ABSTRACT

HUANGFU, WEI-CHUN. Osmotic Stress Activates JNK While Blocking NF-κB Pathway, and Modulates Immune Responses. (Under the direction of Dr. Jun Ninomiya-Tsuji.)

Osmotic stress activates mitogen-activated protein kinases (MAPKs), including c-Jun N-terminal kinase (JNK) and p38, which play important roles in cellular stress responses. Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the MAPK kinase kinase (MAPKKK) family and can activate JNK and p38. TAK1 can also activate IκB kinase (IKK) that leads to degradation of IκB and subsequent NF-κB activation. We found that TAK1 is important for osmotic stress-induced activation of JNK but is not an exclusive mediator of p38 activation. Furthermore, we found that although TAK1 was highly activated upon osmotic stress, it could not induce degradation of IκB or activation of NF-κB. These results suggest that TAK1 activity is somehow modified to function specifically in osmotic stress signaling, leading to the activation of JNK but not of IKK. To elucidate the mechanism underlying this modulation, our group screened for potential TAK1-binding proteins. We determined that TAO2 (thousand-and-one amino acid kinase 2) associates with TAK1 and can inhibit TAK1-mediated activation of NF-κB but not of JNK. It was observed that TAO2 could interfere with the interaction between TAK1 and IKK. Furthermore, TAO2 knockdown reduced osmotic stress-induced activation of JNK, suggesting that TAO2-TAK1 mediates JNK activation. In contrast, TAO2 knockdown moderately increased NF-κB activation following osmotic stress. These results suggest that TAO2 directs TAK1 to JNK pathway while preventing the TAK1-mediated NF-κB activation.

Although TAO2 knockdown slightly upregulates NF-κB, activation of NF-κB is still largely suppressed under osmotic stress, suggesting that NF-κB pathway is regulated through not only TAO2 but also other mechanisms. To determine the TAO2-independnent mechanisms, we

investigated osmotic stress-mediated modification of NF-κB pathway. Even though NF-κB was not activated in response to osmotic stress, we unexpectedly found that osmotic stress quickly activated IκB kinase that phosphorylated IκB. However, the phosphorylated IκB under osmotic stress was poorly ubiquitinated. We found that ubiquitination of β-catenin as well as IκB, both of which are targets of the SCF type E3 ligase, were impaired under osmotic stress. Therefore, it is likely that osmotic stress down-regulates NF-κB through inhibiting SCF type E3 ligase(s). Furthermore, we found that osmotic stress interfered with interleukin 1 (IL-1)-induced ubiquitination of IκB and ultimately blocked NF-κB-mediated expression of cytokine/chemokine. NF-κB is a central regulator of inflammatory immune responses. Hypertonic condition is known to inhibit inflammation and hypertonic saline resuscitation is clinically used to prevent inflammation after trauma. The results in this study have revealed that hypertonicity-induced immune suppression is at least in part through inhibition of ubiquitination of IκB.

Taken together, data in this thesis demonstrate that osmotic stress suppresses NF-κB pathway through at least two-independent mechanisms, namely TAO2-mediated modification of TAK1 and inhibition of IκB ubiquitination, which modify immune responses.

OSMOTIC STRESS ACTIVATES JNK WHILE BLOCKING NF-κB PATHWAY, AND MODULATES IMMUNE RESPONSES

by

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BIOGRAPHY

Wei-Chun HuangFu was born in Taipei, Taiwan on November 27, 1976. She was the oldest of two in the family. She graduated from National Tsing-Hua University in Hsinchu, Taiwan in 1998 with a bachelor degree in Atomic Science. For two years Wei-Chun worked as a research assistant at Institute of Chemistry, Academia Sinica in Taiwan before relocating to Durham, North Carolina, to pursue graduate study in environmental toxicology. She received a Master of Environmental Management at Nicholas School of the Environment from Duke University in 2002. In the same year, she joined the department of Environmental and Molecular Toxicology at North Carolina State University to further pursue her Ph.D. in molecular toxicology under the mentorship of Dr. Jun Ninomiya-Tsuji. Following the completion of her Ph.D., Wei-Chun will begin to work as a postdoctoral fellow at University of Pennsylvania.

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This project would not have been possible without the support of many people. Many thanks to my advisor Dr. Jun Ninomiya-Tsuji for giving me the great opportunity to work with her on the exciting projects as well as training me to become a scientist. Also thanks to my committee members, Dr. Robert Smart, Dr. Yoshiaki Tsuji, and Dr. Robert Rose, who offered invaluable guidance and support.

Special thanks should be given to my colleagues, Dr. Emily Omori, Dr. Maiko Inagaki, Dr. Rie Sakamoto, Dr. Taisuke Kajino, JaeYoung Kim, Noriyuki Uemura, and Pete Broglie, for giving me advice and sharing great ideas with me in my research. And finally, words alone cannot express the thanks I owe to my parents, and I-Hsuan, my fiance, who endured this long process with me, always offering support and love.

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GENERAL DISCUSSION

GENERAL INTRODUCTION

(Some paragraphs are adapted from my two manuscripts and modified)

Mitogen activated kinase cascades and osmotic stress

Mitogen activated kinase (MAPK) cascades play important roles in many extracellular stimuli-activated intracellular signaling pathways (Davis, 2000; Johnson and Lapadat, 2002; Pearson et al., 2001). These stimuli include not only growth factors and cytokines but also chemical and physical stresses such as ultraviolet light irradiation and osmotic stress. Among the three well-characterized MAPK subfamilies, extracellular signal-regulated kinase (ERK), JNK, and p38 MAPK, JNK and p38 are the major regulators of stress-induced cellular responses. In mammalian cell cultures, high osmolarity generated by adding salt or sugar into the culture medium activates JNK and p38, which in turn up-regulates a number of genes involved in cellular stress responses, such as production of osmolytes in renal cells (Sheikh-Hamad and Gustin, 2004). In addition, the JNK and p38 pathways regulate cell survival and cell death (Nakano et al., 2006; Varfolomeev and Ashkenazi, 2004; Wada and Penninger, 2004). JNK and p38 are activated by the MAPK kinases (MAPKKs) MKK4/MKK7 and MKK3/MKK6, respectively. The MAPKKs are normally activated by members of the MAPKK kinase (MAPKKK) family that includes MEKKs, mixed lineage kinases (MLKs), apoptosis-stimulating kinase 1 (ASK1), and others in response to engagement of cell surface receptors or stresses. Although the MAPKKKs are believed to confer signal and stimuli specificity, the particular MAPKKKs that are essential to each chemical and physical stress have not yet been well defined.

Transforming growth factor-β-activated kinase 1

Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the MAPKKK family and plays an essential role in tumor necrosis factor (TNF), interleukin 1 (IL-1), and Toll-like receptor (TLR) signaling pathways (Ninomiya-Tsuji et al., 1999; Omori et al., 2006; Sato et al., 2005; Shim et al., 2005; Takaesu et al., 2000; Takaesu et al., 2001). TAK1 functions as a MAPKKK in these pathways and is indispensable for the activation of JNK and p38. Furthermore, TAK1 is also essential for TNF-, IL-1-, and TLR ligand-induced activation of IκB kinase (IKK), which leads to the subsequent degradation of IκB and activation of transcription factor NF-κB (please see "NF-κB and immune responses" below for further details). TAK1-/- or TAK1Δ/Δ (expressing a kinase-dead TAK1) (see Manuscript I page 36, Fig. 2A) cells do not activate JNK, p38, or NF-κB upon treatment with TNF, IL-1, or TLR ligands (Sato et al., 2005; Shim et al., 2005). Several earlier studies have shown that TAK1 can be activated by chemical and physical stresses, including osmotic stress (Cheung et al., 2003; Shirakabe et al., 1997; Singhirunnusorn et al., 2005). However, the role of TAK1 in stress signaling has not yet been defined.

TAO2 (thousand-and-one amino acid kinase 2)

Thousand-and-One Amino Acid Kinase 1 (TAO1) and its related kinase Thousand-and-One Amino Acid Kinase 2 (TAO2) belong to the Ste20 MAPKKK kinase (MAP4K) family kinases, many of which phosphorylate and activate MAPKKK. Although TAO1 and TAO2 are structurally closely related to MAP4K, Dr. Cobb's group has defined that they function as MAPKKK. TAO1 and TAO2 directly associate with and phosphorylate MKK3 MAPKK (Chen and Cobb, 2001; Chen et al., 1999; Hutchison et al., 1998). TAO1 and TAO2 have been

implicated in osmotic stress signaling as well as G protein coupled receptor signaling via activation of the MAPK pathway (Chen et al., 2003). However, the regulation and physiological roles of TAO1 and TAO2 remain to be elucidated.

Hypertonicity suppresses immune responses

It has long been known that use of hypertonic fluid for resuscitation after trauma has some benefits compare to isotonic fluid (Dubick et al., 2006). Hypertonic solutions have been shown to reduce inflammation associated with resuscitated hemorrhage shock, which results in reduction of organ failure caused by systemic inflammation (Angle et al., 1998; Powers et al., 2005; Zakaria el et al., 2006). Administration of hypertonic saline increases serum osmolality and blocks production of proinflammatory cytokines such as TNF in an animal model system (Oreopoulos et al., 2004). *In vitro* studies have revealed that hypertonic condition inhibits activation of neutrophils and macrophages (Cuschieri et al., 2002; Junger et al., 1998; Rizoli et al., 1999). However the molecular mechanism underlying this inhibition has not been determined. Understanding the mechanism of how osmotic stress inhibits inflammation is required to develop an effective therapeutic strategy.

Note- Hypertonicity v.s. Hyperosmolarity

Tonicity is different from osmolarity. Tonicity is the ability of a solution to cause water movement. It applies to the impermeable solutes such as sodium chloride (NaCl) and sorbitol that cannot pass the plasma membrane thus contributes to tonicity. If the solution is hypertonic, the water concentration is higher inside the cell. Thus water will move from inside to out side of the cell. On the other hand, osmolarity is a measure of the osmotically active particles in a solution

regardless of permeability of the solutes since it takes into account of both permeable and impermeable solutes. Osmotic stress to the cells actually occurs not under all hyperosmotic conditions but only when the concentration of impermeable solutes in the solution is too high to draw water out of the cells (=hypertonic condition). It is well established that osmotic shock such as NaCl and sorbitol lead to the activation of stress-activated MAPK, including JNK (Galcheva-Gargova et al., 1994; Rosette and Karin, 1996).

NF-κB and immune responses

NF-κB is the major transcription factor to regulate inflammatory genes (Rothwarf and Karin, 1999). In response to many receptors involved in immunity including T-cell receptors (TCRs) and B-cell receptors (BCRs), TNFR, CD40, and the Toll/IL-1R family, NF-κB regulates the expression of cytokines, growth factors, and effector enzymes (Hayden and Ghosh, 2004). In unstimulated cells, a complex of NF-κB and its inhibitory protein IκB resides in the cytoplasm. IκB masks the nuclear localization signal of NF-κB (Ziegler and Ghosh, 2005). Proinflammatory stimuli such as IL-1 and TNF initiate the intracellular signaling leading to activation of IκB kinase (IKK) complex consist of IKKα, IKKβ and IKKγ/NEMO. IKK in turn phosphorylates IκB at Ser-32 and Ser-36 that are recognized by β-transducin repeats-containing protein (βTrCP), a subunit of the Skp1.Cullin1.Roc1.F-box protein complex (SCF) type E3 ubiquitin ligase (Karin and Ben-Neriah, 2000). Phosphorylated IκB is ubiquitinated through the SCF E3 ligase complex and degraded through 26S proteosomes. NF-κB then translocates into the nucleus and functions as a transcription factor for many proinflammatory genes including cytokines and chemokines.

Ubiquitin-proteosome pathway

Ubiquitin-proteosome pathway is a major pathway to degrade old, damaged, or misfolded proteins and sometime recycle the proteins in cells. It also functions in controlling many regulatory proteins including oncoproteins, transcription factors, and cell cycle proteins (Varshavsky, 1997). In general, the attachment of ubiquitin marks proteins for rapid degradation and degradation of a protein via the ubiquitin pathway is a multi-step process. First, ubiquitin is activated by attachment to the ubiquitin-activating enzyme, E1. The ubiquitin is then transferred to the ubiquitin-conjugating enzyme (E2). And the E2 enzyme collaborates with E3 protein-ubiquitin ligase to attach the ubiquitin molecule to the lysine residues of the substrate. In yeast, plants, and animals, a single E1 activates ubiquitin, whereas many E2s and multiple families of E3 enzymes were characterized (Haas and Siepmann, 1997). Different members of the E2 and E3 families recognize different substrate proteins, and these different combinations of E2 and E3 enzymes can selectively target specific cellular proteins for degradation by the ubiquitin-proteasome pathway. There are several types of E3 ligases, SCF, HECT, and RING finger types. SCF βTrCP recognizes two phosphorylated serine amino acid residues of IκB and β-catenin and targets them for degradation.

TAO2 regulation of TAK1 in osmotic stress signaling

In the first part of the study, we demonstrate the important role of TAK1 in osmotic stress signaling using TAK1Δ/Δ cells. We found that TAK1 was important for osmotic stress-induced activation of JNK but not of p38. We also found that although TAK1 was highly activated upon osmotic stress treatment, this did not result in IκB degradation or subsequent activation of NF-κB. These results suggest that TAK1 is regulated to activate only the MAPK cascades and not the

NF-κB pathway in osmotic stress signaling. We attempted to elucidate the mechanism underlying this regulation of TAK1. We found that a TAK1-binding kinase, TAO2 (thousand-and-one amino acid kinase 2) blocked the TAK1-mediated NF-κB pathway by preventing the interaction of TAK1 with IKK, yet did not inhibit TAK1-induced activation of JNK. These results suggest that binding by TAO2 may modulate the specific cellular responses of TAK1.

Osmotic stress inhibits ubiquitination of IκB

Although TAO2 is a major regulator of TAK1 in osmotic stress signaling, we found that downregulation of TAO2 cannot dramatically increase activation of NF-κB in response to osmotic stress. This suggests that there is a TAO2-independent mechanism to inhibit activation of NF-κB. In the second part of the study, we investigated regulation of NF-κB pathway in response to osmotic stress. Surprisingly, we found that osmotic stress activates IKK and IκB is phosphorylated. However, the ubiquitination and subsequent degradation of IκB was strikingly impaired under hypertonic condition. Furthermore, hypertonic preconditioning blocked IL-1-induced IκB degradation. IL-1-induced ubiquitination of IRAK was not altered in the presence or absence of osmotic stress, which indicates that osmotic stress does not generally inhibit ubiquitination. We found that ubiquitination of β-catenin was also impaired by osmotic stress. These results suggest that osmotic stress interferes with function of SCF E3 ligase(s) and thereby blocks NF-κB pathway.

RESEARCH HYPOTHESES

Manuscript I

TAK1 MAPKKK is activated by TGF-β family ligands and many cytokines as well as stresses. TAK1 plays essential roles in IL-1, TNF, and TLR signaling pathways by activating both JNK and NF-κB. TAK1 pathways play a crucial role in regulating the genes that mediate inflammation. TAK1 is also activated by stresses including osmotic stress. However, the role of TAK1 in stress signaling has yet to be defined.

TAK1 is important for osmotic stress-mediated JNK activation. In response to IL-1 or TNF, TAK1 is activated and can in turn activate both JNK and NF-κB. However, osmotic stress selectively activates JNK without activating NF-κB. It is plausible that a TAK1 binding protein may modulate TAK1 to direct JNK but to prevent activation of NF-κB.

We hypothesize that TAK1 when activated by osmotic stress, recruits an unidentified protein, resulting in activation of JNK but not NF-κB.

Specific aims:

1) To determine whether TAK1 is essential for osmotic stress-induced JNK and p38 activation.
2) To determine the mechanism by which TAK1 is modified to activate JNK but not NF-κB.

Manuscript II

Recent studies have suggested that hypotonic saline exerts anti-inflammatory effects, which is beneficial in preventing organ failure. NF-κB is a major mediator of inflammation signaling. In the second manuscript, we further investigated other mechanisms that prevent NF-κB activation in hypotonic condition. Our preliminary results demonstrated that osmotic stress inhibits IκB degradation thereby blocking NF-κB activation. We hypothesize that hypotonic condition reduces inflammation through this mechanism. The research was focused on determining how IκB was stabilized under the condition of osmotic stress and delineating relationship between osmotic stress and inflammation.

Specific aims:

1) To determine the mechanism by which degradation of IκB is impaired under conditions of osmotic stress.
2) To determine whether osmotic stress could block IL-1-induced NF-κB activation.
3) To determine whether osmotic stress could block IL-1-induced cytokine/chemokine production.

MANUSCRIPT I

Osmotic Stress Activates the TAK1-JNK Pathway While Blocking TAK1-mediated NF-κB Activation: TAO2 Regulates TAK1 Pathways

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ABSTRACT

Osmotic stress activates MAPKs, including JNK and p38, which play important roles in cellular stress responses. Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the MAPK kinase kinase (MAPKKK) family and can activate JNK and p38. TAK1 can also activate IκB kinase (IKK) that leads to degradation of IκB and subsequent NF-κB activation. We found that TAK1 is essential for osmotic stress-induced activation of JNK but is not an exclusive mediator of p38 activation. Furthermore, we found that although TAK1 was highly activated upon osmotic stress, it could not induce degradation of IκB or activation of NF-κB. These results suggest that TAK1 activity is somehow modified to function specifically in osmotic stress signaling, leading to the activation of JNK but not of IKK. To elucidate the mechanism underlying this modulation, we screened for potential TAK1-binding proteins. We found that TAO2 (thousand-and-one amino acid kinase 2) associates with TAK1 and can inhibit TAK1-mediated activation of NF-κB but not of JNK. TAO2 can inhibit TAK1-mediated activation of NF-κB but not of JNK. We observed that TAO2 can interfere with the interaction between TAK1 and IKK and thus may regulate TAK1 function. TAK1 is activated by many distinct stimuli, including cytokines and stresses, and regulation by TAO2 may be important to activate specific intracellular signaling pathways that are unique to osmotic stress.

INTRODUCTION

MAPK cascades play important roles in many extracellular stimuli-activated intracellular signaling pathways (Davis, 2000; Johnson and Lapadat, 2002; Pearson et al., 2001). These stimuli include not only growth factors and cytokines but also chemical and physical stresses such as ultraviolet light irradiation and osmotic stress. Among the three well characterized MAPK subfamilies, extracellular signal-regulated kinase (ERK), JNK, and p38 MAPK, JNK and p38 are the major regulators of stress-induced cellular responses. In mammalian cell cultures, high osmolarity generated by adding salt or sugar into the culture medium activates JNK and p38, which in turn up-regulates a number of genes involved in cellular stress responses, such as production of osmolytes in renal cells (Sheikh-Hamad and Gustin, 2004). In addition, the JNK and p38 pathways promote cell death, which is important for the elimination of dysfunctional cells (Nakano et al., 2006; Varfolomeev and Ashkenazi, 2004; Wada and Penninger, 2004). JNK and p38 are activated by the MAPK kinases (MAPKKs) MKK4/MKK7 and MKK3/MKK6, respectively. The MAPKKs are normally activated by members of the MAPKK kinase (MAPKKK) family that includes MEKKs, mixed lineage kinases (MLKs), apoptosis-stimulating kinase 1 (ASK1), and others in response to engagement of cell surface receptors or stresses. Although the MAPKKKs are believed to confer signal and stimuli specificity, the particular MAPKKKs that are essential to each chemical and physical stress have not yet been well defined.

Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the MAPKKK family and plays an essential role in tumor necrosis factor (TNF), interleukin 1 (IL-1), and Toll-like receptor (TLR) signaling pathways (Ninomiya-Tsuji et al., 1999; Omori et al., 2006; Sato et al.,

2005; Shim et al., 2005; Takaesu et al., 2000; Takaesu et al., 2001). TAK1 functions as a MAPKKK in these pathways and is indispensable for the activation of JNK and p38. Furthermore, TAK1 is also essential for TNF-, IL-1-, and TLR ligand-induced activation of IκB kinase (IKK), which leads to the subsequent degradation of IκB and activation of transcription factor NF-κB. TAK1-/- or TAK1Δ/Δ (expressing a kinase-dead TAK1) (Fig. 2A) cells do not activate JNK, p38, or NF-κB upon treatment with TNF, IL-1, or TLR ligands (Sato et al., 2005; Shim et al., 2005). Several earlier studies have shown that TAK1 can be activated by chemical and physical stresses, including osmotic stress (Cheung et al., 2003; Shirakabe et al., 1997; Singhirunnusorn et al., 2005). However, the role of TAK1 in stress signaling has not yet been defined.

In this study, we demonstrate the essential role of TAK1 in osmotic stress signaling using TAK1Δ/Δ cells. We found that TAK1 is essential for osmotic stress-induced activation of JNK but not of p38. We also found that although TAK1 is highly activated upon osmotic stress treatment, this did not result in IκB degradation or subsequent activation of NF-κB. These results suggest that TAK1 is regulated to activate only the MAPK cascades and not the NF-κB pathway in osmotic stress signaling. We attempted to elucidate the mechanism underlying this regulation of TAK1. We demonstrate here that a TAK1-binding kinase, TAO2 (thousand-and-one amino acid kinase 2) blocks the TAK1-mediated NF-κB pathway by preventing the interaction of TAK1 with IKK, yet did not inhibit TAK1-induced activation of JNK. These results suggest that binding by TAO2 may modulate the specific cellular responses of TAK1.

MATERIALS AND METHODS

Cell Culture and Reagents—Human embryonic kidney 293 cells, 293 IL-1RI cells that stably expresses IL-1 receptor I (Cao et al., 1996), and TAK1Δ/Δ mouse embryonic fibroblasts (MEFs) (Sato et al., 2005) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone) at 37 °C in 5% CO_2. TAK1 wild type and Δ/Δ keratinocytes (Omori et al., 2006) were cultured in Ca^{2+}-free Eagle's minimal essential medium (BioWhittaker) supplemented with 4% Chelex-treated bovine growth serum, 10 ng/ml human epidermal growth factor (Invitrogen), and 0.05 mM calcium chloride at 33 °C in 8% CO_2. For transfection studies, 293 cells (1×10^6 or 1.6×10^5) were seeded onto 10-cm or 6-well (3.5-cm) dishes, respectively, and transfected with various expression vectors by the standard calcium phosphate precipitation method 24 h after seeding. 36 h after transfection, cells were harvested in 0.5% Triton X-100 lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 μM aprotinin. To induce osmotic stress, cells were treated with NaCl (0.5 or 0.7 M). For cytokine stimulation, 10 ng/ml IL-1-β or 20 ng/ml TNF-α (Roche Applied Science) was used. Stress agents were added 3-30 min prior to harvest.

Expression Plasmids—Expression vectors for HA-tagged and FLAG-tagged TAO1 and TAO2 were generated from the plasmids pCMV5-HA-TAO1(1-1001), pCMV5-Myc-TAO2(1-993), and pCMV5-Myc-TAO2(1-993)D169A (provided by Dr. Melanie Cobb, University of Texas, Southwestern Medical Center, Dallas) (Chen and Cobb, 2001; Chen et al., 1999; Chen et al., 2003; Hutchison et al., 1998) . To generate the pCMV-HA-TAO1 vector, an SacI/NheI-digested

fragment from pCMV5-HA-TAO1(1-1001) and a synthesized 50-bp deoxyoligonucleotide consisting of a 5' EcoRI site, an ATG codon, and TAO1 sequences to the first SacI restriction site were subcloned into the pCMV-HA mammalian expression vector. The gene insert was verified by sequencing. To generate the pCMVHA-and pCMV-FLAG-TAO2(1-993) and pCMV-HA-and pCMV-FLAG-TAO2(1-993)D169A vectors, EcoRI/BamHI-digested fragments from pCMV5-Myc-TAO2(1-993) and pCMV5-Myc-TAO2(1-993)D169A were subcloned into the pCMV-HA and pCMV-FLAG mammalian expression vectors. TAO2(1-993) was used instead of full-length TAO2 due to poor expression of the full-length gene (Chen et al., 2003). Mammalian expression vectors encoding T7-tagged TAK1 (T7-TAK1), T7-tagged TAB1 (T7-TAB1), and HA-tagged IKKα (HA-IKKα) were described previously (Kishida et al., 2005; Uemura et al., 2006).

Antibodies—Polyclonal rabbit antibodies to TAK1 (anti-TAK1) and TAB1 (anti-TAB1) were described previously (Ninomiya-Tsuji et al., 1999). Polyclonal rabbit antibody against TAO2 (anti-TAO2) was produced against peptides corresponding to amino acids 21-33 (Operon Biotechnologies). Anti-phospho-SAPK/JNK (Thr-183/Tyr-185), anti-phospho-p38 (Thr-180/Tyr-182), anti-phospho-IκBα (Ser-32), and anti-phospho-TAK1 (Thr-187) rabbit polyclonal antibodies (Cell Signaling) were used to detect the phosphorylated forms of JNK, p38, IκBα, and TAK1. Anti-JNK1 (FL), anti-p38 (N-20), anti-IκBα (C-21), anti-IKK (H-744), and anti-NF-κB p65 (C-20) polyclonal antibodies (Santa Cruz Biotechnology) and anti-HA (HA.11) monoclonal antibody (Covance), anti-HA (Y-11) polyclonal antibody (Santa Cruz Biotechnology), anti-T7 monoclonal antibody (Novagen), and anti-FLAG M2 monoclonal antibody (Sigma) were used for immunoprecipitation and immunoblotting.

Immunoprecipitation-Immunoblotting—Cells were lysed in the 0.5% Triton X-100 lysis buffer described above. Cellular debris was removed by centrifugation at 10,000 x *g* for 5 min at 4 °C, and the supernatant was collected. Proteins from the lysates were immunoprecipitated with various antibodies. Immunoprecipitates and whole cell extracts were resolved by SDS-PAGE and transferred to Hybond-P membranes (Amersham Biosciences). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse immunoglobulin G (IgG) using the ECL Western blotting system (GE Healthcare).

Electrophoretic Mobility Shift Assay (EMSA)—The binding reactions contained radiolabeled ^{32}P-NF-κB oligonucleotide probe (Promega), 40 μg of cell extracts, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 500 ng of poly(dI-dC) (GE Healthcare), and 10 μg of bovine serum albumin to a final volume of 15 μl. The reaction mixtures were incubated at 25 °C for 30 min, separated by 5% (w/v) polyacrylamide gel, and visualized by autoradiography.

In Vitro Activation—*In vitro* activation was performed as described previously (Janssens et al., 2005; Tinel and Tschopp, 2004). Briefly, 293 cells were washed twice with 1x phosphate-buffered saline and resuspended in hypotonic buffer (20 mM HEPES-KOH, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT (pH 7.5) supplemented with protease inhibitors (10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 20 μM aprotinin). Resuspended cells were lysed by passing through a 22-gauge needle 10 times. Cellular debris was removed by centrifugation at 10,000 x *g* for 20 min at 4 °C followed by filtration at 0.45 μm. Clear cell lysates

were collected and incubated at 37 °C for the indicated times or left untreated on ice. After incubation, an equivalent volume of hypotonic buffer containing 0.1% Nonidet P-40 and 300 mM NaCl was added.

NF-κB Reporter Assay—293 cells (1.6 x 10^5 cells/well) were seeded onto 6-well (35-mm) dishes 24 h before transfection. Cells in each well were transfected with 0.1 μg of an Ig-κB-luciferase reporter plasmid, 0.1 μg of a control reporter plasmid pAct-β-Gal (β-galactosidase under the control of the β-actin promoter), and various expression constructs by the calcium phosphate precipitation method. The total amounts of DNA in each well were kept constant by supplementing with empty vector. After 36 h, cells were treated with or without TNF (20 ng/ml) for 12 h prior to harvesting using reporter lysis buffer (Promega), and luciferase activity was assessed via luminometry. Transfection efficiencies were normalized by β-galactosidase activities from pAct-β-Gal. The NF-κB luciferase activity was expressed as the mean of the normalized luciferase activity from duplicated experiments.

In Vitro Kinase Assays—TAK1 was immunoprecipitated, and the immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 1 μg of bacterially expressed His-MKK6 in 10 μl of kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$ at 25 °C for 2 min. IKK was immunoprecipitated, and the immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol) and 1 μg of bacterially expressed GST-IκB in 10 ml of kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 100 μM ATP at 30 °C for 30 min. Samples were then separated by 10% SDS-PAGE and visualized by autoradiography.

siRNAs—siRNAs were purchased from Ambion. siRNA used as control was Silencer® negative control number 1 siRNA. Human TAO2 siRNAs used were pre-designed siRNAs 118287 (TAO2 siRNA 1) and 118285 (TAO2 siRNA 2) (Ambion). 293 cells were plated onto 6-well plates and transfected with siRNAs (30-50 nmol) using siPORT amine transfection agent (Ambion). Cells were incubated for 48 h post-transfection and were stimulated with 0.5 M NaCl. The reduction of TAO2 expression was monitored by real time PCR using human TAO2-specific primer (Qiagen).

RESULTS

We have demonstrated previously that TAK1 activates JNK, p38, and NF-κB in the TNF and IL-1 signaling pathways (Ninomiya-Tsuji et al., 1999; Sato et al., 2005; Takaesu et al., 2003). Ablation of TAK1 markedly impairs TNF- and IL-1-induced activation of JNK, p38, and NF-κB. TAK1 is activated not only by proinflammatory cytokines but also in response to physical and chemical stresses, including osmotic stress. We found that among several physical and chemical stresses, including oxidative stress, arsenic, and ultraviolet light irradiation, osmotic stress is the most potent activator of TAK1 in human embryonic kidney 293 and mouse embryonic fibroblasts (MEFs) (data not shown). To begin to define the role of TAK1 in osmotic stress signaling, we examined activation of TAK1, JNK, p38, and NF-κB following treatment by IL-1 or osmotic stress. 293 IL-1RI cells that stably express the IL-1 receptor were treated with IL-1 or high osmolarity. The activation of TAK1 was monitored by *in vitro* kinase assay using MKK6 as an exogenous substrate (Fig. 1A) TAK1 was activated by osmotic stress at higher levels relative to activation by IL-1. Activation of TAK1 can be also assessed by the appearance of phosphorylated forms of TAK1 and its binding partner TAK1-binding protein 1 (TAB1), resulting in slower migration on SDS-PAGE (Kishimoto et al., 2000) (Fig. 1A, 2nd panel). Phosphorylation of TAK1 and TAB1 was also observed upon osmotic stress. JNK and p38 were activated following TAK1 activation at higher levels in cells subjected to osmotic stress compared with IL-1-treated cells (Fig. 1A, 3rd and 4th panels). Activation of NF-κB was monitored by phosphorylation and degradation of IκB (Fig. 1A, 5th and bottom panels). In contrast to JNK and p38, only minor phosphorylation and degradation of IκB were observed in

osmotic stress-treated cells. To further confirm this lack of NF-κB activation by osmotic stress, we examined DNA binding of NF-κB by electrophoretic mobility shift assay (EMSA) in 293 IL-1RI and parental 293 cells (Fig. 1B). We could not detect any activation of NF-κB in cells subjected to osmotic stress (Fig. 1B, top panel). Under these conditions, IκB phosphorylation was minor, and no IκB degradation was observed (Fig. 1B, middle and bottom panels). These results demonstrate that osmotic stress activates JNK and p38 but not NF-κB, even though TAK1 is activated.

To define the essential role of TAK1 in osmotic stress signaling, we utilized TAK1Δ/Δ MEFs that express a truncated form of TAK1 lacking kinase activity (Fig. 2A). This cell line was established by infecting a Cre-expressing retroviral vector into MEFs in which both TAK1 alleles are flanked by *loxP* (Sato et al., 2005). We examined activation of JNK and p38 following osmotic stress (Fig. 2B). We found that activation of JNK was greatly impaired in these TAK1-deleted cells, whereas p38 was activated at similar or slightly lower levels. These results suggest that TAK1 plays a major role in the activation of JNK in osmotic stress signaling. To determine whether this role of TAK1 is specific to fibroblasts or if TAK1 functions generally as a mediator of JNK activation in osmotic stress, we used TAK1Δ/Δ keratinocytes, which were isolated from mice having a skin epidermis-specific deletion of TAK1 (Omori et al., 2006). Similar to our results with MEFs, we found that osmotic stress could not activate JNK in TAK1-deficient keratinocytes, whereas p38 was activated at slightly lower levels compared with wild type keratinocytes (Fig. 2C). These results demonstrate that TAK1 is an indispensable intermediate in osmotic stress-induced JNK activation. TAK1 and other signaling intermediates likely function redundantly to activate p38 in osmotic stress signaling.

TAK1 can activate JNK, p38 and NF-κB when activated by IL-1 and TNF treatment or when overexpressed. However, our results show that activation of TAK1 by osmotic stress does not lead to the activation of NF-κB. This prompted us to speculate that under some conditions TAK1 activity may be modulated so that it does not activate NF-κB. One possibility is that TAK1 forms a complex with a molecule that interferes with the interaction between TAK1 and the IKK-NF-κB pathway. To identify such a putative molecule, we performed a yeast two-hybrid screen with TAK1 as bait as described previously (Shibuya et al., 1996; Takaesu et al., 2000). Along with TAB1 and TAB2, which are well characterized TAK1-binding partners, we isolated TAO1 as a binding partner of TAK1. TAO1 shares substantial homology with a closely related kinase TAO2 (also called prostate-derived STE20-like kinase, PSK1) (Chen and Cobb, 2001; Chen et al., 1999; Hutchison et al., 1998). TAO1 and TAO2 are of particular interest because they have been reported to be involved in osmotic stress signaling via activation of the MAPK pathway (Chen and Cobb, 2001). We conducted a co-immunoprecipitation assay to assess the interaction of TAK1 with TAO1 and TAO2 (Fig 3, A and B). Both TAO1 and TAO2 were shown to bind with TAK1 as shown by immunoprecipitation analysis. Interaction of TAO2 with TAK1 was independent of its catalytic activity, because a kinase-dead version of TAO2 (TAO2D169A) could also bind to TAK1. Because TAO2 showed a relatively higher affinity to TAK1 compared with TAO1 in several independent experiments, we conducted subsequent experiments using TAO2. We next investigated whether TAO2 and TAK1 could form a complex under more physiological conditions. We raised antibody against TAO2 and attempted to examine the interaction of endogenous TAK1 with TAO2. Although our TAO2 antibody could weakly detect endogenous TAO2 (see Fig. 6A), the efficiency was not sufficient to examine the co-precipitation assay. We therefore ectopically expressed TAO2 and examined its interaction with endogenous

TAK1. We could not detect any co-precipitation of TAO2 with TAK1 in the presence or absence of osmotic stress when we used a lysis buffer containing 0.5% Triton X, which we usually use for immunoprecipitation and immunoblotting. We anticipated that the TAK1-TAO2 complex is unstable under this lysis condition. It has been reported that some endogenous protein complexes can assemble spontaneously in cell lysates after incubation at 37°C, such as in the case of the apoptosome and caspase 2-containing complex (Janssens et al., 2005; Read et al., 2002; Tinel and Tschopp, 2004). We performed co-precipitation assay with endogenous TAK1 following a similar incubation step at 37 °C. TAO2 was found in the TAK1 complex after incubation for 3 h (Fig. 3C). The interaction of TAO2 with TAK1 was specific, as no TAO2 was found in the control precipitates. These results suggest that TAO2 interacts with TAK1 *in vivo*.

It has been demonstrated that TAO2 can activate JNK and p38 (Chen and Cobb, 2001; Chen et al., 1999; Moore et al., 2000). However, the role of TAO2 in the NF-κB pathway has not been examined. We next tested whether TAO2 influences TAK1-mediated NF-κB activation. TAK1 is highly active when ectopically expressed in culture cells together with its activator subunit TAB1 and can activate JNK, p38, and NF-κB (Kishimoto et al., 2000; Ninomiya-Tsuji et al., 1999; Ono et al., 2001; Shibuya et al., 1996). We therefore examined NF-κB-dependent reporter activity in 293 cells co-expressing TAO2 with TAK1 and TAB1. TAO2 alone was unable to activate NF-κB. Furthermore, activation of NF-κB by ectopic expression of activated TAK1 was markedly reduced by co-expression of TAO2 (Fig. 4A). To assess whether TAO2 kinase activity is required for its effect on TAK1, we used a kinase-dead mutant of TAO2. We found that TAO2 could inhibit TAK1-mediated activation of NF-κB independent of its kinase activity. We next tested whether TAO2 could inhibit the TNF-induced NF-κB pathway by

examining TNF activation of IKK and NF-κB-dependent transcription (Fig. 4, B and C). We found that ectopic expression of TAO2 inhibited activation of IKK and subsequent transcription. These results suggest that TAO2 associates with TAK1 and prevents activation of the TAK1-mediated NF-κB pathway. To determine whether the action of TAO2 on TAK1 is specific to the TAK1-mediated NF-κB pathway, we next examined JNK activation. We co-expressed TAO2 with an activated form of TAK1 (TAK1 + TAB1) and monitored activation of JNK (Fig. 4D). Consistent with the earlier study, we found that TAO2 alone could activate JNK (Moore et al., 2000). Furthermore, TAO2 enhanced TAK1-mediated activation of JNK. These results suggest that the TAK1-TAO2 complex can activate JNK but that binding by TAO2 blocks the TAK1-mediated NF-κB pathway.

To determine the mechanism by which TAO2 interferes with the TAK1-NF-κB pathway, we asked whether TAK1 activity is altered when TAO2 is co-expressed (Fig. 5A). We found that TAK1 activity was not changed by co-expression of TAO2, suggesting that association of TAO2 does not directly alter the activity of TAK1. This is consistent with results showing that TAO2 did not inhibit the TAK1-mediated JNK activation described above (Fig. 4D). We have demonstrated previously that TAK1 forms a complex with the IKKs (Kishida et al., 2005) and that this is important for TAK1-mediated activation of the IKK-NF-κB pathway. We therefore speculated that TAO2 might affect the interaction of TAK1 with IKK. We transfected expression vectors for TAK1, IKK, and increasing amounts of TAO2 and conducted co-precipitation assays in 293 cells to examine the relative amounts of TAK1 present in complexes with IKK (Fig. 5B). We found that increasing TAO2 blocked the formation of the TAK1-IKK complex. To further confirm the effect of TAO2 under more physiological conditions, we examined endogenous

interaction of TAK1 with IKK in the presence and absence of ectopic expression of TAO2 (Fig. 5C). Because the interaction of endogenous TAK1 with IKK is unstable, we conducted co-precipitation assays using the 37 °C incubation step as described above. We found that TAO2 blocked the interaction of IKK with TAK1. These results collectively suggest that TAK1, when associated with TAO2, is sequestered away from the IKK complex, thereby activating the JNK but not the NF-κB pathway.

We finally assessed whether TAO2 plays an essential role in osmotic stress signaling by using TAO2 siRNAs. Two siRNAs showed ability to reduce expression of TAO2 by about 60% (Fig. 6A). We examined the JNK and NF-κB activation upon osmotic stress in the TAO2 knockdown cells using these siRNAs (Fig. 6, B and C). Two independent TAO2 siRNAs reduced osmotic stress-induced JNK activation, which suggest that TAO2 plays at least partially an essential role in the osmotic stress-JNK pathway. Activation of TAK1 was determined by detecting the essential phosphorylation of its activity (Singhirunnusorn et al., 2005). We found that osmotic stress-induced activation of TAK1 was not altered by TAO2 knockdown (Fig. 6B, *left side, bottom panel*). These results suggest that TAO2 is not an upstream kinase of TAK1 in the osmotic stress signaling pathway but rather functions in parallel with TAK1 to lead to JNK activation. NF-κB was slightly activated upon osmotic stress in TAO2 knockdown cells (Fig. 6C). These results further support our conclusion that TAO2 facilitates the TAK1-JNK pathway but blocks the TAK1-NF-κB pathway. However, the induction of NF-κB was marginal in TAO2 knockdown cells. This suggests that NF-κB activation might be blocked by not only TAO2 but also through other mechanisms.

DISCUSSION

TAK1 can be activated by diverse stimuli, including the proinflammatory cytokines TNF, IL-1, Toll-like receptor ligands, and physical and chemical stresses. The role of TAK1 in each of these signaling pathways has been investigated using cells from genetically engineered TAK1-deficient mice, as well as with siRNA gene knockdown methods. TAK1 has been shown to be an indispensable intermediate of TNF, IL-1, and Toll-like receptor signaling in mammalian cells (Omori et al., 2006; Sato et al., 2005; Shim et al., 2005; Takaesu et al., 2003). In *Drosophila*, TAK1 is essential for activation of MAPK in response to innate immune stimuli and plays a redundant role together with MEKK and MLK in stress signaling pathways (Chen et al., 2002). We have demonstrated here that TAK1 is an essential intermediate in osmotic stress induction of JNK in mammalian cells. We also showed that TAK1 is not an exclusive mediator of osmotic stress-induced p38 activation. TAK1 and other MAPKKK family members such as MEKK1 and MLK are likely to function redundantly in the p38 pathway in response to osmotic stress in mammalian cells.

TAK1 is activated in response to many extracellular stimuli, yet TAK1 can initiate cellular responses that are unique to each stimulus. This suggests that TAK1 may be modified by some mechanism to selectively activate different downstream pathways. We have demonstrated here that TAO2 can interact with TAK1 and is responsible for the selective activation of JNK *versus* the NF-κB pathway. TAO2 is activated by osmotic stresses (Chen and Cobb, 2001; Chen et al., 1999); therefore, the TAK1-TAO2 complex may function to mediate osmotic stress signaling. Previously, Mochida *et al.* reported that the MAPKKK ASK1 (apoptosis-stimulating

kinase 1) interacts with and inhibits TAK1 by blocking the interaction of TAK1 with tumor necrosis factor receptor-associated factor 6 in the IL-1 signaling pathway (Mochida et al., 2000). ASK1 is activated by oxidative stress and participates in stress-induced apoptosis (Ichijo et al., 1997; Tobiume et al., 2001). Therefore, it may be possible that the TAK1-ASK1 complex mediates oxidative stress signaling that leads to apoptosis by activating JNK while inhibiting NF-κB.

In this study, we show that TAO2 knockdown only marginally induces NF-κB activation upon osmotic stress. This suggests that TAO2 is not solely responsible for blocking the NF-κB pathway under osmotic stress conditions. The TAO2-related kinase TAO1 may play redundantly to sequester TAK1 from the IKK complex. In addition, to securely block NF-κB activation upon osmotic stress, it is likely that the NF-κB pathway is negatively regulated through several other mechanisms.

NF-κB is a major activator of cell survival signaling (Hayden and Ghosh, 2004; Karin and Lin, 2002). In contrast, activation of JNK and p38 has been generally correlated with apoptotic as well as necrotic cell death (Chang et al., 2006; Nakano et al., 2006; Varfolomeev and Ashkenazi, 2004; Ventura et al., 2004; Wada and Penninger, 2004). Proinflammatory cytokines such as TNF activate both NF-κB and JNK pathways through TAK1. Activation of both pathways is important for gene expression involving inflammatory responses. In inflammatory signaling, cell death is not a desired outcome. Therefore, it is believed that the proinflammatory cytokine-induced NF-κB pathway functions not only to activate inflammatory genes but also aid to cell survival by masking the pro-apoptotic actions of JNK. In contrast, cell death may be a desired outcome in

response to excessive physical and chemical stress, and in these cases NF-κB is not activated and JNK is able to induce cell death. Such cell death is important to eliminate damaged and dysfunctioning cells from the body. Thus, TAK1 regulation in osmotic stress signaling, which activates only JNK while blocking the NF-κB pathway, is important for osmotic stress-induced cell death.

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FIGURE LEGENDS

Fig. 1. Osmotic stress activates TAK1 and JNK but not NF-κB.

(A) 293 cells were subjected to 0.7 M sodium chloride or 5 ng/ml IL-1 for 3 or 15 min. Catalytic activity of endogenous TAK1 was measured in *in vitro* kinase assay using MKK6 as an exogenous substrate (top panel). Activation-coupled migration shift of TAK1 and TAB1 is also shown (2nd panel). Activation of JNK and p38 were monitored by phospho-specific antibodies (3rd and 4th panels). Activation of the NF-κB pathway is monitored by phosphorylation (5th panel) and degradation (bottom panel) of IB. IP, immunoprecipitation; IB, immunoblotting.

(B) 293 IL-1RI and 293 cells were treated with 10 ng/ml IL-1 or 0.5 M sodium chloride for 3-30 min. NF-κB activation was monitored by EMSA (top panel). Activation of the NF-κB pathway was also monitored by transient phosphorylation (2nd panel) and degradation (bottom panel) of IB. Asterisk, nonspecific binding.

Fig. 2. TAK1 is important for osmotic stress-induced JNK but not p38.

(A) Schematic representation of TAK1 deletion.

(B) Wildtype (WT) and TAK1Δ/Δ MEF cells were subjected to osmotic stress (0.5 M NaCl) for 10 or 30 min. Cell extracts were subjected to immunoblotting with anti-phospho-JNK (top panel) and anti-phospho-p38 (3rd panel). The amounts of endogenous JNK, p38, and TAK1 are shown in the 2nd, 4th, and bottom panels, respectively. IB, immunoblotting.

(C) Wild type and TAK1Δ/Δ keratinocytes were subjected to osmotic stress (0.5 M NaCl) for 10 or 30 min. Cell extracts were subjected to immunoblotting with anti-phospho-JNK (top panel)

and anti-phospho-p38 (3rd panel). The amounts of endogenous JNK, p38, and TAK1 are shown in the 2nd, 4th, and bottom panels, respectively.

Fig. 3. TAOs interact with TAK1.

(A) 293 cells were transfected with the expression vectors for T7-TAK1 together with HA-TAO1 as indicated. Left panels, TAO1 was immunoprecipitated with anti-HA, and co-precipitated TAK1 was detected (top panel). The amount of T7-TAK1 in the whole cell extracts (WCE) is shown in the bottom panel. Right panels, TAK1 was immunoprecipitated with anti-T7, and co-precipitated TAO1 was detected (top panel). The amount of HA-TAO1 in the whole cell extracts is shown in the bottom panel. IP, immunoprecipitation; IB, immunoblotting.

(B) 293 cells were transfected with the expression vectors for T7-TAK1 together with either HA-TAO2 or a HA-tagged kinase dead version of TAO2 (HA-TAO2-D169A). Left panels, TAO2 was immunoprecipitated with anti-HA and co-precipitated TAK1 was detected (top panel). The amount of T7-TAK1 in the whole cell extracts (WCE) is shown in the bottom panel. Right panels, TAK1 was immunoprecipitated with anti-T7, and co-precipitated TAO2 was detected (top panel). The amount of HA-TAO2 in the whole cell extracts is shown in the bottom panel.

(C) 293 cells transiently expressing FLAG-TAO2 were lysed and incubated for the indicated times at 37 °C to allow spontaneous formation of the TAK1-TAO2 complex. Anti-HA was used for a control precipitation. Anti-TAK1 and anti-HA immunoprecipitates were analyzed for the presence of TAO2 by immunoblotting.

Fig. 4. TAO2 inhibits TAK1-mediated activation of NF-κB pathway.

(A) 293 cells were transfected with NF-κB-dependent luciferase reporter together with expression vector for HA-TAO2 or the kinase-dead version of TAO2 (HA-TAO2-D169A) and TAK1 + TAB1 (active TAK1). An internal control reporter (pAct-β-Gal) was used to normalize the transfection efficiency. Expression of TAK1 and TAB1 was monitored by immunoblotting (bottom panel). IB, immunoblotting.

(B) 293 cells were transfected with increasing amounts of HA-TAO2 or with 5 μg of empty vector. Cells were either left untreated or treated with 20 ng/ml TNF for 15 min. IKKα was immunoprecipitated from the lysates with anti-IKKα, and the immunoprecipitates were subjected to *in vitro* kinase assay using GST-IκBα as substrate (top panel). The amount of IKKα in the immunoprecipitates is shown in the middle panel. The amount of TAK1 and TAO2 in the whole cell extracts (WCE) is shown in the bottom panel. IKK activity was quantitated and normalized to the levels of IKKα amounts. Relative activation levels of IKK are shown (fold). IP, immunoprecipitation.

(C) 293 cells were transfected with an NF-κB-dependent luciferase reporter together with an expression vector for HA-TAO2. Cells were either left untreated or treated with 20 ng/ml TNF. An internal control reporter (pAct-β-Gal) was used to normalize the transfection efficiency.

(D) 293 cells were transfected with expression vectors for HA-TAO2 and T7-TAK1+ T7-TAB1 (active TAK1). Activated JNK was detected with anti-phospho-specific JNK (P-JNK) (top panel). The amounts of JNK, T7-TAK1 + T7-TAB1, and HA-TAO2 in the whole cell extracts are shown in the 2nd, 3rd, and 4th panels, respectively. Signal intensity of phospho-JNK was quantitated and normalized to the levels of total JNK amounts. Relative activation levels of JNK are shown (fold).

Fig. 5. TAO2 does not inhibit the catalytic activity of TAK1 but blocks the interaction of TAK1 with IKK.

(A) 293 cells were transiently transfected with fixed amounts of expression vectors for T7-TAK1 (1 μg) and T7-TAB1 (1 μg) and increasing amounts of HA-TAO2 as indicated. TAK1 was immunoprecipitated from the lysates with anti-TAK1, and the immunoprecipitates were subjected to *in vitro* kinase assay using His-MKK6 as substrate (top panel). The amount of T7-TAK1 in the immunoprecipitates is shown in the middle panel. The amount of HA-TAO2 in the whole cell extracts (WCE) is shown in the bottom panel. TAK1 activity was quantitated and normalized to the levels of TAK1 amounts. Relative activation levels of TAK1 are shown (fold). IP, immunoprecipitation; IB, immunoblotting.

(B) 293 cells were transiently transfected with fixed amounts of expression vectors encoding T7-TAK1 (0.5 μg) and HA-IKKα (0.1 μg) and increasing amounts of FLAG-TAO2. HA-IKKα was immunoprecipitated from the lysates with anti-HA (2nd panel), and co-precipitated T7-TAK1 was detected with anti-T7 (top panel). The amounts of T7-TAK1 and FLAG-TAO2 in the whole cell extracts (WCE) are shown in the 3rd and 4th panels, respectively.

(C) 293 cells were transfected with an empty vector or an expression vector for HA-TAO2. Cells were lysed and incubated for the indicated times at 37 °C to allow spontaneous formation of the TAK1-IKK or TAK1-HA-TAO2 complexes. Endogenous TAK1 was immunoprecipitated, and the precipitates were analyzed for the presence of endogenous IKKα or HA-TAO2 by immunoblotting.

Fig. 6. Effect of TAO2 siRNA.

(A) 293 cells were transfected with control siRNA and TAO2 siRNAs (TAO2 siRNA #1 and #2) using increasing amounts of transfection reagent. TAO2 expression was determined by real time PCR using human TAO2 primers. The amount of TAO2 mRNA relative to that from control siRNA transfected cells is shown. The TAO2 protein was detected with anti-TAO2. β-Catenin was used as a loading control. IB, immunoblotting.

(B) 293 cells were transfected with control siRNA and TAO2 siRNAs (TAO2 siRNA #1, left panels; TAO2 siRNA #2, right panels) using 10μl of transfection reagent. Cells were subjected to osmotic stress (0.5 M NaCl), and cell extracts were immunoblotted with anti-phospho-JNK (top panel) and anti-phospho-TAK1 (3rd panel). The amounts of endogenous JNK and TAK1 are shown in the 2nd and bottom panels, respectively. Signal intensity of phospho-JNK was quantitated and normalized to the levels of total JNK amounts. Relative activation levels of JNK are shown (fold). Representative experiments of four are shown.

(C) 293 cells were transfected with control siRNA and TAO2 siRNAs. Cells were subjected to osmotic stress (0.5 M NaCl), and cell extracts were subjected to monitor NF-κB activation by EMSA. The amount of p65 NF-κB subunit is shown as a loading control. A representative result of three is shown.

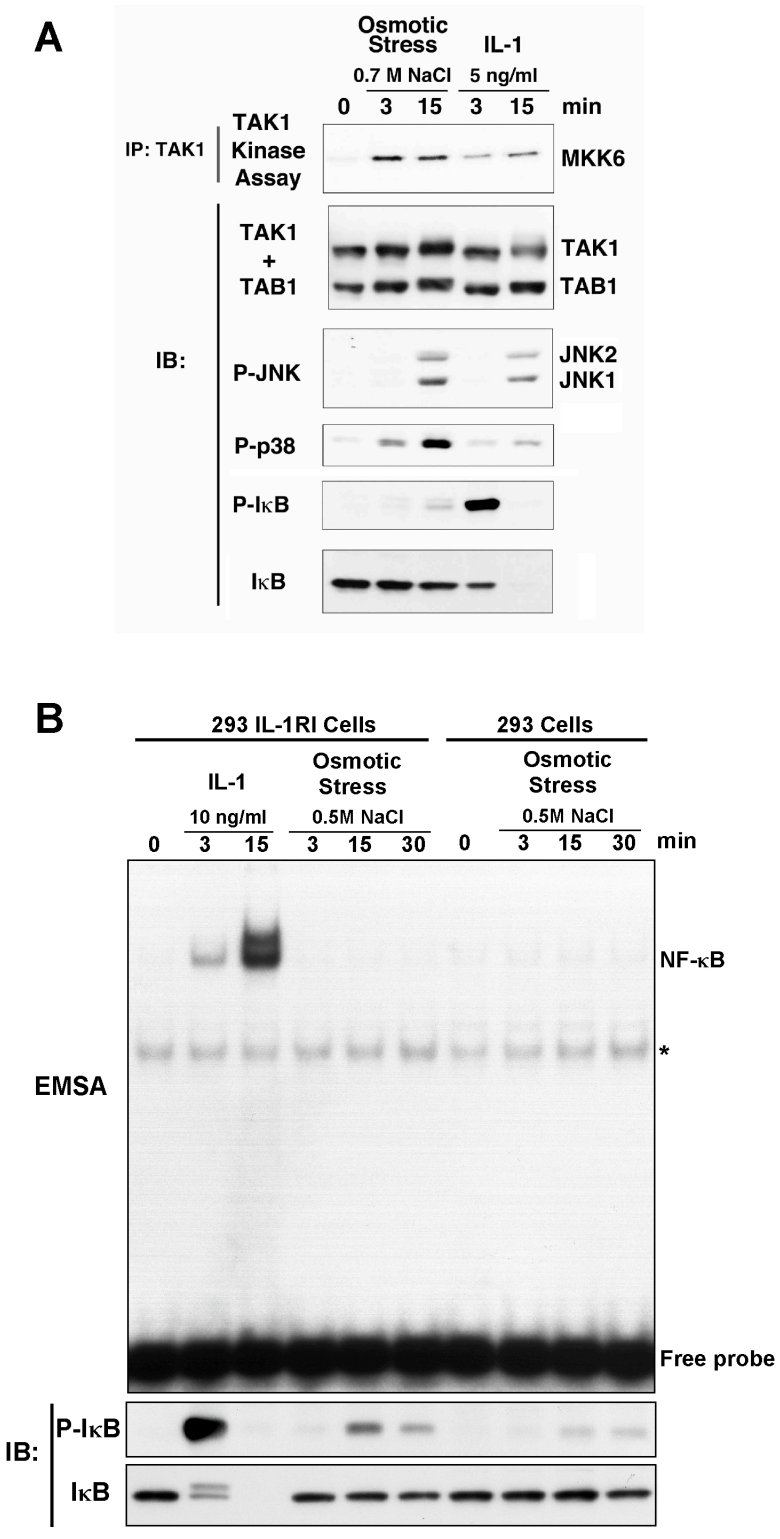


Fig. 1. Osmotic stress activates TAK1 and JNK but not NF-κB.

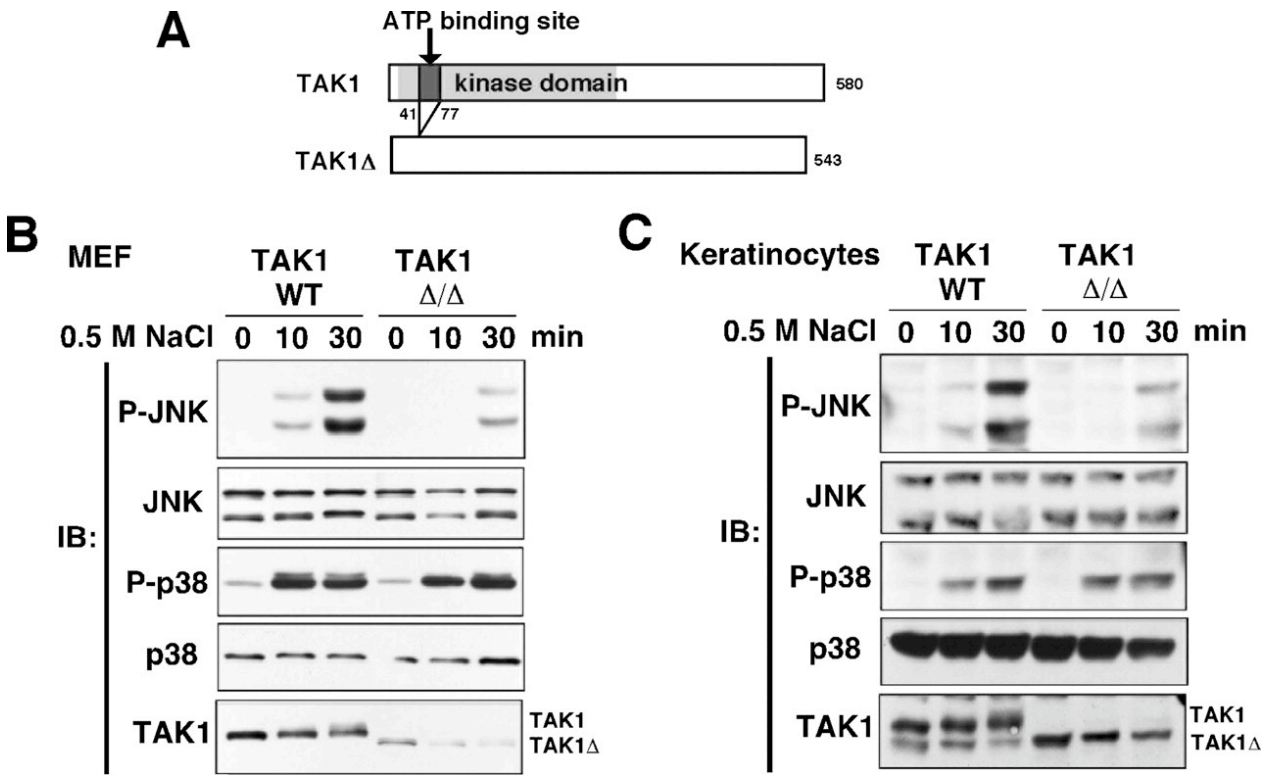


Fig. 2. TAK1 is important for osmotic stress-induced JNK.

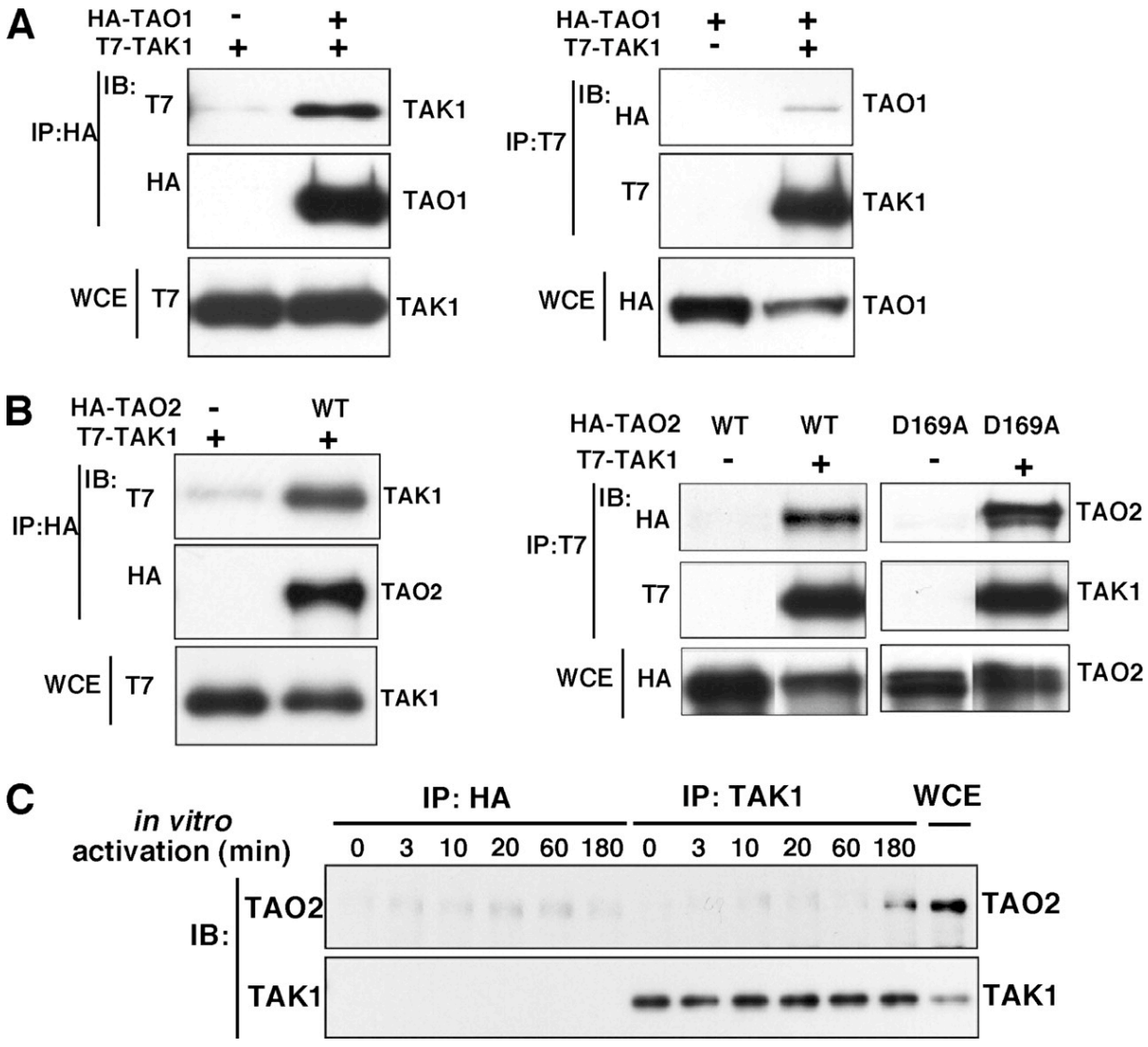
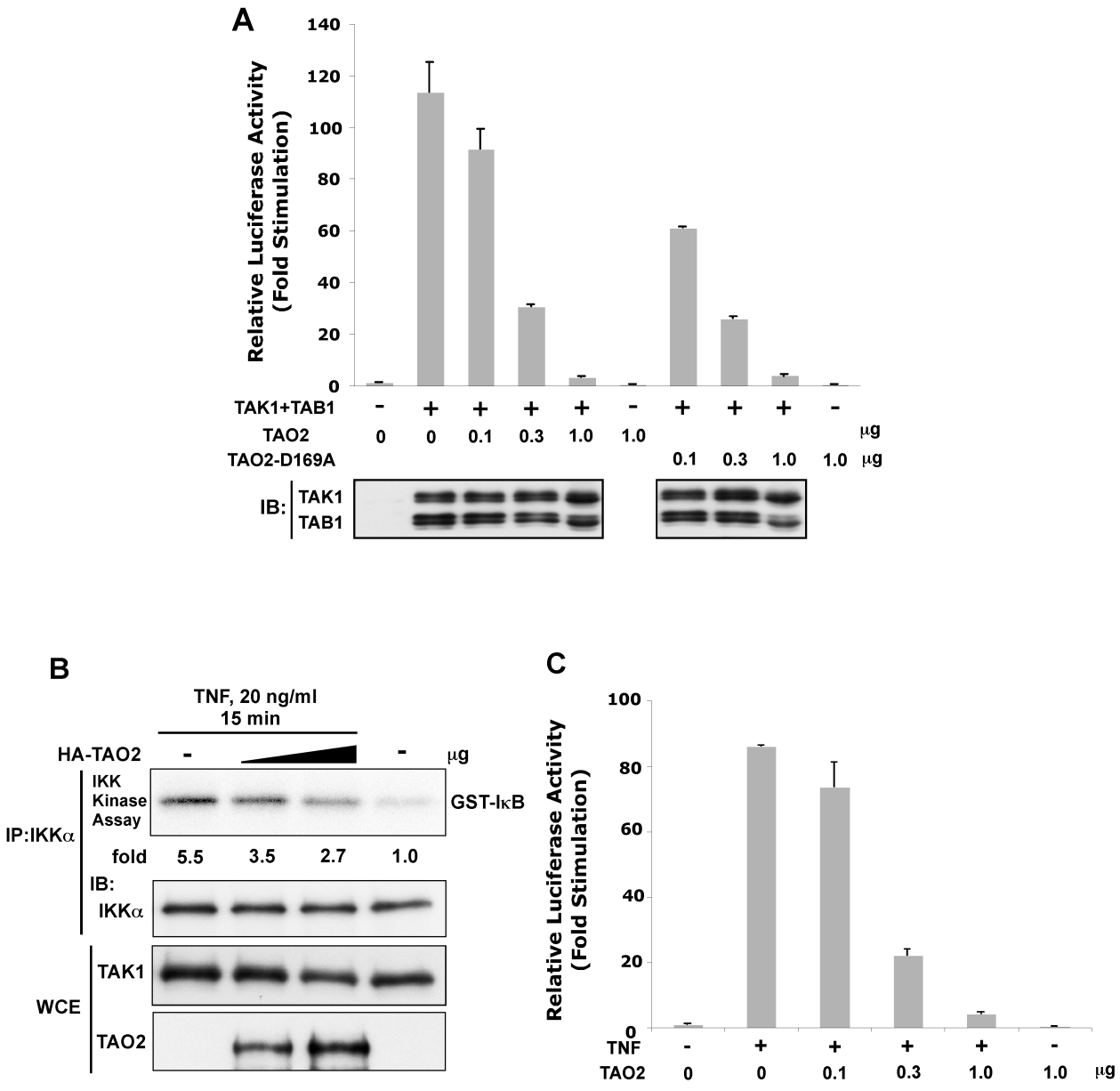


Fig. 3. TAOs interact with TAK1.



<u>Fig. 4.</u> TAO2 inhibits TAK1-mediated activation of NF-κB pathway.

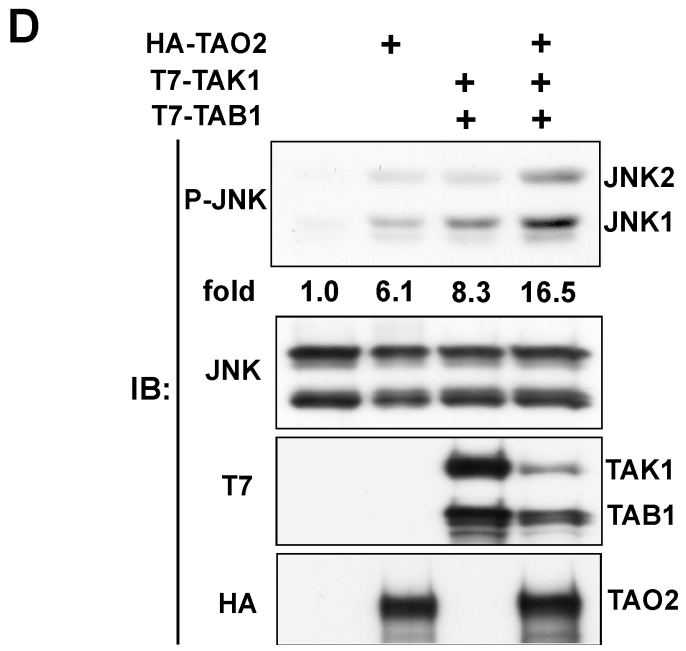
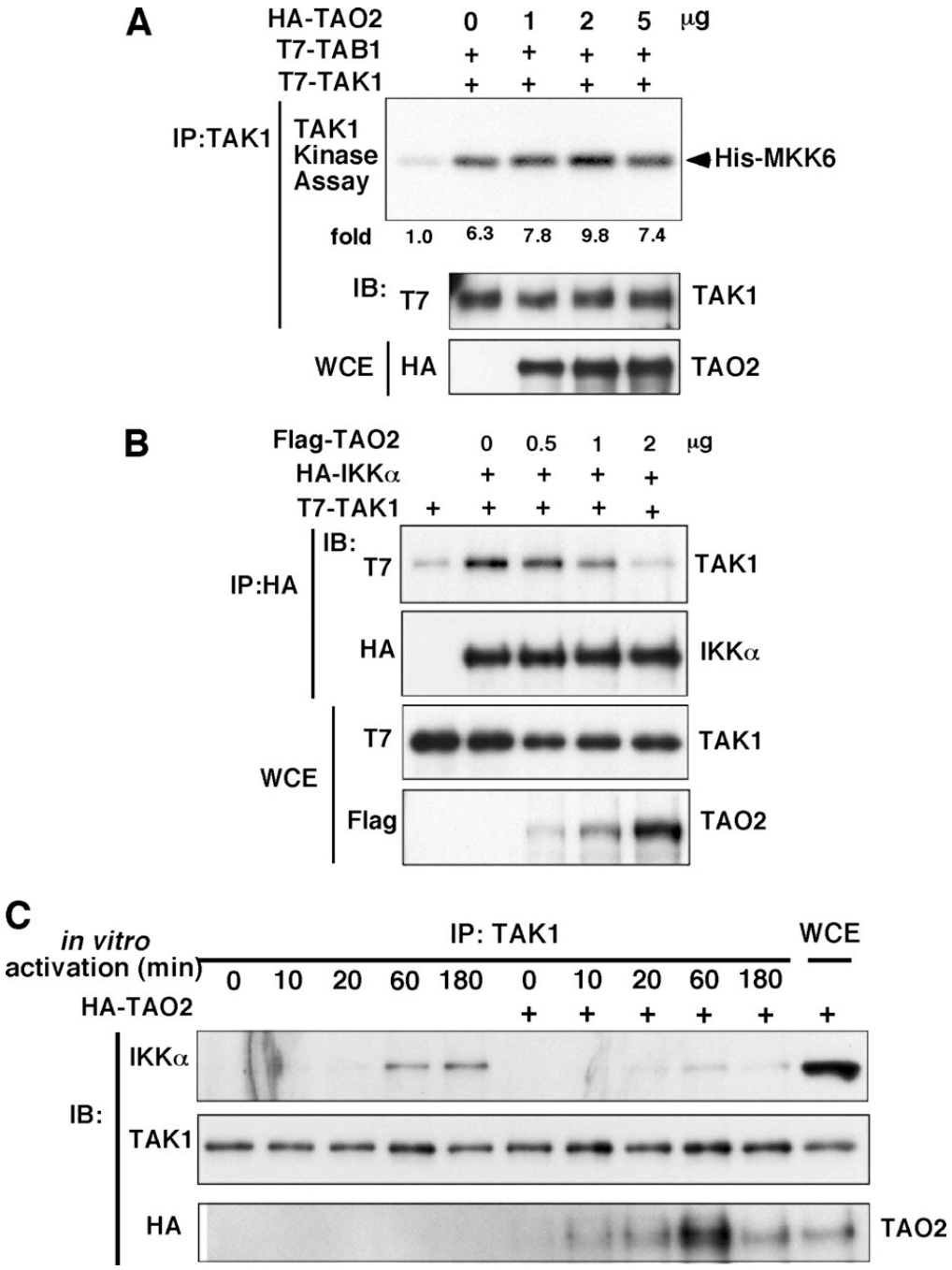


Fig. 4. TAO2 inhibits TAK1-mediated activation of NF-κB pathway.



<u>Fig. 5.</u> TAO2 does not inhibit the catalytic activity of TAK1 but blocks the interaction of TAK1 with IKK.

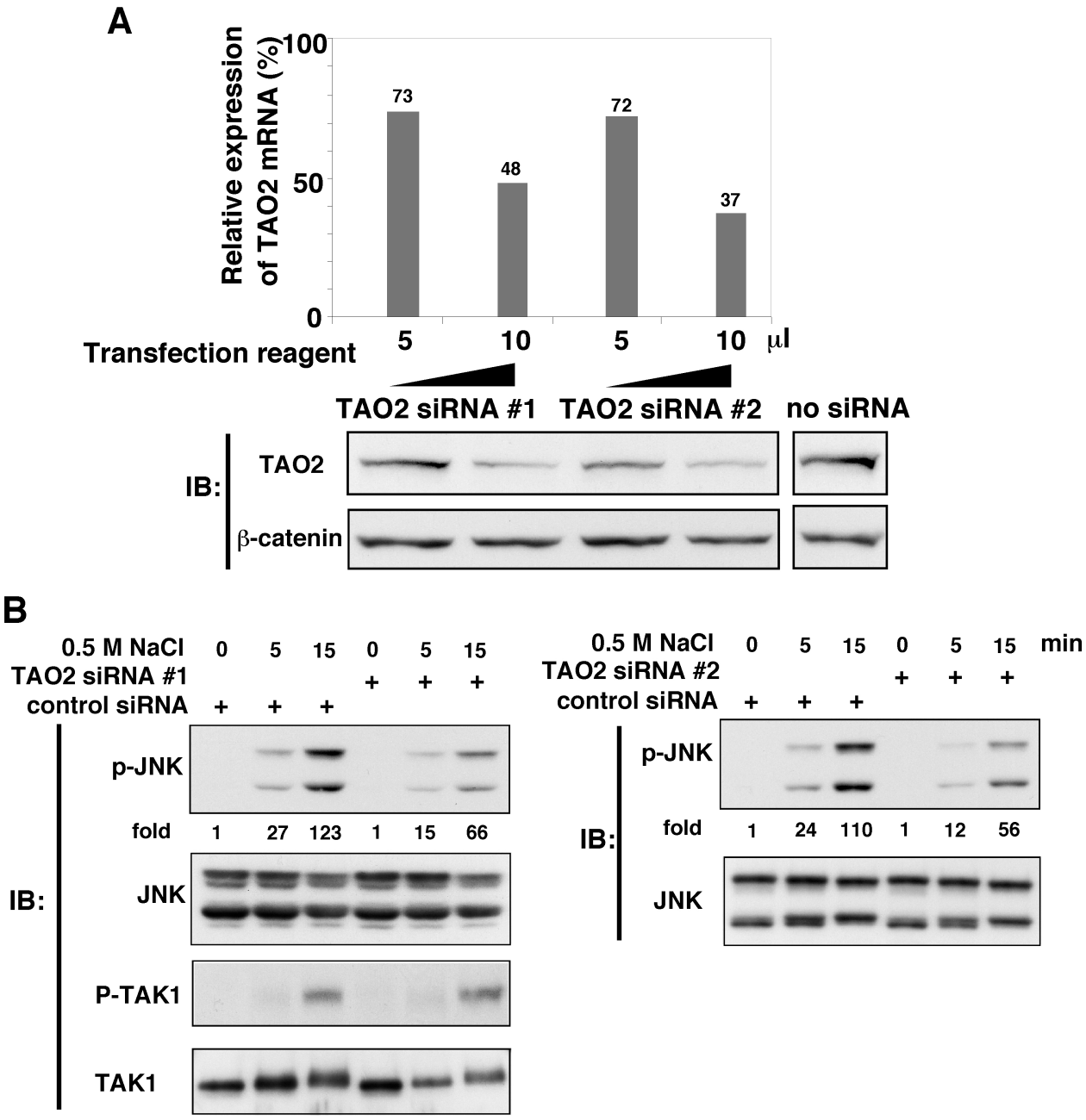


Fig. 6. Effect of TAO2 siRNA.

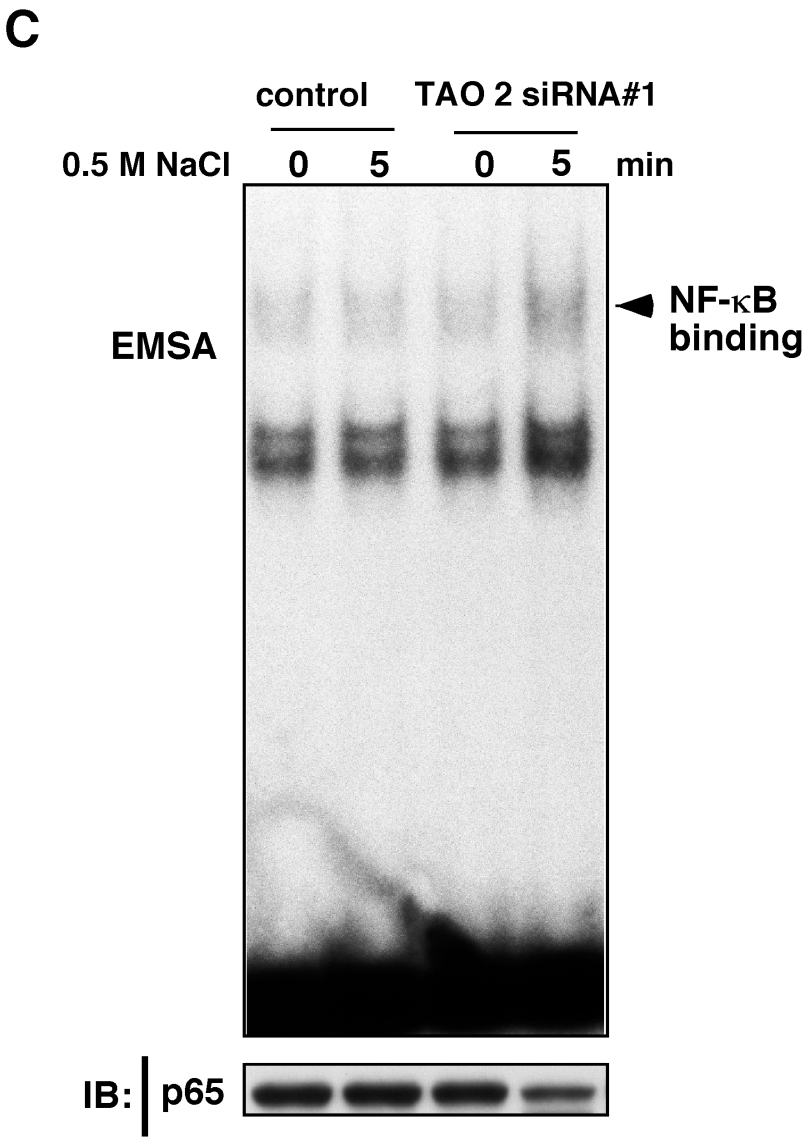


Fig. 6. Effect of TAO2 siRNA.

MANUSCRIPT II

Osmotic stress blocks NF-κB-dependent inflammatory responses by inhibiting ubiquitination of IκB

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ABSTRACT

The inhibitory effects of hypertonic conditions on immune responses have been described in clinical studies; however, the molecular mechanism underlying this phenomenon has yet to be defined. Here we investigate osmotic stress-mediated modification of the NF-κB pathway, a central signaling pathway in inflammation. We unexpectedly found that osmotic stress activated IκB kinase and leading to phosphorylation of IκB, even though NF-κB was not activated. Osmotic stress-induced phosphorylated IκB was poorly ubiquitinated. We found that ubiquitination of β-catenin as well as IκB, both of which are targets of the SCF type E3 ligase, were impaired under osmotic stress conditions, while ubiquitination of other proteins was intact. Osmotic stress interfered with interleukin 1-induced ubiquitination of IκB and ultimately blocked NF-κB-mediated expression of cytokine/chemokines. Thus, osmotic stress down-regulates NF-κB through the inhibition of SCF type E3 ligase(s), and this is likely to be a major mechanism of immune suppression caused by hypertonic conditions.

INTRODUCTION

It has long been known that the use of hypertonic fluid for resuscitation after trauma has some benefits compare to isotonic fluid (Dubick et al., 2006). Hypertonic solutions such as NaCl and mannitol have been shown to reduce inflammation associated with resuscitated hemorrhage shock, thereby reducing organ failure caused by systemic inflammation (Angle et al., 1998; Powers et al., 2005; Zakaria el et al., 2006). In an animal model system, administration of hypertonic saline increases serum osmolarity and blocks the production of proinflammatory cytokines such as TNF (Oreopoulos et al., 2004). *In vitro* studies have revealed that hypertonic condition inhibits the activation of neutrophils and macrophages (Cuschieri et al., 2002; Junger et al., 1998; Rizoli et al., 1999). However the molecular mechanism underlying this inhibition has not been determined. Understanding the mechanism by which osmotic stress inhibits inflammation may be useful in developing effective therapeutic strategies based on this biological effect.

NF-κB is the major transcription factor to regulate inflammatory genes (Rothwarf and Karin, 1999). In unstimulated cells, a complex of NF-κB and its inhibitory protein IκB resides in the cytoplasm. IκB masks the nuclear localization signal of NF-κB (Ziegler and Ghosh, 2005). Proinflammatory stimuli such as interleukin 1 (IL-1) and tumor necrosis factor (TNF) initiate an intracellular signaling cascade leading to activation of the IκB kinase (IKK) complex, which consists of IKKα, IKKβ and IKKγ/NEMO. IKK in turn phosphorylates IκB at Ser-32 and Ser-36 which are recognized by βTrCP, a subunit of the SCF type E3 ligase (Karin and Ben-Neriah, 2000). Phosphorylated IκB is ubiquitinated through the SCF E3 ligase complex and degraded by

26S proteosomes. NF-κB then translocates into the nucleus and functions as a transcription factor for many proinflammatory genes including cytokines and chemokines.

Osmotic stress initiates intracellular signaling primarily through MAPK cascades, which induce cellular responses involved in defending cells from changes in environmental osmolarity. The most upstream components of the MAPK cascades are MAPK kinase kinases (MAPKKK) that phosphorylate and activate MAPK kinase (MAPKK). In addition, several MAP3Ks are involved in other signaling pathways such as the NF-κB pathway. A sub-group of MAP3Ks including TAK1 and MEKKs can activate IKK, leading to NF-κB activation (Ninomiya-Tsuji et al., 1999; Schlesinger et al., 1998). However, even though osmotic stress strongly activates MEKKs and TAK1, activation of NF-κB is normally undetectable under osmotic stress conditions (HuangFu et al., 2006). Therefore, it seemed likely that there is an inhibitory mechanism that blocks NF-κB activation under conditions of osmotic stress. We have previously shown that the TAO2 kinase can interfere with the interaction of TAK1 and IKK, resulting in the inhibition of NF-κB pathway. However, knockdown of TAO2 was observed to have only a partial effect on relieving inhibition of NF-κB (HuangFu et al., 2006). This suggests that NF-κB activation is blocked by additional mechanisms that function under conditions of osmotic stress and may contribute to inhibiting the pro-inflammatory effects of stimuli such as TNF and IL-1 under osmotic stress.

In this study, we investigated the regulation of NF-κB pathway in response to osmotic stress. Surprisingly, we found that IKK could be activated by osmotic stress and that IκB was phosphorylated. However, the ubiquitination and subsequent degradation of IκB were strikingly

impaired under hypertonic condition. Furthermore, IL-1-induced IκB degradation was blocked by hypertonic preconditioning. IL-1-induced ubiquitination of IRAK was not altered in the presence or absence of osmotic stress, which indicates that osmotic stress does not inhibit ubiquitination generally. IκB and β-catenin are ubiquitinated by the same SCF type E3 ligase. We found that ubiquitination of β-catenin was also impaired by osmotic stress. These results suggest that osmotic stress interferes with function of SCF E3 ligase(s) and thereby blocks the NF-κB pathway.

RESULTS

Osmotic stress induces IκB phosphorylation but not degradation

We have previously demonstrated that 0.5-0.7 M NaCl strongly activates JNK and p38 MAPKs, while IκB degradation and NF-κB activation are not detectable (HuangFu et al., 2006). However, under the same experimental condition, we showed that phosphorylation of IκB is detectable to some extent (HuangFu et al., 2006). To further investigate this phosphorylation, we examined phosphorylation of IκB following treatment by another osmotic stressor, 0.4 M sorbitol and a non-osmotic stressor, ultraviolet C (UVC) light irradiation in human embryonic kidney 293 cells. Sorbitol 0.4 M has a milder effect on cells than 0.5-0.7 M NaCl and cells can survive for more than one day. UVC (60 J/m2) stress is a potent activator of MAPK cascades, DNA damage and ultimately cell death (Bode and Dong, 2003). As shown previously, 0.5 M NaCl induced phosphorylation of IκB but not degradation of IκB or activation of NF-κB (Fig. 1A). Sorbitol 0.4 M was a weaker activator compared to 0.5 M NaCl, but none-the-less induced phosphorylation of IκB at 15 min after stimulation. This phosphorylated IκB was stable for at least 1-2 hr after 0.4 M sorbitol treatment. We found that IκB started degrading at 2 h-post stimulation of 0.4 M sorbitol, and NF-κB was subsequently activated (Fig. 1B right panels). UVC strongly activated JNK, which is similar to 0.4 M sorbitol; however, UVC did not induce phosphorylation of IκB until 2 h after the exposure (Fig. 1B left panel). This delayed NF-κB activation is consistent with earlier studies and is likely to be mediated by UV-induced reactive oxygen species (Mabb et al., 2006). Importantly, the time courses of IκB phosphorylation and degradation and activation of NF-κB in UVC exposed cells were very well correlated, whereas osmotic stress-induced IκB degradation

was significantly delayed by at least 1-2 hr after the phosphorylation of IκB. These results indicate that degradation of phosphorylated IκB is impaired under osmotic stress.

Osmotic stress blocks protein ubiquitination and degradation mediated by SCF E3 complex

We sought to determine why degradation of IκB is impaired under conditions of osmotic stress. We first confirmed whether osmotic stress could activate IKK as in the case of other NF-κB activating stimuli. The proinflammatory cytokines TNF and IL-1 induce activation of IKK, which in turn phosphorylates IκB at Ser-32 and Ser-36. These phosphoserines are recognized by βTrCP, a subunit of SCF type E3 ligase. We performed an *in vitro* kinase assay using GST-IκB and a mutant version GST-IκB AA that contains Ser to Ala substitutions at Ser-32 and Ser-36 (Fig. 2A). We observed that osmotic stress activated IKK, leading to phosphorylation at Ser-32 and Ser-36. Osmotic stress could activate IKK at a level comparable to that observed in TNF treated cells. Phosphorylated IκB is normally ubiquitinated by SCF-βTrCP, therefore we asked whether phosphorylated IκB is ubiquitinated following osmotic stress. We stimulated cells with 0.7 M NaCl or TNF in the presence or absence of a proteasome inhibitor MG132, and IκB was analyzed by immunoblotting (Fig. 2B). We observed that both osmotic stress and TNF induced phosphorylation of IκB (Fig. 2B, left 6 lanes). When cells were stimulated by TNF in the presence of MG132, phosphorylated IκB appeared as a ladder of slowly migrating bands, indicating polyubiquitination of IκB (Fig. 2B, right most lane). In contrast, no slowly migrating form of IκB was detected in cells subjected to osmotic stress, even though the level of IκB phosphorylation was similar in cells treated with either TNF or osmotic stress. This result indicates that ubiquitination is blocked under osmotic stress. This raises the possibility that osmotic stress inhibits SCF-β-TrCP E3 ubiquitin ligase that is responsible for ubiquitination of

several proteins including IκB. β-catenin, a signaling molecule in the Wnt pathway, is another target of SCF-β-TrCP. We examined whether osmotic stress alters ubiquitination status of β-catenin (Fig. 3A). Cells were treated with MG132 to accumulate the ubiquitinated form of β-catenin. The slowly migrating ladder of bands that indicates ubiquitinated β-catenin was diminished with osmotic stress. These results indicate that osmotic stress is likely to inhibit IκB ubiquitination through interfering with function of specific E3 ligase(s) including SCF-βTrCP. Earlier studies reported that osmotic stress rather stimulates ubiquitin-dependent degradation of some proteins such as cyclin D and the transcription factor c-Jun (Casanovas et al., 2000; Xia et al., 2007). To determine whether osmotic stress is a general inhibitor of ubiquitination, we tested for the effect of osmotic stress on ubiquitination mediated through the other mechanism. IRAK is a signaling molecule in IL-1 pathway, and is phosphorylated and eventually heavily ubiquitinated for degradation following IL-1 stimulation (Yamin and Miller, 1997). We pretreated 293 cells stably expressing IL-1 receptor 1 (293 IL-1RI cells) with MG132, and examined IL-1-induced ubiquitination of IRAK with and without 0.4 M sorbitol treatment (Fig. 3B). IRAK was heavily ubiquitinated 5-30 min after IL-1 stimulation even under osmotic stress. Thus, osmotic stress is not a non-specific inhibitor for protein ubiquitination.

Osmotic stress inhibits IL-1-induced activation of NF-κB

NF-κB is a major mediator of inflammatory signaling. Our results demonstrate that osmotic stress inhibits E3 ligase thereby blocking NF-κB activation. This raises the possibility that hypertonic condition reduces inflammation through this mechanism. We asked whether osmotic stress could block IL-1-induced NF-κB activation. 293 IL-1RI cells were treated with 0.4 M sorbitol (Fig. 4A) and 0.5 M NaCl (Fig. 4B), and IL-1-induced phosphorylation of IκB and

activation of NF-κB were monitored. IL-1 stimulation induced phosphorylation of IκB, rapid degradation of IκB and subsequent re-accumulation of IκB (Fig. 4A, lanes 1 and 6-8 and Fig. 4B lanes 1-3). However, in the presence of 0.4 M sorbitol, the amount of phosphorylated IκB was strikingly increased and the total amount of IκB was decreased slowly upon IL-1 stimulation (Fig. 4A, lanes 3-5). Concomitantly, IL-1-induced activation of NF-kB was significantly reduced by 0.4 M sorbitol treatment (Fig. 4A, bottom panel). JNK was additively activated upon 0.4 M sorbitol plus IL-1 stimulation (Fig. 4A, third panel). 0.5 M NaCl completely blocked IL-1-induced degradation of IκB and activation of NF-κB (Fig. 4B). We next examined for IL-1-induced ubiquitination of IκB. 293 IL-1RI were transfected with an expression vector for Myc-tagged ubiquitin and stimulated with IL-1 in the presence of MG132. Ubiquitination of phosphorylated IκB was monitored by immunoblotting with anti-Myc (Fig. 4C). MG132 inhibited degradation of phosphorylated IκB and we could observe accumulation of polyubiquitinated IκB at 30 min after IL-1 stimulation (Fig. 4C, lane 8). Treatment of 0.4 M sorbitol reduced ubiquitination of IκB upon IL-1 stimulation. Collectively, these results demonstrate that osmotic stress inhibits IL-1-induced NF-κB pathway by blocking IκB ubiquitination.

Osmotic stress inhibits IL-1-induced expression of IL-8 and TNF

Finally, we asked whether osmotic stress inhibits IL-1-induced inflammatory responses. We examined IL-1-induced cytokine/chemokine production following pretreatment with increasing concentration of sorbitol (Fig. 5). Cells treated with 0.2 M sorbitol were morphologically indistinguishable from untreated cells. Cells treated with 0.4 M sorbitol shrunk at 4-8 hr after treatment but remained viable for at least one day (data not shown). We measured

IL-8 and TNF mRNA levels by quantitative real time PCR after 2 hour-exposure to IL-1. Expression of IL-8 was greatly increased by IL-1, and this increase was effectively blocked about 60% by 0.2 M sorbitol (Fig. 5, upper panel). IL-1 increased the expression of TNF about 10 fold and 0.2-0.3 M sorbitol reduced the TNF mRNA levels about 20-30% (Fig. 5, lower panel). This inhibitory effect was dose-dependent, and 0.4 M sorbitol completely abolished expression of both IL-8 and TNF.

DISCUSSION

The use of hypertonic solutions to reduce acute or chronic inflammation in some pathogenic conditions potentially has benefits, because it could be inexpensive and safer alternative to anti-inflammatory drugs. Understanding the molecular mechanisms by which the hypertonic condition prevents inflammation is essential to develop strategies to utilize hypertonic solutions. Our results demonstrate that osmotic stress inhibits SCF E3 ligase–mediated protein ubiquitination, resulting in the stabilization of IκB and inhibition of NF-κB activation, which blocks induction of inflammatory cytokines/chemokines. How specific is osmotic stress-mediated inhibition of ubiquitin E3 ligase? We here show that osmotic stress abolishes ubiquitination of β-catenin as well as IκB, suggesting that SCF-βTrCP is inhibited by osmotic stress. Among the targets of other types of SCF, p27Kip degradation is impaired by osmotic stress (unpublished data, W-C H). However, degradation of c-Jun and cyclin E, which are also targets of other SCF type ligases, are not blocked but rather are enhanced by osmotic stress (Casanovas et al., 2000; Xia et al., 2007). We confirmed that c-Jun degradation is not impaired by osmotic stress (data not shown). We also show here ubiquitination of IRAK, which is mediated by a RING type E3 ligase (Schauvliege et al., 2006), is not altered by osmotic stress. Therefore, osmotic stress specifically inhibits a subset of SCF type E3 ligases. Studies to define the molecular mechanisms underlying this specific inhibition will further provide integral information to develop strategies for the therapeutic use of hypertonic solutions.

We here show osmotic stress activates IKK. What kinase is responsible for this activation? Osmotic stress activates several MAPKKKs including the MEKKs and TAK1, all of which can

activate IKK. We have previously shown that TAO2, another MAPKKK, inhibits the interaction of TAK1 with IKK and thereby prevents TAK1-mediated activation of IKK in response to osmotic stress (HuangFu et al., 2006). Therefore, TAK1 cannot be responsible for osmotic stress-induced IKK activation. Indeed, we found that osmotic stress still induced IκB phosphorylation in TAK1-deficient mouse embryonic fibroblasts (unpublished data, W-C, H). Thus, MEKKs are likely to activate IKK in response to osmotic stress. In osmotic stress signaling pathway, MAPK pathway mediated by MAPKKKs appears to be very important to upregulate protective molecules against hypertonic environment. Therefore, osmotic stress triggers strong activation of MAPKKKs to effectively upregulate MAPK cascades. In contrast, NF-κB-dependent inflammation appears not to be a desired outcome of osmotic stress. However, IKK could activated as a byproduct of strong activation of MAPKKKs. The TAK1-NF-κB pathway is inhibited by TAO2 and other MAPKKK-NF-κB pathways may be inhibited through several mechanisms including blockage of IκB ubiquitination. These fine-tuning mechanisms may be essential to induce appropriate cellular to hypertonic conditions.

MATERIALS AND METHODS

Cell culture and transfection - Human embryonic kidney 293 cells, 293 IL-1RI cells that stably expresses the IL-1 receptor I (Cao et al., 1996) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Bovine Growth Serum (Hyclone) at 37^{o}C in 5% CO_2. For transfection studies, 293 IL-1RI cells (1 x 10^6) were seeded onto 10 cm dishes and transfected with expression vectors for Myc-tagged ubiquitin (pcDNA3.1-Myc-Ub, provided by Dr. Tanaka) by the standard calcium phosphate precipitation method 24 hr after seeding. 36-48 hr after transfection, cells were harvested in 0.5% Triton X-100 lysis buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM $MgCl_2$, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and 20 μM aprotinin).

Treatment of cells with stress inducers and other reagents—To induce osmotic stress, cells were treated with NaCl (0.5 M or 0.7 M) or sorbitol (0.2-0.4 M). For cytokine stimulation, 5 ng/ml IL-1β or 20 ng/ml TNF-α (Roche Diagnostics) was used. To treat cells with UVC, cells were exposed to 254 nm ultraviolet irradiation (UV Crosslinker, Spectronics Corporation). Carbobenzoxyl-leucinyl-leucinyl-leucinal (MG132) was purchased from Calbiochem.

Antibodies—Anti-phospho-SAPK/JNK (Thr-183/Tyr-185) rabbit polyclonal antibody (Cell Signaling), Anti-JNK1 (FL) polyclonal antibody (Santa Cruz), anti-IκB-α (C-21) polyclonal antibody (Santa Cruz), anti-IκB-α polyclonal antibody (Cell Signaling), anti-phospho-IκB-α (Ser32/36) (5A5) monoclonal antibody (Cell Signaling), anti-IKKα (H-744) polyclonal antibody

(Santa Cruz), anti-cMyc (9E10) monoclonal antibody (Santa Cruz), anti-IRAK polyclonal antibody (Cao et al., 1996), anti-α-Tubulin (TU-02) monoclonal antibody (Santa Cruz), and anti-β-catenin monoclonal antibody (BD), were used for immunoprecipitation and immunoblotting.

Immunoprecipitation-immunoblotting - Cells were lysed in the 0.5% Triton X-100 lysis buffer described above. Cellular debris was removed by centrifugation at 10,000 g for 5 min at 4^{o}C, and the supernatant was collected. Proteins from the lysates were immunoprecipitated with various antibodies. Immunoprecipitates and whole cell extracts were resolved by SDS-PAGE, and transferred to Hybond-P membranes (Amersham Biosciences). The membranes were immunoblotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse immunoglobulin G (IgG) using the ECL Western Blotting System (GE Healthcare).

Electrophoretic mobility shift assay (EMSA) - The binding reactions contained radio-labeled [^{32}P]-NF-κB oligonucleotide (Promega) probe, 40 μg of cell extracts, 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl [pH 7.5], 500 ng poly (dI-dC) (GE Healthcare) and 10 μg BSA to a final volume of 15 μl. The reaction mixtures were incubated at 25^{o}C for 30 min and separated by 5% (w/v) polyacrylamide gel and visualized by autoradiography.

In Vitro Kinase Assays – IKK complex was immunoprecipitated with anti-IKKα and the immunoprecipitates were incubated with 5 μCi of [γ-32P]-ATP (3,000 Ci/mmol) and 1 μg of bacterially expressed GST-IκB or GST- IκB AA in 10 μl of kinase buffer containing 10 mM

HEPES (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$ at 30 ^oC for 30 min. Samples were then separated by 10% SDS-PAGE and visualized by autoradiography.

Real Time PCR—Total RNA was used for quantitative real-time PCR. GAPDH was used as an internal control. Each PCR was carried out in triplicate in a 50 μl volume using SYBR Green Master Mix (Applied Biosystems) in the ABI Prism 7000 sequence detection system. The human primers used for real-time PCR analysis were as follows: GAPDH forward, 5'-GAAGGTCGGAGTCAACGGATT-3'; GAPDH reverse, 5'-GGATCTCGCTCCTGGAAGATGGT-3'; IL-8 forward, 5'-AGCTGGCCGTGGCTCTCT-3'; IL-8 reverse, 5'-CTGACATCTAAGTTCTTTAGCACTCCTT-3'; TNFα forward, 5'-TCTGCCTGCTGCACTTTGG-3'; TNFα reverse, 5'-GCCAGAGGGCTGATTAGAGAGA-3'.

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FIGURE LEGENDS

Fig. 1. Osmotic stress induces IκB phosphorylation but not degradation.

(A) 293 cells were treated with 0.5 M NaCl for 5 or 15 min. Activation of the NF-κB pathway was monitored by degradation (middle panel), phosphorylation (top panel) of IκBα, and EMSA (bottom panel). IB, immunoblotting.

(B) 293 cells were exposed to UVC (60 J/m^2) (left panels) or treated with 0.4 M sorbitol (right panels) for 5 min- 7 hr. Activation of the NF-κB pathway was monitored by phosphorylation (top panels), degradation (2nd panels) of IκBα and EMSA (bottom panels). Activation of JNK was also monitored by phospho-specific JNK antibody (3rd panels). The amount of JNK is also shown (4th panels). IB, immunoblotting.

Fig. 2. Osmotic stress activates IKK but does not induce IκB ubiquitination.

(A) 293 cells were treated with 0.7 M NaCl or 20 ng/ml TNFα and IKK complex was immunoprecipitated with anti-IKKα. The immunocomplexes were subjected to *in vitro* kinase assay using GST-tagged wild type (IκBα WT) or a mutant version of IκBα (IκBα AA) as an exogenous substrate (upper panel). Immunoprecipitated IKKα was detected by immunoblotting (lower panel). IP, immunoprecipitation; IB, immunoblotting.

(B) 293 cells were incubated with a vehicle (DMSO) or an inhibitor of 26 S proteasome (MG132, 10 μM) for 6 h. Cell were then stimulated with 0.7 M NaCl or 20 ng/ml TNF. Phosphorylated IκB and polyubiquitinated IκB were detected by immunoblotting with anti-phospho IκB (upper panel). Total amount of IκB was detected with anti-IκB (lower panel). Asterisk indicates a nonspecific band. IB, immunoblotting.

Fig. 3. Osmotic stress does not affect ubiquitination of IRAK but inhibit ubiquitination of β-catenin.

(A) 293 cells were incubated with 0.4 M sorbitol and MG132 (10 μM) or MG132 alone for 4 hr. β-catenin was detected by immunoblotting (IB) (upper panel). TAK1 immunoblotting is shown as a loading control (lower panel). Two parts from the same exposure are shown (upper panel).

(B) 293 IL-1 RI cells were pre-incubated with 0.4 M sorbitol and MG132 (10 μM) or MG132 alone for 30 min followed by stimulation with 5 ng/ml IL-1 for 5-30 min. IRAK and polyubiquitinated IRAK were detected with anti-IRAK (top panel). Activation of JNK was monitored by anti-phospho JNK (middle panel). Levels of α-Tubulin in the lysates are shown as a loading control (bottom panel). IB, immunoblotting.

Fig. 4. Osmotic stress inhibits IL-1-induced activation of NF-κB.

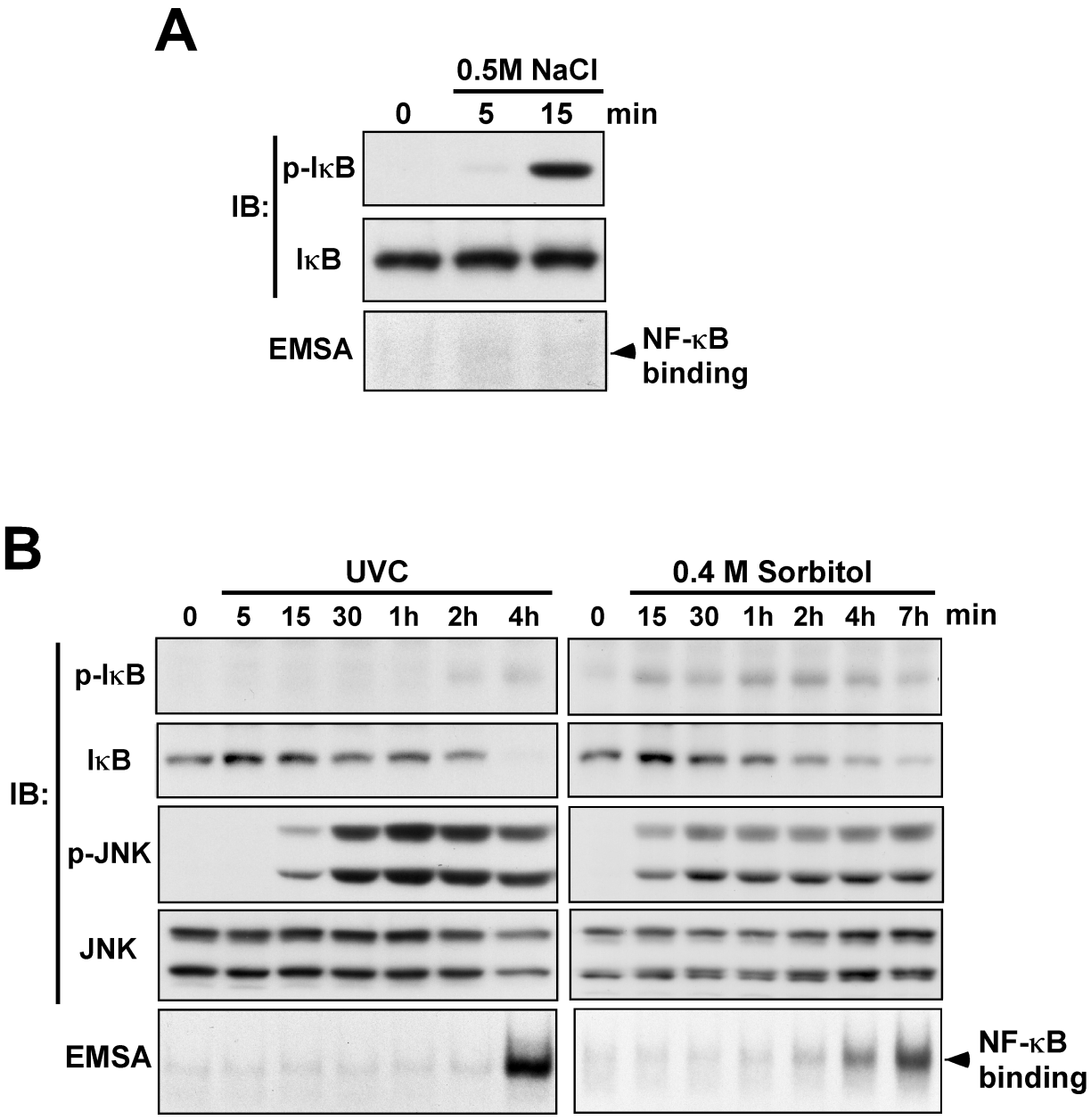
(A) 293 IL-1 RI cells were pre-treated with or without 0.4 M sorbitol for 1 hour and subsequently stimulated with 5 ng/ml for 5-30 min. Activation of NF-κB pathway was monitored by phosphorylation (top panel), degradation (middle panel), of IκBα, and EMSA (bottom panel). Activation of JNK was also monitored by phospho-JNK antibody (3rd panels). IB, immunoblotting.

(B) 293 IL-1 RI cells were treated with 5 ng/ml IL-1 alone for 3 or 15 min, or treated with 0.5 M NaCl for 15 min together with (lane 5) or without (lane 4) 5 ng/ml IL-1. Activation of the NF-κB pathway was monitored by phosphorylation (top panel), degradation (middle panel) of IκBα, and EMSA (bottom panel). Two parts from the same exposures are shown (top and middle panels). IB, immunoblotting.

(C) 293 IL-1 RI cells were transfected with expression vectors for Myc-tagged ubiquitin. At 36 hour post-transfection, cells were incubated with MG132 (20 μM) plus 0.4 M sorbitol or MG132 alone for 1 hr and subsequently stimulated with 5 ng/ml IL-1. Cell lysates are immunoprecipitated with anti-phospho IκBα and immunoblotted with anti-Myc (upper panel). The immunoprecipitated phosphorylated IκBα is also shown (lower panel). IP, immunoprecipitation; IB, immunoblotting.

Fig. 5. Osmotic stress attenuates IL-1-induced production of IL-8 and TNF.

293 IL-1 RI cells were pre-treated with or without increasing concentrations of sorbitol for 1 hour and subsequently stimulated with 5 ng/ml IL-1 for 2 hr. The levels of IL-8 (upper panel) or TNF (lower panel) mRNA were measured by quantitative real time PCR. Means of three independent samples and S.D. are shown.



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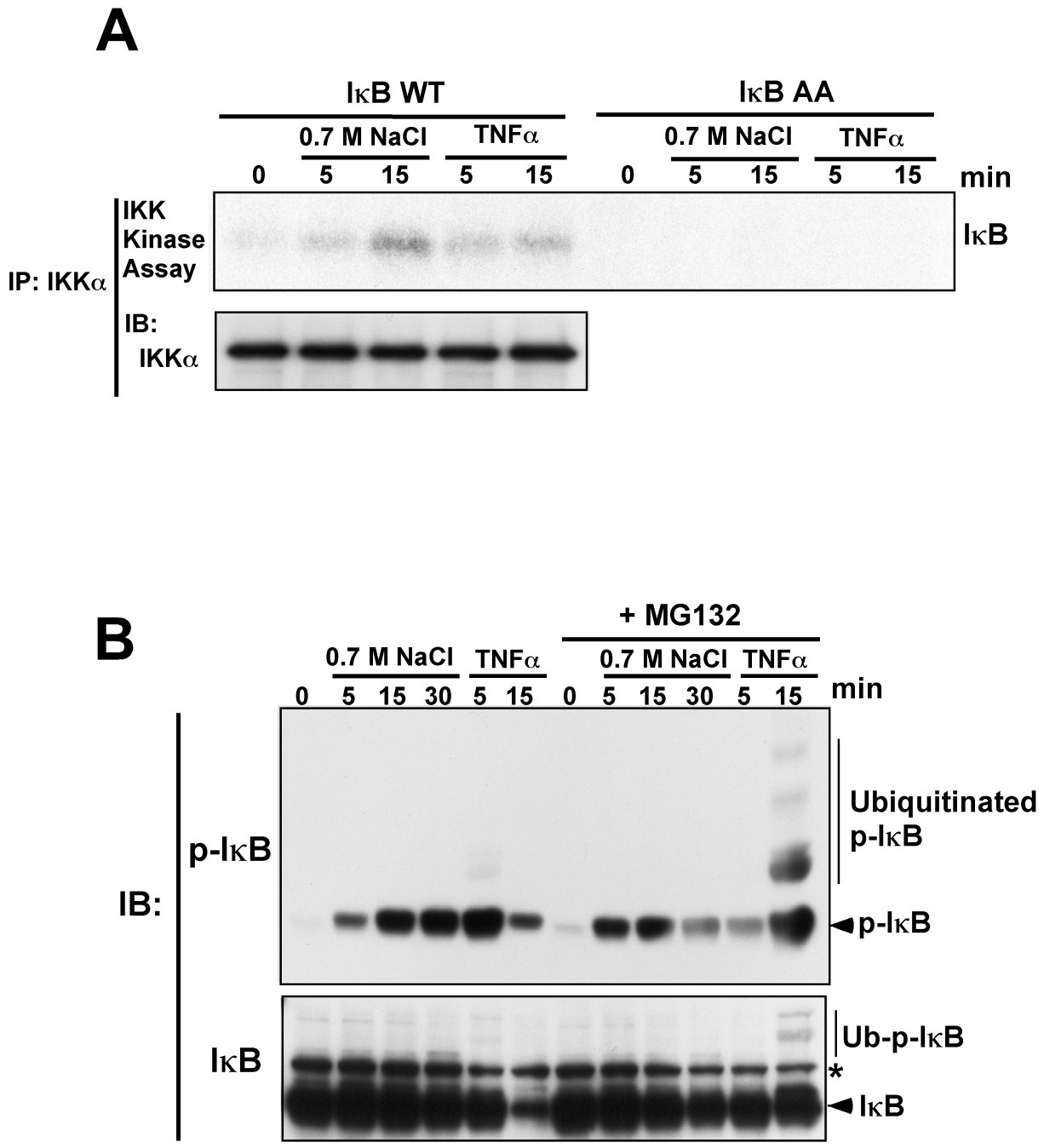
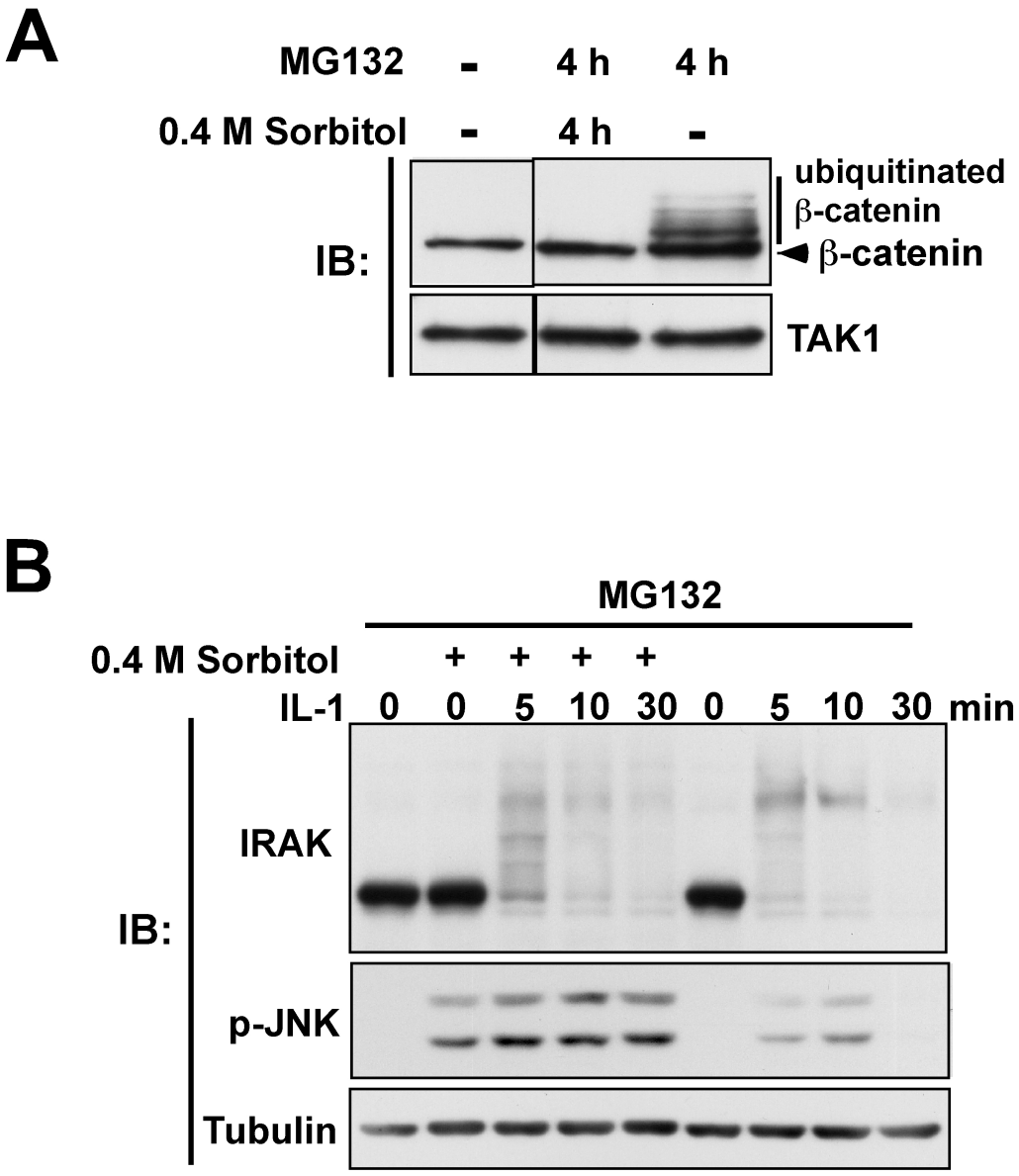


Fig. 2. Osmotic stress activates IKK but does not induce IκB ubiquitination



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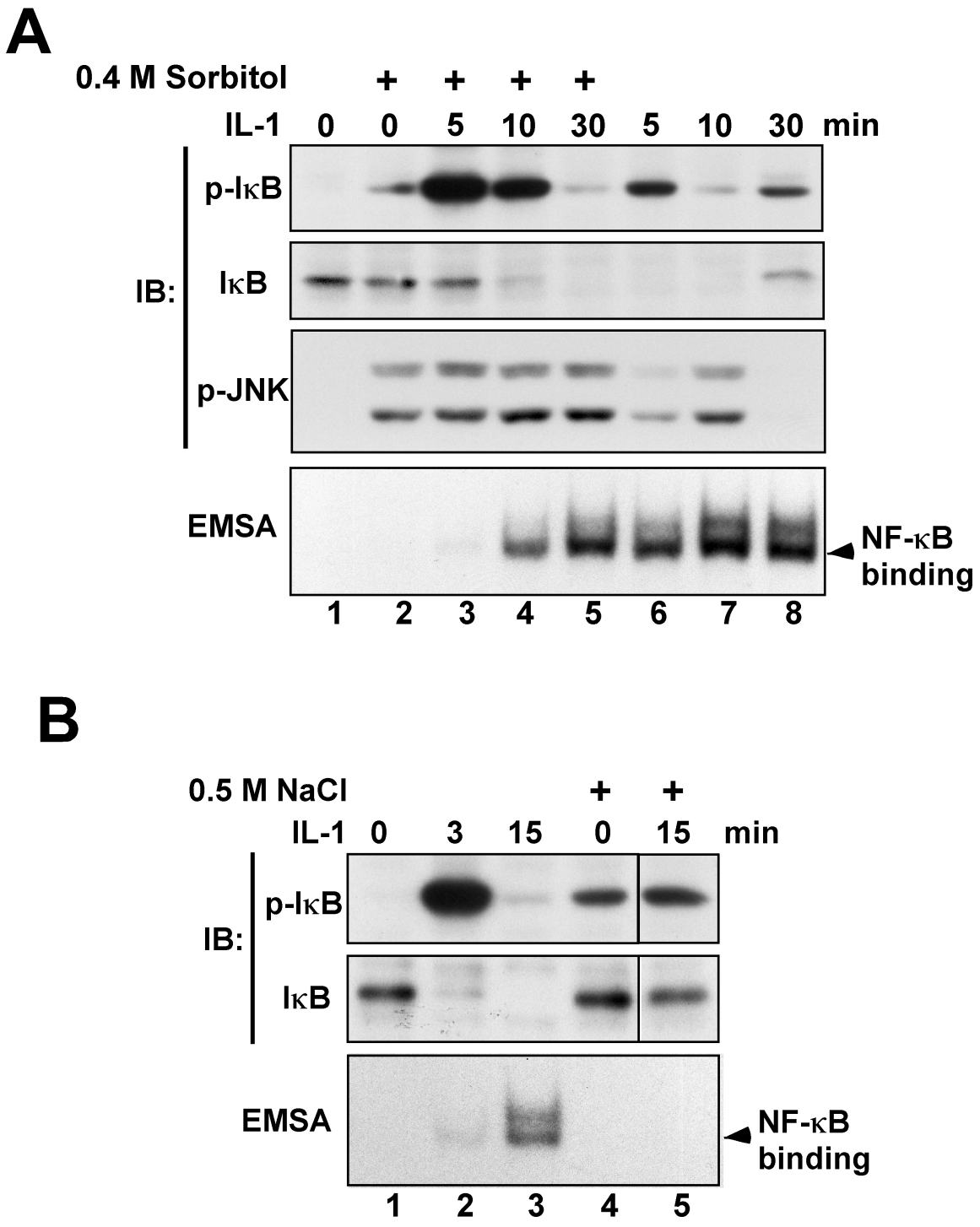

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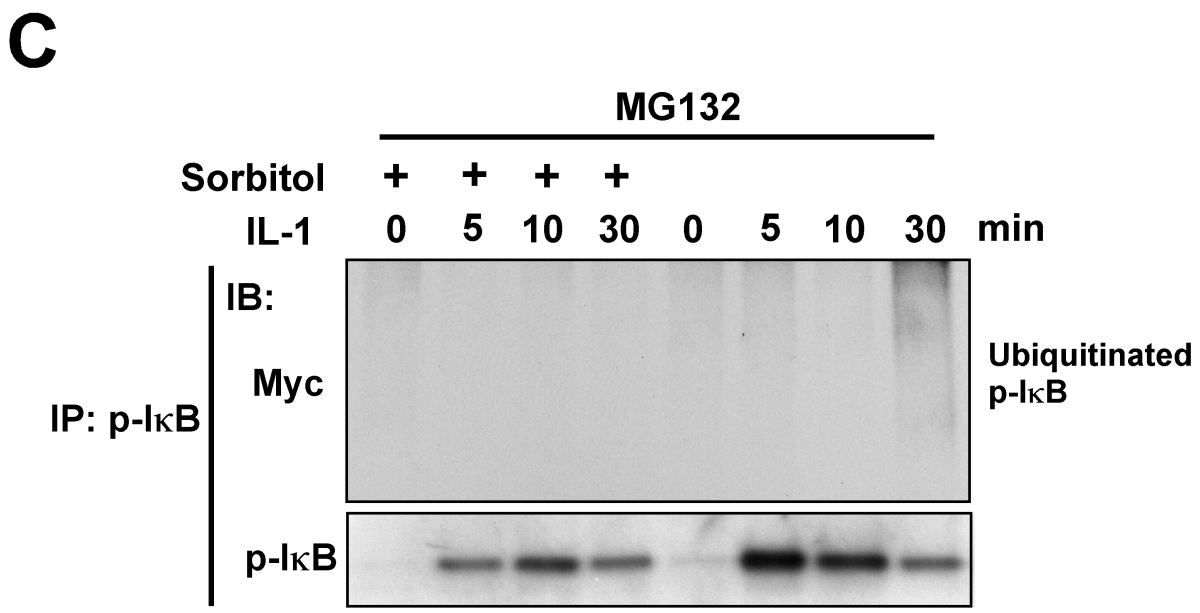

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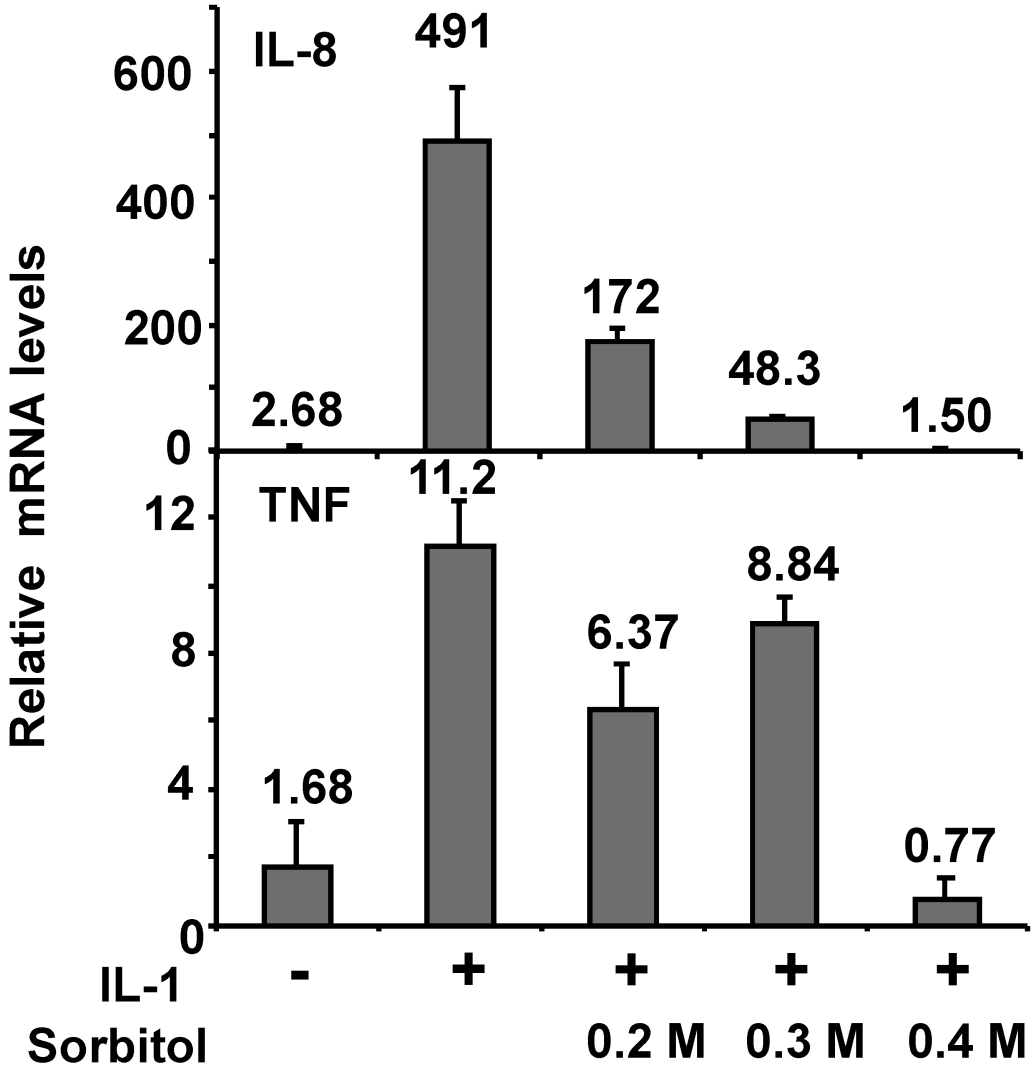

Fig. 5. Osmotic stress attenuates IL-1-induced production of IL-8 and TNF.

GENERAL DISCUSSION

(Some paragraphs are adapted from my two manuscripts and modified)

TAK1 is activated by many extracellular stimuli including osmotic stress

TAK1 can be activated by diverse stimuli, including the proinflammatory cytokines TNF, IL-1, Toll-like receptor ligands, and physical and chemical stresses. The role of TAK1 in each of these signaling pathways has been investigated using cells from genetically engineered TAK1-deficient mice, as well as with siRNA gene knockdown methods. TAK1 has been shown to be an indispensable intermediate of TNF, IL-1, and Toll-like receptor signaling in mammalian cells (Omori et al., 2006; Sato et al., 2005; Shim et al., 2005; Takaesu et al., 2003). In *Drosophila*, TAK1 is essential for activation of MAPK in response to innate immune stimuli and plays a redundant role together with MEKK and MLK in stress signaling pathways (Chen et al., 2002). We have demonstrated here that TAK1 is an important intermediate in osmotic stress induction of JNK in mammalian cells. We also showed that TAK1 is not an exclusive mediator of osmotic stress-induced p38 activation. TAK1 and other MAPKKK family members such as MEKK1 and MLK are likely to function redundantly in the p38 pathway in response to osmotic stress in mammalian cells.

Regulatory mechanisms to modify TAK1 signaling

TAK1 is activated in response to many extracellular stimuli, yet TAK1 can initiate cellular responses that are unique to each stimulus. This suggests that TAK1 may be modified by some mechanism to selectively activate different downstream pathways. We have demonstrated here that TAO2 can interact with TAK1 and is responsible for the selective

activation of JNK *versus* the NF-κB pathway. TAO2 is activated by osmotic stresses (Chen and Cobb, 2001; Chen et al., 1999); therefore, the TAK1-TAO2 complex may function to mediate osmotic stress signaling. Previously, Mochida *et al.* reported that the MAPKKK ASK1 (apoptosis-stimulating kinase 1) interacts with and inhibits TAK1 by blocking the interaction of TAK1 with tumor necrosis factor receptor-associated factor 6 (TRAF6) in the IL-1 signaling pathway (Mochida et al., 2000). ASK1 is activated by oxidative stress and participates in stress-induced apoptosis (Ichijo et al., 1997; Tobiume et al., 2001). Therefore, it may be possible that the TAK1-ASK1 complex mediates oxidative stress signaling that leads to apoptosis by activating JNK while inhibiting NF-κB.

NF-κB signaling *versus* JNK/p38 signaling

NF-κB is a major activator of cell survival signaling and inflammation (Hayden and Ghosh, 2004; Karin and Lin, 2002; Rothwarf and Karin, 1999). Activation of JNK and p38 are involved in many biological processes including inflammation, cell proliferation, differentiation and apoptotic/necrotic cell death (Chang et al., 2006; Davis, 2000; Nakano et al., 2006; Pearson et al., 2001; Varfolomeev and Ashkenazi, 2004; Ventura et al., 2004; Wada and Penninger, 2004). Proinflammatory cytokines such as TNF activate both NF-κB and JNK pathways through TAK1. Activation of both pathways is essential for gene expression involving inflammatory responses. In contrast, upregulation of inflammatory genes do not occur upon osmotic stress, but rather inflammation is inhibited by osmotic stress as described below. This is likely due to lack of NF-κB activation. It can be assumed that JNK/p38 pathway in the absence of NF-κB activation induces adaptive responses against hypertonic condition including production of osmolytes and inhibition of cell growth without inducing

inflammation. Alternatively, JNK may function to promote cell death in response to osmotic stress. In the case where NF-κB (cell survival signal) is not activated, JNK may be able to induce cell death upon osmotic stress. However, the role of JNK in apoptosis is controversial and may be tissue- and stimulus-specific. It has been demonstrated that JNK activation can promote cell survival as well as mediate apoptosis in a temporal manner (Ventura et al., 2006; Weston and Davis, 2007). We have examined whether lack of JNK activation in TAK1-deficient cells prevent cells from death upon osmotic stress (supplementary Figure S1). We found that osmotic stress kills cells regardless of TAK1 status. Therefore, TAK1-JNK pathway is not likely a major pathway to cell death in response to osmotic stress.

TAO2 is not solely responsible for inhibition of NF-κB activation

In the first study, we show that TAO2 knockdown only marginally induces NF-κB activation upon osmotic stress. This suggests that TAO2 is not solely responsible for blocking the NF-κB pathway under osmotic stress conditions. The TAO2-related kinase TAO1 may play redundantly to sequester TAK1 from the IKK complex. In addition, to securely block NF-κB activation upon osmotic stress, it is likely that the NF-κB pathway is negatively regulated through several other mechanisms. We have therefore attempted to determine such mechanisms in my second study.

Osmotic stress inhibits SCF E3 ubiquitin ligases

Our results demonstrate that osmotic stress inhibits SCF E3 ligase–mediated protein ubiquitination, resulting in stabilization of IκB and blockage of NF-κB activation. Through this mechanism, osmotic stress blocks induction of inflammatory cytokines/chemokines. What

is the specificity of osmotic stress-mediated inhibition of ubiquitin E3 ligase? We here show that osmotic stress abolishes ubiquitination of β-catenin as well as IκB, suggesting that SCF-βTrCP is inhibited by osmotic stress. Among targets of other types SCF, p27Kip degradation is impaired by osmotic stress (supplementary Figure S2). However, degradation of c-Jun and cyclin E, which are also targets of other SCF type ligases, is not blocked or rather enhanced by osmotic stress (Casanovas et al., 2000; Xia et al., 2007). We confirmed that c-Jun degradation is not impaired by osmotic stress (data not shown). We also show here ubiquitination of IRAK that is mediated by a RING type E3 ligase (Schauvliege et al., 2006) is not altered by osmotic stress. Therefore, osmotic stress specifically inhibits a subset of SCF type E3 ligases. Studies to define the molecular mechanism underlying this specific inhibition will further provide integral information to develop the strategy of therapeutic use of hypertonic solution.

What is the IKK kinase in osmotic stress signaling?

We here show that osmotic stress also activates IKK. Since osmotic stress activates several MAPKKK including MEKKs and TAK1, all of which can activate IKK. We have shown that TAO2, another MAPKKK, inhibits interaction of TAK1 with IKK and thereby prevents TAK1-mediated activation of IKK in response to osmotic stress in the first study. Therefore, TAK1 is not responsible for osmotic stress-induced IKK activation. Indeed, we found that osmotic stress still induces IκB phosphorylation in TAK1 deficient mouse embryonic fibroblasts (supplementary Figure S3). Thus. MEKKs are likely to activate IKK in response to osmotic stress.

Fine-tuning of stress responses

In osmotic stress signaling pathway, MAPK pathway mediated by MAPKKKs appears to be very important to upregulate protective molecules against hypertonic environment. Therefore, osmotic stress triggers strong activation of MAPKKKs to effectively upregulate MAPK cascades. In contrast, NF-κB-dependent inflammation appears not to be desired outcomes of osmotic stress. However, because of strong activation of MAPKKKs, IKK seems to be activated as a byproduct. TAK1-NF-κB pathway is inhibited by TAO2 and other MAPKKK-NF-κB pathways may be inhibited through several mechanisms including blockage of IκB ubiquitination. To induce appropriate cellular responses against hypertonic condition, such fine-tuning mechanisms should be essential.

Model of osmotic stress signaling

Taken our results all together, we propose a model of osmotic stress signaling pathways (supplementary Figure S4). Osmotic stress activates multiple MAPKKKs including TAK1, TAO2 and MEKKs. TAK1 and TAO2 form a complex, which plays an essential role to activate JNK pathway. TAO2 interferes with TAK1 binding to IKK thereby inhibiting TAK1-induced activation of IKK-NF-κB pathway. MEKKs are likely to activate p38 and IKK. Although IKK phosphorylates IκB, ubiquitination of IκB is suppressed under osmotic stress through inhibition of SCF E3 liganses. JNK and p38 pathways upregulate cellular responses and protect cells from hypertonic condition. Inhibition of SCF E3 ligase reduces inflammatory responses and may participate in growth arrest through preventing degradation of inhibitor of cyclin dependent kinase, p27Kip.

Significance and implication of these studies

Osmotic stress is a major problem in single cell organism such as yeast. Although higher eukaryotes are not directly affected by fluctuations of environmental osmolarity, many cells still need to response to osmotic stress. Under physiological conditions, osmotic stress takes place in renal epithelial cells, where cells produce osmolytes to protect shrinkage of cells. Dysregulation of osmotic stress signaling causes renal dysfunction associated with disease such as high blood pressure. Under pathological conditions, many cells can be exposed to hypertonic condition. High glucose condition in diabetes affects a number of biological processes including immune responses. Defining the osmotic signaling pathway is essential to understand the physiological and pathological cellular responses involving many diseases. From a therapeutic standpoint, use of hypertonic solutions potentially has benefits. Hypertonic solutions can reduce acute or chronic inflammation in some pathogenic conditions, and it could be inexpensive and safer alternative of anti-inflammation drugs. Understanding the molecular mechanism of how hypertonic condition prevents inflammation is essential to develop strategy for use of hypertonic solutions.

Causation vs. association

Whether the osmotic stress-mediated immune suppression is caused by osmotic stress or just associated with osmotic stress? Association does not imply a direct causal connection between the associated variables.

I believe that osmotic stress causally inhibits NF-κB pathway. The reasons are as follows:

1) Osmotic stress does not generally inhibit cell signaling, i.e. osmotic stress activates JNK.

2) Osmotic stress does not generally inhibit protein ubiquitination. Ubiquitination of cyclin E and c-Jun is rather enhanced by osmotic stress.

3) Mild osmotic stress does not induce cell death or affect cell proliferation but still inhibits NF-κB signaling.

We could further strengthen the causation relationship by identifying specific molecules that alter the particular subset of E3 ligase function under osmotic stress.

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SUPPLEMENTARY FIGURES

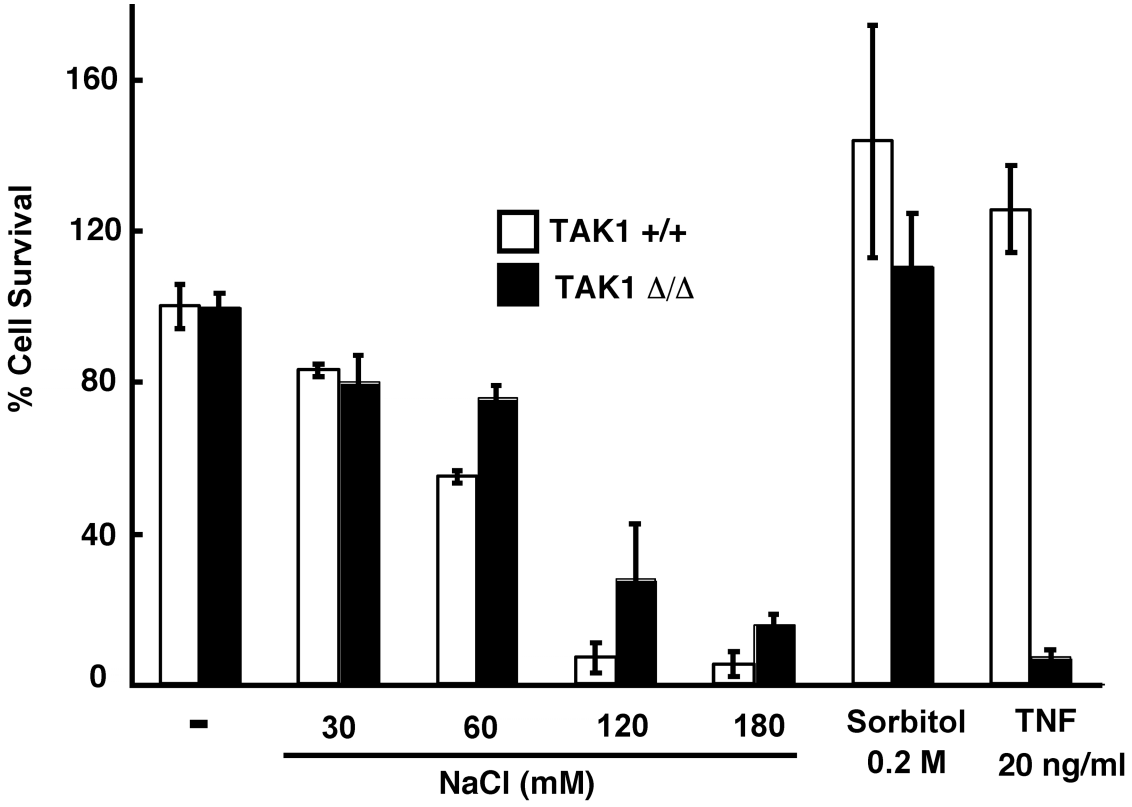

Fig. S1. TAK1-JNK pathway is dispensable for osmotic stress-induced cell death. Wildtype (WT) and TAK1Δ/Δ MEF cells were stimulated with osmotic stress (NaCl and sorbitol) or TNF for 24 h. For osmotic stress, indicated concentrations of solutes (NaCl and sorbitol) were added to the culture medium containing 150 mM NaCl (isotonic). Viability was assessed with crystal violet staining. Data show the means and S.D. (n=3).

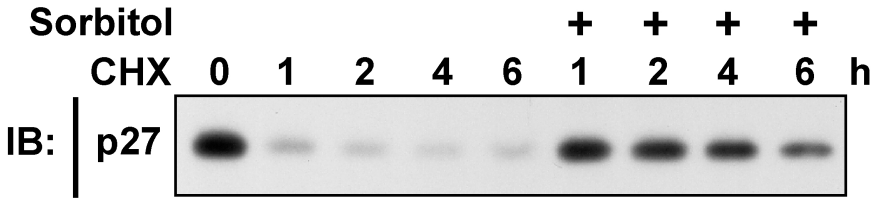

Fig. S2. p27Kip degradation is impaired by osmotic stress.

Cycloheximide (CHX) chase analysis of degradation of p27Kip in 293 cells. Levels of p27 at the indicated time points after adding CHX (50 μg/ml) to cells in the absence or presence of 0.4 M sorbitol were analyzed by immunoblotting with anti-p27. IB, immunoblotting.

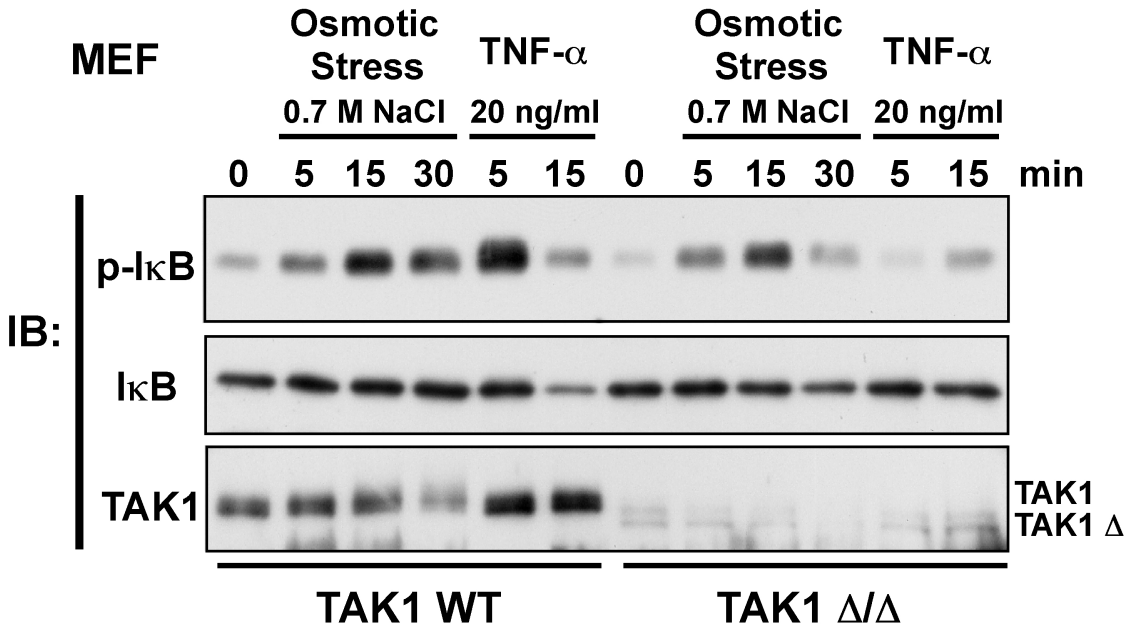

Fig. S3. Osmotic stress induces IκB phosphorylation in TAK1 deficient MEF cells. Wildtype (WT) and TAK1Δ/Δ MEF cells were treated with 0.7 M NaCl or 20 ng/ml TNF-α for 5-30 min. Phosphorylated IκB was detected with anti-phospho IκB (top panel). The amount of IκB was detected with anti-IκB (middle panel). TAK1 expression was detected with anti-TAK1 (bottom panel). IB, immunoblotting.

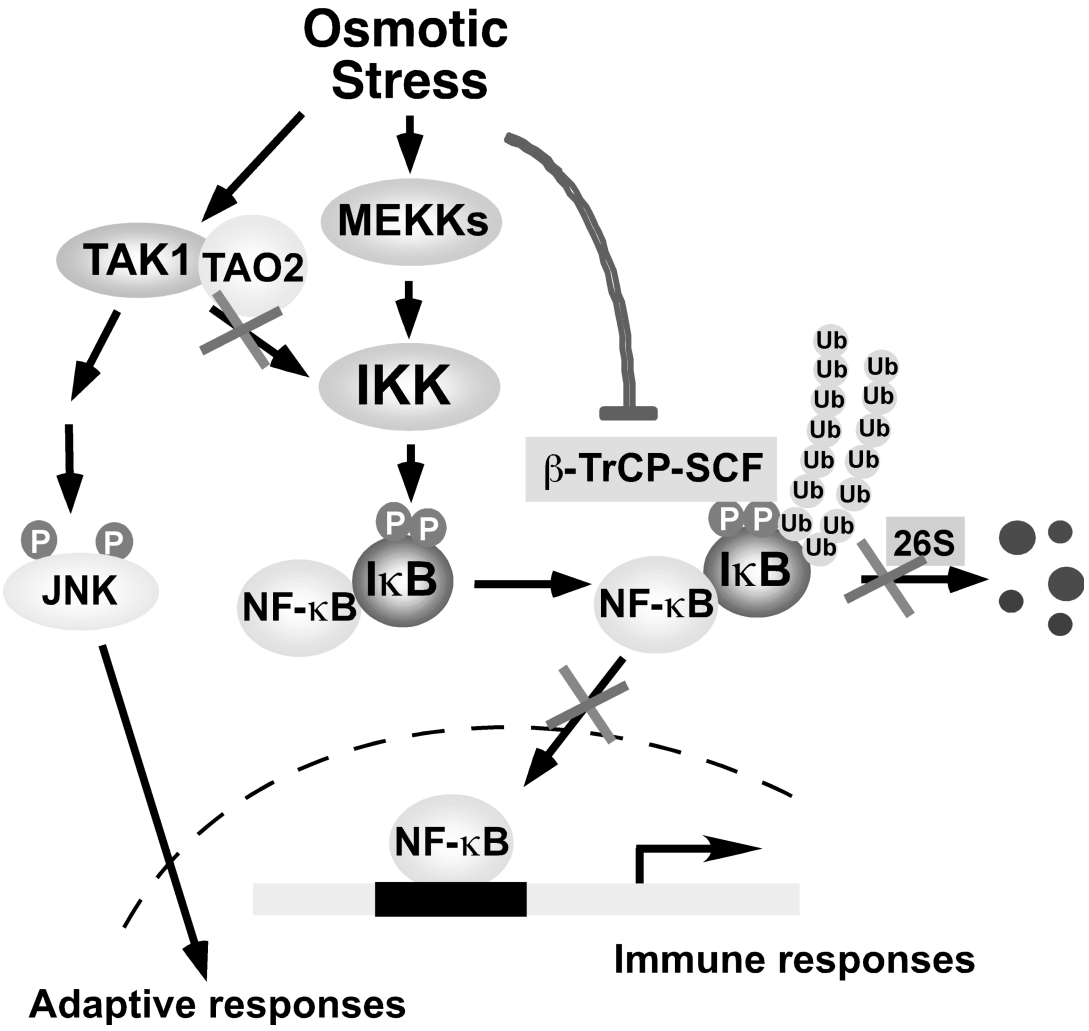

Fig. S4. Model

Upon osmotic stress stimulation, two pathways that prevent NF-κB activation are initiated. TAK1 and TAO2 form a complex, which plays an essential role to activate JNK pathway. TAO2 interferes with TAK1 binding to IKK thereby inhibiting TAK1-induced activation of IKK-NF-κB pathway. MEKKs are likely to activate IKK. Although IKK phosphorylates IκB, ubiquitination of IκB is suppressed under osmotic stress through inhibition of SCF E3 liganses. JNK pathway upregulates cellular responses and protects cells from hypertonic condition. Inhibition of SCF E3 ligase reduces inflammatory responses and may participate in growth arrest.