NFAT5 Induction and Its Role in Hyperosmolar Stressed Human Limbal Epithelial Cells

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Purpose. To introduce a tonicity response gene regulator, NFAT (nuclear factor of activated T-cell)-5 and determine its expression mechanism and specific roles in human limbal epithelial cell (HLECs) subjected to hyperosmolar stress.

Methods. NFAT5 expression was determined in various hyperosmolar conditions in HLECs by RT-PCR and Western immunoblot analyses. NFAT5 translocation during hyperosmolar stress was observed by immunocytochemistry. NFAT5-related signal transduction activity was measured on the basis of inhibition of NF-κB (nuclear factor-κB), and MAPK activity. TNF-α and IL-1β, -6, and -8 levels were determined after inhibition of NFAT5 and/or NF-κB. Hyperosmotic apoptotic cell death, with or without inhibition of NFAT5, was measured by flow cytometry.

Results. NFAT5 was induced and translocated to the nucleus under conditions of hyperosmolar stress. It was inhibited by SB239063, a p38 MAPK inhibitor. Among the inflammatory cytokines induced in hyperosmolar stress conditions, IL-1β and TNF-α levels were significantly reduced after inhibition of NFAT5. Of interest, even after 48 hours of hyperosmolar stress, 45% of HLECs survived. HLEC apoptosis increased markedly as a result of NFAT5 suppression. Moreover, most of the HLECs underwent cell death on dual inhibition of NF-κB and NFAT5.

Conclusions. NFAT5 is induced and translocates to the nucleus in HLECs undergoing hyperosmolar stress through activation of p38. IL-1 β and TNF-α are induced via NFAT5 activation. Our data collectively indicate that NFAT5 may be an important gene regulator and survival factor in hyperosmolar stressed HLECs. (*Invest Ophthalmol Vis Sci.* 2008;49:1827–1835) DOI: 10.1167/iovs.07-1142

Osmotic stress is one of the common stresses on the kidney, skin, respiratory track, and eye and is an important mechanism of tissue damage in those organs.[1–4] On the ocular surface, hyperosmolarity leads to a significant increase in the production of proinflammatory cytokines, such as interleukin (IL)-1, TNF-α, IL-8,[4] and matrix metalloproteinase (MMP)-9, -1, -13, and -3,[5] and induces pathologic changes in the corneal epithelium.[6] However, exactly how cells detect these hyperosmotic conditions and how they respond to the stimuli remains unresolved. Li et al.[5] reported that MMP stimulation by hyperosmolar stress is mediated by activation of JNK. Also, MAPK activation by hyperosmolarity induces HLEC apoptosis.[7] However, activator protein (AP)-1 or transcription factors of the rel family (e.g., NF-κB, NFAT1, NFAT5) in hyperosmolar stress has not been studied in HLECs.

The NFAT (nuclear factor of activated T-cells) transcription factor family contains five members: NFAT1 (NFATp), -2 (NFATc), -3, -4, (NFATx), and NFAT5/TonEBP (tonicity response element-binding protein).[8–10] These proteins are identified as transcriptional regulators of cytokine gene expression and play important roles in modulating T-cell differentiation, B-cell activation via antigen receptors, and calcium flux.[11,12] Genes encoding NFAT proteins are transcribed in almost every tissue, although the expression of individual proteins is fairly tissue-restricted.[13,14] NFAT1, -2, and -4 are mainly expressed in immune cells, and their roles in immune responses are well characterized.[11,12] NFAT5, a member of the rel/NF-B/NFAT family, was originally identified as a transcription factor involved in cellular responses to hypertonic stress.[8,9] NFAT1 to -4 proteins are activated by the Ca^{2+}/calcineurin pathway. In contrast, NFAT5 activity is regulated in a calcineurin-independent manner, because it lacks the N-terminal homology region containing the regulatory motif.[8,15] NFAT5 is activated by phosphorylation under a hyperosmotic environment.[15] The protein transcriptionally regulates the expression of target genes responsible for the metabolism of organic osmolytes, including aldose reductase,[10] taurine transporter (TauT),[16] betaine/GABA transporter (BGT-1),[17] and sodium/myo-inositol transporter.[18,19] NFAT5 also induces molecular chaperones, such as heat shock protein 70-2 (Hsp 70-2),[20] and 94-kDa osmotic stress protein (Osp94).[21] Inhibition of NFAT5 leads to increased susceptibility to hypertonic stress in immune[17] and renal cells.[22]

The causes of keratoconjunctivitis sicca (KCS) are multifactorial and can be related to deficiencies in any one of the components of the ocular surface and tear film, adequate hormonal, and neuronal regulatory mechanisms.[4] As a consequence, the ocular surface is susceptible to the excessive tear evaporation or decreased tear secretion that result in a hyperosmolar tear film. Considering the evaporative nature of water on the ocular surface and the mechanism of KCS, hyperosmotic stress may be a common and important factor in the damage to the surface epithelium of the cornea and conjunctiva. However, to our knowledge, there are no documented reports on NFAT5 in ocular tissue. In this study, we investigated whether NFAT5 is activated by hyperosmolar stress in cultured corneal epithelial cells, and determined its role in the induction of proinflammatory cytokines and stress-induced apoptosis in human limbal epithelial cells (HLECs).

Materials and Methods

Reagents and Antibodies

The NFAT5 antibody for immunoblot analysis and cytostaining was purchased from Abcam (Cambridge, UK). Dispase II was acquired from

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Supported by faculty research grant 6-2006-0043 from the Yonsei University Research Fund.

Submitted for publication September 2, 2007; revised November 13 and December 4, 2007; accepted March 3, 2008.

Disclosure: **J.H. Lee**, None; **M. Kim**, None; **Y.S. Im**, None; **W. Choi**, None; **S.H. Byeon**, None; **H.K. Lee**, None

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Roche Applied Science (Mannheim, Germany). Cholera toxin (subunit A), DMSO, hydrocortisone, transferrin, SP600125, SB239063, U0153, and human insulin were obtained from Sigma-Aldrich (Poole, UK). Human interleukin (IL)-1β, -6, and -8 and tumor necrosis factor (TNF)-α were from R&D Systems (Minneapolis, MN). Activated human caspase-3, the tyrosine kinase inhibitor K252a, IκB kinase inhibitor peptide (IKKI), the NF-κB inhibitor SN50, and its inactive control peptide SN50M, were from Calbiochem (San Diego, CA). Anti-phospho-ERK (Thr202/Tyr204), anti-human RelA/p65, phospho-RelA/p65 of NF-κB (Ser^{536}), MG132, and LY294002 were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-JNK (SAPK; Thr183/Tyr185) and anti-human p38/SAPK2 monoclonal antibodies (clone 2F11) were acquired from Biosource International (Camarillo, CA).

Human Limbal Epithelial Cell Culture and Hyperosmotic Stimulation

Human corneal limbal tissue was harvested from donor corneal buttons after keratoplasty, in accordance with the tenets of the Declaration of Helsinki and with the permission of the institutional review board. Corneal limbal tissues were prepared and cultured, using a published method.[23] For hyperosmolar stress, the cells were cultured for an additional period (6–48 hours) in equal volumes of serum-free medium ranging from 305 to 500 mOsM, achieved by adding 0, 30, 50, 70, or 90 mM sodium chloride (NaCl). The actual osmolarity of the medium was confirmed with an osmometer (Model 3320; Advanced Instrument, Inc., Norwood, MA).

Semiquantitative RT-PCR

RNA isolation and semiquantitative RT-PCR were performed according to a previous report.[23] Primer sequences specific for IL-1β, -6, and -8; TNF-α; and NFAT5 were designed from the published human gene sequences (Table 1).

Western Immunoblot Analysis

For Western blot analysis, adherent cells were washed with ice-cold PBS, and lysed with cell lysis buffer (20 mM HEPES [pH 7.2], 10% glycerol, 10 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% Triton X-100) on ice for 30 minutes. The lysates were sonicated, the cell homogenates centrifuged at 15,000*g* for 10 minutes at 4°C, and the supernatant fractions stored at −70°C before use.

Protein concentrations in the resultant supernatant fractions were determined by using the Bradford reagent. Equal amounts of protein (30 μg) were boiled in Laemmli sample buffer and resolved by 8% SDS-PAGE. The proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore, Billerica, MA) and probed overnight with the primary antibodies NFAT5 (dilution, 1:2000), Erk (1:3000), JNK (1:3000), p38 (1:1500), RelA (1:1000), pRelA (1:800), IL-1β (1:4000), TNF-α (1:3000), IL-6 (1:2000), and IL-8 (1:2000). Immunoreactive bands were detected with horseradish peroxidase–conjugated secondary antibodies and visualized by enhanced chemiluminescence. For analysis of NF-κB translocation, the cells were subjected to hyperosmolarity at various time points, and nuclear fractions prepared using a nuclear extraction kit (Panomics, Fremont, CA), according to the manufacturer's instructions. The protein content in the nuclear fractions was measured by using the BCA assay, and equal amounts were immunoblotted with anti-phospho-NF-κB p65 (Ser^{536}) (Cell Signaling Technology).

NF-κB, AP-1, and NFAT-1c Reporter Assays

HLECs were plated in six-well plates at a concentration of 5×10^5 cells/well, and transfected with 4 μg of NF-κB-, AP1-, or NFAT-1-promoter luciferase reporter vector using 10 μL transfection reagent (Lipofectamine 2000; Invitrogen, Carlsbad CA) in DMEM. Luciferase constructs for AP-1, NF-κB, and NFAT1 were kindly provided by Hong-Hee Kim (Seoul National University, Seoul, Korea). After 4 hours, the medium was replaced with DMEM containing 10% FBS and incubated for 14 hours at 37°C in a CO_2 incubator. The cells were collected by scraping, resuspended in DMEM/10% FBS, and replated in 48-well plates at a concentration of 1×10^5 cells/well. Next, the cells were washed three times with PBS, and the medium was altered to serum-free DMEM. After exposure to hyperosmolar stress for 24 hours, the cells were lysed in lysis buffer (Glo; Promega, Madison, WI), and luciferase activity was measured with a luminometer.

Immunocytochemistry

The cells were fixed for 5 minutes in 3.7% formaldehyde and permeabilized in 0.5% Triton X-100 for 8 minutes. Single- or double-labeled immunofluorescence analysis was performed. As controls, samples were run without primary antibody or with irrelevant IgG to ascertain nonspecific binding of secondary antibodies. For experiments, the samples were incubated with RelA p65 or NFAT5 antibody for 2 hours at room temperature, followed by 1 hour with FITC-conjugated secondary antibodies. After they were rinsed with PBS, the samples were examined by confocal microscope (TSE SPE; Leica Microsystems, Wetzlar, Germany).

cDNA Gene Array Analysis

To determine NFAT5 regulatory genes, a gene microarray (GEArray; SuperArray Inc., Bethesda, MD) was used to determine the regulation of gene expression corresponding to 96 genes involved in the inflammatory cytokines and receptors (OHS-11; for a gene table see http://www.superarray.com). Confluent HLECs were treated with hyperosmolarity for 24 hours, with or without transfection of small interfering (si)NFAT5 or control siRNA before hyperosmolar medium. Then, the cells were collected and RNA was isolated from the cell by a modified GITC (guanidine isothiocyanate) technique. Briefly, 5 μg of total RNA was reverse transcribed into biotin-16-dUTP–labeled cDNA probes using a commercial method (AmpoLabling-LPR; SuperArray Inc.), according to the manufacturer's protocol. The microarray membranes were prehybridized at 60°C for 1 to 2 hours. Hybridization was performed by incubation of the membranes with biotin-labeled cDNA probes at 60°C overnight. The hybridized membranes were washed twice in 2× SSC with 1% SDS and then in 0.1× SSC with 0.5% SDS, further incubated with alkaline phosphatase–conjugated streptavidin, and finally developed with chemiluminescent substrate (CDP-Star; Sigma-Aldrich). The x-ray films and the spots were then digitized

TABLE 1. RT-PCR Primer Designs

Gene	Forward	Reverse	BP*	Ref.
IL-1b	TGAACTGAAAGCTCTCCACC	CTGATGTACCAGTTGGGGAA	297	4
TNF-a	TCAGCCTCTTCTCCTTCCTG	TGAAGAGGACCTGGGAGTAG	324	4
IL-6	CCTTCTCCACAAGCGCCTTC	GGCAAGTCTCCTCATTGAATC	327	24
IL-8	ATGACTTCCAAGCTGGCCGT	TGTGGTCCCTCTCAATCACTC	179	4
NFAT5	AATCGCCCAAGTCCCTCTAC	GGTGGTAAAGGAGCTGCAAG	211	25
GADPH	GCCAAGGTCATCCATGACAAC	GTCCACCACCCTGTTGCTGTA	511	23

Ref., reference source.

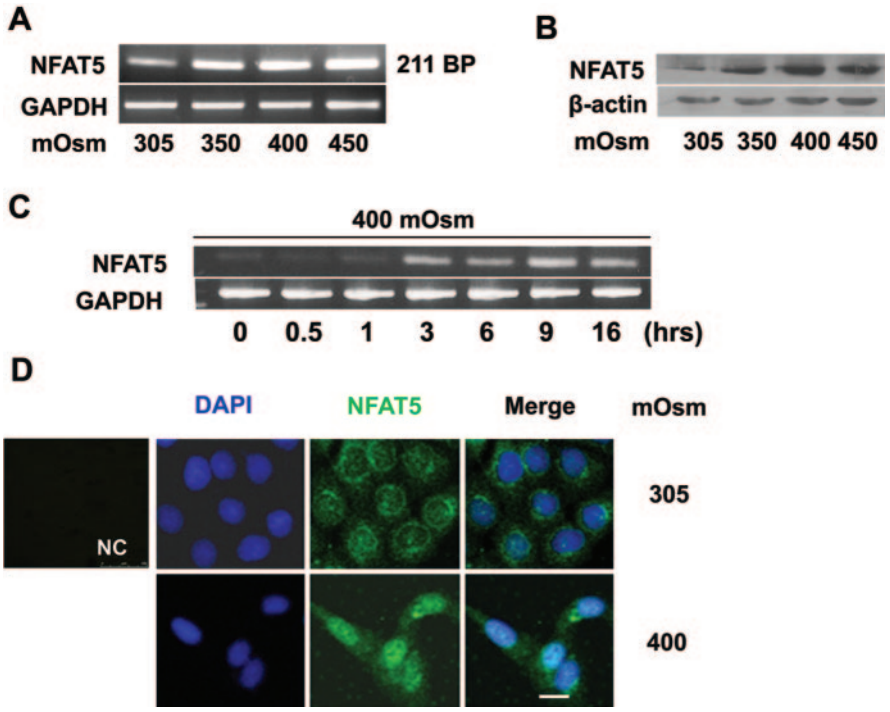


FIGURE 1. NFAT5 induction by hyperosmotic stress. Expression activity of NFAT5 was determined in HLECs in various osmotic conditions, by RT-PCR (**A**) at 12 hours and immunoblot (**B**) at 24 hours. (**C**) Transcription activity of NFAT5 was determined in HLECs at 400 mOsM in a time-dependent manner. (**D**) NFAT5 translocation was determined using immunocytochemistry. The cells were exposed to 400 mOsM for 3 hours, fixed, and incubated with the NFAT5 antibody for 14 hours. Images were obtained with a confocal microscope. *Green*: NFAT5, *blue*: DAPI. NC; primary antibody–depleted negative control.

(ScanAnalyze software (Eisen Lab, Berkeley, CA). The relative expression level of each gene was determined by comparing the signal intensity of each gene in the array after normalization to the signal of the housekeeping gene.

Apoptosis Analysis

The cells were washed with PBS, and incubated in serum-free DMEM under conditions of hyperosmolar stress or with siNFAT5 for various intervals until 48 hours. For apoptosis analysis, the cells were washed and incubated for 15 minutes at room temperature in the presence of annexin V labeled with FITC and propidium iodide. In total, 10,000 cells were excited at 488 nm, and emission measured at 530 and 585 nm for FITC and PI fluorescence, respectively. The cells were analyzed on a flow cytometer (FACScan; BD Biosciences, Franklin Lakes, NJ).

Statistical Analysis

Based on a normal distribution of the data, the Student's *t*-test or Mann-Whitney rank sum test were applied for statistical comparison of assay results between groups. ANOVA was used when more than one comparison was made. $P < 0.01$ was considered statistically significant. All the data were yielded from three independent experiments.

RESULTS

Effect of Hyperosmotic Stress on NFAT5

At 12 hours after treatment with various concentrations of NaCl to induce hyperosmolarity, NFAT5 expression was elevated in the HLECs. Moreover, even under relatively weaker hyperosmotic conditions (350 mOsM), NFAT5 induction was evident (Figs. 1A, B). At 400 mOsM, NFAT5 mRNA expression was induced in HLECs from 3 hours after treatment (Fig. 1C). Under hyperosmotic conditions, both elevated production and translocation of NFAT5 from the cytosol to nucleus were observed in HLECs (Fig. 1D).

Activities of AP1, NFAT1, NF-κB, and MAPKs during Hyperosmolar Stress

The data clearly indicate that NFAT5 is induced in hyperosmolar conditions and translocates to the nucleus. To determine

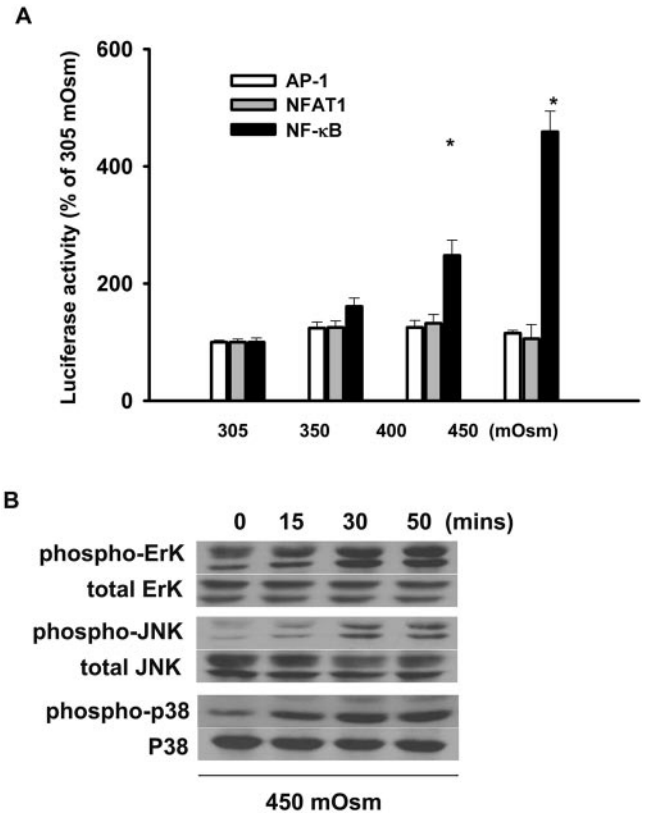


FIGURE 2. Activity of AP1, NFAT1, NF-κB, and MAPKs under hyperosmolar stress conditions. (**A**) After transduction of luciferase constructs of AP-1, NFAT1, or NF-κB, the HLECs were incubated in various hyperosmolar conditions for 24 hours. The transcription activity of each construct was determined via luciferase activity measured with a luminometer. Data are the mean ± SD of results in 12 wells from three independent experiments. (**B**) Phosphorylation of Erk, JNK, and p38 in 450 mOsM was measured at various time points by immunoblot analysis. $^*P < 0.001$: compared with the 305-mOsM condition.

the responses of other inflammatory regulators to hyperosmotic conditions and delineate their relationship with NFAT5, we additionally measured the activities of AP-1, NF-κB, NFAT1, and MAPK during hyperosmolar stress. NF-κB activity was elevated by hyperosmolar stress as confirmed with experiments using luciferase constructs (Fig. 2A). However, AP1 and NFAT1 activities were not elevated at 450 mOsM until 24 hours. After 24 hours of exposure to hyperosmolar stress, NF-κB activity was increased to more than four times that of the resting basal activity. We also observed increased phosphorylation of all three MAPK types within 30 minutes at 450 mOsM hyperosmolar stress (Fig. 2B).

Suppression of NFAT5 Induction and Translocation by p38 MAPK Inhibitors

Because all three types of MAPKs were induced by hyperosmolar stress, we investigated the relationship between NFAT5 and MAPK proteins. Pretreatment with 20 μm U0125, or SP600125 (Erk or JNK inhibitors) before hyperosmolar stress did not affect hyperosmolarity-induced NFAT5 production (Fig. 3A). However, after pretreatment with 10 μM SB239063, a p38 MAPK inhibitor, NFAT5 expression was completely inhibited (Figs. 3A, 3B). Moreover, we found that NFAT5 induction was inhibited by SB239063 in a dose-dependent manner (Fig. 3B), confirming that this system is regulated by p38 MAPK. For specific inhibition of NFAT5 and for use as a negative control, siRNA for NFAT5 (5′-UAA GAU UUC UGG CAC UCC UGC UGG C-3′ and 5′-GCC AGC AGG AGU GCC AGA AAU CUU A-3′) was designed. Also, a negative control siRNA was used (Stealth RNAi Negative Control Duplex Medium GC; Invitrogen). The inhibitory activity of the designed siNFAT5 was confirmed in HLECs (Fig. 3C).

LY294002 or MG132, identified as inhibitors of NFAT5 in other cell types,[13] did not suppress NFAT5 activity in HLECs (Fig. 3D). To determine the effect of NFAT5 translocation by the above inhibitors, we investigated NFAT5 translocation through confocal microscopy. Nuclear translocation of NFAT5 was inhibited only pretreatment of SB239063. However the translocation of NFAT5 did not changed in 10 μM of either MG132 or LY294002 (Fig. 3E).

Determination of the Relationship between NF-κB and NFAT5 in Hyperosmolar Conditions

The transcriptional activities of both NF-κB and NFAT5 increased under hyperosmolar conditions. We attempted to determine whether there was any correlation between the two regulators in hyperosmolarity-induced signal transduction. The phosphorylated Rel A (p65) component of NF-κB was increased in the nuclei of hyperosmolar stressed HLECs (Fig. 4A). From 5 minutes after subjection to 450 mOsM, phosphorylated p65 RelA was observed in the nucleus, which continued for 30 minutes. However, nuclear translocation and transcription activity (data not shown) of p65 RelA were not affected by transfection of siNFAT5, and vice versa. Specifically, hyperosmolarity-induced NFAT5 expres-

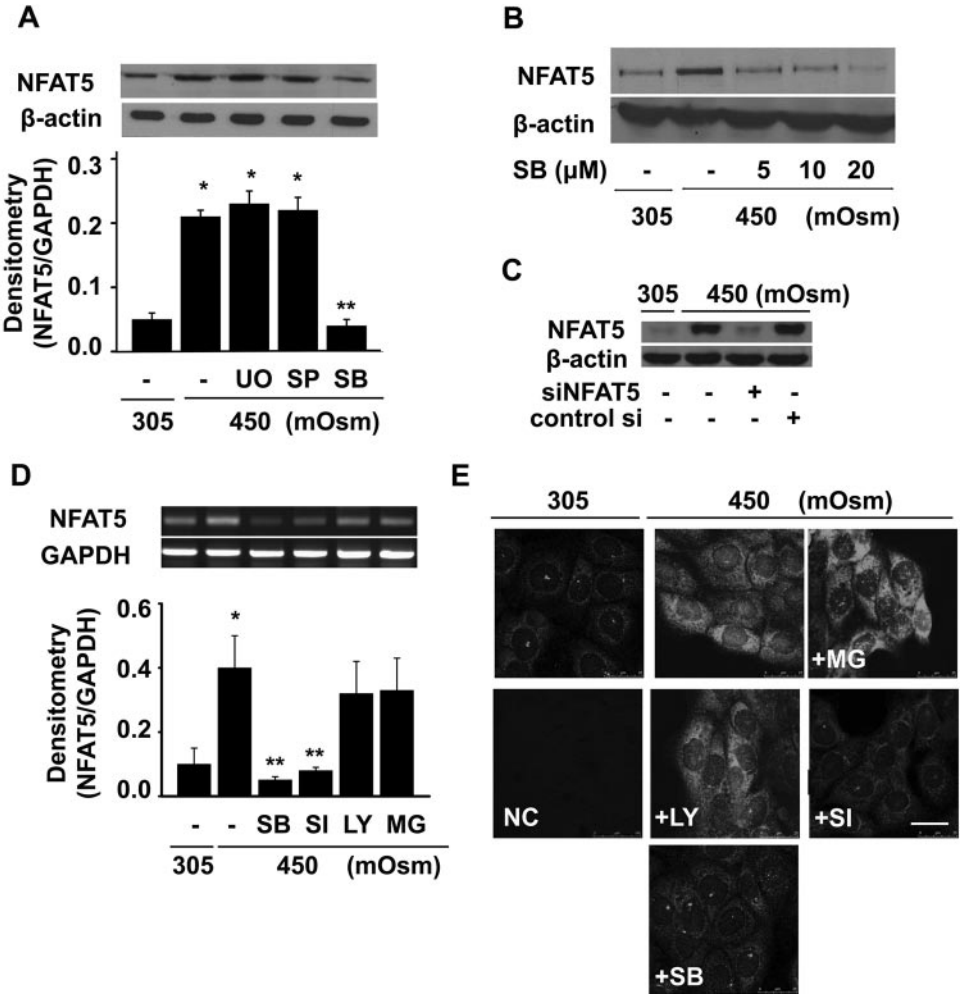


FIGURE 3. NFAT5 induction and translocation was suppressed by inhibition of p38 MAPK. (**A**) NFAT5 expression was determined by immunoblot at 24 hours of exposure to hyperosmolar stress, after 30 minutes of pretreatment with 20 μM U0125, SP600125, or SB239063. (**B**) NFAT5 production was determined after 30 minutes of pretreatment with 5, 10, and 20 μM SB239063 in hyperosmolar stressed HLECs. (**C**) The specific inhibitory effect of siNFAT5 was determined and compared with that of control siRNA in a hyperosmolar condition 24 hours after transfection of RNAi. (**D**, **E**) NFAT5 transcription (**D**) and nucleus translocation (**E**) were analyzed after pretreatment with or without 10 μM SB239063, LY294002, or MG132 and with our without or siNFAT5 transfection at 6 hours after hyperosmolarity. $^{*}P < 0.001$: compared with the 305-mOsM condition. $^{**}P < 0.001$: indicating an inhibitory effect compared with the 450-mOsM condition. NC, primary antibody–depleted negative control.

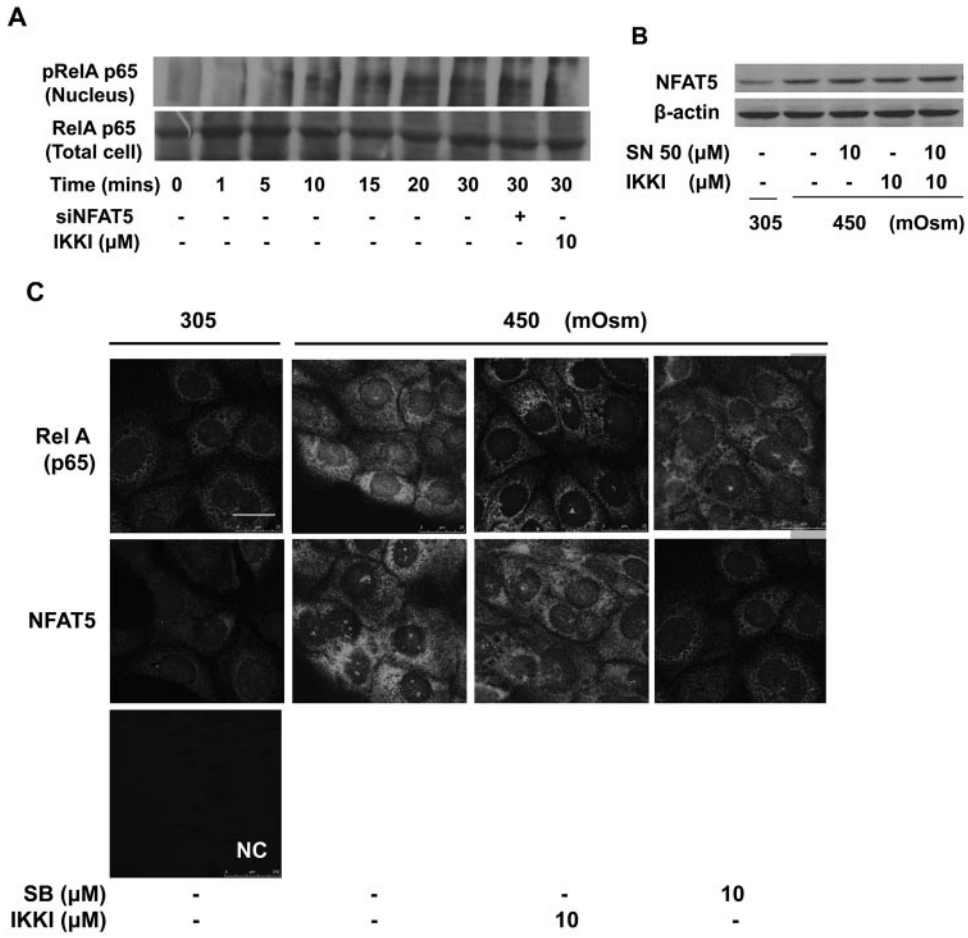


FIGURE 4. Determination of the relationship between NF-κB and NFAT5 in a hyperosmolar stress condition. (**A**) RelA component of NF-κB translocation by hyperosmolar stress was determined with immunoblot at various time points, with or without siNFAT5 treatment. The hyperosmolar stressed HLECs, with or without siNFAT5 transfection were subjected to various time points, and nuclear fraction was prepared. Then, SDS-PAGE and immunoblot for RelA (p65) were performed for both nuclear fraction and total cell lysates, respectively. (**B**) NFAT5 expression was measured by immunoblot in 24 hours hyperosmolar conditions, after pretreatment with 10 μM SN50 or IκB inhibitor (IKKI). (**C**) RelA (p65) and NFAT5 translocation were determined with confocal microscopy at 6 hours after hyperosmolar stress with 30 minutes of pretreatment with either 10 μM of IKKI or SB 239064 (SB) (*blue*: DAPI, *green*: RelA (upper row), NFAT5 (lower row). NC; primary antibody depleted negative control. Bar, 25 μm.

sion was not inhibited by 10 μM SN50 or IκB kinase inhibitor (Fig. 4B). With confocal microscopy, the RelA p65 translocation was not affected by SB239063. Also, NFAT5 nuclear translocation was not changed by 10 μM IκB kinase inhibitor (Fig. 4C). Our data indicate no cross-talk or inhibitory or positive relationships between NF-κB and NFAT5 in hyperosmolar stress conditions.

Induction of Proinflammatory or Inflammatory Cytokines by NFAT5 and NF-κB

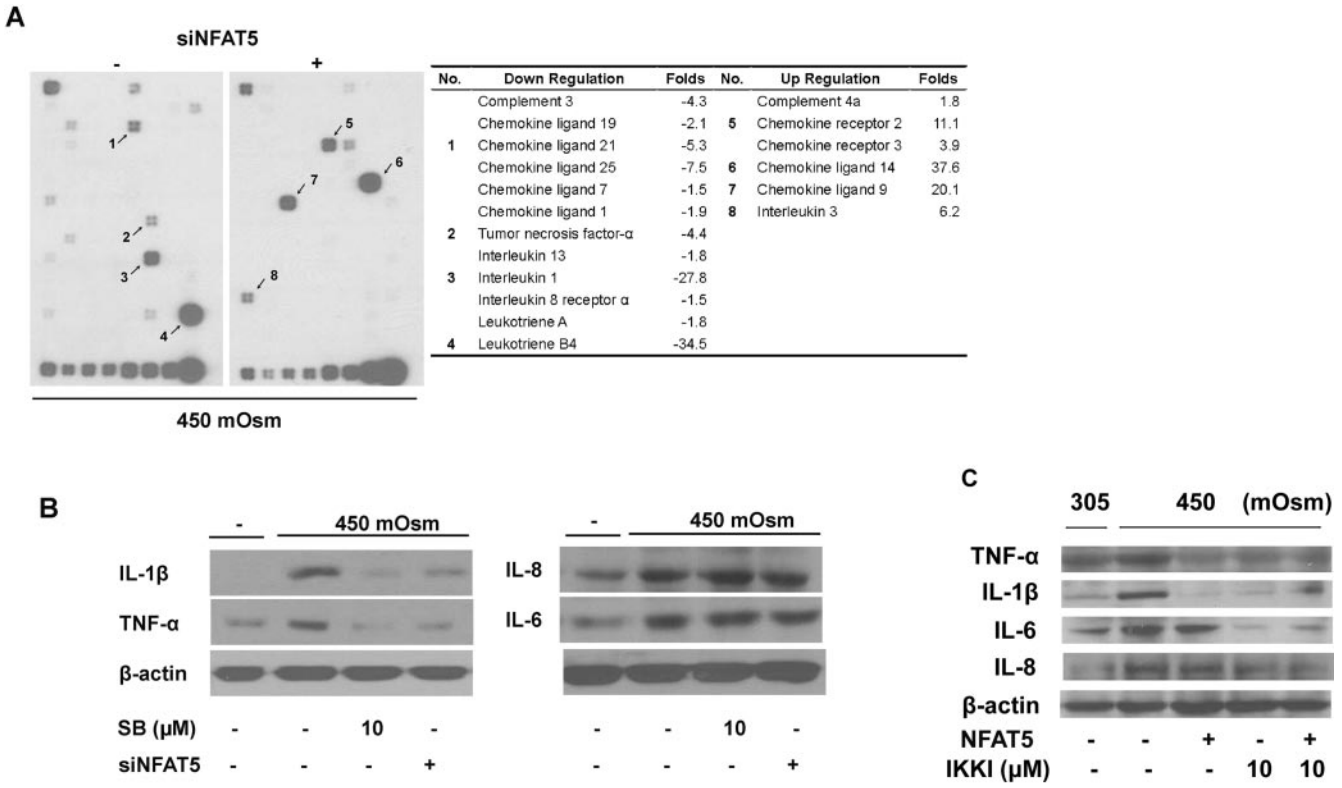
We sought to determine whether NFAT5 affects the hyperosmolarity-induced cytokines using gene microarray (GEArray; SuperArray Inc.) and Western immunoblot analyses. Genes with more than a fourfold increase and decrease in expression were considered significant in the selection criteria. These oligoarray experiments were repeated three times, and representative images are shown in Figure 5A. Comparison of inflammatory cytokines by inhibition of NFAT5 in the hyperosmolar condition showed decreased expression of four genes (IL-1, TNF-α, chemokine ligand 21, and leukotriene B4) and increased expression of four genes (chemokine receptor 2, chemokine ligand-14 and -9, and IL-3). Western immunoblot data also show that hyperosmolarity-induced TNF-α and IL-1β were significantly decreased on inhibition of the p38-NFAT5 complex with 10 μM of SB239063 or siNFAT5 (Fig. 5B). However, IL-6 and -8 expression levels were not significantly affected by SB239063 or siNFAT5. We found NF-κB to be an important regulator of IL-6, and -8 expression in hyperosmolar stressed HLECs (Fig. 5C). On simultaneous inhibition of both NFAT5 and NF-κB, hyperosmolarity-induced inflammatory cytokines were completely suppressed.

NFAT5 Inhibition of Apoptotic Cell Death Triggered by Hyperosmolar Stress

Under conditions of hyperosmolar stress, HLEC apoptosis was significantly increased with time or an osmotic pressure gradient (data not shown). In isotonic conditions, there are no differences in the apoptotic cell proportion between the control vector and NFAT5 siRNA-transfected cells. However, apoptotic cells were significantly increased in NFAT5 siRNA-transfected HLECs in hyperosmotic condition, compared with control vector–transfected cells by 24 hours (Fig. 6A). Moreover, the apoptotic cell fraction was also significantly increased with SN50, an inhibitor of NF-κB, in hyperosmotic conditions (Fig. 6B). In total, 74.3% of cells in 450-mOsM conditioned medium displayed gated apoptosis, after simultaneous inhibition of both NF-κB and NFAT5.

DISCUSSION

NFAT5 is enhanced in HLECs after NaCl-induced hyperosmolar stress. This gene regulator increases proinflammatory cytokine production and plays an important role in preventing cell death by hyperosmolar stress via control of p38 MAPK activation. NFAT5 is a fundamental regulator of the response to osmotic stress in mammalian cells.[22] The gene was initially identified in 1999 as a novel member of the Rel family, which comprises NF-κB and NFAT proteins.[17] Although some of the structural and functional features of NFAT5 are a hybrid between these two major Rel protein groups, the protein has unique characteristics that support its classification as a third type of Rel transcription factor.[1,14] Since its discovery, NFAT5



No.	Down Regulation	Folds	No.	Up Regulation	Folds
	Complement 3	-4.3		Complement 4a	1.8
	Chemokine ligand 19	-2.1	5	Chemokine receptor 2	11.1
1	Chemokine ligand 21	-5.3		Chemokine receptor 3	3.9
	Chemokine ligand 25	-7.5	6	Chemokine ligand 14	37.6
	Chemokine ligand 7	-1.5	7	Chemokine ligand 9	20.1
	Chemokine ligand 1	-1.9	8	Interleukin 3	6.2
2	Tumor necrosis factor-α	-4.4			
	Interleukin 13	-1.8			
3	Interleukin 1	-27.8			
	Interleukin 8 receptor α	-1.5			
	Leukotriene A	-1.8			
4	Leukotriene B4	-34.5			

FIGURE 5. Induction of proinflammatory or inflammatory cytokines by NFAT5. (**A**) RNA was isolated from HLECs after 6 hours in the 450-mOsM condition, with or without siNFAT5 transfection. Then, gene microarray analysis of mRNA extracted from conditioned HLEC was performed. (**B**) The protein levels of IL-1β, TNF-α, IL-6, and IL-8 were determined at 24 hours of exposure to 450 mOsM. To investigate whether NFAT5 affects cytokine production, specific wells of hyperosmolar-stressed HLECs were pretreated with 10 μM of SB239063 (SB) or siNFAT5 transfection, 30 minutes before exposure to hyperosmolar stress. (**C**) Western immunoblot data with the same target cytokines and inhibitors as in (**B**). The cells were pretreated with 10 μM SB239063 or IκB inhibitor (IKKI) or both, 30 minutes before exposure to hyperosmolar stress and then subjected at 24 hours after hyperosmotic treatment.

has been analyzed mostly in the context of the hypertonicity stress response. The advent of mouse models deficient in NFAT5 and other recent advances have confirmed a fundamental osmoprotective role of this factor in mammals,[22] and have revealed features that suggest a wider range of functions, such as inflammatory cytokine production and immune cell development.[1,3]

In our study, hyperosmolarity-induced inflammatory cytokines were partially inhibited on NFAT5 neutralization and were totally inhibited by simultaneously blocking NF-κB and NFAT5 in HLECs. Moreover, NFAT5 inhibits IL-1β and TNF-α transcription more specifically than IL-6 or -8. An earlier study has shown that the activation mechanisms of NFAT5 are distinct from those of NF-κB. NFAT5 activation is associated with kinases, such as p38, fyn,[2] protein kinase A,[26] and ATM,[27] which regulate both cytosol-to-nuclear translocation and transcriptional activation. However, the translocation and phosphorylation of NF-κB are somewhat complicated and are mainly mediated by TRAF-2/5 and -6, which are activated by proinflammatory cytokines or lipopolysaccharide.[28] Moreover, no data on cross-talk between the two regulators are currently available.[1,28] These findings collectively support the present results, which show independent activation of NFAT5 and NF-κB by hyperosmolar stress and no cross-talk or adaptive pathway between the two molecules.

We have already investigated HLEC apoptosis by hyperosmolar stress and confirmed an increased level of apoptotic gene (e.g., Bax), activated caspase-3, and the decreased level of antiapoptotic gene Bcl-xL.[29] During prolonged and strong hyperosmolar stress, some HLECs underwent apoptosis, whereas other cells survived. However, after NFAT5 and NF-κB were blocked, most of the cells died. Although it is difficult to account for this phenomenon in our study, MAPK activation may partially explain how HLECs endure osmotic stress. Concerning the activation of MAPK in osmolar changes, previous studies showed that MAPK components modulate regulatory cell volume responses needed for restoration of isotonic cell volume during exposure to anisosmotic challenges in corneal epithelial cells.[30,31] Capo-Aponte et al.[30] reported that Erk, p38, and SAPK/JNK are activated by a tonicity-dependent hyposmotic condition and a consequent change in the K-Cl cotransporter. Under conditions of hyperosmolarity, all three MAPK types were activated. In general, JNK activation was associated with cellular apoptosis. On the other hand, activation of p38 and Erk was generally related to antiapoptosis.[15] We therefore hypothesize that exposure to hyperosmotic stress stimulates cell survival by p38 MAP kinase, whereas prolonged exposure activates SAPK/JNK, ultimately leading to apoptosis.[32,33] Apart from strong apoptotic signals, such as JNK, hypertonic stress may also activate cellular signaling cascades, leading to the simultaneous promotion of cell survival so that only a limited number of cells undergo death under these conditions. Many mitogenic and/or antiapoptotic signaling molecules, including receptor tyrosine kinases,[34] the Src Family of nonreceptor tyrosine kinases[35]; protein kinases A, C, and G[36,37]; and p21 activated kinases[38] are stimulated in mammalian cells exposed to hyperosmotic stress. Indeed, all three MAP kinase families—p38 MAPK, SAP/JNK, and ERK1/2—are activated by hyperosmolarity in several types of mammalian cells.[39,40]

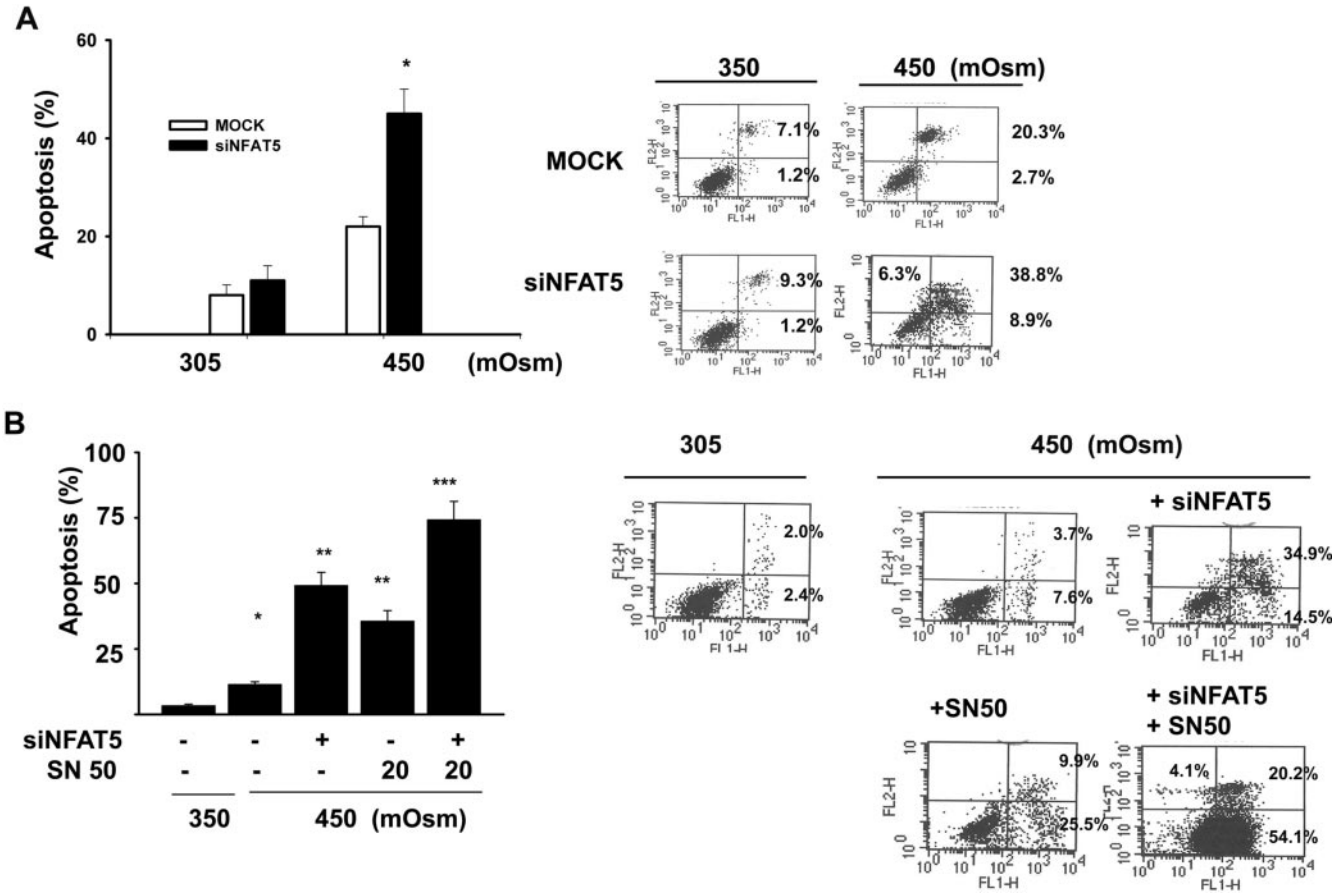


FIGURE 6. NFAT5 inhibited apoptotic cell death caused by hyperosmolar stress. (**A**) HLEC apoptosis was determined with flow cytometry. HLECs were transfected with either siNFAT5 or a control siRNA plasmid (MOCK). Cell apoptosis was measured 24 hours after hyperosmolar stress. (**B**) HLEC apoptosis was analyzed 24 hours after hyperosmolar stress, after pretreatment with 20 μM SN50 or siNFAT5 transfection or both. Data are expressed as the mean ± SD of results in 9 to 12 wells from two independent experiments. $^{*}P < 0.001$: compared with the 305-mOsM condition. $^{**}P < 0.001$: significant difference from the 450-mOsM condition. $^{***}P < 0.001$: compared with the siNFAT5 or SN50-treated condition.

Exposure of cells to a hyperosmotic environment results in the osmotic efflux of water, reduction in cell volume, cell shrinkage, and increase in the concentration of all intracellular constituents. The resultant biochemical disequilibrium gives rise to a wide spectrum of deleterious effects on cell function and, in the event homeostasis is not restored, ultimately results in apoptotic cell death.[41] Bildin et al.[42] reported that sufficient activation of the p38 MAPK is essential for maintaining rabbit corneal epithelium volume in hyperosmotic condition. This previous study is consistent with our result of hyperosmolarity-induced p38 activation. The advance provided by findings in the present study is the additional evidence of activation of NFAT5 through p38 and its protective role from hyperosmolarity-induced cell damage and apoptosis. We did not investigate the precise mechanism by which NFAT5 activation prevents apoptotic cell death during hyperosmolar stress. However, a small number of NFAT5 target genes have been identified to date.[1] These genes either enhance the intracellular concentration of compatible osmolytes, or, in the case of Hsp70, presumably facilitate normal protein folding in an intracellular environment that is altered as a result of osmotic loss of water or increase in intracellular ionic strength.[3,15,20]

In addition to the standard osmoprotective response, NFAT5 stimulates the transcription of at least two cytokine genes, IL-1β and TNF-α, in HLECs subjected to osmotic stress. Both cytokines play an important role in cellular apoptosis, survival, and regulation of immune and inflammatory responses of various cell types.[43–45] We speculate that these cytokines potentiate immune responses to pathogens in the vulnerable status. Moreover, considering that both cytokines are regulated by NF-κB in various cell types including HLECs,[46,47] NFAT5 possibly influences immune and/or inflammatory responses under hyperosmolar conditions at least partially through these cytokines.

It has been suggested that tear hyperosmolarity is the primary cause of discomfort, inflammation and ocular surface cell apoptosis.[48,49] In view of hyperosmolarity-induced NFAT5 and NF-κB activation and its antiapoptotic role in HLECs, both gene regulators may be the important factors for inhibition of cell damage from hyperosmolar stress. If this protective mechanism occurs on KCS patients, minimal ocular surface changes may be expected. However, KCS patients frequently show epithelial metaplasia and decreased cellularity of epithelial and Goblet cells.[48] In vivo, some dry-eye–specific molecules may act to reverse the effect of hyperosmolarity on NFAT5; hence, there still may damage of the epithelial cells. Further studies are needed to elucidate the mechanism in more detail.

In conclusion, NFAT5 induction and translocation to the nucleus in the hyperosmolar status occurs via activation of p38. NFAT5 expression is associated with increasing levels of proinflammatory cytokines and the prevention of apoptosis. Further investigation is needed to determine the precise mechanism of NFAT5 induction in hyperosmolar stress and consequent inhibition of cellular apoptosis and proinflammatory cytokine production.

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