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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 could activate IκBα kinase but did not activate NF-κB. Osmotic stress-induced phosphorylated IκBα was not ubiquitinated, and osmotic stress inhibited interleukin 1-induced ubiquitination of IκBα and ultimately blocked expression of cytokine/chemokines. Thus, blockage of IκBα ubiquitination is likely to be a major mechanism for inhibition of inflammation by hypertonic conditions.**

Keywords: NF-κB; Cytokine; Osmotic stress; Inflammation

1. Introduction

It has long been known that the use of hypertonic fluid for resuscitation after trauma has some benefits compared to isotonic fluid [1]. Hypertonic solutions have been shown to reduce inflammation associated with resuscitated hemorrhage shock, thereby reducing organ failure caused by systemic inflammation [2–4]. In an animal model system, administration of hypertonic saline increases serum osmolarity and blocks the production of proinflammatory cytokines such as tumor necrosis factor (TNF) [5]. In vitro studies have revealed that hypertonic condition inhibits the activation of neutrophils and macrophages [6–8]. However, the molecular mechanism(s) 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 [9]. In unstimulated cells, a complex of NF-κB and its inhibitory protein IκB proteins resides in the cytoplasm. IκB masks the nuclear localization signal of NF-κB [10]. Proinflammatory stimuli such as interleukin 1 (IL-1) and TNF initiate an intracellular signaling cascade leading to activation of the IκB kinase (IKK) complex, which consists of IKK-α, IKK-β and IKK-γ/NEMO. IKK in turn phosphorylates IκBα at Ser-32 and Ser-36, which are recognized by β-TrCP, a subunit of the SCF type E3 ligase [11]. Phosphorylated IκBα 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 motigen-activated protein kinase (MAPK) cascades. The most upstream components of the MAPK cascades are MAPK kinase kinases (MAP3Ks), and a sub-group of MAP3Ks including TAK1 and MEKKs can activate not only MAPK cascades but also IKK, leading to NF-κB activation [12,13]. However, even though osmotic stress strongly activates MEKKs and TAK1, activation of NF-κB is normally undetectable under osmotic stress conditions [14]. 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 the NF-κB pathway. However, knockdown of TAO2 was observed to have only a partial effect on relieving inhibition of NF-κB [14]. This suggests that NF-κB activation is blocked by additional mechanisms that function under conditions of osmotic stress. In this study, we investigated the regulation of NF-κB pathway in response to osmotic stress.

2. Materials and methods

2.1. Cell culture and transfection

Human embryonic kidney 293 cells, 293 IL-1RI cells that stably express the IL-1 receptor I [15] were maintained as described previously [14]. Mouse keratinocytes were described previously [16]. Bone marrow-derived macrophages (BMDMs) were prepared from femora and tibiae of C57BL/6 mice. Bone marrow cells were flushed out from bone marrow cavity and cells were suspended in macrophage-medium (DMEM containing 10% BGS, 30% L929-conditioned medium). 293

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Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin 1; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; BMDM, bone marrow-derived macrophages; EMSA, electrophoretic mobility shift assay; JNK, c-Jun N-terminal kinase

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IL-1RI cells were transfected with expression vectors for Myc- or HA-tagged ubiquitin (pcDNA3.1-Myc-Ub, pcDNA3.1-HA-Ub, provided by Dr. Tanaka) by the standard calcium phosphate precipitation method.

2.2. Antibodies

Anti-phospho-SAPK/JNK (Thr-183/Tyr-185) rabbit polyclonal antibody (Cell Signaling), Anti-JNK1 (FL) polyclonal antibody (Santa Cruz), anti-IκBα (C-21) polyclonal antibody (Santa Cruz), anti-IκBα (Ser32) polyclonal antibody (Cell Signaling), anti-phospho-IκBα (Ser32/36) (5A5) monoclonal antibody (Cell Signaling), anti-IKK-α (H-744) polyclonal antibody (Santa Cruz), anti-cMyc (9E10) monoclonal antibody (Santa Cruz), anti-IRAK polyclonal antibody [15], anti-α tubulin (TU-02) monoclonal antibody (Santa Cruz), and anti-β-catenin monoclonal antibody (BD), were used for immunoprecipitation and immunoblotting.

2.3. 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.

2.4. Immunoprecipitation–immunoblotting, electrophoretic mobility shift assay (EMSA), in vitro kinase assays

These assays were performed as described previously [14]. To inhibit ubiquitin protases, 50 mM iodoacetamide was added to lysis buffer in some experiments.

2.5. Real-time PCR

Total RNA was used for quantitative real-time PCR. GAPDH was used as an internal control. SYBR Green Master Mix or Taqman gene expression assay system (Applied Biosystems) were used. 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′; TNF-α forward, 5′-TCTGCCTGC TGCACTTTGG-3′; TNF-α reverse, 5′-GCCAGAGGGCTGATTAGAGAGA-3′. Human TAO2 specific primers were purchased from Qiagen. The mouse primers and probes for Taqman system were purchased from Applied Biosystems.

3. Results and discussion

We have previously demonstrated that 0.5–0.7 M NaCl strongly activates c-Jun N-terminal kinase (JNK), while IκBα degradation and NF-κB activation are not detectable [14]. However, under the same experimental condition, we showed that phosphorylation of IκBα is detectable to some extent [14]. In this study, we investigated the significance of this phosphorylation. We used two osmotic stressors to assess the relationship between osmotic stress and the NF-κB pathway. NaCl (0.5–0.7 M) is a highly potent inducer of osmotic stress and was used to verify the strongest effect of osmotic stress. Alternative osmotic stressor sorbitol has a milder effect than NaCl and cells usually can survive for more than 12 h after 0.4 M sorbitol treatment. Sorbitol (0.4 M) was used to observe a longer-lasting effect of osmotic stress, which may be more relevant to physiological conditions. We used 293 cells stably expressing IL-1 receptor I (293 IL-1RI), which respond highly to IL-1 and moderately to TNF. 293 IL-1RI cells also respond to several stresses. IL-1 induced phosphorylation of IκBα, rapid degradation of IκBα and activation of NF-κB (Fig. 1, lanes 1–4). At 60 min after IL-1 stimulation, IκBα was re-accumulated due to NF-κB-dependent transcriptional activation of IκB [17]. IL-1 could very effectively induce phosphorylation of IκBα (Fig. 1, lanes 1–4). TNF could also induce phosphorylation and degradation of IκB, and activated NF-κB (Fig. 1, lanes 11–13), although the levels of induction were lower than those in IL-1-stimulated cells. In contrast, 0.5 M and 0.7 M NaCl induced phosphorylation of IκBα but did not significantly induce degradation of IκBα, and no activation of NF-

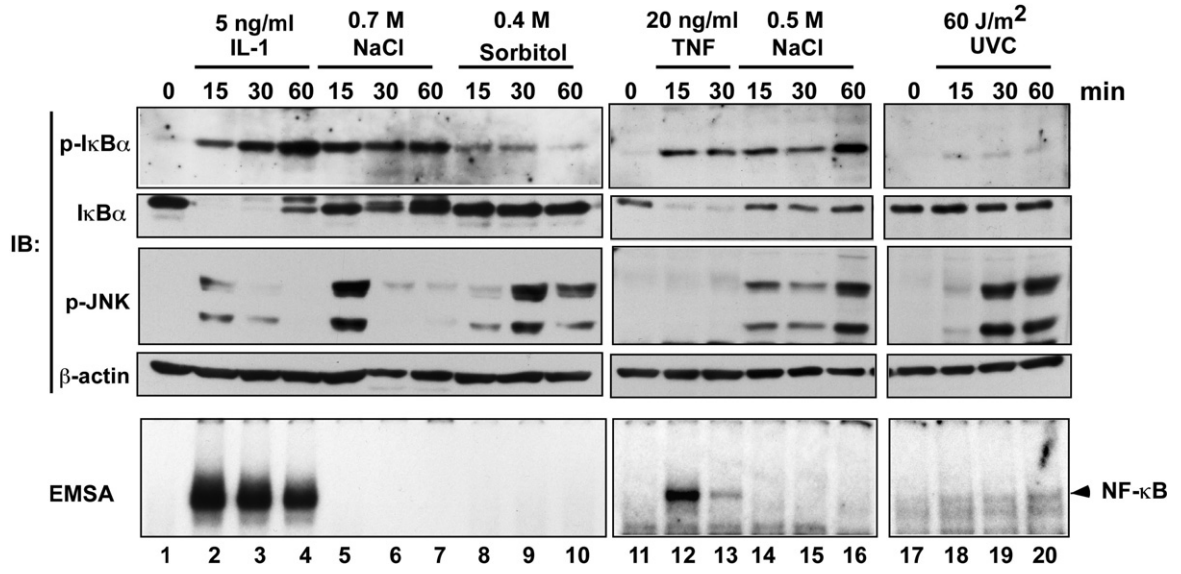

Fig. 1. Osmotic stress induces IκB phosphorylation but not degradation. 293 IL-1RI cells were treated with 5 ng/ml IL-1, 0.7 M NaCl, 0.4 M sorbitol, 20 ng/ml TNF or 0.5 M NaCl, or exposed to UVC (60 J/m^2). Cells were harvested at the time points indicated after treatment or exposure. Activation of the NF-κB pathway was monitored by phosphorylation (top panels), degradation (second panel) of IκBα, and EMSA (bottom panels). Activation of JNK was monitored by phospho-JNK antibody (third panel). The amount of β-actin is also shown as loading control (fourth panels). IB, immunoblotting.

κB was detected (Fig. 1, lanes 5–7 and 14–16). Sorbitol (0.4 M) also induced phosphorylation of IκBα, although induction was weak compared to stress induced by 0.5 M NaCl (Fig. 1, lanes 8–10). This phosphorylated IκBα was not degraded, and NF-κB was not activated. In addition, we examined the effect of a non-osmotic stressor, ultraviolet C (UVC) light irradiation in order to verify the specificity of this IκBα phosphorylation. UVC (60 J/m^2) stress is a potent activator of MAPK cascades, DNA damage and ultimately cell death [18]. UVC strongly activated JNK, similar to 0.5 M NaCl and 0.4 M sorbitol; however, UVC only marginally induced phosphorylation of IκBα in 293 IL-1RI cells (Fig. 1, lanes 17–20). These results demonstrate that osmotic stress induces phosphorylation of IκBα but not activation of NF-κB, and that this effect on the NF-κB pathway may be induced specifically by osmotic stress.

We then sought to determine why degradation of IκBα 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. We performed an in vitro kinase assay using GST- IκBα and a mutant version GST- IκBα AA, which contains Ser to Ala substitutions at Ser-32 and Ser-36 (Fig. 2A). We used 0.7 M NaCl and TNF, because they could induce phosphorylation of IκBα at the similar levels (Fig. 2A, third panel). 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 IκBα is normally ubiquitinated by SCF-β-TrCP; therefore, we asked whether phosphorylated IκBα is ubiquitinated following osmotic stress. We stimulated cells with 0.7 M NaCl or TNF in

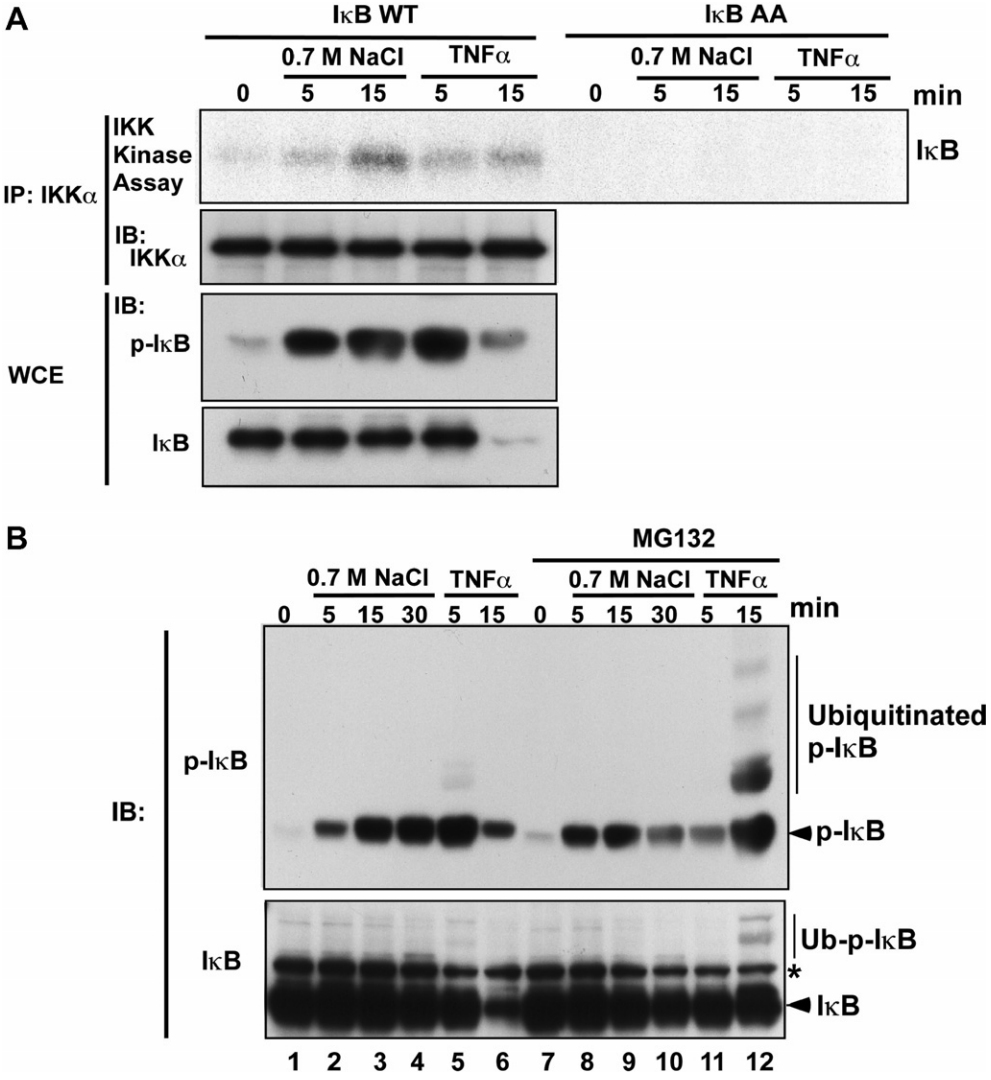

Fig. 2. Osmotic stress activates IKK but does not induce ubiquitination of IκB (A) 293 cells were treated with 0.7 M NaCl or 20 ng/ml TNF-α, and IKK complex was immunoprecipitated with anti-IKKα. The immunocomplexes were subjected to an in vitro kinase assay using GST-tagged wild-type (IκB WT) or a mutant version of IκBα (IκB AA) as an exogenous substrate (top panel). Immunoprecipitated IKK-α was detected by immunoblotting (second panel). Phosphorylated IκBα and total amount of IκBα were detected by immunoblotting with anti-phospho IκB (third panel) and anti-IκB (bottom panel). IP, immunoprecipitation; IB, immunoblotting; WCE, whole cell extracts. (B) 293 cells were transfected with an expression vector for HA-tagged ubiquitin and incubated with vehicle (DMSO) or MG132, 10 μM for 6 h. Cells were then stimulated with 0.7 M NaCl or 20 ng/ml TNF. Phosphorylated IκBα and polyubiquitinated IκBα were detected by immunoblotting with anti-phospho IκB (upper panel). Total IκB was detected with anti-IκB (lower panel). Asterisk indicates a non-specific band. IB, immunoblotting.

the presence or absence of the proteasome inhibitor MG132, and IκBα was analyzed by immunoblotting (Fig. 2B). NaCl (0.7 M) could induce phosphorylation of IκBα at the level similar to that in TNF stimulated cells (Fig. 2B, lanes 1–6). When cells were stimulated by TNF in the presence of MG132, phosphorylated IκBα appeared as a ladder of slowly migrating bands, indicating polyubiquitination of IκBα (Fig. 2B, lane 12). In contrast, no slowly migrating form of IκBα was detected in cells subjected to osmotic stress. These results indicate that ubiquitination of IκBα is blocked by osmotic stress.

Our results raise the possibility that osmotic stress inhibits SCF-β-TrCP E3 ubiquitin ligase that is responsible for ubiquitination of several proteins including IκBα. β-catenin, a signaling molecule in the Wnt pathway, is another target of SCF-β-TrCP. We therefore examined whether osmotic stress alters the ubiquitination status of β-catenin (Fig. 3A). Cells were treated with MG132 for 4 h to drive the accumulation of the ubiquitinated form of β-catenin. Because 0.5–0.7 NaCl causes cell death within 1–2 h, we co-incubated cells with a milder osmotic stress 0.4 M sorbitol and MG132. Ubiquitination of β-catein can be detected by a slowly migrating ladder of bands [19]. Ubiquitination of β-catenin diminished with 0.4 M sorbitol. To examine that osmotic stress does not generally inhibit ubiquitination, we tested the effect of osmotic stress on ubiquitination mediated through other mechanisms. IRAK is a signaling molecule in the IL-1 pathway that is phosphorylated and eventually heavily ubiquitinated for degradation following IL-1 stimulation [20]. We examined IL-1-induced ubiquitination of IRAK with and without a 1 h-pretreatment of 0.4 M sorbitol or 0.5 M NaCl in 293 IL-1RI cells (Fig. 3B, and data not shown). We found that IRAK was ubiquitinated 5–30 min after IL-1 stimulation regardless of osmotic stress conditions. This indicates that osmotic stress does not generally inhibit protein ubiquitination.

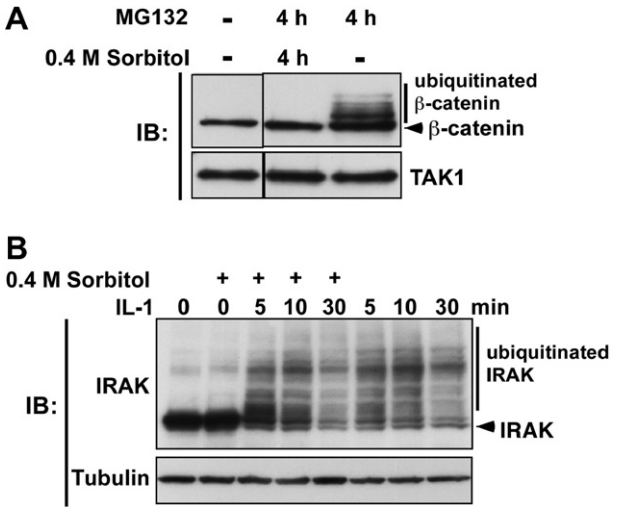

Fig. 3. Osmotic stress inhibits ubiquitination of β-catenin but not of IRAK. (A) 293 cells were incubated with 0.4 M sorbitol and MG132 (10 μM) or MG132 alone for 4 h. β-Catenin was detected by immunoblotting (IB) (upper panel). TAK1 immunoblotting is shown as a loading control (lower panel). Two parts from the same exposure are shown. (B) 293 IL-1 RI cells were pre-incubated with 0.4 M sorbitol for 1 h followed by stimulation with 5 ng/ml IL-1 for 5–30 min. IRAK and polyubiquitinated IRAK were detected with anti-IRAK (top panel). Levels of α-tubulin in the lysates are shown as a loading control (lower panel). IB, immunoblotting.

We next asked whether osmotic stress could block IL-1-induced activation of NF-κB. IL-1 stimulation induced phosphorylation of IκBα, rapid degradation of IκBα and subsequent re-accumulation of IκBα (Fig. 4A, lanes 1 and 6–8). We examined the effect of preconditioning with 0.4 M sorbitol on degradation of IκB and activation of NF-κB (Fig. 4A, lanes 2–5). As shown in Fig. 3B, this preconditioning did not affect IL-1-induced ubiquitination of IRAK. In contrast, the 0.4 M sorbitol preconditioning strikingly increased the amount of phosphorylated IκB but the total amount of IκBα decreased

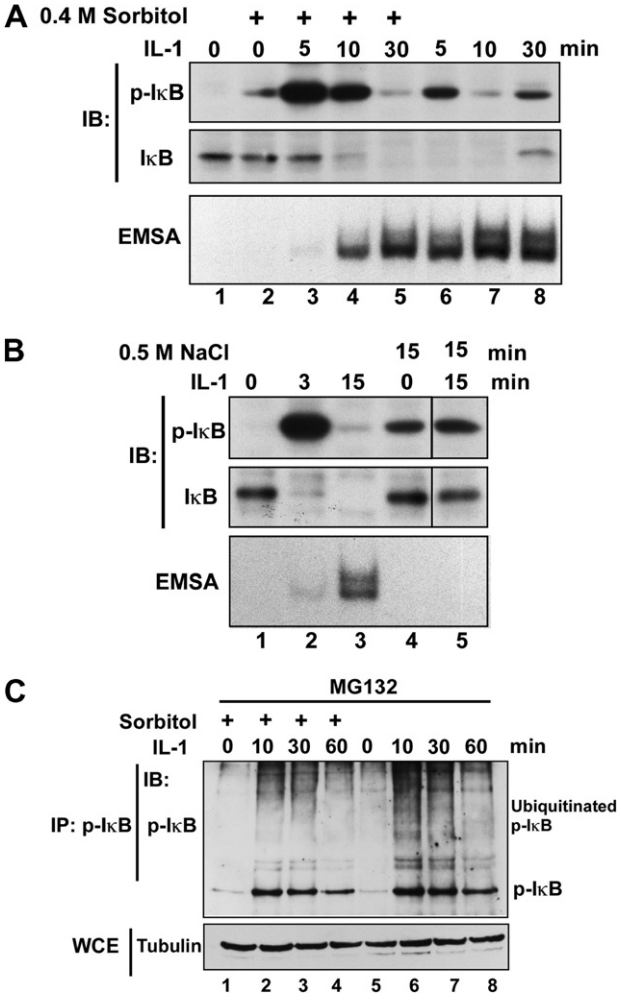

Fig. 4. Osmotic stress inhibits IL-1-induced NF-κB pathway. (A) 293 IL-1 RI cells were pre-treated with or without 0.4 M sorbitol for 1 h and subsequently stimulated with 5 ng/ml IL-1 for 5–30 min. Activation of the NF-κB pathway was monitored by phosphorylation (top panel), degradation (middle panel), of IκBα, and EMSA (bottom panel). (B) 293 IL-1 RI cells were treated with 5 ng/ml IL-1 alone for 3 or 15 min (lanes 2 and 3), 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 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 h post-transfection, cells were incubated with MG132 (10 μM) plus 0.4 M sorbitol or MG132 alone for 1 h and subsequently stimulated with 5 ng/ml IL-1. Cell lysates are immunoprecipitated with anti-phospho IκBα and immunoblotted with anti-phospho IκBα (upper panel). Levels of α-tubulin in the extracts are also shown (lower panel). IP, immunoprecipitation; IB, immunoblotting; WCE, whole cell extracts.

more slowly upon IL-1 stimulation (Fig. 4A, lanes 3–5). IL-1-induced activation of NF-κB was significantly reduced by 0.4 M sorbitol treatment (Fig. 4A, bottom panel). We also examined the effect of a highly potent osmotic stress, 0.5 M NaCl. Co-treatment of 0.5 M NaCl together with IL-1 was sufficient to completely block IL-1-induced degradation of IκB and activation of NF-κB (Fig. 4B, lanes 3 and 5). These demonstrate that osmotic stress blocks IκBα degradation and activation of NF-κB. We then examined whether osmotic stress could block IL-1-induced ubiquitination of IκBα. 293 IL-1RI were transfected with an expression vector for ubiquitin and stimulated with IL-1 in the presence of MG132. Ubiquitination of phosphorylated IκBα was monitored by immunoblotting (Fig. 4C). We observed accumulation of polyubiquitinated IκBα at 10–60 min after IL-1 stimulation (Fig. 4C, lanes 6–8). 0.4 M sorbitol reduced IL-1-induced polyubiquitinated IκBα (Fig. 4C, lanes 2–4). These results demonstrate that osmotic stress inhibits the IL-1-induced NF-κB pathway by blocking IκBα ubiquitination.

Finally, we asked whether osmotic stress inhibits inflammatory responses. 293 IL-1RI cells were used as a model system of IL-1-induced inflammatory responses, and mouse keratinocytes were used to examine the IL-1 responses in physiologically relevant cells. Keratinocytes are major cells in skin epidermis and function as a barrier by physically preventing invasion of microorganisms and by expressing cytokines/chemokines. Macrophages, one of the major cell types to mediate inflammation, were also used. We examined IL-1-induced cytokine/chemokine production following pretreatment with increasing concentrations of sorbitol in 293 IL-1RI and keratinocytes that was isolated from mouse epidermis described previously [16] (Fig. 5A and B). Cells treated with 0.3–0.4 M sorbitol shrunk, but remained viable for at least for 12 h, and expression of unrelated genes including TAO2 kinase, TAK1 kinase, Bcl-2 and BclxL was not markedly altered by sorbitol treatment (Fig. 5A and B, and data not shown). In contrast, expression of chemokine IL-8, MIP2 (mouse ortholog of human IL-8), and cytokine TNF was greatly increased by stimulation with IL-1, and this increase was effectively blocked by 0.3–0.4 M sorbitol (Fig. 5A and B). Bone marrow macrophages (BMDMs) did not effectively respond to IL-1 but was strongly activated by lipopolysaccharide (LPS), which activates the intracellular signaling pathway very much similar to that activated by IL-1 [21]. We treated BMDMs with increasing concentrations of sorbitol and subsequently stimulated by LPS (Fig. 5C). LPS-induced expression of MIP2 and TNF was also reduced by osmotic stress, although osmotic stress was less effective in BMDMs than in 293 IL-1RI cells or keratinocytes. These results suggest that osmotic stress can inhibit inflammation.

Our results demonstrate that osmotic stress inhibits SCF E3 ligase-mediated protein ubiquitination, resulting in the stabil-

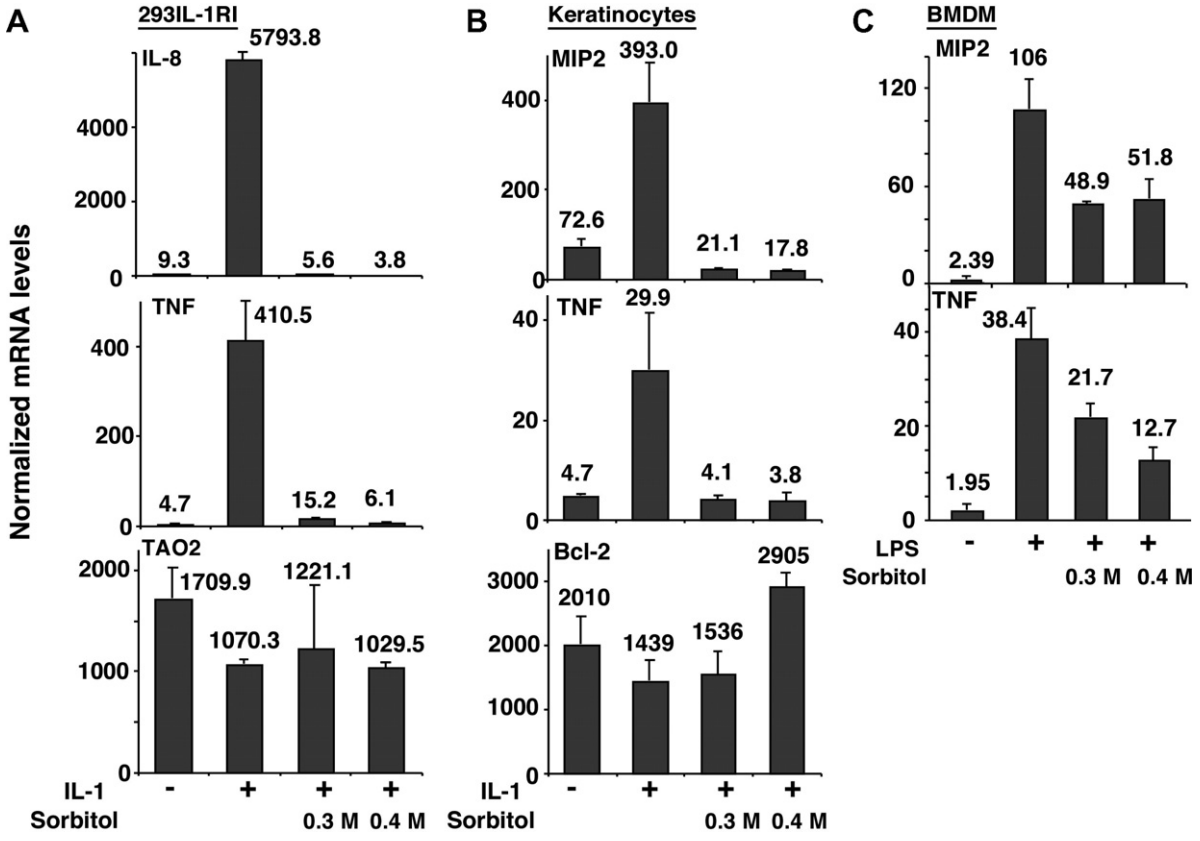

Fig. 5. Osmotic stress inhibits expression of cytokines/chemokines. (A) 293 IL-1 RI cells were pre-treated with or without increasing concentrations of sorbitol for 1 h and subsequently stimulated with 5 ng/ml IL-1 for 3 h. (B) Mouse keratinocytes were pre-treated with or without increasing concentrations of sorbitol for 1 h and subsequently stimulated with 5 ng/ml IL-1 for 3 h. (B) BMDMs were pre-treated with or without increasing concentrations of sorbitol for 1 h and subsequently stimulated with 100 ng/ml LPS for 3 h. The levels of IL-8, MIP2, TNF, TAO2 and Bcl2 mRNA were measured by quantitative real-time PCR. The mRNA levels were normalized to the levels of GAPDH. The means of three independent samples and S.D. are shown.

ization of IκBα and inhibition of NF-κB activation. However, earlier studies have reported that osmotic stress can induce activation of NF-κB and subsequently upregulates production of cytokines in several cell types [22–25]. We also found that 0.4 M sorbitol could induce moderate activation of NF-κB around 4 h after stimulation (Supplementary Fig. 1). However, we should note that IκB was very slowly degraded in the presence of 0.4 M sorbitol, even though IκB was phosphorylated within 15 min. We think that osmotic stress initially inhibits degradation of IκB by blocking ubiquitination of IκB, but later induces degradation of IκB through a delayed-onset mechanism. This mechanism may be regulated by reactive oxygen species as described by Eisner [23]. Expression of TNF was also moderately induced by 0.2–04 M sorbitol, but was much weaker compared to IL-1-induced TNF expression (Supplementary Fig. 1). Moreover, the inhibitory effect on IL-1-induced TNF expression by osmotic stress completely overrode its effect of NF-κB activation (Supplementary Fig. 2). Therefore, it is likely that osmotic stress inhibits inflammatory responses in vivo where IL-1 (and LPS) usually plays as an inflammatory mediator, even though osmotic stress alone is capable of activating NF-κB.

The use of hypertonic solutions to reduce acute or chronic inflammation in some pathogenic conditions potentially has benefits, because it could be an inexpensive and safer alternative to anti-inflammatory drugs. Our results demonstrate that osmotic stress can block IL-1-induced activation of NF-κB by inhibiting ubiquitination of IκBα, which inhibits subsequent upregulation of inflammatory cytokines. Further studies to define the molecular mechanisms of how osmotic stress inhibits SCF-β-TrCP E3 ligase should contribute to develop strategies for the therapeutic use of hypertonic solutions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.11.002.

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