

Hyperosmolar Saline Is a Proinflammatory Stress on the Mouse Ocular Surface

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Purpose. To investigate whether hyperosmolar stress stimulates production of inflammatory mediators and activates the mitogen-activated protein kinase (MAPK) signaling pathways, c-jun N-terminal kinases (JNKs), extracellular-regulated kinases (ERKs), and p38 on the mouse ocular surface. **Methods.** 129SvEv/CD-1 mixed mice were treated with a balanced salt solution (BSS) (305 mOsM) or a hyperosmotic saline solution (HOSS) (500 mOsM). Untreated age-matched mice were used as controls. The concentrations of interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) were measured by enzyme-linked immunosorbent assay. Gelatinase activity was determined by in situ zymography. Corneal and conjunctival epithelia were lysed for Western blot with MAPK antibodies or used for semiquantitative reverse transcription and polymerase chain reaction and gene array. **Results.** Compared with age-matched controls and mice treated with BSS, the concentration of IL-1 β in tear fluid washings and the concentrations of IL-1 β and TNF- α and gelatinolytic activity in the corneal and conjunctival epithelia were significantly increased in mice treated with HOSS for 2 days. The expressions of IL-1 β , TNF- α , and matrix metalloproteinase 9 (MMP-9) messenger RNA by the corneal and conjunctival epithelia were also notably stimulated in mice treated with HOSS. The levels of phosphorylated JNK1/2, ERK1/2, and p38 MAPKs in the corneal and conjunctival epithelia were slightly increased in mice treated with BSS, but markedly increased in mice treated with HOSS. **Conclusions.** These results show that the hyperosmolarity stimulates expression and production of IL-1 β , TNF- α , and MMP-9 and activates JNK, ERK, and p38 MAPK signaling pathways on the mouse ocular surface. These findings suggest that hyperosmolar stress, as it may occur in dry eye, promotes ocular surface inflammation.

Key Words: Epithelium—Hyperosmolarity—Inflammation—Mitogen-activated protein kinase—Ocular surface.

There is increasing evidence that osmotic stress, caused by increased extracellular osmolarity, is a highly relevant challenge to normal cell function in a variety of tissues, including human bronchial epithelial cells,^{1,2} peripheral blood mononuclear cells,³ and corneal epithelial cells.⁴ Hyperosmolarity in the tear film may cause pathologic changes in the corneal epithelium, such as increased desquamation, disrupted intercellular connections, blunting and loss of microvillae, disruptions in cell membranes, and cellular swelling with decreased cytoplasmic density.⁵ A hyperosmotic tear film has been observed in eyes with keratoconjunctivitis sicca (KCS), the ocular surface epithelial disease of dry eye, and tear film hyperosmolarity in dry eye has been postulated to be a key factor in the pathogenesis and diagnosis of KCS.⁶

There is increasing evidence that inflammation is a contributing and exacerbating factor in dry eye disease. Inflammation has been observed in neurturin-deficient mice that develop a naturally occurring and permanent dry eye.⁷ Increased levels of inflammatory mediators, including proinflammatory cytokines and chemokines, have been detected in the tear fluid and conjunctival epithelia of patients with KCS.^{8–10} Increased concentration and activity of matrix metalloproteinases (MMPs) were also observed in the tear fluid of patients with dry eye.^{10,11} These enzymes, such as MMP-9, lyse various substrates, including components of the corneal epithelial basement membrane and tight junction proteins, such as ZO-1 and occludin, which maintain corneal epithelial barrier function.^{12–14} The increased MMP-9 activity in KCS may also be responsible, in part, for the increased corneal epithelial desquamation (punctate epithelial erosions) and corneal surface irregularity.^{15,16} The importance of inflammation in the pathogenesis of dry eye disease is underscored by the clinical improvement in irritation symptoms and signs that have been reported with antiinflammatory therapies, such as cyclosporine A, corticosteroids, and doxycycline.¹⁷

The mitogen-activated protein kinase (MAPK) cascades are well conserved cellular signaling pathways that include the c-jun N-terminal kinases (JNKs), extracellular signal regulated kinases (ERKs), and p38 MAPK. Cellular stress (e.g., hyperosmotic saline) and inflammatory cytokines, such as interleukin 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) activate these MAPK signaling pathways in various cell types, including fibroblasts,^{18,19} vascular endothelial cells,²⁰ and epithelial cells.²¹ JNKs are also known as stress-activated protein kinases (SAPKs) for their response to various cellular stressors.^{22–24} The activated kinases initiate a cascade of protein phosphorylation involving multiple other kinases and activate nuclear transcription factors, such as

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NF κ B (nuclear factor kappa B), AP-1 (activating protein 1), and ATF (activating transcription factor),^{25,26} which stimulate expression of inflammatory cytokines (e.g., IL-1 and TNF- α) and MMPs, such as MMP-1, MMP-9, and MMP-13.^{27,28}

The authors hypothesize that hyperosmolar stress induces ocular surface inflammation and that this process may be mediated through MAPK signaling pathways. This study evaluated the effects of topically applied hyperosmotic saline on the production of inflammatory molecules, such as IL-1 β , TNF- α , and MMP-9, and on the activation of the JNK, ERK, and p38 MAPK signaling pathways on the mouse ocular surface.

MATERIALS AND METHODS

Animals

129SvEv/CD-1 mixed white mice aged 6 to 8 weeks were used for this study. All studies were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and complied with the Declaration of Helsinki. The two treated groups of mice received a 1- μ L drop of balanced salt solution (BSS) (305 mOsM) or a hyperosmotic saline solution (HOSS) (500 mOsM) in both eyes, six times per day, for 2 days. The HOSS was prepared by adding sodium chloride to BSS. Untreated age-matched mice were used as controls. Four to six mice were evaluated per group for each experiment. The experiments were performed at least three times to verify the reproducibility of the results.

Tear Fluid Washings

Tear fluid washings were collected by a previously reported method.⁷ Briefly, 1.5 μ L of phosphate-buffered saline containing 0.1% bovine serum albumin was instilled into the conjunctival sac. The tear fluid and buffer were collected with a 1- μ L glass capillary tube (Drummond Scientific Co., Broomhall, PA) by capillary action from the tear meniscus in the lateral canthus. The 1 μ L of tear fluid washing samples from both eyes of each mouse was pooled and stored at -80°C until enzyme-linked immunosorbent assay (ELISA) was performed.

Enzyme-Linked Immunosorbent Assay

The corneal and conjunctival epithelia from three groups of mice (12 eyes per group), including untreated mice and mice treated with BSS or HOSS, were collected and lysed, and the total protein concentration was measured with a Micro BCA protein assay kit (Pierce, Rockford, IL). The concentrations of IL-1 β and TNF- α in the cell lysates and in the tear fluid washings were determined by Quantikine M Murine ELISA kits for IL-1 β and TNF- α (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol.

In Situ Zymography

In situ zymography was performed to localize the gelatinase activity in the cornea and conjunctiva by using a modification of a previously reported method.²⁹ Fresh whole eyes with their eyelids and conjunctiva were embedded in a mixture of 75% (v) OCT compound (Sakura Finetek USA, Inc., Torrance, CA) and 25% (v) Immu-Mount (Thermo-Shandon, Pittsburgh, PA) and then frozen in liquid nitrogen. Ten-micrometer sections were cut using a cryostat (Leica, Wetzlar, Germany) and stored at -80°C until use. Sections were thawed and incubated overnight with reaction buffer

(0.05 mol/L TrisHCl, 0.15 mol/L NaCl, 5 mM CaCl₂, and 0.2 mM NaN₃, pH 7.6), containing 40 $\mu\text{g}/\text{mL}$ FITC (fluorescein isothiocyanate)-labeled DQ gelatin, which was available in a gelatinase-collagenase assay kit (EnzChek, Molecular Probes, Eugene, OR). As a negative control, 50 μM 1,10-phenanthroline, an inhibitor of metalloproteinases, was added to the reaction buffer before the FITC-labeled DQ gelatin was applied to frozen sections. Proteolysis of the FITC-labeled DQ gelatin substrate yields cleaved gelatin-FITC peptides that are fluorescent. The localization of fluorescence indicates the sites of net gelatinolytic activity. After incubation, the sections were washed three times with phosphate-buffered saline for 5 minutes and counterstained with 1 $\mu\text{g}/\text{mL}$ Hoechst 33342 dye (Sigma, St. Louis, MO) in an antifade Gel/Mount (Fisher, Atlanta, GA), and a 22 \times 50 mm cover slip was applied. The gelatinolytic activity was localized by a Nikon Eclipse E400 fluorescent microscope (Garden City, NY). Images were acquired by a Nikon DMX 1200 digital camera.

Western Blot Analysis

The corneal and conjunctival epithelia were collected and pooled from each treatment group, and they were lysed in radio immunoprecipitation assay lysis buffer containing 50 mM TrisHCl, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM EDTA, 0.1% SDS (sodium dodecyl sulfate), and an EDTA-free protease inhibitor cocktail tablet (Roche Applied Science, Indianapolis, IN). The cell extracts were centrifuged at 14,000g for 15 minutes at 4°C , and the supernatants were used. The total protein concentrations of the cell extracts were determined by a Micro BCA protein assay kit (Pierce). The protein samples (50 μg per lane) were mixed with 6 \times SDS reducing sample buffer and boiled for 5 minutes before loading. Proteins were separated by SDS polyacrylamide gel electrophoresis (4%–15% TrisHCl, gradient gels from Bio-Rad, Hercules, CA), and transferred electronically to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat milk in TTBS (Tris buffered saline, 50 mM Tris, pH 7.5, 0.9% sodium chloride, and 0.1% Tween-20) for 1 hour at room temperature and then incubated for 2 hours at room temperature with a 1:1,000 dilution of rabbit antibody against phospho-p38 MAPK (Cell Signaling, Beverly, MA), 1:100 dilution of rabbit antibody against phospho-JNK, or 1:500 dilution of monoclonal antibody against phospho-p44/42 ERK (Santa Cruz Biotechnology, Santa Cruz, CA). After three washings with TTBS, the membranes were incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody goat antirabbit IgG (1:2,000 dilution, Cell Signaling, Beverly, MA), or goat antimouse IgG (1:5,000 dilution, Pierce). After washing the membranes four times, the signals were detected with an ECL advance chemiluminescence reagent (Amersham, Piscataway, NJ), and the images were acquired by a Kodak image station 2000R (Eastman Kodak, New Haven, CT). The membranes were stripped in 62.5 mM Tris HCl (pH 6.8), containing 2% SDS and 100 mM β -mercaptoethanol, at 60°C for 30 minutes. Then, they were reprobed with a 1:100 dilution of a rabbit antibody against JNK (Santa Cruz Biotechnology) or a 1:1,000 dilution of rabbit antibodies against ERK or p38 MAPK (Cell Signaling). These three antibodies detect phosphorylated and nonphosphorylated forms, which represent the total levels of these MAPKs. The signals were detected and captured as described earlier.

TABLE. Mouse primer sequences used for RT-PCR

Gene	Genebank accession	Sense primer	Antisense primer	PCR Product
IL-1 β	M15131	TGAGCTGAAAGCTCTCCACC	CTGATGTACCAGTTGGGGAA	297 bp
TNF- α	M11731	TCAGCTCTTCTCATTCTCTG	TGAAGAGAACCTGGGAGTAG	333 bp
MMP-9	NM_013599	CGACGACGACGAGTTGTG	CTGTGGTGACGGCCGAATAG	128 bp
GAPDH	M32599	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	498 bp

RNA Isolation and Semiquantitative Reverse Transcription and Polymerase Chain Reaction

Total RNA from the corneal and conjunctival epithelia collected and pooled from 10 eyes per group for each experiment was isolated by an acid guanidinium thiocyanate-phenol-chloroform extraction method³⁰ and stored at -80°C until use. Gene expression was analyzed by reverse transcription and polymerase chain reaction (RT-PCR)^{30,31} by using a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as an internal control. In brief, first-strand complementary DNA (cDNA) was synthesized from 0.5 μg of total RNA with MuLV reverse transcriptase. PCR amplification of the first-strand cDNAs was performed with specific primer pairs for murine IL-1 β , TNF- α , MMP-9, and GAPDH messenger RNA (mRNA) (Table 1). Semiquantitative RT-PCR was established by terminating reactions at intervals of 24, 28, 32, 36, and 40 cycles for each primer pair to ensure that the PCR products formed were within the linear portion of the amplification curve.

Gene Array

Gene array analysis was performed using a nonrad-GEArray Q Series kit for mouse inflammatory cytokines and receptors (SuperArray Bioscience Corporation, Frederick, MD), according to the manufacturer's instructions. This array is designed to profile the expression of cytokine and receptor genes associated with an inflammatory response and contains control sequences (PUC18 and blanks as negative controls and β -actin and GAPDH as loading controls). In brief, first-strand cDNA was synthesized from 3 μg of total RNA from the corneal epithelia by reverse transcriptase, amplified, and labeled with biotin-16-dUTP by linear polymerase reaction (LPR) with DNA polymerase. The GE-Array membranes were incubated with prehybridization solution at 60°C for 2 hours and then hybridized with the biotin-labeled cDNA probes overnight at 60°C in a hybridization oven with continuous agitation. After washing and blocking, the biotin-labeled probes on the array membranes were bound to alkaline phosphatase-conjugated streptavidin (AP) and were detected by CDP-Star chemiluminescent substrate. The array images were acquired by a CCD camera in Kodak image station 2000R (Eastman Kodak).

Statistical Analysis

Based on the normality of the data distribution, the *t* test or Mann-Whitney test was used for statistical comparison of assay results between groups. Analysis of variance (ANOVA) was used to determine whether there were significant differences among the three groups. The Tukey post-hoc multiple comparisons test was used for determining significant between-group differences.

RESULTS

The proinflammatory potential of HOSS on the ocular surface of mice was evaluated by measuring the levels of inflammatory mediators that have been found on the ocular surface in human dry eye (IL-1 β , TNF- α , and MMP-9) and activated SAPK signaling pathways on the ocular surface of mice after exposure to HOSS.

IL-1 β Levels in Tear Fluid Washings

Tear fluid washings (pooled from both eyes of each mouse) were collected from 12 mice before (untreated) and after treatment with BSS or HOSS for 2 days. The concentration of the proinflammatory cytokine IL-1 β in these washings was measured by ELISA. The IL-1 β concentration increased from 29.13 ± 9.28 pg/mL before treatment to 43.49 ± 40.23 pg/mL in the BSS-treated group and 169.42 ± 83.92 pg/mL after treatment with HOSS ($n = 12$ in all groups, $P < 0.0001$ by ANOVA) (Fig. 1). Post-hoc analyses showed that the concentration for the HOSS treatment group was significantly higher than that for the untreated control and BSS treated groups ($P < 0.001$).

Production of IL-1 β and TNF- α by the Corneal and Conjunctival Epithelia

Determined by ELISA, the IL-1 β concentration in the corneal epithelia increased from 5.67 ± 1.28 pg/mg total protein in untreated corneal epithelia to 6.08 ± 1.32 pg/mg in corneal epithelia treated with BSS and 8.52 ± 2.27 pg/mg in corneal epithelia treated with HOSS ($n = 12$ in all groups, $P < 0.0004$ by

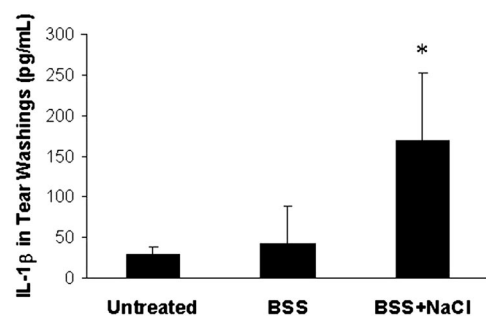


FIG. 1. IL-1 β concentration in tear fluid washings of mice before (untreated) and after treatment with balanced salt solution (BSS) or hyperosmotic saline solution (HOSS) (BSS+NaCl) for 2 days by enzyme-linked immunosorbent assay ($n = 12$ for each group). Data are mean and standard deviation (error bars) of three separate experiments with four mice per group ($*P < 0.0001$ by analysis of variance). Post-hoc analyses with Tukey multiple comparisons test showed that the concentration for the HOSS treatment group was significantly higher than that for the untreated control and BSS treated groups ($P < 0.001$).

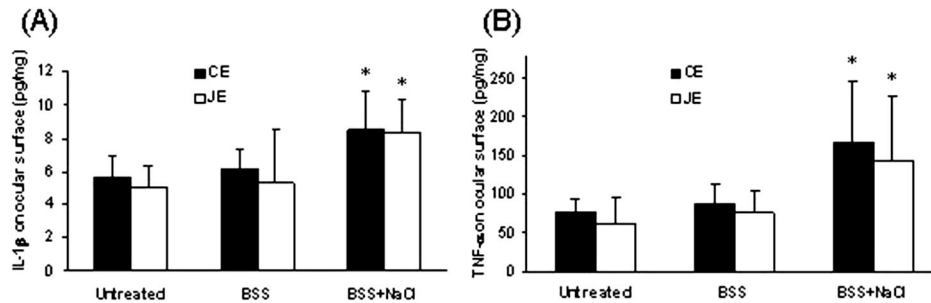


FIG. 2. IL-1 β (A) and TNF- α (B) concentrations in the corneal epithelia (CE) and conjunctival epithelia (JE) of untreated mice (untreated) and mice treated with balanced salt solution (BSS) or hyperosmotic saline solution (HOSS) (BSS+NaCl) measured by enzyme-linked immunosorbent assay. Data show mean and standard deviation (error bars) of three separate experiments with four eyes per group (* $P < 0.01$ by analysis of variance, $P < 0.05$ for HOSS vs. untreated and BSS by post-hoc analysis with Tukey multiple comparisons test).

ANOVA; $P < 0.01$ for HOSS vs. untreated and BSS by post-hoc analysis) (Fig. 2A). The level of IL-1 β in the conjunctival epithelia also increased after osmotic stress, from 5.05 ± 1.27 pg/mg in untreated eyes to 5.29 ± 3.29 pg/mg in eyes treated with BSS and 8.37 ± 2.00 pg/mg in eyes treated with HOSS ($n = 12$, $P < 0.01$ by ANOVA, $P < 0.01$ for HOSS vs. untreated and BSS by post-hoc analysis) (Fig. 2A).

Similar findings were obtained for the concentrations of TNF- α in the corneal and conjunctival epithelia with significant between-group differences noted for each tissue ($P < 0.01$ by ANOVA) (Fig. 2B). The concentration for the HOSS group was significantly higher than that for the BSS and untreated groups in the corneal and conjunctival epithelia ($P < 0.05$, Tukey post-hoc test).

Gelatinolytic Activity on the Ocular Surface Epithelia

In situ zymography showed that mice treated with HOSS for 2 days had higher gelatinolytic activity in the corneal (Fig. 3) and conjunctival epithelia (Fig. 3) compared with untreated mice and mice treated with BSS.

Expression of IL-1 β , TNF- α , and MMP-9 mRNA by the Ocular Surface Epithelia

The levels of RNA transcripts encoding IL-1 β , TNF- α , MMP-9, and the housekeeping gene GAPDH in the corneal or conjunctival epithelia were evaluated by semiquantitative RT-PCR by using pooled total RNA samples obtained from three groups (10 eyes per group) of untreated mice and mice treated with BSS or HOSS for 2 days. The levels of transcripts of IL-1 β , TNF- α , and MMP-9 in the corneal and conjunctival epithelia of mice treated with BSS were similar to untreated control eyes, but they were notably increased in mice treated with HOSS (Fig. 4). As further evidence, gene array analysis was performed by using total RNAs from the corneal epithelia of these three groups of mice. A number of inflammatory cytokines and cytokine receptor transcripts were found to be stimulated when compared with housekeeping genes (β -actin and GAPDH) in the corneal epithelia of the HOSS treated group. Specifically, the expression of IL-1 β , IL-1 receptor 1 (IL-1R1) and receptor 2 (IL-1R2), TNF- α , and TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) mRNA were notably

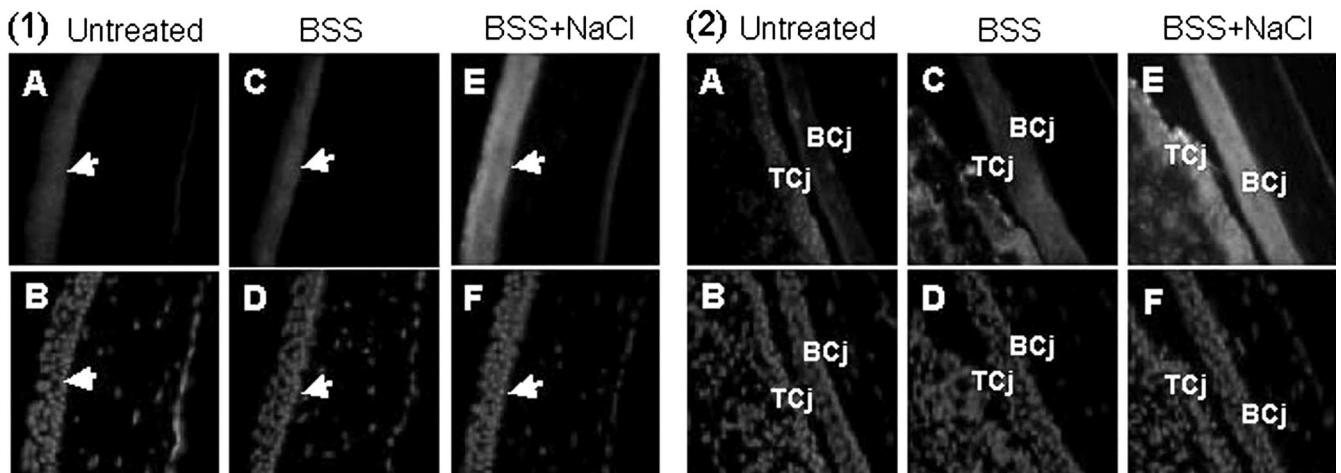


FIG. 3. Representative in situ zymograms showing gelatinolytic activity in the corneal epithelia (1) and conjunctival epithelia (2) of untreated mice (A) and mice treated with balanced salt solution (BSS) (C) or hyperosmotic saline solution (BSS+NaCl) (E) with counterstaining by Hoechst 33342 dye (B, D, and F, respectively). Arrows show the corneal epithelium. BCj, bulbar conjunctiva; TCj, tarsal conjunctiva (original magnification, $\times 400$).

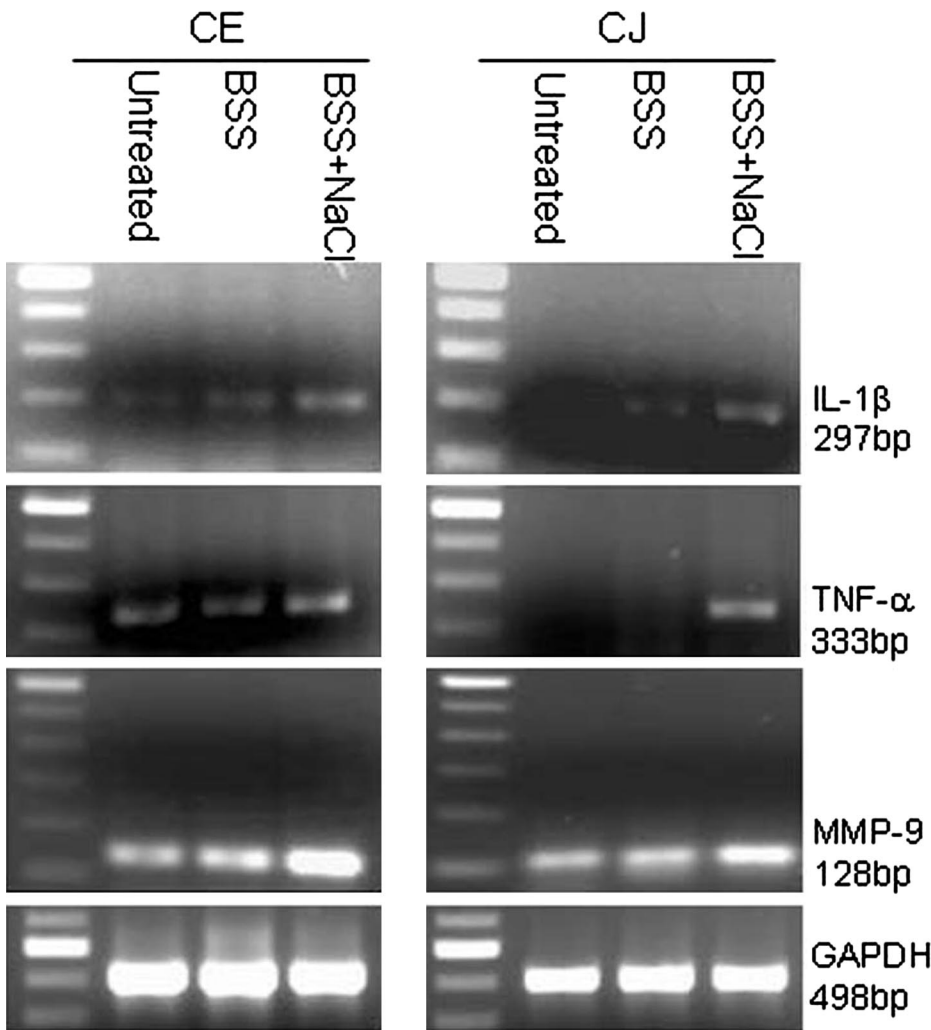


FIG. 4. Representative semiquantitative reverse transcription and polymerase chain reaction profiles showing the expression of IL-1 β , TNF- α , and MMP-9 mRNA by corneal epithelia (CJ) and conjunctival epithelia (JE) in age-matched untreated mice and mice treated with balanced salt solution (BSS) or hyperosmotic saline solution (BSS+NaCl) by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messenger RNA as an internal control.

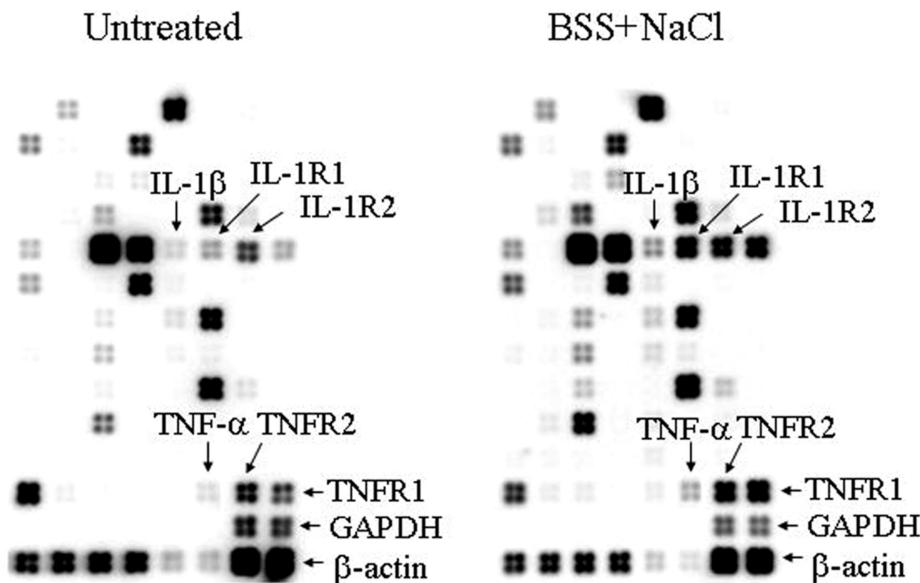


FIG. 5. Representative gene arrays for mouse inflammatory cytokines and receptors showing levels of IL-1 β , IL-1R1, IL-1R2, TNF- α , TNFR1, and TNFR2 messenger RNA transcripts by corneal epithelia in untreated mice and mice treated with hyperosmotic saline solution (BSS+NaCl). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β -actin mRNA was used as internal controls.

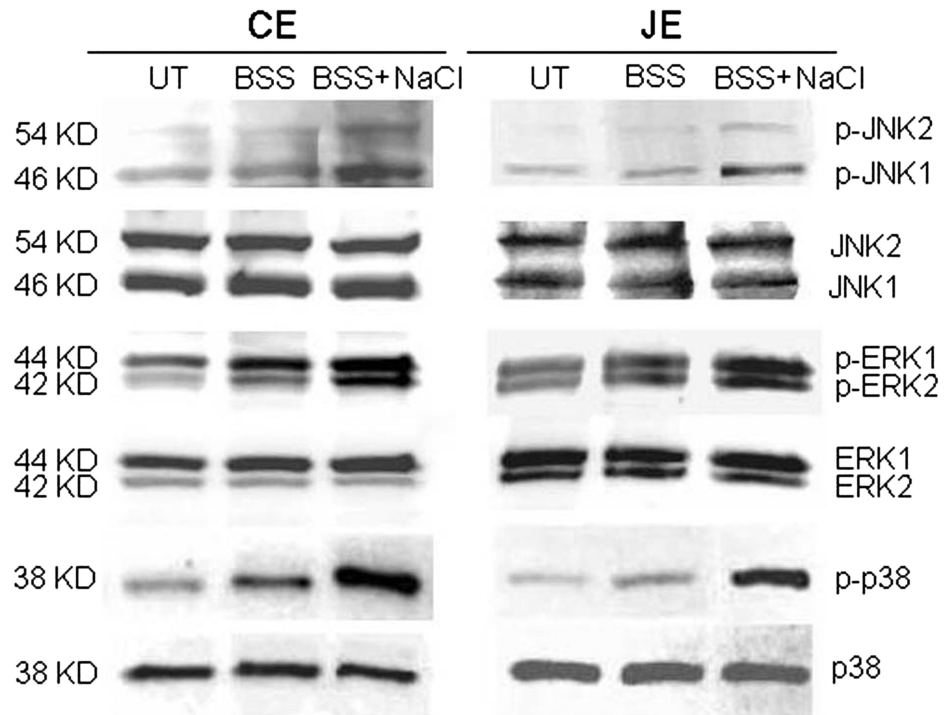


FIG. 6. Representative Western blots showing the levels of phospho-JNKs (p-JNK1 and p-JNK2), total JNKs (JNK1 and JNK2), phospho-ERKs (p-ERK1, p-ERK2), total ERKs (ERK1 and ERK2), phospho-p38 (p-p38), and total p38 (p38) in the corneal epithelia (CE) and conjunctival epithelia (JE) from untreated mice (UT) and mice treated with balanced salt solution (BSS) or hyperosmotic saline solution (BSS+NaCl).

increased. Figure 5 shows the entire array of these inflammatory cytokines and their receptors in untreated mice and mice treated with HOSS as an example.

Activation of JNK, ERK, and p38 MAP Kinases in the Ocular Surface Epithelia

The activation of the JNK, ERK, and p38 MAP kinase pathways was evaluated by immunoblotting with phospho-specific antibodies against the activated forms of these kinases by using pooled extracts of corneal or conjunctival epithelia from the three treatment groups (8 eyes per group). As shown in Figure 6, the levels of phosphorylated (p)-JNK1, p-JNK2, p-ERK1, p-ERK2, and p-p38 in the corneal and conjunctival epithelia were slightly increased in the BSS-treated mice than in the untreated mice; however, they were markedly increased in mice treated with HOSS compared to the other groups. In contrast, levels of total JNK1/2, ERK1/2, and p38 did not change. These findings show that multiple MAPK signaling pathways are activated on the ocular surface by the stress of HOSS.

DISCUSSION

This study presented evidence that hyperosmotic stress stimulates production of inflammatory factors (IL-1 β , TNF- α , and MMP-9) and activates MAPK signaling pathways in the mouse ocular surface epithelia. These findings mimic the proinflammatory effects of experimental dry eye on the ocular surface. HOSS appears to induce more rapid production of IL-1 β and TNF- α by the cornea and conjunctiva. The concentrations of these cytokines were not observed to be significantly increased above baseline concentrations until 5 and 10 days of experimental dry eye.

Hyperosmolarity Induces Ocular Surface Inflammation

Hyperosmolarity has been reported to significantly increase production of inflammatory cytokine IL-1 and chemokine IL-8 in human peripheral blood leukocytes³ and bronchial epithelial cells.¹ The current study provided convincing evidence that ocular surface inflammation develops in response to hyperosmotic stress in mice. The concentration of IL-1 β in the tear fluid washings and gelatinolytic activity on the ocular surface epithelia were observed to significantly increase in mice treated with HOSS (Figs. 1 and 3). Because 1.5 μ L of phosphate-buffered saline was instilled into the tear fluid before collecting the tear fluid washings, the real concentration of IL-1 β in the tear fluid should be much higher than what was measured in the tear fluid washings. The increased IL-1 β concentration that was detected in the tear fluid washings could come from lacrimal glands or the stressed ocular surface epithelia. The concentrations of IL-1 β and TNF- α significantly increased in the corneal and conjunctival epithelia of mice treated with HOSS (Fig. 2). In addition, semiquantitative RT-PCR showed that the levels of IL-1 β , TNF- α , and MMP-9 mRNA in the corneal and conjunctival epithelia noticeably increased in mice treated with HOSS, compared with untreated mice and mice treated with BSS (Fig. 4). The gene array data provide further support that hyperosmolarity induces inflammation at the transcriptional levels by showing increased expression of inflammatory cytokines (IL-1 β and TNF- α) and their receptors (IL-1R1, IL-1R2, TNFR1, and TNFR2) in the corneal epithelia of mice treated with HOSS (Fig. 5).

Interleukin 1 is a potent inducer of other inflammatory cytokines, such as IL-6 and TNF- α , and chemokines, such as IL-8.³² In mice, IL-1 and TNF- α stimulate the production of key chemoattractants, KC (chemokine) and MIP-2 (microphage inflammatory protein-2).^{33,34} IL-1 and TNF- α also stimulate the production of

MMPs by epithelial and inflammatory cells.^{31,35} The gelatinase MMP-9 is one of the most important MMPs on the ocular surface. The concentration and activity of MMP-9 was found to be significantly higher in the tear fluid of patients with KCS, with the highest levels observed in patients who developed sterile ulceration.¹¹ IL-1 and MMP-9 produced by the ocular surface epithelial cells may mediate initial events in the inflammatory cascade of dry eye through a dynamic interplay between them.³⁶ This study used multiple methods to show conclusively that hyperosmotic stress induces the production of these key inflammatory factors on the ocular surface. The findings implicate hyperosmotic stress as one of the initiating stimuli in the pathogenesis of KCS.

Hyperosmotic Tear Film Activates MAPK Signaling Pathways

Mammalian cells respond to environmental stress, such as hyperosmolarity, by activating a variety of protein kinases that are critical for cellular signal transmission, such as the epidermal growth factor receptor, tyrosine kinase, and different members of the MAPK family.³⁷ MAPKs are important cell signaling mediators that play vital roles in cellular responses to stress. ERK MAPK is strongly activated by growth factors, such as platelet-derived growth factor, and many other stimuli that mediate cell proliferation, differentiation, and survival.³⁸ In contrast, the JNK and p38 MAPK cascades are strongly activated by cellular stresses and by proinflammatory agents, such as endotoxin, IL-1, and TNF- α .³⁹⁻⁴¹ Each MAPK pathway is activated by phosphorylation of threonine and tyrosine residues by upstream dual-specificity MAPK kinases (MKKs). ERK is activated by MKK1 and MKK2, p38 by MKK3 and MKK6, and JNK by MKK4 and MKK7. The mechanism of activation and the functional role of each MAPK cascade depend on the particular cell type and the type of stimuli used.⁴² JNK activation has been previously reported in osmotically shocked Chinese hamster ovary cells.²³ In this study, the activated forms of *p*-JNK1/2, *p*-ERK1/2, and *p*-p38 MAP kinases were markedly increased in the corneal and conjunctival epithelia of mice treated with HOSS (Fig. 6).

MAPK signaling pathways have been shown to play a central role in regulating a wide range of inflammatory responses in many different cell types. Cross-talk between ERK and p38 MAPK mediates selective suppression of proinflammatory cytokine production by TGF- β .⁴³ Activation of JNK/c-Jun and ERK1/2 MAPK signal transduction pathways leads to activation of murine peritoneal macrophages.⁴⁴ p38 MAPK activity mediates TNF- α and MIP-2 release⁴⁵ and appears to be involved in the stimulated release of IL-1 β and the sustained neutrophilic response in rat airway inflammation.⁴⁶ In addition, MAPK cascade signaling pathways are known to regulate production and activity of MMPs through activation of transcription factors, such as NF κ B, AP-1, and ATF, in different target cells.^{27,47-49} These findings suggest that the three activated MAPK pathways could mediate the production of proinflammatory cytokines and MMP-9 by stressed ocular surface epithelia in dry eye.

The mechanism of activation of MAPKs in mice treated with HOSS is still unknown. The JNK, ERK, or p38 MAPK signaling pathways have been reported to be activated by inflammatory cytokines, such as IL-1 β and TNF- α ,^{39,41,42} which are also known to stimulate expression and activity of MMPs in various cell types.^{27,28,31} In a preliminary study, it was found that hyperosmotic media stimulate the expression of the inflammatory cyto-

kines (IL-1 β and TNF- α) and MMP-9 by a JNK pathway in cultured human corneal epithelial cells.⁵⁰ Therefore, ocular surface stresses in dry eye, such as hyperosmolarity, could activate MAPK cellular stress pathways³⁶ and induce the production of inflammatory factors, which in turn also trigger MAPK activation and stimulate the expression of MMP-9. Further studies are required to define the relationship between MAPK activation and stimulated production of IL-1 β and TNF- α on the mouse ocular surface.

In conclusion, the findings of the current study have shown that hyperosmotic stress stimulates ocular surface inflammation and activates three MAPK intracellular signaling pathways: JNKs, ERKs, and p38. These findings may establish a link between the hyperosmolar tear film of dry eye and the induction of ocular surface inflammation in KCS.

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