Na,K-ATPase Response to Osmotic Stress in Primary Dog Lens Epithelial Cells

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**Purpose.** Na,K-ATPase activity increases in lens cells exposed to hypertonic stress. To test whether the increase in activity involves stimulation of Na,K-ATPase expression, dog lens epithelial cells were subjected to hypertonic stress, and the time course of Na,K-ATPase protein and mRNA response was measured.

**Methods.** Primary cultures of dog lens epithelial cells were maintained in isotonic or hypertonic media over the course of several days. Rubidium-86 uptake measurements, immunoreactive protein, and northern blot analysis were performed.

**Results.** Dog lens epithelial cells exposed to hypertonic stress from culture medium supplemented with 150 mM NaCl or 250 mM cellobiose showed a twofold increase in Na,K-ATPase activity. The increase in activity was blocked by cycloheximide and was reversible when the cells were returned to isotonic medium. This activity was unaffected by the aldose reductase inhibitor, tolrestat. Na,K-ATPase protein and mRNA levels increased in cells exposed to medium containing 150 mM NaCl. Northern blot analysis showed that the alpha-1 and beta-1 mRNA levels increased as early as 6 hours and maximally increased 1.5-fold to twofold by 12 to 24 hours.

**Conclusions.** Elevation of Na,K-ATPase activity in dog lens epithelial cells exposed to hypertonic stress was associated with increased expression of Na,K-ATPase subunit mRNAs and was dependent on protein synthesis. These results suggest that upregulation of the enzyme activity is the result of an induction of Na,K-ATPase. Invest Ophthalmol Vis Sci. 1995;36:88–94.

Na,K-ATPase is a ubiquitous membrane-bound enzyme that pumps Na⁺ and K⁺ across the cell membrane. It consists of two subunits, alpha and beta, found in equal molar ratios in the complex. Each subunit has several isoforms expressed in a tissue-specific manner. The alpha subunit is the catalytic subunit and contains the ATP and cardiac glycoside-binding sites. The role for the beta subunit has not been determined; however, its presence is required for activity. Na,K-ATPase has an important role in maintaining a proper electrochemical gradient across the cell membrane, in Na⁺-coupled cotransport systems, and in cell volume regulation.

Na,K-ATPase plays a critical role in lens homeostasis. Perturbation of the Na,K-pump can alter the levels of sodium and potassium, increase intracellular water content, and eventually cause loss of lens transparency. Pharmacologic agents, such as the Na,K-ATPase inhibitor ouabain, have been shown to affect the metabolism of the lens in organ culture and the metabolism of cultured bovine lens epithelial cells. By disrupting the Na⁺ and K⁺ gradients established across the cell membrane, this agent blocks the cellular transport of the organic osmolytes, taurine and myo-inositol. This effect has also been observed in hypertonically stressed human lens cells in which the enhanced accumulation of myo-inositol and taurine were minimized by ouabain treatment.

Regulation of Na,K-ATPase by specific hormones, ionic interventions, or pharmacologic agents can induce functional Na,K-pump sites and cause enhanced gene expression. Na⁺ directly regulates Na,K-ATPase gene expression in cardiocytes. Thyroid hormone elevates Na,K-ATPase mRNA in kidney mesangial cells and hypertonic stress has been shown to upregulate Na,K-ATPase expression in renal cortex cells.
We have reported the upregulation of aldose reductase and alphaB-crystallin mRNA and protein in cells cultured in hypertonic media, including dog lens epithelial (DLE) cells. Sorbitol and myo-inositol (MI) also increase in hypertonic conditions as a result of increased mRNA and activity of aldose reductase and sodium-dependent myo-inositol transporters. Recently, enhanced MI accumulation in hypertonicity stressed human lens epithelial cells was shown to be correlated with elevated Na,K-ATPase activity. The purpose of the present study was to examine the effect of hypertonic stress on Na,K-ATPase activity and to elucidate the mechanism involved. We report here that Na,K-ATPase activity, protein, and subunit mRNAs increase in hypertonically stressed DLE cells and that this increase is due to an induction or stabilization of Na,K-ATPase gene expression and not enzyme activation.

METHODS

Cell Culture

Lenses were dissected from eyes of young beagle dogs that were killed according to National Institutes of Health guidelines. Epithelial cells were isolated and cultured in Dulbecco’s modified Eagle’s medium with low glucose, 1 g/l, as previously described. Confluent cultures in the third passage were used in all experiments. Four to six plates (60 mm dishes) were used for western and northern blot analyses, and cells in 12-well plates were used in the enzyme assays. Cell cultures were exposed to various experimental conditions, including culture medium made hypertonic with the addition of 150 mM NaCl, (600 mOsm/kg), or 250 mM cellobiose (550 mOsm/kg), or hypertonic medium (600 mOsm/kg) with 5 μM cycloheximide. At the end of the experimental manipulations, the cells were washed with phosphate-buffered saline plus experimental components at 4°C. The cell homogenate was centrifuged at 1,000 g for 10 minutes at 4°C. The supernatant fraction was then added, and the samples were centrifuged for 20 minutes at 100,000 g for 60 minutes at 4°C. The resulting pellet was dissolved in 0.3 M sodium acetate and then extracted with phenol:chloroform (1:1) and then centrifuged; Beckman Instruments, Palo Alto, CA). The immunoblot analysis was performed using a Vectastain ABC kit according to the manufacturer’s protocols (Vector Laboratories, Burlington, CA). The primary Na,K-ATPase antibody was purchased from Accurate Chemical & Scientific (Westbury, NY) and was used at a dilution of 1:85.

Enzyme Assay

Ouabain-inhibited rubidium-86 (86Rb) uptake (Na,K-ATPase activity) was quantitated in the epithelial cells cultured in media made hypertonic by the addition of 150 mM NaCl or 250 mM cellobiose. Additional experiments were performed in the presence of 5 μM cycloheximide or 10 μM tolrestat (Wyeth–Ayerst Research, Princeton, NJ). For these experiments, ouabain was added to half the cultures 60 minutes before the addition of radiolabel. Cells in 12-well plates were incubated for 3 to 15 minutes with 86Rb, 1 μCi/ml of media, 8.4 mCi/mg. After the incubation with radiolabel, the cells were harvested as described above. 86Rb levels were quantitated by scintillation counting (TriCarb 2200 CA; Packard, Downers Grove, IL), and Na,K-ATPase activity was determined as the difference in 86Rb levels, normalized per milligram of cell protein, between cultures treated with and without ouabain. Results were expressed as the mean and standard deviation of the measurements from three separate experiments.

In preliminary experiments, the extent and rate of 86Rb uptake was independent of previous exposure to ouabain, which ranged from 15 to 60 minutes. Further, 86Rb uptake uninhibited by ouabain, 775 ± 50 DPM/mg protein/3 minutes, was not significantly different between control and hypertonically stressed cells. In control and hypertonically stressed cells, ouabain-inhibited uptake (Na,K-ATPase activity) was about 40% and 65%, respectively, of total 86Rb uptake measured in the absence of ouabain.

Western Blot Analysis

The cell homogenate was centrifuged at 1,000 g for 10 minutes at 4°C. The supernatant fraction was then centrifuged at 100,000 g for 60 minutes at 4°C. The pellet was dissolved in 250 μl of homogenizing buffer. Cell proteins (10 μg per lane) were separated by electrophoresis in SDS-polyacrylamide gels on the Novex mini-gel electrophoresis system (Novex, Encinitas, CA), and the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). The immunoblot analysis was performed using a Vectastain ABC kit according to the manufacturer’s protocols (Vector Laboratories, Burlington, CA). The primary Na,K-ATPase antibody was purchased from Accurate Chemical & Scientific (Westbury, NY) and was used at a dilution of 1:85.

RNA was isolated from the frozen cell pellets following the procedure of Davis et al. Briefly, the frozen cell pellet was solubilized immediately after the addition of guanidinium isothiocyanate. Cesium chloride was then added, and the samples were centrifuged for 20 hours at 174,000 g, 20°C (SW41 rotor, L8-80M Ultracentrifuge; Beckman Instruments, Palo Alto, CA). The resulting pellet was dissolved in 0.3 M sodium acetate and then extracted with phenol:chloroform (1:1) and...
precipitated in ethanol at -70°C for 30 minutes and spun at 12,000g for 30 minutes. The resulting RNA pellet was resuspended in a 1.1% agarose gel in the presence of formaldehyde as described by Davis et al.23 The RNA was transferred to a nylon membrane (ICN Biomedicals, Cleveland, OH), and the blots were probed with random primed 32P-labeled DNA (Gibco BRL, Gaithersburg, MD). Probes to the alpha-1 and beta-1 subunits of Na,K-ATPase were made using the polymerase chain reaction (PCR). PCR primers were designed from the rat (alpha) and the dog (beta) DNA sequence (24 and 25, respectively). The two primer sequences used to make the alpha probe were 5' TGGAACTCTGACTCAAGACGGGATGACAGT 3' and 5' GGAGATAAGACCGCACGAAGACAGGTTATC 3'. The two primer sequences used to make the beta probe were 5' GATTGTGGTACATTATGCATGC 3' and 5' CGGTCTTTCTCATGTAACC 3'. RT-PCR (Perkin-Elmer, Norwalk, CT) was performed on dog kidney endothelial RNA to make a 637-base pair insert for alpha and a 496-base pair insert for beta. The PCR products were cloned into the pCRl000 vector (Invitrogen, San Diego, CA), and the inserts were sequenced using the Promega fmol sequencing system (Promega, Madison, WI) and 33P-ATP (DuPont NEN, Boston, MA). The 18S rRNA probe was a gift of Dr. B. Holmes (Cornell University, Ithaca, NY). The mRNA was quantified by scanning the x-ray films on an UltraVision UC111200S (UMAX Technologies; MacNews, Evanston, IL) using Photoshop and NIH Image software.

RESULTS

Activity Response

Primary cultures of dog lens epithelial cells were exposed to 150 mM NaCl for as long as 4 days (Fig. 1). There was approximately a twofold increase in Na,K-ATPase activity, as measured by 86Rb uptake. The maximal response was evident within 24 hours of treatment and was maintained through the course of the experiment (96 hours). A similar increase was observed when 250 mM cellobiose was used as the osmotic agent. The increase in Na,K-ATPase activity observed from hypertonic stress was blocked when cells were exposed to hypertonic medium and 5 μM cycloheximide for 24 hours. Cell viability, as measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide,26 and Na,K-ATPase activity in control cells were unaffected by cycloheximide treatment (data not shown).

Protein Response

Total epithelial cell proteins were separated by SDS-PAGE. The proteins were transferred to nitrocellulose and identified using a polyclonal antibody to Na,K-ATPase. Figure 3 shows that both the alpha (100-kD) and beta (35-kD) subunits increased in cells grown in media supplemented with 150 mM NaCl.

RNA Response

Complete sequence for the dog alpha-1 cDNA has not been previously reported. We report here a 577-nucleotide sequence for dog kidney alpha-1 (Fig. 4). It is similar to the human HEK293 alpha-1 sequence,27 the pig kidney alpha-1 sequence,28 and the rat brain alpha-1 sequence.24 It shares significant nucleotide homology with human (89%), pig (89%), and rat (85%).
FIGURE 2. Na,K-ATPase activity (86Rb uptake) in dog lens epithelial cells in the presence of the aldose reductase inhibitor, tolrestat, and after removal of osmotic stress. Primary lens cells were grown in the presence of 150 mM NaCl with or without 10 μM tolrestat. For reversal experiments, lens cells were grown in the presence of 150 mM NaCl for 3 days, at which time the NaCl was removed and the cells were placed in isotonic media for 2 days before assaying activity. The ouabain-inhibited 86Rb uptake (Na,K-ATPase activity) was determined and graphed as a percent of control. Data are expressed as the mean ± standard deviation (bars) of the results from three separate experiments.

FIGURE 3. Western blot analysis of Na,K-ATPase. Primary dog lens epithelial cells were grown in 150 mM NaCl and harvested at 0 (lane 1), 12 (lane 2), 24 (lane 3), 48 (lane 4), and 72 (lane 5) hours. Total lens proteins (10 μg/lane) were electrophoresed under denaturing conditions. The proteins were transferred to nitrocellulose. The alpha (100-kD) and beta (35-kD) subunits of Na,K-ATPase were detected using a polyclonal antibody.

FIGURE 4. Nucleotide sequence comparison of the dog kidney alpha-1 clone to the human alpha-1 (24), the pig kidney alpha-1 (25), and the rat brain alpha-1 (22). The numbering pertains to the dog nucleotide sequence that does not include the rat-derived polymerase chain reaction primers. The human sequence begins at nucleotide 1782, the pig sequence begins at nucleotide 1491, and the rat sequence begins at nucleotide 1701. The small letters represent base changes, and the dots represent sequence identity. At the amino acid level, the homology is even greater: human 95%, pig 94%, and rat 92%. The sequence of the dog beta-1 PCR clone (data not shown) was 100% identical to the previously published sequence.25 Northern blots of total RNA from lens epithelial cells cultured in 150 mM NaCl were probed with DNA specific (as seen by sequence homology described above) to the alpha-1 and beta-1 subunits of Na,K-ATPase. The alpha-1 subunit mRNA was elevated in cells exposed for 6 hours to hypertonic media (Fig. 5). This increased level of mRNA (approximately 1.5-fold) was maintained throughout the time course (48 hours). The beta-1 probe detected an increase in two mRNA bands, a major band and a larger, less prominent upper band. Although the increase in the major...
FIGURE 5. Northern blot of Na,K-ATPase alpha-1 and beta-1 subunit mRNAs. Primary lens epithelial cells were grown in 150 mM NaCl and harvested at 0, 6, 12, 24, and 48 hours. Cells were also grown for 48 hours in isotonic media (48°C). Total RNA (7.5 µg) was run on a 1.1% agarose gel in the presence of formaldehyde. The RNA was transferred to a nylon membrane and hybridized sequentially with DNA probes corresponding to the alpha-1 and beta-1 subunits of Na,K-ATPase and to an 18S rRNA control probe, used to verify accuracy of RNA loading.

The beta-1 mRNA band was not as rapid as that of the alpha-1 subunit, by 24 hours the level of induction of both the major beta-1 band and alpha-1 band was similar (approximately 1.5-fold). The upper band of the beta-1 mRNA was barely visible in unstimulated cells; however, by 24 hours in 150 mM NaCl, the level of this mRNA had doubled.

DISCUSSION

Primary cultures of dog lens epithelial cells respond to hypertonic stress by upregulation of Na,K-ATPase at the mRNA, the protein, and the activity levels. The alpha-1 subunit mRNA increased by 1.5-fold, and the multiple beta-1 mRNAs increased by 1.5-fold to twofold. Multiple beta mRNA bands have been reported previously. The bands are found in various proportions in different tissues. These mRNAs are the result of different mRNA processing, and all encode the same protein. Both bands were observed to respond to osmotic stress. The level of induction of the alpha and beta mRNAs is similar to that reported for renal cortex cells when stressed by NaCl.

A twofold increase in Na,K-ATPase activity in response to osmotic stress was produced by both NaCl and cellobiose. Thus, it appears that the Na,K-ATPase increase is a general response to hypertonic stress and not just due to the increase in Na+. Recent reports have also shown an increase in Na,K-ATPase activity due to osmotic stress in other cell types (human renal cortex cells, human lens and retinal pigment epithelial cells), but to slightly lower levels than we observed.

The increase in Na,K-ATPase activity is blocked by the addition of cycloheximide. This suggests that new protein synthesis is necessary to achieve the elevated levels of activity. Thus, osmotic stress appears to stimulate de novo synthesis of Na,K-ATPase. This synthesis of new protein is consistent with the observed increase of alpha and beta protein subunits on Western blot analysis. In addition, the increase in alpha and beta mRNAs suggest that osmotic stress is producing an increase in Na,K-ATPase gene expression, although stabilization of mRNA cannot be ruled out by our data. A similar increase in Na,K-ATPase mRNA levels in renal cells produced in response to osmotic stress was blocked by the addition of actinomycin D and, thus, appears to be a result of increased gene expression. The stimulation of activity in DLE cells is reversible. The removal of osmotic stress returns Na,K-ATPase levels to baseline within 48 hours.

In studies on animal and cultured cell models of diabetic complications, an interrelationship has been shown to exist between sorbitol, MI, and Na,K-ATPase. Administration of ARIs that block the cellular accumulation of sorbitol and depletion of MI preserve Na,K-ATPase activity. Our previous work on DLE cells has demonstrated that sorbitol and MI are also coordinately regulated by hypertonic stress. Both sorbitol and MI showed increased levels by 24 hours in dog lens epithelial cells stimulated by NaCl. The addition of the ARI tolrestat (Alredase) prevented the accumulation of sorbitol, whereas MI accumulation was unaffected. The accumulation of these compatible organic osmolytes increases cellular turgor, an adaptive response to prevent water loss. We have now observed that Na,K-ATPase activity, as well as protein and mRNA, increase during hypertonic stress in lens cells. Under these conditions, blocking aldose reductase activity with tolrestat had no effect on Na,K-ATPase activity. From these studies, it would appear that Na,K-ATPase, MI, and polyol respond to both hypertonic and diabetic stress in a coordinate manner, albeit the response in hypertonic stress is an upregulation of transport proteins and an accumulation of compatible organic osmolytes.

In summary, Na,K-ATPase activity, protein, and mRNA levels increase in response to osmotic stress. The increase in activity is reversible by removing the
osmotic stress, and the increased activity is blocked by cycloheximide. Therefore, increased Na,K-ATPase activity in cells exposed to hypertonic stress is most likely the result of the induction of Na,K-ATPase expression.

Key Words
Na,K-ATPase activity, Na,K-ATPase mRNA, hypertonic stress, lens cell culture

References
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