Chronic Hyperosmolality Increases NHE3 Activity In OKP Cells

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Abstract

This study investigated the effect of chronic hypertonicity on the OKP cell Na/H antiporter, encoded by Na/H exchanger 3 (NHE3). Chronic (48 h) increases in extracellular glucose, mannitol, or raffinose concentration caused a significant increase in Na/H antiporter activity, while increases in urea concentration were without effect. This effect was seen with changes in osmolality of only 20 mOsm/liter, a magnitude that is observed clinically in poorly controlled diabetes mellitus. Increases in mannitol concentration acutely inhibited and chronically stimulated Na/H antiporter activity. The increase in Na/H antiporter activity induced by hypertonic incubation was resistant to 10^{-7} and 5×10^{-6} M but inhibited by 10^{-4} M ethylisopropyl amiloride, consistent with regulation of NHE3. In addition, hypertonicity increased total cellular and plasma membrane NHE3 protein abundance twofold, with only a small increase in NHE3 mRNA abundance. We conclude that chronic pathophysiologically relevant increases in tonicity lead to increases in NHE3 protein abundance and activity. This may be responsible for increased proximal tubule apical membrane Na/H antiporter activity in poorly controlled diabetes mellitus, which could then contribute to hypertension, glomerular hyperfiltration and diabetic nephropathy. (*J. Clin. Invest.* 1998. 101:170–177.) Key words: Na/H antiporter • diabetes mellitus • hyperglycemia • hypertension • hypertonicity

Introduction

The proximal tubule is responsible for the majority of NaCl reabsorption in the kidney, and thus is important in the regulation of extracellular fluid volume and blood pressure. NaCl absorption in this segment is mediated by an Na/H antiporter functioning in parallel with a Cl/base exchanger (1, 2). A number of physiologic regulators of NaCl absorption, such as angiotensin II, endothelin, dopamine, and adrenergic catecholamines have been demonstrated to regulate NaCl absorption through regulation of the apical membrane Na/H antiporter (3). While five mammalian Na/H antiporter gene isoforms have been cloned (4–9), present evidence suggests that a major fraction of apical membrane Na/H exchange mediating transepithelial transport is encoded by Na/H exchanger 3 (NHE3)[1] (10, 11). OKP cells express an apical membrane Na/H antiporter that is encoded by NHE3 and is regulated in a similar fashion to that of the proximal tubule apical membrane Na/H antiporter (12).

Diabetes mellitus is associated with hypertension and renal disease. In rats, induction of diabetes mellitus causes an increase in renal brush border membrane Na/H antiporter activity (13). Increased activity was dependent on the control of the diabetes in that insulin therapy reversed the effect. In this study, we examined the effect of chronic increases in media glucose concentration on Na/H antiporter activity in OKP cells. The results demonstrate that exposure of OKP cells to pathophysiologically relevant increases in glucose concentration for 48 h, causes an increase in Na/H antiporter activity that persists after the cells are removed from the hyperglycemic media. This effect is shown to be due to cell shrinkage in that it is elicited by increases in mannitol or raffinose concentration, but is not elicited by increases in urea concentration. The increase in Na/H antiporter activity is associated with an increase in NHE3 protein abundance.

Methods

Materials and supplies. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted as follows: penicillin and streptomycin from Whittaker M.A. Bioproducts, Inc. (Walkersville, MD); acetoxymethyl derivative of 2′7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), and ethylisopropyl amiloride (EIPA) from Molecular Probes, Inc. (Eugene, OR); culture media from GIBCO BRL (Gaithersburg, MD); and enhanced chemiluminescence kit from Amersham Corp. (Arlington Heights, IL).

Cell culture. OKP cells, a gift from K. Hruska (Washington University, St. Louis, MO), are a clonal line of the opossum kidney (OK) cell line, originally described by Cole et al. (14). OKP cells were passaged in DME (100 mg/dl, 5.55 mM glucose) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). For experimentation, OKP cells were grown to confluence and rendered quiescent by serum deprivation for 24 h before study. Cells were then maintained in serum-free media with additives as indicated for 1–48 h, and then studied. In all studies, control and experimental cells were from the same passage and were assayed on the same day.

Measurement of intracellular pH and Na/H antiporter activity. Continuous measurement of cytoplasmic pH (pH_i) was accomplished using the intracellularly trapped pH-sensitive dye, BCECF, as previously described (15). Cells were loaded with 10 μM acetoxymethyl ester of BCECF for 35 min at 37°C, and pH_i estimated from the ratio of fluorescence with excitation at wavelengths of 500 and 450 nm, with 530 nm emission in a computer-controlled spectrofluorometer (8000C; SLM Instruments, Inc., Urbana, IL). The BCECF excitation fluorescence ratio was calibrated intracellularly using the K/nigericin approach as described (16).

Patrice Ambühl and Morimasa Amemiya contributed equally to the studies reported in this paper.

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1. *Abbreviations used in this paper:* BCECF, 2′7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; EIPA, ethylisopropyl amiloride; MAP, mitogen activated protein; NHE3, Na/H exchanger 3; OK, opossum kidney.

Na/H antiporter activity was assayed as the initial rate of Na-dependent pH_i increase after an acid load in the absence of CO_2/HCO_3, as previously described (15). Cells were acidified with 13 μM nigericin in Na-free solution. The initial rate of pH_i increase (dpH_i/dt) upon Na addition was calculated by drawing a tangent to the initial deflection. To calculate buffer capacity, cells were pulsed with 10 mM ammonium chloride in the Na-free perfusate at the trough pH_i, as described (15). Incubation in hypertonic glucose or mannitol for 48 h had no effect on buffer capacity. Results are therefore reported as dpH_i/dt.

Immunoblot. Cells were rinsed with ice cold PBS three times and dounce homogenized in isotonic Tris-buffered saline (in mM: NaCl, 150; Tris-HCl, 50; pH 7.5, EDTA, 5) containing proteinase inhibitors (in μg/ml: PMSF, 100; Aprotinin, 4; Leupeptin, 4). Nuclei were removed by centrifugation at 13,000 *g* at 4°C. Membranes were pelleted by centrifugation at 100,000 *g* for 20 min. The resulting pellet was resuspended in Tris-buffered saline and total protein content determined by the method of Bradford. 15 μg of protein were diluted 1:5 in 5× SDS loading buffer (1 mM Tris HCl, pH 6.8, 1% SDS, 10% glycerol, 1% 2 mercaptoethanol), size fractionated by SDS PAGE (7.5% gel), and electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, blots were probed in the same buffer for 1 h with a polyclonal anti–opossum NHE3 antibody (antiserum 5683, generated against a maltose binding protein/NHE3 [aa 484–839] fusion protein) at a dilution of 1:300. Blots were washed in 0.05% Tween 20 in PBS one time for 15 min and two times for 5 min, incubated with a 1:10,000 dilution of peroxidase-labeled sheep anti–rabbit IgG in 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, washed as above, and then visualized by enhanced chemiluminescence. NHE3 protein abundance was quantitated by densitometry (Molecular Dynamics ImageQuant Software version 3.3; Molecular Dynamics, Sunnyvale, CA). This procedure labeled a 90-kD band that was not seen when antibody was preincubated with fusion protein or when preimmune serum replaced the anti-NHE3 antiserum.

To measure plasma membrane NHE3, we used surface biotinylation, as described (17–19). Monolayers were placed on ice and washed with ice-cold PBS with 0.1 mM $CaCl_2$ and 1.0 mM $MgCl_2$ twice. The apical surface was then exposed to 1.5 mg/dl sulfoNHS-SS-biotin (Pierce Chemical Co., Rockford, IL) in 10 mM triethanolamine (pH 7.5), 2 mM $CaCl_2$, and 150 mM NaCl for 1 h at 4°C. After labeling, filters were washed four times with quenching solution (PBS containing 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 100 mM glycine) for 20 min at 4°C. Cells were then extracted with RIPA buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 50 mM β-glycerophosphate, 50 mM NaF, 1 mM Na orthovanadate, 2.5 mM EDTA, 5 mM EGTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5% Na deoxycholate, 0.1% SDS, 100 μg/ml PMSF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin), samples precipitated with streptavidin-coupled agarose, and the precipitate subjected to SDS-PAGE and blotting with anti-NHE3 antibodies.

RNA blotting. RNA was extracted using RNeasy (QIAGEN, Inc., Chatsworth, CA). 15 μg of total RNA was size fractionated by agarose–formaldehyde gel electrophoresis and transferred to nylon membranes. The radiolabeled NHE3 probe was synthesized from a full-length OKP NHE3 cDNA (12) by the random hexamer method, and the 18S probe was an end-labeled oligonucleotide. Prehybridization, hybridization, and washing were done as previously described (20). Filters were exposed to film overnight at −70°C and labeling quantitated by densitometry. Changes in NHE3 abundance were normalized for changes in 18S rRNA abundance.

Statistics. Data are reported as mean±SEM. Statistical significance was assessed using an unpaired Student's *t* test or analysis of variance (ANOVA) as appropriate.

Results

Chronic increases in [glucose] increase Na/H antiporter activity. To determine the effect of chronic exposure to increased glucose concentration, quiescent OKP cells were preincubated in low glucose DME (100 mg/dl glucose) or in a similar medium with [glucose] raised to 460 mg/dl (Δ osmolality = 20 mOsm/liter) for 48 h. After this preincubation, cells were incubated in isoosmotic media (low glucose DME) for 45 min and Na/H antiporter activity assayed. As shown in Fig. 1, cells preincubated in media with a high glucose concentration demonstrated a 39% increase in Na/H antiporter activity. Fig. 2 shows a dose–response curve, plotting Na/H antiporter activity as a function of preincubation glucose concentration. Progressive increases in media glucose concentration led to progressive increases in Na/H antiporter activity, which achieved a maximal effect at a glucose concentration 360 mg/dl above that of control media.

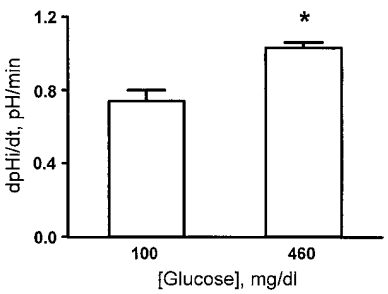

Figure 1. Increases in media glucose concentration increase Na/H antiporter activity. Cells were preincubated in media glucose concentrations of 100 or 460 mg/dl for 48 h. Na/H antiporter activity was then assayed and is plotted as dpH_i/dt (pH U/min). $n = 4$; $^*P <$ 0.01 versus control.

Chronic increases in extracellular fluid tonicity increase Na/H antiporter activity. This effect of glucose could be due to a specific effect of glucose or to an increase in extracellular fluid osmolality. To differentiate between these possibilities, we compared the effects of 48 h preincubation in media made hyperosmolar by addition of 20 mM glucose or mannitol. As can be seen in Fig. 3, the effects of glucose and mannitol were similar. Cells preincubated in high glucose demonstrated a 63% increase and cells preincubated in mannitol exhibited a 57% increase in Na/H antiporter activity. In that mannitol is not metabolized and does not enter cells, these results suggest

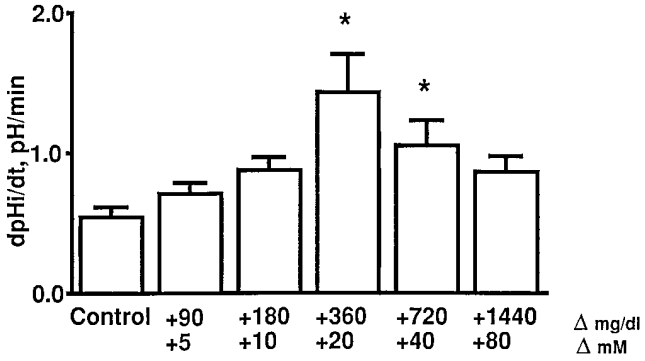

Figure 2. Increases in media glucose concentration activate the Na/H antiporter: dose dependence. Control cells were preincubated in a media glucose concentration of 100 mg/dl, while experimental cells were preincubated in increasing glucose concentrations for 48 h. Na/H antiporter activity is plotted as a function of the increase in glucose concentration in mg/dl and in mM. Control, $n = 12$; +5 mM, $n = 13$; +10 mM, $n = 11$; +20 mM, $n = 12$; +40 mM, $n = 10$; +80 mM, $n = 11$. $^*P < 0.05$ versus control.

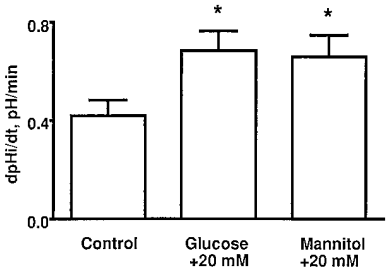

Figure 3. Increases in extracellular fluid osmolality increase Na/H antiporter activity. Media osmolality was increased by addition of 20 mM glucose or mannitol for 48 h. Na/H antiporter activity is plotted. Control, $n = 17$; glucose, $n = 22$; mannitol, $n = 20$. $^*P < 0.05$ versus control.

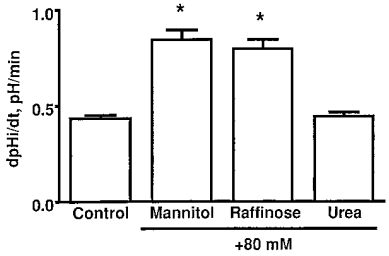

Figure 5. Hypertonicity increases Na/H antiporter activity. Medium osmolality was increased by addition of 80 mM mannitol, raffinose, or urea for 48 h. Na/H antiporter activity is plotted. Control, $n = 7$; mannitol, $n = 8$; raffinose, $n = 4$; urea, $n = 7$. $^*P < 0.001$.

that the effect of glucose is due to an increase in extracellular osmolality.

Fig. 4 shows a dose–response curve for the mannitol effect. Unlike the response to glucose, increases in osmolality above 20 mOsm/liter led to further increases in Na/H antiporter activity. These results suggest that chronic increases in extracellular fluid osmolality lead to progressive increases in Na/H antiporter activity. Very high concentrations of glucose may have a toxic effect, or glucose uptake at high concentrations may dissipate the osmotic gradient.

To further address the solute specificity of the effect, we compared 48 h preincubation in media made hyperosmolar by addition of 80 mM mannitol, raffinose, or urea. As seen in Fig. 5, mannitol and raffinose, two solutes that cannot enter cells, both caused similar adaptive increases in Na/H antiporter activity (98% and 86% increase, respectively). However, urea, which enters cells more freely, did not elicit an increase in Na/H antiporter activity. Similarly, preincubation in media made hypertonic by addition of 20 mM urea had no effect on Na/H antiporter activity (data not shown). These results suggest that cell shrinkage is responsible for the increase in Na/H antiporter activity.

In the studies presented thus far, cells preincubated in high extracellular fluid osmolality for 48 h were exposed to isotonic solutions for 45 min before assay. This was done to eliminate any effects of hypertonicity during the assay, but may have led to acute cell swelling, which could possibly be responsible for the increased Na/H antiporter activity. To examine this, we performed studies wherein after 48 h preincubation in solutions of high osmolality, cells were then exposed to similar hyperosmolar solutions during assay of the Na/H antiporter. In Fig. 6 *A*, osmolality was increased by addition of 20 mM mannitol, while in Fig. 6 *B*, osmolality was increased by addition of 80 mM mannitol. The first bar represents control cells, the second bar represents cells preincubated in hypertonic media for 48 h and then assayed in isotonic media, and the last bar represents cells preincubated and assayed in hypertonic media. In all cases preincubation in hypertonic media increased Na/H antiporter activity. Thus, when cell swelling before assay was prevented, Na/H antiporter activity was still increased by chronic hypertonic incubation.

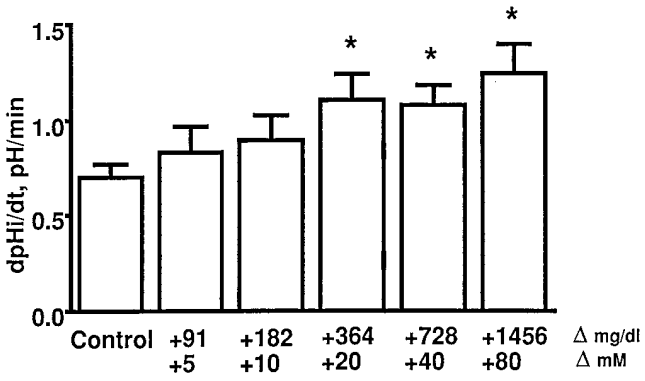

Figure 4. Mannitol increases Na/H antiporter activity: dose dependence. Cells were preincubated in control media or in media where osmolality was increased by mannitol addition for 48 h. Na/H antiporter activity is plotted as a function of the amount of mannitol added, expressed as mg/dl or mM. Control, $n = 8$; +5 mM, $n = 9$; +10 mM, $n = 9$; +20 mM, $n = 9$; +40 mM, $n = 9$; +80 mM, $n = 9$. $^*P < 0.05$ versus control.

Fig. 7 shows a complete time course wherein cells were preincubated and assayed in hypertonic solution. At 1 h, hypertonicity resulted in inhibition of Na/H antiporter activity, as previously reported (21–23). From 6 to 24 h, net inhibition disappeared and there was a tendency to increase Na/H antiporter activity. This became significant at 48 h. This gradual transition from net inhibition to net stimulation likely represents a loss of the acute inhibitory effect which is not present at 48 h (Fig. 6), and an increase in the chronic stimulatory effect. Given this time course, it is possible that the magnitude of stimulation would increase further with greater duration of hypertonic incubation.

Hypertonicity activates NHE3. To prevent any effects of acute cell swelling, the remaining studies were performed with 48 h hypertonic preincubation followed by assay in hypertonic media. First, we addressed whether chronic hypertonic incubation increased the activity of an EIPA–resistant Na/H antiporter. Previously, we have shown that OKP cells express an Na/H antiporter whose IC_{50} for EIPA is 4.5 μM when assayed with 15 mM Na (20). This kinetic behavior is consistent with that of NHE3, and agrees with the finding that OKP cells express NHE3 mRNA (12). Nevertheless, it is possible that the increase in Na/H antiporter activity induced by chronic hypertonicity is mediated by a different isoform. To test this, kinetics for EIPA inhibition were performed on cells preincubated in control or hypertonic media for 48 h. For these studies, Na/H antiporter activity was measured with 15 mM Na. In this setting, 10^{-7} and 5×10^{-6} M EIPA would be predicted to completely inhibit NHE1 and other EIPA-sensitive isoforms, while having no effect on NHE3. 10^{-4} M EIPA should inhibit all Na/H antiporter isoforms. As shown in Fig. 8, 10^{-7} and 5×10^{-6} M EIPA had no significant effect on Na/H antiporter ac-

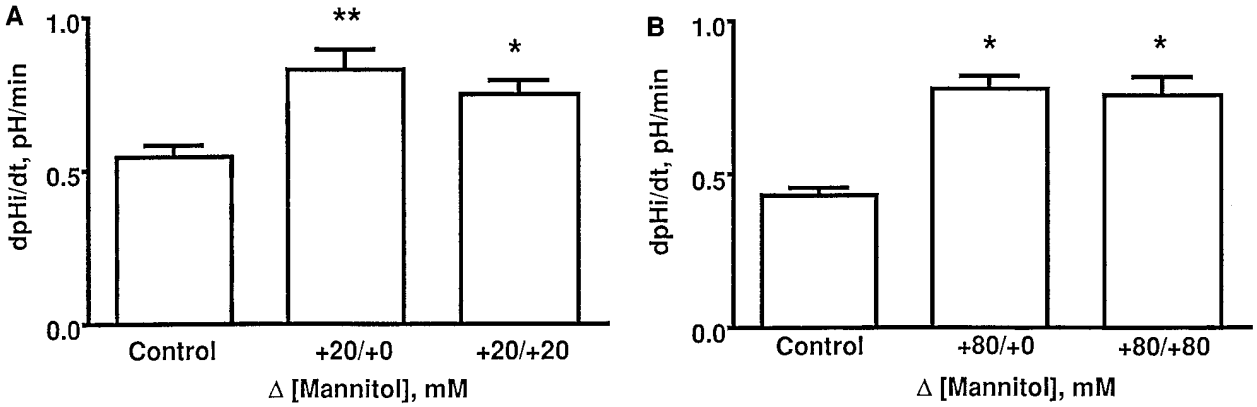

Figure 6. Chronic hypertonicity increases Na/H antiporter activity: effect of tonicity during assay. Cells were preincubated in control media and assayed in control media (*Control*), preincubated in hypertonic media and assayed in control media (*+20/+0* or *+80/+0*) or preincubated in hypertonic media and assayed in hypertonic media (*+20/+20* or *+80/+80*). Preincubation was for 48 h, and assay was 45 min in duration. Na/H antiporter activity is plotted. (*A*) Osmolality was increased by 20 mOsm/liter. Control, $n = 4$; +20/+0, $n = 4$; +20/+20, $n = 5$. $*P < 0.05$; $**P < 0.01$ versus control. (*B*) Osmolality was increased by 80 mOsm/liter. Control, $n = 4$; +80/+0, $n = 5$; +80/+80, $n = 5$. $*P < 0.001$ versus control.

tivity measured under basal conditions or after hypertonic preincubation, and had no effect on the hypertonicity-induced increase in antiporter activity. On the other hand, 10^{-4} M EIPA completely inhibited all of these parameters. Thus, these studies demonstrate that chronic hypertonic incubation increases the activity of an EIPA-resistant Na/H antiporter, likely NHE3.

An increase in NHE3 activity could be related to an increase in NHE3 protein abundance. To examine this, Western blots were performed with anti-NHE3 antiserum. Fig. 9 shows results of preincubating OKP cells in media made hypertonic by addition of mannitol. Preincubation in 80 mM mannitol for 48 h caused a 131±18% increase in NHE3 protein abundance, while preincubation in 20 mM mannitol for 48 h caused a 48±5% increase in NHE3 protein abundance (Fig. 9 *A*). At 24 h, 20 mM mannitol caused a 132±21% increase and 80 mM mannitol caused a 115±30% increase in NHE3 abundance (Fig. 9 *B*).

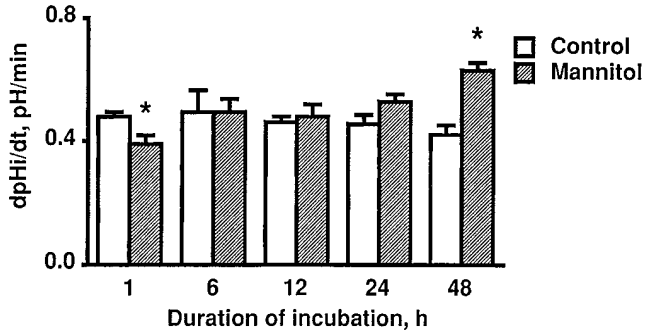

Figure 7. Hypertonicity increases Na/H antiporter activity: time course. Cells preincubated in media made hypertonic by addition of 80 mM mannitol were compared with control cells. Cells were preincubated and assayed under isotonic (*Control*) or hypertonic (*Mannitol*) conditions. Na/H antiporter activity is plotted. Control: 1 h, $n = 5$; 6 h, $n = 4$; 12 h, $n = 4$; 24 h, $n = 5$; 48 h, $n = 4$. Mannitol: 1 h, $n = 4$; 6 h, $n = 4$; 12 h, $n = 4$; 24 h, $n = 5$; 48 h, $n = 4$. $*P < 0.05$ versus control.

The observation that hypertonicity caused an increase in NHE3 protein abundance but no increase in activity at 24 h was surprising. Therefore, we used membrane biotinylation to measure plasma membrane NHE3 abundance at 24 and 48 h. Results are shown in Fig. 10. Hypertonicity caused a 135±59% increase in plasma membrane NHE3 at 48 h ($P < 0.02$). However, hypertonicity did not affect plasma membrane abundance at 24 h (−38±20%, ns). Thus, at 24 h there is an increase in NHE3 protein abundance but no change in activity because the transporter has not trafficked to the plasma membrane. At 48 h NHE3 moves to the plasma membrane and results in increased activity.

Increases in media urea concentration were without effect on Na/H antiporter activity (Fig. 5). Preincubation in 80 mM urea also did not affect NHE3 protein abundance (Δ NHE3 abundance = −19±12%; Fig. 11).

An increase in NHE3 protein abundance can be due to an increase in NHE3 mRNA abundance, or can be due to direct effects on protein synthesis or degradation. Next, we examined whether chronic preincubation in hypertonic media caused an increase in NHE3 mRNA abundance. Preincubating OKP cells in media made hypertonic by addition of 80 mM mannitol caused a 38±14% increase at 24 h and a 36±13% increase at 48 h in NHE3 mRNA abundance normalized for 18S rRNA abundance (Fig. 12). Similar results were obtained if glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. While this effect was consistent, it was small and its physiologic significance is uncertain. The approximately twofold increases in NHE3 protein abundance and activity induced by hypertonicity seem to be too large to be accounted for by the small 35–40% change in NHE3 mRNA abundance.

To examine whether the effect of hypertonic preincubation requires protein synthesis, studies were performed with cycloheximide. 100 μM cycloheximide was added to cells 1 h before control or experimental preincubation. This concentration of cycloheximide inhibits 94% of [^{3}H]leucine incorporation in OKP cells (15). In addition, this concentration of cycloheximide inhibited the chronic effects of cAMP and dexamethasone on Na/H antiporter activity in OKP cells (15, 24). However, as

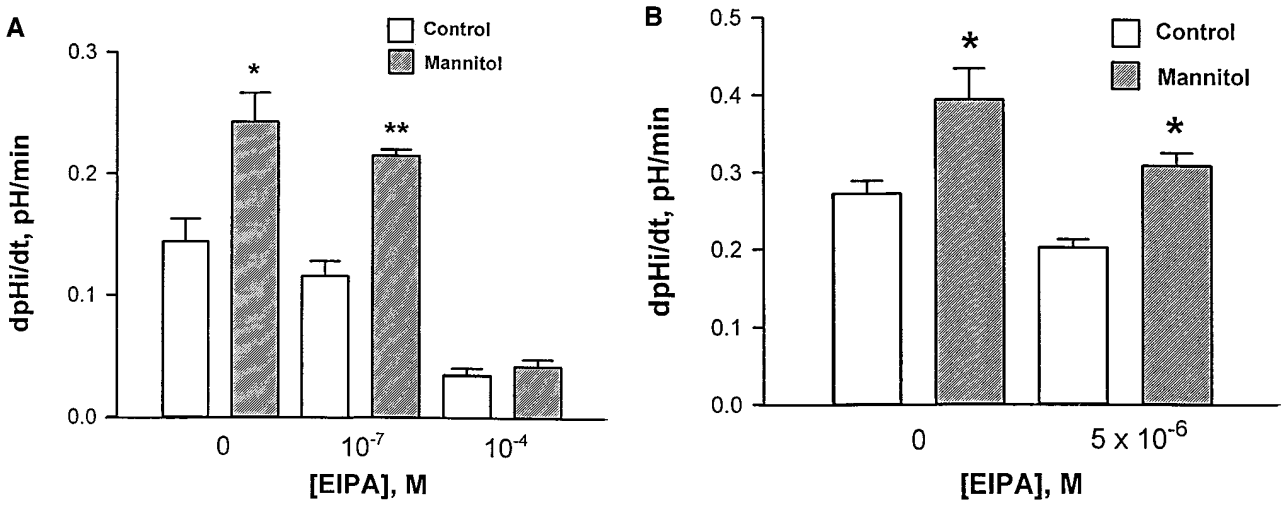

Figure 8. Chronic hyperosmolality increases the activity of an EIPA resistant Na/H antiporter. Cells were preincubated in control media or media made hypertonic by addition of 80 mM mannitol. Cells were preincubated and assayed under isotonic (*Control*) or hypertonic (*Mannitol*) conditions. Antiporter activity was assayed with 15 mM Na, in the absence of EIPA, or in the presence of 10^{-7} M EIPA (*A*), 5×10^{-6} M EIPA (*B*), or 10^{-4} M EIPA (*A*). Na/H antiporter activity is plotted. $n = 4$. *$P < 0.05$ versus control; **$P < 0.001$ versus control.

shown in Fig. 13, the effect of hypertonic preincubation was seen even in the presence of cycloheximide. The significant decrease in basal activity induced by cycloheximide may be due to continued degradation of NHE3 protein in the absence of synthesis, and agrees with our previous results (15). Given the significant change in basal activities, it is difficult to compare the magnitudes of the stimulation in the presence and absence of cycloheximide. Nevertheless, it is clear that at least a component of the hypertonic effect occurs by mechanisms independent of protein synthesis.

Discussion

Diabetes mellitus is associated with renal NaCl retention and an expanded extracellular fluid volume. Volume expansion is responsible for hypertension and hyporeninemic hypoaldosteronism, and may contribute to the altered glomerular hemodynamics responsible for diabetic nephropathy. The incidence of diabetic nephropathy is increased by poor metabolic control and chronically high plasma glucose levels. An effect of hyperglycemia to increase proximal tubular NHE3 activity could explain all of these effects, and would thus be of significant clinical importance. In rats with diabetes mellitus, proximal tubule apical membrane Na/H antiporter activity is increased (13). This effect was reversed by insulin treatment suggesting that hyperglycemia may be responsible. However, the increased Na/H antiporter activity was also prevented by administration of $NaHCO_3$, raising the possibility that ketoacidosis was responsible. In that $NaHCO_3$ administration may inhibit Na/H antiporter activity, it remains possible that hyperglycemia per se causes a chronic adaptive increase in Na/H antiporter activity in the renal proximal tubule.

In OKP cells increases in media glucose concentration caused an increase in Na/H antiporter activity over 48 h, which

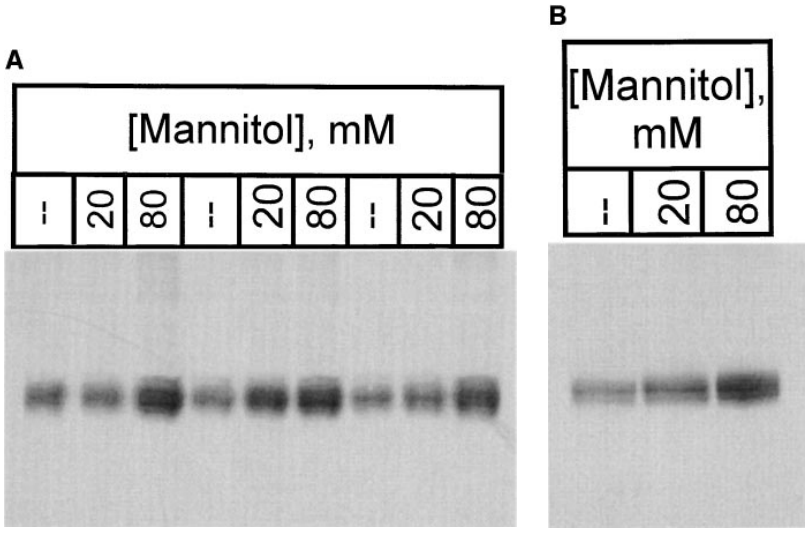

Figure 9. Mannitol increases NHE3 protein abundance. Cells were incubated in control media or media made hypertonic by addition of 20 or 80 mM mannitol. Western blotting was then performed with anti-NHE3 antisera. (*A*) 48 h incubation. (*B*) 24 h incubation. $n = 8$.

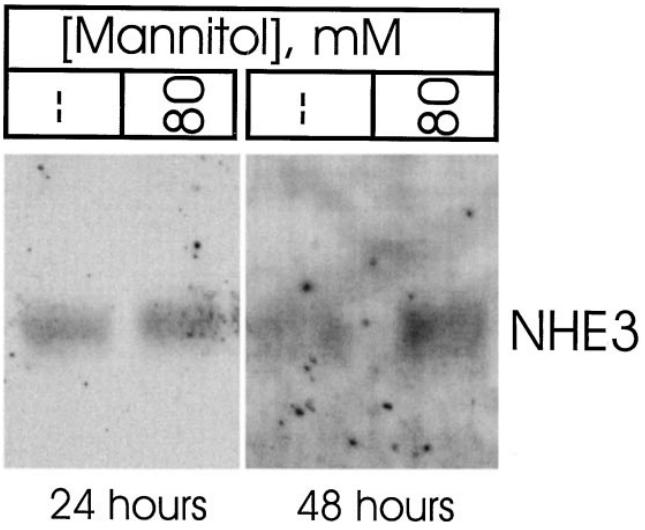

Figure 10. Plasma membrane NHE3 is increased by hypertonicity at 48 h, but not at 24 h. Cells were incubated in control media or media made hypertonic by addition 80 mM mannitol. Plasma membrane proteins were then labeled with biotin and precipitated with agarose-conjugated streptavidin. Precipitates were blotted with anti-NHE3 antisera. (*A*) 48 h incubation. (*B*) 24 h incubation. $n = 5$.

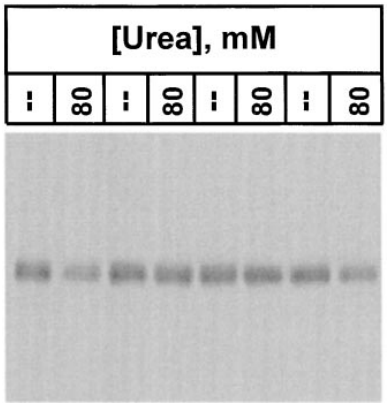

Figure 11. Urea does not increase NHE3 protein abundance. Cells were incubated in control media or media made hypertonic by addition of 80 mM urea ($n = 4$). Western blotting was then performed with anti-NHE3 antisera.

was significant with increases in glucose concentration of 360 mg/dl, corresponding to 20 mOsm/liter. This increase in glucose concentration is pathophysiologically significant, in that it is seen in patients with poorly controlled diabetes mellitus. This effect was not unique to glucose, but was seen with increases in media osmolality elicited by addition of mannitol or raffinose. By contrast, increases in media osmolality elicited by addition of a more cell permeant solute, urea, had no effect on Na/H antiporter activity, suggesting that this effect is mediated by cell shrinkage.

The increase in Na/H antiporter activity induced by hypertonicity was EIPA-resistant, suggesting that it involved an increase in NHE3 activity. At 24 h there was an increase in NHE3 protein abundance but no increase in plasma membrane NHE3 or activity. At 48 h there were increases in total cellular NHE3, plasma membrane NHE3, and activity. While hypertonicity did induce a small increase in NHE3 mRNA abundance, this likely was not of sufficient magnitude to explain the increase in NHE3 protein abundance. The increase in protein abundance is most likely due to a direct effect of hypertonicity on NHE3 protein synthesis or degradation. In addition, studies with cycloheximide demonstrate that at least part of the response to hypertonicity can occur in the absence of protein synthesis.

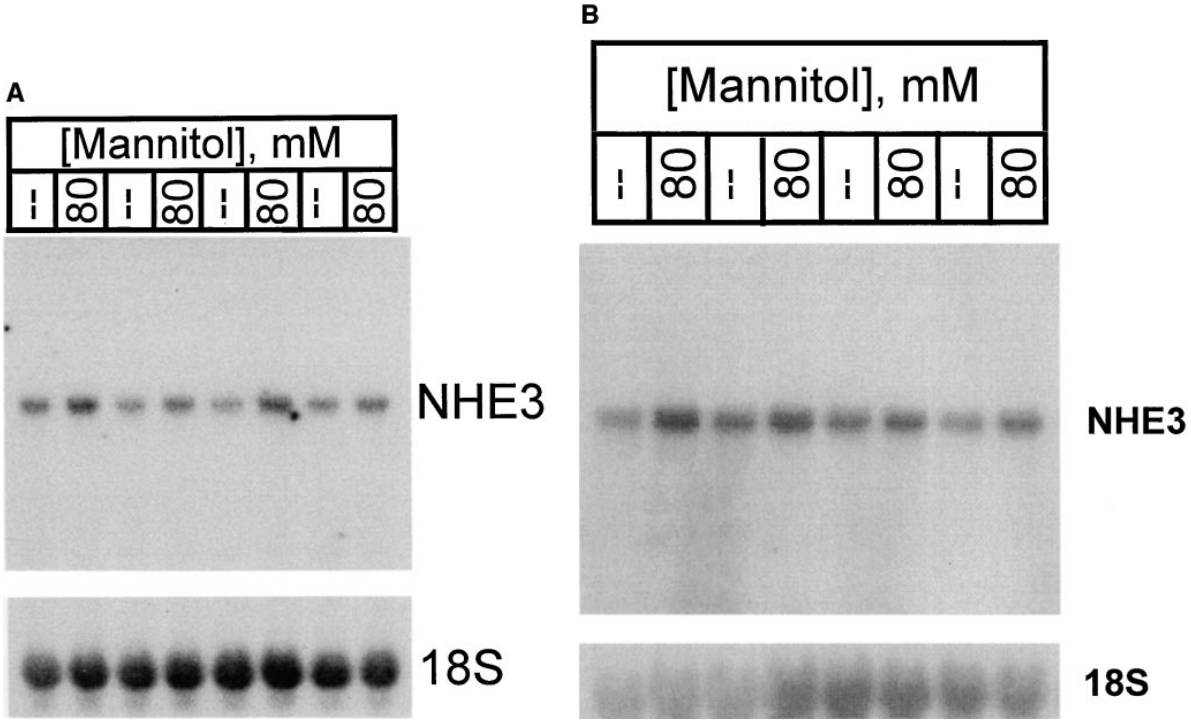

Figure 12. Mannitol increases NHE3 mRNA abundance. Cells were incubated in control media or media made hypertonic by addition of 80 mM mannitol for 24 (*A*, $n = 4$) or 48 h (*B*, $n = 11$). Blots were probed for NHE3 mRNA and 18S rRNA abundance.

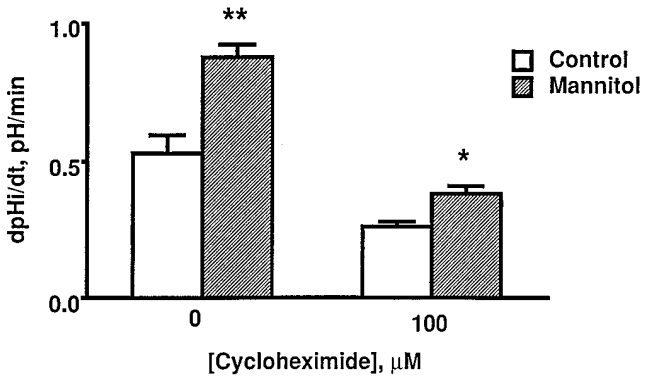

Figure 13. Increases in media osmolality increase Na/H antiporter activity in the absence of protein synthesis. Cells were preincubated in control media or media made hypertonic by addition of 80 mM mannitol. Cycloheximide was absent or present at 100 mM throughout the incubation. Cells were preincubated and assayed under isotonic (*Control*) or hypertonic (*Mannitol*) conditions. Na/H antiporter activity is plotted. [Cycloheximide] = 0, $n = 8$; [cycloheximide] = 100 mM, $n = 9$. $^*P < 0.05$ versus control; $^{**}P < 0.001$ versus control.

Increases in extracellular fluid osmolality acutely increase the activity of NHE1 and NHE2 expressed in antiporter-deficient Chinese hamster ovary cells (23). In the case of NHE1, hypertonicity-induced increases in Na/H antiporter activity are not associated with changes in NHE1 phosphorylation (25). NHE4 expressed in antiporter-deficient cells demonstrates no Na/H antiporter activity under isosmolar conditions, but is induced to express Na/H antiporter activity by acute increases in osmolality (26). Thus, the activities of NHE1, NHE2, and NHE4 are increased acutely by hyperosmolality. This response provides a mechanism for increased Na uptake in cell volume defense during hyperosmolality.

By contrast, NHE3 expressed in antiporter-deficient cells is inhibited acutely by hypertonic cell shrinkage (23). In agreement with this, the Na/H antiporter activities of rabbit proximal tubule suspensions, thick ascending limb, LLC-PK1 cells, and OK cells, cells which all express NHE3, are inhibited acutely by hyperosmolality (21, 22, 27). In all of these studies, changes in extracellular fluid osmolality have been large, frequently around 200 mOsm/liter. While these changes are physiologically relevant for the renal medulla, they are not physiologically relevant to cells in the renal cortex such as the proximal tubule. Nevertheless, in agreement with these results we found that hypertonicity acutely inhibits Na/H antiporter activity in OKP cells.

Chronic increases in extracellular fluid osmolality of 200 mOsm/liter for 72 h caused an increase in Na/H antiporter activity in a mouse inner medullary collecting duct cell line (28). This was accompanied by an increase in NHE2 mRNA abundance and a decrease in NHE1 mRNA abundance. In vascular smooth muscle cells, chronic increases in extracellular fluid glucose concentration of 15 mM caused an increase in Na/H antiporter activity and NHE1 mRNA abundance (29). This effect, which was due to glucose per se rather than osmolarity, may contribute to vasoconstriction and hypertension in diabetics.

The mechanism by which hypertonicity is sensed and elicits changes in cell function have not been determined in mammalian cells. In yeast cells, a cascade has been defined wherein hyperosmolality is sensed by transmembrane proteins (SlnI and ShoI), which activate parallel pathways leading to sequential activation of PbsII (a mitogen activated protein [MAP] kinase) and HogI (a MAP kinase) (30–32). Indeed, p38 and Jun NH2-terminal kinase (JNK), mammalian MAP kinases, are activated by hypertonicity and can complement mutations in HogI in yeast (33, 34). Activation of JNK by hypertonicity has recently been shown to be due to hypertonicity-induced clustering of receptors for epidermal growth factor, tumor necrosis factor, and interleukin-1, possibly triggered by perturbations of the cell surface (35).

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