Osmosensitive Taurine Transporter Expression and Activity in Human Corneal Epithelial Cells

Ryo Shioda,1 Peter S. Reinach,2 Tatsubiro Hisatsune,1 and Yusei Miyamoto1

PURPOSE. To characterize in SV40-immortalized human corneal epithelial cells (tHCEC) osmosensitive taurine transporter gene and protein expression as well as its functional activity. To evaluate whether medium supplementation with taurine improves cell viability during a hypertonic challenge.

METHODS. tHCEC were preincubated for up to 48 hours in hypertonic DMEM medium (i.e., up to 500 mosmol/kg). Taurine uptake was monitored through measurements of intracellular [3H]taurine accumulation. Gene and protein expression was detected by Northern and Western blot analyses, respectively. An amino acid analyzer measured intracellular cold taurine content. The live/dead assay evaluated with confocal microscopy determined cell viability.

RESULTS. Na+-dependent taurine uptake occurred in an isotonic (310 mosmol/kg) medium. The apparent Michaelis-Menten constant, $K_m$, for taurine was 4.6 $\mu$M, and uptake increased as a function of exposure time and rises in osmolality. Exposure for 12 hours to a 450 mosmol/kg medium increased uptake by 4.1-fold. However, after 48 hours of exposure to this medium, taurine uptake returned to its isotonic level. With time, biphasic changes occurred in taurine transporter gene and protein expression and taurine transport activity as well as elevating intracellular taurine content by 4.5-fold. Taurine medium supplementation for 48 hours improved cell viability.

CONCLUSIONS. tHCEC express Na+-dependent osmosensitive taurine transport activity. The hypertonic-induced biphasic effects on gene and protein expression as well as transport activity suggest feedback regulation of these responses. Rises in intracellular taurine do not appear to be essential for osmoregulation, but instead enhance cell survival perhaps through a membrane stabilizer or an antioxidant effect. (Invest Ophthalmol Vis Sci. 2002;43:2916–2922)

The corneal epithelium provides an essential barrier function in protecting the cornea from damage by noxious and infectious agents.1 Such challenge to this function includes corneal epithelial exposure to a hypertonic stress. This condition may be a component of the ocular surface disease dry eye because the tears may become hypertonic in this syndrome.2 In this case, barrier function may be compromised as the result of disruption of the tight junctions and/or epithelial cell volume shrinkage.5–7 Loss of this function could lead to corneal swelling and losses in transparency. As in many other epithelia, there is evidence in the corneal epithelium that hypertonicity activates a regulatory volume response, which offsets shrinkage by stimulating Na+K2Cl cotransport activity.5–7 Such a response is referred to as a regulatory volume increase (RVI) and results in increases in intracellular osmolyte accumulation.8,9 This RVI response in many other epithelia can in part be the consequence of the activation of the uptake of organic osmolytes such as taurine.10 Even though in human tears taurine has been reported to be present at 1.2 mM,11 there have been no studies on corneal epithelium describing either taurine transport or a possible functional role for this amino acid in this tissue.

In some other epithelia, one of the physiological roles of taurine, a β-aminoisulfonic acid, includes being a compatible osmolyte.10,12–14 For example, in the Madin-Darby canine kidney (MDCK) cell line, their taurine content increases twofold after a rise in medium osmolality from 300 to 600 mosmol/kg.14 However, it was not indicated whether the measured doubling of taurine intracellular content was adequate for taurine to provide an essential role as an osmolyte in restoring osmotic equilibrium between the internal and external milieu. Such increases are known to be in part accountable for by stimulation of a cell inward directed sodium- and chloride-dependent taurine transporter. This transporter has been identified in a host of epithelia and mediates NaCl:taurine transport with a stoichiometric ratio of 2:1:1.15 In MDCK cells and a rabbit renal papillary epithelial cell line (PAP-HT25), adaptation to imposed hypertonic stress includes transcription of genes that encode proteins (specific enzymes and transporters) directly involved in the metabolism and transport of organic osmolytes such as sorbitol, betaine, and inositol.12,16 Furthermore, taurine transporter gene expression is upregulated by hypertonicity in Caco-2 cells,17 MDCK cells,1,4 hepatocytes,18 H4IIE cells,19 and astrocytes.19 In Caco-2 cells, this hypertonicity-induced upregulation is dependent on both the culturing time and the level of hyperosmolality.17 In all cases, these increases are associated with rises in taurine uptake from the bathing medium. However, it was not possible to document until very recently that hypertonicity-induced increases in taurine transporter gene expression and functional activity may also be associated with an increase in taurine transporter protein expression. This limitation existed because of lack of availability of a selective antitaurine transporter antibody.

We chose to probe for osmosensitive taurine transport activity in tHCEC because one of us showed that during chronic exposure of tHCEC to osmotic stress these cells partially adapt to such a challenge.7 It was possible that increases in taurine uptake may have been providing increased osmolyte levels because cell proliferation persisted at a diminished level despite inhibition of another osmosensitive membrane uptake mechanism: the Na+K2Cl cotransporter. Another possible protective function of an osmosensitive uptake process is that it could contribute to the expression of responses that help counter hypertonic-induced oxidative stress. For example, in murine renal medullary cells hypertonicity induces as a result of oxidative stress increases in the expression of heme oxygenase-1.20 It is conceivable that hypertonicity-induced rises in taurine could support such a protective role because it is...
known to support cell survival during such a challenge.\textsuperscript{21} For taurine to provide either one or both of these functions in tHCEC, a osmosensitive taurine transport process would elicit rises in intracellular taurine accumulation. If such rises are significant, they could contribute to a RVI response needed for osmoregulation and/or affect other processes important for eliciting increases in cell survival. Either one or both of these responses would help sustain corneal epithelial barrier function during exposure to chronic hypertonic stress.

We describe here in tHCEC the time-dependent effects of rises in medium osmolality on taurine transporter gene and protein expression as well as its functional activity. To determine the physiological relevance of such expression and activity to their ability to adapt to a hypertonic challenge, we assessed the effects of medium supplementation with taurine on intracellular taurine levels and cell survival.

**Materials and Methods**

**Materials**

\[1,2,3,4\] Taurine was purchased from Amersham Pharmacia (Amersham, Buckinghamshire, United Kingdom). SV40 large T antigen-transformed HCEC were a generous gift from Kaoru Araki-Sasaki and the Santen Pharmaceutical Co., Ltd. (Osaka, Japan). Antitaurine transporter antibody was purchased from Alpha Diagnostic International (San Antonio, TX).

**Cell Culture**

tHCEC were cultured in Dulbecco’s modified Eagle’s medium (DMEM/HAM’S F-12 nutrient mixture (Iwaki, Tokyo, Japan), supplemented with 10% fetal bovine serum (Iwaki), 5 μg/ml insulin (Invitrogen, Groningen, The Netherlands), 0.1 μg/ml cholera toxin (Sigma, St. Louis, MO), 10 ng/ml human fibroblast growth factor (Collaborative Biomedical Products, Bedford, MA), and 40 μL/ml gentamicin (Invitrogen). The cultures were maintained under 95% air and 5% CO\(_2\) at 37°C. The medium was replaced every 2 days. The cultures were passed using 0.05% trypsin-EDTA (Invitrogen).

**tHCEC Uptake**

tHCEC were initially grown for 4 days in isotonic medium and then were exposed for up to another 48 hours to either isotonic or a hypertonic culture medium. The culture medium was removed and the cells were washed once with uptake buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.8 mM MgSO\(_4\), 5 mM glucose, and 15 mM Tris/HCl, pH 7.5). The osmolality of uptake buffer was adjusted to that of corresponding culture medium using sucrose. Taurine uptake was initiated by adding 1 mL of the uptake buffer containing 25 nM radiolabeled taurine. Uptake measurements were performed after incubations for up to 1 hour at 37°C. At the end of a period, uptake was terminated by removal of the medium by aspiration followed by three washes with uptake buffer that contained equimolar replacement of NaCl with KCl. Cells were then lysed with 1 mL of 0.2 M NaOH and 1% SDS, and radioactivity was quantified by scintillation counting. Influx data were normalized to cellular protein determined by a modified Lowry assay.

**Northern Blotting Analysis**

Total RNA was isolated using TRIzol Reagent (Gibco BRL), and then poly(A)+ RNA was isolated with Oligotex-dT30 Super (TaKaRa, Shiga, Japan), as described by the manufacturer. Electrophoresis was performed by loading equal amounts of poly(A)+ RNA per lane on 1% agarose, 2.2 M formaldehyde gel, followed by capillary transfer to Hybond N° (Amersham Pharmacia). The human taurine transporter translated region expressed in human placenta labeled with digoxigenin was used as the probe (Roche, Mannheim, Germany). Taurine transporter mRNA was detected with a protocol provided with the RNA nucleic acid detection kit (Roche). β-Actin RNA levels were evaluated to validate loading equivalence.

**Western Blotting**

Protein extracts were solubilized in a sample buffer (20 mM Tris, pH 6.8, 1% SDS, 1 M glycerol) by incubation at 37°C for 15 minutes. Protein concentration was measured with a modified Lowry assay. One hundred micrograms of proteins were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer to a nitrocellulose membrane (Millipore, Bedford, MA) using a semidy blottter (200 mA, 45 minutes, for a 35-cm\(^2\) gel) with a Tri-glycine transfer buffer (25 mM Tris, 190 mM glycine, 20% [v/v] methanol, pH 8.3). Blotted proteins were probed with antitaurine transporter antibody and detected by ECL Plus (Amersham Pharmacia).

**Intracellular Taurine Concentration**

About five million cells were trypsinized and then centrifuged at 2000 rpm. The resulting pellet was dissolved with 300 μL of 5% trichloroacetic acid. After cent rifuging at 13,000 rpm for 5 minutes, the supernatant was filtered through 0.20-μm pores (Millipex-LG Syringe Driven Filter Unit, Millipore). Intracellular taurine amount was measured with the L-8500A Amino Acid Analyzer (Hitachi, Tokyo, Japan). To measure intracellular water, 1 × 10\(^7\) cells were centrifuged at 13,000 rpm for 4 minutes. The supernatant was completely discarded, and the pellet was weighed and then dried using a freeze dryer (Iwaki). Water volume was calculated as the difference between total and dry weight. Intracellular taurine concentration was calculated by dividing taurine content by the corresponding intracellular water volume.

**Live/Death Assay**

After subculturing for 4 days, the cells were first exposed for another 2 days to 450 mosmol/kg hypertonic medium. The taurine concentration of isotonic culture medium was 100 μM, and it was increased through the addition of sufficient taurine to obtain a final concentration of 1 mM. At the end of this incubation period, the live/death assay was performed as described in the LIVE/DEAD Kit provided by Molecular Probes (LIVE/DEAD Reduced Biohazard Viability/Cytotoxicity Kit; Molecular Probe, Eugene, OR). Cells were washed once with HEPES-buffered saline solution (HBSS: 135 mM NaCl, 1 mM KCl, 1 mM MgSO\(_4\), 1.8 mM CaCl\(_2\), and 10 mM HEPES, pH 7.4) and incubated for 15 minutes with SYTO 10 green fluorescent nucleic acid dye and DEAD Red nucleic acid stain in HBSS (each of them diluted 1:500) at room temperature. After fixing with 4% glutaraldehyde in HBSS for 15 minutes, live and dead cells were visualized with confocal laser scanning microscopy equipped with an Air/Kr laser (Leica TCN NT, Mannheim, Germany). The images were captured and stored for data analysis using software provided with the confocal microscope. For each culture condition, six 55-mm dishes were used. The number of red staining cells was counted in a field at a magnification of ×100. Cell viability was characterized as the number of red staining/total cells. The data are shown as mean ± SD of six dishes per each condition. Significance (i.e., \(P < 0.05\)) was evaluated using Student’s t test (unpaired data).

**Results**

Taurine transport activity is coupled with sodium and chloride influx in a stoichiometric ratio of 2Na+:Cl:taurine.\textsuperscript{10} To determine in tHCEC whether such a transport process is expressed, the effect was measured on taurine transport of isotonic replacement in the uptake buffer of sodium chloride with chloride chloride. Figure 1 shows that Na+-dependent taurine uptake linearly increased over a 60-minute period. However, replacement of NaCl with chloride chloride virtually eliminated taurine uptake, suggesting the presence of sodium-dependent taurine transport activity in tHCEC. To further validate that this uptake is reflective of taurine transporter activity, the individual effects were measured of known competitors of this pro-
cess. The results shown in Figure 2 reveal that each of them had its predicted inhibitory effect on taurine uptake. Both 5 mM hypotaurine and β-alanine decreased taurine uptake by as much as approximately 90%. Furthermore, a similar decline occurred in the presence of unlabeled 5 mM taurine. In contrast, other known noncompetitors (i.e., either 2-amino isobutyric acid (AIB), Leu or Pro) of this uptake process had no significant inhibitory effect on influx. To evaluate the kinetics of taurine transport, its uptake was characterized under isotonic conditions (i.e., 310 mosmol/kg) as a function of taurine medium concentration in the range from 0.25 to 10 μM. The Eadie-Hofstee plot shown in Figure 3 of taurine uptake/[taurine] versus uptake was linear, suggesting that a single transport system is involved in mediating taurine uptake. Evaluation of the kinetic parameters with this procedure revealed that the apparent Michaelis-Menten constant, $K_t$ and $V_{max}$ were 4.6 μM and 52 pmol/mg of protein per 15 minutes, respectively.

Because exposure to an acute hypertonic stress in a number of tissues increases taurine transport activity, 10,12,13,18,19 we probed for such a response in tHCEC. This was done by measuring taurine transport after culture for 12 hours in the medium of interest. The results shown in Figure 4 indicate that taurine transporter activity over the range from 310 to 500 mosmol/kg increased in a sigmoid-like manner to reach a level at 500 mosmol/kg that was 4.7-fold higher than its rate under isotonic conditions. The culture time was determined that is needed to elicit hypertonic-induced increases in taurine transport. This was done by culturing the tissue in 450 mosmol/kg medium over different periods for up to 48 hours followed by measuring taurine transport for 15 minutes. This osmolality was chosen because human tear film tonicity measurements indicated that it can rise up to 450 mosmol/kg. 2 As can be seen in Figure 5, the time dependence of uptake appears to have an inverted parabolic-like appearance because taurine transport reached a maximum after 12 hours followed by a decline over the next 36 hours to its level measured at 310 mosmol/kg.

To validate that these increases in taurine transporter activity reflect an increase in medium tonicity, other osmotic agents with reflection coefficients equal to or less than that of sucrose were singularly used as osmotic replacements. Figure 6 shows
that supplementation with either 450 mosmol/kg sorbitol or sucrose, having the highest reflection coefficients of the group, induced increases in taurine uptake that were larger than those measured with mannitol or urea, which are more cell permeant. Mannitol only increased uptake by twofold, whereas culturing with either sorbitol or sucrose caused uptake to increase up to more than threefold. In contrast, the lack of increase over the isotonic level observed with urea is reflective of its ability to rapidly equilibrate across membranes.

The time-dependent effects of exposure to 450 mosmol/kg medium on taurine transporter expression were evaluated with Northern blot analysis. Figure 7 shows that there were initially large increases in the normalized level of its expression that reached a maximum value after 6 hours of incubation. Subsequently, its level progressively waned and returned to a level after 48 hours similar to that measured during exposure to isotonic medium. The corresponding levels of protein expression evaluated with Western blot analysis are shown in the bottom panel of Figure 7. Similarly, as with taurine transporter gene expression, there were initially corresponding increases in protein expression followed by declines to levels higher than the isotonic value. It is of interest to note that these changes in part mirrored those seen with Northern blot analysis except that the changes in gene expression preceded those in protein expression. However, at 48 hours relative to...
their isotonic controls only protein expression remained higher than the corresponding level of gene expression.

To evaluate whether during hypertonic stress the changes seen in taurine transporter gene and protein expression had corresponding effects on intracellular taurine accumulation, its concentration was measured at the same times as those used for Northern and Western blot analysis. Figure 8 shows that intracellular taurine concentration increased in a linear-like manner by nearly fivefold after 24 hours and nearly remained at this elevated level for another 24 hours. Consistent with the changes seen in taurine transporter gene and protein expression as well as in taurine transporter activity the rises in intracellular taurine concentration were time delayed from these other effects. However, it is noteworthy that the taurine concentration remained elevated after reaching a maximal level after 24 hours, whereas gene and protein expression as well as influx declined markedly after reaching their maxima.

We evaluated the possible supportive effect of increased taurine accumulation on cell viability during exposure to 450 mosmol/kg hypertonic stress for 48 hours. Figure 9 shows that supplementation of the medium with 1 mM taurine significantly increased cell viability during such exposure. This is evident because in the supplemented medium the Dead ratio decreased by 45% from that measured in the unsupplemented medium. In addition, the intracellular taurine concentration was 2.4 times higher in the 1 mM taurine-supplemented medium than in the unsupplemented medium (Fig. 9C). This increase suggests that during hypertonic stress increases in medium and intracellular taurine concentration may improve cell viability.

**DISCUSSION**

We obtained clear evidence that in tHCEC there is net taurine transport activity under isotonic conditions based on measurements of time-dependent increases in intracellular taurine accumulation. Such uptake is Na\(^+\) dependent, and it has both comparable kinetics and very similar substrate affinities to those described for net taurine uptake in a variety of other tissues in which this process has a stoichiometric relationship of 2Na:Cl:taurine.\(^{10}\) Specifically, we found that the apparent Michaelis-Menten constant of the transporter for taurine, \(K_m\), was 4.6 \(\mu\)M, which is very close to that reported in human placenta (i.e., 6 \(\mu\)M),\(^{23}\) Caco-2 cells (i.e., 4.8 \(\mu\)M),\(^{24}\) and human retinal pigmented epithelial cells (i.e., 2.0 \(\mu\)M).\(^{25}\) Furthermore, the transporter's specificity for taurine and other \(\beta\)-amino acids is the same as that described in the aforementioned tissues. Other evidence for this transporter's identity is that its apparent molecular weight of approximately 60 kDa is close to the one calculated based on the established amino acid sequence of the taurine transporter.\(^{25}\) Taurine transport function is responsible for establishing a 100-fold taurine concentration gradient between the cell interior and the external medium (i.e., 1 mM/0.01 mM). However, the importance of the maintenance of this gradient to cellular function under isotonic conditions is not yet apparent in tHCEC. It is conceivable that taurine may have a function in maintaining ocular surface health as its concentration in the tears is orders of magnitude higher than that reported for 10 other amino acids detected in tears.

Corneal epithelial cells can adapt to a hypertonic stress by restoring their cell volume to its isotonic level through the stimulation of membrane ion transporters mediating net uptake of osmolytes. Such effects are sufficient to restore a close match between the intracellular and extracellular osmolalities. One transport process known to be stimulated by chronic hypertonic exposure in SV40 immortalized rabbit corneal epithelial cells (tRCEC) is the Na:K:2Cl cotransporter.\(^{3,4}\) It was shown that this response includes upregulation of NKCC gene and protein expression. These effects make a substantial contribution to the RVI response because inhibition of the Na:K:2Cl cotransporter with bumetanide significantly suppressed the RVI response. However, unlike in (tRCEC) neither upregulation of NKCC gene and protein expression nor its functional activity was described in the same human corneal epithelial cell line as used in the present study. The inability of basal NKCC activity in tHCEC to offset osmotic induced shrinkage
was also indicated by the small RVI response to an acute hypertonic challenge after chronic exposure for up to 48 hours to a hypertonic medium. This response was partially inhibited by bumetanide, but a remaining component was not sensitive to bumetanide. Our rationale to probe in tHCEC for osmosensitive taurine transporter activity was twofold. First, we sought to determine whether net taurine transport could be a contributor to the remaining bumetanide-insensitive component of RVI recovery. Secondly, osmosensitive taurine transporter activity in tHCEC was of greater interest because preliminary results with tRCEC revealed lower rates of taurine transporter activity under both isotonic and hypertonic conditions than in tHCEC.

There are several lines of evidence showing that taurine transport is osmosensitive. The results shown in Figure 4 indicate that net taurine transport was simulated as a function of exposure to hyperosmolality. After 12 hours at 500 mosmol/kg, it maximally increased 4.7-fold above its isotonic value. The time dependence for taurine transport to increase at 450 mosmol/kg shown in Figure 5 revealed that it initially increased up to 3.8-fold after 12 hours, but then declined during the next 36 hours to its isotonic control value. These changes are consistent with our finding that after 24 hours of exposure to 450 mosmol/kg medium intracellular taurine concentration increased fivefold from 0.9 to 4.5 mM. This small increment is consistent with our finding that the RVI response after chronic exposure to a 450 mosmol/kg medium is much less than that required for matching the internal and external osmolality. It appears at this osmolality that matching of these osmolalities can occur provided the RVI response increases intracellular osmolality by 140 mosmol/kg (i.e., 450–310 mosmol/kg). However, the increase we measured in intracellular taurine concentration was only 3% of the difference that is needed for equilibration of the intracellular and extracellular osmolalities. Even if the reflection coefficients in corneal epithelial cells for sucrose are somewhat unique compared to other tissues, the increase in intracellular taurine concentration was far less than that needed for equilibration. It has been instead suggested that taurine may provide an antioxidant function or a membrane protection function. Alternatively, this relatively small increase in taurine concentration may activate another unknown function required for the RVI response.

The osmosensitivity of the taurine transporter was further documented based on the results obtained from Northern and Western blot analyses. The results shown in Figure 7 reveal that exposure to 450 mosmol/kg caused the levels of taurine transporter mRNA to initially increase and reach a maximal value at 6 hours. It then declined after another 18 hours to a level that was lower than the control isotonic value. During the subsequent 24 hours, its gene expression slightly increased, but did not return to the level seen at 6 hours. These changes in taurine transporter gene expression were in part mirrored by changes in taurine transporter protein expression at 450 mosmol/kg except that they reached a maximum at 12 hours (i.e., 6 hours later than those seen in the Northern blot analysis). One difference between the two patterns of expression was that at 24 and 48 hours the mRNA expression decreased below the level of the control, whereas even though protein expression decreased, it did not fall below its isotonic level. The pattern of protein expression is consistent with the associated changes in transport activity because they also reached a maximal value at 12 hours, indicating that stimulation of taurine transport may solely result from increases in transporter number rather than an increase in transporter affinity for taurine. On the other hand, at 48 hours transporter protein expression remained higher than its isotonic value even though taurine transport fell to its isotonic value. Therefore, modulation of taurine transport activity may at some time points also involve changes in the kinetic parameters. An alternative is that the slight decline in taurine accumulation from 24 to 48 hours could result from increases in taurine efflux.

Because the increases in intracellular taurine are too small during hypertonic stress for them to have an important role as an osmolyte, we determined whether medium supplementation with taurine improves cell viability during such a challenge. The results shown in Figure 9 reveal that supplementation with 1 mM taurine of the 450 mosmol/kg medium significantly increased cell viability after 48 hours of culture. It should be noted that the endogenous level of taurine in the medium was 100 μM. Had it been possible to culture the cells in a taurine-free system, the protective value of taurine supplementation could have been larger than the one seen here. To our knowledge, this is the first time that taurine supplementation has been shown to provide a protective function against hypertonic-induced cell death, perhaps reflecting an antioxidant or membrane stabilization effect of taurine.

References


