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

MORIMASA AMEMIYA,¹ KAORU TABEI,¹ EIJÌ KUSANO,¹ YASUSHI ASANO,² AND ROBERT J. ALPERN²

¹Division of Nephrology, Department of Internal Medicine, Jichi Medical School, Tochigi, Japan 329-0498; and ²University of Texas Southwestern Medical Center, Dallas, Texas 75235-8856

This is a brief summary of the article.
LOW-K⁺ INCUBATION INCREASES NHE3 ACTIVITY

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 QuikHyb 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 American. DNA and 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 QuikHyb 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.

Statistics. Data are reported as means ± SE. Statistical significance was assessed by using the unpaired Student t-test or ANOVA for pHi 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⁺ concn ([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 assayed with 15 mM Na⁺ and 10⁻⁴ or 10⁻³ 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 10⁻³ 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 pHi recovery in OKP cells incubated in control and low-K⁺ media and inhibited the effect of

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 10⁻³ 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 10⁻³ 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 pHi recovery in OKP cells incubated in control and low-K⁺ media and inhibited the effect of

low-K⁺ activity in OKP cells. OKP cells were incubated in Na⁺/H⁺ antiporter activity in opossum kidney, clone P (OKP) cells. OKP cells were incubated for 24 h in medium with 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.

Fig. 2. Chronic incubation in low-K⁺ medium increases Na⁺/H⁺ antiporter activity (time course). OKP cells were incubated for indicated times with medium containing 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.

Fig. 3. Chronic incubation in low-K⁺ medium activates NHE3 in OKP cells. OKP cells were incubated in Na⁺/H⁺ antiporter activity in opossum kidney, clone P (OKP) cells. OKP cells were incubated for 24 h in medium with 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.

Fig. 1. Chronic incubation in low-K⁺ medium increases Na⁺/H⁺ antiporter activity (time course). OKP cells were incubated for indicated times with medium containing 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.

Fig. 2. Chronic incubation in low-K⁺ medium increases Na⁺/H⁺ antiporter activity (time course). OKP cells were incubated for indicated times with medium containing 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.

Fig. 3. Chronic incubation in low-K⁺ medium activates NHE3 in OKP cells. OKP cells were incubated in Na⁺/H⁺ antiporter activity in opossum kidney, clone P (OKP) cells. OKP cells were incubated for 24 h in medium with 5.4 (open bars) or 2.7 mM K⁺ (hatched bars), after which Na⁺/H⁺ antiporter activity (y axis) was assayed. n = 4; *P < 0.05, **P < 0.01 for value at 5.4 mM vs. that at 2.7 mM.
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 (\(\ast\)P < 0.01). After this, pH\(_i\) started to recover. There were no differences in pH\(_i\) between cells in control and low-K\(^+\) media at 8 and 24 h (8 h: control, 7.18 \(\pm\) 0.02; low K\(^+\), 7.17 \(\pm\) 0.02; 24 h: control, 7.17 \(\pm\) 0.04; low K\(^+\), 7.16 \(\pm\) 0.03, n = 6).

To confirm the importance of a decrease in pH\(_i\), we examined the effect of isotonic addition of sodium propionate, a weak acid that causes cell acidification. Incubation of OKP cells in 5 mM sodium propionate decreased pH\(_i\) by 0.09 \(\pm\) 0.01 units at 20 s (n = 6, \(\ast\)P < 0.001 vs. control) and increased Na\(^+\)/H\(^+\) antiporter activity at 8 and 24 h (Fig. 7).

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 \(\pm\) 0.3-fold increase; \(\ast\)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 \(\pm\) 0.1% increase, \(\ast\)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; \(\ast\)P < 0.01. **P < 0.05 for value at 5.0 mM K\(^+\) vs. that at 2.5 mM K\(^+\).
Fig. 8. This was blocked by 10^-4 M EIPA but not by 10^-3 M EIPA, indicating a role for NHE3.

Tyrosine kinase pathways mediate NHE3 activation in response to intracellular acidification. We previously reported that tyrosine kinase pathways 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^+/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 pH recovery occurring during incubation in low-K^+ medium. Of interest, a 2-h preincubation in herbimycin A had no effect on early pH recovery in OKP cells incubated in low-K^+ medium (Fig. 10).

DISCUSSION

K^+ depletion causes an increase in apical membrane Na^+/H^+ antiporter and basolateral membrane Na^+/HCO_3^-cotransporter activity (23). To study the mechanisms responsible for NHE3 activation in low-K^+ medium, these cells expressed NHE3, the same isoform expressed in 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 (6, 8), an effect similar to the effect of metabolic acidosis in proximal tubule. In the present study, incubation of OKP cells in low-K^+ medium caused a 31% increase in NHE3 activity.

Metabolic acidosis and K^+ depletion both cause a decrease in pH 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 suggested that 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, NHE3 activity was unchanged at 3 h and increased by 10.2% at 24 h.

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, this increase in NHE3 mRNA abundance was associated with an increase in total cellular NHE3 protein abundance. The increase in NHE3 mRNA abundance induced by incubation in low-K^+ medium was clearly very small if present. This protein synthesis-independent activation may be mediated by trafficking or posttranslational modification. At 24 h, NHE3 activity was unchanged at 3 h and increased by 10.2% at 24 h.

The increase in NHE3 activity induced by incubation in low-K^+ medium was clearly very small if present. This protein synthesis-independent activation may be mediated by trafficking or posttranslational modification. At 24 h, NHE3 activity was unchanged at 3 h and increased by 10.2% at 24 h.
been concluded that the key signal is 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 NH₄Cl 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 pHᵢ recovery mediated by NHE3 during the initial 2,000 s (Fig. 8) occurs at a decreased pHᵢ and is likely due to allosteric activation of NHE3 by intracellular acidosis (9). The observation that early recovery of pHᵢ 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 low K⁺ and sodium propionate is dependent on tyrosine kinases. OKP cells were incubated in 5.4 mM K⁺, 2.7 mM K⁺, or 5 mM sodium propionate (5.4 mM K⁺) for 8 (A) and 24 (B) h, with or without 10 mM herbimycin A. Then Na⁺/H⁺ antiporter activity (y axis) was measured. At 8 h, n = 5; at 24 h, n = 4. *P < 0.05, **P < 0.01 vs. values at 5.4 mM K⁺.

![Fig. 9. Activation of NHE3 by low K⁺ and sodium propionate is dependent on tyrosine kinases. OKP cells were incubated in 5.4 mM K⁺, 2.7 mM K⁺, or 5 mM sodium propionate (5.4 mM K⁺) for 8 (A) and 24 (B) h, with or without 10 mM herbimycin A. Then Na⁺/H⁺ antiporter activity (y axis) was measured. At 8 h, n = 5; at 24 h, n = 4. *P < 0.05, **P < 0.01 vs. values at 5.4 mM K⁺.](http://ajpcell.physiology.org/)

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.

We gratefully acknowledge the technical help of K. Inose and Y. Watanabe.

These studies were supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by National Institute of Diabetes and Digestive and Kidney Diseases Grant R37-DK-39298.

Address for reprint requests: M. Amemiya, Internal Medicine, Nephrology Division, Jichi Medical School, 3311-1 Yakushiji Minami-kawachi-Machi Kawachi-Gun Tochigi-Ken, Japan 329-0498.

REFERENCES


