ET_β receptor activation leads to activation and phosphorylation of NHE3


ET_β receptor activation leads to activation and phosphorylation of NHE3. Am. J. Physiol. 276 (Cell Physiol. 45): C938–C945, 1999.—In OKP cells expressing ET_β endothelin receptors, activation of Na^+_/H^+ antiporter activity by endothelin-1 (ET-1) was resistant to low concentrations of ethylisopropyl amiloride, indicating regulation of Na^+_/H^+ exchanger isoform 3 (NHE3). ET-1 increased NHE3 phosphorylation in cells expressing ET_β receptors but not in cells expressing ET_A receptors. Receptor specificity was not due to demonstrable differences in receptor-specific activation of tyrosine phosphorylation pathways or inhibition of adenylyl cyclase. Phosphorylation was associated with a decrease in mobility on SDS-PAGE, which was reversed by treating immunoprecipitated NHE3 with alkaline phosphatase. Phosphorylation was first seen at 5 min and was maximal at 15–30 min. Phosphorylation was maximal with 10^-9 M ET-1. Phosphorylation occurred on threonine and serine residues at multiple sites. In summary, ET-1 induces NHE3 phosphorylation in OKP cells on multiple threonine and serine residues. ET_β receptor specificity, time course, and concentration dependence are all similar between ET-1-induced increases in NHE3 activity and phosphorylation, suggesting that phosphorylation plays a key role in activation.

THE ENDOTHELINS ARE A FAMILY OF THREE 21-AMINO ACID PEPTIDES (ET-1, ET-2, AND ET-3) THAT SERVE AS PARACRINE/AUTOCRINE FACTORS BY INTERACTING WITH TWO RECEPTORS (ET_A AND ET_β). ET-1 HAS BEEN SHOWN TO INCREASE THE ACTIVITY OF THE PROXIMAL TUBULAR APICAL MEMBRANE Na^+_/H^+ antiporter (13, 15), WHICH MEDIATES THE MAJORITY OF PROXIMAL TUBULAR NaCl AND NaHCO3 ABSORPTION. FIVE PLASMA MEMBRANE Na^+_/H^+ EXCHANGER (NHE) ISOFORMS HAVE BEEN DONED (NHE1–NHE5). THE PROXIMAL TUBULAR APICAL MEMBRANE Na^+_/H^+ antiporter IS ENCODED PREDOMINANTLY BY NHE3, BASED ON LOCALIZATION OF NHE3 PROTEIN EXPRESSION (3, 8), LOCALIZATION OF NHE3 mRNA EXPRESSION (26, 31), INHIBITOR KINETICS (25, 32, 37), REGULATION BY GLUCOCORTICOIDS AND ACIDOSIS (1, 6, 37, 40), AND INHIBITION OF 61% OF PROXIMAL TUBULE HCO3 ABSORPTION IN NHE3 KNOCKOUT MICE (29).

OKP cells express NHE3, and in these cells NHE3 is stimulated by glucocorticoids and acidosis, similar to its regulation in vivo (4, 5). In OKP cells expressing the ET_β but not the ET_A receptor, ET-1 increases Na^+_/H^+ antiporter activity (12). This agrees with binding studies that suggest that the ET_β receptor is the predominant endothelin receptor of the renal proximal tubule (30). The present studies examine whether ET-1-induced Na^+_/H^+ antiporter activation is associated with NHE3 activation and phosphorylation. The results demonstrate that ET-1 activates NHE3. Binding of ET-1 to ET_β receptors, but not to ET_A receptors, causes a two- to threefold increase in NHE3 phosphorylation with a time and dose dependence that parallels that of the change in activity. Phosphorylation occurs at multiple sites involving serine and threonine residues. The nature of the receptor specificity is not due to receptor-specific activation of tyrosine phosphorylation pathways or inhibition of adenylyl cyclase.

METHODS

Materials. All chemicals were obtained from Sigma Chemical (St. Louis, MO) unless otherwise noted as follows. Penicillin and streptomycin were from Whittaker MA Bioproducts (Walkersville, MD); culture media and G418 were from Gibco BRL (Grand Island, NY); Triton X-100 and protein G-agarose were from Calbiochem (La Jolla, CA); horseradish peroxidase (HRP)-labeled anti-mouse IgG, enhanced chemiluminescence (ECL) kit, and cAMP RIA kit were from Amer sham (Arlington Heights, IL); [35S]methionine-cystine was from DuPont NEN (Boston, MA); anti-phosphotyrosine monoclonal antibody PY20 was from Santa Cruz Biotechnology (Santa Cruz, CA); 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-AM and ethylisopropyl amiloride (EIPA) were from Molecular Probes (Eugene, OR); and ET-1 was from Peptides International (Louisville, KY).

Cell culture. Studies were performed in clonal cell lines generated by stable transfection of OKP cells with either pMEhETA or pMEhETB plasmids, which contain the cDNAs for the ET_A and ET_β receptors, respectively, driven by an Sralpha promoter (OKPETA and OKPETB cells, respectively) (12). Cells were passaged in high-glucose (450 mg/dl) DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml), and 200 µg/ml G418. For experimentation, G418 was removed at the time of splitting, 5–7 days before cells were studied. When confluent, cells were rendered quiescent for 48 h before study by the removal of serum and placed in low-glucose (100 mg/dl) DMEM.

To measure levels of cAMP production, cells were treated with 2 mM IBMX during treatment with ET-1 or vehicle. After protein precipitation with addition of 100 µl TCA/well, the supernatant was extracted with water-saturated ether and used to measure cAMP by RIA, as described (12).

Na^+_/H^+ antiporter activity. Cell pH was measured in a temperature-controlled spectrofluorometer (SLM 8000C) as the ratio of BCECF fluorescence with excitation at 500 nm to that at 450 nm (530-nm emission wavelength), as previously described (17). To measure Na^+_/H^+ antiporter activity, cells
were acidified by addition of nigericin in Na⁺-free media. Na⁺/H⁺ antiporter activity was then measured as the initial rate of cell alkalinization in response to Na⁺ addition, as previously described (10).

Western blotting. Cultured cells were washed in PBS three times, scraped in RIPA buffer [in mM: 150 NaCl, 50 Tris·HCl (pH 7.4), 2.5 EDTA, 5 EGTA, 50 β-glycerophosphate, 5 NaF, 1 sodium orthovanadate, 1 phenylmethylsulfonyl fluoride (PMSF), 0.5 dithiothreitol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 μg/ml pepstatin, 5 μg/ml leupeptin, and 5 μg/ml aprotinin], incubated at 4°C for 45 min, and centrifuged at 10,000 g for 15 min. Supernatants were diluted with RIPA buffer to 3 mg protein/ml (Bradford method; Bio-Rad), then mixed with an equal volume of 2× SDS loading buffer [5 mM Tris·HCl (pH 6.8), 1% SDS, 10% glycerol, and 1% -mercaptoethanol], boiled 5 min, size fractionated by SDS-PAGE on 7.5% gels, and electrophoretically transferred to nitrocellulose. After being blocked with 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, blots were probed with a polyclonal anti-opossum NHE3 antibody [anti- serum 5683, generated against a maltose binding protein-NHE3 (amino acids 484–639) fusion protein] at a dilution of 1:200 (2). Blots were washed in 0.05% Tween 20 in PBS once for 15 min and twice for 5 min, incubated with a 1:5,000 dilution of HRP-labeled donkey anti-rabbit IgG in 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, washed as above, and then visualized by ECL. This procedure labeled a 90-kDa band that was not seen when antibody was preincubated with fusion protein or when preimmune serum replaced the anti-NHE3 antisum (2). To measure protein tyrosine phosphorylation, cell extracts were treated as above but blotted with 1 μg/ml mouse monoclonal anti-phosphotyrosine antibody PY20 and HRP-labeled sheep anti-mouse IgG.

Immunoprecipitation. Cells were extracted in RIPA buffer as above; 700 μl of cell extract were mixed with 15 μl of polyclonal anti-NHE3 antiserum, rocked overnight at 4°C, mixed with 25 μl protein G-agarose, rocked for 2 h, pelleted at 10,000 g for 30 s, and washed three times with RIPA buffer. The pellet was suspended in 60 μl of 1× SDS loading buffer, boiled for 5 min, and subjected to SDS-PAGE.

To assess the effect of dephosphorylation on NHE3 mobility, immunoprecipitated NHE3 was washed in 1 ml of reaction buffer [50 mM Tris (pH 8.5), 2 mM PMSF, 0.1% -mercaptoethanol, and 8 mM MgCl₂], and suspended in 30 μl of reaction buffer with 30 units of alkaline phosphatase (type VII-T from bovine intestine) at 37°C for 1 h. (Native brush-border alkaline phosphatase does not interfere with this reaction, as it is inactivated during extraction in RIPA buffer.) The reaction was quenched by diluting the sample 1:2 in 2× SDS buffer, and the sample was subjected to SDS-PAGE and Western blotting with NHE3 antisera.

To measure protein tyrosine phosphorylation, cells were washed in methionine- and cystine-free medium for 1 h and metabolically labeled by incubation with 275 μCi [35S]methionine-cystine in the same medium for 2 h before study. After treatment with ET-1 or vehicle (0.1% acetic acid), extracts from metabolically labeled cells were prepared as described above, and proteins were immunoprecipitated with 6 μg/ml mouse monoclonal anti-phosphotyrosine antibody PY20. Tyrosine phosphorylation was then determined by autoradiography.

Phosphorylation. Cells were washed in TBS [137 mM NaCl, 2.7 mM KCl, and 25 mM Tris (pH 7.4)] three times, incubated in phosphate-free DMEM for 60 min, washed in TBS twice, and incubated with [32P]orthophosphate (200 mCi/ml) in phosphate-free DMEM for 2.5 h. Cells were then treated with 10⁻⁸ M ET-1 or vehicle for 35 min and washed with TBS three times, and NHE3 was immunoprecipitated as above. The immunoprecipitate was subjected to SDS-PAGE, ³²P incorporation was measured by autoradiography, and results were normalized for NHE3 abundance as measured by Western blot. All phosphorylation experiments were performed in triplicate.

Phosphoamino acid analysis. After autoradiography of polyacrylamide gels, the NHE3 band was extracted in 50 mM NH₄HCO₃ with 0.1% (wt/vol) SDS and 50 μl/ml β-mercaptoethanol as described (9). TCA precipitated, washed with 90% ethanol and then acetone, and air dried. The phosphoprotein was then hydrolyzed in 200 μl of 6 N HCl for 2 h, lyophilized, suspended in 10 μl of loading buffer [15 parts pH 1.9 electrophoresis buffer (50 mM 88% formic acid and 156 ml glacial acetic acid in 2 liters deionized water) and 1 part 1 mg/ml phosphoserine, phosphothreonine, and phosphotyrosine standards] and centrifuged at 10,000 rpm for 1 min, and 8 μl of supernatant were spotted onto the TLC plate. The sample was then electrophoresed in the first dimension in pH 1.9 buffer (1.5 kV; Hunter thin-layer electrophoresis system) and in the second dimension in pH 3.5 buffer (100 ml glacial acetic acid and 10 ml pyridine in 2 liters of water; 1.3 kV), and labeled phosphoamino acids were identified by phosphoimaging and alignment with ninhydrin standards.

Phosphopeptide analysis. Phosphopeptide analysis was performed by two-dimensional gel electrophoresis-chromatography following trypsin digestion, as described (9). After ³²P incorporation, immunoprecipitation, SDS-PAGE, and transfer of the sample to nitrocellulose, the filter was washed in 0.5% polyvinylpyrrolidone-360 in glacial acetic acid at 37°C for 30 min, then in 2 ml of water four times, and then in 2 ml of 50 mM NH₄HCO₃ twice. The sample was then incubated four times for 2 h each time in 200 μl of 50 mM NH₄HCO₃ with 15 μl of trypsin (1 mg/ml 0.1 M HCl) at 37°C; 300 μl of water were added, the membrane was removed, and the phosphopeptide was subjected to cycles of 500-μl water washes and lyophilization until no salt residues were visible. The sample was then washed in 200 μl of pH 1.9 electrophoresis buffer, lyophilized, resuspended in 20 μl of the same buffer, and spotted onto a TLC plate. The sample was electrophoresed in the first dimension in pH 1.9 buffer for 30 min and then subjected to chromatography in the second dimension for 12 h in 375 ml n-butanol, 250 ml pyridine, 75 ml glacial acetic acid, and 300 ml water, and peptides were localized by phosphoimaging.

RESULTS

Binding of ET-1 to the ET₉ receptor activates NHE3. We previously showed that 10⁻⁸ M ET-1 causes a 25–40% increase in Na⁺/H⁺ antiporter activity in donal OKP cells stably transfected with a cDNA encoding the ET₉ receptor (OKPETB cells) (12). Although resting OKP cells express an EIPA-resistant Na⁺/H⁺ antiporter encoded by NHE3, it is possible that endothelin activates a different NHE isoform that is not detectable at baseline. To address this, we examined the EIPA sensitivity of Na⁺/H⁺ antiporter activity in the absence and presence of 10⁻⁸ M ET-1 in OKPETB6 cells (OKPETB cells, clone 6). Studies were performed in the presence of 15 mM Na⁺ and in the absence or presence of 10⁻⁷ M EIPA. With 15 mM Na⁺, this concentration of EIPA should inhibit the amiloride-sensitive antiporter isoforms, NHE1 and NHE2, but should not inhibit the amiloride-resistant NHE3. As shown in Fig. 1, 10⁻⁷ M EIPA did not inhibit Na⁺/H⁺ antiporter activity in the absence or presence of ET-1. In addition, 10⁻⁷ M EIPA
did not inhibit the ET-1-induced increase in Na\(^+/\)H\(^+\) antiporter activity. In the absence of EIPA ET-1 increased Na\(^+/\)H\(^+\) antiporter activity by 39%, whereas in the presence of EIPA ET-1 increased Na\(^+/\)H\(^+\) antiporter activity by 43%. Thus ET-1 increases the activity of NHE3 in OKP cells.

Binding of ET-1 to the ET\(_B\) receptor causes NHE3 phosphorylation. To examine whether phosphorylation of NHE3 plays a role in the ET-1-induced increase in NHE3 activity, clonal OKP cell lines expressing the ET\(_B\) receptor were preincubated in \(^{32}\)PO\(_4\) and treated with vehicle or ET-1, and NHE3 was immunoprecipitated. Phosphorylation was measured by autoradiography and normalized for NHE3 abundance as measured by Western blot (Fig. 2). Addition of \(10^{-8}\) M ET-1 to OKPET\(_B\)6 cells for 35 min caused a 2.3 ± 0.6-fold increase in NHE3 phosphorylation (P < 0.03), which was associated with a decrease in mobility on SDS-PAGE (Fig. 2A). Similar results were obtained in OKPET\(_B\)5 cells (Fig. 2B), in which ET-1 caused a 2.1 ± 0.4-fold increase in NHE3 phosphorylation (P < 0.03).

Binding of ET-1 to the ET\(_A\) receptor does not lead to NHE3 phosphorylation. In contrast to cells expressing the ET\(_B\) receptor, ET-1 did not regulate Na\(^+/\)H\(^+\) antiporter activity in clonal OKP cells transfected with a cDNA encoding the ET\(_A\) receptor (OKPET\(_A\)6 and OKPET\(_A\)9 cells) (12). To examine whether this correlated with NHE3 phosphorylation, these clones were studied. ET-1 (\(10^{-8}\) M) had no effect on phosphorylation as assessed by \(^{32}\)P content or mobility shift in OKPET\(_A\)9 cells (Fig. 3A) or OKPET\(_A\)6 cells (Fig. 3B). In OKPET\(_A\)9 cells ET-1 induced a 2 ± 1% increase, and in OKPET\(_A\)6 cells ET-1 induced an 18 ± 28% decrease, in NHE3 phosphorylation (not significant). Thus binding of ET-1 to the ET\(_B\) receptor leads to activation and phosphorylation of NHE3, whereas binding of ET-1 to the ET\(_A\) receptor does not result in either of these effects.

**Signaling pathways responsible for receptor specificity.** The mechanism responsible for the receptor specificity of NHE3 activation and phosphorylation is not clear. We previously showed that addition of ET-1 to OKP cells expressing ET\(_B\) receptors caused an increase in intracellular Ca\(^{2+}\) concentration, increased protein tyrosine phosphorylation, and decreased cAMP generation (11, 12). One possible explanation for receptor specificity is that only ET\(_B\) receptors activate these signaling pathways. We previously showed that addition of ET-1 to ET\(_A\)-expressing cells caused similar increases in cell Ca\(^{2+}\) concentration (12).

To examine whether failure of the ET\(_A\) receptor to induce tyrosine phosphorylation was responsible for receptor specificity, we performed Western blots with anti-phosphotyrosine antibodies in OKPET\(_A\)9 cells. As shown in Fig. 4, \(10^{-8}\) M ET-1 induced tyrosine phosphorylation of proteins of 210, 130, 125, 110, and 68 kDa. This pattern is similar to that seen with addition of
ET-1 to OKPET B6 cells (11) and is typical of a focal adhesion kinase pattern.

To further examine this, we metabolically labeled cells with [35S]methionine and measured tyrosine phosphorylation by immunoprecipitation with anti-phosphotyrosine antibodies. As shown in Fig. 5, similar patterns of phosphorylation were seen in OKPETB6 (Fig. 5A) and OKPETA9 (Fig. 5B) cells.

We previously showed that ET-1 inhibited cAMP production in OKPETB6 cells (12). In the present studies, we examined whether ET-1 had a similar effect in cells expressing ET₆ receptors. As shown in Fig. 6, 10⁻⁸ M ET-1 caused similar inhibition of cAMP production in OKPETB6 and OKPETA9 cells.

Time course and concentration dependence. All remaining studies were performed in OKPETB6 cells. To examine whether the apparent decrease in mobility induced by ET-1 was due to phosphorylation, immunoprecipitates were treated with alkaline phosphatase. As shown in Fig. 7, treatment with alkaline phosphatase significantly decreased the change in mobility on SDS-PAGE, confirming that it was due to phosphate incorporation. The upper band in Fig. 7 is a nonspecific band that is occasionally seen on anti-NHE3 Western blots with our antibody.

Using the change in mobility as an index of phosphorylation, we examined the time course and dose dependence of phosphorylation. ET-1 (10⁻⁸ M) induced a mobility shift that was first seen at 5 min and was
maximal at 15–30 min (Fig. 8). This is similar to the
time course of ET-1 on NHE3 activity in which we
previously found a small effect at 5 min that became
maximal at 12 min (12).

As shown in Fig. 9, the ET-1-induced mobility shift at
35 min was not seen with 10^{-2} M ET-1 and was
maximal between 10^{-9} and 10^{-7} M ET-1. Again, this is
similar to the concentration dependence observed by
measuring the effect of ET-1 on NHE3 activity in these
cells, in which no effect was seen with 10^{-10} M ET-1 and
maximal stimulation was seen with 10^{-9} M and 10^{-8} M
ET-1 (12).

1,2-Bis(2-aminophenoxy)ethane-N,N',N' ',N'-tetraacetic acid (BAPTA), which inhibits cell Ca^{2+} increases,
and herbimycin A, a tyrosine kinase inhibitor, inhibit
ET-1-induced NHE3 activation (11). When added to-
together, BAPTA and herbimycin A inhibited ET-1-
induced ^32P incorporation into NHE3 (control, 2.7-fold
increase; inhibitors, 20% decrease).

Phosphoamino acid analysis and phosphopeptide
mapping. ET-1 induced a 3.2-fold increase in phospho-
threonine content and a 2.5-fold increase in phosphoser-
ine content, with only a 1.4-fold increase in phosphoty-
rosine content (Fig. 10). Phosphopeptide mapping
identified increased phosphorylation in three to five
peptides (Fig. 11). ^32P incorporation into peptides A, I,
and J [as designated by Wiederkehr et al. (36)] was seen
only in ET-1 treated cells. ^32P incorporation into pep-
tides C and D was seen in control cells but appeared to
increase in ET-1-treated cells (Fig. 11). Thus ET-1
induces phosphorylation of NHE3 at multiple sites on
threonine and serine residues.

DISCUSSION

At low concentrations, ET-1 stimulates volume and
presumably Na^+ absorption in the proximal tubule
(14). This effect is likely related to the observation that
ET-1 activates the proximal tubule apical membrane
Na^+/H^+ antiporter, encoded by NHE3 (13, 15). In OKP
cells expressing ETB receptors ET-1 increases Na^+/H^+
antiporter activity, whereas in cells expressing ETA
receptors there is no effect on antiporter activity (12).
The present studies demonstrate that ET-1 binding to the ET \(_B\) receptor leads to activation of NHE3 and phosphorylation of NHE3 involving multiple serine and threonine residues. Phosphorylation is associated with a decrease in NHE3 mobility on SDS-PAGE. Although the present studies do not prove that phosphorylation plays a role in the increase in NHE3 activity, the many similarities between NHE3 phosphorylation and activation suggest a relationship. Both ET-1-induced increases in activity and phosphorylation occur in cells expressing ET\(_B\) receptors but not in cells expressing ET\(_A\) receptors. In addition, activation and phosphorylation have a similar ET-1 concentration dependence and time course. Last, BAPTA and herbimycin A inhibit activation and phosphorylation of NHE3.

The specificity for the ET\(_B\) receptor agrees with the observation that proximal tubules express ET\(_B\) receptors (30). Nevertheless, the mechanism responsible for this specificity is unclear. We previously showed that 10^{-8} M ET-1 caused an increase in cell Ca\(^{2+}\), an increase in protein tyrosine phosphorylation, and inhibition of cAMP production in OKP cells expressing ET\(_B\) receptors (11, 12). Previous studies showed that the increase in cell Ca\(^{2+}\) also occurred in ET\(_A\)-expressing cells (12). The present studies demonstrate that ET-1 causes similar patterns of protein tyrosine phosphorylation and adenylyl cyclase inhibition in ET\(_A\)- and ET\(_B\)-expressing cells.

Thus the mechanism for the specificity of the ET\(_B\) receptor in causing phosphorylation and activation of NHE3 is not immediately clear. A number of possibilities exist. First, it is possible that the ET\(_B\) and ET\(_A\) receptors cause spatially distinct cell Ca\(^{2+}\) increases. Second, it is possible that a key tyrosine kinase substrate that is only phosphorylated in response to ET\(_B\) receptor activation and is responsible for NHE3 activation does not appear in Figs. 4 and 5. Last, it is possible that receptor specificity lies in an additional signaling pathway that is unique to the ET\(_B\) receptor. One possibility is that the ET\(_B\) receptor, but not the ET\(_A\) receptor, binds an NHE3 regulatory protein. This would be analogous to regulation of NHE3 by the \(\beta_2\)-adrenergic receptor. Binding of agonist causes the receptor to bind and sequester NHE3 regulatory factor (NHE-RF), leading to activation of NHE3 (16). Activation of NHE3 by ET-1 could require activation of calmodulin kinase and a tyrosine kinase and binding of NHE-RF or a related protein to the ET\(_B\) receptor. This would explain the requirements for these signaling pathways and for the ET\(_B\) receptor.

Regulation of Na\(^+\)/H\(^+\) antiporter activity by phosphorylation was first demonstrated for NHE1 activation by growth factors (27). Activation of distinct signaling pathways resulted in similar patterns of NHE1 phosphorylation at five sites in the cytoplasmic domain (28). In PS120 cells, regulation of NHE3 by growth factors and phorbol esters was not accompanied by changes in phosphorylation, whereas serum did lead to phosphorylation at two sites (39). In contrast, in AP-1 cells, phorbol esters and cAMP led to phosphorylation of NHE3 (20, 24, 36).

On the basis of results with inhibitors, regulation of NHE3 by endothelin is mediated by Ca\(^{2+}\)/calmodulin kinase and tyrosine kinase pathways rather than protein kinase A- or protein kinase C-related pathways.
Porter activity in wild-type mice but has no effect in threonine kinases that phosphorylate NHE3. Ca2+/calmodulin involves binding of the complex to amino acids 636–656 of the cytoplasmic domain of NHE1 (7, 33). It is not clear whether a similar interaction plays a role in regulation of NHE3 (23, 34). In addition, our studies suggest that, although tyrosine kinase pathways play a significant role, NHE3 phosphorylation on tyrosine is not significant. Thus tyrosine kinases most likely activate upstream pathways that converge on one or more serine/threonine kinases that phosphorylate NHE3. Ca2+/calmodulin kinase may also activate upstream pathways or may interact directly with NHE3. In the present studies, we found that BAPTA and herbimycin A together inhibited ET-1-induced NHE3 phosphorylation.

Phosphorylation could increase activity by activating NHE3 resident in the apical membrane or could regulate trafficking of NHE3 into and out of the apical membrane. Vasopressin-induced insertion of aquaporin-2 into the apical membrane is accompanied by phosphorylation of aquaporin-2 (21, 22). Mutation of the phosphorylated serine (Ser-256) leads to inhibition of trafficking (19). In preliminary studies, we have found that ET-1 leads to trafficking of NHE3 into and out of the apical membrane in OKPT6 cells (unpublished observation).

By regulating NHE3, the endothelins could play important roles in acid-base regulation. Acidosis leads to increased expression of c-Fos and c-Jun transcription factors that are key regulators of ET-1 expression (18, 38). Renal interstitial levels of ET-1 are increased in acidosis, and blockade of ETB receptors impairs regulation of distal tubule function in acidosis (35). In preliminary studies, we have found that acid feeding increases renal cortical apical membrane Na+/H+ antiporter activity in wild-type mice but has no effect in ETB receptor-deficient mice (unpublished observation). Thus acidosis-induced increases in ET-1 expression could lead to phosphorylation and activation of NHE3.

We thank Martha Ferguson for technical assistance. These studies were supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-39298 and DK-48482, the Department of Veterans Affairs, and a grant from the Texas Coordinating Board. M. Yanagisawa is an investigator of the Howard Hughes Medical Institute.

Address for reprint requests and other correspondence: R. J. Alpern, Office of the Dean, Southwestern Medical School, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235-9003 (E-mail: robert.alpern@email.swmed.edu).

Received 20 October 1998; accepted in final form 8 January 1999.

REFERENCES


