Hypertonic Stress Increases NaK ATPase, Taurine, and Myoinositol in Human Lens and Retinal Pigment Epithelial Cultures

Tomihisa Yokoyama, Li-Ren Lin, Bhargavan Chakrapani, and Venkat N. Reddy

Purpose. Recent evidence suggests that taurine and myoinositol may serve as organic osmolytes in a number of cells, including lens and retinal pigment epithelia, but the mechanism for their increased accumulation in response to hypertonic stress is not known. To assess whether NaK ATPase contributed to the elevated levels of taurine and myoinositol in cells exposed to hypertonic media, we measured the activity of NaK ATPase, which is known to be implicated in the transport of these substances, in human lens and retinal pigment epithelia cultured in isotonic and hypertonic media.

Methods. Primary cultures of human lens epithelial (HLE) and human retinal pigment epithelial (HRPE) cells were maintained in isotonic and hypertonic media for varying periods of time, and the activity of NaK ATPase and the levels of taurine and myoinositol were measured in cells cultured under two different conditions. The possible involvement of the transport enzyme in the accumulation of the two osmolytes was also investigated by inhibiting the enzyme with ouabain.

Results. When primary cultures of HLE and HRPE were exposed to hypertonic medium containing NaCl (600 mOsm) or cellobiose (500 mOsm) for 72 hours, the concentration of taurine and myoinositol in HLE cells increased by 218% and 558% of control, respectively, in NaCl medium, whereas the corresponding increases in cellobiose medium were 147% and 439%. In HRPE cells, the increase in myoinositol levels in the two hypertonic media was more dramatic than that in taurine. Concomitant with the increase in the concentration of the osmolytes, there was an increase in NaK ATPase activity in both cell types. Although the accumulation of taurine in HLE cells in hypertonic media in a 6-hour culture was essentially prevented by $10^{-8}$ mmol/l ouabain, myoinositol levels were affected to a lesser, but still significant, extent. In HRPE cells, which were cultured for 24 hrs in the presence of $10^{-6}$ mmol/l ouabain, there was a more direct correlation between the inhibition of NaK ATPase and the decreased accumulation of taurine and myoinositol in the hypertonic media.

Conclusion. Although the exact mechanism by which NaK ATPase activity increases in response to hypertonic stress remains to be established, the increased activity of the enzyme is related to the enhanced accumulation of the organic osmolytes, taurine, and myoinositol, in HLE and HRPE cells cultured in hypertonic medium.

A preliminary report of these findings was presented at the Annual Meeting of the Association for Research in Vision and Ophthalmology, Sarasota, Florida, May 1992.

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A number of studies have shown that exposure of mammalian cells to hypertonic stress results in an increase in intracellular small organic solutes.1 Sorbitol, myoinositol, betaine, and taurine have been identified as osmolytes in renal medulla cells.2,3 Recently, we have shown the presence of polyol pathway in primary cultures of human lens epithelial (HLE) and human
retinal pigment epithelial (HRPE) cells. Furthermore, when HRPE cells are cultured in hypertonic media by the addition of 150 mM NaCl, there is an induction of aldose reductase (AR), a key enzyme in the polyol pathway; there is also a corresponding increase in mRNA with a concomitant increase in the accumulation of polyol. HRPE cells also accumulate myoinositol and free amino acids as a result of hypertonic stress. These findings demonstrated that HRPE cells have a regulatory system governing intracellular osmolarity similar to that observed in renal tissue. However, the mechanism by which cellular organic osmolytes increases in response to hypertonic stress is not known.

A common feature of the lens, retinal pigment epithelium, and renal tissue is that they are target tissues involved in diabetic complications. It has been proposed that the initial mechanisms involved in diabetic complications of these tissues is intracellular hypertonicity and the resultant hydration from polyol accumulation. Taurine and myoinositol transport systems in the lens have been shown to be dependent on extracellular Na. Na- and K-stimulated ATPase, a membrane-bound enzyme, plays a critical role in maintaining sodium and potassium ion gradients across the membrane by extruding sodium from and pumping potassium into cells. A specific inhibitor of this enzyme, ouabain, is known to inhibit taurine and myoinositol uptake in rabbit lens in vitro, suggesting that the uptake of both osmolytes into the lens is influenced by NaK ATPase-dependent electrochemical gradients of sodium and potassium ions. In this report, we present evidence that the primary cultures of HLE and HRPE cells respond to extracellular hypertonicity by increasing the activity of the transport enzyme, NaK, which in turn may enhance the accumulation of taurine and myoinositol in the cell.

MATERIALS AND METHODS

Primary cultures of HLE (through passage 3) and HRPE cells (through passage 5 or 6) were established according to the methods described previously. Four hundred thousand cells per well were transferred to 6 well plates and cultured for 3 days with Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% and 20% fetal bovine serum (Gibco, Grand Island, NY) for HLE and HRPE cells, respectively. The culture medium was then replaced with DMEM supplemented with either 150 mM NaCl (total osmolarity, 600 mOsm) or 200 mM cellobiose (total osmolarity, 500 mOsm) and cultured for an additional 6 to 72 hrs. Before scraping, the cells were rinsed 3 times with isotonic NaCl solution. The research followed the tenets of the Declaration of Helsinki, informed consent was obtained, and the research was approved by our institutional review board on human investigation.

For the analysis of taurine, cells were scraped from the dish and collected into 600 μl of 0.1 N HCl containing 0.2 mM L-methionine sulfonate as an internal standard. The cells were sonicated with a Branson Cell Disrupter 200 (Danbury, CT), centrifuged at 7,000 g for 15 min, and the supernatant was filtered with an Ultrafree-MC filter (Millipore Corp., Millipore Products Division, Bedford, MA). The free amino acids in the filtrate were derivatized with phenylisothiocyanate and analyzed with a reverse phase HPLC System, PICO TAG Amino Acid Analysis System (Millipore Corporation, Waters Chromatography Division, Bedford, MA).

For the determination of myoinositol, the cells were scraped into 400 μl of 2% ZnSO4, sonicated, and neutralized by adding an equivalent amount of 2% Ba(OH)2. The samples were centrifuged and the supernatants lyophilized. Just before the analysis with a gas chromatograph, lyophilized samples were derivatized with Tri-Sil Z (Pierce, Rockford, IL). The gas chromatograph assay conditions for myoinositol were the same as previously used.

For the assay of NaK ATPase, HLE and HRPE cells were scraped and collected in 200–500 μl of deionized water and sonicated. The cell homogenates were used as an enzyme preparation. NaK ATPase activity was measured according to the method described previously. Composition of the reaction mixture used was: Tris (100 mM), ATP (2 mM), Mg2+ (1 mM), K+ (5 mM), Na+ (58 mM), CN− (10 mM), EDTA (0.1 mM), ouabain (1 mM). NaK ATPase activity was calculated from the difference in inorganic phosphate liberated during the reaction with or without ouabain. Because large variations were observed in primary cultures established from different eyes, cells derived from the same eye were employed for control and experiment involving the studies of various parameters. The data are expressed as μmol/L of inorganic phosphate liberated per 10⁶ cells. In each experiment, the cell number was determined from another batch of cells cultured under the same conditions as the experiment.

RESULTS

Effect of Hypertonic Stress

The effect of hypertonicity on taurine, myoinositol, and NaK ATPase in HLE and HRPE cells was investigated by culturing them in media supplemented with either 150 mM NaCl (600 mOsm) or cellobiose (500 mOsm) for a period of 72 hrs. The results are summarized in Table 1. Because these experiments involved the use of primary cultures rather than cell lines, there were large variations with respect to cell size and cellu-
TABLE 1. Effects of Hypertonic Stress on Intracellular Concentration of Taurine and Myoinositol and Na⁺-K⁺ ATPase Activity in HLE and HRPE Cells cultured for 72 hr

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Taurine (nmol/10⁶ Cells)</th>
<th>Percent of Control</th>
<th>Myoinositol (nmol/10⁶ Cells)</th>
<th>Percent of Control</th>
<th>Na⁺-K⁺ ATPase (μmol Pi/hr/10⁶ Cells)</th>
<th>Percent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLE cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (isotonic)</td>
<td>32.2 ± 5.1 (3)</td>
<td>100</td>
<td>111 ± 32 (4)</td>
<td>100</td>
<td>0.120 ± 0.064 (6)</td>
<td>100</td>
</tr>
<tr>
<td>Hypertonic (150 mmol/l NaCl)</td>
<td>70.3 ± 2.8 (3)*</td>
<td>218</td>
<td>618 ± 41 (3)*</td>
<td>558</td>
<td>0.184 ± 0.018 (6)*</td>
<td>153</td>
</tr>
<tr>
<td>Control (isotonic)</td>
<td>29.2 ± 1.8 (3)</td>
<td>100</td>
<td>182 ± 35 (5)</td>
<td>100</td>
<td>0.172 ± 0.054 (5)</td>
<td>100</td>
</tr>
<tr>
<td>Hypertonic (200 mmol/l cellobiose)</td>
<td>43.0 ± 6.7 (3)*</td>
<td>147</td>
<td>799 ± 152 (5)*</td>
<td>439</td>
<td>0.283 ± 0.112 (4)</td>
<td>165</td>
</tr>
<tr>
<td>HRPE cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (isotonic)</td>
<td>29.8 ± 1.8 (3)</td>
<td>100</td>
<td>27 ± 1 (4)</td>
<td>100</td>
<td>0.072 ± 0.018 (7)</td>
<td>100</td>
</tr>
<tr>
<td>Hypertonic (150 mmol/l NaCl)</td>
<td>41.5 ± 2.5 (3)*</td>
<td>141</td>
<td>333 ± 34 (4)*</td>
<td>1254</td>
<td>0.123 ± 0.024 (8)*</td>
<td>171</td>
</tr>
<tr>
<td>Control (isotonic)</td>
<td>14.9 ± 0.6 (3)</td>
<td>100</td>
<td>34 ± 13 (6)</td>
<td>100</td>
<td>0.064 ± 0.024 (7)</td>
<td>100</td>
</tr>
<tr>
<td>Hypertonic (200 mmol/l cellobiose)</td>
<td>34.8 ± 0.3 (3)*</td>
<td>233</td>
<td>465 ± 42 (6)*</td>
<td>1368</td>
<td>0.118 ± 0.036 (8)*</td>
<td>184</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of three to eight determinations. Numbers in parentheses are sample sizes.
* P < 0.05 as compared to control.

lar content. For this reason, the values for different controls under similar experimental conditions are not necessarily the same. Therefore, in comparing the effect of hypertonicity or ouabain on a specific parameter, it was necessary to use the same batch of cells for control and experiment. In HLE cells cultured in DMEM supplemented with NaCl, the intracellular levels of taurine and myoinositol increased to 218% and 558% of control, respectively. The corresponding values in HRPE cells cultured under the same condition were 141% and 1254% of control. The extent of increase in myoinositol was more dramatic than that of taurine in both types of cells. However, myoinositol accumulation under hypertonic stress was much greater in HRPE cells than in HLE cells.

The activities of NaK ATPase in HLE and HRPE cells, measured at the end of the culture period using cell homogenates as an enzyme preparation, were found to be 0.120 μmol/l inorganic phosphate (Pi)/hr·10⁶ cells and 0.072 μmol/l Pi/hr·10⁶ cells, respectively. Figure 1 shows the time course of the effect of hypertonic NaCl (600 mOsm) on HRPE cells. The activity of the enzyme appeared to be unaffected for the first 12 hrs of culture. However, at the end of 24 hrs, there was nearly a twofold increase (209%) in enzyme activity, and it was maintained approximately at this level until the end of 72-hr culture. This increased activity after 24 hrs was found to be significant (P < 0.05). When the cells were cultured in a medium supplemented with 150 mM NaCl for 72 hrs, NaK ATPase activities in HLE and HRPE cells were 153% and 171% of control, respectively (Table 1). Hypertonicity of the medium produced by the addition of 200 mmol/l cellobiose (500 mOsm), a nonionized and membrane-impermeable molecule, also resulted in an increase in the concentration of taurine and myoinositol as well as the activity of the transport enzyme similar to those in NaCl-supplemented media (Table 1). Although the extent of increase in taurine varied somewhat under two hypertonic conditions, the increase in myoinositol concentration and NaK ATPase activity in
both types of cells cultured in the cellobiose-supplemented medium were comparable to those in the NaCl-supplemented medium.

**Effect of Ouabain**

In preliminary experiments, it was found that HLE cells were much more sensitive to ouabain than HRPE cells and showed morphologic abnormalities and cell death when cultured in a $10^{-6}$ M ouabain-containing medium for 6 hrs. However, HRPE cells maintained normal morphology and cell growth when cultured in the same concentration of ouabain even for 24 hrs. Therefore, the effect of ouabain on the biochemical parameters was examined by employing two different concentrations of the inhibitor. HLE cells were exposed to $10^{-8}$ M ouabain and cultured for 6 hrs, whereas HRPE cells were exposed to $10^{-6}$ M ouabain and cultured for 24 hrs. The results, summarized in Table 2, show that when HLE cells were exposed to $10^{-8}$ M ouabain for 6 hrs in an isotonic medium, NaK ATPase activity was inhibited by 33% of control, and the increased enzyme activity induced by hypertonic stress was also prevented. Although intracellular taurine concentration was unaffected in the presence of ouabain in isotonic media, the elevation of taurine concentration in hypertonic media was prevented by ouabain and remained near the control value. However, at this concentration of ouabain ($10^{-8}$ M), myoinositol levels decreased slightly in the isotonic medium (by 16% of control, not significant) and myoinositol's accumulation from hypertonic stress was minimized.

In HRPE cells cultured for 24 hrs, in which a higher concentration of ouabain ($10^{-6}$ M) was employed, NaK ATPase activity was strongly inhibited (by 90%). Under hypertonic stress, the enzyme activity was twice that of the cells cultured in the isotonic medium but decreased to 26% of control in the presence of ouabain. Taurine and myoinositol concentrations in HRPE cells cultured in the isotonic medium containing $10^{-6}$ M ouabain were 42% and 21% of control, respectively, and their increase by hypertonic stress was prevented by the addition of $10^{-6}$ M ouabain to the medium.

In summary, the results suggest that the increase in NaK ATPase occurs simultaneously with the enhanced accumulation of taurine and myoinositol in cells subjected to hypertonic stress.

**DISCUSSION**

The results of this study further confirm the previous findings that HRPE cells can accumulate significant amounts of taurine and myoinositol when cultured in DMEM supplemented with 150 mM NaCl. The increase in cellular concentration of taurine and myoinositol due to hypertonic stress was also observed in HLE cells (Table 1). Hypertonicity elicited by adding 200 mM cellobiose, a nonionized and membrane impermeable molecule, increased intracellular concent-

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**TABLE 2. Effects of Ouabain on Na$^+$-K$^+$ ATPase Activity and Intracellular Concentration of Taurine and Myoinositol in HLE and HRPE Under Different Culture Conditions**

<table>
<thead>
<tr>
<th>Culture Medium</th>
<th>Na$^+$-K$^+$ ATPase (μmol Pi/hr/10⁶ Cells)</th>
<th>Percent of Control</th>
<th>Taurine (nmol/10⁶ Cells)</th>
<th>Percent of Control</th>
<th>Myoinositol (nmol/10⁶ Cells)</th>
<th>Percent of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLE cells (6 hr culture)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (isotonic)</td>
<td>0.124 ± 0.021 (4)</td>
<td>100</td>
<td>20.4 ± 0.7 (3)</td>
<td>100</td>
<td>111 ± 32 (4)</td>
<td>100</td>
</tr>
<tr>
<td>Control + 0.01 μmol/l OUA</td>
<td>0.083 ± 0.035 (3)</td>
<td>67</td>
<td>20.0 ± 3.2 (3)</td>
<td>98</td>
<td>93 ± 17 (4)</td>
<td>84</td>
</tr>
<tr>
<td>Hypertonic</td>
<td>0.198 ± 0.046 (4)*</td>
<td>157</td>
<td>29.1 ± 3.6 (3)*</td>
<td>143</td>
<td>618 ± 41 (3)*</td>
<td>557</td>
</tr>
<tr>
<td>Hypertonic + 0.01 μmol/l OUA</td>
<td>0.108 ± 0.025 (4)†</td>
<td>87</td>
<td>18.5 ± 2.0 (3)†</td>
<td>91</td>
<td>476 ± 30 (4)†</td>
<td>429</td>
</tr>
<tr>
<td>HRPE cells (24 hr culture)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control (isotonic)</td>
<td>0.058 ± 0.016 (6)</td>
<td>100</td>
<td>17.1 ± 0.8 (3)</td>
<td>100</td>
<td>20.5 ± 3.8 (4)</td>
<td>100</td>
</tr>
<tr>
<td>Control + 1 μmol/l OUA</td>
<td>0.006 ± 0.025 (6)*</td>
<td>10</td>
<td>7.1 ± 0.1 (3)*</td>
<td>42</td>
<td>4.2 ± 0.6 (4)*</td>
<td>21</td>
</tr>
<tr>
<td>Hypertonic</td>
<td>0.121 ± 0.028 (8)*</td>
<td>209</td>
<td>37.8 ± 3.7 (3)*</td>
<td>221</td>
<td>70.4 ± 12.6 (4)*</td>
<td>344</td>
</tr>
<tr>
<td>Hypertonic + 1 μmol/l OUA</td>
<td>0.015 ± 0.031 (7)†</td>
<td>26</td>
<td>14.3 ± 0.6 (3)*†</td>
<td>84</td>
<td>19.6 ± 3.0 (4)†</td>
<td>96</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD of three to eight determinations. Hypertonic medium was made by adding 150 mmol/l NaCl to normal culture medium. In experiments of myoinositol in HLE cells, cells were pre-cultured in normal medium or hypertonic medium for 66 hr. The media then were replaced with isotonic media with and without OUA and cultured for an additional 6 hr. Numbers in parentheses are sample sizes.

OUA = ouabain.

*P < 0.05 as compared with control.
†P < 0.05 as compared to hypertonic (Student's t-test).
toration of taurine and myoinositol in both types of cells. These results indicate that taurine and myoinositol act as osmolytes in HLE and HRPE in response to hypertonicity.

To provide a clue to the possible mechanism involved, NaK ATPase activity was determined in HLE and HRPE cells cultured in DMEM supplemented with either 150 mM NaCl or 200 mM cellobiose. The hypertonic stress resulted in an increase in NaK ATPase activity in both cell types, suggesting that the enhanced accumulation of the osmolytes in these cells may be the result of elevated transport enzyme activity. The observed increase in NaK ATPase may be either the result of an activation or a possible induction of the enzyme in both types of cells exposed to hypertonic stress. When NaK ATPase activity was inhibited by its specific inhibitor, ouabain, accumulation of taurine and myoinositol in HLE and HRPE cells was prevented or minimized. These results indicate that increased NaK ATPase has a close relationship with the increased intracellular concentration of taurine and myoinositol induced by hypertonicity in both HLE and HRPE cells.

Taurine and myoinositol transport in lens has been reported to depend on extracellular sodium and is inhibited by ouabain, a specific inhibitor of NaK ATPase. Recently, Miyamoto et al have characterized the uptake of taurine in the apical membrane vesicle from bovine retinal pigment epithelia. The uptake was stimulated markedly by the presence of an inwardly directed NaCl gradient but not an Na gradient alone across the membrane. Transporters of taurine and myoinositol in Madin-Darbin canine kidney (MDCK) cells have been expressed in Xenopus laevis oocytes. Poly(A) + RNA from MDCK cells cultured in a hypertonic solution elicited higher taurine and myoinositol uptake activities than from MDCK cells cultured in an isotonic solution, suggesting increased activity of taurine and myoinositol transporters.

Although the results of the present study show an elevated level of NaK ATPase associated with the increased accumulation of taurine and myoinositol in the cells exposed to hypertonic stress, the data do not provide evidence as to whether hypertonicity affects activation of the transport enzyme or its induction. Because hypertonic conditions, elicted by 150 mM NaCl or by 200 mM cellobiose, increased NaK ATPase activity approximately to the same extent, sodium ion influx may occur under both conditions. The increased sodium ion in the cell may result in the activation or in the induction of NaK ATPase. The latter possibility appears likely in view of a recent report on the expression of NaK ATPase in dog lens epithelial cells exposed to hypertonic media. In the aforementioned study, changes in enzyme activity appeared to result from de novo synthesis because activity was not increased when protein synthesis was blocked with cyclohexamide. It is also possible that in addition to the observed changes in the activity of NaK ATPase, hypertonicity may increase the activity of specific transporters of taurine and myoinositol, leading to higher uptake of these two organic osmolytes. Thus, there is a need to examine further the effect of hypertonicity on the activity of the specific transporters responsible for taurine and myoinositol uptake, which may lead to a clarification of the precise mechanism involved.

Key Words
human lens epithelium, human retinal pigment epithelium, tissue culture, hypertonic stress, NaK ATPase, taurine, myoinositol

References
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