Sodium salicylate inhibits activation of the transcription factor NF-κB by blocking the phosphorylation and degradation of the NF-κB inhibitor IκBα. We previously demonstrated that salicylate inhibits IκBα degradation induced by tumor necrosis factor (TNF) but not by interleukin-1 (IL-1) and implicated p38 mitogen-activated protein kinase activation by salicylate in the inhibition of TNF-induced IκBα phosphorylation. Both TNF and IL-1 rapidly activate the IκB kinase (IKK) complex, containing the catalytic subunits IKKα and IKKβ, which directly phosphorylates IκB proteins. Others have recently suggested that salicylate inhibits NF-κB activation by directly binding to IKKβ. To clarify the mechanism whereby salicylate inhibits IKK activity, we examined its effects upon cytokine-induced IKK activity in intact cells and in vitro. Treatment of intact cells with salicylate inhibited TNF-induced but not IL-1-induced IKK activity, and this inhibition was prevented by the p38 inhibitor SB203580. In contrast, inhibition of IKK activity by salicylate in vitro was neither selective for TNF nor affected by SB203580. In vitro, salicylate treatment comparably inhibited the kinase activity of overexpressed IKKα and IKKβ and also decreased p38 kinase activity. Therefore, direct inhibition of IKK activity in vitro does not reflect the inhibitory mechanism of salicylate in intact cells, which involves interference with TNF signaling.

The transcription factor NF-κB is an important regulator of genes involved in immune and inflammatory responses. The NF-κB pathway is activated by a variety of stimuli, including the cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1) and bacterial lipopolysaccharide. In most mammalian cells, the NF-κB dimer is sequestered in the cytoplasm by an inhibitory IκB isoform such as IκBα, IκBβ, or IκBe (1). Stimulus-induced phosphorylation of IκB proteins on two conserved N-terminal serine residues leads to IκB degradation via the ubiquitin-proteasome pathway allowing for the release of NF-κB and its nuclear translocation. TNF-induced NF-κB activation is initiated by oligomerization of the type 1 or type 2 TNF receptors. This leads to the recruitment of cytoplasmic signaling proteins, such as TNF receptor-associated death domain, TNF receptor-associated factor 2 and receptor interacting protein, and activation of kinases including MAP/ERK kinase kinase 1 (MEKK1) and the NF-κB-inducing kinase (NIK) (2–5). Both NIK and MEKK1 can activate a multiprotein IκB kinase (IKK) complex of 700–900 kDa that is responsible for directly phosphorylating IκB isoforms (3, 6–8). The IKK complex consists of two catalytic components, IKKα and IKKβ, as well as a regulatory IKKγ subunit, which may facilitate interaction with upstream signaling factors (9–11). Other proteins, such as IKK complex associated protein (12), have been shown to interact with the IKK complex, and their precise roles in the activation of the IKK complex remain to be defined.

Salicylate and its acetylated derivative, aspirin (acetylsalicylic acid), represent the oldest known nonsteroidal anti-inflammatory drugs. A well-characterized mechanism of action for aspirin involves the acetylation of cyclooxygenase isoforms, leading to irreversible inhibition of prostaglandin synthesis (13). In the intact organism, aspirin is rapidly deacetylated to salicylate, which is a relatively weak inhibitor of cyclooxygenases (14). Nonetheless, both aspirin and salicylate are potent anti-inflammatory agents, used in the treatment of chronic inflammatory disorders such as rheumatoid arthritis (14, 15).

Sodium salicylate (NaSal) and aspirin can inhibit the activation of NF-κB by preventing the phosphorylation and degradation of IκBα (16, 17), and inhibition of NF-κB may explain some of the clinically documented anti-inflammatory effects seen with high concentrations of salicylates (14, 15). We have demonstrated that NaSal rapidly and persistently activates p38 MAP kinase and that NaSal-induced p38 activation is important for its ability to inhibit TNF-induced IκBα phosphorylation and degradation (18, 19). Furthermore, we have demonstrated that p38 activation may play a more general role in the inhibition of TNF-induced NF-κB activation (20). A recent report has suggested that NaSal and aspirin may inhibit NF-κB by an alternative mechanism, via direct binding to the IKK complex (21). In this study, we sought to clarify the mechanism by which NaSal inhibits IKK activity. We demonstrate that treatment of intact cells with NaSal selectively inhibits IKK activity induced by TNF, yet not by IL-1, and this inhibition is prevented by the p38 inhibitor SB203580. However, we find that inhibition of IKK activity in vitro is not selective and cannot be prevented by SB203580, suggesting that direct inhibition of the IKK complex is not likely to be the mechanism by which NaSal exerts its inhibitory effect upon NF-κB activation in vivo.

*EXPERIMENTAL PROCEDURES*

Cell Culture and Reagents— COS-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS)
and were serum-starved for 18-24 h in DMEM with 0.5% FBS. Normal human diploid FS-4 fibroblasts were cultured in Eagle's minimsal medium (MEM) supplemented with 5% FBS and were serum-starved for 2-3 days in MEM with 0.25% FBS before use in experiments.

NaSal, sodium meta-arsenate, and myelin basic protein (MBP) were purchased from Sigma. NaSal and arsenite were dissolved in distilled water, unless otherwise indicated. Recombinant human TNF-α was provided by Masafumi Tsujimoto of the Suntory Institute for Biomedical Research, Osaka, Japan. Recombinant human IL-1α was obtained from the National Cancer Institute. The p38 inhibitor SB203580 (22) was purchased from Calbiochem and dissolved in Me2SO. The rabbit polyclonal anti-IκBα and anti-IκKα (H-744) antibodies were from Santa Cruz Biotechnology. The M2 monoclonal anti-FLAG antibody was from Kodak Scientific Imaging Systems.

Plasmids and Transfections—Full-length FLAG-tagged IKKα and IKKβ cloned into the pfR5 vector were provided by David Goeddel (6, 23). FLAG-p38α cloned into the pcDNA3 vector was provided by Jiahuai Han (24). Full-length NIK cloned into the pcDNA3 vector was a gift from David Wallach (2). Full-length IκBα in the pGEX-2T vector was provided by John Hiscott (25). A fragment encoding the first 62 amino acids of IκBα was amplified from this construct by polymerase chain reaction and cloned into the EcoRI and NotI sites of the pGEX-4T1 vector. Glutathione S-transferase (GST)-IκBα (amino acids 1-62) was subcloned purified from Escherichia coli DH5α with glutathione-agarose for use in immunokinase assays.

Transfections were performed in COS-1 cells plated at a density of approximately 2.5 × 10^5 cells per well in 6-well plates. Cells were transfected the following day using LipofectAMINE (Life Technologies, Inc.) in a total volume of 1 ml of serum-free DMEM. 5 h post-transfection, 1 ml of serum-free DMEM was added to each well. Cells were incubated for an additional 24 h, prior to stimulation as indicated.

Immunokinase Assays—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 (18, 20). To immunoprecipitate the endogenous IKK complex, equal amounts of lysates were incubated 1–2 h at 4 °C with anti-IKKα antibody followed by collection of immune complexes for 2 h at 4 °C using protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). For immunoprecipitation of transfected FLAG-IKKα, FLAG-IKKβ, or FLAG-p38α, equal amounts of lysates were incubated with anti-FLAG M2 antibody followed by collection of immune complexes using protein G-agarose beads (Life Technologies, Inc.). The immunoprecipitates were subsequently washed three times with lysis buffer and once with kinase buffer without ATP. Assays for IKK activity were performed for 30 min at 30 °C in kinase buffer consisting of 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, 1 mM ATP, 10 μCi of [γ-32P] ATP, and 5 μg of GST-IκBα (1–62) as substrate. Assays for p38 kinase activity were performed under identical conditions in kinase buffer containing 5 μg of MBP as substrate. Kinase reactions were terminated by the addition of 2× protein sample buffer, and phosphorylated GST-IκBα or MBP was visualized following SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography.

To determine the effect of NaSal treatment in vitro upon IKK activity, aliquots of immunoprecipitates were incubated with various concentrations of NaSal in kinase buffer for 30 min on ice. Kinase assays were then performed as described above in the continuous presence of NaSal.

Immunoblotting—Immunoblot analysis was performed as described (20). Briefly, whole cell lysates were fractionated by SDS-PAGE, 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. Antibody-antigen complexes were detected with the aid of horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and a chemiluminescent substrate development kit (Kirkegaard and Perry Laboratories).

RESULTS

**NaSal Inhibits TNF-induced but Not IL-1-induced IKK Activity in Intact Cells**—We and others have reported that NaSal inhibits IκBα degradation induced by TNF but not by IL-1 (19, 20, 26). A preponderance of evidence suggests that both TNF and IL-1 induce IκBα phosphorylation and degradation via activation of the IKK complex (23, 27). To determine whether blockade of IκBα degradation correlates with inhibition of IKK activity, we treated COS-1 cells with various concentrations of NaSal prior to stimulation with TNF or IL-1. An antibody to IKKα was used to immunoprecipitate endogenous IKK complex from whole cell lysates (21), which were then assayed for in vitro kinase activity using GST-IκBα as substrate. NaSal inhibited TNF-induced IKK activity in a dose-dependent manner with an IC_{50} of approximately 5 mM (Fig. 1A, top panel). Separate aliquots of lysates were subjected to immunoblot analysis (IB) with an antibody against IκBα (bottom panel). COS-1 cells were left untreated or treated with TNF (20 ng/ml) or IL-1α (4 ng/ml) for 10 min. IKKα was immunoprecipitated from whole cell lysates, and immunoprecipitates were divided into five equal fractions for incubation with the indicated concentrations of NaSal for 30 min, as described under "Experimental Procedures." These fractions were then assayed for in vitro kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography.

**FIG. 1. Effects of NaSal treatment in vitro and in vivo upon TNF- and IL-1-induced IKK activity.** A, COS-1 cells were treated with the indicated concentrations of NaSal for 1 h and then either left untreated or treated with TNF (20 ng/ml) or IL-1α (4 ng/ml) for 10 min. Aliquots of whole cell lysates were immunoprecipitated with an antibody against IκKα. Immunoprecipitates (IP) were assayed for in vitro kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography (top panel). Separate aliquots of lysates were subjected to immunoblot analysis (IB) with an antibody against IκBα (bottom panel). B, COS-1 cells were left untreated or treated with TNF (20 ng/ml) or IL-1α (4 ng/ml) for 10 min. IKKα was immunoprecipitated from whole cell lysates, and immunoprecipitates were divided into five equal fractions for incubation with the indicated concentrations of NaSal for 30 min, as described under "Experimental Procedures." These fractions were then assayed for in vitro kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography.

**Effects of Salicylate on IKK Activity**

It seemed difficult to reconcile the notion that NaSal inhibits IκBα degradation via a rapid and persistent activation of p38 MAP kinase (18, 19, 28). We have shown that inhibition of TNF-induced IκBα phos-
Effects of Salicylate on IKK Activity

**Fig. 2.** SB203580 reverses the inhibition of TNF-induced IKK activation by NaSal in *vivo* but not in *vitro*. A, COS-1 cells were incubated for 1.5 h in the presence of SB203580 (10 μM) or Me₂SO vehicle followed by incubation for 30 min in the presence or absence of NaSal (20 mM). Cells were then either left untreated or treated with TNF (20 ng/ml) for 10 min. Whole cell lysates were immunoprecipitated with an antibody against IKKα. Immunoprecipitates (IP) were assayed for *in vitro* kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography (top panel). Separate aliquots of lysates were subjected to immunoblot analysis (IB) with an antibody against IκBα (bottom panel). B, COS-1 cells were left untreated or treated with TNF (20 ng/ml) for 10 min. IKKα was immunoprecipitated from whole cell lysates, and immunoprecipitates were divided into six equal fractions for incubation with either SB203580 (10 μM) or Me₂SO for 10 min followed by incubation with the indicated concentrations of NaSal for 30 min. These fractions were then assayed for *in vitro* kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography.

**Fig. 3.** Effects of *in vivo* and *in vitro* treatment with NaSal upon TNF-induced IKK activity in FS-4 fibroblasts. A, FS-4 cells were treated with the indicated concentrations of NaSal for 1 h and then either left untreated or treated with TNF (20 ng/ml) for 10 min. Cell lysates were immunoprecipitated with an antibody against IKKα, and immunoprecipitates (IP) were subjected to an *in vitro* kinase assay using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography (top panel). Separate aliquots of lysates were subjected to immunoblot analysis (IB) with an antibody against IκBα (bottom panel). B, FS-4 cells were left untreated or treated with TNF (20 ng/ml) for 10 min. IKKα was immunoprecipitated from whole cell lysates, and immunoprecipitates from each group were divided into five equal fractions for incubation with the indicated concentrations of NaSal for 30 min. These fractions were then assayed for *in vitro* kinase activity using GST-IκBα as substrate followed by 12% SDS-PAGE and autoradiography.

**NaSal Inhibits Both IKKα and IKKβ Kinase Activity in *Vitro***—To determine whether the inhibitory effect of NaSal in *vitro* is selective for either the IKKα or IKKβ catalytic components of the IKK complex, we transfected COS-1 cells with expression vectors encoding FLAG-IKKα or FLAG-IKKβ. Because the kinase activity of overexpressed IKKβ is at least 20 times higher than that of IKKα (23), we cotransfected NIK along with IKKα to augment its *in vitro* kinase activity. FLAG-tagged IKKs were immunoprecipitated from whole cell extracts and used in an *in vitro* kinase assay with GST-IκBα as substrate in the presence of 0, 5, or 20 mM NaSal. NaSal dose-dependently inhibited IKKα activity in the presence or absence of cotransfected NIK in addition to inhibiting IKKβ activity in *vitro* (Fig. 4, top panel). Levels of immunoprecipitated IKKα and IKKβ were equivalent in all lanes as demonstrated by immunoblot analysis (Fig. 4, bottom panel).

**NaSal Inhibits p38 Kinase Activity in *Vitro***—To determine whether the *in vitro* inhibitory effect of NaSal is specific for IKKs or whether other kinases are also affected, we analyzed the effect of NaSal treatment upon *in vitro* p38 kinase activity. COS-1 cells were transfected with FLAG-tagged p38α and either left untreated or treated with arsenite for 15 min to activate p38. FLAG-tagged p38 was immunoprecipitated from whole cell extracts and used in an *in vitro* kinase assay with MBP as substrate in the presence of 0, 5, or 20 mM NaSal. Arsenite stimulated p38 kinase activity above baseline, and both baseline and arsenite-stimulated p38 activity were inhibited dose-dependently by NaSal treatment in *vitro* (Fig. 5, top panel). Expression of immunoprecipitated FLAG-p38 was equivalent in all lanes (Fig. 5, bottom panel). We also observed that *in vitro* treatment with 5 and 20 mM NaSal inhibited the kinase activity of endogenous p38 activated by TNF treatment (data not shown). Because treatment of cells with NaSal has been shown to activate p38 MAP kinase (18, 19), the inhibitory effect of NaSal upon p38 activity supports the argument that the effect of NaSal upon kinase activity in *vitro* does not reflect the action of NaSal in intact cells.
Effects of Salicylate on IKK Activity

It has been shown that NaSal and aspirin inhibit NF-κB activation by preventing the phosphorylation and subsequent degradation of IκBα (16, 17). NaSal inhibits IκBα phosphorylation and degradation induced by TNF but not by IL-1 (19, 26). The ability of NaSal to inhibit TNF-induced IκBα phosphorylation is dependent upon NaSal-induced p38 MAP kinase activation, and p38 may play a more general role in the inhibition of TNF-induced NF-κB activation (19, 20). Recently, Yin et al. (21) have reported that NaSal and aspirin may inhibit the IKK complex by directly binding to IKK6, thereby interfering with ATP binding. Because both TNF and IL-1 activate the IKK complex (23, 27), such a mechanism of NF-κB inhibition by NaSal is inconsistent with the observations that NaSal inhibits TNF-induced but not IL-1-induced IκBα degradation (19, 26) and that the inhibition by NaSal is p38-dependent (19, 20). To clarify the mechanism by which NaSal inhibits IκBα phosphorylation, we analyzed the effects of NaSal upon cytokine-induced IKK activity in intact cells and in vitro. We conclude that the direct inhibitory effect of NaSal upon IKK activity in vitro does not reflect the mechanism whereby NaSal inhibits IKK activity in intact cells.

Studies of IKKα and IKKβ knockout mice indicate that only IKKβ is required for cytokine-induced IκBα degradation, whereas IKKα appears to be important for epidermal differentiation and skeletal morphogenesis (reviewed in Refs. 29 and 30). Because both TNF- and IL-1-induced IKK activation is impaired in IKKβ−/− cells (31–33), direct inhibition of IKKβ by NaSal would be expected to impair IKK activation induced by either TNF or IL-1. However, our results demonstrate that treatment of intact cells with NaSal inhibits TNF-induced IKK activity but does not appreciably affect IL-1-induced IKK activity (Fig. 1A). This observation, in concert with our previous data (19, 20), suggests that NaSal may inhibit NF-κB activation by selectively targeting a component of the TNF signaling pathway leading to IKK activation. On the other hand, treatment of immunoprecipitated IKK with NaSal in vitro dose-dependently inhibited IKK activity from either TNF- or IL-1-treated cells (Fig. 1B). In addition, we observed that NaSal did not appreciably inhibit either TNF-induced IKK activity or IκBα degradation in intact FS-4 fibroblasts (Fig. 3A), whereas in vitro treatment of IKK immunoprecipitated from FS-4 cells with 20 mM NaSal led to a strong inhibition of IKK activity (Fig. 3B). The inhibitory effect of NaSal in vitro is therefore not indicative of its inhibitory profile in whole cells in at least two distinct cell systems.

Because the p38 MAP kinase inhibitor SB203580 can prevent the ability of NaSal to inhibit TNF-induced IκBα phosphorylation and degradation (19, 26), we analyzed the effect of this inhibitor on the ability of NaSal to affect TNF-induced IKK activity. Consistent with previous studies, we observed that incubation of cells with SB203580 significantly prevented the NaSal-mediated inhibition of TNF-induced IKK activity (Fig. 2A). SB203580 is a specific inhibitor of p38α and p38β isoforms and fails to inhibit the activity of numerous other kinases (34). However, some reports indicate that high concentrations of SB203580 may exert effects unrelated to inhibition of p38 (35–37). It seemed possible that SB203580, a low molecular weight imidazole compound, might directly interact with the IKK complex and thereby block NaSal binding. We have ruled out a direct effect of SB203580 on the IKK complex by demonstrating that incubation of immunoprecipitated IKK with SB203580 in vitro, prior to incubation with NaSal, did not reverse the inhibition by NaSal (Fig. 2B). This finding further supports the conclusion that the direct inhibition of IKK activity in vitro does not reflect the mechanism of IKK inhibition by NaSal in vivo.

It is likely that the effect of SB203580 in vivo reflects a genuine role for NaSal-induced p38 activation in the inhibition of IKK activity. In support of this notion, diverse stimuli that persistently activate p38, such as hyperosmolarity, H2O2, staurosporine, and vitamin C, similarly inhibited TNF-induced IκBα phosphorylation and degradation in a manner preventable by SB203580 (20, 26, 38). Furthermore, we demonstrated that a constitutively active MAP kinase kinase 6 mutant, which activates p38, decreased TNF-induced IκBα phosphorylation and NF-κB reporter activity (20), and this inhibition occurs at the level of receptor interacting protein or TNF receptor-associated factor 2 in the TNF signaling pathway (data not shown). Other cellular effects of NaSal have been attributed to p38 activation, such as the induction of apoptosis in fibroblasts and induction of adipocyte differentiation (18, 28),
suggesting that NaSal may exert multiple biological effects through activation of p38.

Although it has been reported that NaSal and aspirin directly inhibit IKKβ but fail to affect IKKα (21), we observed that NaSal inhibited the activity of both immunoprecipitated IKKα and IKKβ in vitro (Fig. 4). We observed significant in vitro and in vivo inhibitory effects of NaSal only at millimolar concentrations, although Yin et al. (21) have demonstrated an IC50 for inhibition of the IKK complex, and IKKβ specifically, at concentrations ranging from 50–100 μM. We cannot explain this discrepancy; however, numerous groups have documented significant effects of NaSal upon NF-κB function, MAP kinase activity, induction of apoptosis, and gene expression only at concentrations of NaSal in the 5–20 mM range (16–18, 39–41). Although these concentrations exceed the 1–2 mM serum levels that may be achieved in patients undergoing high dose salicylate therapy (15), they may nonetheless be consistent with a role for p38-mediated NF-κB inhibition in the anti-inflammatory effects of salicylates. As organic acids, salicylates accumulate at the mildly acidic environments prevailing at sites of inflammation (14, 15, 42). Salicylates are uncharged at low pH and can readily cross membranes, yet deprotonate and become trapped as anions in the more neutral environment found within cells (42). Therefore, it is likely that local concentrations of salicylates at sites of inflammation may reach levels sufficient for NF-κB inhibition. Furthermore, studies employing catalytically inactive IKK mutants indicate that slight reductions in IKK activity may correlate with significant decreases in NF-κB-mediated gene expression (8, 9), suggesting that the reduction in TNF-induced IKK activity observed at 1–5 mM NaSal (Fig. 1A) is likely to translate into biologically relevant NF-κB inhibition.

Interestingly, we noted that millimolar concentrations of NaSal significantly inhibited p38 MAP kinase activity in vitro (Fig. 5). However, treatment of numerous cell types with millimolar concentrations of NaSal leads to p38 activation rather than to its inhibition (18, 19, 28). This finding further underscores the notion that the effect of NaSal upon in vitro kinase activity does not reflect the mechanism by which NaSal affects various kinases within the cell. Furthermore, the inhibitory action on p38 in vitro indicates that the effect of NaSal upon in vitro kinase activity is not limited to the IKKs. In agreement with this conclusion, it was recently reported that 20 mM NaSal inhibits RSK2 kinase activity in vitro (43). Overall, our results emphasize the need to exercise caution when attributing the actions of pharmacological agents in intact cells to effects observed in vitro without thoroughly considering potential interactions with various signal transduction components.

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Inhibition of IκB Kinase Activity by Sodium Salicylate in Vitro Does Not Reflect Its Inhibitory Mechanism in Intact Cells
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