Cell Stress and MKK6b-mediated p38 MAP Kinase Activation Inhibit Tumor Necrosis Factor-induced IκB Phosphorylation and NF-κB Activation*  

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Tumor necrosis factor (TNF) exerts many actions through activation of the transcription factor NF-κB. NF-κB is sequestered in the cytosol by an inhibitory subunit IκB, which is inducibly phosphorylated by an IκB kinase complex and subsequently degraded. Sodium salicylate (NaSal) can block NF-κB activation by inhibiting IκBα phosphorylation. Recently, we used the specific p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 to demonstrate that inhibition of TNF-induced IκBα phosphorylation requires NaSal-induced p38 activation. We demonstrate that NaSal similarly inhibits TNF-induced IκBα degradation in a p38-dependent manner. To further examine the role of p38, we determined whether other agents that activate p38 can block TNF-induced IκB phosphorylation and degradation. Sorbitol, H₂O₂, and arsenite each blocked IκBα phosphorylation induced by TNF, and SB203580 reversed the inhibitory effects of sorbitol and H₂O₂, but not arsenite. In addition, sorbitol and H₂O₂ blocked TNF-induced but not interleukin-1-induced IκBα phosphorylation, whereas arsenite inhibited IκBα phosphorylation induced by TNF and interleukin-1. Transient expression of MAP kinase kinase (MKK) 6b(E), a constitutive activator of p38, reduced both TNF-induced phosphorylation of IκBα and NF-κB-dependent reporter activity. However, MKK7(D), a constitutive activator of c-Jun N-terminal kinases, failed to inhibit these TNF actions. Thus, sustained p38 activation by various stimuli inhibits TNF-induced IκB phosphorylation and NF-κB activation.

Association of the inflammatory cytokines tumor necrosis factor (TNF)† and interleukin-1 (IL-1) with their respective cell surface receptors triggers intracellular signal transduction cascades that may culminate in gene activation (1–3). Among the events leading to enhanced gene expression by these cytokines is activation of the transcription factor NF-κB, important in the regulation of genes involved in immune and inflammatory responses. NF-κB exists as a homo- or heterodimer of members of the Rel family of proteins, consisting of p50/p105, p52/p100, p65/RelA, c-Rel, and RelB. These proteins contain an N-terminal ~300-amino acid region known as the Rel homology domain that is responsible for dimerization, DNA binding, and interaction with an inhibitory IκB subunit, such as IκBα, IκBβ, or IκBε (4). In most cell types, an IκB protein sequesters NF-κB in the cytoplasm. IκBα may be inducibly phosphorylated on two serine residues at its N terminus and degraded by the ubiquitin-proteasome pathway, leading to the release of NF-κB and its translocation to the nucleus.

The pathway by which TNF leads to NF-κB activation involves the TNF-induced oligomerization of the type I TNF receptor, allowing for the recruitment of cytoplasmic proteins, including TNF receptor-associated death domain (TRADD), TNF receptor-associated factor 2 (TRAF2), and receptor-interacting protein (RIP), and activation of kinases, such as MAP ERK kinase kinase 1 (MEKK1) and the NF-κB-inducing kinase NIK (5, 6). IL-1 activates NF-κB by recruiting the IL-1 receptor accessory protein (IL-1RACp) to the IL-1 receptor, enabling the association of MyD88 and the serine/threonine kinases IL-1 receptor-associated kinase (IRAk) and IRAK-2 (7, 8). The IRAks interact with TRAF6, which associates with and leads to activation of NIK (9–12). Thus, NIK is a point of convergence for TNF and IL-1 signaling leading to NF-κB activation. Both NIK and MEKK1 have been shown to activate a multiprotein IκB kinase (IKK) complex of 700–900 kDa containing the catalytic subunits IKKa and IKKβ (11–16). IKKa and IKKβ directly phosphorylate the appropriate N-terminal serines of IκBα and IκBβ (17, 18).

Mitogen-activated protein (MAP) kinases are proline-directed serine/threonine kinases that are important mediators of cellular responses to a variety of stimuli. The three major subfamilies of MAP kinases in mammalian cells are the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAP kinases. Each of these MAP kinase subfamilies is activated by specific upstream MAP kinase kinases (MKks), which dually phosphorylate MAP kinases on a threonine and tyrosine residue separated by an intervening amino acid characteristic for each MAP kinase subfamily (i.e. TEY for ERKs, TPY for JNKs, and TGY for p38 MAP kinases) (19, 20). Peptide growth factors and phorbol esters preferentially activate ERKs, whereas cellular stresses, Sal, sodium salicylate; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GST, glutathione S-transferase.
such as hyperosmolarity or reactive oxygen species, as well as lipopolysaccharide and certain cytokines, most potently activate the JNK and p38 kinases. Activation of MAP kinase family members leads to phosphorylation of numerous downstream cellular effectors, including protein kinases, such as MAPKAP-K1, MAPKAP-K2/3, Mnk1/2, and MSK1, and transcription factors, including SAP-1, Elk-1, c-Jun, ATF-2, MEF2C, and CHOP (reviewed in Refs. 21 and 22).

We have recently demonstrated that the nonsteroidal anti-inflammatory agent sodium salicylate (NaSal) can rapidly and persistently activate p38 MAP kinase (23, 24). Through the use of the pyridinyl imidazole compound SB203580, a specific p38 inhibitor (25, 26), we have shown that the ability of NaSal to induce apoptosis in normal human FS-4 fibroblasts is dependent upon NaSal-induced p38 activation (23). NaSal has been demonstrated to inhibit NF-κB activation by inhibiting IκBα phosphorylation (27, 28), and we have shown that NaSal-induced p38 activation is important for its ability to inhibit TNF-induced IκBα phosphorylation and degradation (24). Here we demonstrate that NaSal similarly inhibits TNF-induced degradation of IκBα in a p38-dependent manner. In addition, we show that stress stimuli, such as hyperosmolar sorbitol and H2O2, inhibit TNF-induced phosphorylation and degradation of IκBα in a p38-dependent manner but have a much weaker inhibitory effect upon IL-1-induced IκBα phosphorylation and degradation. Expression of a dominant active MKK6b(E) mutant, which specifically activates p38 MAP kinases, partially inhibits IκBα phosphorylation and NF-κB-dependent transcription induced by TNF, whereas expression of an MKK7(D) mutant, which specifically activates JNKs, fails to inhibit these actions. Our results provide evidence that sustained p38 MAP kinase activation plays a role in the inhibition of TNF-induced NF-κB activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HT-29 human colon adenocarcinoma cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 8% fetal bovine serum (FBS) and were serum-starved for 24 h in DMEM with 0.25% FBS before use in experiments. COS-1 African green monkey kidney cells were cultured in DMEM with 10% FBS, and were serum-starved for 2–3 days in minimal essential medium with 0.25% FBS before use in experiments. COS-1 African green monkey kidney cells were cultured in DMEM with 10% FBS and were serum-starved for 18–24 h in DMEM with 0.5% FBS, except when used in transfections.

**Reagents**—NaSal, sodium metabisulfite, sorbitol, and hydrogen peroxide (H2O2) were purchased from Sigma. NaSal and arsenite were dissolved in distilled water, and sorbitol was dissolved in tissue culture medium. Recombinant human TNF-α was provided by Masafumi Tsujiimoto of the Suntory Institute for Biomedical Research (Osaka, Japan). Recombinant human IL-1α was obtained from the NC1, National Institutes of Health. The p38 inhibitor SB203580 (26) was generously supplied by John C. Lee and Peter Young (SmithKline Beecham) or purchased from Calbiochem and was dissolved in dimethyl sulfoxide (Me2SO). Control experiments demonstrated that the treatment of cells with Me2SO alone had no effect on the ability of all agents tested to inhibit TNF-induced IκBα phosphorylation and degradation. The rabbit polyclonal anti-IκBα, anti-IκBβ, and anti-p38 antibodies and the mouse monoclonal anti-c-Myc (9E10) antibody were purchased from Santa Cruz Biotechnology. Alternatively, a peroxidase-conjugated 9E10 antibody (Roche Molecular Biochemicals) was used for direct detection in immunoblots. The rabbit polyclonal anti-phospho-p38 (recognizing p38 dually phosphorylated on Thr-180 and Tyr-182), anti-phospho-IκBα (recognizing IκBα phosphorylated on Ser-32) and monoclonal anti-phospho JNK (recognizing the 46- and 54-kDa JNK isoforms dually phosphorylated on Thr-183 and Tyr-185) antibodies were obtained from New England Biolabs.

**Plasmids and Transfections**—Full-length constitutively active human MKK6b(E) and MKK7(D) mutants cloned in the vector pcDNA3 were described previously (29, 30). pSVK3-mIκBα was a generous gift from John Hiscott (McGill University) (31, 32). IκBα was tagged at the C terminus with a Myc epitope (encoding EQKLISEEDL) by polymerase chain reaction and cloned into the pSVK3 vector. An NF-κB-luciferase construct containing a 4× NF-κB binding element from the immunoglobulin κ enhancer (33) upstream of a minimal angiotensinogen promoter was kindly provided by David Ron (New York University Medical Center). All transfections were performed in COS-1 cells, using a calcium phosphate precipitation method (34). Cells were transfected with the indicated plasmids using LipofectAMINE (Life Technologies, Inc.) in a total volume of 1 ml serum-free DMEM. Five hours posttransfection, 1 ml of DMEM with 20% FBS was added to each well. Cells were refed with DMEM containing 10% FBS the next day and incubated for 24 h following initial serum addition, prior to stimulation with the indicated agents. The pcDNA3 vector was used to equalize the total amount of DNA transfected for each experiment.

**Luciferase Assays**—COS-1 cells were transfected as described above, except that 1 ml of serum-free DMEM was added to each well 5 h posttransfection, and cells were refed with serum-free DMEM the next day. Following the indicated treatments, cells were lysed in 250 µl of 1× reporter lysis buffer (Promega) and assayed for luciferase activity in a reaction mixture containing 25 µl glycylglycine (pH 7.8), 15 mM MgSO4, 1 mM ATP, 0.1 µg/ml bovine serum albumin, and 1 mM dithiothreitol. Luciferase activity was measured with a Lumat LB 9507 luminometer (EG&G Berthold) using 1 ml n-luciferin (PharMingen) as substrate.

**NF-κB- and p38-JNK Kinase Assays**—Kinase assays were performed as described previously (23, 34). Briefly, whole cell lysates were generated using a buffer consisting of 1% Igepal, 50 mM Hepes (pH 7.5), 100 mM NaCl, 2 mM EDTA, 1 mM pyrophosphate, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 100 mM NaF. Protein concentrations were estimated by the Bradford method (Bio-Rad). To assay JNK activity, equal amounts of total protein were incubated for 1–2 h at 4 °C with glutathione S-transferase (GST)-c-Jun (amino acids 1–223) coupled to glutathione agarose beads (35). The beads were subsequently washed three times with lysis buffer, then incubated for 30 min at 30 °C in kinase buffer containing 20 mM Hepes (pH 7.6), 20 mM MgCl2, 20 mM β-glycerophosphate, 10 mM NaF, 0.2 mM sodium orthovanadate, 0.2 mM dithiothreitol, 10 µM ATP, and 10 µM of γ-[32P]ATP. The kinase reaction was terminated by the addition of 2× sample buffer, and phosphorylated GST-c-Jun was visualized following SDS-polyacrylamide gel electrophoresis and autoradiography.

**Immunoblotting**—Immunoblot analysis was performed as described previously, with some modifications (23, 24, 36). Briefly, whole cell extracts were fractionated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membranes (Millipore) and blocked for 1 h at room temperature in TBS (10 mM Tris [pH 7.5], 150 mM NaCl) containing 0.5% Tween-20 and 5% nonfat milk. Membranes were then incubated overnight at 4 °C with primary antibody. The anti-phospho-IκBα, anti-phospho-p38, anti-phospho, anti-c-Myc (9E10), and anti-IκBβ antibodies were each used at a dilution of 1:1000 in TBS-5% bovine serum albumin. The anti-phospho JNK antibody was used at 1:2000, and the anti-IκBα antibody was used at 1:250 in TBS-5% bovine serum albumin. Antibody-antigen complexes were detected with the aid of horseradish peroxidase-conjugated protein A, horseradish peroxidase-conjugated goat anti-rabbit IgG, or horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad), and a chemiluminescent substrate development kit (Kirkegaard and Perry Laboratories).

**RESULTS**

**Effect of NaSal on TNF-induced IκBα and IκBβ Degradation**—It has been demonstrated that activation of NF-κB by TNF, lipopolysaccharide, phorbol ester, or viral infection can be prevented by treatment with NaSal (28, 37–39) and that this inhibition occurs at the level of IκBα phosphorylation (24, 27). Because IκBα is another major IκB isoform responsible for inhibition of NF-κB nuclear translocation (40), we sought to determine whether NaSal could similarly inhibit IκBβ degradation. Degradation of IκBα can be induced by haptotactic lipo-lysaccharide (40), but in our cell systems by TNF (41, 42). We observed that in HT-29 cells, TNF induced partial degradation of IκBβ by 15 min, with IκBβ levels remaining decreased for at least 60 min (Fig. 1A, top panel). When the same lysates were immunoblotted with an antibody directed against IκBα, a nearly complete degradation of IκBα was seen by 15 min (Fig. 1A, bottom panel). Unlike IκBα, IκBβ was reappeared within 30–60 min, as reported for other systems (43).
We have previously shown that NaSal inhibits TNF-induced IκB phosphorylation and degradation in a manner preventable by the specific p38 inhibitor SB203580 (24), implicating a requirement for p38 activation by NaSal in the inhibition of IκB phosphorylation. To determine whether NaSal-mediated inhibition of TNF-induced IκB degradation was similarly p38-dependent, HT-29 cells were treated with SB203580 prior to the addition of NaSal. After a 15-min incubation with TNF, cells were harvested, and lysates were immunoblotted for IκBα and IκBβ. NaSal inhibited TNF-induced degradation of both IκBα and IκBβ, and this inhibition was reversed by pretreatment with SB203580 (Fig. 1B), suggesting that NaSal employs a similar p38-dependent mechanism to inhibit degradation of these two IκB isoforms.

Cell Type-specific Inhibition of IκBα Degradation by NaSal—We have observed that NaSal inhibits IκBα degradation in a manner reversible by SB203580 in HT-29, COS-1, and 293T cell lines (Ref. 24, Figs. 1 and 2, and data not shown). However, NaSal only weakly inhibited TNF-induced IκBα degradation in normal human FS-4 fibroblasts; nevertheless, this weak inhibition was reversed by SB203580 pretreatment, as was the stronger inhibition in COS-1 cells (Fig. 2A). As previously demonstrated (23, 24), NaSal produced a rapid and sustained activation of p38 in both FS-4 and COS-1 cells (Fig. 2B, top panels). The finding that NaSal activated p38 in the two cell types, but more strongly inhibited IκBα degradation in COS-1 cells than in FS-4 cells, suggested that p38 activation by NaSal may be required, but not sufficient, for maximal inhibition of TNF-induced IκBα degradation.

Because JNK and p38 MAP kinases are coordinately regulated in many systems (44, 45), we considered the possibility that activation of both p38 and JNK may be necessary for maximal inhibition of IκBα degradation induced by TNF. In previous reports, we demonstrated that NaSal fails to activate JNK in FS-4 cells yet activates both JNK and p38 in COS-1 and HT-29 cells (23, 46). Treatment with NaSal for time periods ranging from 15 to 60 min increased phosphorylation of the 46- and 54-kDa isoforms of JNK in COS-1 cells but not in FS-4 cells (Fig. 2B, bottom panels). Phosphorylation of these JNK isoforms in COS-1 cells correlated with increased in vitro JNK kinase activity (Ref. 46 and data not shown). NaSal did not produce activation of ERKs over this time course in either FS-4 or COS-1 cells (Ref. 36 and data not shown). Thus, it appeared plausible that NaSal-induced activation of JNK, together with p38, may contribute to the strong inhibition of TNF-induced IκBα degradation observed in COS-1 (Fig. 2A) and HT-29 cells (Fig. 1B).

Multiple Stress Stimuli Inhibit TNF-induced IκBα Phosphorylation and Degradation—In order to assess the relative importance of p38 and JNK in the inhibition of TNF-induced IκBα degradation, we examined the effect of various stress stimuli on TNF-induced IκBα degradation in FS-4 cells. To induce activation of both p38 and JNK, FS-4 fibroblasts were treated for 15–60 min with hyperosmolar sorbitol, H2O2, or the sulfhydryl reagent arsenite. Consistent with other reports (47–50), treatment with each of these agents produced a strong p38 activation at the time points examined, as indicated by site-specific p38 phosphorylation (Fig. 3A, top panel). Activation of endogenous JNK kinase activity was most rapid and intense following sorbitol treatment and was also marked after treatment with H2O2 or arsenite (Fig. 3A, bottom panel).

To determine whether these stress stimuli inhibit TNF-induced IκBα degradation, FS-4 cells were treated for 30 min with sorbitol, arsenite, or H2O2, followed by treatment with TNF for 15 min. All three agents completely inhibited IκBα degradation induced by TNF (Fig. 3B). To determine whether this inhibition was dependent upon activation of p38, as previously demonstrated for NaSal, FS-4 cells were incubated with the specific p38 inhibitor SB203580 prior to treatment with the various agents, followed by TNF stimulation. SB203580 reversed the inhibitory effects of sorbitol and H2O2, but not that of arsenite (Fig. 3B), suggesting that activation of p38 by sor-
bital and H$_2$O$_2$ is required for the inhibitory effect of these agents. A similar, albeit weaker, inhibition of IκBα degradation was also observed when cells were treated with the protein synthesis inhibitor anisomycin, and this inhibition was also reversed by SB203580.2

TNF stimulation induces IκBα phosphorylation at serines 32 and 36, followed by ubiquitination and subsequent degradation of IκBα by the 26 S proteasome (51). Therefore, inhibition of IκBα degradation could occur at the level of IκBα phosphorylation, or it could be due to inhibition of ubiquitination or proteasomal degradation. To determine whether IκBα phosphorylation is inhibited by stress stimuli, FS-4 cells were treated for 30 min with sorbitol, arsenite, or H$_2$O$_2$, followed by treatment for 5 min with TNF. Each of these agents inhibited TNF-induced IκBα phosphorylation, as manifested by the appearance of a slower migrating (i.e. hyperphosphorylated) form of IκBα (Fig. 3C, top panel), or visualized by an antibody specific for Ser-32-phosphorylated IκBα (Fig. 3C, bottom panel). Pretreatment with SB203580 reversed the inhibition of IκBα phosphorylation by sorbitol and H$_2$O$_2$, yet not by arsenite, consistent with what was observed for IκBα degradation in Fig. 3B. Therefore, as observed previously for NaSal (24, 27), stress stimuli inhibit TNF-induced IκBα degradation at the level of

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2 D. Alpert and J. Vilček, unpublished results.

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**Fig. 4.** Differential effect of stress stimuli on TNF- and IL-1-induced IκBα phosphorylation and degradation. A, selectivity of inhibition of TNF- and IL-1-induced IκBα degradation by stress stimuli. Serum-starved FS-4 cells were incubated for 30 min with 0.3 M sorbitol, 1 mM H$_2$O$_2$, or 0.5 mM arsenite, followed by incubation for 15 min with TNF (20 ng/ml) or IL-1α (4 ng/ml). Lysates were immunoblotted with an antibody against IκBα. B, selectivity of inhibition of TNF- and IL-1-induced IκBα phosphorylation by stress stimuli. Serum-starved FS-4 cells were incubated for 30 min with 0.3 M sorbitol, 1 mM H$_2$O$_2$, or 0.5 mM arsenite, followed by incubation for 5 min with TNF (20 ng/ml) or IL-1α (4 ng/ml). Lysates were immunoblotted with an antibody against IκBα (top panel) or antibody against Ser-32-phosphorylated IκBα (pIκBα, bottom panel).
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We also analyzed the effects of MKK6b(E) and MKK7(D) overexpression on TNF-induced NF-κB luciferase reporter. TNF produced a potent activation of a 4× NF-κB luciferase reporter (Fig. 5, D and E) but failed to increase reporter activity in cells transfected with an equivalent 4× NF-κB luciferase construct in which the κB binding sites were mutated (data not shown). MKK6b(E) cotransfection decreased TNF-induced activation by approximately 2-fold (Fig. 5D). Unlike MKK6b(E), MKK7(D) expression failed to decrease TNF-induced NF-κB-dependent luciferase activity (Fig. 5E). At the highest dose of cotransfected MKK7(D), TNF-induced NF-κB reporter activity was actually further increased. Collectively, these data show that persistent p38 activation, but not JNK activation, results in a partial inhibition of TNF-induced IκB phosphorylation and NF-κB-dependent transcription.

In order to test whether simultaneous activation of both p38 and JNK is more effective in inhibiting TNF-induced IκB phosphorylation than p38 activation alone, COS-1 cells were transfected with MKK6b(E) alone, MKK7(D) alone, or both activated MKKs together, and TNF-induced IκB-MyC phosphorylation and NF-κB-dependent luciferase activity were analyzed. Consistent with our previous observations, MKK6b(E) expression appreciably decreased TNF-induced IκB-MyC phosphorylation, whereas MKK7(D) had no significant effect. The combination of MKK6b(E) and MKK7(D) did not further decrease IκB-MyC phosphorylation below the level seen with MKK6b(E) alone (Fig. 6A). Similarly, the combination of MKK6b(E) and MKK7(D) failed to decrease TNF-induced NF-κB luciferase activity more strongly than MKK6b(E) alone (Fig. 6B). These results suggest that simultaneous activation of p38 and JNK in this system does not enhance p38-mediated inhibition of TNF-induced IκB phosphorylation and NF-κB-dependent transcription.

We also addressed the question of whether treatment with NaSal for 30 min prior to stimulation with TNF would decrease IκB-MyC phosphorylation and NF-κB-dependent transcription more strongly than MKK6b(E). As observed previously for endogenous IκBα, NaSal decreased TNF-induced phosphorylation of transfected IκBα. MKK6b(E) also decreased IκBα-MyC phosphorylation, but the level of phosphorylation was further decreased upon treatment of MKK6b(E)-transfected cells with NaSal (Fig. 6C). Consistent with this, TNF-induced NF-κB luciferase activity was decreased by approximately 50% in the presence of MKK6b(E), as compared with a decrease of greater than 75% after treatment with NaSal. Importantly, NaSal was able to further decrease the inhibition of TNF-induced luciferase activity in cells transfected with MKK6b(E) (Fig. 6D). These results suggest that p38 activation alone does not account for all of the inhibitory action of NaSal on TNF-induced IκB phosphorylation and NF-κB-dependent transcription.

DISCUSSION

Several reports have demonstrated that NaSal and aspirin inhibit NF-κB activation induced by various agents (28, 37–39),...
and correspondingly decrease expression of various NF-κB-depen
dent genes (27, 38, 39, 58). We have previously reported that the
ability of NaSal to inhibit TNF-induced NF-κB activation at the
level of IκB phosphorylation requires NaSal-induced p38 activation (24).
Our current data suggest a more general role for p38 activation in the
inhibition of TNF-induced IκB phosphorylation and degradation and the
resulting NF-κB activation. Firstly, we have shown that multiple stress stimuli
that activate p38 can inhibit IκB phosphorylation. Both hyper
molosar sorbitol and H2O2 inhibit IκB phosphorylation and degradation
induced by TNF in a manner reversed by the p38 inhibitor SB203580 (Fig. 3).
Secondly, we have demonstrated that MKK6b(E), which selectively activates p38,
partially inhibits TNF-induced phosphorylation of cotransfected IκBα and activation of
an NF-κB-luciferase reporter construct (Fig. 5). In addition, similar to the selective
inhibition of TNF-induced IκBα phosphorylation and degradation by NaSal (24),
we observed that both sorbitol and H2O2 inhibit TNF-induced, but not IL-1-induced, phosphorylation of IκBα (Fig. 4).

IκBβ is another major IκB family member that, like IκBα,
contains two N-terminal serines (Ser-19 and Ser-22) that are
inducibly phosphorylated prior to proteasomal degradation
(42). Both IκBα and IκBβ are direct substrates for IKKα and
IKKβ (17, 18). Consistent with this, we observed that TNF-
induced IκBβ degradation was also inhibited by NaSal in a
p38-dependent manner (Fig. 1B). It will be of interest to deter-
mine whether NaSal inhibits stimulus-induced phosphoryla-
tion and degradation of IκBα, a recently identified member of
the IκB family that is structurally similar to IκBα and IκBβ
(59, 60).

As inhibition of TNF-induced IκBα degradation by NaSal
was notably weaker in FS-4 cells than in COS-1 cells, even
though NaSal activated p38 in both cell lines (Fig. 2), we
considered the possibility that p38 activation by NaSal may
not be sufficient for maximal inhibition of IκBα degradation.
We found that NaSal activated JNK in COS-1 cells, but not in FS-4
cells, raising the possibility that both p38 and JNK activation
might be required for maximal inhibition of IκBα degradation.
We also observed that sorbitol and H2O2 inhibited TNF-
induced IκBα degradation more strongly than NaSal in FS-4
cells, consistent with their ability to simultaneously activate
JNK and p38 (38). However, overexpression in COS-1 cells of
MKK7(D), which selectively activates JNKs, failed to inhibit
TNF-induced IκBα phosphorylation and NF-κB activation (Fig.
5, C and E), and overexpression of both MKK6b(E) and
MKK7(D) failed to decrease TNF-induced IκBα phosphoryla-
tion and NF-κB activation more strongly than MKK6b(E) alone
(Fig. 6, A and B). Thus, we failed to obtain direct evidence for
a role of JNK activation in the inhibition of TNF-induced IκBα
phosphorylation and NF-κB activation.

Our observation that NaSal treatment further decreased the
inhibition of IκBα phosphorylation and NF-κB activation ob-
served with MKK6b(E) (Fig. 6) also suggests that there may be
an additional mechanism(s) of inhibition by NaSal. While this
report was in preparation, Yin et al. (61) provided evidence that
NaSal and aspirin may directly bind to IKKβ and inhibit its
activity by competing for ATP binding. However, the observa-
tions of Yin et al. (61) are inconsistent with our previous dem-

Fig. 6. Effects of MKK6b(E) and MKK7(D) expression on IκBα phosphorylation and NF-κB-dependent transcription. A, coexpression of
MKK6b(E) and MKK7(D) does not further decrease TNF-induced IκBα phosphorylation. COS-1 cells were transfected with 0.3 μg of IκBα-Myc in
combination with 0.4 μg of MKK6b(E), 0.4 μg of MKK7(D), or both. Twenty-four hours after transfection, cells were left untreated or treated
for 5 min with TNF (20 ng/ml) prior to harvest. Lysates were immunoblotted with an antibody against Ser-32-phosphorylated IκBα (top panel)
or against the Myc epitope tag (bottom panel). Comparable results were obtained in at least three independent experiments. B, MKK6b(E)
and MKK7(D) coexpression does not further decrease TNF-induced NF-κB-luciferase activity. COS-1 cells were transfected with 0.2 μg of 4×
NF-κB-luciferase in combination with 0.4 μg of MKK6b(E), 0.4 μg of MKK7(D), or both. The next day, cells were left untreated or treated with TNF
(20 ng/ml) for 6 h prior to harvest. Luciferase activity was determined and normalized to total protein concentration, and luciferase activity induced
by TNF in vector-transfected cells was normalized to 100%. Mean ± S.D. from three independent transfections each performed in duplicate is
shown. C, effect of NaSal on TNF-induced IκBα phosphorylation in the presence of MKK6b(E). COS-1 cells were transfected with 0.3 μg of
IκBα-Myc and 0.4 μg of MKK6b(E) or pcDNA3. Twenty-four hours posttransfection, cells were incubated for 30 min in the presence or absence
of NaSal (20 mM) and subsequently left untreated or treated with TNF (20 ng/ml) for 5 min prior to harvest. Lysates were immunoblotted with an
antibody against Ser-32-phosphorylated IκBα (top panel) or against the Myc tag (bottom panel). Comparable results were obtained in three
independent experiments. D, effect of NaSal on TNF-induced NF-κB luciferase activity in the presence of MKK6b(E). COS-1 cells were transfected
with 0.2 μg of 4× NF-κB-luciferase and 0.4 μg of MKK6b(E). The next day, cells were incubated for 30 min in the presence or absence of
NaSal (20 mM) and then left untreated or treated with TNF (20 ng/ml) for 6 h prior to lysis. Luciferase activity was assayed and calculated as in B.
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![Diagram showing p38-dependent inhibition of IκB phosphorylation.](image)

**Fig. 7. Model for p38-dependent inhibition of IκB phosphorylation.** Both TNF and IL-1 lead to NF-κB activation by inducing IκB phosphorylation, but their receptor proximal signaling pathways are distinct. We observed that NaSal, hyperosmotic stress, and oxidative stress inhibit TNF-induced but not IL-1-induced IκBα phosphorylation and degradation in a p38-dependent manner, suggesting that the target of p38 lies between the TNF receptors and the elements immediately upstream of NIK. It is also possible that p38 activation may lead to phosphorylation of an inhibitor, such as TRAF-interacting protein (TRIP), which could sequester TRAF2 from the TNF receptor signaling complex. Arsenite inhibits IκBα phosphorylation and degradation by both TNF and IL-1, and its effect appears not to require activation of p38.

Our observation that NaSal selectively inhibits TNF-induced but not IL-1-induced IκBα phosphorylation and degradation (24), because direct inhibition of IKKβ should inhibit both TNF- and IL-1-induced IκBα phosphorylation (12). In addition, hyperosmotic sorbitol and H₂O₂, which display a similar inhibitory profile to NaSal (Figs. 3 and 4), are unlikely to directly inhibit IKKβ by competing for ATP binding. Nevertheless, it is probable that NaSal inhibits NF-κB activation at multiple levels, via both p38-dependent and p38-independent mechanisms.

Our observation that H₂O₂ can inhibit TNF-induced phosphorylation and degradation of IκBα was initially surprising, given that H₂O₂ and other reactive oxygen species mediate activation of NF-κB in various cell systems (62). However, certain cell types are refractory to activation of NF-κB by H₂O₂, even at doses as high as 5 mM (63, 64). In FS-4 cells, H₂O₂ induced minimal phosphorylation and degradation of IκBα over a time course of 1 h (data not shown). In agreement with our findings, it has recently been demonstrated in Jurkat cells that pretreatment with H₂O₂ inhibits TNF- and phorbol ester-induced IκBα phosphorylation and degradation, suggesting that chronic oxidative stress can inhibit NF-κB activation (65). Our data further suggest that the mechanism by which chronic oxidative stress inhibits TNF-induced IκBα phosphorylation and degradation involves the activation of p38.

Unlike the other stress stimuli tested, arsenite potently inhibited IκBα phosphorylation induced by both TNF and IL-1, and its effects were not reversed by SB203580. Arsenite is a redox-active sulfhydryl reagent that interacts strongly with protein thiols (66), and it has been shown to affect various MAP kinases and MAP kinase phosphatases (47, 50, 67). In vitro, pretreatment of nuclear extracts with arsenite inhibits NF-κB binding to a consensus κB oligonucleotide, indicating that critical sulfhydryls of the NF-κB heterodimer are required for its interaction with DNA (68). To our knowledge, the ability of arsenite to inhibit the phosphorylation and degradation of IκBα in vivo has not been previously reported. Because it inhibits phosphorylation of IκBα induced by both TNF and IL-1, it is possible that arsenite inhibits IκBα phosphorylation downstream of the convergence of the TNF and IL-1 signaling pathways, leading to NF-κB activation (Fig. 7). However, we cannot rule out the possibility that arsenite may inhibit TNF- and IL-1-induced IκBα phosphorylation at multiple levels.

In view of our evidence that p38 inhibits NF-κB activation, it initially appeared paradoxical that TNF can activate both p38 (45) and NF-κB. However, careful analysis of the kinetics of p38 activation and IκBα phosphorylation by TNF in FS-4 cells demonstrated that TNF-induced IκBα phosphorylation precedes p38 activation, rendering it unlikely that p38 activation by TNF would inhibit IκBα phosphorylation (Ref. 24 and data not shown). Additionally, numerous reports have demonstrated a role for TNF-induced p38 activation in the transactivation of certain promoters by NF-κB (69–72). Although we did observe a slight activation of NF-κB luciferase activity in the presence of MKK6b(E) alone (Fig. 5B), MKK6b(E) reduced TNF-induced IκBα phosphorylation and NF-κB activation by approximately 50% (Figs. 5 and 6). These seemingly incongruous effects of p38 activation upon NF-κB activity may be attributable to the kinetics of p38 activation by stress stimuli versus TNF. TNF induces a transient activation of p38, and this has been associated with cell survival (73) and transactivation of NF-κB (69). However, the stimuli examined in our experiments (NaSal, sorbitol, and H₂O₂) and MKK6b(E) transfection all produce persistent p38 activation, which has frequently been associated with apoptosis (23, 44, 49, 74). It has been shown that inhibition of NF-κB function can promote apoptosis in several systems (75–77), suggesting that sustained p38 activation may contribute to apoptosis by inhibiting IκBα phosphorylation and degradation.

Our observation that sustained p38 activation by stimuli such as NaSal, hyperosmolarity, and H₂O₂ can inhibit TNF-induced but not IL-1-induced IκBα phosphorylation leads us to propose a model in which p38 activation inhibits IκB phosphorylation at a target between the TNF receptors and the elements immediately upstream of NIK (Fig. 7). Potential targets include the TNF receptors, TRADD, TRAF2, and RIP. It is possible that p38 may directly phosphorylate one or more of these proteins and thereby inhibit the interactions required to induce activation of the IKK complex. Alternatively, as p38 can directly phosphorylate various kinases (21, 22, 78), it may be acting indirectly through another kinase to modify a particular target(s) in the TNF signaling pathway. Another possibility is that p38 may activate an inhibitor of TNF signaling, such as the recently identified TRAF-interacting protein TRIP. TRIP specifically interacts with TRAF2 and inhibits TNF- and TRAF2-mediated, but not IL-1-mediated, NF-κB activation (79). Overall, our data show that a variety of stress stimuli activate signaling pathways that converge at the level of p38 activation to inhibit IκBα phosphorylation and NF-κB activation. The precise mechanisms of this inhibitory effect are currently under investigation in our laboratory.

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