalin as the sole rat kidney sialoglycoprotein containing poly alpha2,8 deaminoneuraminic acid. *J Am Soc Nephrol.* 1999; 10:203–209. [PubMed: 10215318]

Fig. 1.

Signaling pathway for the chronic effect of insulin on Na^+/H^+ exchanger isoform 3 (NHE3). **A:** opossum kidney (OK) cells were pretreated with wortmannin (10^{-7} M) or vehicle and then subjected to 10^{-7} M insulin for 24 h. **B:** OK cells were transfected with either wild-type serum- and glucocorticoid-dependent kinase 1 (SGK1) or kinase-dead K127M SGK1, which functioned as a dominant-negative, and then subjected to 10^{-7} M insulin or vehicle for 24 h. For all of the above experiments, NHE3 activity was measured fluorometrically as Na^+ -dependent cell pH recovery after an acid load. Bars and error bars represent means and SE, respectively, from independent experiments ($n = 15$ for each condition). Statistical significance of differences was assessed by ANOVA (* $P < 0.05$).

Fig. 2.

Expression of SGK1 in the proximal tubule. *A*: rat renal cortex was fixed and stained with anti-SGK1 antibody. Specificity of staining was confirmed by blocking with immunogenic peptide (amino acids 399–412 of human SGK1, GKSPDSVLVTASVK; *right*). *B*: staining of SGK1 (green) and actin (red) in OK cells. *C*: rat proximal tubules were microdissected free of all attached tissues, and SGK1 transcript was detected by RT-PCR in proximal straight tubules (PST), proximal convoluted tubules (PCT), and cortical collecting duct (CCD). NHE3 transcript served as a control for microdissection quality.

Fig. 3.

Regulation of SGK1 in OK cells. **A:** primary sequence of opossum (*Didelphis virginiana*) SGK1. Total OK cellular RNA was reverse-transcribed, PCR-amplified with degenerate primers, and cloned as described in EXPERIMENTAL PROCEDURES. Alignment with mouse and human SGK1 is shown. Putative phosphorylation sites are marked with an asterisk. Representative experiments demonstrating SGK1 transcript and protein by RNA (**B**) and immunoblotting (**C**) in OK cells are shown. **B:** quiescent confluent OK cells were treated with hydrocortisone (HC; 10^{-6} M), insulin (I; 10^{-6} M), or hypertonic medium (mannitol; to 450 mosmol/l) for the stated time and harvested for RNA blotting. **C:** quiescent confluent OK cells were treated with hydrocortisone (10^{-6} M) for the stated duration, and SGK1 protein was detected by immunoblotting. **D:** OK cells were transfected with murine SGK1, treated with the stated agonists, and phosphorylation of SGK1 serine 78 was assayed by immunoblotting with phosphopeptide-specific antisera. Changes in phosphorylation were normalized to SGK1 protein. Each experiment was repeated at least twice.

Fig. 4.

Effect of insulin on NHE3 phosphorylation. *A*: endogenous ATP pool in OK cells was labeled with [^{32}P]orthophosphate pulse, cells were then treated with insulin (10^{-6}M , 2 h) or vehicle, and native NHE3 was immunoprecipitated, resolved on SDS-PAGE, and analyzed with immunoblotting and autoradiography. The phospho-NHE3 signals were compared with total NHE3 antigen. Bars and errors bars represent means and SE, respectively; $n = 9$ for each condition. *B*: for separate visualization of individual phosphorylation sites, ^{32}P -labeled native NHE3 isolated by immunoprecipitation was proteolyzed with trypsin, and the tryptic peptides were resolved in 2 dimensions (electrophoresis and thin-layer chromatography) and imaged with autoradiography. Agonists were insulin (10^{-6}M , 2 h, *top*) or combined hydrocortisone and insulin (each 10^{-6}M , 2 h, *bottom*); $n = 3$ for each condition.

Fig. 5.

Signal transduction pathways of acute (2 h) effect of insulin on NHE3. **A:** OK cells were pretreated with wortmannin (10^{-7} M) or vehicle and then subjected to 10^{-7} M insulin for 2 h. **B:** OK cells were transfected with either wild-type or dominant-negative SGK1 and subjected to 10^{-6} M insulin for 2 h (or vehicle). **C:** OK cells were transfected with dominant-negative (Q67L) or constitutively active (T23N) TC10 and then subjected to 10^{-6} M insulin for 2 h (or vehicle). **D, top:** OK cells were treated for 2 h with 10^{-6} M insulin and assayed for phosphorylation of native Akt. **Bottom:** OK cells were transfected with either wild-type or dominant-negative Akt (K179M) and treated with 10^{-6} M insulin for 2 h. Statistical significance of differences was assessed by ANOVA (* $P < 0.05$); $n = 15$ for each condition.

Fig. 6.

Effect of acute insulin (2 h) stimulation on NHE3-associated proteins. A: OK cells were treated with insulin (10^{-6} M, 2 or 24 h) or vehicle, and total cell surface and cell surface megalin-associated NHE3 were assayed as described in EXPERIMENTAL PROCEDURES.

B: effect of insulin on NHE3 phosphorylation, NHE3-calcineurin homologous protein (CHP) association, and phosphorylation of the NHE3-associated CHP. After cotransfection with NHE3 and CHP, quiescent OK cells were treated with insulin (10^{-6} M, 2 h) after a [^{32}P]orthophosphate pulse, and CHP was coimmunoprecipitated with NHE3. NHE3 and CHP phosphorylation and antigen in the immune complex were then analyzed by autoradiography and immunoblotting, respectively; $n = 6$ for each condition.