

# Involvement of TonEBP/NFAT5 in osmoadaptative response of human retinal pigmented epithelial cells to hyperosmolar stress

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**Purpose:** Macular edema, a frequently encountered complication of diabetic retinopathy (DR), results from alterations of the blood retinal barrier (BRB) and leads to modifications of the retinal pigmented epithelium (RPE) functions. Osmolar changes of the surrounding medium could be responsible for modifications of the RPE functions leading to disturbance of retinal homeostasis. The expression, activation and function of the key hyperosmolar response factor Tonicity Enhancer Binding Protein (TonEBP also called nuclear factor of activated T-cell 5 - NFAT5) was investigated in ARPE-19 cells, derived from human RPE, in response to hyperosmolar stimulation.

**Methods:** ARPE-19 cells were exposed to hyperosmolar medium. TonEBP mRNA and protein levels were quantified by qRT-PCR and semi-quantitative Western blot. TonEBP nuclear translocation was investigated by immunofluorescence. TonEBP transactivation activity was measured using a reported plasmid containing TonEBP binding sites.

**Results:** In response to hyperosmolar stimulation of ARPE-19 cells, a dose-dependent increase in TonEBP mRNA and protein levels, as well as TonEBP nuclear translocation were observed. TonEBP transactivation activity was further demonstrated using a reporter plasmid containing TonEBP binding sites. A dominant negative form of TonEBP abolished NaCl-induced increase in TonEBP transactivation activity, and inhibited the increase of the target genes aldose reductase and sodium-dependent taurine transporter mRNA levels. SB203580, an inhibitor of two of the p38 protein kinase's isoforms (p38 $\alpha$  and p38 $\beta$ ) inhibited the TonEBP nuclear translocation and transactivation activity in ARPE-19 cells exposed to hyperosmolar stimulation.

**Conclusions:** Our data demonstrates the involvement of TonEBP in the mechanisms responsible for osmoadaptation to hyperosmolar stress in RPE cells. Given the emerging role of TonEBP in different pathological pathways, these data open new perspectives for the analysis of the mechanisms involved in the modification of functions of the RPE during macular edema.

Diabetic retinopathy (DR), the primary cause of acquired blindness in developed countries, affects more than 4 million people over 40 years old in the United States [1]. The incidence of DR will rise in parallel with the increasing prevalence of type 2 diabetes, now considered a pandemic [2]. DR is characterized by complex microvascular alterations responsible, among others, for macular edema. Macular edema results from abnormal liquid accumulation within the central part of the retina.

The pathophysiological mechanism of macular edema results from an alteration of the blood retinal barrier's (BRB) impermeability [3]. The BRB is a functional entity that regulates the movement of water, ions, and solutes. The inner BRB results principally from the presence of tight junctions between the non-fenestrated endothelial cells of the retinal

capillaries, reinforced by extensions from Müller cells and astrocytes. The outer BRB results from tight junctions between RPE cells, preventing paracellular flux and ensuring the maintenance of a trans-epithelial osmolar gradient created by ionic channels and pumps [4]. Under physiologic conditions, water follows this trans-epithelial osmolar gradient and flows from the sub-retinal space to the choroid, via the RPE. Under pathological conditions such as DR, the ruptured inner [5,6] and outer [7] BRB lead to the accumulation of proteins and solutes adjacent to the RPE cells. This, in turn, leads to an increase in the osmolar pressure to which the RPE cells are exposed, and thus the formation of macular edema [5,6]. Thereafter, following the resorption of the edema, probably with the help of the RPE cells, exudates corresponding to the precipitated proteins persist [4]. This suggests that RPE cells are again exposed to hyperosmolar conditions.

Cell protection in response to hyperosmolar stress involves various mechanisms in which tonicity enhancer binding protein/osmolar response element binding protein

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TABLE 1. PRIMERS CHARACTERISTICS.

Gene	Forward primer (5'-3') Reverse primer (5'-3')	Accession number	Amplicon size (bp)	Efficiency (%)
TonEBP	GAAGTGGACATTGAAGGCACT CTGGCTTCGACATCAGCATT	NM_138714.3	119	97
AR	GTCTCCTGCTCAACAACGG ACACATGGGCACAGTCGAT	NM_001628.2	135	83
TauT	AGATCATCATAGGCCAGTACAC TAGACATTCAGGAGGGACACA	NM_001134367.1	119	88
SEAP	GTGAACCGCAACTGGTACTC GTACTTTTCGGCCTCCACCTA	pSEAP2-Basic2 Clontech 4.7 kb (*)	129	87
HPRT1	TGGCGTCGTGATTAGTGATG CTCGAGCAAGACGTTCAAGT	NM_000194.2	137	99
B2M	AGATGAGTATGCCTGCCGTG TCATCCAATCCAAATGCGGC	NM_004048.2	120	103
ATP5B	AGAGGTCCCATCAAAAACCAAAC AAAAGCCCAATTTTGCCACC	NM_001686.3	152	98
YWHAZ	ACAAAAGACGGAAGGTGCTG TCTGCTTGTGAAGCATTGGG	NM_145690.2	141	100
MDH1	AGATGACAGCTGGCTCAAGG ACACAAACTCTCCCTCTGGG	NM_005917.2	161	83

\* SEAP primers were determined within the coding sequence of the SEAP gene contained in the pSEAP2-Basic vector (GenBank Accession no U89937) from Clontech (Mountain View, CA), between bases 130–1636.

(TonEBP/OREBP) [8,9], also called nuclear factor of activated T-cell 5 (NFAT5), a member of the Rel family of transcription factors, plays a crucial role. The activation of TonEBP leads to transactivation of ion channel/transporter and enzyme genes, thus inducing the subsequent increase in intracellular organic osmolytes [10,11]. Recently, TonEBP induction has been reported in the mouse retina during experimental diabetic retinopathy and in ARPE-19 cells exposed to high glucose concentrations [12]. Furthermore, RPE cells exposed to hyperosmolar stress notably modify the expression of sodium-dependent taurine transporter (TauT) and aldose reductase (AR), suggesting that the osmoprotective response is conserved in RPE cells [13,14]. In addition to inducing an osmoprotective cell response, hyperosmolar stress also induces other cellular effects, including the cell cycle in several cell types [10] as well as ARPE-19 cells [15]. Therefore, the aim of our study was to investigate the expression, activity, and function of TonEBP in the osmoadaptive response of the human retinal pigmented epithelial cell line ARPE-19 to hyperosmolar stress, within the frame of macular edema occurring during the course of diabetic retinopathy.

## METHODS

**Materials:** The antibodies for anti-phospho p38, anti-phospho AKT, and AKT were obtained from Cell Signaling (Danvers, MA). The antibody for NFAT5 was from Novus Biologicals (Littleton, CO). ShTonEBP and control shRNA came from Origene (Rockville, MD). The dominant negative form of TonEBP (DN-TonEBP) construct was received from Dr. B.C. Ko (University of Hong Kong, China) [16], and the pSEAP-TonE construct was a gift from Dr. W. Neuhofer (University of Heidelberg, Germany) [17].

**Cells:** ARPE-19 cells were purchased from ATCC - LGC Standards (Molsheim, France). Short tandem repeat analysis was performed using the Geneprint 10 System (Promega, Madison, WI) according to the manufacturer's manual (IdentiCell, Aarhus, Denmark), shown in Appendix 1. ARPE-19 cells were grown in Dulbecco's Modified Eagle Medium (DMEM)/HAM-F12 (Life Technologies, Carlsbad, CA) medium containing 10% fetal calf serum, 100 UI/ml streptomycin-penicillin, and 4 mM glutamine, and passaged twice a week.

To perform transient ARPE-19 transfection, cells at 80% of confluence were trypsinized and then resuspended in Ingenio electroporation medium (Mirus, Madison, WI) before they were transfected by electroporation using a Gene

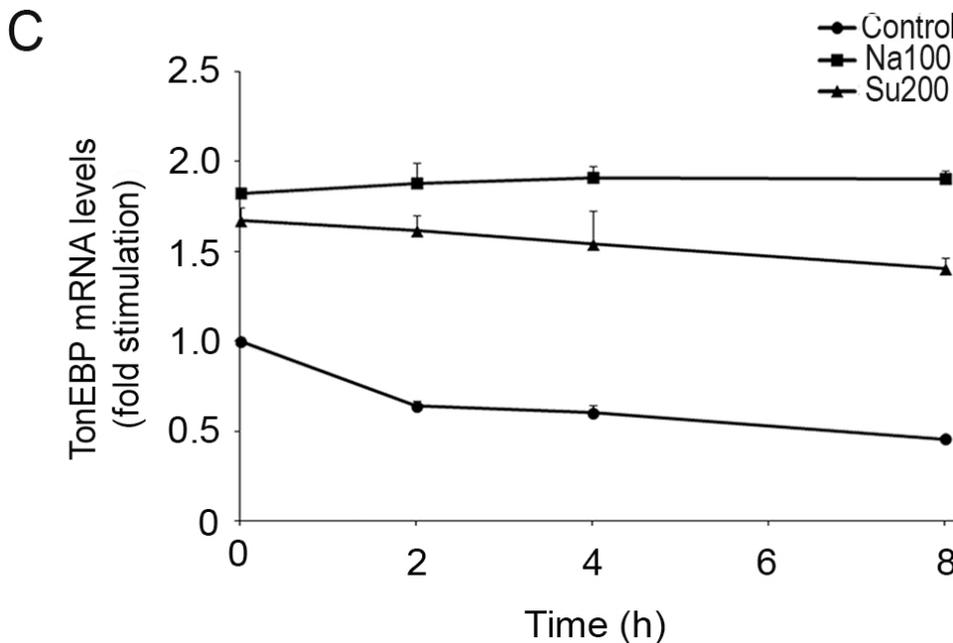
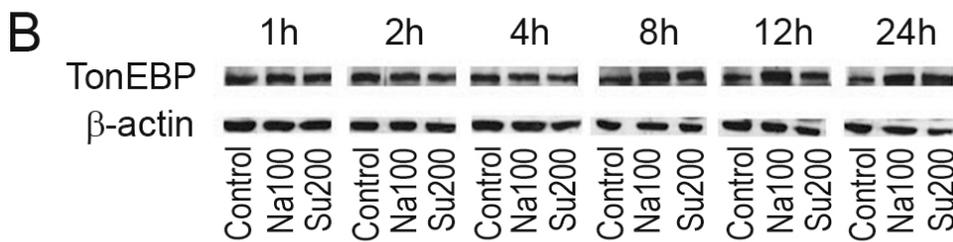
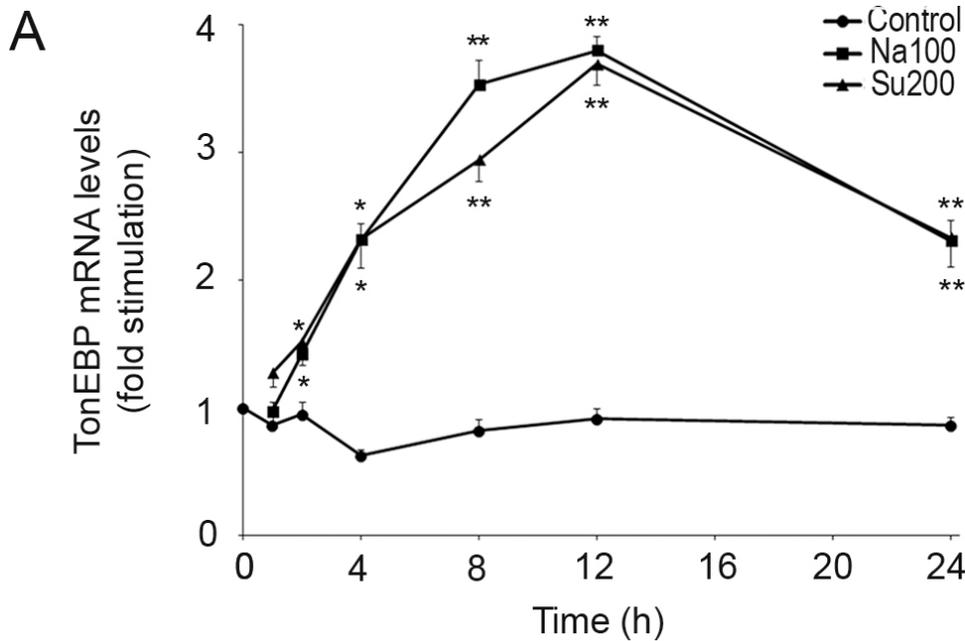


Figure 1. Kinetics of TonEBP expression in ARPE-19 cells exposed to hyperosmolar stress. **A, B:** ARPE-19 cells were incubated for 0, 1, 2, 4, 8, 12, or 24 h with iso-osmolar medium (control) or media containing the additional presence of 100 mM NaCl (Na100) or 200 mM sucrose (Su200). **C:** ARPE-19 cells were incubated for 4 h under iso-osmolar or hyperosmolar medium (Na100 or Su200), after which 1 µg/ml of actinomycin D (ActD) was added. Tonicity enhancer binding protein (TonEBP) mRNA levels were determined with real-time quantitative PCR (RT-qPCR) at 0, 2, 4, 6, and 8 h following the addition of ActD. **A, C:** TonEBP mRNA levels were measured with RT-qPCR as described in the Methods section. Data are expressed as relative TonEBP mRNA levels (in fold stimulation) to the 0 h time point set to 1. Data are the mean ± standard error of the mean (SEM; n=3) and are expressed as TonEBP mRNA levels following normalization with appropriate reference genes (*HPRT1*, *B2M*, *ATP5B*). Data were analyzed using repeated-measures ANOVA and Dunnett’s post-hoc tests. \*; p<0.05 and \*\*p <0.01 indicate statistical significance compared to time 0 h. **B:** The TonEBP protein levels were determined with semiquantitative western blot analysis. β-actin was used as an internal control of protein expression. Data are representative of three independent experiments.

**TABLE 2. KINETICS OF TONEBP PROTEIN EXPRESSION IN ARPE-19 CELLS EXPOSED TO HYPEROSMOLAR STRESS.**

Condition	Iso-osmolar	Na100	Su200
1h	100.0±0.0%	96.3±4.6%	101.0±2.6%
2h	100.3±5.0%	102.7±3.8%	98.3±1.3%
4h	100.3±0.3%	99.7±6.3%	114.7±4.1%
8h	100.7±0.7%	116.0±8.5%	111.3±7.2%
12h	99.7±0.9%	113.0±0.6%*	112.7±1.5%*
24h	99.3±0.7%	112.7±2.2%*	120.7±4.9%*

ARPE-19 cells were incubated for 1, 2, 4, 8, 12 or 24h with iso-osmolar or hyperosmolar (Na100, Su200) medium. TonEBP protein levels were determined by semiquantitative western blot analysis.  $\beta$ -actin was used as an internal control of protein expression. Band densities were estimated using an imaging system as described under Material and Methods. TonEBP protein level (ratio of TonEBP band density over  $\beta$ -actin band density) was expressed as percent of the control value (iso-osmolar condition, set to 100%) and are the mean  $\pm$  SEM (n=3). For both NaCl and sucrose stimulation, statistical analyses were performed using repeated measures ANOVA and Dunnett's post-hoc tests. \* indicates  $p < 0.05$  compared to Iso-osmolar condition at similar incubation time.

Pulser X cell (Bio-Rad Laboratories, Hercules, CA) with H<sub>2</sub>O, pcDNA3.1 (a control plasmid), DN-TonEBP coding for a truncated TonEBP protein inhibiting its activity, or a reporter plasmid of the transactivation activity of TonEBP containing two binding sites for TonEBP regulating the transcription of the reporter gene coding for a secreted embryonic alkaline phosphatase SEAP (pSEAP-TonE). Cells were treated for various time periods in the absence (control) or presence of various additional NaCl or sucrose concentrations and in the absence (dimethyl sulfoxide, DMSO) or presence of p38 kinase inhibitor SB203580.

**SEAP activity assessment with luminescence:** pSEAP-TonE was a gift from Prof. W. Neuhofer [17]. Briefly, two TonEBP responsive elements were cloned upstream of the secreted human placental alkaline phosphatase (SEAP) coding region, in the pSEAP2-Basic vector (GenBank Accession # U89937) from Clontech (Mountain View, CA). Cells were transfected transiently as described above. After 24 h, cells were treated for 24 h either in iso-osmolar or hyperosmolar (additional 100 mM NaCl) medium. The cell culture medium supernatant was collected, cleared with centrifugation, and stored at -20 °C until assayed. SEAP activity in the cell culture medium was assayed using the Phospha-Light™ SEAP Reporter Gene Assay System (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions, where 120  $\mu$ l of culture medium was diluted in an equal volume of kit 1X dilution buffer. After treatment as described in the user's manual, samples were assayed in duplicates of 50  $\mu$ l. Samples were measured in a Wallac Victor<sup>2</sup> Luminescence Multilabel Counter (model 1420-042b, PerkinElmer, Waltham, MA). Luminescent results were calculated from a standard curve (ranging from 0 to 5 mU/ml of standard

SEAP activity) of standard SEAP supplied in the Phospha-Light™ SEAP Reporter Gene Assay System. Results were then expressed as the relative modulation compared to the control iso-osmolar condition.

**Gene expression analysis using real-time quantitative PCR:** RNA extraction from ARPE-19 cells, RNA concentration and purity, and cDNA synthesis were performed as previously described [15]. Primers were designed for real-time quantitative PCR (RT-qPCR) as previously described [15] to ensure optimal DNA polymerization efficiency and amplification specificity, as well as optimal amplicon length (100 bp to 180 bp; Table 1). Possible genomic DNA amplification by the primers was verified by performing q-PCR in the presence of 2.5 ng genomic DNA. The modulation of gene expression was investigated using RT-qPCR. The RT-qPCR reaction was performed as previously described using 2.5 ng cDNA as the template and the SYBR Green MasterMix (WOUB MESA BLUE qPCR MasterMix Plus SYBR Low Rox, Eurogentec, Seraing, Belgium) [15]. Data were analyzed using StepOne software version 2.1 (Applied Biosystems) and normalized using selected appropriate stable reference genes using the geNorm algorithm [18]. All RT-qPCRs were performed in accordance with the Minimal Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines [19]. All non-target negative controls were performed using molecular biology grade water– RNase/DNase-free water instead of cDNA. Data are expressed as relative gene expression over the control and are the mean  $\pm$  standard error of the mean (SEM) of several independent experiments.

**Immunofluorescent labeling:** ARPE-19 cells, grown on gelatinized glass slides, were rinsed three times for 5 min with PBS (10 mM Na<sub>2</sub>HPO<sub>4</sub>, 154 mM NaCl, pH 7.4), fixed for 10

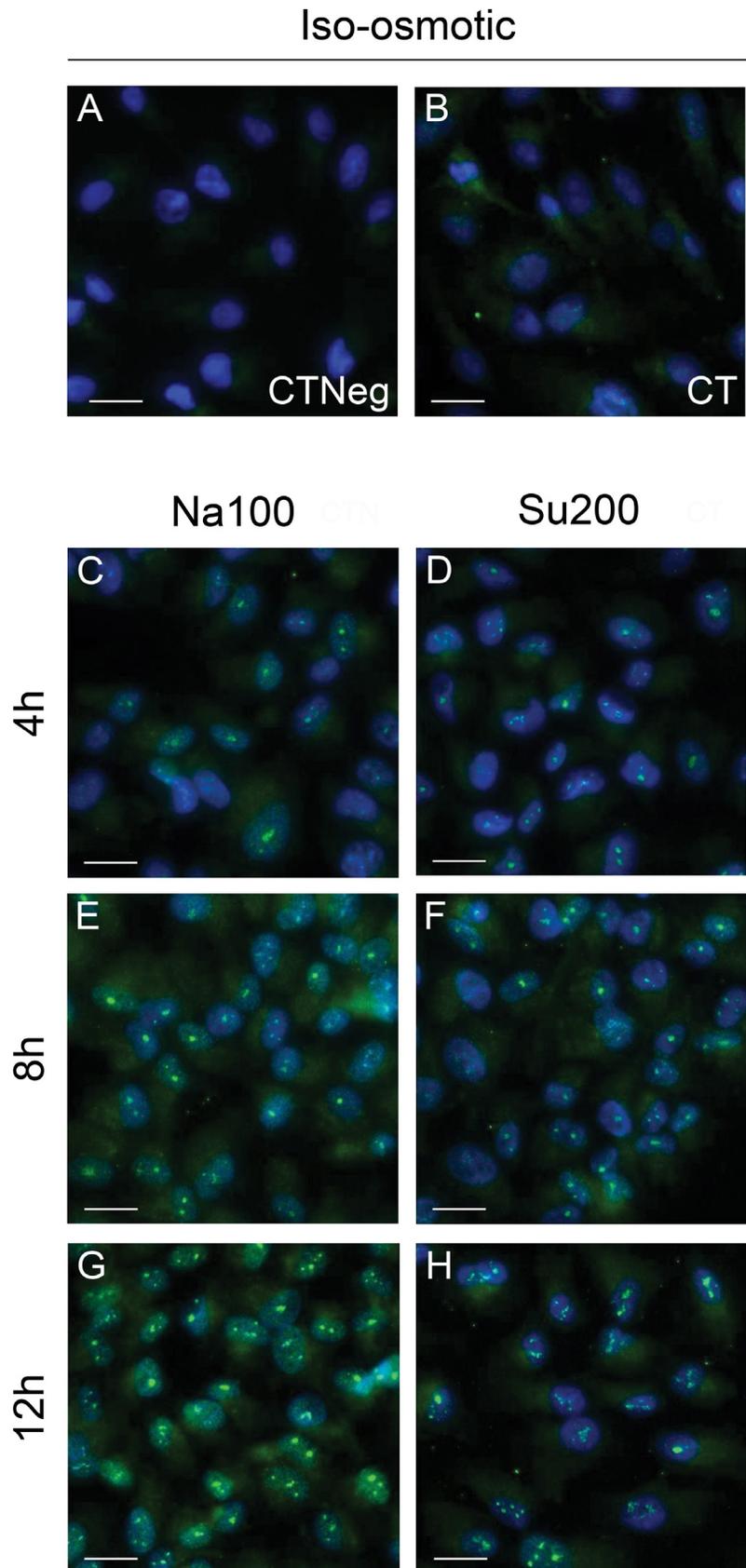


Figure 2. Kinetics of TonEBP nuclear translocation in ARPE-19 cells exposed to hyperosmolar stress. ARPE-19 cells were incubated for 4, 8, or 12 h with iso-osmolar medium (control) or media containing the additional presence of 100 mM NaCl (Na100) or 200 mM sucrose (Su200). Cells were then fixed and exposed to immunofluorescent staining of tonicity enhancer binding protein (TonEBP) (in green) as described in the Methods section. **A:** Negative control (CTNeg) was performed in the sole presence of secondary antibodies. **B:** Cells were incubated under iso-osmolar conditions for 0 h (CT). **C, E, G:** Cells were incubated with Na100. **D, F, H:** Cells were incubated with Su200. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars represent 20  $\mu$ m. Pictures were taken at 40X magnification. Data are representative of three independent experiments.

**TABLE 3. KINETICS OF TONEBP NUCLEAR TRANSLOCATION IN ARPE-19 CELLS EXPOSED TO HYPEROSMOLAR STRESS.**

Condition	4 h	8 h	12 h
Iso-osmolar	0.00±0.00%	0.67±1.15%	0.33±0.58%
Na100	70.67±5.86%*	80.33±11.93%&	100.00±0.00%#
Su200	60.33±5.51%*	81.67±10.07%&	100.00±0.00%#

ARPE-19 cells were incubated for 4, 8 or 12 h with iso-osmolar or hyperosmolar (Na100 or Su200) medium. Cells were then fixed and exposed to immunofluorescent staining of TonEBP as described under Material and Methods. The percentage of ARPE-19 cells displaying a nuclear localization of TonEBP protein was determined by cell count following immunofluorescent staining. Data represent the mean ± SEM (n=3) and are expressed as the percentage of ARPE-19 cells displaying a nuclear localization of TonEBP protein. Statistical analyses were performed using repeated measures ANOVA and Dunnett's post-hoc tests. p<0.05 compared to Iso-osmolar condition at 4h: \*; 8 h: &; 12 h: #.

min in 100% methanol at -20 °C, and rinsed three times for 5 min with PBS. ARPE-19 cells were incubated overnight at 4 °C with the primary antibodies against TonEBP (Novus Biologicals, Littleton, CO, dilution 1:200), followed by incubation with biotinylated anti-rabbit immunoglobulin (IgG; GE Healthcare UK Limited, Buckinghamshire, UK, dilution 1:600) and streptavidin-cyanin2 (Jackson ImmunoResearch Laboratories, West Grove, PA, dilution 1:200). Slides with cells were mounted using Prolong Gold Antifade glue with 4',6-diamidino-2-phenylindole (DAPI; staining the nuclei in blue; Invitrogen, Carlsbad, CA). As negative controls, ARPE-19 cells were incubated with the secondary antibody alone. Images were taken at 40X magnification using an Axiocam MRB fluorescent microscope (Zeiss, Göttingen, Germany).

**Protein preparation and western blot analysis:** ARPE-19 cells were harvested in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP-40) containing 0.5 mM sodium fluoride, 1 mM sodium orthovanadate, and a cocktail of protease inhibitors (Complete EDTA free, Roche Diagnostics, Indianapolis, IN). Harvested ARPE-19 cells were immediately frozen in liquid nitrogen, then thawed, and submitted to 10 min centrifugation at 15,000 ×g at 4 °C. The supernatant containing the total proteins was collected. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Total proteins, in the presence of 5% β-mercaptoethanol, were analyzed with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) polyacrylamide gels. Proteins were transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated for 1 h in PBS containing 0.1% Tween-20 and 8% milk and then rinsed three times for 10 min in PBS containing 0.1% Tween-20. The proteins were then immunolabeled using specific primary antibodies. The bound primary antibodies were detected using secondary

anti-mouse or anti-rabbit antibodies (GE Healthcare, Little Chalfont, UK) and an enhanced chemiluminescence (ECL) chemiluminescence detection kit (PerkinElmer). Band densities were estimated using an imaging system and Quantity One software (Bio-Rad Laboratories). The TonEBP protein level (ratio of TonEBP band density over β-actin band density) was expressed as the percentage of the control value (set to 100%) and represents the mean ± standard error of the mean (SEM; n=3).

**Statistical analysis:** Statistical analyses were performed using repeated-measures ANOVA and Dunnett's post-hoc tests, the conformity *t* test or the paired *t* test. The tests were considered statistically significant when the *p* value was less than 0.05.

## RESULTS

**Kinetics of TonEBP expression in ARPE-19 cells exposed to hyperosmolar stress:** ARPE-19 cells were incubated under iso-osmolar medium or hyperosmolar medium (medium containing additional 100 mM NaCl (Na100) or 200 mM sucrose (Su200)) for 0, 1, 2, 4, 8, 12, or 24 h. The TonEBP mRNA and protein levels were then measured with RT-qPCR and western blot, respectively, p<0.05.

Compared to the control condition, Na100 and Su200 significantly increased TonEBP mRNA levels from 2 h (1.4 and 1.5 fold, respectively) up to 12 h of stimulation (3.8 and 3.7 fold, respectively; Figure 1A). However, at 24 h following Na100 and Su200, TonEBP mRNA levels had decreased compared to those measured at 12 h (2.3 and 2.3 fold, respectively, p<0.01), but nevertheless remained significantly higher than those quantified at 2 h (Figure 1A).

TonEBP protein levels, normalized to β-actin, significantly increased at 12 h and 24 h in response to Na100 (113.0±0.6%, p<0.05; 112.7±2.2; n=3) and Su200 (112.7±1.5%,

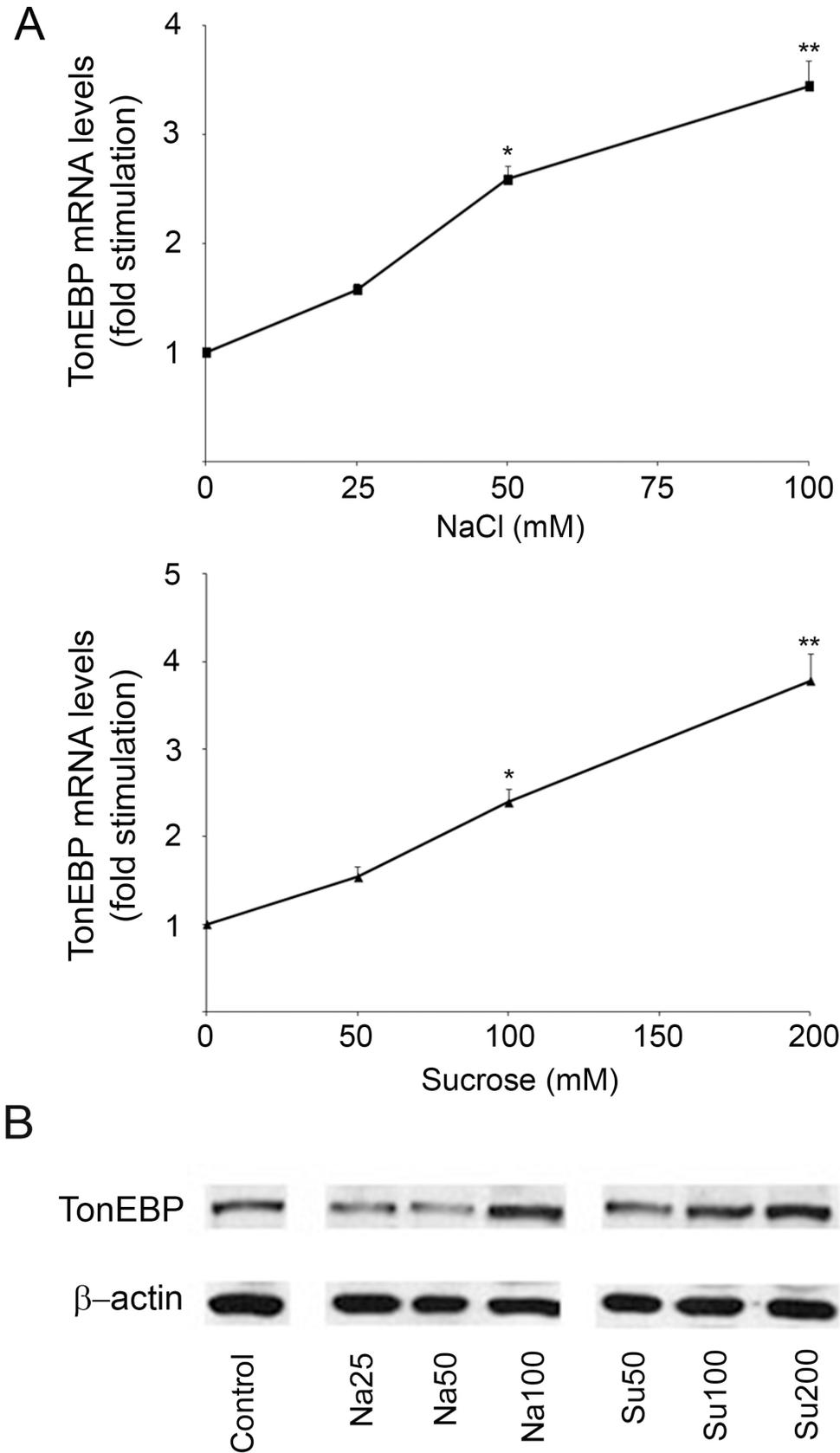


Figure 3. Dose–response curve of hyperosmolar stress on TonEBP expression in ARPE-19 cells. ARPE-19 cells were incubated for 12 h with iso-osmolar medium (control) or media containing the additional presence of increasing concentrations of NaCl (Na25, Na50, Na100) or sucrose (Su50, Su100, Su200). **A**: Tonicity enhancer binding protein (TonEBP) mRNA levels measured with real-time quantitative PCR (RT-qPCR) under increasing concentrations of NaCl. **B**: TonEBP mRNA levels measured with RT-qPCR under increasing concentrations of sucrose. **A, B**: Data are expressed as relative TonEBP mRNA levels (in fold stimulation) over the iso-osmolar condition (Na0 or Su0) set to 1. The data are the mean ± standard error of the mean (SEM; n=3) following normalization with the appropriate reference genes (*HPRT1*, *B2M*, *ATP5B*). Data were analyzed using repeated-measures ANOVA and Dunnett’s post-hoc tests. \*: p<0.05 and \*\*p <0.01 indicate statistical significance compared to the iso-osmolar medium (Na0 or Su0). **C**: The TonEBP protein levels were determined with semiquantitative western blot analysis. β-actin was used as an internal control of protein expression. Data are representative of three independent experiments.

**TABLE 4. DOSE-RESPONSE CURVE OF HYPEROSMOLAR STRESS ON TonEBP PROTEIN EXPRESSION IN ARPE-19 CELLS.**

Condition	TonEBP protein expression (%)
Iso-osmolar	100.0±0.0%
Na25	109.0±1.9%
Na50	123.2±1.5% *
Na100	125.5±10.4% *
Su50	106.9±0.9%
Su100	132.2±18.9%
Su200	199.1±34.0% *

ARPE-19 cells were incubated for 12 h with iso-osmolar or hyperosmolar (Na25, Na50, Na100, Su50, Su100 and Su200) medium. TonEBP protein levels were determined by semiquantitative western blot analysis.  $\beta$ -actin was used as an internal control of protein expression. Band densities were estimated using an imaging system as described under Material and Methods. TonEBP protein level (ratio of TonEBP band density over  $\beta$ -actin band density) was expressed as percent of the control value (iso-osmolar condition, set to 100%) and are the mean  $\pm$  SEM (n=3). For both NaCl and sucrose stimulation, statistical analyses were performed using repeated measures ANOVA and Dunnett's post-hoc tests. \* indicates  $p < 0.05$  compared to Iso-osmolar condition.

$p < 0.05$ ; 120.7 $\pm$ 4.9; n=3) compared to the control condition (99.7 $\pm$ 0.9%; 99.3 $\pm$ 0.7%; n=3; Figure 1B, Table 2).

To determine whether the increased TonEBP mRNA level resulted from decreased TonEBP mRNA degradation or increased transcriptional activity, the stability of TonEBP mRNA was measured in the presence of the transcriptional inhibitor actinomycin D (ActD). Cells were treated with iso-osmolar or hyperosmolar medium (Na100 or Su200) for 4 h, after which 1 mg/ml ActD was added. TonEBP mRNA levels were quantified at 0, 2, 4, 6, and 8 h after the addition of ActD (Figure 1C). TonEBP half-life (around 6 h under the iso-osmolar condition) significantly increased in response to hyperosmolar stress (>24 h). These data show that the increase in the TonEBP mRNA levels in response to hyperosmolar stress is largely due to increased mRNA stability.

**Kinetics of TonEBP nuclear translocation in ARPE-19 cells exposed to hyperosmolar stress:** ARPE-19 cells were incubated under iso-osmolar medium, Na100 or Su200 for 4, 8, or 12 h. The percentage of ARPE-19 cells displaying nuclear localization of TonEBP protein was determined by cell count following immunofluorescent staining.

Under the iso-osmolar condition, the TonEBP protein was strictly localized to the cell cytoplasm at all times studied (Figure 2, Table 3). In response to Na100 and Su200, the percentage of cells displaying nuclear translocation of

TonEBP significantly increased following 4 h (70.67 $\pm$ 5.86% and 60.33 $\pm$ 5.51%, respectively,  $p < 0.05$ , n=3) up to 12 h of stimulation (Figure 2 and Table 3). All cells presented nuclear TonEBP labeling following 12 h stimulation with Na100 and Su100 (both 100.00 $\pm$ 0.00%,  $p < 0.05$ , n=3; Figure 2, Table 3).

**Dose-response curve of hyperosmolar stress on TonEBP expression:** ARPE-19 cells were incubated for 12 h with iso-osmolar medium or hyperosmolar medium of increasing additional concentrations of NaCl (25 mM (Na25), 50 mM (Na50), 100 mM (Na100)) or sucrose (50 mM (Su50), 100 mM (Su100), 200 mM (Su200)). TonEBP mRNA and protein levels were then measured with RT-qPCR and western blot, respectively.

The TonEBP mRNA levels increased dose-dependently in response to the increasing concentrations of NaCl and sucrose. The TonEBP mRNA levels were significantly increased from Na50 (2.6 fold,  $p < 0.05$ ) and Su100 (2.4 fold,  $p < 0.05$ ) up to Na100 (3.4 fold,  $p < 0.05$ ) and Su200 (3.8 fold,  $p < 0.05$ ; Figure 3A). The TonEBP protein levels, normalized to those of  $\beta$ -actin, significantly increased in response to Na50 (123.2 $\pm$ 1.5%,  $p < 0.05$ ; n=3), Na100 (125.5 $\pm$ 10.4%,  $p < 0.05$ ; n=3), and Su200 (199.1 $\pm$ 34.0%,  $p < 0.05$ ; n=3) compared to the iso-osmolar condition (100.0 $\pm$ 0.0%; Figure 3B, Table 4).

**Dose-response curve of hyperosmolar stress on TonEBP nuclear translocation:** ARPE-19 cells were incubated for 4 h or 12 h in the presence of iso-osmolar medium or hyperosmolar medium of increasing additional concentrations of NaCl (Na25, Na50, Na100) or sucrose (Su50, Su100, Su200). The percentage of ARPE-19 cells displaying nuclear localization of the TonEBP protein was determined by cell count following immunofluorescent staining.

At 4 h and 12 h, the TonEBP protein was strictly localized to the cell cytoplasm under the iso-osmolar condition (Figure 4 and Table 5). Following 4 h of stimulation with Na25, Na50, Su50, and Su100, TonEBP was weakly localized in the cell nucleus (0.67 $\pm$ 1.15%, 6.33 $\pm$ 5.86%, 2.00 $\pm$ 2.65%, and 6.67 $\pm$ 4.04%, respectively, n=3; Figure 4 and Table 5). However, following 4 h of stimulation with Na100 and Su200, the TonEBP protein significantly translocated to the cell nucleus (77.67 $\pm$ 17.50% and 46.00 $\pm$ 14.73%,  $p < 0.05$ ; n=3). Following 12 h of stimulation with hyperosmolar media, TonEBP translocation increased following incubation with Na25 and Su50 (71.67 $\pm$ 9.61% and 67.00 $\pm$ 7.00%, respectively,  $p < 0.05$ , n=3) and reached the maximum at Na100 and Su200 (both 100.00 $\pm$ 0.00%,  $p < 0.05$ , n=3; Figure 4 and Table 5).

**Kinetics of AR and TauT mRNA expression in ARPE-19 cells exposed to hyperosmolar stress:** ARPE-19 cells were incubated with iso-osmolar medium, Na100 or Su200 for 0, 1, 2, 4,

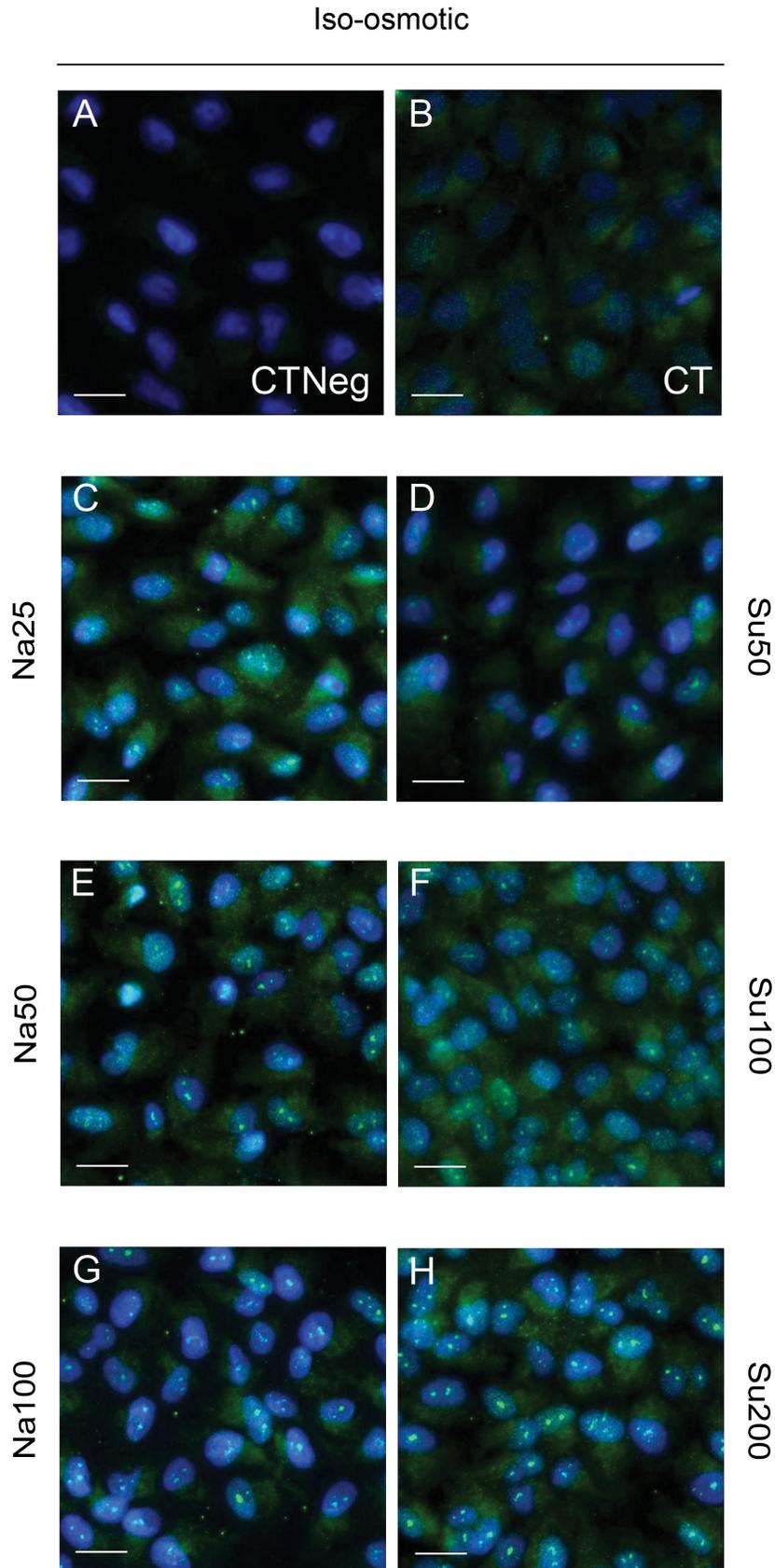


Figure 4. Dose–response curve of hyperosmolar stress on TonEBP nuclear translocation in ARPE-19 cells. ARPE-19 cells were incubated for 4 h with iso-osmolar medium (CT). Cells were incubated for 4 h in media containing the additional presence of increasing concentrations of NaCl (Na25, Na50, Na100; C, E, G) or sucrose (Su50, Su100, Su200; D, F, H). Negative control (CTNeg) was performed in the sole presence of secondary antibodies. Cells were then fixed and exposed to immunofluorescent staining of tonicity enhancer binding protein (TonEBP) (in green) as described in the Methods section. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; blue). Scale bars represent 20  $\mu$ m. Pictures were taken at 40X magnification. Data are representative of three independent experiments.

8, 12, or 24 h, and the levels of AR and TauT mRNA expression were measured with RT-qPCR. In response to Na100 and Su200, the AR mRNA levels significantly increased from 8 h and 12 h, respectively, up to 24 h ( $p < 0.05$ , Figure 5A). In response to Na100, the TauT mRNA levels were significantly increased at 8 h and 12 h ( $p < 0.05$ , Figure 5B). In response to Su200, the TauT mRNA levels were significantly increased at 24 h ( $p < 0.05$ , Figure 5B).

*A dominant negative form of TonEBP blocks the transactivation activity of TonEBP:* ARPE-19 cells were transiently transfected with either pSEAP-TonE (a reporter plasmid containing two binding sites for TonEBP regulating the transcription of the reporter gene coding for a secreted embryonic alkaline phosphatase (SEAP)) and pcDNA3.1 (used as a control plasmid) plasmids or with pSEAP-TonE and DN-TonEBP (a dominant negative form of TonEBP) plasmids, before being incubated for 24 h in the absence (control) or presence of additional 100 mM NaCl (Na100). SEAP activity was then measured with chemiluminescence in the cell supernatant.

SEAP activity was significantly increased in response to Na100 compared to the control condition  $p < 0.05$ . DN-TonEBP induced a significant decrease of 86% in Na100-induced SEAP activity ( $p < 0.05$ , Figure 6).

*Effects of DN-TonEBP on NaCl-induced TauT and AR expression:* ARPE-19 cells were transiently transfected with either H<sub>2</sub>O (control) or with DN-TonEBP before being treated for 8 h in the absence (control) or presence of additional 100 mM NaCl (Na100). The levels of AR and TauT mRNA expression were measured with RT-qPCR.

DN-TonEBP induced a significant 45% reduction in NaCl-induced AR mRNA levels, compared to the control cells (from  $6.09 \pm 0.89$  fold to  $3.34 \pm 0.65$  fold,  $p < 0.05$ ,  $n=5$ ; Figure 7A). In addition, DN-TonEBP induced a significant 71% reduction in NaCl-induced TauT mRNA levels, compared to the control cells (from  $9.06 \pm 1.43$  fold to  $2.65 \pm 0.41$  fold,  $p < 0.01$ ,  $n=5$ ; Figure 7B).

*Involvement of p38 protein kinase in TonEBP activation and subsequent transactivation activity induced by hyperosmolar stress in ARPE-19 cells:* ARPE-19 cells were preincubated for 1 h in the presence of either 0.1% DMSO or 10  $\mu$ M SB203580 (an inhibitor of p38 protein kinase activation) diluted in DMSO. ARPE-19 cells were then incubated for various time periods in iso-osmolar or hyperosmolar Na100 media in the remaining presence of either DMSO or protein kinase inhibitors. The percentage of ARPE-19 cells displaying a nuclear localization of TonEBP protein was determined by cell count following immunofluorescent staining after a 4 h exposure with iso-osmolar or hyperosmolar N100 media. SEAP activity was measured in cell supernatant by luminescence after a 24 h exposure with iso-osmolar or hyperosmolar N100 media. The levels of AR and TauT mRNA expression were measured by RT-qPCR after 8 h exposure with iso-osmolar or hyperosmolar N100 media. Under iso-osmolar conditions, SB203580 did not induce nuclear localization of the TonEBP protein (both  $0.00 \pm 0.00\%$ ;  $n=3$ ). However, SB203580 inhibited by 93% TonEBP nuclear translocation in response to 4 h stimulation with Na100 ( $7.00 \pm 2.07\%$ ;  $n=3$ ) compared to cells incubated in the absence of the inhibitor ( $100.00 \pm 0.00\%$ ,  $n=3$ ; Figure 8A). In addition, SB203580 did

**TABLE 5. DOSE-RESPONSE CURVE OF HYPEROSMOLAR STRESS ON TONEBP NUCLEAR TRANSLOCATION IN ARPE-19 CELLS.**

Condition	4 h	12 h
Iso-osmolar	0.00±0.00%	0.00±0.00%
Na25	0.67±1.15%	71.67±9.61%#
Na50	6.33±5.86%	89.67±5.51%#
Na100	77.67±17.50%*	100.00±0.00%#
Su50	2.00±2.65%	67.00±7.00%#
Su100	6.67±4.04%	83.33±9.07%#
Su200	46.00±14.73%*	100.00±0.00%#

ARPE-19 cells were incubated for 4 h or 12 h with iso-osmolar or hyperosmolar (Na25, Na50, Na100, Su50, Su100, Su200) medium. Cells were then fixed and exposed to immunofluorescent staining of TonEBP as described under Material and Methods. The percentage of ARPE-19 cells displaying a nuclear localization of TonEBP protein was determined by cell count following immunofluorescent staining. Data represent the mean  $\pm$  SEM ( $n=3$ ) and are expressed as the percentage of ARPE-19 cells displaying a nuclear localization of TonEBP protein. For both NaCl and sucrose stimulation, statistical analyses were performed using repeated measures ANOVA and Dunnett's post-hoc tests. \* and # indicate  $p < 0.05$  statistical significance compared to Iso-osmolar condition at 4h and 12h, respectively.

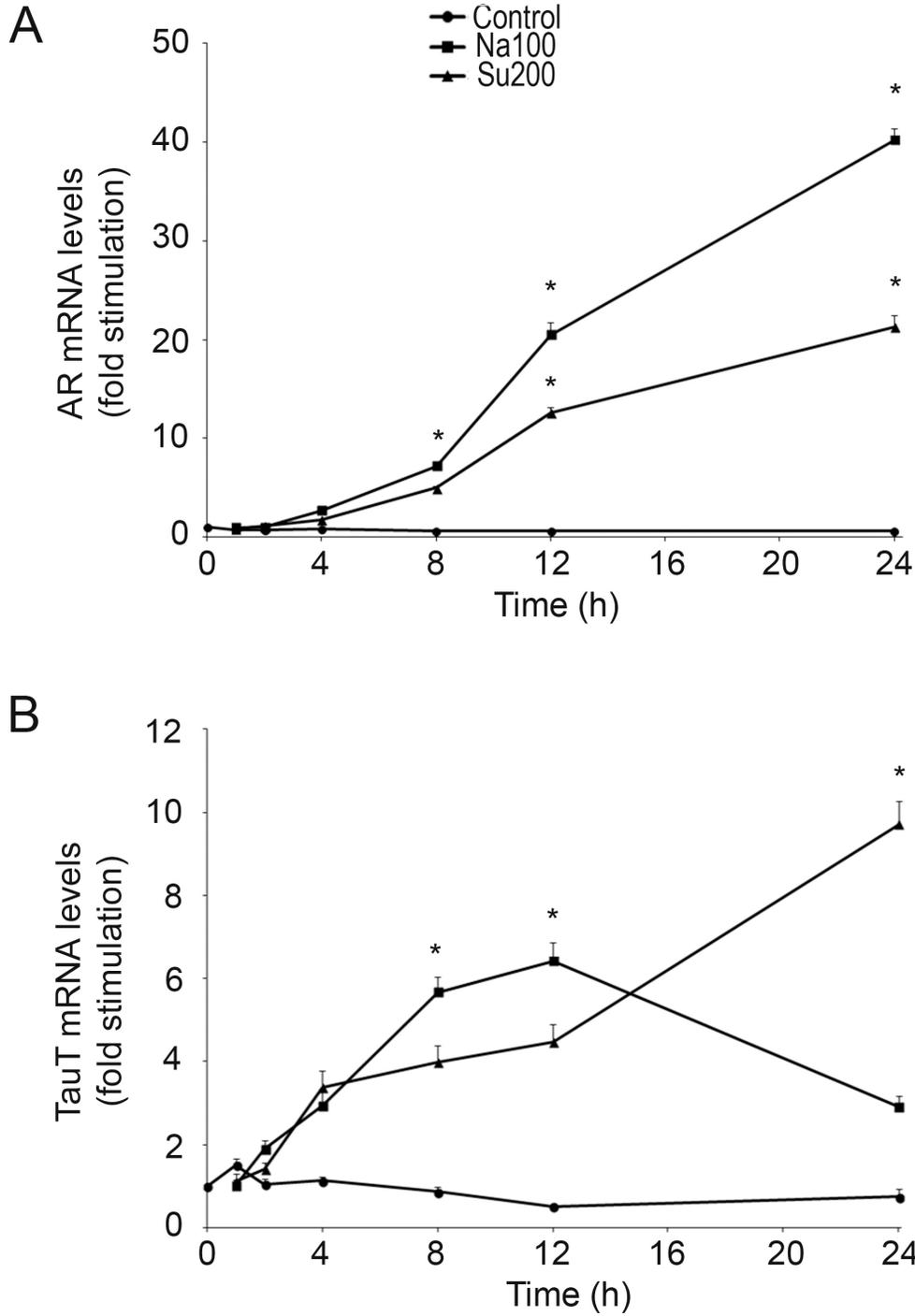


Figure 5. Kinetics of AR and TauT mRNA expression in response to hyperosmolar stress. ARPE-19 cells were incubated for 0, 1, 2, 4, 8, 12, or 24 h with iso-osmolar medium (control) or media containing the additional presence of 100 mM NaCl (Na100) or 200 mM sucrose (Su200). Genes mRNA levels were measured with real-time quantitative PCR (RT-qPCR) as described in the Methods section. Data are expressed as (A) relative aldose reductase (AR) or (B) sodium-dependent taurine transporter (TauT) mRNA levels (in fold stimulation) over the 0 h time point set to 1. The data are the mean  $\pm$  standard error of the mean (SEM; n=3) and are expressed as TauT and AR mRNA levels following normalization with the appropriate reference genes (*HPRT1*, *B2M*, *ATP5B*). Data were analyzed using repeated-measures ANOVA and Dunnett's post-hoc tests. \*p<0.05 indicates statistical significance compared to time 0 h.

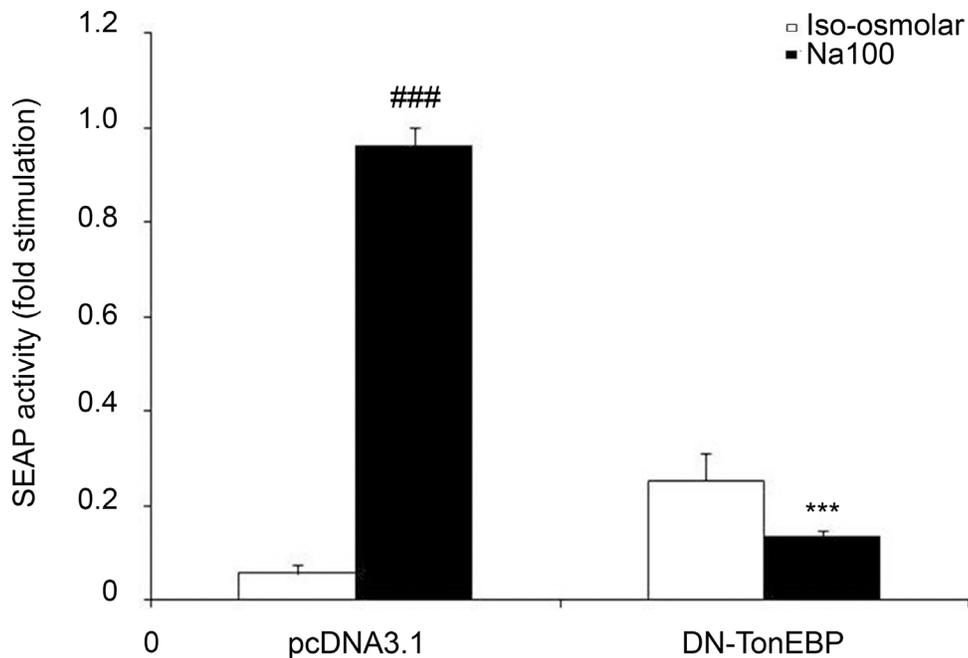


Figure 6. Effects of DN-TonEBP on the transactivation activity of TonEBP. ARPE-19 cells were transiently transfected with either 10  $\mu$ g of pSEAP-TonE plasmid and 10  $\mu$ g of pcDNA3.1 plasmid (control plasmid) or with 10  $\mu$ g of pSEAP-TonE plasmid and 10  $\mu$ g of DN-TonEBP plasmid, before being incubated for 24 h in the absence (control) or presence of 100 mM additional NaCl (Na100). The activity of secreted embryonic alkaline phosphatase (SEAP) was measured with luminescence in the cell culture supernatant. Data are expressed as relative activity (in fold stimulation) over the iso-osmolar condition and are the mean  $\pm$  standard error of the mean (SEM; n=3). Statistical analysis was

performed with the conformity *t* test (###*p*<0.005) that compared the control in Na100 to the control in the iso-osmolar condition, and a paired *t* test (\*\**p*<0.005) was used to compare the dominant negative form of tonicity enhancer binding protein (DN-TonEBP) TonEBP in Na100 to DN-TonEBP in the iso-osmolar condition.

not modify SEAP activity under the iso-osmolar condition (control), whereas SB203580 significantly inhibited Na100-induced SEAP activity compared to the control (*p* <0.05, Figure 8B). Furthermore, although SB203580 had no effect on AR (*p* <0.05) and TauT mRNA levels under the iso-osmolar conditions, SB203580 significantly inhibited Na100-induced AR but not TauT mRNA levels compared to the control (Figure 8C,D).

## DISCUSSION

Tightly regulated water movement through RPE cells is required for normal retinal function [4]. Under several pathophysiological situations, the osmolar gradient can be disturbed. During diabetic macular edema, for example, rupture of the inner and outer BRB leads to protein accumulation in the retina leading to an increase in oncotic pressure and water accumulation in the sensory retina and the subretinal space [20]. Thus, it is likely that RPE cells are exposed to hyperosmolar stress during diabetic macular edema [20]. However, the osmoprotective response of RPE cells to hyperosmolar stress remains poorly understood, although stimulation of certain osmoprotective genes, including TauT (Gene ID: 6533; OMIM 186854) [13] and AR (Gene ID: 231; OMIM 103880) [14], has been shown. In renal cells, the mechanisms responsible for osmoadaptation to

hyperosmolarity is well-established [10] and largely imply the activation of TonEBP, a transcription factor of the Rel family, and the subsequent transactivation of osmoprotective genes allowing the accumulation of osmolytes in the cell cytoplasm to reduce the intracellular ionic force [10]. In ARPE-19 cells, TonEBP induction under high glucose challenge has been demonstrated [12]. In addition, it has also been recently shown that TonEBP is involved in the hyperosmolar induction of several aquaporins and vascular endothelial growth factor in RPE cells [21]. However, the involvement of TonEBP in osmoadaptation of RPE cells to hyperosmolar stress response has not been demonstrated. The present study clearly shows the involvement of TonEBP in the mechanisms responsible for adaptation to hyperosmolarity in ARPE-19 cells. TonEBP mRNA and protein levels are stimulated in a time- and dose-dependent manner in response to hyperosmolar stress. Furthermore, hyperosmolar stress induces TonEBP nuclear translocation in RPE cells in a time- and dose-dependent manner as well. In addition, hyperosmolar stress induced the transactivation activity of TonEBP and osmoprotective genes expression in ARPE-19 cells. Finally, a dominant negative form of TonEBP abolished NaCl-induced increase in TonEBP transactivation activity and decreased NaCl-induced AR and TauT mRNA level increases. Finally, p38 inhibition blocks hyperosmolar-induced TonEBP nuclear translocation and

transactivation activity, suggesting that p38 is likely involved in hyperosmolar-induced TonEBP phosphorylation.

The hyperosmolar stress-induced increase in TonEBP mRNA levels occurs early, as of 2 h up to 12 h, due to increased mRNA stability. Our data are in agreement with those obtained on the kidney showing increased TonEBP mRNA stability in response to hyperosmolar stress [7,22]. Moreover, the TonEBP protein levels increased in a delayed manner compared with the mRNA levels, as of 12 h up to 24 h of hyperosmolar stimulation. In addition, in response to hyperosmolar stress, TonEBP transactivation activity was significantly increased, and the kinetics of the AR and TauT

mRNA levels follows the increase in the TonEBP mRNA and TonEBP protein levels, as well as TonEBP nuclear translocation. A dominant negative form of TonEBP abolished the hyperosmolar-induced increase in TonEBP transactivation activity. Therefore, in ARPE-19 cells, hyperosmolar stress induced TonEBP nuclear translocation and transactivation activity. However, our data cannot exclude a role for other transcription factors in hyperosmolar-induced increases in AR and TauT mRNA levels as the inhibitory effect of DN-TonEBP was partial.

p38 is likely involved in hyperosmolar-induced TonEBP phosphorylation and subsequent nuclear translocation and

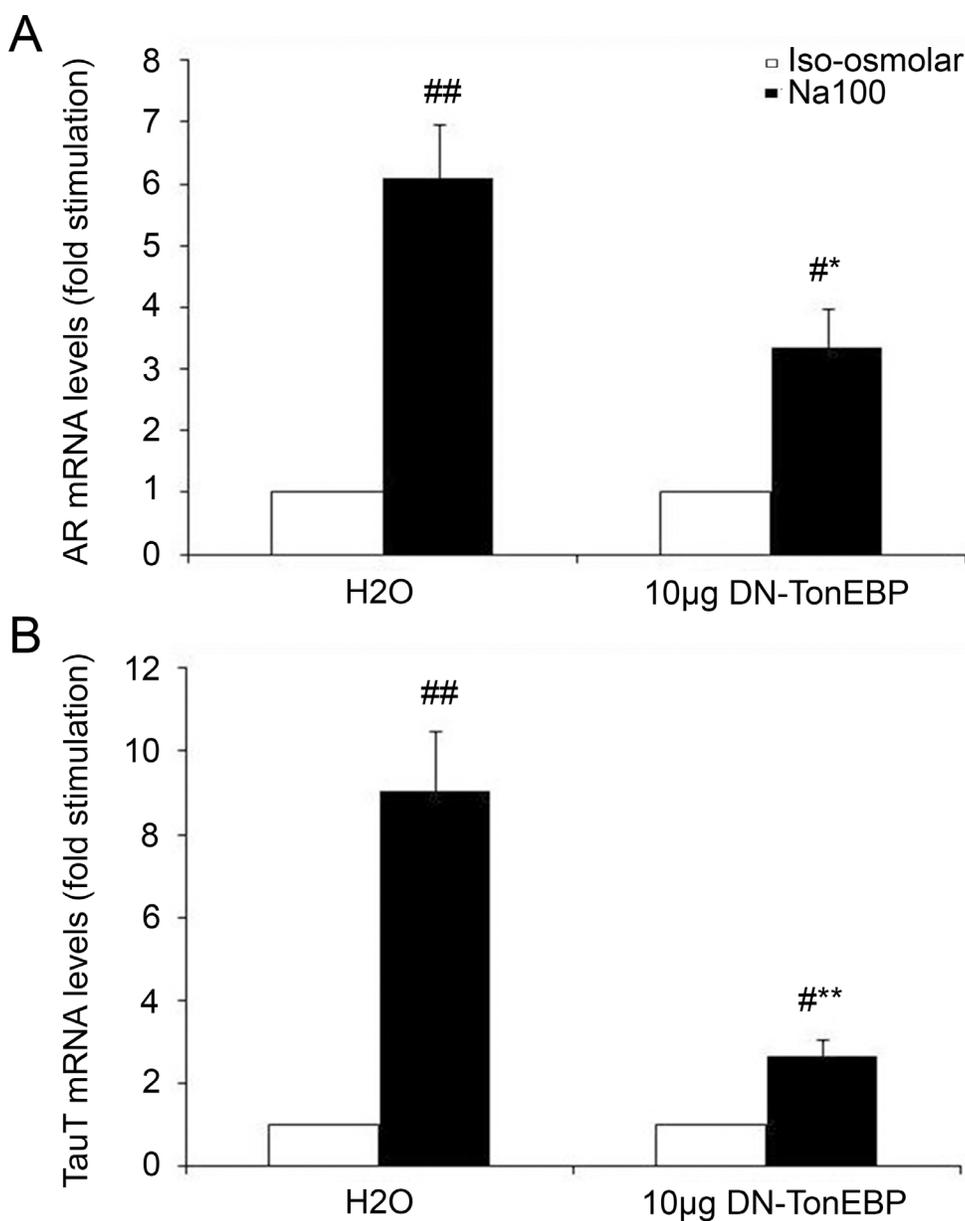


Figure 7. Effects of DN-TonEBP on NaCl-induced TauT and AR expression. ARPE-19 cells transiently transfected with either H<sub>2</sub>O or 10 µg of DN-TonEBP were incubated for 8 h with iso-osmolar medium (control, CT) or media containing the additional presence of 100 mM NaCl (Na100). (A) Aldose reductase (AR) and (B) sodium-dependent taurine transporter (TauT) mRNA levels were measured with real-time quantitative PCR (RT-qPCR) as described in the Methods section. The data are the mean ± standard error of the mean (SEM; n=5) and are expressed as gene mRNA levels (in fold stimulation) over the iso-osmolar condition set to 1 for H<sub>2</sub>O and a dominant negative form of tonicity enhancer binding protein (DN-TonEBP) following normalization with the appropriate reference genes (*YWHAZ*, *ATP5B*, *MDHI*). Statistical analysis was performed with the conformity *t* test (#p<0.05; ##p<0.01) that compared the Na100 condition to the iso-osmolar condition, and a paired *t* test (\*p<0.05; #\*\*p<0.01) as used to compare DN-TonEBP in Na100 to DN-TonEBP in the iso-osmolar condition.

transactivation activity. An inhibitor of p38 alpha and p38 beta isoforms, but not of p38 gamma and p38 delta [23-25], inhibited hyperosmolar-induced TonEBP nuclear translocation, transactivation activity as well as AR and, to a lesser extent (i.e., statistically not significant), TauT mRNA levels.

The absence of a significant inhibitory effect of SB203580 on hyperosmolar-induced TauT mRNA levels might result from the opposite effects of p38 delta compared to p38 alpha in response to hyperosmolar stress [26], or the effects of p38 alpha and/or p38 beta on other transcription factors.

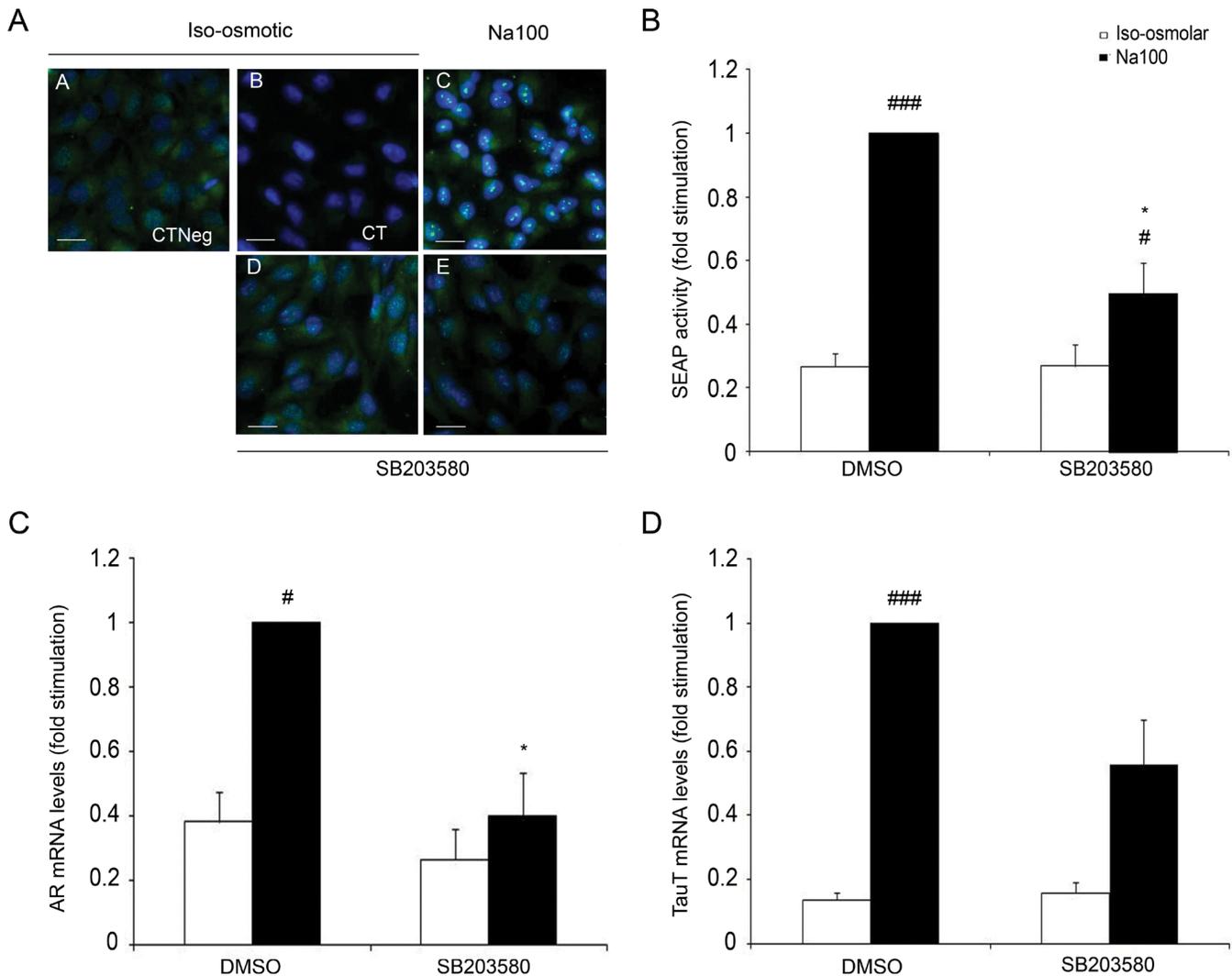


Figure 8. Involvement of p38 protein kinase in TonEBP activation and subsequent transactivation activity induced by hyperosmolar stress in ARPE-19 cells. Cells were preincubated for 1 h in the presence of 0.1% dimethyl sulfoxide (DMSO) or 10  $\mu$ M SB203580 and then incubated for various times with iso-osmolar medium (CT; open columns) or medium containing the additional presence of 100 mM NaCl (Na100; closed columns). (A) Tonicity enhancer binding protein (TonEBP) translocation, (B) secreted embryonic alkaline phosphatase (SEAP) activity, and (C) quantification of aldose reductase (AR) and (D) sodium-dependent taurine transporter (TauT) mRNA levels were performed following 4, 24, 8, and 8 h incubation, respectively, as described in the Methods section. A: TonEBP was labeled in green, while cell nuclei were labeled in blue. Scale bars represent 20  $\mu$ m. Pictures were taken at 40X magnification. Data are representative of three independent experiments. B: SEAP data are expressed as relative activity (in fold stimulation) over the control (DMSO) in Na100 and are the mean  $\pm$  standard error of the mean (SEM; n=3). C, D: Data are expressed as relative gene mRNA levels (in fold stimulation) over the DMSO iso-osmolar condition set to 1. The data are the mean  $\pm$  SEM (n=3) and are expressed as gene mRNA levels following normalization with the appropriate reference genes (*HPRT1*, *B2M*, *ATP5B*). B, C, D: Statistical analysis was performed using the conformity *t* test (\* $p$ <0.05) that compared SB203580 Na100 with control Na100, a paired *t* test (## $p$ <0.005) that compared SB203580 with SB203580 in the iso-osmolar condition, and a second paired *t* test that compared DMSO in the iso-osmolar condition and SB203580 in the iso-osmolar condition.

Additional studies are required to further investigate the contribution of each p38 isoform and/or possible involvement of other transcription factors in hyperosmolar-induced TonEBP transactivation activity.

In summary, our data demonstrate the involvement of TonEBP in the mechanisms responsible for osmoadaptation to hyperosmolar stress in RPE cells. Given the emerging role of TonEBP in different pathologies, these data open up new perspectives for the analysis of the mechanisms involved in the modification of functions of the RPE during macular edema.

## APPENDIX 1. STR ANALYSIS.

To access these data, click or select the words "[Appendix 1](#)".

## ACKNOWLEDGMENTS

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