Downregulation of the human taurine transporter by glucose in cultured retinal pigment epithelial cells

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Stevens, Martin J., Yoshiyuki Hosaka, Jennifer A. Master, Sandra M. Jon, Thommey P. Thomas, and Dennis D. Larkin. Downregulation of the human taurine transporter by glucose in cultured retinal pigment epithelial cells. Am. J. Physiol. 277 (Endocrinol. Metab. 40): E760–E771, 1999.—In diabetes, activation of the aldose reductase (AR) pathway and alterations of glucose-sensitive signal transduction pathways have been implicated in depletion of intracellular taurine, an endogenous antioxidant and compatible osmolyte. Cellular taurine accumulation occurs by an osmotically induced, protein kinase C (PKC)-regulated Na+-taurine cotransporter (hTT). The effects of ambient glucose on taurine content, hTT activity, and hTT gene expression were therefore evaluated in low and high AR-expressing human retinal pigment epithelial cell lines. In low AR-expressing cells, 20 mM glucose decreased taurine content, hTT activity, and hTT gene expression by 10.22±0.33.4 on July 7, 2017 http://ajpendo.physiology.org/ Downloaded from
potential PKC and protein kinase A (PKA) phosphorylation sites (19). In cell culture models, the TT has been shown to be regulated by signal transduction pathways (3, 21, 22), hypertonicity (52, 53), and extracellular taurine levels (11, 21, 22). Although nonionic “nonperturbing” compounds respond coordinately to osmolar stress to buffer injurious shifts in intracellular electrolyte and water composition (39), sorbitol, myo-inositol, and betaine are not substrates for the hTT and are not known to directly affect hTT activity (18).

The effects of hyperglycemia on hTT gene expression and activity are unknown. The studies reported in this communication explore the effects of pathophysiological hyperglycemia and glucose-sensitive signal transduction pathways on hTT gene expression and activity in low and high AR-expressing cultured human retinal pigment epithelial (hRPE) cells (13, 48, 50). Taurine is the most abundant free amino acid in the retina (43), with levels in RPE cells approaching 20 mM (23). In the absence of hyperglycemia, taurine is present in hRPE cells at levels in excess of those of the other principal retinal osmolytes, myo-inositol and sorbitol (48), consistent with an important role for taurine in osmoregulation within the RPE. The RPE regulates the entry of taurine from the blood to the neural retina, has no taurine synthetic capacity (30), and therefore relies on the hTT to accumulate taurine. In the retina, taurine inhibits protein phosphorylation (31) and oxidative damage (42), and taurine deficiency can result in retinal degeneration (23). The results contained herein demonstrate that glucose rapidly and specifically decreases taurine content, hTT activity, and hTT mRNA abundance by non-AR-related posttranscriptional “metabolic” as well as AR-related transcriptionally mediated “osmotic” mechanisms.

MATERIALS AND METHODS

Cell culture media (MEM and taurine concentrations = 0 µM), Hanks’ balanced salt solution, trypsin-EDTA, and antibiotics were obtained from Gibco (Grand Island, NY); bovine calf serum (BCS) ([taurine] = 80–120 µM) was from HyClone Laboratories (Logan, UT); culture dishes, wells, and flasks were from Falcon (Lincoln Park, NJ); taurine, bicinchoninic acid, copper sulfate, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), dioctanoylglycerol (DiC8), α-phorbol deoxyst configur (α-PDD), isobutylmethylxanthine (IBMX), forskolin, isoproterenol, and dibutyryl cAMP were from Sigma Chemical (St. Louis, MO); and H-89 was from Biomol (Plymouth Meeting, PA). Calphostin C was from Kamiya Biomedical (Thousand Oaks, CA), and bisindolylmaleimide (BIM) was from LC laboratories (Woburn, MA). Radioisotopes were from Amersham (Arlington Heights, IL). Measurements were normalized to cell protein determined by the bicinchoninic acid method with BSA as the standard.

Cell Culture Techniques and Experimental Design

Primary cultures of hRPE cells were established as previously described (8), using postmortem eyes obtained from the Michigan Eye Bank. hRPE cell lines were passaged at a density of 40–100,000 cells/cm² in 25-cm² or 75-cm² flasks in MEM with 2 mM L-glutamine containing 20% BCS ([taurine] adjusted to 50 µM) and 5 mM glucose at 37°C in a humidified 95% air-5% CO₂ atmosphere. Passaged cells were plated at initial densities of 5,000–20,000 cells/cm² in 6-well plates or 150-mm-diameter culture dishes to yield near-confluent cultures at the end of experiments.

Passages 10–20 of two cell lines differing in AR gene regulation (13, 48) but obtained from subjects of similar age and sex (both subjects were nondiabetic females and were 50 and 54 yr of age at death) were compared. The freshly plated cells were allowed to attach in standard growth medium for 24 h and then were grown in MEM with 5% BCS ([taurine] adjusted to 50 µM for 3 days at 37°C to allow equilibration to take place). After 3 days, the cells were exposed to the various experimental conditions specified in the text and legends to Figs. 1–12, and taurine content, uptake, or hTT mRNA measurements were performed as we will describe. The phenotypic characteristics were monitored microscopically, functionally [rod outer segment phagocytosis (8)], and immunocytochemically [alpha-acetylated low-density lipoprotein receptor protein (8)]. Neither cell morphology nor survival was detectably altered by the experimental conditions employed in these studies. Ten to thirty millimolar glucose simulated pathophysiological isosmotic hyperglycemia in which flux through the AR pathway would be stimulated primarily by mass action rather than by gene induction (13).

Preparation of hTT cDNA from hRPE mRNA by PCR Amplification

A partial cDNA encoding the hTT was obtained by PCR amplification of oligo(dT)-primed cDNA prepared from total cellular RNA from low AR-expressing hRPE 47 cells after 24 h of hypertonic stress in medium supplemented with 300 mM mannitol. Oligonucleotide primers derived from the coding portion of the hTT cDNA (nucleotides 84–1008) (19) were used to amplify a 900-bp fragment, which had little sequence homology with other Na⁺- or Cl⁻-dependent neurotransmitter transporters. A single major product was observed. The nucleotide sequence was determined on both strands and was identical to the published hTT sequence (19). The purified 900-bp cDNA insert for hRPE hTT was subsequently utilized as a probe for evaluation of changes in hTT mRNA levels.

Preparation of hTT Genomic Targets for Nuclear Run-On Studies

In preliminary studies, low level hybridization to the 900-bp hTT cDNA target prevented evaluation of transcriptional change, which initiated the acquisition of a 5'-extended hTT genomic clone for use as a target. To this end, a human peripheral blood leukocyte genomic DNA library in lambda fix (1.2 × 10⁶ recombinant phage) was screened at high stringency by filter hybridization with the 900-bp hTT cDNA. Candidate clones were plaque purified, and large scale phage DNA preparations were digested with restriction endonucleases and mapped by Southern blotting with different 5'-hTT cDNA probes or synthetic oligonucleotides. Restriction fragments hybridizing with the probes were subcloned and sequenced, which confirmed their identity. Two hTT genomic fragments were selected as potential targets in run-on studies. Two hTT genomic fragments were single copy and suitable for nuclear run-on studies. To increase signal intensity, these two fragments were pooled to create a single target.

Northern Analysis Techniques

Total cellular RNA was isolated and purified from cultured hRPE cells by differential extraction with acid phenol (48) after exposure to various experimental conditions (below). Ten micrograms of hRPE cell RNA or 2.5 µg of a 0.24- to 9.5-kb
RNA ladder (Life Technologies, Gaithersburg, MD) were resolved on 2.2 M formaldehyde-1% agarose gel and transferred by capillary blotting to nylon membranes (Zetabind; Cuno, Meridian, CT). Methylene blue staining confirmed uniformity of loading and transfer and integrity of RNA, and the filters were fixed in vacuo at 80°C for 2 h. Probes were labeled with α[32P]-dCTP by use of random primers to a specific activity in excess of 10^9 dpm/μg. Filters were prehybridized at 65°C in 0.5 M NaH₂PO₄ (pH 7.0), 1 mM EDTA, 7% SDS, and 1% BSA (fraction V) for 4–6 h before addition of denatured probe. After 18 h, filters were washed twice with 40 mM NaH₂PO₄ (pH 7.0), 1 mM EDTA, and 7% SDS at 65°C, and then four times with 40 mM NaH₂PO₄ (pH 7.0), 1 mM EDTA, and 1% SDS at 65°C. Hybridization was quantitated using a phosphorimager (Molecular Dynamics) or by exposure at −80°C to preflashed X-Omat AR (Kodak) film with intensifying screens, and multiple exposures of the autoradiogram were obtained and quantitated by laser densitometry. Filters were sequentially hybridized, stripped, and rehybridized with [32P]-labeled cDNA probes for the hTT, human AR (48), chicken β-actin (6), and/or glyceraldehyde-3-phosphate dehydrogenase mRNA (1) as previously described (48).

Isolation of Nuclei

Nuclei (10⁶ per time point) from hRPE cells exposed to the various experimental conditions were harvested and washed with PBS. Pelleted cells were suspended in resuspension buffer (RSB) (10 mM Tris pH 7.5, 10 mM NaCl, 5 mM MgCl₂) to a final concentration of 10 ml/10⁷ cells and pelleted again. The supernatant was aspirated, and the cells were suspended in an appropriate concentration of NP-40 in RSB (hRPE cells at 0.3%) and pelleted. The supernatant was aspirated, and the pellet was suspended in 210 µl of nuclear freezing buffer (NFB)/5× 10⁷ cells. Aliquots (210 µl) were snap-frozen in liquid nitrogen. An aliquot of nuclei was stained with trypan blue in NFB, examined, and counted in a hemocytometer chamber. Recovery of nuclei was >80%, without clumping or excessive cytoplasmatic tags. No nuclei were stored in liquid nitrogen for longer than 2 wk.

Evaluation of hTT Transcriptional Rate

A 210-µl aliquot of frozen hRPE nuclei (~3 × 10⁷) was thawed on ice, and 5× transcription buffer was added to a final concentration of 5 mM Tris (pH 8.0), 150 mM KCl, 2.5 mM MgCl₂, and 250 µM ATP, CTP, and GTP. Three hundred microcuries of α[32P]-UTP (3,200 Ci/mM, final concentration 0.3 µM) were added, and the nuclei were incubated at 30°C for 30 min. The nuclei were incubated with RNase-free DNase at 37°C for 30 min and then incubated with 100 µg of proteinase K in 1% SDS, 5 mM EDTA at 42°C for 1 h. After phenol/chloroform extraction, the RNA was precipitated and suspended in TE, and residual unincorporated nucleotides were removed with minispin columns of G-50. The RNA was partially hydrolyzed with 0.2 N NaOH on ice for 10 min and precipitated. The pelleted RNA was dissolved in hybridization buffer consisting of 10 mM TES (pH 7.4), 10 mM EDTA, 0.3 M NaCl, 0.2% SDS, 1× Denhardt’s, and 0.25 mg/ml Escherichia coli RNA. Identical volumes of hybridization buffer containing the same amount of labeled RNA were hybridized at 65°C for 48 h with denatured DNAs fixed to nitrocellulose filters. Two hTT genomic fragments were pooled to create a single target to increase signal intensity. The negative controls were pBluescript plasmid DNA, p285 DNA, and human genomic DNA. After stringent hybridization and washing, hybridization was quantitated using a phosphorimager.

Biochemical Measurements

Taurine uptake. Cells were cultured in 6-well plates to reach 80% confluence, exposed to various experimental conditions, and then washed three times in 2-ml aliquots of prewarmed (37°C) taurine uptake buffer (in mM: 20 HEPES, 140 NaCl, 5.4 KCl, 1.0 CaCl₂, 0.8 MgSO₄, and 5 glucose) and equilibrated in 3 ml of the uptake buffer at 37°C. Uptake was initiated by adding uptake buffer containing [3H]taurine (1–1,000 µM and 1–10 µCi/ml), with the amount of label increased at higher taurine concentrations to offset the decrease in specific activity. Preliminary studies (not shown) demonstrated that increasing the glucose content of the uptake buffer to 30 mM did not acutely affect hTT activity, confirming that glucose per se does not directly interfere with taurine uptake. Aliquots of the original labeled media were counted to determine specific activity. Uptake was measured over 15 min (at which time uptake was linear) unless otherwise specified, in the presence or absence of appropriate concentrations of ligands or vehicle, as described in text and Figs. 1–12. Na⁺-independent uptake was measured by substituting NaCl with equimolar concentrations of choline chloride. The radioactive buffer was aspirated, and the cells were washed in ice-cold uptake buffer in the absence of radioactive taurine, extracted at room temperature in 1 ml of 0.1 M NaOH, and sonicated; uptake was measured by scintillation spectrometry and expressed as picomoles of taurine per milligram protein per minute. At 1 µM [3H]taurine, the Na⁺-independent component was negligible (<1% of total uptake) and was not routinely subtracted from total uptake for the measurement of Na⁺-independent uptake.

Cell taurine and sorbitol content. Taurine was measured by reversed-phase HPLC after precolumn derivatization with o-phthalaldehyde (20). Briefly, cells were sonicated in 1 ml of 6% trichloroacetic acid (TCA) and centrifuged at 4,000 g for 10 min. The supernatants were purified on washed dual-bed ion-exchange columns (2.5 cm AG 1-X8 100–200 mesh (Bio-Rad, Richmond, CA) in the chloride form over 2.5 cm AG 50W-X8 200/400 mesh (Bio-Rad) in the hydroxide form) by elution with 2 ml of water and lyophilized. Samples and standards were dissolved in 100 µl of water before HPLC analysis on a Waters system (Waters, Milford, MA) equipped with a model 501 pump, a 717 autosampler, a 3.9 × 150-mm Nova-Pak C₁₈ column, and a model 470 scanning fluorescence detector. Isocratic elution was carried out at a flow rate of 2 ml/min, using 43% solvent A (0.05 M NaH₂PO₄ pH 5.3, plus 5 M NaOH) combined with 57% solvent B (0.05 M NaH₂PO₄ in 75% methanol-water). Glutamine, added after ion exchange chromatography, was used as the internal standard. Standard curves were linear over the concentration range in hRPE samples, and recovery of taurine was >90% hRPE cell sorbitol was determined, as previously described, by gas-liquid chromatography of aldonitrile acetate derivatives from lyophilized aliquots of protein-free filtrates of hRPE cells sonicated in 5% w/vol TCA with α-methyl mannoorphanoside as an internal standard (48). Standard curves were generated daily, and the recovery-corrected values were expressed as nanomoles per milligram protein. Cell protein was measured with bichinchoninic acid (48).

Statistical Analysis

Data are expressed as means ± SE of at least three determinations for a representative experiment. Each experiment was replicated in triplicate. Differences among experimental groups were detected by analysis of variance, and the differences between groups were assessed by the Student-
Newman-Keuls test. Significance was defined at the 0.05 level.

RESULTS

Comparison of the Relationship of Basal AR and hTT Gene Expression in High and Low AR-Expressing hRPE Cells

To explore the relationships between basal AR and hTT gene expression, steady-state AR and hTT mRNA levels were compared in high (hRPE 91) and low (hRPE 47) AR-expressing hRPE cell lines that had been maintained in 5 mM glucose. Basal expression of the AR gene was twofold higher in hRPE 91 cells than in the hRPE 47 cell line (Fig. 1). In both cell lines, the hTT cDNA probe hybridized to a single distinct transcript of the expected molecular size (6.9 kb) (45). However, in the high AR-expressing hRPE 91 cells, basal hTT mRNA levels were 46 ± 5% (P < 0.01) lower than in the hRPE 47 cell line (Fig. 1).

Effect of Elevated Glucose on Taurine Content and hTT Gene Expression in Low AR-Expressing hRPE Cells

Effects on taurine content and steady-state hTT mRNA abundance. The time-dependent changes in taurine content were measured in low AR-expressing hRPE 47 cells exposed to 20 mM glucose for 2–24 h. Taurine content was decreased by 32% (P < 0.05) within 2 h of glucose exposure (20.7 ± 1.4 nmol/mg protein vs. 14.2 ± 0.6 nmol/mg protein in 5 and 20 mM glucose, respectively). This decrease was unaffected by the addition of the AR inhibitor sorbinil (10 µM) [13.3 ± 0.5 nmol/mg protein, nonsignificant (NS)]. Taurine content remained depressed (by 25%, P < 0.05) and AR uncorrectable even after 24 h of glucose exposure. To explore the AR-unrelated effects of glucose on hTT gene expression, the acute time-dependent effects of 5–30 mM glucose were assessed in hRPE 47 cells, which do not accumulate detectable levels of sorbitol within the first 12 h of exposure to glucose (8). In 20 mM glucose, hTT mRNA levels began to decline by 2 h, reaching maximal depression by 12 h (Fig. 2A). This decrease was neither affected by sorbinil nor reproduced by 30 mM mannitol (not shown). The reduction of mRNA was concentration dependent between 5 and 30 mM glucose, because hTT mRNA levels were decreased to 84 ± 5% (P < 0.05) of basal values in 10 mM glucose and were maximally decreased at concentrations of glucose >15 mM at 12 h (57 ± 17% of basal, P < 0.05; Fig. 2B). No changes in AR mRNA levels were observed over the same time course. Thus physiological levels of glucose appear to rapidly and specifically decrease steady-state hTT mRNA levels by a mechanism that is at least in part independent of AR-pathway activity.
**E764 Taurine Transporter Regulation by Glucose**

**Target Layout**

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**Fig. 3.** Effect of glucose on hTT transcriptional rate in low AR-expressing hRPE cells. Nuclear run-on analysis of hTT gene transcription was performed with nuclei isolated from hRPE cells maintained in 5 or 20 mM glucose for 6 and 12 h. The labeled nascent transcripts were purified free of genomic DNA and unincorporated label, partially hydrolyzed, precipitated, and hybridized under stringent conditions with denatured DNAs fixed to nitrocellulose filters. Pooled hTT genomic DNA fragments were used to detect hTT gene transcription. The negative controls were pBluescript plasmid DNA (pBS), p28S DNA, and human genomic DNA. Hybridization signals were normalized to genomic DNA. hTT gene transcription was unaffected by exposure to 20 mM glucose for up to 12 h in low AR-expressing cells. Similar results were obtained in 3 independent experiments.

**Fig. 4.** Effect of glucose on hTT mRNA stability. Low AR-expressing hRPE 47 cells were exposed to 10 µg/ml actinomycin D for 2–8 h in 5 mM (top panels) or 20 mM (bottom panels) glucose. RNA was isolated, Northern blots were prepared, and the filter was sequentially hybridized with the 900-bp hTT cDNA probe and chicken β-actin cDNA. Cell density was unaffected by the experimental conditions. Hybridization was quantified using a phosphorimager. hTT mRNA abundance decreased rapidly in 20 mM glucose compared with 5 mM glucose. Similar results were obtained in 3 independent experiments.

**Fig. 5.** Substrate kinetics of taurine uptake in hRPE 47 cells. Taurine uptake measurements were made at different concentrations of taurine in the presence (filled symbols) or absence (open symbols) of Na⁺. The Na⁺-dependent taurine uptake (•) was obtained by subtracting the Na⁺-independent uptake from the total uptake. Inset: Eadie-Hofstee plot for Na⁺-dependent taurine uptake. V(TT) uptake divided by [taurine]. Values are means ± SE of 3 cell samples, with similar results obtained in 3 independent experiments.

**Kinetic Characterization of hTT Activity and Effects of Elevated Glucose on Taurine Transport in Low AR-Expressing hRPE Cells**

**Kinetic characterization.** Uptake of 1 µM [³H]taurine remained linear for 30 min (data not shown), permitting reliable estimation of the rate of uptake during an initial 15-min interval. Composite initial uptake varied in curvilinear fashion with respect to [³H]taurine concentration from 1 to 1,000 µM (Fig. 5). Composite uptake consisted of two components: a nonsaturatable Na⁺-independent component and Na⁺-dependent uptake that by Eadie-Hofstee analysis (Fig. 5, inset) was saturated with a Michaelis-Menten constant (K_m) value of 3.9 ± 0.2 µM and a maximum velocity (V_max) value of 285.9 ± 7.3 pmol·min⁻¹·mg⁻¹. The kinetics of this high-affinity transporter were in agreement with another report in hRPE cells (28). It is therefore reasonable to ascribe the measured saturable Na⁺-dependent [³H]taurine uptake to hTT activity in hRPE cells. The physiological levels of plasma taurine are reported to be in the range of 50 µM (17); therefore, net taurine uptake from plasma would predominantly reflect the Na⁺-dependent component. Subsequent experiments were performed with [³H]taurine = 1 µM, well below the K_m for this transporter.

**Glucose effects.** Exposure of low AR-expressing cells to 30 mM glucose decreased hTT activity in a time-dependent fashion (Fig. 6). Transporter activity was maximally decreased to 66.3 ± 2.4% of control after 4 h of glucose exposure (P < 0.01; Fig. 6). This decrease in transporter activity was unaffected by the addition of 10 µM sorbinil and was not reproduced by equimolar concentrations of mannitol (Fig. 6). Kinetic characterization of hTT activity after exposure to 5 or 30 mM glucose for 6 h is shown in Fig. 7. Eadie-Hofstee analysis of triplicate cell preparations demonstrated
significantly decreased the apparent V_{\text{max}} of transport.

Effects of Known Glucose-Sensitive Signal Transduction Pathways on hTT Activity in Low AR-Expressing hRPE Cells

To explore regulation of the hTT by PKC, initial (15 min) Na\(^{+}\)-dependent \(^{3}\text{H}\)taurine uptake was measured in hRPE 47 cells treated acutely with PKC activators or PKC inhibitors, or chronically with concentrations of PKC agonists designed to downregulate PKC. Activation of PKC by exposure to 100 nM TPA for 15 min markedly decreased Na\(^{+}\)-dependent 1 µM \(^{3}\text{H}\)taurine uptake to 31 ± 3% of control values (P < 0.01). Dic8, a nonphorbol PKC-activating diacylglycerol (DAG) (50 µM × 30 min), also decreased 1 µM \(^{3}\text{H}\)taurine uptake to 75 ± 2% of control (P < 0.01). The PKC-inactive phorbol ester α-PDD (100 nM × 15 min) failed to alter 1 µM \(^{3}\text{H}\)taurine uptake (99.7 ± 3.1% of control, P = NS). The effect of Dic8 was found not to be dependent on its conversion to phosphatidic acid, because the DAG kinase inhibitor R-59949 (20 µM × 30 min) did not attenuate the decrease in hTT activity by Dic8 (79 ± 2% of control, P < 0.01). Conversely, PKC inhibition with calphostin C (100 nM × 2 h) and BIM (2 µM × 15 min) increased 1 µM \(^{3}\text{H}\)taurine uptake to 117 ± 1 and 126 ± 4% of control, respectively (P < 0.01). PKC downregulation (1 µM TPA × 24 h) also increased apparent hTT activity (126 ± 1% of control, P < 0.01). These data are consistent with the hypothesis that hTT activity is tonically suppressed by basal PKC activity and that PKC activation inhibits apparent hTT activity. To evaluate whether the effects of glucose on hTT activity in hRPE 47 cells involved PKC, the effects of the PKC inhibitor BIM were explored in cells exposed to 20 mM glucose. The glucose-induced decrease in hTT activity was not observed in the presence of BIM (Fig. 8).

Regulation of the hTT by PKA was explored by measuring the initial (15 min) Na\(^{+}\)-dependent uptake of 1 µM \(^{3}\text{H}\)taurine in hRPE 47 cells treated acutely (30–60 min) with cAMP analogs, PKA inhibitors, or compounds associated with endogenous cAMP accumulation. Dibutyl cAMP (DBcAMP, 1 mM × 60 min), a PKA-activating nonmetabolized cell-permeable cAMP analog, decreased hTT activity (59 ± 4% of control, P < 0.01). Receptor-mediated activation of the cAMP signal pathway by the β-adrenergic agonist isoproterenol (10 µM × 30 min), as well as the cAMP phosphodiesterase inhibitor IBMX (0.5 mM × 60 min), also inhibited hTT activity (77 ± 5 and 76 ± 5% of control, respectively, P < 0.01). In agreement with another report (3), PKA inhibition with H-89 (10 µM × 30 min) was also paradoxically found to inhibit hTT activity (72 ± 3% of control, P < 0.01). These data suggest that PKA regulates hTT activity in a complex fashion, with normal unstimulated PKA activity permissive for normal hTT activity. Neither PKA activation nor inhibition
caldhostin C (100 nM × 4 h) or BIM (2 µM × 4 h, not shown) was, however, without measurable effect on hTT mRNA abundance. Calphostin C was found to partially attenuate the TPA-mediated increase in hTT mRNA abundance (1.8 ± 0.3-fold above control, P < 0.01 vs. TPA alone) and completely prevented the increase mediated by DiC8 (1.0 ± 0.5-fold above basal) (Fig. 9). Neither PKA activation using DBcAMP, isoproterenol, or IBMX nor PKA inhibition with H-89 (for time periods from 2 to 12 h) was found to affect hTT mRNA levels (not shown). These data suggest that hTT gene expression is regulated by PKC but not PKA. However, neither PKC activation nor inhibition could reproduce the effects of elevated glucose on hTT gene expression, suggesting that the glucose-induced down-regulation of hTT gene expression is not directly mediated by a change in composite cellular PKC activity.

Effect of Elevated Glucose on Intracellular Sorbitol and Taurine Content in High AR-Expressing hRPE Cells

To explore the consequences of sorbitol accumulation on intracellular taurine content, the effects of 20 mM glucose with and without the ARI sorbinil (10 µM) were explored in high AR-expressing cells after 2–4 days [when intracellular sorbitol content had increased significantly (8, 48)]. Intracellular sorbitol and taurine content was then measured. In 5 mM glucose, intracellular taurine content did not change over the experimental period. However, in 20 mM glucose, intracellular sorbitol content progressively increased and taurine content progressively declined to 37 ± 2% of basal by 48 h (P < 0.01; Fig. 10). Sorbinil blocked the accumulation of sorbitol and prevented the decline in taurine levels. These data suggest that, in high AR-expressing hRPE cells, elevation of media glucose may lead to intracellular taurine depletion through an AR-related mechanism.
Effect of Elevated Glucose on hTT Gene Expression and Activity in High AR-Expressing Cells

To explore the effects of high glucose on hTT gene expression in high AR-expressing cells, changes in steady-state hTT mRNA abundance were assessed in these cells on exposure to 5 and 20 mM glucose with and without 10 µM sorbinil for 48 h. In 5 mM glucose, the low level of hTT mRNA was reduced by 50 ± 5% (P < 0.01) in 20 mM glucose, and this decrease was prevented by sorbinil (Fig. 11A). No changes in AR mRNA levels were observed over the same time course. The corresponding changes in hTT activity in these cells is shown in Fig. 11B. Twenty millimolar glucose decreased hTT activity to 32 ± 3% of control (P < 0.01), which was corrected by ARI. To explore the mechanism of the glucose-induced ARI-sensitive decrease in hTT mRNA abundance, nuclear run-on analysis was performed to assess changes in hTT transcriptional rate.

Fig. 11. Effects of 20 mM glucose and ARI on hTT and AR mRNA abundance (A) and hTT activity (B) in high AR-expressing hRPE cells. High AR-expressing hRPE 91 cells were plated at a density designed to achieve 80% confluence at the end of the experiment and then were exposed to 20 mM glucose ± ARI (10 µM sorbinil) for 48 h. RNA was isolated, and Northern blots were prepared. The filter was sequentially hybridized with the 900-bp hTT cDNA probe, a partial human AR cDNA, and chicken β-actin cDNA. Parallel experiments assessed the initial (15 min) Na⁺-dependent [3H]taurine uptake under the same experimental conditions. Values are means ± SE from 3–12 independent experiments performed in triplicate wells. *P < 0.01 vs. 5 mM glucose control; †P < 0.01 vs. 20 mM glucose. 20 mM Glucose decreased hTT mRNA abundance and activity by an ARI-sensitive mechanism. Similar results were obtained in 3 independent experiments.

In hRPE 91 cells, hTT gene transcription was decreased by 91 ± 6% (P < 0.01) in 5 mM glucose compared with hRPE 47 cells, was not further depressed in 20 mM glucose, but increased 27 ± 8-fold (P < 0.01) in 20 mM glucose with the addition of ARI (Fig. 12) to levels that were 3.2-fold above hRPE 47 cells. These data suggest that in high AR-expressing hRPE cells, increased polyol pathway flux decreases hTT mRNA abundance by suppressing hTT transcriptional rate.

DISCUSSION

The studies reported herein explore potential mechanisms whereby glucose could interfere with taurine accumulation at the level of taurine transporter gene expression and activity in cultured hRPE cells. These cells in vivo exhibit morphological and biochemical changes in response to diabetes (36). In low AR-expressing cultured hRPE cells, glucose decreased taurine content before intracellular sorbitol had detectably accumulated (8). Moreover, in these cells, glucose decreased hTT gene expression in a time- and concentration-dependent fashion that was unaffected by ARI and correlated with a decrease in hTT mRNA stability. Changes in hTT activity paralleled hTT mRNA levels, and kinetic studies suggested that 20 mM glucose decreased the apparent V_max of transport. PKC activation inhibited hTT activity but paradoxically increased hTT mRNA abundance, whereas PKC inhibition stimulated hTT activity and did not affect hTT mRNA levels. PKA activation and inhibition both decreased hTT activity, and neither affected hTT gene expression. The pattern of hTT regulation by glucose was considerably different in high AR-expressing cells. In high AR-expressing cells in 5 mM glucose, hTT transcription was decreased compared with low AR-expressing cells. hTT mRNA abundance was also lower in high vs. low
AR-expressing cells in 5 mM glucose. Exposure of high AR-expressing cells to 20 mM glucose decreased taurine content, hTT activity, and hTT mRNA abundance without further suppressing hTT gene transcription: these effects were prevented by ARI. This suggests that AR activity downregulates hTT at the transcriptional level in high AR-expressing hRPE cells. Thus studies in low and high AR-expressing hRPE cells suggest that glucose downregulates taurine transport by AR-independent and AR-dependent mechanisms at the level of hTT transcription, mRNA stability, and perhaps other posttranscriptional or posttranslational mechanisms.

In low AR-expressing hRPE cells, elevated glucose decreased hTT gene expression, activity, and taurine content before sorbitol accumulation and without sensitivity to ARI, suggesting an osmotically independent mechanism. These data provide novel evidence that, in addition to being an osmotically regulated gene, the hTT is critically affected by glucose-sensitive metabolic pathways. The attenuated magnitude of the reduction of hTT activity and taurine depletion in low AR-expressing vs. high AR-expressing hRPE cells implicates the additional osmotic consequences of excess intracellular sorbitol accumulation. Depletion of intracellular taurine in the absence of a reciprocal rise in intracellular sorbitol content suggests that osmotic equilibrium is maintained under conditions of high glucose in low AR-expressing cells by changes in other osmotically active solutes such as myo-inositol (8, 48), betaine (5, 57), and inorganic ions (5, 39). In contrast to hRPE 91 cells (8, 48, 50), myo-inositol is not depleted in low AR-expressing hRPE cells exposed to 20 mM glucose (48), and an hRPE betaine transport system has not been reported. Further studies are required to address the interrelationships of osmotically active solutes in hRPE cells in response to pathophysiological concentrations of glucose.

In low AR-expressing hRPE cells, high glucose resulted in destabilization of hTT mRNA without decreasing hTT gene transcription, suggesting that in these cells the inhibitory effects of glucose on hTT activity may be predominantly posttranscriptional. This would parallel the posttranscriptional effects of glucose on the regulation of several hepatic glucogenic, glycolytic, and lipogenic enzymes. For example, in cultured hepatocytes and hepatoma cells, glucose accelerates the degradation of phosphoenolpyruvate carboxykinase (EC 4.1.1.32) mRNA (35). Decreased mRNA stability has also been invoked in the accelerated disposal of AR mRNA in response to hypotonicity (47). The mechanisms by which glucose may destabilize hTT mRNA are unknown. The sequence of the full 3'-untranslated region (UTR) of the hTT cDNA, including the polyadenylation site, has not yet been reported. Although regulation of mRNA stability in general is less well understood than the factors regulating gene transcription, sequence elements in mRNAs that provoke rapid poly(A) shortening and mRNA degradation have been identified in other genes. For example, AU-rich sequences in the 3'-UTR that appear to destabilize transcripts (46) and stimulate deadenylation have been identified in the c-fos coding region and 3'-UTR (54). In addition, proteins have been identified that bind to these AU-rich elements (34) and increase mRNA instability (4) or stability (34). Therefore, high glucose may regulate the formation or binding of proteins to elements in hTT mRNA, thereby decreasing its stability. Alternatively, elevated glucose may facilitate the generation of hTT mRNAs with nonsense codons (44), unspliced introns (12), or extended 3'-UTRs (44) that decrease their stability. In the studies reported herein, act-D was used to determine hTT mRNA half-life. Because the hTT half-life in 5 mM glucose is too long for an accurate determination using act-D (there was no detectable decline in hTT mRNA abundance in 5 mM glucose even after 24 h (data not shown)), hTT mRNA stability will need to be determined without transcriptional blockade by hybrid selection of [32P]UTP-labeled cytoplasmic RNA (pulse chase) (47). Future studies aimed at identifying elements regulating hTT mRNA stability are also required to begin to understand the metabolic effects of glucose on hTT.

hRPE cells are polarized cells that transport taurine from choroidal blood into the neural retina (17, 23). Taurine may also regulate the function of the RPE itself, because it can stimulate phagocytosis (41), a process that is known to be impaired by high glucose in hRPE cells (8). Kinetic characterization of the hTT in hRPE cells demonstrated a single high-affinity transport system, which is consistent with other reports in intact baboon RPE preparations (14) and in cultured hRPE cells (28). In contrast, kinetic characterization of bovine RPE cells has demonstrated two high-affinity saturable Na+-dependent transport systems located on the apical and basolateral membranes (23). Lower-affinity transporters have also been identified in bovine RPE cells, with the greatest number of sodium-insensitive carriers (which potentially mediate taurine efflux) localized to the basolateral surface (23). In our studies, only the high-affinity apical hRPE taurine uptake system was characterized, because hTT activity measurements were performed on cells attached to impermeable plastic supports. Because the direction and magnitude of taurine transport across the RPE are highly dependent on the relative taurine concentrations in the apical and basolateral compartments (23), and glucose exposure results in changes of hRPE intracellular taurine content secondary to decreased hTT activity, it seems reasonable to speculate that hyperglycemia could not only result in hRPE taurine depletion but also disrupt the directionality of taurine transport across the RPE. Additional studies using cells cultured on permeable supports are required to explore the effects of glucose on the vectorial taurine transport system.

Changes in signal transduction pathways could potentially play an important role in the overall effect of glucose on hTT activity. TT activity is known to be differentially regulated by PKC, PKA, and Ca2+-/calmodulin-dependent protein kinases (3, 21, 22). Con
sistent with our data, PKC activation has been reported to inhibit TT activity in HT-29 and Caco-2 human carcinoma cells (3) and LLC-PK1 cells (22). In HT-29 and Caco-2 cells (3), the inhibitory effects of TPA are associated with a decrease in $V_{\text{max}}$ and an increase in $K_m$ Our studies in hRPE 47 cells revealed, however, that elevated glucose selectively decreases hTT $V_{\text{max}}$ without affecting the $K_m$. The role of PKA in mediating the effects of glucose is, however, unclear, as both PKA activation and inhibition decreased hTT activity. These paradoxical findings are in agreement with studies of PKA in Caco-2 cells (3). Because the hTT has multiple potential PKA (and PKC) phosphorylation sites (19), the seemingly paradoxical effects of PKA on hTT activity may reflect differential responses to PKA-mediated phosphorylation at different sites on the hTT protein. In any event, neither activation nor inhibition of PKC or PKA pathways was found to reproduce the glucose-induced decrease in hTT mRNA abundance. PKC inhibition with BIM, however, increased hTT activity and therefore compensated for the reduction in hTT gene expression. It is tempting to speculate, therefore, that in low AR-expressing cells exposed to high glucose, alterations in glucose-sensitive signal transduction pathways may through posttranslational mechanisms contribute to the rapid component of the decrease in hTT activity that precedes the decline in hTT mRNA abundance. With more prolonged glucose exposure, decreased hTT mRNA instability may also contribute to impaired taurine transport.

In high AR-expressing hRPE cells in 5 mM glucose, levels of steady-state hTT mRNA were suppressed compared with low AR-expressing cells, declined further on exposure to 20 mM glucose, and were corrected by ARI. Changes in hTT gene transcriptional rate in general paralleled mRNA abundance, although a further reduction in hTT gene transcription in 20 mM glucose was not detected, implicating additional posttranscriptional mechanism(s). This implies that AR overexpression sufficiently augments AR activity such that even euglycemic concentrations of glucose fully suppress hTT gene transcription. ARI increased hTT gene transcription to levels above those in low AR-expressing cells. Thus, in high AR-expressing hRPE cells, the ARI-sensitive component of hTT gene transcription may compensate for the posttranscriptional effects of glucose (it is unclear however, whether the AR-inhibitable effect of 20 mM glucose on hTT mRNA abundance is associated with AR-inhibitable changes in hTT mRNA stability). These changes are consistent with the role of the hTT as an osmotic stress response protein (52, 53) and suggest that hTT gene expression is suppressed at a transcriptional level in response to changes in the intracellular/extracellular osmotic gradient secondary to intracellular sorbitol accumulation. The mechanism(s) whereby intracellular osmotic perturbations secondary to sorbitol accumulation lead to alterations of hTT gene expression are, however, unknown.

In conclusion, these studies demonstrate that glucose rapidly and specifically decreases intracellular taurine content, hTT activity, and mRNA abundance by metabolic posttranscriptional and osmotic transcriptional mechanisms. Downregulation of the hTT in concert with altered expression of other osmoregulatory genes such as AR (13, 48) may exacerbate glucotoxicity in diabetes.

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