Effect of acetylsalicylic acid on endogenous IκB kinase activity in lung epithelial cells

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Yoo, Chul-Gyu, Seunghee Lee, Choon-Taek Lee, Young Whan Kim, Sung Koo Han, and Young-Soo Shim. Effect of acetylsalicylic acid on endogenous IκB kinase activity in lung epithelial cells. Am J Physiol Lung Cell Mol Physiol 280: L3–L9, 2001.—The anti-inflammatory effect of acetylsalicylic acid (ASA) has been thought to be secondary to the inhibition of prostaglandin synthesis. Because doses of ASA necessary to treat chronic inflammatory diseases are much higher than those needed to inhibit prostaglandin synthesis, a prostaglandin-independent pathway has been emerging as the new anti-inflammatory mechanism of ASA. Here, we examined the effect of ASA on the interleukin (IL)-1β- and tumor necrosis factor (TNF)-α-induced proinflammatory cytokine expression and evaluated whether this effect is closely linked to the nuclear factor (NF)-κB pathway. A high dose of ASA blocked IL-1β- and TNF-α-induced IL-8 expression, respectively. ASA inhibited TNF-α-induced activation of NF-κB by preventing phosphorylation and subsequent degradation of IκB-α in a prostaglandin-independent manner. TNF-α-induced activation of IκB kinase was also suppressed by ASA pretreatment. These observations suggest that the anti-inflammatory effect of ASA in lung epithelial cells may be due to suppression of IκB kinase activity, which thereby inhibits subsequent phosphorylation and degradation of IκB-α, activation of NF-κB, and proinflammatory cytokine expression in lung epithelial cells.

nuclear factor-κB; interleukin-8

OVER THE LAST DECADE, many studies of basic biological characteristics of inflammation and tissue injury have implicated proinflammatory cytokine-mediated tissue injury in the pathogenesis of a wide variety of inflammatory disorders including sepsis, acute respiratory distress syndrome, and multiorgan dysfunction syndrome. As a result, anti-inflammatory agents, which inhibit the expression of proinflammatory cytokines, have been tried as the specific therapy for these diseases (4).

Acetylsalicylic acid (ASA) is a nonsteroidal anti-inflammatory drug (NSAID) used in the treatment of many inflammatory diseases. It is rapidly deacetylated to salicylate in the intact organism. Its ability to inhibit arachidonic acid metabolites by blocking cyclooxygenase (COX) and prostaglandin H synthase has been regarded as the main anti-inflammatory mechanism. However, doses of ASA necessary to treat chronic inflammatory diseases are much higher than those needed to inhibit prostaglandin synthesis (21, 25). In addition, nonacetylated salicylates, which do not interfere with prostaglandin synthesis, are still effective anti-inflammatory agents when used in high doses (21, 25). These findings have led to the speculation that the anti-inflammatory effect of ASA may be mediated by a prostaglandin-independent pathway.

Nuclear factor (NF)-κB is a ubiquitous inducible transcription factor involved in immune, inflammatory, stress, and developmental processes. It is sequestered in the cytoplasm in an inactive state by association with the inhibitory molecule IκB-α. NF-κB is rapidly activated in response to various stimuli including viral infection, lipopolysaccharide, ultraviolet (UV) irradiation, and proinflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β (2, 3, 11). TNF-α leads to the sequential activation of the downstream NF-κB-inducing kinase (NIK) and the recently isolated TNF-α-inducible IκB kinase (IKK) complex (9, 15, 23, 26, 29). When activated, IKK directly phosphorylates Ser32 and Ser36 of IκB-α, triggering ubiquitination at Lys21 and Lys22 and rapid degradation of IκB-α in 26S proteasomes (2, 3, 11). This process liberates NF-κB, allowing it to translocate to the nucleus. In the nucleus, NF-κB binds to its cognate κB site and transactivates the downstream genes. Most genes for inflammatory mediators such as TNF-α, IL-2, IL-6, IL-8, lynphotoxin, granulocyte-macrophage colony-stimulating factor, interferon-β, and adhesion molecules have κB sites in the 5’-flanking region (2, 3, 11).

Recent reports (14, 16, 20, 24) suggest that high doses of salicylates show an anti-inflammatory effect through the inhibition of NF-κB activation in monocytic, lymphocytic, and endothelial cells. However, the
anti-inflammatory effect of ASA and its mechanism of action in lung epithelial cells are poorly understood. In the present study, we investigated the effect of ASA on proinflammatory cytokine expression and evaluated whether this effect is closely linked to NF-κB/IκB-α regulation in lung epithelial cells. First, we found that a high dose of ASA blocked TNF-α-induced IL-8 mRNA and protein expression. Second, ASA pretreatment inhibited TNF-α-induced activation of NF-κB by preventing the degradation of IκB-α. Finally, TNF-α-induced activation of endogenous IKK and subsequent phosphorylation of IκB-α were suppressed by ASA pretreatment. These observations suggest that the anti-inflammatory effect of ASA in lung epithelial cells may be due to the blocking of IκB-α phosphorylation by suppressing IKK activity, thereby inhibiting subsequent degradation of IκB-α, activation of NF-κB, and proinflammatory cytokine expression.

**METHODS**

**Cell culture.** BEAS-2B cells, representing normal human bronchial epithelial cells, were maintained as a monolayer in keratinocyte growth medium (Clonetics, Walkersville, MD), and A549 cells, representing type II alveolar epithelial cells, were maintained in RPMI 1640 medium containing 10% fetal bovine serum, 60 μg/ml of penicillin, and 100 μg/ml of streptomycin at 37°C under 5% CO₂.

**Reagents.** Recombinant human TNF-α and an ELISA kit for IL-8 were purchased from R&D Systems (Minneapolis, MN). A stock solution of TNF-α was prepared in distilled water, and aliquots were stored at −70°C until used. Rabbit polyclonal anti-IκB-α, anti-p65, and anti-IKK-α antibodies and recombinant glutathione S-transferase (GST)-IκB-α were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phosphorylated IκB-α antibody (Ser32) was supplied by New England Biolabs (Beverly, MA). Goat anti-rabbit secondary antibody conjugated with horseradish peroxidase and T4 polynucleotide kinase were purchased from Promega (Madison, WI). Rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody was obtained from Jackson ImmunoResearch (West Grove, PA). Protein G Sepharose beads and an enhanced chemiluminescence kit were supplied by Amershams Pharmacia Biotech (Uppsala, Sweden). Protease inhibitors were obtained from Roche (Mannheim, Germany). ASA, indomethacin, and prostaglandin E₂ were obtained from Sigma (St. Louis, MO). The proteasome inhibitor N-carbonyl-L-lysine (Calbiochem, La Jolla, CA). GST-IκB-α (containing amino acids 1–317) and 10 μCi of [γ-³²P]ATP were purchased from ICN Pharmaceuticals (Costa Mesa, CA). A random-priming kit was purchased from Stratagene (La Jolla, CA).

**Northern blot analysis.** Total cellular RNA was isolated with TRIzol reagent. Equal amounts of total RNA (20 μg/lane) from each sample were loaded into each lane of 1.0% agarose-2% formaldehyde gels and capillary transferred to the membrane. The RNA was cross-linked to the nylon membrane by 1,500-J UV irradiation in a UV cross-linker (Stratagene). The human cDNA for IL-8 was radiolabeled with [α-³²P]dCTP with a random-priming kit. After hybridization of the membranes for 2 h at 45°C in hybridization buffer, radiolabeled cDNA probe (1 × 10⁶ counts·min⁻¹·ml⁻¹·final concentration) was added and incubated overnight at 45°C. The membranes were then washed at 45, 50, and then 55°C. The membranes were exposed to X-ray film in a cassette with an intensifying screen at −70°C.

**IL-8 ELISA.** Cells (1 × 10⁴) were grown in 96-well culture plates in equal numbers. The supernatants were collected and stored at −70°C until analyzed. IL-8 concentrations were quantified with an ELISA kit according to the manufacturer's specifications.

**Western blot analysis.** Cytoplasmic, nuclear, and whole cell extracts were prepared as previously described (28). Twenty micrograms of protein were resolved by 10% SDS-PAGE and transferred to nitrocellulose. The membranes were blocked with 5% skim milk-PBS-0.1% Tween 20 for 1 h before overnight incubation at room temperature withrabbit polyclonal anti-p65 antibody, anti-IκB-α antibody, or antibody specific for phosphorylated IκB-α diluted 1:1,000 in 5% skim milk-PBS-0.1% Tween 20. The membranes were washed three times in 1× PBS-0.1% Tween 20 and incubated with goat-anti-rabbit horseradish peroxidase-conjugated antibody dilution 1:2,000 in 5% skim milk-PBS-0.1% Tween 20 for 1 h. After successive washes, the membranes were developed with an enhanced chemiluminescence kit.

**Immunofluorescent staining for NF-κB.** The cells grown in two-well chamber slides were fixed and permeabilized as previously described (28). The cells were incubated with rabbit polyclonal anti-p65 antibody diluted 1:100 in 1% BSA for 30 min. The cells were incubated with rhodamine isothiocyanate-conjugated goat anti-rabbit immunoglobulin G antibody diluted 1:100 in 1% BSA for 30 min. After being mounted with 50% glycerol, the slides were analyzed with a fluorescence light microscope.

**Electrophoretic mobility shift assays.** NF-κB DNA binding activity was assessed as previously described (28). Briefly, nuclear extracts were incubated for 20 min at room temperature with a radiolabeled NF-κB consensus sequence in the κ light chain enhancer in B cells (5'-AGTTGAGGGACTTTCCAGGC-3'). In competition experiments, a 50-fold molar excess of unlabeled oligonucleotide was added to the binding reaction. In supershift experiments, 0.4 μg of anti-p65 or anti-p50 antibody was added and allowed to react for 45 min at room temperature. DNA-protein complexes were resolved on 4% nondenaturing polyacrylamide gels. The gels were dried and autoradiographed at −70°C.

**IKK assay.** IKK activity was assessed with an in vitro kinase assay as previously described (28). In brief, the IKK complex was immunoprecipitated with an anti-IKK-α antibody diluted 1:100. The immunoprecipitates were incubated at 30°C for 30 min in a kinase buffer containing 0.5 μg of GST-IκB-α (containing amino acids 1–317) and 10 μCi of [γ-³²P]ATP. Kinase reaction products were subjected to SDS-PAGE in 10% gels followed by transfer to a nitrocellulose membrane and autoradiography.

**RESULTS**

ASA blocks IL-1β- and TNF-α-induced proinflammatory cytokine expression. To determine whether ASA shows anti-inflammatory effects in lung epithelial cells, we first analyzed the effect of ASA on proinflammatory cytokine expression. To evaluate whether ASA inhibits cytokine production dose dependently, A549 cells were pretreated with medium or various amounts of ASA (2.5, 5, 10, or 20 mM) for 2 h and then stimulated with IL-1β or TNF-α. IL-8 concentrations in the culture supernatants were assayed by ELISA after 18 h of TNF-α stimulation. TNF-α increased IL-8 production in the absence of ASA. Both IL-1β- and TNF-
α-induced IL-8 production were reduced by pretreatment with a high dose of ASA (Fig. 1A). To evaluate whether the reduction in TNF-α-induced IL-8 production was due to the decrease in mRNA expression, the cells were pretreated with 20 mM ASA for 2 h and then stimulated with IL-1β or TNF-α for 4 h. IL-1β-induced TNF-α and IL-1β- and TNF-α-induced IL-8 mRNA expression were assayed by Northern blot analysis. Although TNF-α mRNA was hardly detectable in untreated cells, IL-1β induced a marked increase in TNF-α mRNA 4 h after stimulation, and this increase was blocked completely in the presence of ASA (Fig. 1B). Both IL-1β- and TNF-α-induced IL-8 mRNA expression were also suppressed by ASA pretreatment (Fig. 1B). To exclude the possibility that this effect of ASA is due to its cytotoxicity, the cells were incubated in the presence of 2.5, 5, 10, or 20 mM ASA for 2.5 h. Cell viability was evaluated by MTT assay. Cell viability did not change in both cells at all doses used (data not shown). These observations indicate that ASA shows anti-inflammatory effects in lung epithelial cells by inhibiting proinflammatory cytokine production.

NF-κB activation is inhibited by ASA. Because most of the proinflammatory cytokine genes including IL-8 contain κB-binding motifs in their promoter regions, we questioned whether the inhibition of proinflammatory cytokine expression by ASA is due to the blocking of TNF-α-induced activation of NF-κB. NF-κB activation was assayed by two approaches: one measured the nuclear translocation of NF-κB and the other assessed the NF-κB-DNA binding activity by electrophoretic mobility shift assay. The expression of NF-κB was assayed by Western blot analysis for the p65 subunit of NF-κB in cytoplasmic and nuclear extracts from cells stimulated with TNF-α in the presence and absence of ASA. Although the majority of p65 was located in the cytoplasmic fraction in the basal state, p65 increased in the nuclear fraction 30 min after TNF-α stimulation, which was completely blocked by ASA pretreatment (Fig. 2A). Total cellular expression of p65 was not affected by ASA pretreatment (Fig. 2A). We next investigated the subcellular localization of NF-κB by immunofluorescent staining. There was a strong nuclear staining of p65 30 min after stimulation with TNF-α in both BEAS-2B and A549 cells compared with the cytoplasmic distribution in untreated cells. This nuclear translocation of p65 by TNF-α was blocked by ASA pretreatment as demonstrated by the cytoplasmic staining pattern (Fig. 2B). We next evaluated the effect of ASA on the NF-κB-DNA binding activity by electrophoretic mobility shift assay. Nuclear extracts from TNF-α-stimulated cells had more active NF-κB available to bind to the κB probe compared with extracts from untreated cells. This TNF-α-induced increase in

Fig. 1. Acetylsalicylic acid (ASA) blocked interleukin (IL)-1β- and tumor necrosis factor (TNF)-α-induced proinflammatory cytokine expression. A: dose-dependent effect of ASA on the production of IL-8 by IL-1β (left) or TNF-α (right). A549 cells were treated with medium alone or indicated doses of ASA for 2 h and then stimulated with IL-1β (5 ng/ml) or TNF-α (5 ng/ml) for 18 h in the continued presence (+) and absence (−) of ASA. The concentrations of IL-8 in supernatant fluid were quantitated by ELISA. Data are means ± SD from 3 different experiments. B: effect of ASA on proinflammatory cytokine mRNA expression. A549 cells were treated with medium alone or ASA for 2 h and then stimulated with IL-1β or TNF-α for 4 h. IL-1β-induced TNF-α and IL-1β- and TNF-α-induced IL-8 mRNA expression were assayed by Northern blot analysis. Results are representative of 3 different experiments.
NF-κB-DNA binding activity was inhibited by 20 mM ASA (Fig. 2C). When a 50-fold molar excess of unlabelled double-strand NF-κB oligonucleotide was added to the binding reaction, the retarded band disappeared, suggesting the specificity of binding. Supershift assay showed the presence of the p50 and p65 subunits of NF-κB (Fig. 2C). These results indicate that suppression of proinflammatory cytokine expression by ASA is due to the blockade of NF-κB activation.

ASA suppresses IκB-α degradation in a prostanoid-independent manner. Because NF-κB exists as an inactive form bound to the inhibitory protein IκB-α in the cytoplasm, the degradation of IκB-α must occur in order for NF-κB to translocate to the nucleus. We next analyzed the effect of ASA on the IL-1β- and TNF-α-induced degradation of IκB-α. IL-1β- and TNF-α-induced degradation of IκB-α was blocked by high doses of ASA (Fig. 3A). ASA is known to be a weak inhibitor of COX activity in respiratory epithelial cells. To evaluate whether the inhibition of COX activity stabilizes IκB-α, we examined the effect of indomethacin, which is a potent COX inhibitor, on the TNF-α-induced degradation of IκB-α. IκB-α degradation by TNF-α was not blocked by indomethacin pretreatment at all doses used (10, 50, 100, and 500 μM; Fig. 3B). To evaluate whether ASA stabilizes IκB-α in a prostanoid-dependent manner, we next examined the effect of exogenously applied prostaglandin E2 on the IκB-α stabilizing effect of ASA. Exogenously applied prostaglandin E2 did not reduce the blocking effect of ASA on the TNF-α-in-
ASA blocks activation of IKK. Cytokine-induced IκB-α phosphorylation is mediated by the IKK complex. To evaluate whether the decrease in phosphorylated IκB-α in ASA-treated cells is due to the inhibition of IKK activity, the effect of ASA on IKK activity was assayed with GST-IκB-α as a substrate after TNF-α stimulation with various doses of ASA. TNF-α induced a marked increase in phosphorylated GST-IκB-α after 5 and 10 min of stimulation in BEAS-2B and A549 cells, respectively, which implies activation of the IKK complex. TNF-α-induced phosphorylation of IκB-α was completely blocked by ASA pretreatment at doses of 10 mM in BEAS-2B cells and 20 mM in A549 cells, suggesting different sensitivities to ASA in different cell lines (Fig. 5). This inhibition of IKK activity by ASA was not due to a decrease in IKK-α protein levels because immunoblot analysis demonstrated comparable IKK-α expression at all conditions (data not shown). This was further confirmed by immunoblotting with an antibody specific to phosphorylated IκB-α (data not shown). These observations suggest that stabilization of IκB-α by ASA may be due to the inhibition of IκB-α phosphorylation.

ASA blocks activation of IKK. Cytokine-induced IκB-α phosphorylation is mediated by the IKK complex. To evaluate whether the decrease in phosphorylated IκB-α in ASA-treated cells is due to the inhibition of IKK activity, the effect of ASA on IKK activity was assayed with GST-IκB-α as a substrate after TNF-α stimulation with various doses of ASA. TNF-α induced a marked increase in phosphorylated GST-IκB-α after 5 and 10 min of stimulation in BEAS-2B and A549 cells, respectively, which implies activation of the IKK complex. TNF-α-induced phosphorylation of IκB-α was completely blocked by ASA pretreatment at doses of 10 mM in BEAS-2B cells and 20 mM in A549 cells, suggesting different sensitivities to ASA in different cell lines (Fig. 5). This inhibition of IKK activity by ASA was not due to a decrease in IKK-α protein levels because immunoblot analysis demonstrated comparable IKK-α expression at all conditions (data not shown).
Salicylates have been shown to inhibit the transcription of genes such as adhesion molecules and inducible nitric oxide synthase, which are involved in the inflammation process (10, 20). Because proinflammatory cytokine-mediated tissue injury has been implicated in the pathogenesis of a wide variety of inflammatory disorders and transcription of most proinflammatory cytokine genes is dependent on NF-κB activation, it is very likely that the anti-inflammatory effect of salicylates is closely related to the suppression of proinflammatory cytokine expression. Although ASA and sodium salicylates inhibit NF-κB activation in monocyctic, lymphocytic, and endothelial cells (14, 16, 20, 24), TNF-α-induced activation of NF-κB was not blocked by salicylates in cultured cardiac fibroblasts (10). The effect of salicylates on the activation of NF-κB differs according to cell type. At present, few data about the anti-inflammatory effect of ASA and its mechanism of action in lung epithelial cells have been presented, whereas the importance of the role of lung epithelial cells in lung inflammation is increasing.

In this study, we demonstrated that ASA inhibited IL-1β- and TNF-α-induced proinflammatory cytokine expression by blocking NF-κB activation in lung epithelial cells. This effect of ASA can be generalized to respiratory epithelial cells because the same effects were observed in both BEAS-2B and A549 cells, which represent bronchial and alveolar epithelial cells, respectively. Considering the important role of proinflammatory cytokines in the inflammatory process, this result suggests an anti-inflammatory effect of ASA in lung epithelial cells. We also found that blocking of NF-κB activation was due to the stabilization of IκB-α. This result coincides with previous reports in monocyctic, lymphocytic, and endothelial cells (14, 16, 20, 24).

Prostanoids are important mediators of airway inflammation, and their synthesis is mediated by COX. NSAIDs, which potently inhibit COX activity, reduced IL-8 production in a prostanoid-dependent manner in airway smooth muscle cells (18), and a study (19) has shown that inhaled NSAIDs are protective in airway inflammation. Because ASA is known to inhibit COX activity, we examined whether the IκB-α stabilizing effect of ASA is prostanoid dependent. Indomethacin, which potently inhibits COX-induced prostaglandin E2 production, did not block TNF-α-induced degradation of IκB-α, and the IκB-α stabilizing effect of ASA was not overcome by exogenously applied prostaglandin E2. The most likely explanation for this result is that the anti-inflammatory effect of ASA is prostanoid independent in lung epithelial cells.

Because NF-κB is sequestered in the cytoplasm by IκB-α, activation of NF-κB requires degradation of IκB-α. The first step of degradation involves phosphorylation of IκB-α by the IKK complex. The IKK complex is made up of several kinases including IκKα and IκKβ, and it requires phosphorylation by NIK to become activated. Phosphorylated IκB-α undergoes ubiquitination and finally degradation through a proteasome pathway. Because TNF-α-induced phosphorylation of IκB-α was not observed in the presence of the proteasome inhibitor in this study, the IκB-α stabilizing effect of ASA is likely to occur at the level of IκB-α phosphorylation by either inhibition of IKK activity or activation of phosphatase. Because our immune complex kinase assay showed that ASA suppressed endogenous IKK activity, it is likely that ASA blocks IκB-α phosphorylation by suppressing IKK activation rather than by activating a phosphatase.

The TNF-α and IL-1β-induced NF-κB/IκB signaling pathway involves distinct pathways. TNF-α stimulation recruits TNF receptor-associated factor (TRAF)-2 and the receptor-interacting protein (12, 13), whereas IL-1β uses the IL-1 receptor (IL-1R) accessory protein and the IL-1R-associated kinase to transmit signals to TRAF-6 (6, 7). The TNF-α and IL-1β pathways converge on NIK to activate the IKK complex. Thus the target to block cytokine-induced degradation of IκB-α could be the receptor-interacting protein, TRAF-2, TRAF-6, NIK, or IKK. However, because ASA pretreatment in this study blocked both TNF-α- and IL-1β-induced phosphorylation of IκB-α by inhibiting the activation of IKK, it seems likely that ASA interferes with a common signal upstream or parallel to IKK.

It has been reported that in vitro treatment with salicylates or ASA inhibited IKK-β but failed to affect IKK-α (27). In this study, our results demonstrated that treatment of intact cells with ASA inhibited TNF-α-induced endogenous IKK activity. Although we used anti-IKK-α antibody for immunoprecipitation, it cannot be concluded that ASA blocks the function of endogenous IKK-α because IKK-α and IKK-β form a complex and IKK-β can be immunoprecipitated with anti-IKK-α antibody. The concentrations of ASA that blocked TNF-α-induced IKK activation in our experiments were 10 and 20 mM in BEAS-2B and A549 cells, respectively, which is much higher than the usual therapeutic serum concentration. Only high concentrations of ASA blocked
nuclear translocation of NF-κB in endothelial cells (20, 24). Therefore, it seems likely that this in vitro effect of ASA cannot be applied to the anti-inflammatory effect in vivo. Salicylates accumulate in the mildly acidic environments prevailing at sites of inflammation (1, 5, 25). Salicylates are unchanged at low pH and can readily cross membranes (5). Therefore, the local concentrations of ASA could be much higher than those of serum and would be sufficient to suppress IKK activation and subsequent NF-κB-dependent expression of proinflammatory cytokines.

In this study, we have shown that high doses of ASA inhibit proinflammatory cytokine production by blocking NF-κB activation in lung epithelial cells. We demonstrated that this inhibitory effect of ASA on NF-κB activation is secondary to the stabilization of IkB-α by blocking the phosphorylation of IkB-α and its subsequent degradation. This blocking of IkB-α phosphorylation by ASA was due to the inhibition of IKK activity. Because proinflammatory cytokine function in redundant and overlapping ways through the so-called cytokine “cascade” or “network,” it would be necessary to modulate the entire cytokine network at the same time to achieve an anti-inflammatory response. Because transcription of most proinflammatory cytokine genes is regulated by NF-κB activation, our data showing that inhibition of IKK activation by ASA resulted in the stabilization of IkB-α and inhibition of NF-κB activation suggest that the IKK complex could be an excellent molecular target for a new anti-inflammatory therapy.

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