**Hydroosmotic Stress–Induced TRPM2 Channel Activation Stimulates NLRP3 Inflammasome Activity in Primary Human Corneal Epithelial Cells**

Qinxiang Zheng,1 Qiufan Tan,1,2 Yueping Ren,1 Peter S. Reinach,1 Ling Li,1 Chaoxiang Ge,1 Jia Qu,1 and Wei Chen1

1School of Ophthalmology and Optometry, Wenzhou Medical University, Zhejiang, China
2Yiwu Maternal and Child Health Hospital, Jinhua, Zhejiang, China

**Correspondence:** Wei Chen, The School of Ophthalmology and Optometry, Wenzhou Medical University, 270 Xueyuan West Road, Wenzhou, Zhejiang 325027, People’s Republic of China; chenweit@hotmail.com.

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**PURPOSE.** The purpose of this study was to determine whether either a hyperosmotic or oxidative stress induces NLRP3 inflammasome activation and increases in bioactive IL-1β secretion through transient receptor potential melastatin 2 (TRPM2) activation in primary human corneal epithelial cells (PHCECs).

**METHODS.** Real-time PCR, Western blots, and immunofluorescent staining were used to evaluate TRPM2 and NLRP3, ASC, caspase-1, and IL-1β mRNA and protein expression levels, respectively. A CCK-8 assay evaluated cell viability. Hyperosmotic 500 mOsm and oxidative 0.5 mM H2O2 stresses were imposed. TRPM2 expression was inhibited with a TRPM2 inhibitor, 20 μM N-(p-amylcinnamoyl) anthranilic acid (ACA), or TRPM2 siRNA knockdown.

**RESULTS.** In the hypertonic medium, TRPM2, NLRP3, ASC, caspase-1, and IL-1β gene and protein expression levels rose after 4 hours (P ≤ 0.043), whereas ACA preincubation suppressed these rises (P ≤ 0.044). Similarly, H2O2 upregulated TRPM2 protein expression by 80%, and induced both NLRP3 inflammasome activation and increased bioactive IL-1β secretion (P ≤ 0.036), whereas ACA pretreatment suppressed these effects (P ≤ 0.029). TRPM2 siRNA transfection reduced TRPM2 gene expression by 70% (P = 0.018) in this hyperosmotic medium and inhibited the increases in NLRP3, caspase-1, and IL-1β gene (P ≤ 0.028) and protein expression (P ≤ 0.037).

**CONCLUSIONS.** TRPM2 activation by either a hyperosmotic or oxidative stress contributes to mediating increases in NLRP3 inflammasome activity and bioactive IL-1β expression because inhibiting TRPM2 activation or its expression blunted both of these responses in PHCECs. This association points to the possibility that TRPM2 is a viable target to suppress hyperosmotic-induced corneal epithelial inflammation.

Keywords: human corneal epithelial cells, dry eye, TRPM2, NLRP3, IL-1β

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Corneal epithelial layer integrity and function are essential for sustaining normal vision. Injury and/or pathogenic infiltration of this outermost protective layer can induce visual function losses due to opacification.1 Even if the changes are limited to altering tear film makeup and/or declines in secretion, tear film hyperosmolarity may develop and ultimately induce increases in reactive oxygen species (ROS) formation and inflammation.2 These pathophysiologic effects are characteristic in some cases of dry eye (DE) disease and Sjögren’s syndrome.3–5 In corneal epithelial cells, we showed that hyperosmotic and oxidative stresses induce immune cell activation and inflammation as a consequence of assembly of the ROS-induced NLRP3 inflammasome-IL-1β signaling axis.6–8 If the inflammatory response is self-limiting, it can hasten restoration of wound healing through suppressing pathogenic infiltration.8 Even though some of the sequelae subsequent to hypertonicity-induced ROS generation have been characterized, it is unknown whether transient receptor potential melastatin 2 (TRPM2) channels are a biosensor of hyperosmotic and oxidative stress and trigger activation of this signaling axis in human corneal epithelial cells (HCECs).

Numerous studies showed that some other transient receptor potential (TRP) channel isomers serve as biosensors by mediating responses to variations in different environmental conditions.9–11 These Ca2+-permeable nonselective ionic channels are grouped into six different subfamilies designated as TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPA (ankyrin), TRPP (polycystin), and TRPML (mucolipin) based on their amino acid sequence homology.10,11 Some of the TRP isotypes in the TRPV, TRPM, and TRPA subfamilies are thermosensitive, osmosensitive, and mechanosensitive.12 In HCECs, some of the members of the TRPV, TRPC, TRPM, and TRPA subfamilies mediate responses to environmental challenges that affect corneal epithelial layer renewal and function.13–14 TRPV1, TRPV2, TRPM8, and TRPA1 sense noxious heat, moderate cold, and more extreme cold, respectively, in this tissue.14 Furthermore, TRPV1 and TRPV4 act as osmosensors by inducing inflammatory and volume regulatory behavior to anisosmotic challenges, respectively.15,16
TRPM2 is a biosensor of oxidative and osmotic stresses under physiologic and pathophysiologic conditions in the brain, heart, vasculature, and hematopoietic cells. These challenges can increase TRPM2 expression and promote the expression of NF-κB and generate IL-1β and TNF-α through activation of the JNK-MAPK signaling pathway.17 Unlike all other TRP channels, TRPM2 is unique because it is ligand gated by increases in ribosylated adenosine diphosphate (ADPβR) induced by increases in ROS during exposure to a hypertonic stress in numerous tissues. Specifically, this chain of events triggered by a hypertonic stress increases ROS generation and induces DNA breaks. As a consequence, poly(ADPR) polymerase-1 (PARP-1) is hyperactivated causing increases in ADPβR formation.18 ADPβR can also be elicited enzymatically through NAD glycohydrolases such as CD38.19,20 TRPM2 channels possess an enzymatic domain binding ADPβR that ligand gates TRPM2 causing transient rises in intracellular Ca2+ influx and formation of the NLRP3 inflammasome signaling pathway axis.

TRPM2 activation has diverse effects that include inducing cell death by sustaining Ca2+ influx or by enhancing proinflammatory cytokine production, aggravating inflammatory, and tissue injury. On the other hand, its activation can instead be protective by sustaining mitochondrial function and cell survival for a number of different physiologic and pathophysiologic processes.21 TRPM2 has diverse functional roles in different tissues that could be relevant to corneal epithelial physiology. They include (1) inducing some of the responses to TNF-α activation, which are known to underlie inflammatory corneal responses to chemical injury22; (2) activation of NLRP3 inflammasome signaling axis in auto inflammatory and metabolic diseases6,7; (3) inducing epithelial thermosensitive responses to noxious temperatures of 33°C to 38°C, which occur in warm climates; and (4) transducing environmental and endogenous oxidative stresses inducing ROS generation into pathologic pain through Ca2+ signaling, which is one of the second messenger responses induced by corneal injury.23 This agreement between the known responses induced by different mediators in corneal epithelial cells and those occurring in other tissues expressing TRPM2 prompted us to probe for functional TRPM2 expression in primary human corneal epithelial cells (PHCECs). As we recently demonstrated the involvement of the ROS–NLRP3–IL-1β signaling pathway axis mediating proinflammatory responses to hypertonic stress,6,7 we hypothesized that either a hypertonic or oxidative stress activates TRPM2 and induces NLRP3 inflammasome activation, as well as increases in bioactive IL-1β release in PHCECs.

We show here that exposure to either a hypertonic or an oxidative challenge induces TRPM2 activation because either TRPM2 siRNA knockdown of TRPM2 gene and protein expression or inhibition by N-(p-amlycinnamoyl) anthranilic acid (ACA),24 a potent inhibitor of TRPM2 activation, both reduced stress-induced NLRP3 inflammasome activation and bioactive IL-1β release along with increases in caspase-1 expression in PHCECs. These results suggest that TRPM2 is a potential target to offset inflammatory responses induced by a hypertonic and a resulting oxidative stress that is similar to those reported in some patients afflicted with DE disease.25

**Materials and Methods**

**Cell Culture and ACA Treatment**

Human corneoscleral tissues not qualified for clinical use from 20- to 50-year-old donors were obtained from the Wenzhou University Eye Bank in Zhejiang Province, China. PHCECs were cultured from limbal explants using the same method described by Li et al.26 PHCECs were cultured in the supplemented hormonal epidermal medium (SHEM) supplemented with 5% FBS, 10 ng/mL human epidermal growth factor, 5 μg/mL insulin, 50 μg/mL gentamicin, 1.25 μg/mL amphotericin B, 0.5% DMSO, 0.5 μg/mL hydrocortisone, and 30 ng/mL cholera toxin, and the same. 5% CO2 was replaced every other day. PHCECs cultured in isotonic 310 mOsm medium were switched to a hyperosmotic 500 mOsm medium by supplementing the isotonic medium with 90 mM NaCl. Oxidative stress was imposed instead by incubating the PHCECs in the isotonic medium containing 0.5 mM H2O2 for 30 minutes. PHCECs were pretreated for 2 hours with a TRPM2 inhibitor, ACA (Sigma-Aldrich Corp., St. Louis, MO, USA), at a final concentration of 20 μM, in the 500 mOsm medium or isotonic medium containing 0.5 mM H2O2. Cells were collected after 4 hours of hyperosmotic or oxidative stress and stored in a −80°C freezer for subsequent real-time PCR analysis of TRPM2, NLRP3, ASC, caspase-1, and IL-1β gene expression. TRPM2, NLRP3, caspase-1, and IL-1β protein expression levels were measured following 12-hour incubation under the aforementioned conditions with Western blot analysis and immunofluorescent staining.

**Viability Assay**

Cell density was adjusted to 2.0 × 10⁴ cells/mL and seeded into a 96-well plate (100 μL/well). The wells contained either DMSO (i.e., 0, 0.05%, 0.1%, 0.2%, and 0.5%) or ACA (i.e., 0, 10, 20, 40, and 80 μM in 0.1% DMSO). Measurements of each of these conditions were repeated three times in the same plate. Plates were incubated for 24 hours. Ten microliters of the CCK-8 reagent was added to each well 2 hours before the incubation ended. The optical density (OD; at 490 nm) was measured with a microplate reader (Bio-Tek, Inc., Winooski, VT, USA).

**RNA Interference**

PHCECs were transfected at 70% confluence using Lipofect transfection reagent (SignaGen, Rockville, MD, USA), and siRNA targeted against TRPM2 (sense, 5'-GUCUCGGGACAUCUACUTT-3', and antisense, 5'-AGAUAUGAGUUGCCGA GACTT-3'), or a physiologically irrelevant negative control (NC) siRNA (sense, 5'-UUUCGCGAGUUCGACUTT-3', and antisense, 5'-ACUGAGCAAGUCCGAGAAAT-3'). Each dried-down siRNA was dissolved in nuclease-free water to achieve a concentration of 200 nM. Each dried-down siRNA was dissolved in nuclease-free water to achieve a final concentration of 20 nM. Then, 2 μL siRNA (20 nM) and 2 μL Lipofect were added to a 100-μL buffer system. The mixtures were kept at room temperature for 10 to 15 minutes to form complexes, and equal aliquots were then added into one of the wells of a six-well plate. The cultures were incubated at 37°C in a humidified 5% CO2 incubator. The medium was replaced after 24 hours with either the 310- or 500-mOsm medium that did not contain either a siRNA or the transfection reagent. Cells were collected at 48 hours for mRNA or protein expression analyses.

**Real-Time PCR**

The quantity and integrity of the total extracted RNA from the collected cells (RNA lysis buffer RIT; Applied Biosystems, Grand Island, NY, USA) were evaluated with a NanoDrop spectrophotometer. One microgram RNA from each sample was reverse transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Applied Biosystems) in a 25-μL reaction system according to the manufacturer's instructions. The sequences of the primers were: for TRPM2: sense, 5'-CTGTAGTGGATTCC GAAACATCA-3', and antisense, 5'-CGTGGATCCATTAAGTAGGAGT-3'.
TRPM2 Induces NLRP3 Inflammasome in HCECs

**RESULTS**

TRPM-2 Inhibition Suppresses Hyperosmolar-Induced Increases in NLRP3 Inflammasome and IL-1β Expression

Exposure to a hypertonic stress induced increases in ROS generation in rabbit corneal epithelial cells. As this osmotic challenge elicited NLRP3 inflammasome activation and increased bioactive IL-1β release mediated by increases in caspase-1 expression in this tissue, we determined whether inhibition of TRPM2 activation blunts these downstream responses. First, it was necessary to identify a final noncytotoxic DMSO concentration that solubilized ACA, a liposoluble substance. At a DMSO concentration as high as 0.2%, cell viability was not different from that of the control group at concentrations of 10 and 20 μM ACA. However, with both 40 and 80 μM ACA, the cell viability significantly decreased (P < 0.006). Each experiment was performed in triplicate and in triplicate replicates. **P < 0.01.**

**Statistical Analysis**

SPSS 18.0 (IBM Corp., Armonk, NY, USA) evaluated significance, and GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA) was used to generate figures. Significance between two groups was evaluated using nonpaired Student’s t-test. One-way ANOVA with Bonferroni correction was used for comparison between three or more groups. Differences were defined as significant at P < 0.05.
Following exposure to a 500 mOsm medium for 4 hours, the gene expression levels of TRPM2, NLRP3, ASC, caspase-1, and IL-1β rose significantly by 116%, 123%, 37%, 55%, and 66%, respectively, compared with their corresponding isotonic levels (\( P \leq 0.005 \); Fig. 2A). After 12 hours of exposure to this hyperosmotic stress, the TRPM2, NLRP3, caspase-1 (p10), and IL-1β protein expression levels also rose markedly by 90%, 50%, 33%, and 57%, respectively (\( P < 0.043 \)).

**Figure 2.** TRPM2 inhibitor suppresses hyperosmolarity-induced increases in NLRP3 inflammasome and IL-1β expression. (A) A summary showing that hyperosmotic 500 mOsm medium significantly increased the TRPM2, NLRP3, ASC, caspase-1, and IL-1β mRNA levels relative to their corresponding control levels in isotonic 310 mOsm after 4 hours (\( P \leq 0.005 \)). Pretreatment with 20 μM ACA inhibited hyperosmotic-induced TRPM2 activation and suppressed the rises (\( P \leq 0.015 \)) induced by 500 mOsm + 0.1% DMSO group without ACA. Exposure for 12 hours to the 500 mOsm medium provides the following Western blot results shown in B–E. Each panel provides the results of a representative experiment along with a summary histogram plot shown below of a densitometric scan of Western blots. They indicate that the TRPM2, NLRP3, caspase-1 (p10), and IL-1β protein levels also rose significantly (\( P \leq 0.043 \)) relative to their corresponding levels in isotonic 310 mOsm. In contrast, their protein expression levels decreased by 40% (\( P = 0.028 \)), 36% (\( P = 0.044 \)), 23% (\( P = 0.010 \)), and 46% (\( P < 0.001 \)), respectively, in the 500 mOsm + ACA group. The results in A were normalized to β-actin expression levels. Each experiment was repeated three times in triplicate replicates. *\( P < 0.05 \), **\( P < 0.01 \).
FIGURE 3. TRPM2 inhibition suppresses TRPM2, NLRP3, and IL-1β protein expression levels. The individual effects of 20 µM ACA on TRPM-2 (A), NLRP3 (B), and IL-1β (C) protein levels determined by Western blot analysis shown in Figure 2 were validated based on changes in their corresponding immunofluorescent staining intensity. Under the same hyperosmotic conditions indicated in Figure 2 that resulted in increases in gene and protein expression levels, staining intensities rose. In contrast, 20 µM ACA pretreatment dampened all of these increases induced by exposure to a 500 mOsm medium. Each experiment was repeated three times in triplicate replicates.
respective levels in the isotonic medium (Figs. 2B–2E). On the other hand, ACA pretreatment for 2 hours in isotonic medium followed by replacement with the hyperosmotic stress significantly decreased these aforementioned mRNA level rises by 43%, 29%, 25%, 31%, and 51%, respectively, from those in the absence of the drug (i.e., 500 mOsm + DMSO group; *P < 0.015; Fig. 2A). These declines were accompanied by protein expression level decreases of 40%, 36%, 23%, and 46%, respectively (*P < 0.044; Fig. 2B). Immunofluorescent staining validated these aforementioned protein expression level changes induced by the hyperosmolar stress in the presence and absence of ACA because their relative staining intensity changes followed the same trends as the rises in their aforementioned gene and protein expression levels. Consistent with inhibition by ACA of hyperosmotic-induced TRPM2 upregulation, this drug treatment suppressed all of these signaling mediator increases (Fig. 3).

**H₂O₂ Activates TRPM2 and Induces Rises in NLRP3 Inflammasome and IL-1β Expression**

TRPM2 also acts as an oxidative stress sensor by mediating ROS-induced inflammatory responses to tissue injury and infection. H₂O₂ is a convenient ROS source, which upregulated TRPM2, NLRP3, ASC, caspase-1, and IL-1β gene expression levels by 53%, 66%, 55%, 77%, and 41%, respectively, relative to the levels in its absence (*P < 0.013; Fig. 4A). On the other hand, ACA preincubation eliminated these rises (*P < 0.010), because they fell to levels that were similar to those in the control 310 mOsm + DMSO group (*P ≥ 0.482; Fig. 4A). In agreement with these changes in gene expression levels, the
TRPM2, NLRP3, caspase-1 (p10), and IL-1β protein expression levels also increased in the H2O2-treated group relative to those measured in the control group (P ≤ 0.036) and declined in the H2O2 + ACA group (P ≤ 0.029; Figs. 4B–4E).

**TRPM2 siRNA Knockdown Inhibits Hyperosmolar-Induced NLRP3 Inflammasome and IL-1β Expression Level Rises**

TRPM2 siRNA targeted against the coding region of human TRPM2 reduced TRPM2 mRNA and protein expression levels in the hypertonic medium by 70% and 59%, respectively, compared with the corresponding levels in the negative control (siNC) group (Figs. 5A, 5B). Knockdown efficiency was evaluated by using the expression levels in the 500 mOsm medium as a reference because TRPM2 expression levels were much lower in the isotonic medium. Following TRPM2 siRNA knockdown and exposure to the 500 mOsm hypertonic medium, the NLRP3, ASC, caspase-1, and IL-1β mRNA levels were markedly lower than those in the siNC group (P ≤ 0.028) and comparable to those in the 310-mOsm group. The rises in all of these gene expression levels in the siNC-transfected group were indistinguishable from those in the nontransfected control 500-mOsm group (Fig. 5A). In agreement with these changes in gene expression levels, the TRPM2, NLRP3, caspase-1 (p10), and IL-1β protein expression levels increased significantly in the 500 mOsm group, as well as the 500 mOsm + siNC group, whereas their levels decreased by 56%, 48%, 35%, and 25%, respectively, in the 500 mOsm + TRPM2 siRNA-transfected group from those in the 500 mOsm + siNC group.
FIGURE 6. TRPM2 gene silencing reduces hyperosmotic induced rises in TRPM2, NLRP3, and IL-1β protein expression. Immunofluorescent staining shows that hyperosmotic-induced increases in TRPM2 (A), NLRP3 (B), and IL-1β (C) protein expression levels that were selectively blunted in the 500 mOsm + TRPM2 siRNA group relative to the larger rises in the 500 mOsm + NC siRNA group. Each experiment was repeated three times in triplicate replicates.
primary HCECs. In the current study, TRPM2 is identified as a biosensor of external stresses, it is noteworthy that the staining pattern is not plasma membrane delimited, suggesting a multifunctional role of this channel at the nuclear level.

**Discussion**

We previously showed that the ROS–NLRP3–IL-1β signaling pathway axis has a priming role in environment-induced DE development in both a DE murine model and ocular surface samples of clinical DE patients. Such control is in agreement with other results in which a 500-mOsm stress induced both ROS upregulation and NLRP3 inflammasome activation, as well as increases in bioactive IL-1β secretion in immortalized and primary HCECs. In the current study, TRPM2 is identified as an osmotic and oxidative stress sensor triggering both NLRP3 inflammasome activation and increases in bioactive IL-1β, as well as caspase-1 gene and protein expression levels. TRPM2 involvement is evident since declines in its expression levels had corresponding inhibitory effects on both 500 mOsm and H2O2 stress-induced increases in NLRP3 inflammasome activation, as well as increases in caspase-1 and bioactive IL-1β release. Therefore, TRPM2 along with TRPV1, which is another hyperosmotic sensor, both induce proinflammatory responses that can over a short term hasten restoration of epithelial corneal epithelial function compromised by an environmental stress.

TRPM2 channels are expressed in a broad range of tissues such as the brain, pancreas, spleen, kidney, and a wide range of other cell types, including immunocytes, lymphocytes, neutr-}

Many studies have revealed that a dysfunctional rise in cytoplasmic Ca2+ is the vital intermediate step triggering mitochondrial destabilization, which generates mitochondrial-associated ligands that activate the NLRP3 inflammasome, both in vitro and cryopyrin-associated periodic syndromes (CAPS) patient samples. As the hyperosmotic stress imposed in the current study may be similar to those inducing rises in ROS levels and apoptosis of fibroblasts isolated from keratoconus patients, TRPM2 activation may be involved in triggering these responses. Furthermore, the hypertonic stress we imposed is the same that elevated ROS generation in cultured corneal epithelial cells that are accepted as DE animal models. On the other hand, TRPM2 activation induces Ca2+ signaling in response to endogenous redox oxidative stress and ROS. The widespread expression of this TRP channel subtype accounts for why there is emerging interest in determining its potential as a therapeutic target in pathologic settings accompanied by ROS generation and inflammation. Our results are supportive of the notion that targeting TRPM2 has the potential to identify novel strategies for improving therapeutic management of ocular surface disease.

Numerous studies demonstrated that TRPM2 signaling plays an important role in inducing the pathophysiology conditions underlying immune and inflammatory diseases. TRPM2 contributes to inflammatory and neuropathic pain through aggravating pronociceptive inflammatory responses in mice. In a very recent Alzheimer's disease study, ROS induced DNA damage and rises in ADPR generation, which increased intracellular Ca2+ to a level that induced NLRP3 activation in microglial cells. It was suggested that in type 2 diabetes mellitus, TRPM2 channels are a potential target for alleviating NLRP3 inflammasome activation stemming from hyperglycemia-induced oxidative stress. Our results suggest that the inflammatory conditions associated with some ocular surface diseases in some cases may stem from TRPM2 activation because the osmotic stress we imposed is comparable to the tear film osmolarity of clinical samples obtained from some individuals afflicted with DE disease. Furthermore, in other studies involving different groups of DE patients, their ROS levels were elevated in the ocular surface tissues. This correspondence between TRPM2 activation and inflammatory responses warrants further studies to determine whether suppressing TRPM2 activation with novel techniques improves therapeutic management of inflammation in ocular surface DE disease. One limitation of our study is that we did not validate the functional contribution by TRPM2 activation to mediating responses to hypertonic stress imposed by a desiccating environment in vivo. Such an assessment required using TRPM2 knockout mouse for this purpose. Unfortunately, the sole provider of TRPM2 knockout mice was not responsive to our request to share this resource with us.

Taken together, exposure to a hyperosmotic or oxidative challenge induces TRPM2 upregulation, which in turn induces NLRP3 inflammasome formation and increases in bioactive IL-1β release in HCECs. Therefore, TRPM2 along with previously described TRPV1 channels in this tissue both serve as biosensors of hyperosmotic stress and induce increases in proinflammatory cytokine levels that can be offset by inhibitors of their activity.

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**References**

TRPM2 Induces NLRP3 Inflammasome in HCECs


