Osmoregulation of Taurine Transporter Function and Expression in Retinal Pigment Epithelial, Ganglion, and Müller Cells

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Purpose. To determine whether taurine transporter (TauT) activity and expression are regulated by hyperosmolarity in RPE, ganglion, and Müller cells.

Methods. Uptake of taurine was measured in ARPE-19 cells cultured in DMEM-F12 medium without or with the addition of 50 mM NaCl or 100 mM mannitol. The kinetics of the transport were analyzed. RT-PCR and Northern and Western blot analyses were used to assess TauT mRNA and protein levels. The influence of hyperosmolarity on the uptake of taurine, *myo*-inositol, and γ-aminobutyric acid GABA was studied in RPE, RGC-5, and rMC1 cells.

Results. TauT activity was abundant in RPE and was stimulated (3.5-fold) when the cells were exposed to hyperosmolar conditions (DMEM-F12 culture medium plus 50 mM NaCl or 100 mM mannitol). Peak stimulation of taurine uptake occurred after 17 hours of exposure to hyperosmolar medium. Kinetic analysis revealed that the hyperosmolarity-induced stimulation was associated with an increase in V_{max} of TauT with no change in K_m. TauT mRNA and protein levels increased in RPE cells exposed to hyperosmolar conditions. Hyperosmolarity also stimulated the uptake of *myo*-inositol (~15-fold); GABA uptake was influenced less markedly. Immunofluorescence and functional studies showed that TauT is present in cultured RGC-5 and rMC1 cells. TauT activity was robust in these cells in normal osmolar conditions and increased by approximately twofold in hyperosmolar conditions.

Conclusions. These studies provide the first evidence that hyperosmolarity regulates TauT activity and expression in RPE and that TauT is present in ganglion and Müller cells and is regulated by hypertonicity. The data are relevant to diseases such as diabetes, macular degeneration, and neurodegeneration, in which retinal cell volumes may fluctuate dramatically. (*Invest Ophthalmol Vis Sci.* 2004;45:694–701) DOI:10.1167/iovs.03-0503

Taurine, a β-aminosulfonic acid, is the most abundant retinal amino acid[1] and is essential for maintenance of retinal structure and function.[2,3] Although its role in the retina is uncertain, several functions have been ascribed to taurine including antioxidant defense, protein stabilization, stress responses, and osmoregulation. Regarding osmoregulation, there is evidence that taurine is involved in cell volume homeostasis. Cells achieve osmotic equilibrium by accumulating or losing small organic solutes, such as taurine and *myo*-inositol, accompanied by the flux of osmotically obligated water. Studies in astrocytes showed that exposure to hypotonic conditions result in massive effluxes of taurine[4,5] and *myo*-inositol.[6] Conversely, hypertonic conditions caused a significant influx of these solutes.[7] In retinal pigment epithelium (RPE), Yokoyama et al.[8] demonstrated that hypertonic stress increases Na,K-ATPase activity and leads to accumulation of taurine and *myo*-inositol.

The influx and efflux of solutes, such as taurine and *myo*-inositol, are mediated by transporter proteins. Hypertonicity upregulates the taurine transporter abbreviated TauT (SLC6A6) in several cell types including astrocytes,[7] Caco-2 cells,[9] and hepatocytes.[10] Recent studies in ocular tissues have demonstrated that hyperosmolar stress results in an increased expression of TauT in corneal[11] and lens[12] epithelial cells. The cDNAs for human,[13,14] rat,[15] and mouse[16] TauT have been cloned, and sequence homology places TauT within the gene family of Na^+- and Cl^--dependent neurotransmitter transporters. Human TauT cDNA encodes a protein of 619 amino acids with 12 putative transmembrane domains. It is noteworthy that a mouse model with a disrupted gene coding for TauT (*taut*$^{-/-}$ mice), reported recently by Heller-Stilb et al.,[17] exhibits severe retinal degeneration, suggesting that TauT is critical for normal retinal development and function.

In situ hybridization analysis of mouse retina showed that TauT is expressed in several retinal cell types, including RPE and photoreceptor cells.[16] Our studies have localized TauT protein to RPE, ganglion cells, and the inner nuclear layer.[18] In the present study, we were interested in determining whether hyperosmotic conditions alter the function and expression of TauT in RPE. RPE maintains the adjacent, highly metabolically active photoreceptor cells by transporting nutrients, removing waste, and phagocytosing shed photoreceptor disks.[19] Although TauT has been characterized in RPE,[20–23] the regulation of TauT by hyperosmolarity in RPE has not been studied. The question is relevant because fluctuations in cell volume are associated with diseases such as ischemia and reperfusion during diabetic retinopathy, macular edema, and neurodegeneration.[24] Using functional and molecular biological assays, we investigated TauT regulation in hyperosmotic conditions in ARPE-19 cells, a well-characterized human retinal pigment epithelial cell line.[25] In addition, we examined TauT activity in retinal ganglion and Müller cells. Ganglion cells are the second-order neurons of the visual pathway and are susceptible to apoptotic death in glaucoma and diabetic retinopathy.[26–28] (Moore et al. unpublished observations, 2003). Müller cells are the major glial cells of the retina. They subserve the metabolic, ionic, and extracellular buffering requirements of adjacent neurons and play a key role in clearing toxic substances. Müller

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cell function is altered in diabetic retinopathy.[29,30] With the recent development of the rat ganglion cell line RGC-5[31] and the rat Müller cell line rMC1,[32] we were able to study TauT function in normal and hyperosmolar conditions. Our data show that TauT is stimulated in hyperosmotic conditions in RPE, RGC-5, and rMC1 cells.

Materials and Methods

Reagents were obtained from the following sources: [1,2-^{3}H]-taurine and [α-^{32}P]-dCTP (Amersham Pharmacia Biotech, Piscataway, NJ); [2,3-^{3}H(*N*)]-γ aminobutyric acid (GABA; Dupont/NEN, Boston, MA); [^{3}H(*N*)]-carnitine, [4,5-^{3}H]-leucine, and [2-^{3}H]-*myo*-inositol (Moravek Biochemicals, Brea, CA); taurine, *myo*-inositol, GABA, succinyl concanavalin A (sConA), and anti-β actin antibody (Sigma-Aldrich, St. Louis, MO); DMEM-F12 medium (catalog no. 12320), extraction reagent (TRIzol), and penicillin-streptomycin (Gibco-Invitrogen Corp., Grand Island, NY); fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA); ARPE-19 cells (ATCC, Manassas, VA); rat ganglion cells RGC-5 (Neeraj Agarwal, University of North Texas Health Science Center, Fort Worth, TX); rat Müller cells rMC-1 (Vijay Sarthy, Northwestern University, Chicago, IL); antibody against rat TauT and the immunogenic control peptide (Alpha Diagnostics, Inc., San Antonio, TX); FITC-conjugated anti-rabbit IgG (AffiniPure; Jackson ImmunoResearch Laboratories, West Grove, PA); HRP-conjugated goat anti-rabbit IgG, and an enhanced chemiluminescence (ECL) detection kit (Santa Cruz Biotechnology, Santa Cruz, CA); protease inhibitor cocktail (Complete Mini Tablets; Roche Applied Science, Indianapolis, IN); RNA PCR core kit (Perkin-Elmer, Boston, MA); hybridization solution (ExpressHyb; Clontech, Palo Alto, CA); and ultrasensitive hybridization buffer (ULTRAHyb; Ambion, Austin, TX).

Cell Culture

ARPE-19 and rMC1 cells were cultured in DMEM-F12 medium, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified chamber with 5% CO_2. The culture medium was replaced every other day. After confluence, cultures were passaged by dissociation in 0.05% (wt/vol) trypsin in 0.01 M PBS. RGC-5 cells were passaged initially in DMEM-F12. For differentiation, the method of Krishnamoorthy et al.[31] was followed in which cells were cultured in the absence of serum for 24 hours, after which they were cultured in DMEM-F12 (1% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin) supplemented with 50 μg/mL sConA for 6 to 7 days.

Determination of Hyperosmolarity Effects on Taurine Uptake

Cells were grown in 24-well plates and exposed to various concentrations of NaCl (10, 25, 50, or 75 mM) or mannitol (20, 50, 100, or 150 mM) in the normal culture medium, which increased the osmolarity of the medium by 20, 50, 100, and 150 mOsM, respectively. For uptake experiments, culture medium was removed, and cells were washed twice with uptake buffer (25 mM HEPES/Tris, 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose [pH 7.5]). For experiments assessing the influence of Na^+ and Cl^- on the transport process, the uptake buffer was modified by replacing KCl and $CaCl_2$ with iso-osmolar concentrations of respective gluconate salts and by replacing NaCl with iso-osmolar concentrations of either sodium gluconate or *N*-methyl-D-glucamine (NMDG)-chloride. Uptake was initiated by adding 250 μL of uptake buffer containing radiolabeled substrates. Uptake measurements were performed with 15 minutes incubation at 37°C. At the end of the incubation, uptake was terminated by removal of the medium followed by two washes with ice-cold uptake buffer without the radiolabeled substrates. Cells were solubilized in 0.5 mL of 1% SDS in 0.2N NaOH; radioactivity was quantified by scintillation.

RT-PCR and Northern Blot Analysis of Steady State Levels of TauT mRNA

The expression of TauT mRNA in RPE cells, exposed for 17 hours to normal culture medium or hyperosmolar medium in which the osmolarity was increased by 100 mOsM by addition of either 50 mM NaCl or 100 mM mannitol, was analyzed by semiquantitative RT-PCR and northern hybridization. For RT-PCR, total RNA was prepared using extraction reagent (TRIzol; Invitrogen-Gibco). RT-PCR was performed using primer pairs specific for human TauT (5′-GCTAGCTGCATAGTAGTC-3′ (sense); 5′-TGGAACACAC CTCACTGC-3′ (antisense), nucleotide positions 872-889 and 1751-1769 of human TauT cDNA[14]) and human GAPDH (5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′ (sense); 5′-TTCCCGTTCAGCTCAGGATGACCTTGCCC-3′ (antisense), nucleotide positions 241-270 and 711-740 in human GAPDH cDNA[33]). PCR after reverse transcription was performed over a range of cycles (9–30). The products were size fractionated on agarose gels, transferred onto nylon membranes and subjected to Southern hybridization with probes specific for TauT or GAPDH. These probes were generated by labeling the respective subcloned RT-PCR products with [α-^{32}P]dCTP. The intensity of hybridization signals was quantified using phosphorescence imaging (STORM Phosphorimager; Amersham Biosciences, Sunnyvale, CA). The relationship between the signal intensity and PCR cycle number was analyzed to determine the linear range for the PCR product formation, which was then used for data analysis. For Northern blot analysis, poly(A)$^+$ mRNA was isolated with a commercially available kit (MACS; Miltenyi Biotec, Auburn, CA) and size-fractionated on a denaturing agarose gel. A 1.1-kb cDNA fragment, derived from human TauT cDNA,[14] was labeled with [α-^{32}P]-dCTP and used as the probe. Hybridization was performed in ultrasensitive hybridization solution (ULTRAHyb; Ambion) for 18 hours at 42°C. The blots were washed two times (30 minutes each) at 60°C in a solution containing 2× SSC (0.15 M NaCl and 15 mM sodium citrate [pH 7]) and 0.5% SDS (sodium dodecyl sulfate). The same blot was stripped and reprobed with [^{32}P]-GAPDH cDNA[34] as an internal control. Hybridization signals were quantified using phosphorescence imaging (STORM; Amersham Biosciences). The intensity of the TauT mRNA signal was normalized with that of the GAPDH mRNA signal to correct for potential differences in RNA loading. The TauT mRNA/GAPDH mRNA signal ratio in control cells exposed to normal culture medium was taken as 1 and the changes in TauT mRNA in cells exposed to hyperosmolar conditions were compared to this value.

Western Blot Analysis of TauT Protein Levels in RPE Cells

Confluent cultures of ARPE-19 cells were exposed for 17 hours to normal culture medium without or with the addition of 50 mM NaCl or 100 mM mannitol (an increase of 100 mOsM in the osmolarity of the medium). Cells were washed with 0.01 M PBS and lysed in cold lysis buffer (50 mM Tris-HCl [pH 7.4], containing 1% Triton-X-100, 10 mM EDTA, 2 mM Na_3VO_4, 0.5% deoxycholate, 10 mM sodium pyrophosphate, and 50 mM NaF) containing a protease inhibitor cocktail (1 tablet/10 mL lysis buffer; Complete Mini; Roche Applied Science) and were sonicated for 30 seconds. Cell debris was removed by centrifugation at 20,000*g* for 15 minutes at 4°C. Protein concentration in the supernatant was determined.[34] Equivalent amounts of protein (40 μg) from total cell lysates were boiled in Laemmli's buffer for 5 minutes and analyzed by 7.5% SDS-PAGE.[35] Separated proteins were transferred to nitrocellulose membranes and were blocked for 1.5 hours with TBS-0.1% Tween-20 containing 5% nonfat milk. Membranes were incubated overnight with anti-TauT antibody, probed with HRP secondary antibody (1:3000) for 1.5 hours, and washed and the proteins visualized with the ECL system. Membranes were washed and reprobed with anti-β actin antibody. Densitometric scans of the membranes were performed using a digital imaging system (AlphaImager 2200; Alpha Innotech Corp., San Leandro, CA).

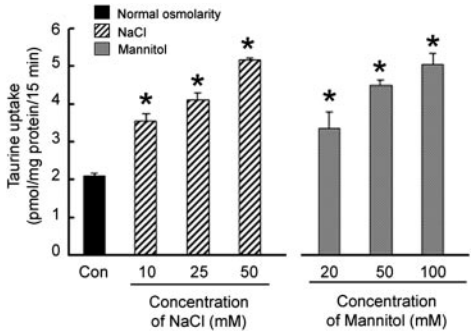

FIGURE 1. Influence of increasing osmolarity on taurine uptake by RPE cells. Confluent ARPE-19 cells were exposed for 8 hours to various concentrations of NaCl or mannitol added to the normal culture medium and the uptake of taurine (80 nM) was determined. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from control ($P < 0.05$).

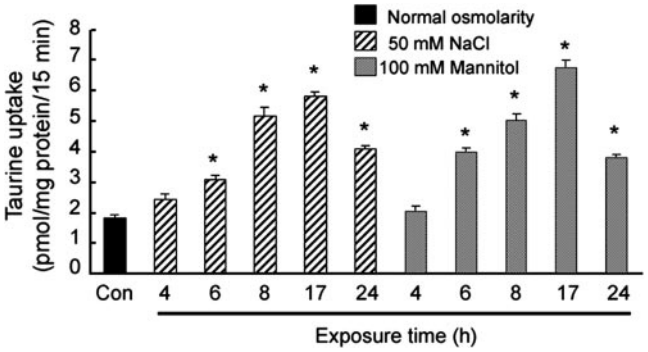

FIGURE 2. Influence of exposure time to hyperosmotic conditions on taurine uptake in RPE cells. Confluent ARPE-19 cells were exposed for various lengths of time (0–24 hours) to either 50 mM NaCl or 100 mM mannitol added to the normal culture medium and the uptake of taurine (80 nM) was determined. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from the control ($P < 0.05$).

Immunocytochemical Detection of TauT in Cultured Retinal Cells

ARPE-19, RGC-5, and rMC-1 cells were cultured on chamber slides. Cells were fixed with ice-cold methanol, blocked with 10% goat serum, and incubated overnight at 4°C with anti-TauT antibody (1:100). As a negative control, the primary antibody was neutralized with an excess of antigenic peptide before use. Additional negative controls included using buffer only and 0.1% normal rabbit serum in place of the primary antibody. Samples were incubated overnight at 4°C with FITC-conjugated anti-rabbit IgG (1:100). Slides were examined using a fluorescent microscope (Axioskop 2; Carl Zeiss Meditec, Oberkochen, Germany) equipped with a digital camera and software (Spot Camera and Spot Software version 3.4.5; Diagnostic Instruments, Inc., Sterling Heights, MI).

Data Analysis

Data were analyzed using the NCSS 97 statistical package (NCSS, Kaysville, UT). In cases of multiple comparisons, ANOVA was used followed by the Tukey-Kramer paired comparison test. $P < 0.05$ was considered significant.

RESULTS

Influence of Hyperosmolarity on Taurine Uptake

Earlier reports demonstrated an active TauT in RPE cells.[20–23] To determine whether TauT function is regulated in RPE by hyperosmotic conditions, ARPE-19 cells were exposed for 8 hours to various concentrations of NaCl (ranging from 10 to 50 mM) or mannitol (ranging from 20 to 100 mM) added to normal culture medium. An increase of 50 mOsM in the osmolarity of the culture medium (addition of 25 mM NaCl or 50 mM mannitol) stimulated taurine uptake significantly (100% and 120%, respectively; Fig. 1). An increase of 100 mOsM in the medium osmolarity (addition of 50 mM NaCl or 100 mM mannitol) stimulated taurine uptake to a greater extent (150% in both cases). Unless otherwise stated, for the remainder of the experiments, cells were exposed to normal culture medium without or with the addition of 50 mM NaCl or 100 mM mannitol.

Influence of Exposure Time to Hyperosmotic Conditions on Taurine Uptake

ARPE-19 cells were incubated in normal or hyperosmolar medium for various periods (0–24 hours) and taurine uptake was measured. By 6 hours, there was a significant increase in taurine uptake in cells exposed to medium containing 50 mM NaCl or 100 mM mannitol (an increase of 100 mOsM in medium osmolarity; Fig. 2). Increasing the exposure time resulted in an increase in taurine uptake through 17 hours, when the greatest stimulation in taurine uptake was observed. By 24 hours, taurine uptake in hyperosmolar conditions had decreased compared with the 17-hour uptake, although the levels were significantly greater than in cells exposed to normal osmolar (control) conditions.

Specificity of Hyperosmolarity Effects on Taurine Uptake

The hyperosmolarity-induced stimulation of taurine uptake in RPE was not a nonspecific effect, as the uptake of other selected nutrients was not affected in RPE cells cultured in hyperosmotic conditions. Incubation of ARPE-19 cells for 17 hours in hyperosmotic medium had no significant effect on the uptake of leucine (30 nM) or carnitine (30 nM; Fig. 3). Taurine uptake increased markedly in identical conditions.

Influence of Hyperosmolarity on the Kinetic Parameters of Taurine Uptake

We analyzed the kinetics of TauT uptake by RPE cells maintained in normal osmolar conditions compared with those

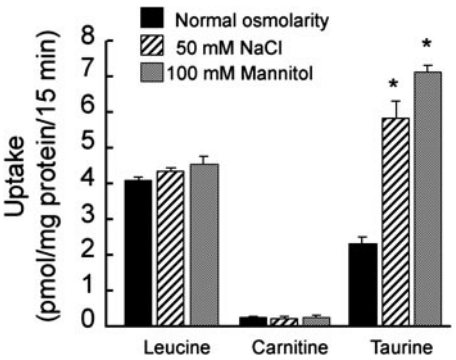

FIGURE 3. Specificity of the stimulation of taurine uptake in RPE cells by hyperosmolarity. Confluent ARPE-19 cells were exposed for 17 hours to either 50 mM NaCl or 100 mM mannitol added to the normal culture medium and the uptake of leucine (30 nM), carnitine (30 nM), and taurine (80 nM) was determined. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from cells incubated in normal osmolar conditions ($P < 0.05$).

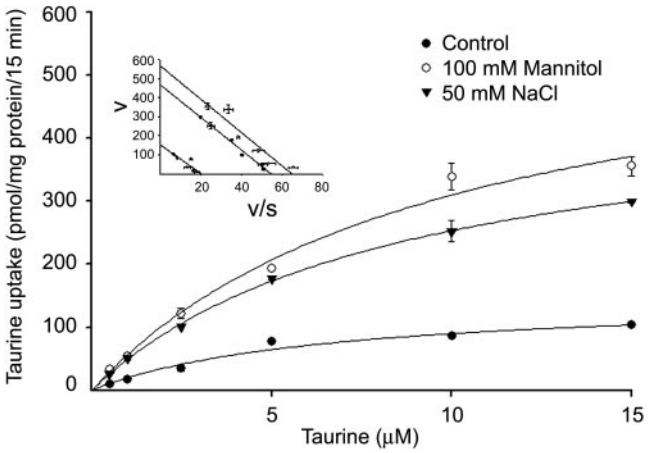

FIGURE 4. Kinetic analysis of taurine uptake in RPE cells cultured in normal and hyperosmolar conditions. Confluent ARPE-19 cells were incubated for 17 hours in normal culture medium with or without (control) the addition of 100 mM mannitol or 50 mM NaCl. Uptake of taurine was measured over a taurine concentration range of 0.5 to 15 μM. Values are means ± SEM of six determinations from two independent experiments. Results are presented as plots describing the relationship between taurine concentration and taurine uptake rate. *Inset*: Eadie-Hofstee plots (*v/s* versus *v*, where *v* is taurine uptake in picomoles per milligram protein per 15 minutes and *s* is taurine concentration in micromolar).

incubated for 17 hours in hyperosmolar conditions. Taurine uptake was measured over a concentration range of 0.025 to 15 μM (Fig. 4). The analysis showed that the increase in TauT activity observed in hyperosmolar conditions was associated with an increase in the maximal velocity of the transporter, with no significant change in substrate affinity. The maximum velocity of taurine uptake was significantly greater in cells exposed to hyperosmolar conditions than in controls cells (471 ± 13 for 50 mM NaCl and 615 ± 83 for 100 mM mannitol versus 148 ± 21 pmol/mg protein/15 minutes in control cells). The Michaelis-Menten constant for taurine remained almost the same in controls cells and in those incubated in hyperosmolar conditions (8.6 ± 0.5 μM with 50 mM NaCl, 9.9 ± 2.6 μM with 100 mM mannitol, and 6.5 ± 2.2 μM in control cells).

Influence of Hyperosmolarity on Steady State Levels of TauT mRNA

RNA samples isolated from ARPE-19 cells that had been incubated in normal or hyperosmolar culture medium (increase of 100 mOsM by addition of 50 mM NaCl or 100 mM mannitol) for 17 hours were used for semiquantitative RT-PCR (total RNA) and for Northern blot analysis (mRNA). As an internal control, the steady state levels of GAPDH mRNA were determined in the samples in parallel. The steady state levels of TauT mRNA increased significantly compared with control cell levels, as assessed by both methods (Fig. 5). The steady state levels of TauT mRNA, after they were normalized with the internal control, were approximately two times higher in cells exposed to hyperosmolar medium compared with control cells exposed to normal culture medium. These results demonstrate that the hyperosmolarity-induced increase in TauT activity is probably due to increased expression of the gene encoding TauT.

Influence of Hyperosmolarity on TauT Protein Levels

Confluent ARPE-19 cells were incubated either in normal culture medium or hyperosmolar culture medium (increase of 100 mOsM by addition of 50 mM NaCl or 100 mM mannitol) for 17 hours and subjected to Western blot analysis with an antibody specific for TauT or β-actin (Fig. 6A). Densitometric scans of membranes (Fig. 6B) showed that cells incubated in hyperosmolar medium had a 45% increase in TauT protein compared with control cells. The β-actin band was used as an internal control for protein loading to normalize the values for TauT protein levels.

Influence of Hyperosmolarity on *myo*-Inositol and GABA Uptake in RPE

Hyperosmolar conditions upregulate the uptake of other osmolytes such as *myo*-inositol in cell types other than RPE.[6] Therefore, we investigated the influence of hyperosmolarity on the uptake of *myo*-inositol in RPE cells. Our previous studies showed that a GABA transporter expressed in RPE apical membrane vesicles can transport taurine to a significant extent.[36] Hence, we tested whether hyperosmolarity influences the uptake of GABA in RPE cells. Cells were incubated for 17 hours in normal or hyperosmolar medium and the uptake of *myo*-inositol (100 nM) or GABA (65 nM) determined. An increase in medium osmolarity of 100 mOsM (by addition of 50 mM NaCl

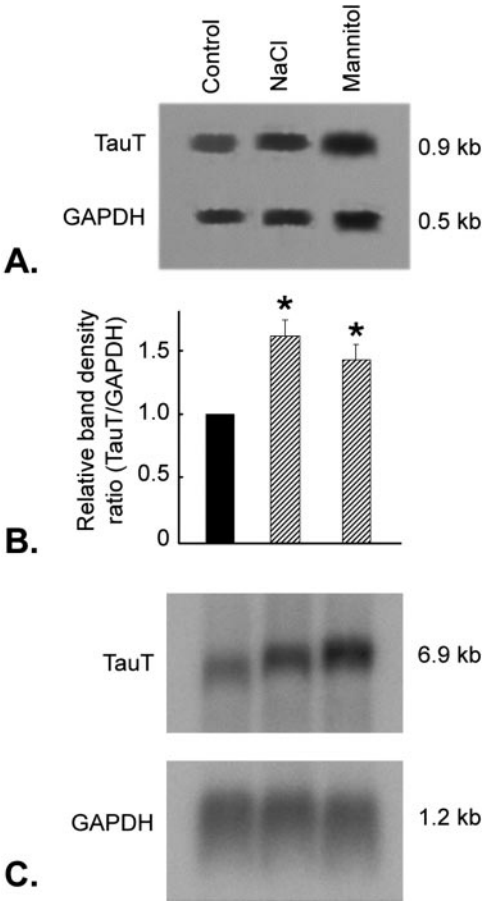

FIGURE 5. Analysis of steady state levels of TauT mRNA in RPE cells cultured in hyperosmolar conditions. Confluent cells were exposed for 17 hours to normal culture medium, with or without (control) the addition of 100 mM mannitol or 50 mM NaCl. RNA was isolated from these cells and used for semiquantitative RT-PCR (total RNA) and Northern blot analysis (mRNA). (**A**) Data from Southern hybridization with ^{32}P-labeled cDNA probes specific for TauT and GAPDH. (**B**) Data shown in (**A**) were subjected to phosphorimaging analysis. The ratios of the TauT-specific band to the GAPDH-specific band in various osmolar conditions are shown; the ratio in control cells was taken as 1. (**C**) Northern blot.

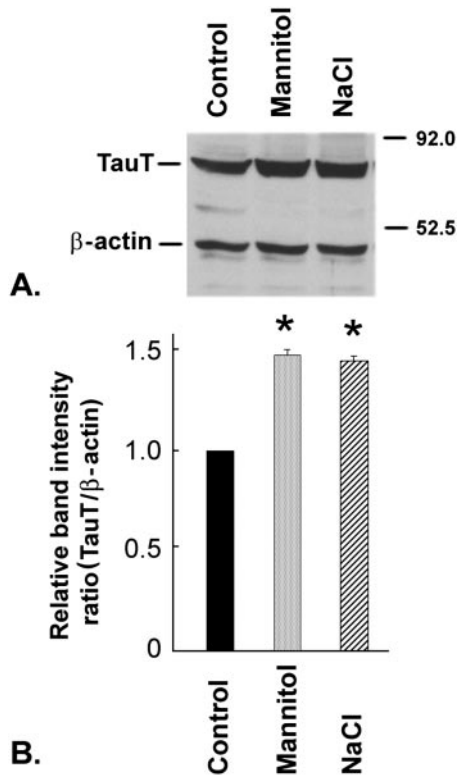

FIGURE 6. Analysis of TauT protein levels in RPE cells cultured in hyperosmolar conditions. Confluent ARPE-19 cells were exposed for 17 hours to normal culture medium with or without (control) the addition of 100 mM mannitol or 50 mM NaCl. Protein was isolated and subjected to immunoblot analysis. (**A**) Representative blot of proteins from RPE cells cultured in various osmolar conditions. TauT has a molecular mass of 70 kDa, and β-actin (internal control) has a molecular mass of 45 kDa. (**B**) Quantitation of the ratio of the TauT band to β-actin band. The ratio in control cells was taken as 1.

or 100 mM mannitol) caused a marked stimulation of *myo*-inositol uptake (~15-fold increase; Fig. 7). GABA uptake was also stimulated in hyperosmolar conditions, but to a much lesser extent.

Immunocytochemical Localization of TauT in Cultured RPE, RGC-5, and rMC1 Cells

ARPE-19, RGC-5, and rMC1 cell lines were grown in chamber slides, and immunocytochemical methods were used to determine whether TauT was present. TauT was detected in

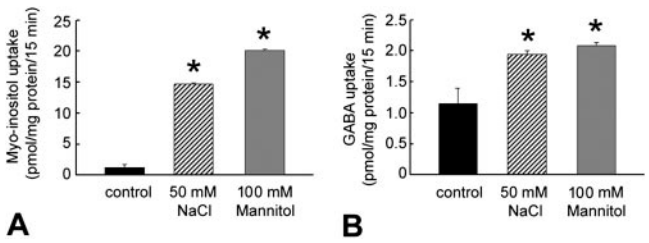

FIGURE 7. Influence of hyperosmolarity on the uptake of *myo*-inositol and GABA in RPE cells. Confluent ARPE-19 cells were maintained for 17 hours in normal culture medium with or without (control) the addition of 50 mM NaCl, or 100 mM mannitol. Uptake of *myo*-inositol (100 nM) (**A**) or GABA (65 nM) (**B**) was determined in these cells. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from control ($P < 0.05$).

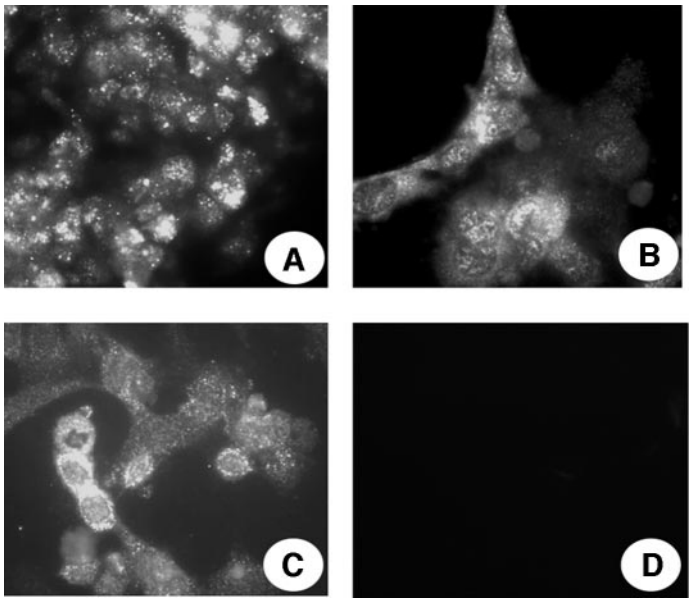

FIGURE 8. Immunolocalization of TauT in cultured human ARPE-19, rat ganglion (RGC-5), and Müller (rMC1) cells. ARPE-19 (**A**), RGC-5 (**B**) and rMC1cells (**C**) were cultured on laminin-coated chamber slides and processed for immunohistochemistry using a primary antibody against TauT followed by an FITC-labeled secondary antibody. (**D**) ARPE-19 cells incubated with antibody that had been preincubated with the peptide (negative control) showing no positive staining. Magnifications, ×600.

ARPE-19 (Fig. 8A), RGC-5 (Fig. 8B), and rMC1 (Fig. 8C) cells. Incubation of cells with antibody that had been incubated with an excess of the antigenic peptide showed no positive immunofluorescence (Fig. 8D). These immunofluorescence data show that TauT is present abundantly in the cultured RPE, ganglion, and Müller cells.

Analysis of TauT Activity in RGC-5 and rMC1 Cells

To determine whether RGC-5 and rMC1 cells have a functional Na^+- and Cl^--dependent TauT, cells were incubated in various buffer conditions and taurine uptake assessed (Table 1). RGC-5 and rMC1 cells demonstrated significant taurine uptake when cells were incubated with taurine (40 nM) in buffer containing NaCl, but virtually no uptake of taurine in the absence of Na^+ but the presence of Cl^- (NMDG chloride buffer), or in the presence of Na^+ but the absence of Cl^- (sodium gluconate buffer). In addition, cells incubated in NaCl buffer containing

TABLE 1. Functional TauT in RGC-5 and rMC1 Cells

	Taurine Uptake pmol/mg Protein/ 15 min	
Buffer	**Ganglion Cells**	**Müller Cells**
NaCl	14.50 ± 0.700	20.95 ± 0.48
NMDG-Cl	0.06 ± 0.001	0.09 ± 0.01
Sodium gluconate	0.12 ± 0.010	2.72 ± 0.12
NaCl + β-alanine	0.33 ± 0.010	0.79 ± 0.09

Values are mean picomoles/milligram protein per 15 minutes ± SE. RGC-5 and rMC1 cells were cultured as described in the text and the uptake of [^{3}H]taurine (80 nM) was measured. Uptake buffer consisted of 20 mM HEPES-Tris (pH 7.5) containing 140 mM NaCl, sodium gluconate or *N*-methyl-D-glucamine (NMDG) chloride. Inhibition of taurine transport in the presence of 2.5 mM β-alanine (in NaCl buffer) was also measured.

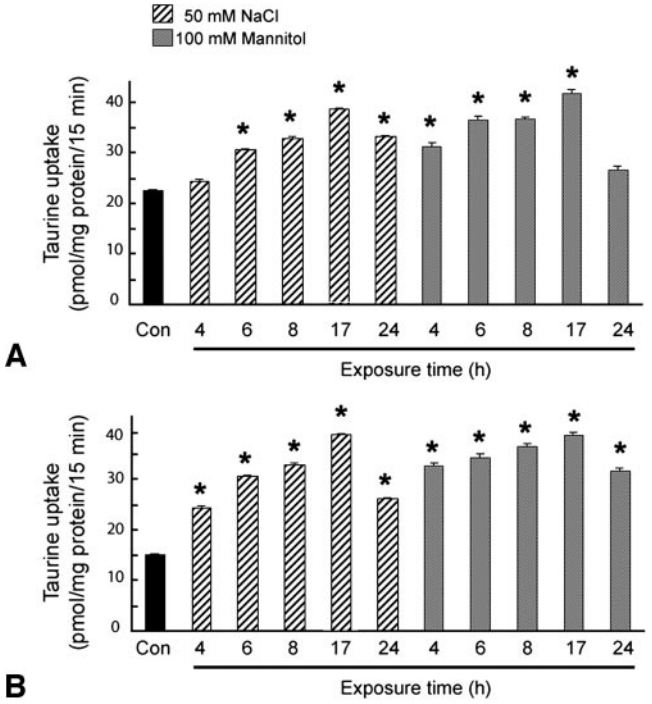

FIGURE 9. Influence of exposure time to hyperosmotic conditions on taurine uptake in RGC-5 and rMC-1 cells. Confluent RGC-5 (**A**) and rMC1 (**B**) cells were exposed for various lengths of time (0–24 hours) to either 50 mM NaCl or 100 mM mannitol added to the normal culture medium (an increase of 100 mOsM in the osmolarity of the medium) and the uptake of taurine (80 nM) determined. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from control ($P < 0.05$).

β-alanine, a known competitive inhibitor of taurine uptake,[14] showed minimal uptake of taurine. These data provide strong evidence of a functional TauT in these retinal cell lines.

Influence of Hyperosmolarity on Taurine Uptake in RGC-5 and rMC1 Cells

Confluent RGC-5 and rMC1 cells were incubated in normal or hyperosmolar culture medium (increase of 100 mOsM by addition of 50 mM NaCl or 100 mM mannitol) for various periods (0–24 hours) and the uptake of taurine (80 nM) assessed. RGC-5 and rMC1 cells demonstrated an increased TauT activity in hyperosmolar conditions (Fig. 9). Within 6 hours of exposure to hyperosmolar conditions, RGC-5 cells demonstrated a marked increase in TauT activity that peaked at 17 hours. Müller cells showed an even more rapid response to hyperosmolar conditions by increasing TauT activity within 4 hours of exposure to hyperosmolar conditions. The basal activity (activity in normal osmolar conditions) of TauT was more robust in RGC-5 and rMC1 cells than in RPE. When incubated in normal culture medium, the uptake of taurine by RPE was approximately 2.5 pmol/mg protein/15 minutes, whereas for ganglion and Müller cells it was approximately 15 to 20 pmol/mg protein/15 minutes.

Influence of Hyperosmolarity on *myo*-Inositol and GABA Uptake in RGC-5 and rMC1 Cells

To determine whether uptake of *myo*-inositol and GABA in RGC-5 and rMC1 cells was regulated also by hyperosmotic conditions, cells were cultured for 17 hours in normal or hyperosmolar medium. The osmolarity of the medium was increased by 100 mOsM by the addition of 50 mM NaCl or 100 mM mannitol. The uptake of *myo*-inositol increased dramatically in RGC-5 cells incubated in hyperosmolar medium compared with cells incubated in normal medium (Fig. 10A). GABA uptake was also enhanced by hyperosmolarity, but to a lesser extent. In rMC1 cells (Fig. 10B) there was a modest but significant increase in *myo*-inositol uptake in cells exposed to hyperosmolar conditions. GABA uptake in rMC1 cells was stimulated slightly but significantly in identical conditions.

DISCUSSION

Our previous studies demonstrated that oxidative stress upregulates TauT in RPE,[18] providing evidence that taurine may act as an antioxidant in these cells. The present study assessed the effects of hyperosmolarity on taurine transport by RPE. Changes in RPE cell volume in vivo may alter the volume and composition of the extracellular (subretinal) space surrounding photoreceptors, and thus isosmotic volume regulation could play a key physiological role in maintaining the integrity and health of the neural retina in normal and pathophysiological conditions.[37] One mechanism used by cells to ensure proper volume homeostasis is by accumulation or release of osmolytes such as taurine. Yokoyama et al.[8] reported an increase in taurine content in RPE cells, when they were cultured in hyperosmolar conditions. These studies did not reveal whether the increase in taurine content was due to increased taurine synthesis or increased influx of extracellular taurine through TauT.

To determine whether TauT activity is increased by hyperosmolar conditions in RPE, we used ARPE-19 cells, which retain many features characteristic of RPE cells in vivo.[25, 38–39] Our studies were performed by increasing the osmolarity of the culture medium with the addition of either NaCl or mannitol and determining the ability of the cells to take up taurine. Addition of either 50 mM NaCl or 100 mM mannitol to the normal culture medium (an increase of 100 mOsM in the osmolarity of the medium) led to a marked increase in taurine uptake. The effects of hyperosmolarity on TauT activity were not immediate. Rather, a significant increase in TauT activity was observed after 8 hours of incubation in hyperosmolar conditions and peaked at 17 hours. Incubation of cells for 17 hours in hyperosmolar conditions led to a 2.5- to 3.5-fold

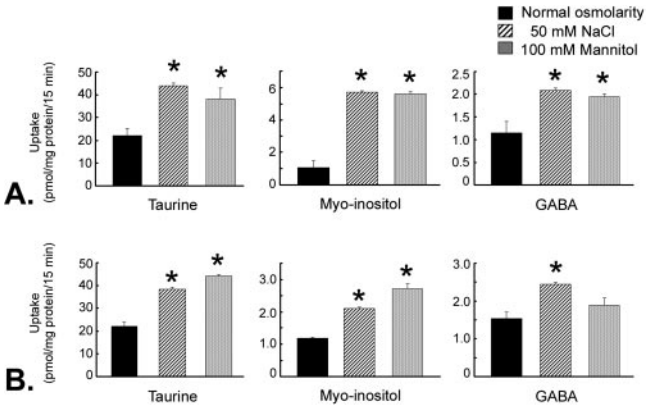

FIGURE 10. Uptake of taurine, *myo*-inositol and GABA in ganglion cells and Müller cells cultured in hyperosmolar conditions. Confluent RGC-5 cells (**A**) or rMC1 cells (**B**) were maintained for 17 hours in normal culture medium with no addition (normal osmolarity), 50 mM NaCl, or 100 mM mannitol (an increase of 100 mOsM in the osmolarity of the culture medium). Uptake of taurine (80 nM), *myo*-inositol (100 nM), or GABA (65 nM) was determined. Data are the mean ± SEM of six determinations from two independent experiments. *Significantly different from control (normal osmolarity; $P < 0.05$).

stimulation of TauT activity. The effects of hyperosmolarity were not nonspecific, as uptake of carnitine and leucine was not affected by a change in osmolarity.

Kinetic analysis showed that the increased TauT activity in hyperosmolar conditions was due to an increase in the maximum velocity of taurine uptake. The substrate affinity was not affected in these conditions. This suggests that transporter density in the plasma membrane may be increased by hyperosmolarity. RT-PCR and Northern blot analysis provided evidence that the steady state levels of TauT mRNA increased approximately twofold in hyperosmolar conditions. Thus, the hyperosmolarity-induced increase in TauT activity is probably due to increased expression of the gene coding for TauT. Western blot analysis demonstrated an increase in TauT protein in cells exposed to hyperosmolar conditions.

We determined whether uptake of *myo*-inositol and GABA in RPE was regulated by hyperosmotic conditions. *myo*-Inositol belongs to the polyol class of chemicals and, like taurine, is a small organic solute that can be lost or accumulated by cells, accompanied by the flux of osmotically obligated water. GABA is an amino acid that is abundant in retina and can act as an osmolyte. Exposure of RPE cells to hyperosmolar conditions led to a marked stimulation of *myo*-inositol uptake (15-fold) and a twofold increase in the uptake of GABA.

Osmoregulation of TauT has been studied in other ocular epithelial cells. Shioda et al.[11] studied the activity and expression of TauT in corneal epithelial cells. Exposure to hypertonic medium for 12 hours led to a fourfold increase in TauT activity that was associated with an increase in gene expression, just as we found for RPE. Similarly, Cammarata et al.[12] studied taurine uptake in lens epithelial cells and found that hyperosmolar conditions led to an increase in the V_{max} of the transporter, but that there was no change in substrate affinity. They reported an upregulation of TauT mRNA after exposure to hyperosmolar conditions. Our studies provide the first evidence that TauT activity in RPE, like corneal and lens epithelial cells, is regulated by hyperosmolarity. These findings are important because there are significant changes in extracellular osmolarity in a broad range of RPE disorders, including retinitis pigmentosa, retinal detachment, and macular degeneration[24,40,41] and in systemic disease such as diabetic retinopathy.[40–42]

In addition to RPE, we asked whether TauT activity in other retinal cell types would respond similarly to hyperosmolar stress. We were interested in ganglion and Müller cells, both of which are affected in diabetes.[26,27,29,43] Because in diabetes elevated glucose levels disturb cellular osmoregulation,[44] it was of interest to determine whether ganglion and Müller cells demonstrate TauT activity. TauT activity has not been reported in either of these cell types, although it was reported in neurons[45] and astrocytes[46] derived from brain. Our earlier immunohistochemical studies in intact retinal tissue detected TauT in retinal ganglion cells and the inner nuclear layer,[18] which contains Müller cells. It is not feasible to study TauT activity of specific cells using intact retinas, and therefore we obtained the ganglion cell line RGC-5[31] and the Müller cell line rMC1,[32] to study taurine uptake in these specific cell types. Our immunocytochemical analyses detected TauT in both cell lines. We demonstrated that RGC-5 and rMC1 cells have a NaCl-dependent TauT whose taurine transport function is inhibited completely by β-alanine, a known substrate of TauT.

Exposure of RGC-5 and rMC1 cells to hyperosmolar conditions resulted in a marked increase in taurine uptake. Interestingly, the basal level of taurine uptake in these cells was significantly greater than in RPE. In normal osmolar conditions, RPE cells took up taurine (80 nM) at an average rate of approximately 2 pmol/mg/protein/15 minutes, whereas the rates for ganglion and Müller cells were 22 pmol/mg protein/15 minutes and 15 pmol/mg protein/15 minutes, respectively in similar conditions. Exposing RGC-5 and rMC1 cells to hyperosmolar conditions stimulated TauT, and the time course of stimulation was similar to that in RPE cells with the peak stimulation occurring at 17 hours. The increase in TauT activity was approximately twofold, which is slightly less than that observed in RPE (~3.5 fold). As with the RPE, incubation of RGC-5 and rMC1 cells in hyperosmolar conditions did not affect all transport systems. For example, the uptake of carnitine and leucine was not affected. However, whereas ARPE-19 cells demonstrated robust uptake of *myo*-inositol, neither RGC-5 nor rMC1 cells had such marked activity. Both cell types appeared to activate TauT preferentially in hyperosmolar conditions, rather than take up *myo*-inositol. As with RPE, the induction of GABA uptake in hyperosmolar conditions was not extensive.

These studies represent the first demonstration that TauT is functional in ganglion and Müller cells, and they provide the first evidence that TauT is regulated by hyperosmolar conditions in these cells and RPE. It is noteworthy that in many call types, failure to adapt to osmotic stress can result in apoptotic cell death. Moreover, the ability of cells to resist osmotic shrinkage by cell volume regulation parallels their resistance to apoptosis after osmotic shock.[47] A recent study indicated that chronic taurine supplementation ameliorates oxidative stress in diabetic rat retina.[48] Taurine supplementation may have therapeutic potential for the treatment of derangement in osmoregulation characteristic of diabetes. Future analysis of the effects of hyperglycemia on the activity of TauT in these retinal cell types should provide insights about the function of this transporter in diabetic conditions. The present study assessed the activity of the transporters for taurine and *myo*-inositol using tracer concentrations of substrate in vitro. It is possible that in an in vivo situation with physiological concentrations of taurine and *myo*-inositol, the alterations in transporter expression may lead to sufficient accumulation of these osmolytes inside of cells, which matches the changes in osmolarity in the extracellular space. Future comprehensive studies to address this issue should establish whether this is indeed the case.

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