

## Forum Original Research Communication

# Regulation of the Human Taurine Transporter by Oxidative Stress in Retinal Pigment Epithelial Cells Stably Transformed to Overexpress Aldose Reductase

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### ABSTRACT

In diabetes, overexpression of aldose reductase (AR) and consequent glucose-induced impairment of antioxidant defense systems may predispose to oxidative stress and the development of diabetic complications, but the mechanisms are poorly understood. Taurine (2-aminoethanesulfonic acid) functions as an antioxidant, osmolyte, and calcium modulator such that its intracellular depletion could promote cytotoxicity in diabetes. The relationships of oxidative stress and basal AR gene expression to Na<sup>+</sup>-taurine cotransporter (TT) gene expression, protein abundance, and TT activity were therefore explored in low AR-expressing human retinal pigment epithelial (RPE) 47 cells and RPE 47 cells stably transformed to overexpress AR (RPE 75). Changes in TT gene expression were determined using a 4.6-kb TT promoter-luciferase fusion gene. Compared with RPE 47 cells, in high AR-expressing RPE 75 cells, TT promoter activity was decreased by 46%, which was prevented by an AR inhibitor. TT promoter activity increased up to 900% by prooxidant exposure, which was associated with increased TT peptide abundance and taurine transport. However, induction of TT promoter activity by oxidative stress was attenuated in high AR-expressing cells and partially corrected by AR inhibitor. Finally, exposure of RPE 75 cells to high glucose increased oxidative stress, but down-regulated TT expression. These studies demonstrate for the first time that the TT is regulated by oxidative stress and that overexpression of AR and high glucose impair this response. Abnormal expression of AR may therefore impair antioxidant defense, which may determine tissue susceptibility to chronic diabetic complications. *Antioxid. Redox Signal.* 7, 1530–1542.

### INTRODUCTION

THE PREVALENCE OF DIABETES is increasing in the United States and now afflicts over 18 million individuals (2), imposing a major challenge for the prevention and treatment of chronic microvascular complications. Although the etiology of the chronic complications is not well understood, re-

cent evidence clearly implicates increased oxidative stress (8, 22, 50, 82) coupled with impaired antioxidant defense (7, 21). In diabetic rodents, for example, increased oxidative stress has been identified in the nerve (5, 22, 82), eye (65), kidney (3), vasculature (41), and heart (34), and antioxidant therapy can attenuate diabetes-induced deficits in these tissues (64).

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In diabetes, activation of aldose reductase (AR) has been viewed as a critical link between hyperglycemia, intracellular osmotic stress, oxidative stress, and tissue damage (38, 79). AR-mediated oxidative stress is multifactorial, mediated by shifts toward more oxidized state of NADP<sup>+</sup>/NADPH and GSH/GSSG redox couples and by causing the compensatory depletion of the osmolyte and endogenous antioxidant taurine (48, 78, 83). AR gene expression is induced by extracellular hyperosmolality (4), an observation that led to the formulation of a "compatible osmolyte hypothesis," which argues that nonionic osmotically active compounds, including sorbitol and taurine, function as alternative organic intracellular osmolytes responding coordinately to changes in external osmolality (6). Osmotic stress alters the intracellular concentration of these alternative osmolytes by the induction or suppression of the synthetic enzymes, *e.g.*, AR in the case of sorbitol (54, 77), or membrane transporters, *e.g.*, Na<sup>+</sup>-taurine cotransporter (TT) (84).

Isotonic hyperglycemia perturbs this osmoregulatory system by promoting intracellular sorbitol accumulation by providing excess substrate for AR rather than by osmotic induction of its gene (81). This may result in transcriptional down-regulation of other osmotically regulated genes, including the TT (81), causing their intracellular depletion. Although initially regarded as metabolically inert, these alternative organic osmolytes are now thought to have important metabolic as well as osmoregulatory roles (64, 67). For example, taurine functions as an important endogenous antioxidant (23, 64, 65), calcium modulator (31, 45), neurotransmitter (11), as well as osmolyte (6, 77, 81), such that its intracellular depletion could promote chronic cytotoxicity in diabetes. Taurine depletion has been identified in the sciatic nerve, retina, and lens of diabetic rodents (62, 64, 65), and taurine replacement attenuates oxidative stress (64) and its downstream consequences in these tissues (48, 60, 64, 66).

We have previously reported the use of stable lines of cultured human retinal pigment epithelial (RPE) cells to explore the regulation of osmosensitive genes in response to high glucose and hypertonicity (27, 77, 80). RPE cell lines have been identified that exhibit quantitative and qualitative differences in AR gene regulation (27, 77). In these cells, the antiparallel excursions of sorbitol with taurine in response to hyperglycemic concentrations of glucose were found to be qualitatively and quantitatively modulated by the regulation of the AR gene (77). However, even under isosmotic euglycemic (5 mM glucose) conditions, overexpression of AR was associated with decreased TT gene expression (80, 81). We now have extended these observations to explore the effect of AR gene expression on the response of the TT to oxidative stress. Oxidative stress was found to increase TT gene expression, but this induction was blunted in high AR-expressing cells. Moreover, exposure of RPE cells to high glucose increased oxidative stress, but down-regulated TT expression. These studies demonstrate for the first time that the TT is regulated by oxidative stress and that overexpression of AR and high glucose impair this response. These studies offer mechanistic insight into the role of abnormal AR gene expression in the development of impaired antioxidant defense, which may determine tissue susceptibility to chronic diabetic complications.

## MATERIALS AND METHODS

### Materials

Cell culture media [minimum essential medium (MEM)] ([taurine] = 0  $\mu$ M), Hanks' balanced salt solution, and Trypsin-EDTA and antibiotics were obtained from GIBCO Inc. (Grand Island, NY, U.S.A.); bovine calf serum ([taurine] = 80–120  $\mu$ M) from Hyclone Laboratories (Logan, UT, U.S.A.); culture dishes, wells, and flasks from Falcon (Lincoln Park, NJ, U.S.A.); bicinchoninic acid, copper sulfate from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and radioisotopes from Amersham (Arlington Heights, IL, U.S.A.). Sorbinil was a gift from Pfizer Inc. (Groton, CT, U.S.A.), and fidarestat was a gift from Sanwa Kagaku Kenkyusho Ltd., Co. (Nagoya, Japan). Values were normalized to cell protein determined by the bicinchoninic acid method with bovine serum albumin (BSA) as the standard.

### Cell culture techniques and experimental design

Primary cultures of RPE cells were established as previously described (12) using postmortem eyes, obtained from the Michigan Eye Bank. RPE cell lines were passaged at a density of 40,000–100,000 cells/cm<sup>2</sup> in 25-cm<sup>2</sup> or 75-cm<sup>2</sup> flasks in MEM with 2 mM L-glutamine containing 20% bovine serum (BS) ([taurine] adjusted to 50  $\mu$ M) and 5 mM glucose at 37°C in a humidified 95% air/5% CO<sub>2</sub> atmosphere. Passaged cells were plated at initial densities of 5,000–20,000 cells/cm<sup>2</sup> in six-well plates or 150-mm diameter culture dishes to yield near-confluent cultures at the end of experiments. The freshly plated cells were allowed to attach in standard growth medium for 24 h and then grown in MEM with 5% BS for 72 h at 37°C to allow equilibration to take place. After 72 h, the cells were exposed to the various experimental conditions specified in the text and figure legends, and biochemical and molecular measurements were performed as described below. The phenotypic characteristics were monitored microscopically, functionally [rod outer segment phagocytosis (12)], and immunocytochemically [acetoacetylated low-density lipoprotein receptor protein (12)]. Neither cell morphology nor survival was altered detectably by the experimental conditions used in these studies.

### Northern analysis techniques

Total cellular RNA was isolated and purified from cultured RPE cells by differential extraction with acid phenol (81) after exposure to various experimental conditions (see below). Ten micrograms of RPE cell RNA or 2.5  $\mu$ g of a 0.24–9.5-kb RNA ladder (Life Technologies, Gaithersburg, MD, U.S.A.) was resolved on 2.2 M formaldehyde–1% agarose gel and transferred by capillary blotting to nylon membranes (Zetabind; Cuno Inc., Meridian, CT, U.S.A.). 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 [ $\alpha$ -<sup>32</sup>P]dCTP using random primers to a specific activity in excess of 10<sup>9</sup> dpm/ $\mu$ g. Filters were prehybridized at 65°C in 0.5 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.0, 1 mM EDTA, 7% sodium dodecyl sulfate

(SDS), and 1% BSA (fraction V) for 4–6 h before denatured probe was added. After 18 h, filters were washed twice with 40 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0, 1 mM EDTA, and 7% SDS at 65°C, and four times with 40 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.0, 1 mM EDTA, and 1% SDS at 65°C. Hybridization was quantified using a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA, U.S.A.) or by exposure at  $-80^\circ\text{C}$  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  $^{32}\text{P}$ -labeled cDNA probes for human AR (81), human taurine transporter (hTT) (81), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as previously described (81).

### Evaluation of TT transcriptional rate

**Preparation of 4.6-kb hTT promoter DNA and the reporter construct.** A 4.6-kb TT genomic DNA fragment extending upstream from the 5' end of exon 1 was polymerase chain reaction (PCR)-amplified (Roche, Expand long template system) from human genomic DNA isolated from RPE cells using the following PCR primers: forward, 5'-AGGCAGGCAAGAGGAGGGTAGTGG-3' reverse, 5'-CCCTCTTGGCGCAGCTTGTCCTCT-3'. 5' *XhoI* and 3' *BglIII* restriction sites were added to the forward and reverse primers, respectively. The PCR product was TA-cloned into PCR 2.1 (Invitrogen Corp., TA cloning kit), and sense and antisense constructs were identified by restriction digests and confirmed by DNA sequencing. The sense orientation fragment was digested out of PCR 2.1 using *KpnI/BglIII* and subcloned into pGL3-Basic (Promega, Madison, WI, U.S.A.) for transfection studies. Cloning success was confirmed by restriction digest and sequencing. RPE cells were seeded at 25,000 cells per well in 24-well cluster dishes and transfected 24 h post seeding with 180 ng of a 4.6-kb fragment of the TT promoter region DNA cloned into the promoterless luciferase vector pGL3-Basic (Promega). In order to standardize for transfection efficiency, RPE cells were cotransfected with 5 ng of pRL-SV40 vector (*Renilla reniformis* luciferase reporter vector; Promega) using LipofectAMINE 2000 (Invitrogen Corp.). An empty pGL-Basic vector was used as a negative control. Cells were allowed to express transfected DNA for 24 h, exposed to various experimental conditions, and then harvested and lysed in 200  $\mu\text{l}$  of lysis buffer, and the luciferase assay was performed using the Promega DLR system and a luminometer. The promoter activity (in relative light units) of the construct under various experimental conditions was determined as the mean of three samples normalized to the pRL-SV40 control.

### Biochemical measurements

**Measurement of sorbitol content.** RPE cell sorbitol content was determined as previously described by gas-liquid chromatography of aldonitrile acetate derivatives from lyophilized aliquots of protein-free filtrates of RPE cells sonicated in 5% (wt/vol) trichloroacetic acid with methyl  $\alpha$ -D-mannopyranoside as an internal standard (77). Standard curves were generated daily and the recovery-corrected values ex-

pressed as nanomoles per milligram of protein. Cell protein was measured by using bicinchoninic acid.

**Measurement of malondialdehyde (MDA) production.** Analysis of MDA was performed using a commercially available kit (Oxis International, Inc., Portland, OR, U.S.A.; catalog no. MDA LPO 586). RPE cell cultures were grown in 15-cm dishes to 80% confluency and treated under experimental conditions. At time of harvest, cell cultures were washed twice with phosphate-buffered saline (PBS), residual PBS was removed by aspiration, and cells were trypsinized. The cell pellet was collected by centrifugation ( $50 \times g$ , 10 min) and washed with PBS, and samples were stored at  $-80^\circ\text{C}$  until ready for analysis. Cell pellets were thawed and homogenized in 100  $\mu\text{l}$  of Tris (100 mM, pH 7) supplemented with 5 mM butylated hydroxytoluene using a glass Dounce homogenizer. The homogenate was collected and centrifuged at 10,000 g (30 min,  $4^\circ\text{C}$ ) to remove solid debris. Samples were then allowed to react with the chromogen *N*-methyl-2-phenylindole (1 h,  $45^\circ\text{C}$ ) and centrifuged. Supernatant absorbance was measured spectrophotometrically at 586 nm and compared with the MDA standards processed in the same run. Results were normalized to protein content.

**Cell taurine content.** Taurine was measured by reversed-phase HPLC after precolumn derivatization with *o*-phthalaldehyde (81). In brief, cells were sonicated in 1 ml of 6% trichloroacetic acid and centrifuged at 4,000 g for 10 min. The supernatants were purified on washed dual-bed, ion-exchange columns [2.5 cm of AG 1-X8 100–200 mesh (Bio-Rad, Richmond, CA, U.S.A.) in the chloride form over 2.5 cm of AG 50W-X8 200–400 mesh (Bio-Rad) in the hydrogen form] by elution with 2 ml of water and lyophilized. Samples and standards were dissolved in 100  $\mu\text{l}$  of water prior to HPLC analysis on a Waters system (Waters, Milford, MA, U.S.A.) equipped with a model 501 pump, a 717 autosampler, a  $3.9 \times 150$  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  $\text{NaH}_2\text{PO}_4$ , pH 5.3, plus 5 M NaOH) combined with 57% solvent B (0.05 M  $\text{NaH}_2\text{PO}_4$  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 RPE samples, and recovery of taurine was >90%.

**Taurine uptake.** Cells were cultured in six-well plates to reach 80% confluence, exposed to various experimental conditions, and then washed three times in 2-ml aliquots of prewarmed ( $37^\circ\text{C}$ ) uptake buffer (20 mM HEPES, 140 mM NaCl, 5.4 mM KCl, 1.0 mM  $\text{CaCl}_2$ , 0.8 mM  $\text{MgSO}_4$ , and 5 mM glucose) and equilibrated in 3 ml of the uptake buffer at  $37^\circ\text{C}$ . Uptake was initiated by adding uptake buffer containing [ $^{14}\text{C}$ ]-taurine (1  $\mu\text{M}$  taurine and 0.1  $\mu\text{Ci}/\text{ml}$ ). Aliquots of the original labeled media were counted to determine specific activity. Uptake was measured over 5 min (at which time uptake was linear).  $\text{Na}^+$ -independent uptake was measured by substituting NaCl with equimolar concentrations of choline chloride. The radioactive buffer was aspirated and the cells 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 of protein per minute. At 1  $\mu$ M [ $^{14}$ C]taurine, the Na<sup>+</sup>-independent component was negligible (<1% of total uptake) and was not routinely subtracted from total uptake for the measurement of Na<sup>+</sup>-dependent uptake.

### *Preparation of TT polyclonal antibodies*

The Michigan Diabetes Research and Training Center Molecular Biology Core prepared recombinant hTT antigen for the production of polyclonal antibodies. Synthetic peptides were prepared using a hydrophilic antigenic C-terminal sequence of the hTT, corresponding to amino acids 581–603 conjugated with heterobifunctional cross-linkers to both keyhole limpet hemocyanin (KLH) and BSA. Rabbits were immunized with the KLH-conjugated antigens, and postimmunization test sera obtained demonstrated substantial titer (1:700,000).

### *Western blot analysis*

Human RPE cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 0.015% sodium deoxycholate, 1  $\mu$ g/ml aprotinin, leupeptin, and pepstatin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared by centrifugation at 14,000 g for 2 min and the supernatants transferred to new tubes. Forty-microgram aliquots of protein extract of RPE were separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blots were blocked in 5% nonfat dry milk in Tris-buffered saline for 1 h and subsequently incubated for 2 h at room temperature with the polyclonal rabbit anti-human TT antibody (diluted 1:3,000). After washing, the blots were incubated with peroxidase-conjugated affinity-purified donkey anti-rabbit IgG (Amersham Pharmacia Biotech, U.K.) at 1:1,000 for 1 h, and the blots were analyzed with a luminol chemiluminescent detection kit (Boehringer Mannheim Biological, Indianapolis, IN, U.S.A.).

### *Statistical analysis*

Data are expressed as means  $\pm$  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 difference between these groups was assessed by the Student–Newman–Keuls test. Significance was defined at the 0.05 level.

## RESULTS

### *Stable transfection of RPE cells to express human AR at a high level*

A cDNA library in pcDNA1 was prepared from size-selected (>1 kb) RNA extracted from hypertonicity-stressed RPE cells. Screening with a partial human AR cDNA (77) yielded multiple recombinant plasmids. Plasmids with

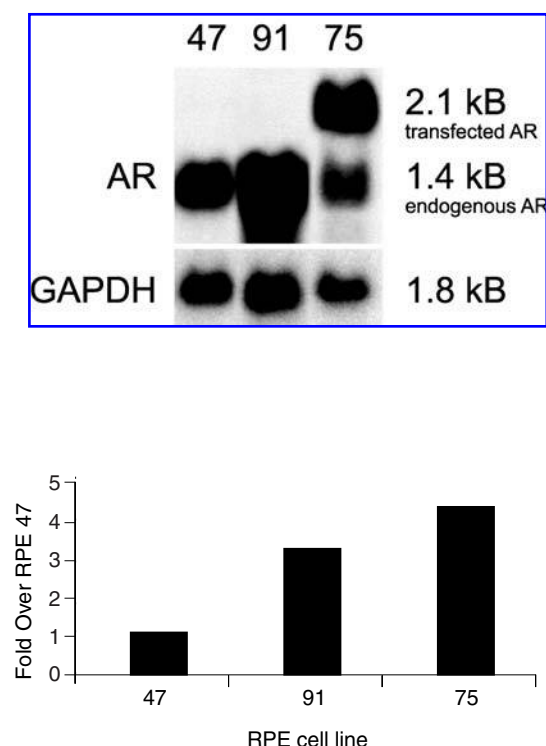
~1.3-kb inserts were selected for nucleotide sequencing using primers flanking the insert to establish that the complete open reading frame was present. Because of the variable lengths of the 3' and 5' untranslated regions, plasmids were screened for function by transiently transfecting COS and RPE cells and measuring AR enzymatic activity (77). A clone with the highest AR activity was selected for stable transformation in low AR-expressing RPE 47 cells. After lethal concentrations of G418 (neomycin) were determined in this cell line, the cells were cotransfected with pRSV-neo-pcDNA1-AR27, and 400 G418-resistant colonies were isolated and screened for high AR gene expression by stimulating with 300 mM glucose for 4 h and measuring sorbitol content. This time period was sufficient for production of excess sorbitol in candidates overexpressing AR, but insufficient to osmotically stimulate transcription of the endogenous gene (77). In the initial assessment of 98 of the G418-resistant clones, four clones were identified that demonstrated greatly elevated (>100-fold higher than untransfected RPE 47 cells) sorbitol content. Three stably transfected clones (designated numbers 72, 74, and 75) were then evaluated for high levels of AR enzymatic activity. All three of these stably transfected clones expressed fourfold elevated AR enzymatic activity relative to untransfected RPE 47 cells (data not shown), and reproduced the basal AR activity of untransfected naturally high AR-expressing RPE 91 cell line.

### *Expression of the AR gene in untransformed and stably transformed RPE cell lines*

In order to compare AR gene expression in parental RPE 47 cells, AR-transformed RPE 75 cells, and naturally high AR-expressing RPE 91 cells (27, 77), total cytoplasmic RNA was extracted from cells exposed to 5 mM glucose for 24 h, and northern blots were prepared, then sequentially hybridized with a partial human AR cDNA, and normalized to GAPDH. In RPE 75 cells, two AR transcripts were observed, a 2.1-kb transcript consistent with the size predicted for the transfected AR gene and a 1.4-kb transcript consistent with expression of the endogenous AR gene (27, 77) (Fig. 1). Basal expression of AR (transfected and endogenous AR genes) gene was increased 3.5-fold versus the parental RPE 47 cells, which reproduced that observed in the naturally high AR-expressing RPE 91 cells. In RPE 75 cells, basal expression of the endogenous AR gene was decreased, however, by 38% compared with that in the parental RPE 47 cells. These data therefore suggest that the RPE 75 cell line reproduces the high basal level of AR gene expression observed in the RPE 91 cells. Moreover, in RPE 75 cells, down-regulation of the endogenous AR gene suggests complex interactions among osmoregulatory genes.

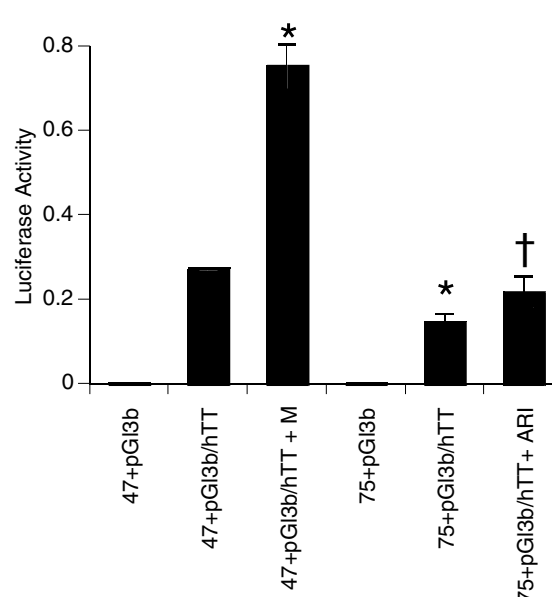
### *Molecular cloning of the TT promoter*

A human peripheral blood leukocyte genomic DNA library was screened with a TT cDNA. Candidate clones were hybridized with more 5' TT cDNAs, and positive clones were purified. Large-scale phage DNA preparations were digested with restriction endonucleases and mapped by Southern



**FIG. 1.** Expression of the AR gene in untransformed and stably transformed RPE cell lines.

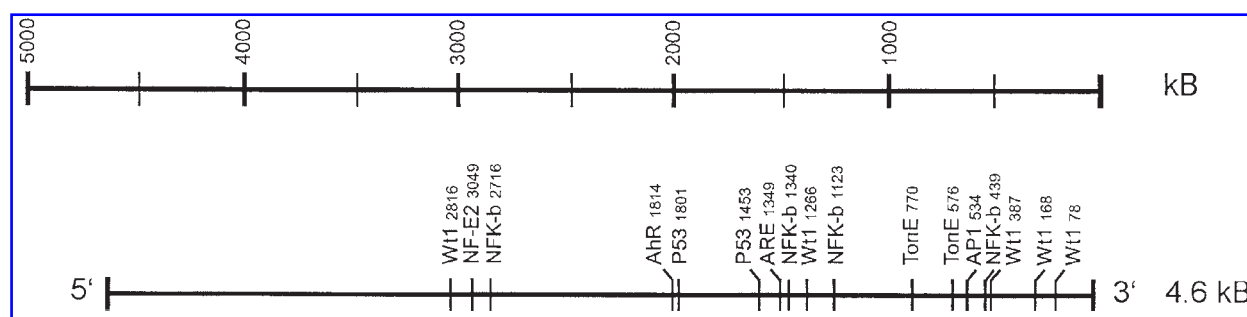
blotting. The 5' end of the first exon was confirmed by specific primer extension and nuclease protection experiments. The structure of the full-length (4.6 kb) TT promoter is shown in Fig. 2. The promoter contains an antioxidant response element (ARE) (70) (GTGACNNNGC) at -1,349, as well as two putative tonicity response elements (TonEs) at -576 and -770. Other sites potentially sensitive to glucose, osmotic, or oxidative stress-mediated regulation include an aryl-hydrocarbon receptor (AhR) (10, 37, 57), four nuclear factor- $\kappa$ B (NF- $\kappa$ B) sites (44), an activator protein-1 (AP-1) site (72) at -534, and a nuclear factor, erythroid derived 2 (NF-E2) site (59, 75, 88).



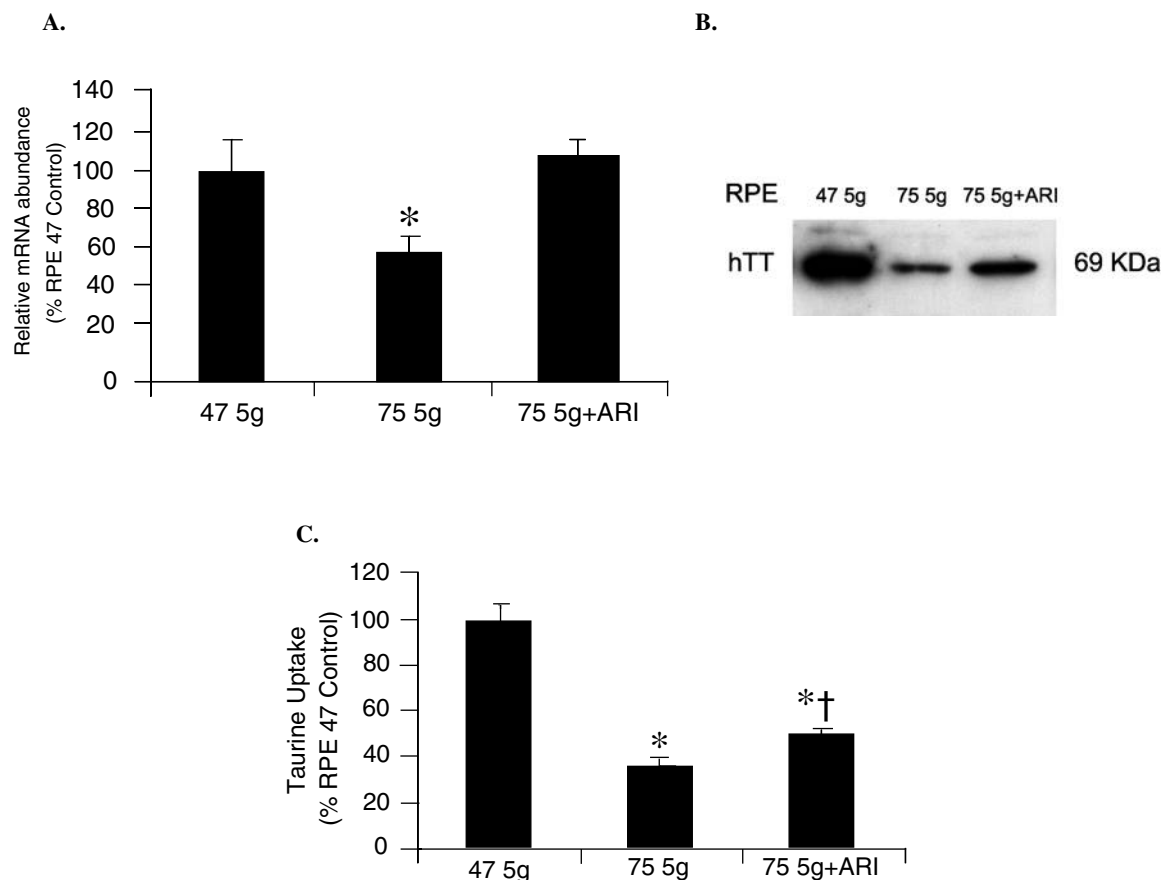
**FIG. 3.** Low AR-expressing RPE 47 cells and high AR-expressing RPE 75 cells were transiently transfected (using LipofectAMINE reagent) with a full-length (4.6 kb) TT promoter-luciferase fusion gene in conditions of 5 mM glucose, and 24 h post transfection exposed to 300 mM mannitol (M) (for 16 h) or ARI (48 h) and luciferase activity measured. Data are shown as means  $\pm$  SEM. \* $p$  < 0.05 versus basal RPE 47 activity; † $p$  < 0.05 versus RPE 75 without ARI.

### TT gene expression in human RPE cells in response to osmotic and oxidative stress

**Effect of tonicity on regulation of the TT promoter.** Compared with the empty pGL-Basic vector, transient transfection of the TT promoter-driven reporter construct into low AR-expressing RPE 47 cells demonstrated robust activity, which was unaffected by AR inhibition (data not shown) and increased threefold ( $p$  < 0.05) after exposure of the cells to 300 mM mannitol (Fig. 3). This induction of TT promoter activity by 300 mM mannitol is consistent with the reported increase in TT steady-state mRNA abundance under these condi-



**FIG. 2.** A human peripheral blood leukocyte genomic DNA library was screened with a TT cDNA. Candidate clones were hybridized with more 5' TT cDNAs, and positive clones were purified. Large-scale phage DNA preparations were digested with restriction endonucleases and mapped by Southern blotting. The 5' end of the first exon was confirmed by specific primer extension and nuclease protection experiments.



**FIG. 4.** (A) Northern blot analysis of TT mRNA abundance was performed on extracts from RPE 75 and 47 cells exposed to 5 mM glucose with and without 100  $\mu$ M sorbinil for 72 h. Northern blots were prepared, then sequentially hybridized with the TT cDNA probe, and normalized to GAPDH. The data shown are the means  $\pm$  SEM of three different cell preparations. Statistical comparison was performed on data from three independent experiments. \* $p$  < 0.05 versus RPE 47 cells. (B) Western blot analysis of TT peptide abundance was performed on protein extracts from RPE 75 and 47 cells exposed to 5 mM glucose with and without 100  $\mu$ M sorbinil for 72 h. The blots were probed sequentially with the polyclonal rabbit anti-hTT antibody or preimmune sera. A 69-kDa protein (the expected TT molecular mass) was detected by western blot analysis when RPE cell protein extracts were probed with the TT antibody. (C) The effect of basal AR gene expression on TT Na<sup>+</sup>-dependent cotransporter activity was evaluated after 72 h in RPE 75 and 47 cells exposed to 5 mM glucose with and without 100  $\mu$ M sorbinil for 72 h. The data shown are the means  $\pm$  SEM of three different cell preparations. Statistical comparison was performed on data from three independent experiments. \* $p$  < 0.05 versus RPE 47 cells; † $p$  < 0.05 versus RPE 75 cells.

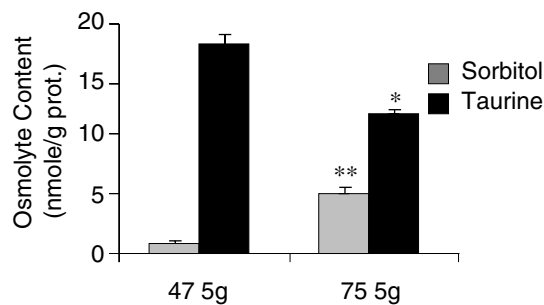
tions. Transfection of the TT promoter construct into RPE 75 cells resulted in a down-regulation of TT promoter activity to levels that were 46% ( $p$  < 0.05) lower than that measured in RPE 47 cells (Fig. 3). In RPE 75 cells, down-regulation of the TT promoter could be prevented by incubation of the cells with an AR inhibitor (ARI) [TT transcription increased by 48% ( $p$  = NS versus RPE 47 cells)] (Fig. 3). In concert, the levels of the TT mRNA, TT peptide, and TT activity were decreased by 41%, 54%, and by 66% in RPE 75 cells, respectively (Fig. 4). These decreases were also partially attenuated by the addition of ARI.

Sorbitol content was increased sevenfold in RPE 75 cells (Fig. 5). Addition of an ARI lowered sorbitol content 96% ( $p$  < 0.01) to levels that were not significantly different from those of RPE 47 cells (data not shown). Consistent with the decrease in TT gene transcription, steady-state mRNA level, peptide abundance, and transporter activity, intracellular taurine content was decreased by 36% in RPE 75 cells (Fig. 5).

Addition of ARI increased levels of taurine by 20% ( $p$  < 0.05), with levels of intracellular taurine of ARI-treated RPE 75 cells remaining 25% ( $p$  < 0.05) below levels in RPE 47 cells. These data confirm osmotic regulation of the TT promoter and that in high AR-expressing cells, intracellular sorbitol accumulation decreases TT transcriptional rate, protein abundance, and activity.

#### *Effect of oxidative stress on regulation of the TT promoter.*

The effect of exposure of RPE 47 cells to a panel of prooxidants on TT promoter regulation was initially explored (Fig. 6). TT promoter transcriptional activity was increased up to 900% by prooxidant exposure, which was associated with increased TT peptide abundance (Fig. 6, inset) and taurine transport (Fig. 7B). In comparison, however, induction of TT promoter transcriptional activity by oxidative stress was attenuated in high AR-expressing RPE 75 cells (Fig. 7A), and taurine

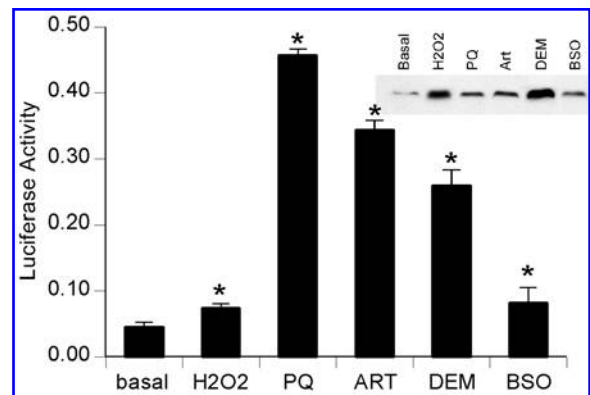


**FIG. 5.** Intracellular sorbitol and taurine contents were evaluated after 72 h in RPE 75 and 47 cells maintained in 5 mM glucose. The data shown are the means  $\pm$  SEM of three different cell preparations. Statistical comparison was performed on data from three independent experiments. \* $p < 0.05$ , \*\* $p < 0.001$  versus RPE 47 cells.

transport was unchanged (Fig. 7B). ARI cotreatment however, restored up-regulation of TT promoter activity and taurine transport in response to oxidative challenge. These data demonstrate for the first time that the TT is up-regulated by oxidative stress and that AR overexpression impairs this response.

#### *Effect of high glucose on MDA levels and TT promoter activity in RPE 75 cells*

High glucose increases production of reactive oxygen species (ROS) in high AR-expressing RPE cells (unpublished observations). The effect of exposure of high AR-expressing RPE 75 cells to 5 and 20 mM glucose for 48 h on MDA content was next explored. MDA content was increased 2.7-fold ( $p < 0.05$ ) under high-glucose conditions, consistent with increased oxidative stress (Fig. 8A) [MDA levels in RPE 47 cells did not increase in 20 mM glucose (data not shown)]. However, despite increased oxidative stress, TT promoter activity was down-regulated (Fig. 8B). Therefore, in high AR-expressing cells, high-glucose exposure increases oxidative

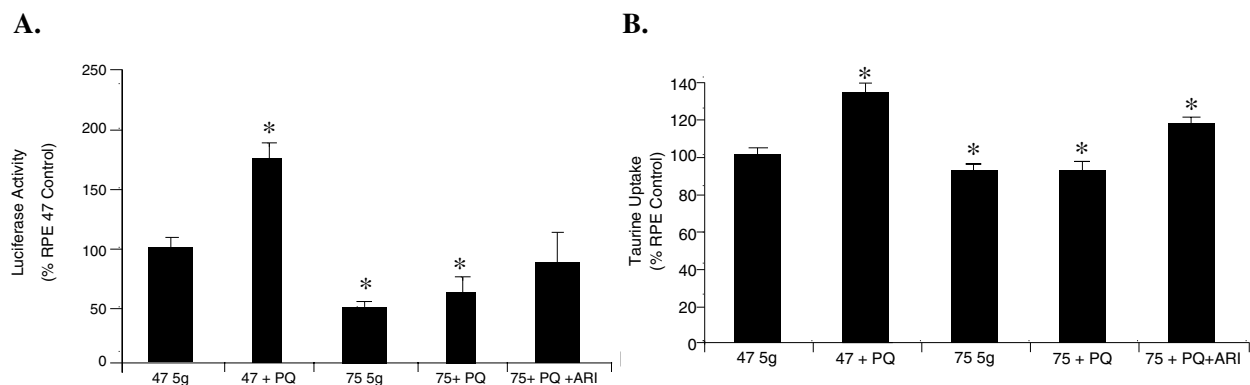


**FIG. 6.** RPE 47 cells were transiently transfected with the full-length (4.6 kb) TT promoter-luciferase gene and 24 h posttransfection exposed to the prooxidants hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (0.5 mM), primaquine (PQ) (50  $\mu\text{M}$ ), artemisinin (ART) (50  $\mu\text{M}$ ), diethyl maleate (DEM) (60  $\mu\text{M}$ ), and buthionine sulfoximine (BSO) (50  $\mu\text{M}$ ). (Inset) Corresponding change in TT peptide abundance and TT activity. Data are shown as means  $\pm$  SEM. \* $p < 0.05$  versus basal RPE 47 activity.

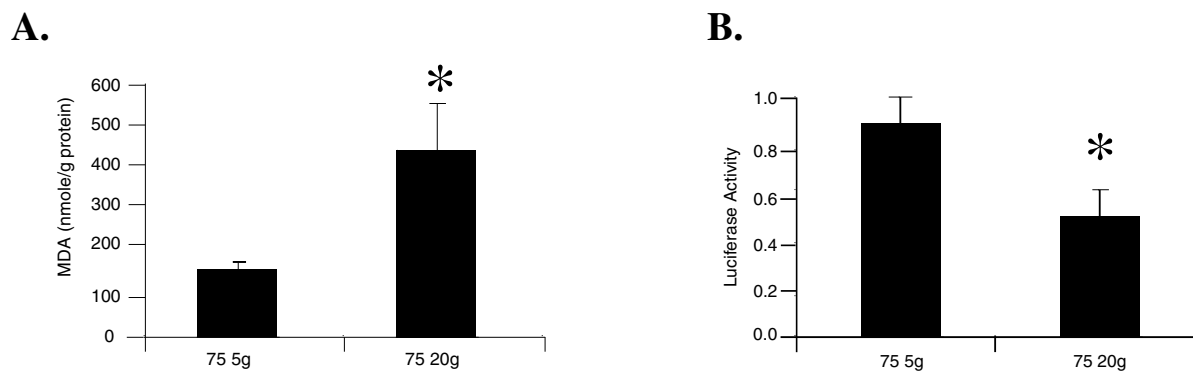
stress, but paradoxically down-regulates antioxidant defense systems.

## DISCUSSION

In diabetes, increased expression of AR has been implicated as the critical link between chronic glucose toxicity and tissue damage (6, 15, 36, 73, 79, 86). The precise pathophysiological mechanisms whereby the reduction of glucose by AR may predispose to the development of diabetic complications is uncertain, but has been invoked to include depletion of the osmolyte and antioxidant taurine (64, 67, 79, 80) through an inhibitory effect on its  $\text{Na}^+$ -dependent cotransporter (79–81). This study therefore explored the relationships of oxidative



**FIG. 7.** (A) RPE 75 cells were transiently transfected with the full-length (4.6 kb) TT promoter-luciferase gene and 24 h posttransfection exposed to the prooxidant primaquine (PQ) (50  $\mu\text{M}$ ). Data are shown as means  $\pm$  SEM. \* $p < 0.05$  versus basal RPE 47 activity. (B) The effect of basal AR gene expression on TT  $\text{Na}^+$ -dependent cotransporter activity was evaluated after 24 h in RPE 75 cells exposed to the prooxidant primaquine (PQ) (50  $\mu\text{M}$ ) with or without ARI fidarestat. Data are shown as means  $\pm$  SEM. \* $p < 0.05$  versus basal RPE 75 activity. The data shown are the mean  $\pm$  SEM of three different cell preparations.



**FIG. 8.** (A) RPE 75 cells were exposed to 5 or 20 mM glucose for 48 h. Total MDA content was assessed using a commercially available kit from Oxis International Inc. Data are shown as means  $\pm$  SEM. \* $p < 0.05$  versus basal RPE 75 basal. (B) RPE 75 cells were transiently transfected with the full-length (4.6 kb) TT promoter-luciferase gene and 24 h post transfection exposed to 5 or 20 mM glucose for 48 h. Data are shown as means  $\pm$  SEM. \* $p < 0.05$  versus mM glucose.

stress and basal AR gene expression to TT gene expression, protein abundance, and TT activity in low AR-expressing human RPE 47 cells and RPE 47 cells stably transformed to overexpress AR (designated RPE 75). In low AR-expressing RPE 47 cells, the TT promoter demonstrated robust activity, which was unaffected by ARI and increased threefold in hypertonicity. In high AR-expressing RPE 75 cells, TT promoter activity was decreased by 46% and this down-regulation was prevented by an ARI. In low AR-expressing cells, TT promoter activity increased up to 900% by prooxidant exposure, which was associated with increased TT peptide abundance. However, induction of TT promoter activity by oxidative stress was attenuated in high AR-expressing cells and partially corrected by ARI. High-glucose exposure increased MDA production, but paradoxically down-regulated TT transcription. These studies demonstrate that the TT is up-regulated by oxidative stress and that overexpression of AR and hyperglycemia impair this response. Glucose metabolism by AR may therefore contribute to impaired antioxidant defense, which may determine tissue susceptibility to chronic diabetic complications.

The recognition that AR gene expression is heterogeneous not only between different tissues, but also between similar cell lines, such as RPE cells isolated from different individuals, has expanded interest in understanding regulation of the gene, not only in response to hypertonicity, but also under isotonic conditions. AR gene expression and activity can vary up to 10-fold under isotonic conditions in different RPE cell lines (27, 77). The high basal level of AR gene expression and activity in the RPE 91 cell line, for example, can be reproduced in other RPE cell lines only after maximal hypertonic conditioning (27, 77). Such heterogeneous expression of the AR gene may precondition the cell to glucose toxicity in the presence of otherwise modest elevations of the ambient glucose level (79, 81) by activating ROS-producing pathways and by impairing antioxidant defense systems, including taurine.

Primary cultures of RPE 47 cells were stably transformed to overexpress AR. The resultant RPE 75 cells in 5 mM glucose achieved approximately fourfold elevation of basal AR gene expression and approximately sevenfold elevation of in-

tracellular sorbitol content, thereby reproducing the degree of AR overexpression observed in the naturally AR-overexpressing RPE 91 cell line (27, 81). Overexpression of AR was found to have no demonstrable effects on cell viability or growth characteristics (data not shown). However, expression of the TT in RPE 75 cells was found to be very sensitive to intracellular sorbitol accumulation, because even in 5 mM glucose, TT gene expression, protein abundance, and activity were decreased and levels of intracellular taurine reduced by ~40%. Therefore AR overexpression may increase cellular susceptibility to glucose-derived oxidative damage, because levels of the important endogenous antioxidant taurine are depleted in response to small elevations of sorbitol-derived intracellular tonicity.

The etiology of the down-regulation of TT in response to alterations in intracellular osmotic stress is unclear. AR, as well as other osmoregulatory genes, is transcriptionally up-regulated by homologous TonEs in their 5' flanking sequence (18, 25, 40, 69), which bind TonE binding proteins (TonEBPs) in response to hypertonic stress (51, 87). Indeed, molecular cloning of the TT promoter revealed two putative TonEs, which may inhibit basal transcription in response to small increases in sorbitol-derived intracellular osmotic stress. TonEs have been shown to cooperatively interact in response to hypertonic stress in the human AR gene (40). A ubiquitous TonEBP cDNA has been cloned (51), which increases fourfold in abundance in response to hypertonicity (87). The DNA-binding domain of TonEBP is 45% homologous to the DNA-binding domains of the nuclear factor of activated T-cell (NFAT) family of transcription factors, but unlike NFAT, can stimulate transcription in the absence of activation AP-1 (47). The TonEBP is phosphorylated at serine and tyrosine residues in hypertonic conditions (9) and becomes localized within the nucleus within 12 h (51). The signal transduction pathways that are involved in the response to hypertonic stress remain poorly characterized, but are thought to involve the p38 kinase pathway (14, 55). The role of TonEs and TonEBPs in the transcriptional response to hypotonic stress is, however, much less clearly defined. In Madin-Darby canine kidney cells, TonEBP abundance and nuclear distribution are down-regulated (87) in response to hypo-

tonicity, implicating tonic activation of TonEBP under isotonic conditions. Presumably, if transcriptional regulation of the TT involves similar TonEs and TonEBPs identified in other osmoregulated genes, this regulatory pathway appears to be highly sensitive to alterations in intracellular tonicity, making it uniquely sensitive to disruption by AR overexpression or elevated glucose. The precise mechanisms whereby AR overexpression down-regulates TT gene expression remain to be explored.

Prooxidant exposure dramatically increased TT transcription and peptide synthesis, consistent with an important role for taurine in the cell antioxidant defense. Analysis of the promoter region of the TT revealed an ARE (5'-TGACTCGGC-3'), which is known to mediate transcriptional activation of genes in cells exposed to oxidative stress (59). These genes include those important in the regulation of cellular redox and the protection from oxidative damage, including glutathione *S*-transferases (19),  $\gamma$ -glutamylcysteine transferase catalytic and regulatory subunits (53), NAD(P)H:quinone oxidoreductase (46), and heme oxygenase-1 (32). Although the regulation of the ARE is not well understood, it is thought to involve a class of transcription factors known as bZIP (basic region plus leucine zipper) proteins, which bind to DNA as part of a homodimer and/or heterodimer complex (59). Indeed, the bZIP transcription factor NF-E2-related factor 2 (Nrf2) mediates ARE-driven response to oxidative stress (30, 88) and has been shown to be important in the transcriptional activation of many genes regulated by oxidative stress (1, 58, 85). It is thought that Nrf2 may mediate both basal and inducible activity of the ARE (49). Nrf2s may heterodimerize with small Maf proteins (16), which can act as transcriptional repressors (16). Finally, Nrf2 activity can be repressed by association with Kelch-like-ECH-associated protein 1 (Keap 1), which is thought to prevent Nrf2 nuclear translocation (59).

The complex regulation of the ARE and the importance of its context, outside its core sequence (59), make it highly susceptible to alterations in the intracellular milieu, as might occur in response to overexpression of AR and/or the presence of hyperglycemia. For example, nuclear translocation of Nrf2 may be regulated by glucose- and insulin-sensitive signal transduction pathways, including protein kinase C (PKC) (29) and phosphatidylinositol 3-kinase. PKC, for example, has been shown to activate the ARE in an Nrf2-dependent manner by direct phosphorylation, thereby stimulating translocation from the cytoplasm to the nucleus (29). In RPE cells, PKC activation increases TT gene transcription (81) and overexpression of AR has been associated with down-regulation of PKC (56), which may therefore influence both basal and inducible ARE activity. The role of the mitogen-activated protein kinase cascades in the regulation of the ARE has also been extensively evaluated, although remains poorly understood. In general, activation of the extracellular regulated kinase pathway has been positively associated with activation of the ARE (88), whereas activation of p38 has been associated with down-regulation of both basal and inducible ARE activity (88). Whether tonicity-mediated activation of the p38 kinase pathway (14, 55) is involved in the down-regulation of basal and oxidative stress-inducible activation

of the TT is currently being explored. Finally, the context of the ARE in the TT may also play an important role in its regulation, because the TT promoter contains a number of regulatory sequences known to be important in regulating gene expression in response to oxidative stress, including AP-1 (68), SP1 (71), NF- $\kappa$ B (44) sites, as well as TonEs (40, 43). Exploration of the potential interactions between the ARE and these sites may help elucidate the mechanisms contributing to aberrant regulation of the TT.

Overexpression of AR and increased sorbitol pathway flux have been linked to the development of diabetic complications (6, 15, 24, 36, 73, 75, 76, 79, 86). Increased AR gene expression has been identified in peripheral blood mononuclear cells in diabetic patients with nephropathy (73) and microvascular complications (76). Increased AR immunoreactivity has been identified in renal glomeruli and peripheral nerve in postmortem specimens from diabetic patients and correlated with the severity of the pathological changes (36). The concept that aberrant overexpression of the AR gene may predispose to the development of diabetic complications has received support by the identification of polymorphisms at the 5' end of the AR gene that are associated with the presence of microvascular complications in certain populations of diabetic subjects (13, 20, 26, 35, 39, 73). In RPE cells, recent studies have shown that AR gene transcription paralleled AR mRNA abundance and activity, implicating increased AR gene transcription as the basis of AR overexpression in high AR-expressing tissues and cells (80, 81). Additionally, pathophysiological elevations of glucose have been reported to increase AR mRNA and protein abundance in RPE cells (28). An association between an (A-C)<sub>n</sub> dinucleotide repeat in the AR gene basal promoter region has been identified in diabetic subjects with nephropathy (73), retinopathy (20, 35, 39), and neuropathy (26) and with increased expression of the AR gene (74). However, this association has been challenged (17, 33), which may reflect heterogeneity in different ethnic populations studied as well as the type of diabetes. More recently, a C(-106)T substitution in the AR gene basal promoter has also been associated with nephropathy (52) and retinopathy (13, 35). In any event in diabetes, overexpression of AR irrespective of the mechanism increases the formation of ROS (61) and impairs antioxidant defense systems such as the TT, thereby predisposing to glucose toxicity.

In summary, these studies demonstrate that the TT is regulated by oxidative stress and that overexpression of AR and hyperglycemia impair this response. Abnormal expression of AR may therefore impair antioxidant defense, which may determine tissue susceptibility to chronic diabetic complications.

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## ABBREVIATIONS

AhR, aryl-hydrocarbon receptor; AP-1, activator protein-1; AR, aldose reductase; ARE, antioxidant response element; ARI, aldose reductase inhibitor; BS, bovine serum; BSA, bovine serum albumin; bZIP, basic region plus leucine zipper; G418, neomycin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione, reduced form; GSSG, glutathione disulfide, oxidized form; hTT, human taurine transporter; KLH, keyhole limpet hemocyanin; MDA, malondialdehyde; MEM, minimum essential medium; NFAT, nuclear factor of activated T-cell; NF- $\kappa$ B, nuclear factor- $\kappa$ B; NF-E2, nuclear factor, erythroid derived 2; Nrf2, NF-E2-related factor 2; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; ROS, reactive oxygen species; RPE, retinal pigment epithelial; RPE 47, retinal pigment epithelial cell clone no. 47; RPE 75, retinal pigment epithelial cell clone no. 75; SDS, sodium dodecyl sulfate; TonE, tonicity response element; TonEBP, TonE binding protein; TT, Na<sup>+</sup>-taurine cotransporter.

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