Incubation of OKP cells in low-K⁺ media increases NHE3 activity after early decrease in intracellular pH

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Incubation of OKP cells in low-K⁺ media increases NHE3 activity after early decrease in intracellular pH. Am. J. Physiol. Renal Physiol. 276: C711–C716, 1999.—Chronic hypokalemia increases the activity of proximal tubule apical membrane Na⁺/H⁺ antiporter NHE3. The present study examined the effect of the incubation of OKP cells (an opossum kidney, clone P cell line) in control medium (K+ concn (K⁺) = 5.4 mM) or low-K⁺ medium (K⁺ = 2.7 mM) on NHE3. The activity of an ethylisopropyl amiloride-resistant Na⁺/H⁺ antiporter, whose characteristics were consistent with those of NHE3, was increased in low-K⁺ cells beginning at 8 h. NHE3 mRNA and NHE3 protein abundance were increased 2.2-fold and 62%, respectively, at 24 h but not at 8 h. After incubation in low-K⁺ medium, intracellular pH (pHᵢ) decreased by 0.27 pH units (maximum at 27 min) and then recovered to the control level. Intracellular acidosis induced by 5 mM sodium propionate increased Na⁺/H⁺ antiporter activity at 8 and 24 h. Herbimycin A, a tyrosine kinase inhibitor, blocked low-K⁺-and sodium propionate-induced activation of the Na⁺/H⁺ antiporter at 8 and 24 h. Our results demonstrate that low-K⁺ medium causes an early decrease in pHᵢ, which leads to an increase in NHE3 activity via a tyrosine kinase pathway.

sodium/hydrogen antiporter; tyrosine kinase; low potassium; proximal tubule; opossum kidney; clone P cells

CHRONIC POTASSIUM DEPLETION stimulates HCO₃⁻ absorption in the renal proximal tubule (3). This is mediated by stimulation of the apical membrane Na⁺/H⁺ antiporter and basolateral Na⁺/HCO₃⁻ cotransporter (23). Because K⁺ depletion leads to cell acidification (1), it seems likely that cell acidification could mediate the effect of K⁺ depletion to increase the activities of these transporters. However, direct evidence in support of this thesis is lacking. Among the five isoforms of the Na⁺/H⁺ antiporter family (NHE1 to NHE5), NHE3 appears to mediate most of proximal tubule transcorticular H⁺ secretion, on the basis of inhibitor sensitivity (17, 27), mRNA localization (21, 26), and regulation by glucocorticoids (10, 30). In addition, NHE3 is localized to the apical membrane of the proximal tubule (7, 11).

OKP cells, an opossum kidney, clone P cell line, express NHE3 (8). The present study examined the effect of incubating OKP cells in low-K⁺ media on NHE3 activity. The results demonstrate that incubation in low-K⁺ media causes a decrease in intracellular pH (pHᵢ) at early time points, which leads to an increase in NHE3 activity. The increase in NHE3 activity was inhibited by herbimycin A, a tyrosine kinase inhibitor.

METHODS

Cell culture. OKP cells, a cell line originally described by Cole et al. (16), were passaged in high-glucose DMEM with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. For experimentation, OKP cells were grown to confluence and rendered quiescent by serum removal for 48 h before the study. In all experiments, control and experimental cells were from the same passage and were assayed on the same day.

Low-K⁺ medium was prepared by mixing 1:1 high-glucose DMEM and a K⁺-free solution (in mM: 110 NaCl, 1.8 CaCl₂, 0.8 MgSO₄, 0.9 Na₂HPO₄). Normal K⁺ medium (control medium) was made by mixing 1:1 high-glucose DMEM and a K⁺-containing solution [in mM: 110 NaCl, 5.4 KCl (same as DMEM), 1.8 CaCl₂, 0.8 MgSO₄, 0.9 Na₂HPO₄]. Both media were titrated to pH 7.4 by NaHCO₃ addition and then filtered.

Measurement of pHᵢ and Na⁺/H⁺ antiporter activity. Continuous measurement of pHᵢ was accomplished by using intracellularly trapped, pH-sensitive dye 2,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxyethyl ester (BCECF-AM), as previously described (8, 18). Cells were loaded with 10 µM BCECF-AM for 30 min at 37°C, and Na⁺/H⁺ antiporter activity was assayed as the initial rate of Na⁺/H⁺-dependent (with Na⁺-containing solution) pHᵢ increase after an acid load (K⁺-nigericin in Na⁺-free solution) in the absence of CO₂/HCO₃⁻. For this assay, the Na⁺-containing solution was composed of (in mM) 130 Na⁺, 5.0 K⁺, 1.1 Ca²⁺, 1.5 Mg²⁺, 140.2 Cl⁻, and 30 HEPES. In Na⁺-free solution, Na⁺ was replaced with choline. To calculate buffer capacity, cells were pulsed with 10 mM NH₄Cl in the Na⁺-free solution at the trough pHᵢ (after washing out the albumin) and the pHᵢ decrease caused by the removal of NH₄Cl, ΔpHᵢ, was used to calculate the cell buffer capacity as previously described (18). There was no difference in buffer capacity between incubation in control vs. low-K⁺ medium at 24 h. Therefore, Na⁺/H⁺ antiporter activity is expressed as dpHᵢ/dt (pH units/min).

Protein blotting. OKP cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 100 µg/ml phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 2 µg/ml pepstatin A), and samples were diluted 1:5 in 5× SDS buffer (1× SDS buffer is 1% SDS, 20% glycerol, 2% 2-mercaptoethanol, 10 mM Tris, pH 6.8). Protein (20 µg) was size fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were sequentially incubated with anti-OKP NHE3 antibody [polyclonal anti-OKP NHE3 antiserum 5683, generated against a maltose binding protein-OKP NHE3 (amino acids 484–839) fusion protein; Ref. 4] and then with a peroxidase-linked sheep anti-rabbit secondary antibody. Labeling was detected by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantitated by densitometry.

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RNA blotting. Total RNA was extracted from OKP cells with ISOGEN (Nippon Gene, Toyama, Japan) and ethanol precipitated. RNA (20 µg) was size fractionated by agarose formaldehyde gel electrophoresis and transferred to nylon membranes. Radiolabeled probes were synthesized from 30 ng of appropriate cDNA by the random-hexamer method. Hybridization signals were normalized with glyceraldehyde-3-phosphate dehydrogenase. For hybridization, filters were prehybridized in QuickHyb hybridization solution (Stratagene, La Jolla, CA) for 1 h at 68°C, hybridized in the same solution containing 0.1 mg/ml salmon sperm DNA and radiolabeled probe at 68°C for 3 h, and washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS at room temperature for 15 min each and in 0.1× SSC with 0.1% SDS at 50°C for 15 min. Filters were exposed to film for the appropriate time at −70°C, and labeling was quantitated by densitometry.

Reagents. All chemicals were obtained from Sigma (St. Louis, MO) except as follows. Culture media were from Gibco-BRL (Grand Island, NY), BCECF-AM was from Dojindo (Kumamoto, Japan), and [α-32P]CTP was from Amerham (Bucks, UK).

Statistics. Data are reported as means ± SE. Statistical significance was assessed by using the unpaired Student t-test or ANOVA for pH and Na+/H+ antiporter activity and the Mann-Whitney test for RNA blotting and immunoblotting.

RESULTS

Low-K+ medium increases Na+/H+ antiporter activity in OKP cells. We first examined whether chronic incubation of OKP cells in low-K+ medium increases Na+/H+ antiporter activity. OKP cells were incubated in control medium ([K+] = 5.4 mM) or in low-K+ medium ([K+] = 2.7 mM) for 24 h. After this incubation, cells were incubated in Na+-containing assay solution for 30 min with BCECF-AM, and Na+/H+ antiporter activity was measured in the presence of a normal K+ concentration. As shown in Fig. 1, Na+/H+ antiporter activity was increased 31% by chronic incubation in low-K+ medium. Figure 2 shows a time course, with Na+/H+ antiporter activity plotted as a function of incubation time. Low-K+ medium stimulated Na+/H+ antiporter activity beginning at 8 h.

Chronic incubation in low-K+ medium activates NHE3 activity in OKP cells. OKP cells express an ethylisopropyl amiloride (EIPA)-resistant type of Na+/H+ antiporter, NHE3 (8). However, it is still possible that incubation in low-K+ medium activates an Na+/H+ antiporter isoform other than NHE3. To address this, Na+/H+ antiporter activity was assayed with 15 mM Na and 10−4 or 10−7 M EIPA after a 24-h preincubation in normal or low-K+ medium. With 15 mM Na, 10−7 M EIPA should inhibit EIPA-sensitive isoforms, such as NHE1 and NHE2, and 10−4 M EIPA should inhibit all NHE isoforms. Na+/H+ antiporter activity was also assayed with 130 mM Na+ and 10−4 M EIPA, a condition in which EIPA should completely inhibit NHE2 (25). As shown in Fig. 3, 10−4 M EIPA with 15 mM Na+ inhibited most of pH recovery in OKP cells incubated in control and low-K+ media and inhibited the effect of
incubation in low-K\(^+\) medium. By contrast, incubation in low-K\(^+\) medium stimulated Na\(^+\)/H\(^+\) antiporter activity in the presence of \(10^{-7}\) M EIPA (15 mM Na\(^+\)) and \(10^{-4}\) M EIPA (130 mM Na\(^+\)). Because both of the conditions should inhibit the EIPA-sensitive type of Na\(^+\)/H\(^+\) antiporter, including NHE2, these results confirm that incubation in low-K\(^+\) medium activates NHE3.

Chronic incubation in low-K\(^+\) medium increases NHE3 mRNA and protein abundance at 24 h. An increase in NHE3 activity could be related to an increase in NHE3 mRNA and/or protein abundance. As shown in Fig. 4, incubation in low-K\(^+\) medium increased NHE3 mRNA abundance 2.2-fold at 24 h. There was, however, no effect on NHE3 mRNA abundance at 8 h. Similarly, incubation in low-K\(^+\) medium increased NHE3 protein abundance by 62% at 24 h but had no effect at 8 h (Fig. 5).

Incubation in low-K\(^+\) medium activates NHE3 activity via a decrease in pH\(_i\). Adam and colleagues (1) found that renal pH\(_i\) was decreased in K\(^+\)-depleted rats. In that acidosis also causes an increase in renal proximal tubule apical membrane Na\(^+\)/H\(^+\) antiporter activity and NHE3 protein abundance (2, 5, 22, 27), cell acidification may be responsible for these effects in both conditions. To examine this possibility, the pH\(_i\) of OKP cells was continuously measured in the control solution (Na\(^+\)-containing assay solution, [K\(^+\)] = 5.0 mM) and low-K\(^+\) solution (same composition as Na\(^+\)-containing solution except [K\(^+\)] = 2.5 mM) after loading cells with BCECF-AM. As shown in Fig. 6, pH\(_i\) in low-K\(^+\) solution decreased to values significantly lower than those for the control solution, with a maximal \(\Delta\)pH\(_i\) of 0.27 pH units at 1,600 s (\(P < 0.001\)).

Next we examined whether NHE3 is responsible for pH\(_i\) recovery. As shown in Fig. 3, in the presence of 15 mM Na\(^+\), \(10^{-4}\) M EIPA inhibited Na\(^+\)/H\(^+\) antiporter activity whereas \(10^{-7}\) M EIPA did not, a result consistent with a role for NHE3. During the initial 2,000 s after incubation in low-K\(^-\) medium, pH\(_i\) recovery was blocked by \(10^{-4}\) M EIPA but not by \(10^{-7}\) M EIPA in the presence of 15 mM Na\(^+\) (Fig. 8A). Under the same conditions, we measured pH\(_i\) recovery at 2 h. pH\(_i\) recovered to control levels at 2 h in the absence of EIPA.

**Fig. 4.** Incubation in low-K\(^+\) medium increases NHE3 mRNA at 24 h but not at 8 h. OKP cells were incubated for 8 and 24 h in medium with 5.4 or 2.7 mM K\(^+\), and then Northern blotting was performed; n = 5. At 8 h, increase was not significant; at 24 h, there was a 2.2 ± 0.3-fold increase; \(P < 0.001\). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

**Fig. 5.** Incubation in low-K\(^+\) medium increases NHE3 protein abundance at 24 h but not at 8 h. OKP cells were incubated for 8 and 24 h in medium with 5.4 or 2.7 mM K\(^+\), and then Western blotting was performed with anti-NHE3 antibodies; n = 5. At 8 h, increase was not significant; at 24 h, there was a 68 ± 0.1% increase, \(P < 0.001\).

**Fig. 6.** Incubation in low-K\(^+\) medium decreases pH\(_i\) in OKP cells. OKP cells were incubated in indicated medium [K\(^+\)], and pH\(_i\) was measured. n = 5; *P < 0.01, **P < 0.05 for value at 5.0 mM K\(^+\) vs. that at 2.5 mM K\(^+\).
Tyrosine kinase pathways mediate NHE3 activation in response to intracellular acidification. We previously reported that cytoplasmic acidification activates tyrosine kinase pathways in renal cells (29). Activation of tyrosine kinase pathways was shown to mediate NHE3 activation by acid (28) and endothelin (14, 15) in OKP cells. To examine the role of tyrosine kinase pathways, OKP cells were incubated with 10^{-6} M herbimycin A (a tyrosine kinase inhibitor); incubation with herbimycin A began 2 h before incubation in low-K^+ medium or sodium propionate, or control medium and continued throughout the experiment. As shown in Fig. 9, the activation of Na^+\text/H^+ antiporter activity by low-K^+ medium or sodium propionate medium was abolished in the presence of herbimycin A at 8 and 24 h. We also examined the role of tyrosine kinases in the early \(pHi\) recovery occurring during incubation in low-K^+ medium. Of interest, a 2-h preincubation in herbimycin A had no effect on early \(pHi\) recovery in OKP cells incubated in low-K^+ medium (Fig. 10).

**DISCUSSION**

K^+ depletion causes an increase in apical membrane Na^+\text/H^+ antiporter and basolateral membrane Na^+\text/3HCO_3 cotransporter activities (23). To study the mechanisms responsible for Na^+\text/H^+ antiporter activation, we examined the effect of incubating OKP cells in low-K^+ medium. These cells express NHE3, the isoform expressed in the apical membrane of the proximal tubule (7, 8, 11). Incubation of OKP cells in acid medium leads to an increase in NHE3 activity and protein abundance, and the effect of metabolic acidosis on proximal tubule NHE3 (5, 27). In the present study, incubation of these cells in low-K^+ medium caused a 31% increase in NHE3 activity.

The increase in NHE3 activity was first seen at 8 h and persisted for 24 h. There was no increase at 4 h. The increase in NHE3 activity induced by incubation in low-K^+ medium at 8 h occurred in the absence of a change in total cellular NHE3 protein abundance or NHE3 mRNA abundance. Although it is possible that small changes in protein and mRNA abundances were missed, the changes are clearly very small if present. This protein synthesis-independent activation may be mediated by trafficking or posttranslational modification. At 24 h activation was associated with an increase in total cellular NHE3 protein and mRNA abundances. This time course agrees with the one that we have previously found by incubation of OKP cells in acid medium. NHE3 activity was unchanged at 3 h and increased from 6 to 24 h, but NHE3 mRNA abundance did not begin to increase until 12 h (8).

Metabolic acidosis and K^+ depletion both cause a decrease in \(pHi\), but have opposite effects on extracellular \(pH\) (1). Because the responses of the renal proximal tubule to acidosis and K^+ depletion are similar, it has...
been concluded that the key signal is \( \text{pHi} \). The present study provides direct support for this hypothesis. First, incubation of OKP cells in low-K\(^+\) medium causes intracellular acidification. Second, in these cells Na\(^+/\)H\(^+\) antiporter activity is regulated similarly by incubation in low-K\(^+\) medium, weak-acid addition, and medium acidification (8), all of which are activities that acidify the cells. Last, herbimycin A, a tyrosine kinase inhibitor, inhibits chronic activation of the Na\(^+/\)H\(^+\) antiporter by acid incubation (28), incubation in low-K\(^+\) medium, and weak-acid addition. We previously found that intracellular acidification induced by medium acidification, weak-acid addition, nigericin addition, or an NH\(_4\)Cl prepulse activates c-Src tyrosine kinase (29). At 8 and 24 h there was no longer a measurable change in cell pH. It is possible that there was a small, unmeasurable change in cell pH. If not, it is unclear what is the signal that maintains the response to incubation in low-K\(^+\) medium.

Immediately after a decrease in medium K\(^+\) concentration, pHi decreases and then recovers. This recovery is mediated by NHE3 but occurs before the increase in NHE3 activity shown in Fig. 2. In the experiments shown in Fig. 2, antiporter activity was assayed in cells incubated in control and low-K\(^+\) media under identical conditions at the same pHi, and it was found that NHE3 activity did not increase at 4 h. The pH\(_i\) recovery mediated by NHE3 during the initial 2,000 s (Fig. 8) occurs at a decreased pH, and is likely due to allosteric activation of NHE3 by intracellular acidosis (9). The observation that early recovery of pH\(_i\) is not dependent on tyrosine kinase pathways also implies an early mechanism for NHE3 activation distinct from the one that is observed at 8 and 24 h.

Activation of NHE3 by cell acidification subserves two distinct functions. First, it defends the cell pH of the proximal tubule cell. In some segments of the proximal tubule, NHE1 is not expressed (12, 19) and NHE3 may be more responsible for cell homeostasis. Second, activation of NHE3 by intracellular acidification defends the pH of the extracellular fluid by stimulating transcellular HCO\(_3^–\) absorption. Activation of the Na\(^+/\)HCO\(_3^–\) cotransporter during metabolic acidosis and K\(^+\) deficiency defends extracellular fluid pH while worsening proximal tubule cell acidification in these conditions.

Activation of NHE3 by K\(^+\) depletion causes serum [HCO\(_3^–\)] to rise to abnormal levels. In addition, K\(^+\) depletion may also be detrimental to blood pressure. In many clinical conditions associated with the activation of the renin-angiotensin system, K\(^+\) deficiency is associated with hypertension. Here, activation of NHE3 will stimulate proximal tubule NaCl absorption, further contributing to hypertension. Conversely, increasing dietary K\(^+\) can attenuate essential hypertension in patients (20). One reported mechanism to explain this is inhibition of proximal tubule and thick ascending limb Na\(^+\) reabsorption (13, 24). The present study suggests that K\(^+\) administration could inhibit NHE3, which contributes to Na\(^+\) absorption in these two segments.

In summary, these studies demonstrate that low extracellular [K\(^+\)] causes an intracellular acidification at early time points, which results in the activation of NHE3 by three distinct mechanisms. Immediately, there is allosteric activation of the antiporter. At 8 h, activation occurs in the absence of a change in cell pH.
and in the absence of increases in NHE3 protein or mRNA abundance. At 24 h, increased NHE3 activity is associated with increases in protein and mRNA abundances. Tyrosine kinase pathways play a role in the activation at 8 and 24 h.

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