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Protein kinase Cμ plays an essential role in hypertonicity-induced heat shock protein 70 expression

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Abbreviations: HSF1, heat shock transcription factor 1; HSP70, heat shock protein 70; TonEBP, the tonicity-responsive enhancer binding protein

Abstract

Heat shock protein 70 (HSP70), which evidences important functions as a molecular chaperone and anti-apoptotic molecule, is substantially induced in cells exposed to a variety of stresses, including hypertonic stress, heavy metals, heat shock, and oxidative stress, and prevents cellular damage under these conditions. However, the molecular mechanism underlying the induction of HSP70 in response to hypertonicity has been characterized to a far lesser extent. In this study, we have investigated the cellular signaling pathway of HSP70 induction under hypertonic conditions. Initially, we applied a variety of kinase inhibitors to NIH3T3 cells that had been exposed to hypertonicity. The induction of HSP70 was suppressed specifically by treatment with protein kinase C (PKC) inhibitors (Gö6976 and GF109203X). As hypertonicity dramatically increased the phosphorylation of PKCμ, we then evaluated the role of PKCμ in hypertonicity-induced HSP70 expression and cell viability. The depletion of PKCμ with siRNA or the inhibition of PKCμ activity with inhibitors resulted in a reduction in HSP70 induction and cell viability. Tonicity-responsive enhancer binding protein (TonEBP), a transcription factor for hypertonicity-induced HSP70 expression, was translocated rapidly into the nucleus and was modified gradually in the nucleus under hypertonic conditions. When we administered treatment with PKC inhibitors, the mobility shift of TonEBP was affected in the nucleus. However, PKCμ evidenced no subcellular co-localization with TonEBP during hypertonic exposure. From our results, we have concluded that PKCμ performs a critical function in hypertonicity-induced HSP70 induction, and finally cellular protection, via the indirect regulation of TonEBP modification.

Keywords: HSP70 heat-shock proteins; NFAT5 protein, human; protein kinase C; protein kinase inhibitors

Introduction

Because all organisms face environmental and pathophysiological stresses, they need to develop appropriate protective mechanisms. One of the best-known protective mechanisms in this regard is the rescue of cells against a variety of insults via the induction of stress response proteins (Flanagan *et al*., 1995; Scliess *et al*., 1999). Inducible HSP70 has been implicated as a molecule which performs a pivotal role in the protection of cells against a variety of stresses. HSP70 synthesis has been identified in response to a broad range of chemicals and biological signals, including hypertonic stress, amino acid analogues, energy metabolism inhibitors, radiation, oxidative stress, and heavy metals (Williams and Morimoto, 1990; Hatayama *et al*., 1993; Abe *et al*., 1995; Wagner *et al*., 1999). This means that HSP70 is involved in a general cellular defense mechanism. Recently, the cytoprotective roles of HSP70 were clearly identified and the molecular mechanisms inherent to the anti-apoptotic function of HSP70 were clearly elucidated (Beere *et al*., 2000; Li *et al*., 2000; Ravagnan *et al*., 2001; Lee *et al*., 2005).

Inducible HSP70 is encoded from both *hsp*70.1 and *hsp*70.3 genes, which evidence a high degree of similarity in their coding sequences and a linked tandem array with the MHC region of the same chromosome. Two inducible *hsp*70 genes differ from each other in their 5'- and 3'-untranslated regions (Walter *et al*., 1994). We observed previously that *hsp*70.1 and *hsp*70.3 genes respond differentially to different types of stress (Lee and Seo, 2002). Hypertonicity induced only *hsp*70.1 ex-

pression, because only *hsp*70.1 harbors tonicity-responsive enhancer (TonE) sites, which have been shown to perform an important function in the response to hypertonicity (Heo *et al*., 2006).

HSP70 accumulation in cells exposed to stress is known to generally depend on the activation of heat-shock factor (HSF) (Abravaya *et al*., 1991; Morimoto *et al*., 1992). The stress-induced rapid activation of HSF is a very common phenomenon. However, it has been recently suggested that inducible HSP70 synthesis appears to be regulated via alternative transcription activators. As the *hsp*70.1 promoter harbors TonE sites, the expression of HSP70 may be regulated by the tonicity-responsive enhancer binding protein (TonEBP) under hypertonic conditions (Woo *et al*., 2002). The regulatory mechanism of *hsp*70 transcription during exposure to hypertonicity remains to be well-defined.

The principal objective of this study was to elucidate the manner in which HSP70 induction is regulated in response to hypertonic conditions. We determined that HSP70 expression is regulated by the transcription factor, TonEBP, via the activation of PKCμ, ultimately resulting in the protection of cells against hypertonic stress conditions.

Materials and Methods

Materials

PD98059, LY293002, H-89, and Gö6976 were purchased from Calbiochem (La Jolla, CA) and GF109203X was from LC Laboratories (Woburn, MA). Anti-HSP70, anti-actin, anti-TonEBP, anti-Lamin, and secondary antibodies were from Santa Cruz (Santa Cruz, CA). Anti-phospho-PKC and anti-PKCμ antibodies were from Cell Signaling Technology (Beverly, MA).

Cell culture

NIH3T3 mouse fibroblast and M-1 human renal epithelial cells were grown in DMEM with 10% FBS, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in a 5% CO_2 humidified atmosphere at 37°C. In order to generate hypertonic condition, NaCl solution was added to the medium to 130 mM final concentration of NaCl.

Preparation of whole cell lysate and Western blot analysis

Cells were washed with PBS, scraped and collected in extraction buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 100 mM NaCl, 1 mM PMSF, 10 mM sodium azide, 1 mM orthovanidate, 20 μg/ml aprotinin, and 3 mM dithiothreitol). The collected cells were incubated on ice for 30 min. The lysate was centrifuged and quantitated with a Bradford Assay Reagents (Bio-Rad, Hercules, CA). Equal amount of proteins was loaded onto an 8 or 10% SDS-PAGE gel. After separation of proteins depending on their molecular weights, it was transferred to a nitrocellulose membrane. The membrane blot was incubated with antibody at 4°C overnight, and washed three times in TBST. Protein bands were detected by sequential treatment with an HRP-conjugated secondary antibody (Santa Cruz, CA), and an enhanced chemiluminescence substrate kit (Amersham Bioscience, NJ).

MTT assay

Cells were applied to each well of 25-well culture plates and incubated overnight. One hundreds μl of 5 mg/ml MTT dissolved in PBS was added to each well and incubated for 4 h. The purple formazan formed by viable cells was dissolved by the addition of DMSO and absorbance was measured at 540 nm with VERSAmax (Molecular Device, Sunnyvale, CA).

Cellular fractionation

Cells were incubated with 400 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM PMSF, 10 mM sodium azide, 1 mM orthovanidate, 20 μg/ml aprotinin, and 1 mM DTT) on ice for 5 min. These samples were mixed with 25 μl of 10% Nonidet P-40 and vortexed for 10 s. After centrifugation at 14,000 rpm for 1 min, the supernatant was saved as cytoplasmic fraction. The pellets were washed once with the same buffer, resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 mM sodium azide, 1 mM orthovanidate, 20 μg/ml aprotinin, and 1 mM DTT), and vortexed on ice for 10 min. After centrifugation at 14,000 rpm for 10 min, the supernatant was waved as nuclear fraction.

Transient transfection

Recombinant plasmid 3-TonE-HSP70 promoter region up to -2 Kb from the transcription start point was obtained from PCR with mouse genomic DNA. PCR product (HSP70 promoter region) was ligated to promoterless EGFP vector (Clontech, Palo Alto, CA), and generated recombinant plasmid, pH70pro-EGFP. Plasmids, pcDNA-PKCα-KD-HA and pcDNA-PKCβI-KD-HA were thankfully obtained from Dr. YS Lee (Korea Institute of Radiological and Medical Sciences, Seoul, Korea). Recombinant plas-

mids were transfected to NIH-3T3 cells using Lipofectamine (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. After transfection, the gene expression was monitored by Western blotting analysis.

Immunostaining and confocal microscopy

Cells were fixed in 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton X-100 for 10 min. The cells were then blocked with 2% BSA in PBS for 1 h, washed with PBS, and incubated with primary antibody diluted 1:200 in PBS containing 2% BSA. Then, cells were washed with PBS three times. Secondary FITC-conjugated antibody (1:200 dilutions in PBS containing 2% BSA) was applied to the coverslip. After washing three times with PBS, the cells were stained with DAPI for the detection of nuclei. After mounting, the cells were examined under a Zeiss confocal laser scanning microscope (Carl Zeiss, Jena, Germany). Images were captured and merged with LSM 5 PASCAL software (Carl Zeiss, Jena, Germany).

Treatment of siRNA

The stealth RNAi duplex targeting nucleotides of the PKCμ cDNA and negative control oligomer (BLOCK iT Oligo) were synthesized by Invitrogen (Carlsbad, CA). The stealth RNAi sequences of the PKCμ were as followed: sense 5'-UGCCAAAGCC-AUCUCAGUAUCUUGG-3' and antisense 5'-CCA-AGAUACUGAGAUGGCUUUGGCA-3'. Cells were transfected with 25 μM of either the PKCμ siRNA duplex or control dsRNA using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real time PCR

SYBR green-based real time PCR technique was used to detect the expression of HSP70 and PKCμ. Total RNA was prepared from NIH-3T3 cells, and 1 μg of total RNA was reverse-transcribed into cDNA in the presence of RT-premix (Bioneer, Daejeon, Korea) and 1 μg of random primers (Invitrogen, Carlsbad, CA) in a total volume of 20 μl at 42°C for 1 h. The PCR mixture consisted of 1 μl cDNA, 10 μl of SYBR green-containing PCR master mixture (Qiagen, Valencia, CA), 0.75 μl of ROX (passive fluorescent dye) and 10 pmol of each primer in a total volume of 20 μl. The specific primers for real time PCR were designed by using the Primer3 software as follows: HSP70.1 (untranslated region in upstream of the start site) sense 5'-GAGACATGGACAAGCAAGCA-3' and antisense 5'-GGTGGTGAGAGTGTGGGACT-3'; PKCμ sense CGACGTCATCATCCATCAAC and antisense ACT-GTGGTACCCTGCTCTGG; GAPDH sense 5'-GG-AGCGAGACCCCACTAACA-3' and antisense 5'-ACATACTCAGCACCGGCCTC-3'. Real time PCR was performed using the ABI7000 thermocycler (Applied Biosciences, Foster City, CA), and the cycling conditions used were 95°C for 15 s, 60°C for 10 s and 72°C for 30 s for 40 cycles, followed by a melting point determination that results in a single peak if the amplification is specific. The ROX-normalized fluorescence measurements were exported to Microsoft Excel. The results were normalized to the housekeeping gene GAPDH mRNA in the same sample. The relative expression of each gene in NIH-3T3 cells was calculated using a derivative of the comparative CT method.

Results

PKC signaling pathway involves hypertonicity-induced HSP70 expression

In order to determine the essential signaling pathway for the hypertonicity-induced HSP70 expression, we treated NIH3T3 cells with a variety of kinase inhibitors, and evaluated the induction of HSP70. When cells were exposed to hypertonic conditions (with the administration of an additional 130 mM NaCl to the medium), the levels of intracellular HSP70 were increased dramatically (Figure 1A). As ERK and p38 were activated in cells exposed to hypertonic conditions (Figure 1B), we attempted to determine whether ERK or p38 were involved in hypertonicity-induced HSP70 expression. Treatment with an ERK inhibitor (PD98059) exerted no effects on hypertonicity-induced HSP70 expresssion (Figure 1C). When we conducted transfection with a dominant negative form of p38 (p38 DN), the induction of HSP70 remained unaltered (Figure 1D). PKA inhibitor (H89) and PI-3K inhibitor (LY293002) also had no effects on the level of HSP70 induction (Figure 1E). However, when cells were treated with a broad range of PKC inhibitor (GF109203X), hypertonicity-induced HSP70 expression was inhibited completely (Figure 1E). In order to verify the inhibitory effects of GF109203X on the hypertonicity-induced expression of HSP70, we transfected the recombinant plasmid, pHSP70pro-EGFP (recombinant plasmid containing HSP70 promoter and EGFP coding regions), into NIH3T3 cells and detected EGFP expression under hypertonic conditions. Whereas the NIH3T3 cells evidenced EGFP protein expression as the result of NaCl

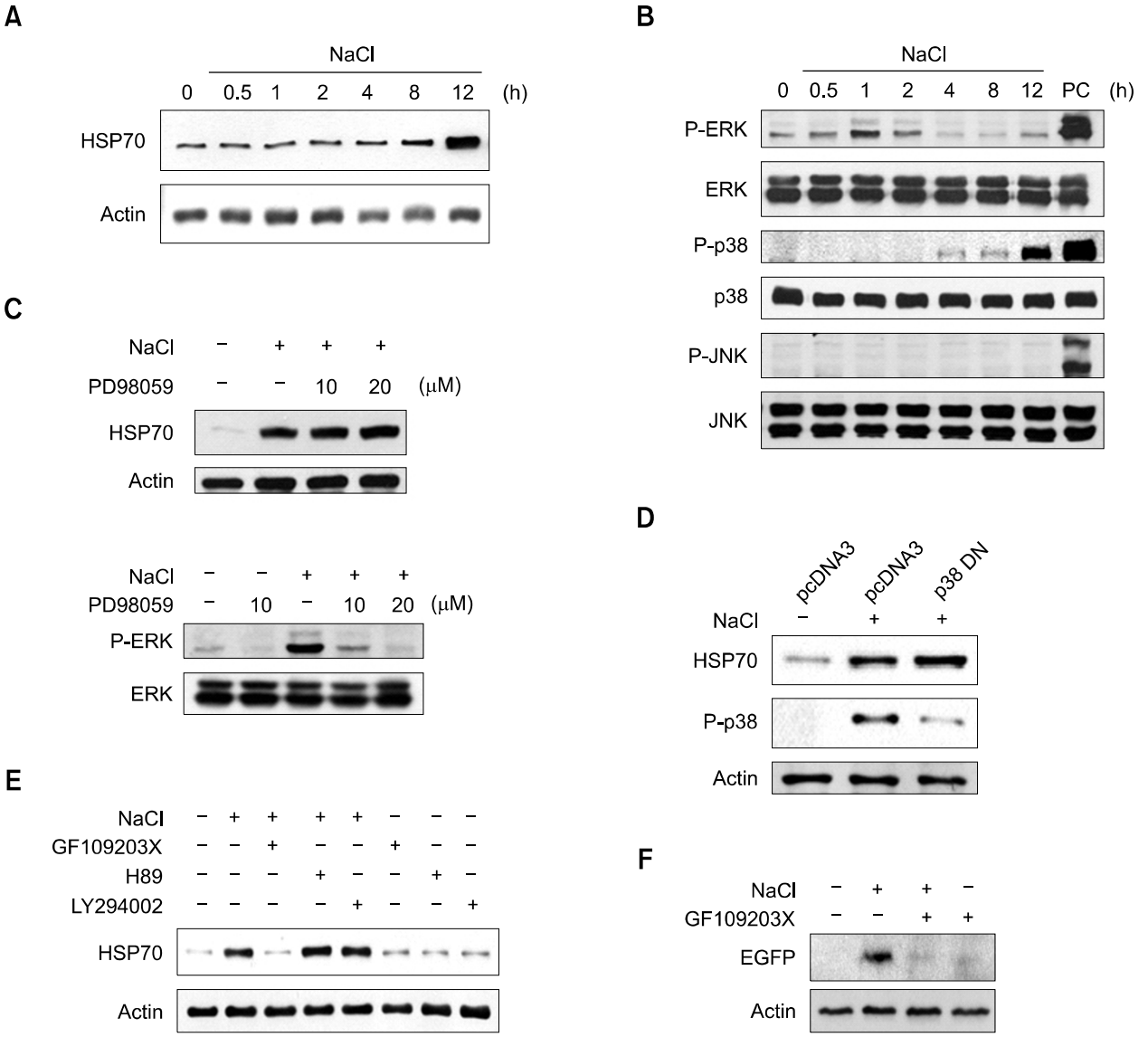

Figure 1. Involvement of PKC in hypertonicity-induced HSP70 expression. (A) HSP70 induction in NIH-3T3 cells exposed to hypertonic conditions. Cells were exposed to hypertonic conditions for the indicated time periods and analyzed via immunoblot assay. (B) Phosphorylation status of ERK, p38, and JNK in hypertonicity. Phosphorylated proteins were detected with phospho-specific ERK, p38, and JNK antibodies. PC designates the positive control sample, which was NIH3T3 cells treated for 30 min at 43°C. (C) The effects of ERK activity on hypertonicity-induced HSP70 expression. ERK inhibitor (10 μM and 20 μM PD98059) was added 1 h prior to exposure to hypertonicity. The cell lysates were prepared, and ERK phosphorylation (lower panel) and HSP70 expression (upper panel) were detected. (D) The effects of dominant negative p38 (p38 DN) on hypertonicity-induced HSP70 expression. The cells were transiently transfected with p38 DN and exposed to hypertonicity. P38 phosphorylation and HSP70 induction were detected at 4 h and 12 h after exposure to hypertonicity, respectively. (E) The effects of PKC inhibitor on hypertonicity-induced HSP70 expression. NIH-3T3 cells were treated with inhibitors (25 μM GF109203X, 2 μM H89, or 10 μM LY294002) for 1 h prior to exposure to hypertonicity for 12 h. Cell lysates were resolved via SDS-PAGE and blotted with anti-HSP70 antibody. (F) Effects of PKC inhibitor on HSP70 promoter activity. Cells were transiently transfected with recombinant plasmid, pHSP70promoter-EGFP (see Materials and Methods) and treated with 25 μM GF109203X prior to hypertonicity treatment. Cell lysates were resolved by SDS-PAGE and blotted with anti-EGFP antibody.

treatment, GF109203X clearly inhibited the hypertonicity-induced expression of EGFP under the control of the HSP70 promoter (Figure 1F).

PKCμ is essential for hypertonicity-induced HSP70 expression and cytoprotection against lethal stress

Because we observed the involvement of PKC in

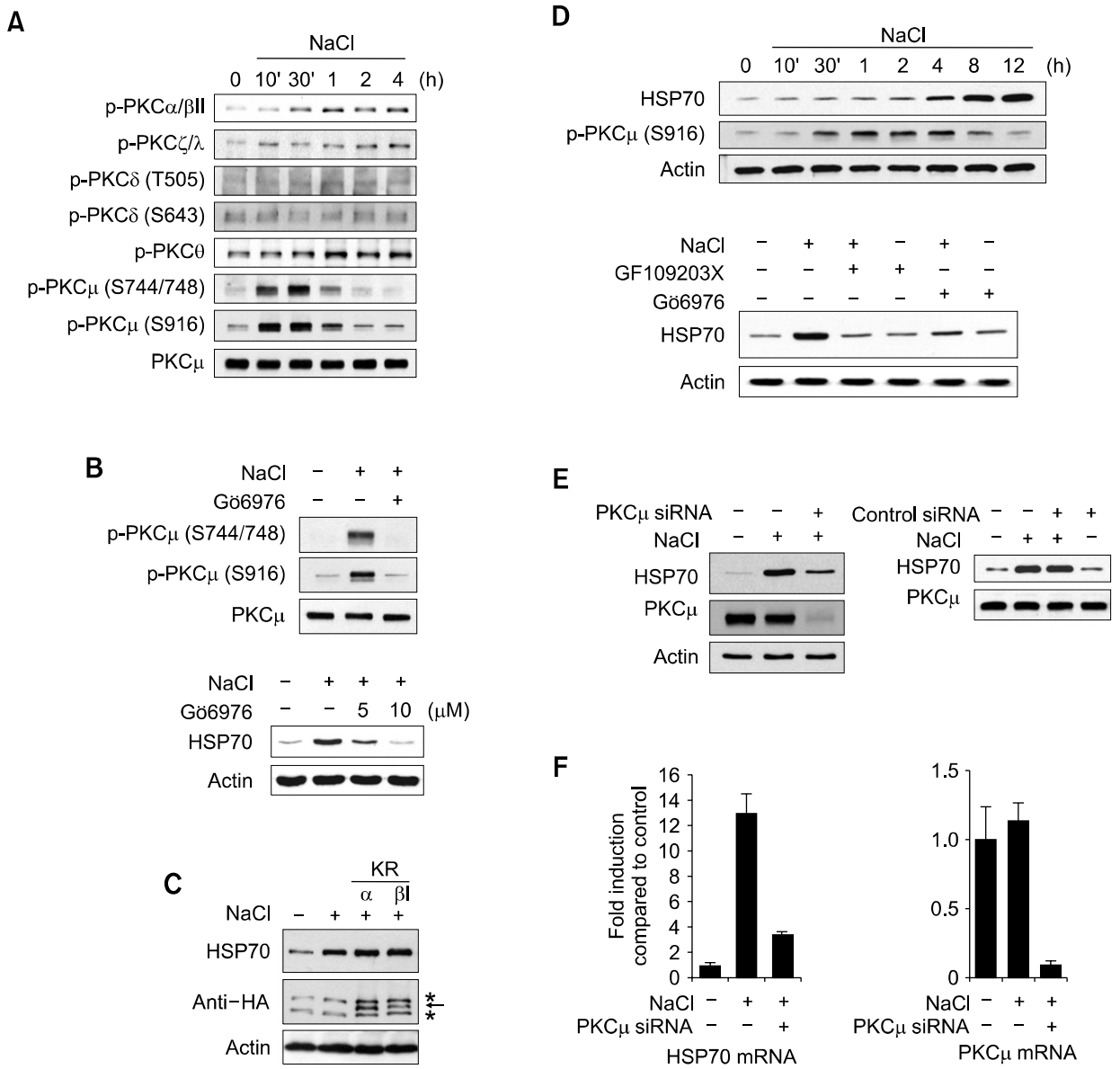

Figure 2. Essential role of PKCμ in hypertonicity-induced HSP70 expression. (A) Phosphorylation status of various PKC isoforms under hypertonic conditions. Cells were maintained in media containing an additional 130 mM NaCl for the indicated time periods. Cell lysates were blotted with anti-p-PKCα/βII, anti-p-PKCζ/λ, anti-p-PKCθ, anti-p-PKCδ, and anti-p-PKCμ antibodies. (B) Effect of PKCμ inhibitor, Gö6976 on hypertonicity-induced HSP70 expression. Cells were pretreated with Gö6976 (5 μM or 10 μM) for 1 h, and then kept in hypertonic media for either 30 min for the detection of p-PKCμ or 12 h for the detection of HSP70. Cell lysates were resolved via SDS-PAGE and blotted with anti-p-PKCμ and anti-PKCμ antibodies (upper panel) or anti-HSP70 antibody (low panel). (C) Effect of kinase-inactive mutants (KR) of PKCα and PKCβI on hypertoniciy-induced HSP70 expression. Cells were transiently transfected with HA-PKCα KR or HA-PKCβI KR constructs. After maintaining transfectant cells in hypertonic media for 12 h, the cell lysates were prepared. Exogenous PKCα and PKCβI (indicated by arrow) and HSP70 were detected with anti-HA antibody. The asterisk designates non-specific signals. (D) Effect of PKCμ inhibitor on HSP70 expression in hypertonically stressed M-1 cells. Human renal epithelial M-1 cells were pretreated with inhibitors (25 μM GF109203X or 10μmM Gö6976) for 1 h and exposed to hypertonicity for the indicated time periods (upper panel) or 12 h (lower panel). Cell lysates were resolved on SDS-PAGE and blotted with anti-HSP70 and anti-p-PKCμ antibodies. (E) Effect of PKCμ knock-down on hypertonicity-induced HSP70 expression. NIH-3T3 cells were transiently transfected with RNAi duplexes (25 μM negative control or 25 μM PKCμ RNAi duplexes). After 48 h of transfection, the cells were exposed to hypertonic conditions for 12 h. The cell lysates were resolved via SDS-PAGE and blotted with anti-HSP70 and anti-PKCμ antibodies. (F) Effect of PKCμ knock-down on hypertonicity-induced HSP70 mRNA transcription. Cells were transiently transfected with PKCμ RNAi duplexes. After 48 h of transfection, the cells were exposed to hypertonic conditions for 4 h. Cells were harvested and real time PCR was conducted as described in Materials and Methods.

hyperonicity-induced HSP70 expression, we attempted to determine which PKC isoforms could be activated under hypertonic conditions. The phosphorylation of PKCμ was most evident under hypertonic stress condition among the various isoforms of PKC (Figure 2A). We then treated the cells with Gö6976, a PKCμ inhibitor, in order to evaluate the involvement of PKCμ in the pathway of hypertonicity-induced HSP70 expression. Treatment with Gö6976 completely inhibited hypertonicity-induced PKCμ phosphorylation. Hypertonicity-induced HSP70 expression was also inhibited completely by treatment with Gö6976, and this effect occurred in a dose-dependent manner (Figure 2B). It has been reported that Gö6976 also inhibits PKC isoforms, including PKCα and PKCβI (Martiny-Baron *et al*., 1993). In order to assess the possibility that PKCα and βI phosphorylation were involved in the pathway inherent to hypertonicity-induced HSP70 expression, we transfected kinase-inactive mutants of PKCα or PKCβI (PKCα KR and PKCβI KR). Both kinase-inactive PKC mutants did not alter the expression of HSP70 under hypertonic conditions (Figure 2C). We conducted the same experiments in human M-1 kidney cells. Under hypertonic stress conditions, PKCμ was phosphorylated and HSP70 was induced in M-1 cells. When GF109203X and Gö6976 were applied to the M-1 cells, the induction of HSP70 was also inhibited completely (Figure 2D).

In order to verify whether PKCμ was involved in hypertonicity-induced HSP70 expression *per se*, we conducted an RNA interference assay. In NIH-3T3 cells transfected with PKCμ siRNA, no PKCμ expression was detected, and hypertonicity-induced HSP70 expression was reduced at both the mRNA and protein levels, as compared to what was observed in cells treated with a negative

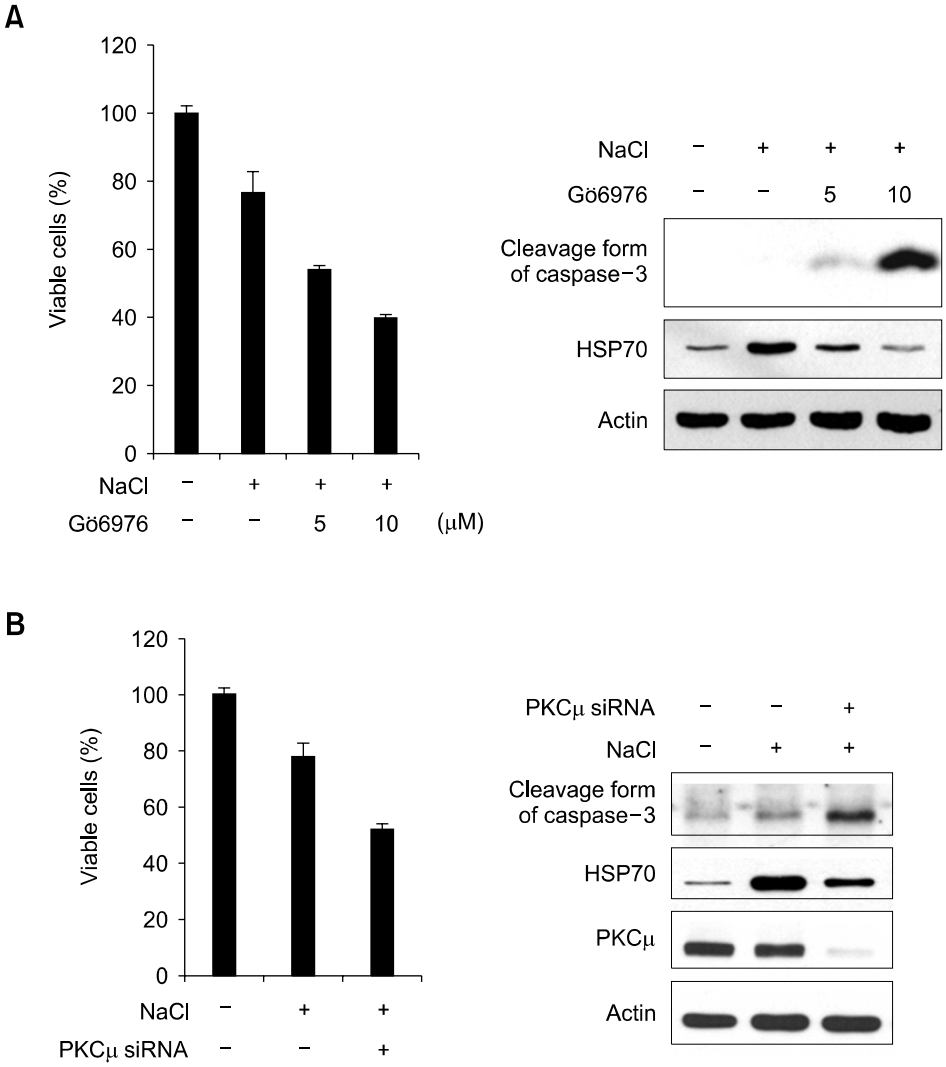

Figure 3. The critical role of PKCμ on cell viability under hypertonic condition. Increase of hypertonicity-induced cell death due to the treatment of PKCμ inhibitor (A) or PKCμ Si duplex (B) NIH-3T3 cells were treated with Gö6976 or transiently transfected with PKCμ RNAi duplexes prior to exposure to hypertonic conditions for 12 h. Cell viability was assessed via MTT assay. Values are expressed as the means ± standard deviation of triplicate determinations. Cell lysates were also resolved via SDS-PAGE and blotted with anti-HSP70 and anti-caspase-3 antibodies.

control siRNA duplex (Figure 2E and F).

Induced HSP70 performs an anti-apoptotic role via the inhibition of caspase-3 activation under lethal conditions (Li *et al*., 2000). We evaluated the effects of PKCμ inhibition or depletion on cell viability under hypertonic conditions. Either the inhibition of PKCμ activity as the result of Gö6976 treatment or PKCμ depletion with PKCμ siRNA resulted in reduced cell viabilities due to the activation of caspase-3 (Figure 3A and B). Our data indicate that PKCμ is crucial for cytoprotection via the mechanism of HSP70 induction and the prevention of caspase-3 activation under hypertonic stress conditions.

PKCμ partially regulates TonEBP modification in the nucleus under hypertonic conditions

HSF1 is a well-known general transcription factor for the induction of HSP70 under a variety of stresses (Morimoto *et al*., 1992). However, it has been previously reported that hypertonicity-induced HSP70 expression was mediated by another transcription factor, TonEBP (Woo *et al*., 2002). First, we attempted to determine whether or not HSF1 was involved in hypertonicity-induced HSP70

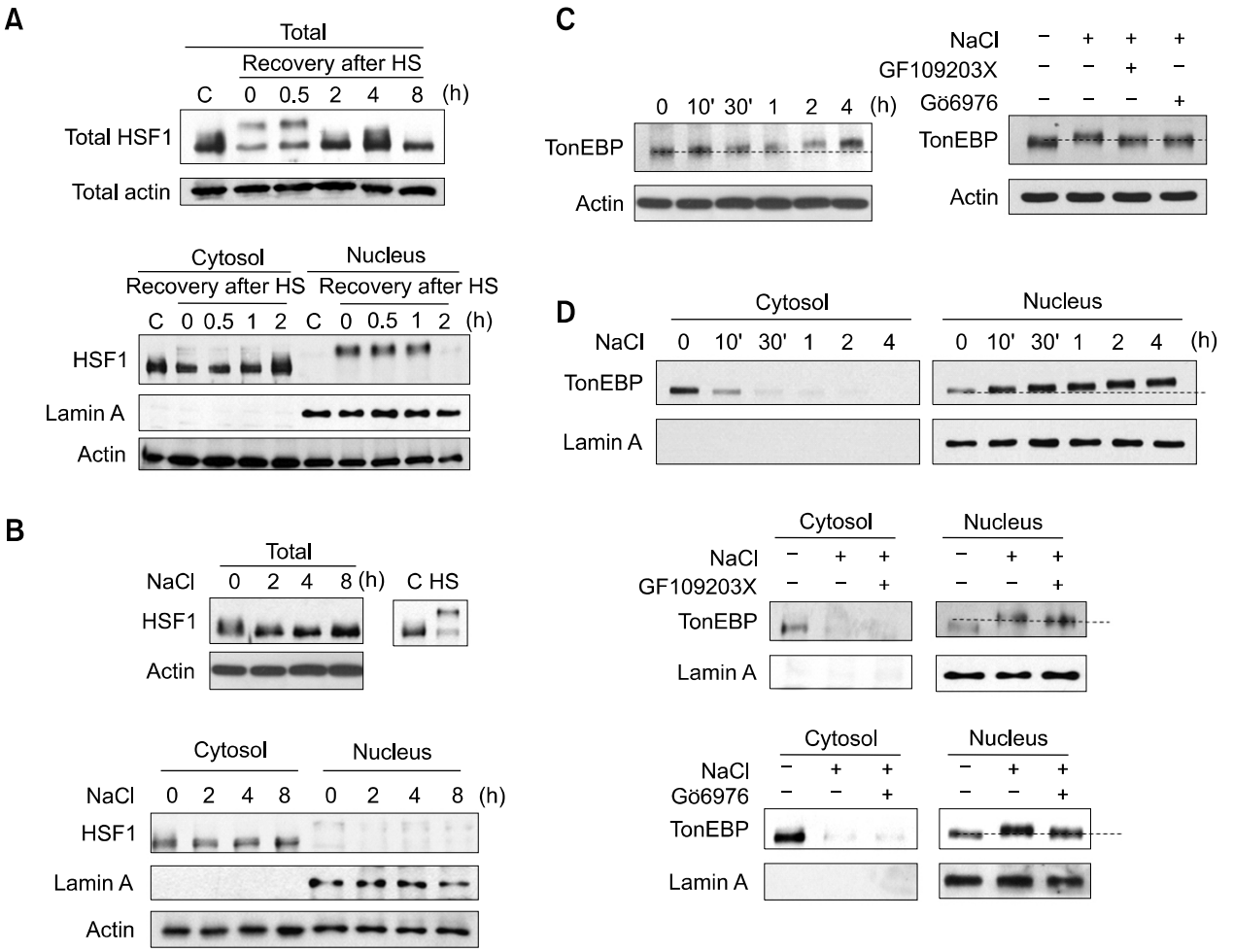

Figure 4. Effect of PKC inhibitors on TonEBP mobility shift under hypertonic conditions. (A) HSF1 mobility shift and translocation to the nucleus by the treatment of heat shock (HS). NIH-3T3 cells were treated with 42°C heat shock for 30 min and recovered at 37°C for the indicated time periods. Then, the whole cell lysates, cytosolic fraction, and nuclear fraction were prepared as described in the Materials and Methods section. Proteins were resolved via SDS-PAGE on 8% low-bias gels and blotted with anti-HSF1 and anti-lamin A antibodies. (B) HSF1 status under hypertonic conditions. NIH-3T3 cells were exposed to hypertonic conditions for the indicated time periods. Prepared whole cell lysates, cytosolic fraction, and nucleus fractions were analyzed via immunoblot assay. (C) Effect of PKC inhibitors on the mobility shift of TonEBP. Cells were exposed to hypertonic conditions either for the indicated time periods without any pretreatment (left panel) or for 2 h with pretreatment of 25 μM GF109203X or 10 μM Gö6976 (right panel). Cell lysates were prepared and analyzed via immunoblot assay. (D) Effect of PKC inhibitors on TonEBP translocation. Cells were maintained in hypertonic media for either the indicated time periods without inhibitor treatment (upper panel) or for 2 h with pretreatment of 25 μM GF109203X (middle panel) and 10 μM Gö6976 (lower panel). Cell lysates were prepared and analyzed via immunoblot assay for the dectection of TonEBP translocation.

expression. HSF1 was immediately modified and translocated into the nucleus during heat shock (HS) treatment, and was relocated to the cytosol and dephosphorylated during the recovery period (Figure 4A). However, HSF1 was neither modified nor translocated into the nucleus under hypertonic conditions (Figure 4B). TonEBP was translocated immediately into the nucleus, then gradually modified in the nucleus under hypertonic conditions (Figure 4C and D). Lamin A was utilized as a marker protein for nuclear fractionation.

As we determined that the phosphorylation of PKCμ was critical for hypertonicity-induced HSP70 expression, we then assessed the effects of PKC inhibitors (GF109203X and Gö6976) on the translocation and modification of TonEBP under hypertonic conditions. Neither GF109203X nor Gö6976 treatment prevented the translocation of TonEBP (Figure 4D). Although GF109203X and Gö6976 did not prevent the translocation of TonEBP from the cytoplasm to the nucleus, TonEBP evidenced a slightly lesser mobility shift in the nuclear fraction, due to the inhibitor treatment (Figure 4C and D). Hypertonicity induced a gradual mobility shift of TonEBP in a time dependent-manner. The inhibition of PKCμ activity induced an incomplete mobility shift of TonEBP, as was shown in Figure 4D.

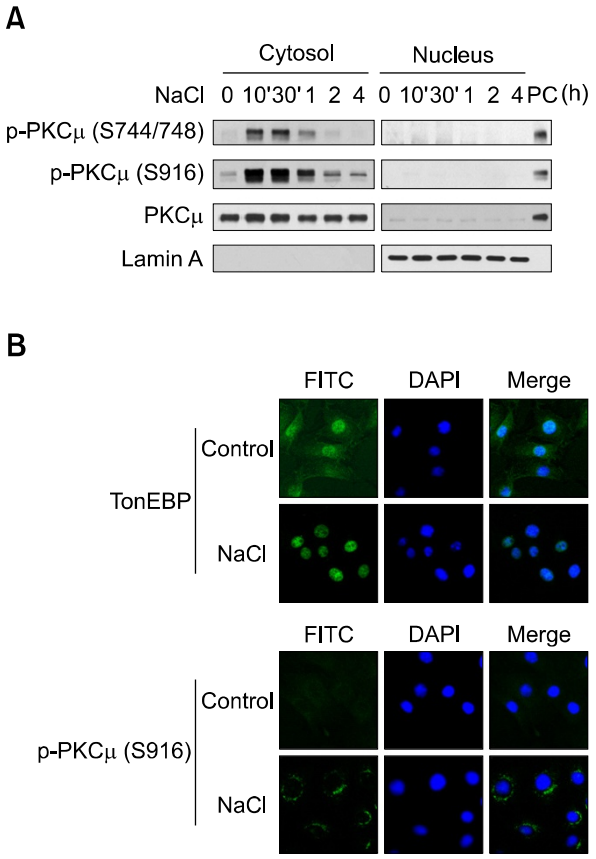

Figure 5. Subcellular localization of PKCμ and TonEBP under hypertonic conditions. (A) Subcellular localization of PKCμ and phosphorylated PKCμ. NIH-3T3 cells were exposed to hypertonic conditions for the indicated time periods. Cytosolic and nuclear fractions were prepared as described in the Materials and Methods section. The cellular fractions were resolved via SDS-PAGE and blotted with anti-PKCμ and anti-phospho PKCμ antibodies. PC designates the positive control sample (cytosol fraction of cells treated with 130 mM NaCl for 30 min). B. Immunofluorescence detection of PKCμ localization. Immunofluorescence stainings were conducted using anti-TonEBP and anti-phospho PKCμ primary antibodies and FITC-conjugated secondary antibodies in cells exposed for 30 min to hypertonicity.

PKCμ indirectly regulates TonEBP modification under hypertonic condition

We determined that PKCμ mediated a complete mobility shift of TonEBP, thereby inducing HSP70 expression under hypertonic conditions. We attempted to determine whether hypertonicity-activated PKCμ could directly regulate TonEBP modification. TonEBP was translocated rapidly into the nucleus under hypertonic conditions, as is shown in Figure 4C. However, phosphorylated PKCμ was not detected in the nuclei (Figure 5A). Phosphorylated PKCμ and TonEBP evidenced different subcellular localization characteristics (Figure 5B). Whereas TonEBP was translocated into the nucleus, phosphorylated PKCμ accumulated in the peri-nuclear region. We observed no physical interaction occurring between PKCμ and TonEBP (data not shown). These results show that PKCμ indirectly regulates the activation of TonEBP, thereby inducing the expression of HSP70.

Discussion

HSP70 is one of the most conserved proteins from bacteria to humans. Whereas HSC70 is expressed constitutively, HSP70 can be induced by a variety of stresses (Morimoto *et al*., 1992; Wagner *et al*., 1999). Induced HSP70 protects cells against stresses by virtue of its ability to chaperone denatured proteins and to prevent the activation of the apoptotic pathway (Lee *et al*., 2005). We reported previously that hyperosmolarity induced only hsp70.1 expression (Lee and Seo, 2002). It has been reported that the hsp70.1 gene harbors TonE cis-acting elements within its promoter region (Heo *et al*., 2006). As the trans-acting factor for the TonE site, TonEBP, was recently identified (Miyakawa *et al*., 1998), it could be surmised that TonEBP is activated in the induction of hsp70.1 gene expression under hypertonic conditions. Several papers have reported that TonEBP stimulates the trans-

cription of HSP70 in response to hypertonicity (Woo *et al*., 2002; Na *et al*., 2003; Wang, 2006; Neuhofer *et al*., 2007). However, until now, there have been no reports concerning the precise signaling pathway of TonEBP activation and HSP70 induction under hypertonic conditions.

In this study, we attempted to address the question as to the manner in which TonEBP is involved in the expression of hsp70.1 under hypertonic conditions. First, in order to determine which kinases mediate hypertonicity-induced HSP70 expression, we administered several kinase inhibitors during the process of hypertonicity-induced HSP70 expression. MAPKs perform critical functions in a variety of signaling pathways in mammalian cells. It has been reported that p38 kinase is an essential kinase for the hypertonic induction of HSP70, and the dual control of p38 and Fyn tyrosine kinase regulates the functioning of TonEBP via the transactivation domain (Sheikh-Hamad *et al*., 1998; Ko *et al*., 2002). ERK has been studied as a signaling molecule for the induction of the *de novo* expression of proteins including HSP70, BGT-1 (sodium/chloride/betain cotransporter 1), SMIT (sodium/ myo-inosito cotransporter), and TauT (sodium/chloride/taurine cotransporter) under hypertonic conditions (Ho, 2003; Uhlik *et al*., 2003; Tsai *et al*., 2007). We determined that hypertonicity activated ERK and p38, but not JNK, during hypertonicity treatment. However, we found no evidence to suggest that MAPKs are involved in the hypertonicity-induced expression of HSP70 (Figure 1B-D). GF109203X (an inhibitor of novel and conventional PKC isoforms) and Gö6976 (an inhibitor of PKCμ, PKCα, and PKCβI isoforms) caused a reduction in TonEBP-dependent HSP70 expression (Figure 1E). More specifically, when cells were transfected with PKCμ siRNA, the induction of HSP70 was inhibited (Figure 2E and 3B). The effects of PKC inhibition on TonEBP activation were also observed. The mobility shift of TonEBP located in the nucleus was affected by treatment with PKC inhibitors (Figure 4C and D). Since it has been established that the PLC/DAG/PKC signaling cascade performs a crucial function in the activation of PKCμ (Rozengurt *et al*., 2005; Wang, 2006), we surmised that the activation of PKCμ by hypertonicity might be mediated by the upstream kinase PKC. To the best of our knowledge, this study is the first report to demonstrate that PKCμ plays an essential role in hypertonicity-induced HSP70 expression.

Even though HSF1 is a general transcription activator for the induction of HSP70 under a variety of stressful conditions (Morimoto *et al*., 1996), we demonstrated that HSF1 was neither activated nor translocated to the nucleus under hypertonic conditions, by way of contrast with heat shock treatment (Figure 4A and B). Instead of HSF1, TonEBP was translocated into the nucleus and then post-translationally modified to respond to hypertonicity (Figure 4 C and D). TonEBP is a member of the Rel family of transcriptional activators, which includes NF-κB and NFAT (nuclear factor of activated T-cells) (Woo *et al*., 2002). TonEBP stimulates the transcription of several genes, including BGT1, SMIT, TauT, and AT (aldorase reductase), to protect cells against the deleterious effects of hypertonicity, which principally occurs via the attenuation of cellular ionic strength (Jeon *et al*., 2006). TonEBP also regulates the induction of HSP70. However, the action mechanism of HSP70, which is induced by TonEBP in hypertonic situations, operates differently. Hypertonicity causes double-stranded DNA breaks and increases mitochondrial ROS generation, finally resulting in apoptosis (Zhou *et al*., 2006). We demonstrated that HSP70 protects against hyperosmolarity-induced apoptosis and cellular damage via the prevention of caspase-3 activation (Lee *et al*., 2005). HSP70 induced via the mechanism of PKCμ and TonEBP activation also prevents the activation of caspase-3, the executioner of the hypertonicity-induced apoptosis pathway, ultimately protecting against apoptotic cell death (Figure 3).

TonEBP is activated via subsequent events, including phosphorylation, dimerization, and nuclear translocation under hypertonic conditions (Dahl *et al*., 2001; Lopez-Rodriguez *et al*., 2001; Lee *et al*., 2002). We observed an upward shift in TonEBP which appeared to be the result of phosphorylation, and this event occurred exclusively in the nucleus (Figure 4C and D). TonEBP is gradually modified in a time-dependent manner under hypertonic conditions. Previous research has shown that TonEBP activation is regulated by several kinases, including p38 and Fyn, ATM, and PKA (Ferraris *et al*., 2002; Ko *et al*., 2002; Irarrazabel *et al*., 2006). However, the kinases that directly phosphorylate TonEBP have yet to be clearly identified (Jeon *et al.,* 2006). In addition, we determined that, although the PKC and PKCμ inhibitors inhibited hypertonicity-induced HSP70 expression almost completely, hypertonicity-induced TonEBP modification was partially affected. Therefore, upstream kinases and the molecular mechanisms inherent to the modification of TonEBP under hypertonic conditions remain to be clearly elucidated.

Finally, to assess whether PKCμ is colocalized with TonEBP in the nucleus, we evaluated the distribution of PKCμ under hypertonic conditions. The activated PKCμ remained in the perinuclear

region, which appeared to have accumulated in the Golgi apparatus (Figure 5). It has been reported that PKCμ is localized in several intracellular compartments, including the plasma membrane, Golgi apparatus, mitochondria, and nucleus (Rey and Rozengurt, 2001; Rey *et al*., 2004; Waldron *et al*., 2004; Storz *et al*., 2005). In particular, the precise functions of PKCμ in the Golgi apparatus in the context of hypertonicity-induced HSP70 expression via the activation of TonEBP will require further study.

PKCμ activates NF-κB to protect the cells from oxidative stress-related cell death. Src-Abl activated by oxidative stress induces phosphorylation of PKCμ and leads activation of downstream IKK-NF-κB signaling (Storz and Toker 2003). Additionally, mitochondrial ROS also activate PKCμ and induce the expression of manganese-dependent superoxide dismutase (MnSOD) through activating NF-κB (Storz *et al*., 2005). Reduced ROS production by antioxidant suppresses NaCl-induced TonEBP activation and BGT1 expression (Zhou *et al*., 2006). Therefore, we suggest that PKCμ is a mediator between ROS and TonEBP activation in hypertonic condition. Since, when we inhibited PKCμ using specific inhibitor or siRNA knock-down method, TonEBP phosphorylation and HSP70 induction were evidently decreased, we could conclude that increased ROS by hypertonicity might be the main cause of PKCμ activation and TonEBP-mediated HSP70 gene expression.

In summary, PKCμ is a novel mediator of hypertonicity-induced HSP70 expression. We have demonstrated herein that the PKC/PKCμ/TonEBP signaling cascade performs a function in inhibiting the hypertonicity-induced apoptotic pathway via the induction of the HSP70 protein.

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