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

Wei-Chun HuangFu[a,1], Kunihiro Matsumoto[b,c], Jun Ninomiya-Tsuji[a,*]

[a] Department of Environmental and Molecular Toxicology, North Carolina State University, Raleigh, NC 27695, United States
[b] Department of Molecular Biology, Graduate School of Science, Nagoya University, Nagoya 464-8602, Japan
[c] Solution Oriented Research for Science and Technology, Japan Science and Technology Agency, Japan

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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

*Corresponding author. Fax: +1 919 515 7169.
E-mail address: Jun_Tsuji@ncsu.edu (J. Ninomiya-Tsuji).

[1]Present address: Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, United States.

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-

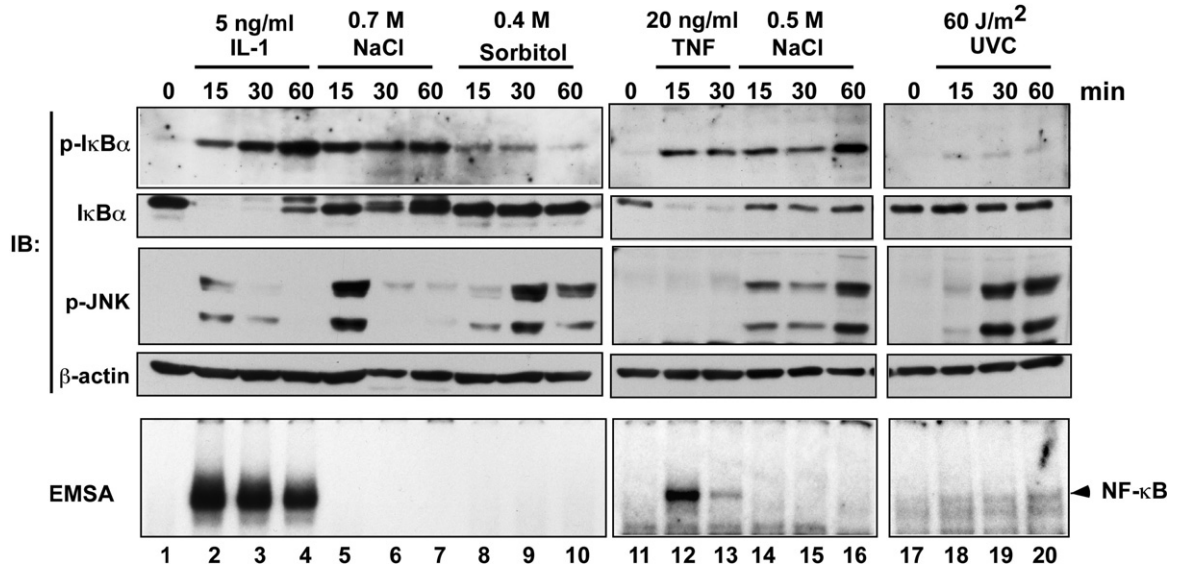

Fig. 1. Osmotic stress induces IκB phosphorylation but not degradation. 293 IL-1RI cells were treated with 5 ng/ml IL-1, 0.7 M NaCl, 0.4 M sorbitol, 20 ng/ml TNF or 0.5 M NaCl, or exposed to UVC (60 J/m^2). Cells were harvested at the time points indicated after treatment or exposure. Activation of the 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.