Sulindac Inhibits Activation of the NF-κB Pathway*

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Sulindac is a non-steroidal anti-inflammatory agent that is related both structurally and pharmacologically to indomethacin. In addition to its anti-inflammatory properties, sulindac has been demonstrated to have a role in the prevention of colon cancer. Both its growth inhibitory and anti-inflammatory properties are due at least in part to its ability to decrease prostaglandin synthesis by inhibiting the activity of cyclooxygenases. Recently, we demonstrated that both aspirin and sodium salicylate, but not indomethacin, inhibited the activity of an IκB kinase β (IκKβ) that is required to activate the nuclear factor-κB (NF-κB) pathway. In this study, we show that sulindac and its metabolites sulindac sulfide and sulindac sulfone can also inhibit the NF-κB pathway in both colon cancer and other cell lines. Similar to our previous results with aspirin, this inhibition is due to sulindac-mediated decreases in IκKβ kinase activity. Concentrations of sulindac that inhibit IκKβ activity also reduce the proliferation of colon cancer cells. These results suggest that the growth inhibitory and anti-inflammatory properties of sulindac may be regulated in part by inhibition of kinases that regulate the NF-κB pathway.

The NF-κB1 pathway regulates the cellular response to a variety of stimuli including cytokines, bacterial and viral infection, and activation of cellular stress pathways (reviewed in Refs. 1 and 2). The NF-κB pathway is also critical for the control of cellular growth properties (3–7). For example, disruption of the gene encoding the p65 member of the NF-κB family leads to severe hepatic apoptosis indicating that at least in some cell types NF-κB is a critical anti-apoptotic factor (5). Inhibition of apoptosis is likely mediated by NF-κB induction of cellular genes such as cellular inhibitor of apoptosis-1 and -2 which inhibit the apoptotic process (4, 6). The fact that high constitutive levels of NF-κB are found in the nucleus of some tumors further implicates activation of this pathway as a potential mechanism to stimulate cellular growth (8, 9).

NF-κB is comprised of a family of related proteins that can either heterodimerize or homodimerize to facilitate their binding to a consensus DNA element to result in activation of gene expression (reviewed in Refs. 1 and 2). The DNA binding and dimerization properties of NF-κB are mediated by a conserved domain in these proteins known as the Rel homology domain. NF-κB is normally sequestered in the cytoplasm of cells where it is bound by a family of inhibitory proteins known as IκB (2, 10). These proteins that include IκBα, IκBβ, and IκBε contain multiple ankyrin repeats that are critical for their inhibitory function. The ability of the IκB to mask the nuclear localization signal of NF-κB prevents the nuclear translocation of these proteins (11). A variety of stimuli including treatment of cells with TNFα, interleukin-1, phorbol esters, lipopolysaccharide, and the viral protein Tax results in activation of the NF-κB pathway (2). These stimuli modulate signal transduction pathways that lead to the ability of upstream kinases including NIK and MEKK1 to activate the IκB kinases IKKα and IKKβ (12–16). Stimulation of the activity of these kinases results in their ability to phosphorylate two conserved serine residues in the amino terminus of the IκB proteins (17–23). This resultant phosphorylation of IκB leads to its ubiquitination and its degradation by the proteasome resulting in the nuclear translocation of NF-κB (19, 20).

Aspirin and sodium salicylate, but not several other anti-inflammatory agents including indomethacin, can inhibit activation of the NF-κB pathway (24–26). Inhibition of the NF-κB pathway by aspirin and salicylate is the result of their specific binding to IκKβ which inhibits its kinase activity (26). The effects of aspirin and salicylate prevent IκB degradation and the nuclear translocation of NF-κB (26). In addition to its role as an anti-inflammatory agent, aspirin can also help to prevent the development of colon cancer (27–31).

Sulindac is a non-steroidal anti-inflammatory agent that is structurally related to indomethacin and inhibits cyclooxygenase activity to prevent prostaglandin synthesis (32–35). In the colon, sulindac is converted by bacteria to the metabolites sulindac sulfide and sulindac sulfone. Sulindac sulfide is the most active metabolite of sulindac and is concentrated in the colonic epithelium at concentrations that are at least 20-fold higher than those seen in the serum which are about 10–15 μM (33). Sulindac sulfide, but not sulindac sulfone, blocks prostaglandin synthesis by non-selective inhibition of cyclooxygenase 1 and cyclooxygenase 2 (33). However, alternative mechanisms of sulindac action other than inhibition of prostaglandin function have been suggested. For example, sulindac sulfone can inhibit mammary carcinogenesis (36), and sulindac sulfide inhibits the proliferation of colon cancer cell lines that do not express cyclooxygenases (37).

Sulindac, like aspirin, has anti-inflammatory properties and has also been demonstrated to induce the regression of adenomatous colon polyps to help prevent the development of colon cancer (27–31, 38–43). The effects of sulindac on preventing colon cancer are likely mediated by stimulating cellular apo-
ptotic pathways (37, 44–48). Since sulindac and aspirin have similar pharmacologic properties, we investigated whether sulindac-like aspirin could also inhibit kinases that regulate the NF-κB pathway to mediate in part its anti-inflammatory and pro-apoptotic properties. In this study, we demonstrate that sulindac and its metabolites, sulindac sulfide and sulindac sulfone, inhibit the activation of the NF-κB pathway by inhibiting IKKβ kinase activity. This result suggests that inhibition of components of the NF-κB pathway may at least in part be involved in the anti-inflammatory properties and the growth properties inhibitory of these agents.

MATERIALS AND METHODS

Cell Culture and Treatment—COS, HCT-15, and HT-29 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% CO2 and 95% air incubator at 37 °C. Aspirin, sodium salicylate, and sulindac were obtained from Sigma and dissolved in 1 m Tris-HCl, pH 8.0, to make 1 m stock solution. In transfection assays, aspirin and salicylate were used at a concentration of 5 mM; sulindac and sulindac sulfone were used at a concentration of 1 mM; sulindac sulfide was used at a concentration of 200 μM, and indomethacin and ibuprofen were used at a concentration of 25 μM. Indomethacin and ibuprofen were dissolved in ethanol to make a 100 mM stock solution (26). All the anti-inflammatory agents were added into cell culture medium for 16–18 h for the luciferase reporter gene expression assay or for 2 h prior to TNF-α treatment and assays of IKK activity (26).

DNA Plasmids and Transient Transfections—The expression plasmids hemagglutinin-tagged IKKα and FLAG-tagged IKKβ, and their constitutively active mutants (SS/EE), were described previously (49). The HIV-1 long terminal repeat luciferase reporter contains the human immunodeficiency virus long terminal repeat with two binding sites for NF-κB. Approximately 80% confluent COS, cells in 60-mm plates were transfected with the 3 μg of DNA using Fugene 6 (Roche Molecular Biochemicals). Cells were harvested 24–36 h after the transfection.

Assay of IKK Kinase Activity—Histidine and FLAG-tagged bacular- expressed IKKα and IKKβ proteins were purified by nickel-agarose chromatography and immunoprecipitated with the M2 monoclonal antibody and then assayed in kinase assays as described (26). Transfected COS cells were suspended in lysis buffer containing 40 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 10 mM β-mercaptoethanol, 10 mM NaF, 1 mM sodium vanadate, and protease inhibitor cocktail (Roche Molecular Biochemicals). Immunoprecipitation was performed with 300 ng of M2 FLAG antibody or 50 μl of 12CA5 supernatant to precipitate the wild-type and mutant hemagglutinin-IKKα and wild-type and mutants FLAG-IKKβ using 200 μg of transfected cell lysate. This was followed by the addition of 20 μl of protein A-agarose. After washing with the lysis buffer, the immunocomplex was then incubated with a kinase assay buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, 10 μM ATP, 10 mM β-mercaptoethanol, 10 mM NaF, 1 mM Na3VO4, 5 μCi of [γ-32P]ATP, and 5 μg of glutathione S-transferase-IκBα (aa 1–54) at 30 °C for 30 min. The kinase reaction mixture was resolved on a SDS-polyacryl- amide gel electrophoresis and detected by autoradiography (26).

Calculation of the IC50 Value of Sulindac—COS cells were treated with various concentrations of sulindac in vivo for 2 h before TNF-α treatment and assays of endogenous IKK activity. To assay endogenous IKK activity, cell lysates (200 μg of protein) were immunoprecipitated with a rabbit polyclonal antibody directed against IKKα (Santa Cruz Biotechnology) that immunoprecipitates the IKKα/IKKβ heterodimer followed by assays of kinase activity using the GST-IκBα substrate (26).

Apoptosis Assays of HCT-15 Cells—Apoptosis assay was measured with Cell Death Detection ELISA™ PLUS kit (Roche Molecular Biochemicals) using procedures suggested by the manufacturer with modifications. Briefly, HCT-15 cells were cultured in 6-well plates at a density of 2 × 104 cells per well overnight. Cells were treated with reagents including aspirin (5 mM), salicylate (5 mM), sulindac (1 mM), ibuprofen (25 μM), and indomethacin (25 μM) for the indicated periods. After incubation, the cells were washed twice with PBS and harvested from the plates. Cells were lysed 30 min at room temperature, and the lysates were centrifuged at 200 × g for 5 min. Then 20 μl of the supernatant was transferred into streptavidin-coated 96-well plates provided by the manufacturer, and 80 μl of the immunoreagents containing anti-histone conjugated with biotin and anti-DNA conjugated with peroxidase was added to each well. After incubation for 2 h at room temperature, plates were washed three times with wash buffer. Peroxidase was determined photometrically with 2,2′-azinodi(3-ethylbenz-thiazoline-6-sulfonate) as substrate using microtiter enzyme-linked immunosorbent assay plate reader at wavelength of 410 nm. The values from duplicate samples were averaged and subtracted from those of background (samples without lysates). The results were expressed as absorbance of the sample (dying/dead cells)/absorbance of the control cells (without treatment).

RESULTS

Sulindac Inhibits NF-κB-directed Gene Expression—Aspirin and sodium salicylate, but not indomethacin, inhibit NF-κB-directed gene expression (24, 25, 49). Recently, we extended these studies and demonstrated that the effects of aspirin and sodium salicylate are mediated by the direct binding of these agents to IKKβ to decrease its kinase activity (26). Since aspirin and sulindac both have anti-inflammatory and anti-proliferative effects, we investigated whether sulindac could also inhibit activation of the NF-κB pathway. Such a result could help explain previous data demonstrating that sulindac sulfide can induce apoptosis in the colon cancer cell line HCT-15 which lacks cyclooxygenases (37).

First, we compared the effects of sulindac, aspirin, salicylate, indomethacin, and ibuprofen on the expression of an HIV-1 long terminal repeat reporter construct that contains two NF-κB sites. The concentration of sulindac necessary to inhibit the proliferation of colon cancer cell lines ranges from 400 to 1200 μM (44, 45, 47). At these concentrations, sulindac does not cause cell death, and its effects on cell proliferation are reversible after removing the drug (44, 45, 47, 48). Approximately 4–6-fold lower concentrations of the sulfide metabolite of sulindac (100–200 μM) inhibit cell proliferation (44, 45, 47). In the analysis described below, we used a concentration of 1,000 μM for both sulindac and sulindac sulfone and a concentration of 200 μM for sulindac sulfide. These concentrations of sulindac and its metabolites resulted in reversible effects on cellular proliferation (data not shown). The concentrations of aspirin (1–5 mM), indomethacin (25 μM), and ibuprofen (25 μM) were based on the pharmacologic concentrations of these agents in the serum of patients required for their anti-inflammatory properties (26).

An NF-κB reporter was transfected into COS cells that were treated with known activators of the NF-κB pathway. These included either TNF-α (50), the MAP3 kinases NIK (51) or MEKK1 (52, 53), or the human T-cell leukemia virus type I transactivator Tax (49). Each of these activators stimulated NF-κB-directed gene expression from 10- to 40-fold (Fig. 1, A and B). These activators did not stimulate the expression of an HIV-1 reporter containing mutated NF-κB sites (data not shown). Treatment of the cells with either aspirin or salicylate reduced TNF-α-directed NF-κB gene expression approximately 4-fold (Fig. 1A). Treatment of the cells with either sulindac or sulindac sulfone also reduced TNF-α induction of NF-κB gene expression about 4-fold (Fig. 1A). Sulindac sulfide resulted in approximately a 10-fold decrease in NF-κB-directed gene expression (Fig. 1A). Similar levels of inhibition were seen with these agents when NF-κB gene expression was activated by NIK expression in cells treated with either aspirin, sodium salicylate, sulindac, or the sulfide and sulfone metabolites of sulindac (Fig. 1A). In contrast, two other non-steroidal anti-inflammatory agents, indomethacin and ibuprofen, did not reduce the TNF-α stimulation of NF-κB-directed gene expression (Fig. 1A).

MEKK1 and Tax activation of the NF-κB pathway was also inhibited 4–6-fold by treatment of cells with either aspirin, sodium salicylate, sulindac, or sulindac sulfone (Fig. 1B). Sulindac sulfide consistently resulted in a 2–3-fold greater level of inhibition of the NF-κB pathway than seen with aspirin, salic-
ylate, or sulindac sulfone (Fig. 1, A and B). Neither indomethacin nor ibuprofen reduced Tax or MEKK1 induction of NF-κB-directed gene expression (Fig. 1B). These results suggest that sulindac and its sulfone and sulfide metabolites, like aspirin and sodium salicylate, inhibit activation of the NF-κB pathway in response to a variety of well characterized inducers of this pathway.

**Sulindac Inhibits NF-κB Nuclear Translocation**—Next it was important to address the mechanism by which sulindac inhibited the NF-κB pathway. Nuclear extract was prepared from either untreated COS cells, COS cells treated with TNFα, or COS cells transfected with NIK. Gel retardation analysis was performed with nuclear extract prepared from these cells using oligonucleotides corresponding to either NF-κB- or SP1-binding sites. TNFα treatment of cells induced binding to the wild-type NF-κB oligonucleotide (Fig. 2A, lanes 1 and 2) but not to a mutant NF-κB oligonucleotide (data not shown). Stimulation of NF-κB binding in response to TNFα was inhibited by incubation of the cells with either aspirin, salicylate, or sulindac (Fig. 2A, lanes 3–5) but not ibuprofen or indomethacin (Fig. 2A, lanes 6 and 7). Similar inhibition of NF-κB binding by treatment of cells with either aspirin, salicylate, or sulindac was seen when the NF-κB pathway was activated by NIK transfection (Fig. 2A, lanes 9–14). None of these agents altered SP1 binding in the gel retardation assay (Fig. 2A, lower panel). These results indicated that sulindac, like aspirin and salicylate, inhibited NF-κB nuclear translocation.

Assays were performed to investigate whether sulindac altered the degradation of IκBα when COS cells were treated with either TNFα or following transfection of COS cells with a
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**Fig. 2.** Sulindac inhibits the nuclear translocation of NF-κB. A, nuclear extract (10 μg) prepared from TNFα-treated (lanes 2–8) or NIK-transfected (lanes 9–15) COS cells in the presence of the same concentration of anti-inflammatory agents used in Fig. 1 were assayed in gel retardation assays using either NF-κB (top panel) or SP1 (lower panel) oligonucleotides as probes. B, cytosolic extracts from TNFα-treated (lanes 1–7) or NIK-transfected (lanes 8–14) cells from A were collected and used to perform Western blot assays with a polyclonal antibody against IKKα, and the extracts from the different treatments are shown in lanes 2–15 as indicated. C, cytosolic extracts were prepared from TNFα-treated (lanes 1–7) or NIK-transfected (lanes 8–15) cells and used to assay for endogenous IKK kinase activity in untreated cells (lanes 1 and 8) or cells treated with the indicated agents (top panel). Cell lysate containing 200 μg of protein was immunoprecipitated with a polyclonal antibody directed against IKKα, and 20 μl of protein A-agarose was added to precipitate the endogenous IKK kinase complexes. Kinase assays containing GST-IκBα (aa 1–54) as a substrate. The endogenous IKK was also analyzed by Western blot analysis using a polyclonal antibody against IKKα (lower panel). Untr., untreated; PBS, phosphate buffered saline; ASA, aspirin; Sal, sodium salicylate; Sulindac, sulindac; Indo, indomethacin; Ibpr, ibuprofen.

NIK expression vector. TNFα treatment markedly reduced IκBα levels at 30 min post-treatment when compared with the IκBα levels seen in untreated COS cells (Fig. 2B, lanes 1 and 2). Treatment of the COS cells with either aspirin, salicylate, or sulindac prevented TNFα-induced IκBα degradation (Fig. 2B, lanes 3–5). In contrast, treatment of cells with either indomethacin or ibuprofen resulted in significant TNFα-induced IκBα degradation (Fig. 2B, lanes 6 and 7). Similar inhibition of IκBα degradation was seen in NIK-transfected cells treated with either aspirin, salicylate, or sulindac (Fig. 2B, lanes 10–12). These results suggested that decreases in either the kinase activity of IKK or the ubiquitination or proteasome-mediated degradation of IκBα could be responsible for sulindac-mediated inhibition of the NF-κB pathway.

Cytoplasmic extracts prepared from the COS cells used in Fig. 2, A and B, were also assayed for IKK activity. Immunoprecipitation was performed with an IKKα antibody that results in the isolation of the IKKα/IKKβ heterodimer (49). These immunoprecipitates were then assayed for kinase activity using a GST-IκBα substrate extending from amino acids 1–54. TNFα treatment of cells stimulated IKK activity (Fig. 2C, lanes 1 and 2), and this stimulation was inhibited by treatment of cells with either aspirin, salicylate, or sulindac (Fig. 2C, lanes 3–5). In contrast, indomethacin and ibuprofen did not inhibit IKK activity (Fig. 2C, lanes 6 and 7). Similar results with these agents were seen in extracts prepared from NIK-transfected COS cells (Fig. 2C, lanes 8–14). A GST-IκBα mutant at serine residues 32 and 36 was not phosphorylated by IKK (data not shown). Western blot analysis indicated no change in IKKα protein levels in these extracts (Fig. 2C, lower panel). These results indicate that sulindac, like aspirin and salicylate, could inhibit IKK activity to prevent IκBα degradation and subsequent NF-κB nuclear translocation.

*Sulindac Inhibits IKK Activity in Colon Carcinoma Cell Lines*—It was important to analyze the concentrations of sulindac that were necessary to inhibit endogenous IKK activity. Sulindac concentrations ranging from 10 μM to 12 μM were incubated with COS cells followed by treatment of these cells with TNFα (Fig. 3). Endogenous IKK kinase activity was then analyzed following immunoprecipitation with an IKKα antibody that precipitates the IKKα/IKKβ heterodimer and a GST-IκBα substrate. This analysis indicated that the IC50 of sulin-
Sulindac was approximately 200 μM which is in the lower concentration range where sulindac inhibits cellular growth properties (Fig. 3).

Next we assayed whether sulindac was able to prevent TNFα-mediated increases in IKK activity in two colon cancer lines, HCT-15 and HT-29. The growth of HCT-15 and HT-29 cells is inhibited by treatment with either sulindac or sulindac sulfide (37, 44). HCT-15 cells do not produce prostaglandins, and sulindac sulfide inhibition of their proliferation is due to a mechanism independent of inhibiting cyclooxygenases (37). Both sulindac and aspirin, but not indomethacin, markedly reduced TNFα-mediated increases in IKK activity in HCT-15 (Fig. 4A, lanes 1–5) and HT-29 (Fig. 4A, lanes 6–10) cells. There was no change in IKKα protein levels in these cells when incubated with aspirin or sulindac (Fig. 4A, lower panel). These results indicate that both sulindac and aspirin can alter TNFα induction of IKK activity in colon carcinoma cell lines.

The ability of these anti-inflammatory agents to induce apoptosis in HCT-15 cells was next assayed. Since HCT-15 cells do not contain cyclooxygenases, we would expect the mechanism of apoptosis to be prostaglandin-independent and thus not affected by treatment with ibuprofen or indomethacin. In contrast, agents that inhibit the NF-κB pathway including sulindac, aspirin, and salicylate might be expected to induce apoptosis in HCT-15 cells. As shown in Fig. 4B, sulindac potently induced apoptosis in approximately 20% of HCT-15 cells after 24 h of incubation. Following 48 h of treatment, aspirin and salicylate also stimulated apoptosis of HCT-15 cells (Fig. 4C). However, neither indomethacin nor ibuprofen induced significant amounts of apoptosis in the HCT-15 cells after either 48 (Fig. 4C) or 72 h (data not shown) of incubation with these agents. These results would be consistent with potential inhibition of the NF-κB pathway leading to increased apoptosis in HCT-15 cells that is independent of changes in prostaglandin synthesis.

Sulindac Specifically Inhibits IKKβ Kinase Activity—To determine whether sulindac inhibited both IKKα and IKKβ kinase activity, we transfected epitope-tagged cDNAs encoding either wild-type or constitutively active forms of these kinases into COS cells. The transfected COS cells were treated with either aspirin, salicylate, sulindac, indomethacin, or ibuprofen (Fig. 5). Following immunoprecipitation of each of these kinases with epitope-specific monoclonal antibodies, they were assayed for their ability to phosphorylate the GST-IκBα substrate. None of these agents altered the kinase activity of either the wild-type or constitutively active IKKα (Fig. 5A, top panel). In contrast, treatment of cells with aspirin, salicylate, or sulindac, but not indomethacin or ibuprofen, resulted in 4–7-fold inhibition of both the wild-type (Fig. 5B, lanes 1–6) and the constitutively active IKKβ (Fig. 5B, lanes 7–12). These results indicate that sulindac, like aspirin and salicylate, specifically inhibited IKKβ kinase activity. There was no effect of these agents on the level of either the transfected IKKα or IKKβ proteins (Fig. 5, A and B, lower panels).

In addition, we assayed the effects of sulindac and its metabolites sulindac sulfide and sulindac sulfone on preventing NIK-mediated increases of both endogenous IKK activity and IKKβ activity. NIK strongly induced endogenous IKK activity (Fig. 5C, lanes 1 and 2), and this was somewhat inhibited by treatment of cells with aspirin and sulindac (Fig. 5C, lanes 3

**Fig. 3. IC₅₀ for in vivo sulindac treatment.** Sulindac concentrations ranging from 10 μM to 12 mM were added to COS cells for 2 h prior to treatment of the cells with TNFα (20 ng/ml) for 10 min. Immunoprecipitation with IKKα antibody was then performed followed by assays of IKK kinase activity using a GST-IκBα substrate. Kinase activity was quantitated by PhosphorImager scans and plotted against the concentration of sulindac in three separate experiments.

**Fig. 4. Sulindac inhibits IKK activity in colon carcinoma cell lines.** A. either HCT-15 (lanes 1–5) or HT-29 (lanes 6–10) colon cancer cell lines were untreated (Untr, lanes 1 and 6) or treated with either PBS (lanes 2 and 7), aspirin (ASA, 5 mM) (lanes 3 and 8), sulindac (Sulin, 1 mM) (lanes 4 and 9), or indomethacin (Indo, 25 μM) (lanes 5 and 10) for 2 h prior to TNFα treatment for 10 min. IKK kinase activity was then assayed following immunoprecipitation with an IKKα antibody (top panel), and this antibody was also used in Western blot analysis (lower panel) (B and C). HCT-15 cells were untreated or treated with aspirin (5 mM), salicylate (5 mM), sulindac (1 mM), ibuprofen (25 μM), or indomethacin (25 μM) for either (B) 24 h or (C) 48 h. The percentage of treated cells undergoing apoptosis as compared with control cells was determined using a cell death detection enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals) for three independent experiments.
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Fig. 5. Sulindac specifically inhibits IKKβ kinase activity. Epitope-tagged expression vectors for IKKα (wild type, WT) (lanes 1–6) or a constitutively active IKKα (SS/EE) (lanes 7–12) (A) or IKKβ (WT) (lanes 1–6) or a constitutively active IKKβ (SS/EE) (lanes 7–12) (B) were transfected into COS cells, and the cells were treated with PBS (lane 1) or the different anti-inflammatory agents (lanes 2–12) as indicated. A and B, the concentration of aspirin (ASA) and salicylate (Sal) was 5 mM; sulindac (Sulindac) was 1 mM, and indomethacin (Indo) and ibuprofen (Ibprof) were 25 μM. C, COS cells were transfected with vector alone (lane 1) or a NIK expression vector (lanes 2–7), and endogenous IKK activity was assayed following immunoprecipitation with IKKα antibody that immunoprecipitates the IKKα/IKKβ heterodimer. The transfections with NIK were treated with either PBS (lane 2), aspirin (1 mM) (lane 2), sulindac (1 mM) (lane 3), sulindac sulfone (1 mM) (lane 5), sulindac sulfide (200 μM) (lane 6), or indomethacin (25 μM) (lane 7). A Western blot of the immunoprecipitated IKKα protein is shown in the bottom panel. D, the epitope-tagged IKKβ cDNA was transfected into COS cells alone (lanes 1) or in the presence of NIK (lanes 2–9). Cells were treated with PBS (lane 2) or with sulindac at a concentration of 200 μM (lane 3) or 1 mM (lane 4), sulindac sulfide at 40 μM (lane 5) or 200 μM (lane 6), sulindac sulfide at 1 mM (lane 7) or 200 μM (lane 8), or indomethacin at 25 μM (lane 9). Kinases were immunoprecipitated with epitope-specific monoclonal antibodies and assayed with a GST-IκBα (aa 1–54) as substrate (top panel). Western blot analysis is shown in the lower panels using the monoclonal antibody, 12CA5, for IKKα and the M2 FLAG monoclonal antibody for IKKβ to assay the expression of these kinases.

and 4) but not indomethacin (Fig. 5C, lane 7). Both sulindac sulfone (Fig. 5C, lane 5) and sulindac sulfide (Fig. 5C, lane 6) potently inhibited IKK kinase activity. The effects of these agents on IKKβ kinase activity were then assayed following immunoprecipitation of an epitope-tagged IKKβ protein. NIK strongly induced IKKβ kinase activity (Fig. 5D, lanes 1 and 2), and its effects were inhibited by 1 mM but not 200 μM of sulindac (Fig. 5D, lanes 3 and 4). Sulindac sulfide inhibited IKKβ activity at 200 but not 40 μM (Fig. 5D, lanes 5 and 6). Sulindac sulfone inhibition of IKKβ activity, like that of sulindac, was seen at 1 mM but not 200 μM (Fig. 5D, lanes 7 and 8), whereas indomethacin treatment did not inhibit IKKβ activity (Fig. 5D, lane 9). There was no effect of sulindac or its metabolites on IKKβ protein levels (Fig. 5D, lower panel). These results indicate that sulindac and its metabolites inhibit IKKβ kinase activity.

We next assayed the effects of sulindac, aspirin, salicylate, and indomethacin on the kinase activity of cDNAs encoding the MAP kinases p38, ERK2, or SAPK (54–56). Thus we could address the ability of sulindac to inhibit a variety of other cellular kinases. Each of these epitope-tagged kinases was transfected into COS cells, and the cells were treated with either anisomycin or 12-O-tetradecanoylphorbol-13-acetate to stimulate the activity of these kinases. Anisomycin treatment increased the kinase activity of both p38 (Fig. 6, lanes 1–6) and SAPK (Fig. 6, lanes 13–18), whereas 12-O-tetradecanoylphorbol-13-acetate treatment increased the activity of ERK2 (Fig. 6, lanes 7–12). Neither aspirin, salicylate, sulindac, or indomethacin treatment of COS cells altered the activity of these kinases (Fig. 6, top panel) or the levels of these epitope-tagged proteins (Fig. 6, lower panel). These results indicate that the ability of sulindac and aspirin to inhibit IKKβ was specific in that the activity of a variety of other kinases was not altered by these agents.

Sulindac Inhibits Baculovirus-produced IKKβ—To determine whether sulindac or other anti-inflammatory agents directly inhibited IKKβ kinase activity, recombinant IKKα and IKKβ proteins produced in baculovirus were assayed in kinase reactions. Neither aspirin, salicylate, sulindac, indomethacin, nor ibuprofen inhibited IKKα kinase activity (Fig. 7A, lanes 1–6). However, aspirin, salicylate, and sulindac, but not indomethacin or ibuprofen, inhibited IKKβ kinase activity from 3- to 5-fold (Fig. 7B, lanes 1–6). Western blot analysis indicated that equivalent amounts of these kinases were present in each of these reactions (Fig. 7A and B, lower panels). These results indicate that IKKβ is a direct target for inhibition of the NF-κB pathway by sulindac, aspirin, and salicylate.

DISCUSSION

Previously, we demonstrated that the anti-inflammatory agents salicylate and aspirin inhibit the NF-κB pathway by direct binding of aspirin and salicylate to IKKβ resulting in their competition for its binding to ATP (26). In contrast, the anti-inflammatory agent indomethacin does not alter NF-κB-
FIG. 6. Sulindac does not inhibit MAP kinase activity. Epitope-tagged expression vectors for the MAP kinases p38 (lanes 1–6), ERK2 (lanes 7–12), or SAPK (lanes 13–18) were transfected into COS cells, and the cells were treated with anisomycin (Aniso) for the p38 and SAPK-transfected cells or phorbol ester for the ERK2-transfected cells for 30 min prior to harvesting. Either aspirin (ASA, 5 mM), salicylate (Sal, 5 mM), sulindac (Sulin, 1 mM), or indomethacin (Indo, 25 μM) were added as indicated for 2 h prior to harvesting the cells. Immunoprecipitated kinases were assayed with GST-ATF2 (aa 1–254) (lanes 1–6), myelin basic protein (lanes 7–12), or GST-c-Jun (aa 1–169) (lanes 13–18) as substrates (top panel). Western blot analysis of each of these immunoprecipitated kinases with epitope-specific monoclonal antibodies is shown in the lower panels. TPA, 12-O-tetradecanoylphorbol-13-acetate.

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FIG. 7. Sulindac specifically inhibits IKKβ kinase activity in vitro. Histidine and influenza epitope-tagged baculovirus-produced IKKα (lanes 1–6) (A) or IKKβ (lanes 1–6) (B) proteins were purified, and approximately 1 μg of each of these kinases was incubated in vitro with either no added agents (lane 1), aspirin (ASA, 1 mM) (lane 2), salicylate (Sal, 1 mM) (lane 3), sulindac (Sulin, 1 mM) (lane 4), or either indomethacin (Indo) or ibuprofen (Ibpr) (25 μM) (lanes 5 and 6) as indicated for 30 min and then assayed for kinase activity with a GST-κB substrate. The lower panel shows Western blot analysis of the IKKα and IKKβ kinases.

directed gene expression. In the current study, we addressed whether the anti-inflammatory agent sulindac, which is structurally related to indomethacin, and its metabolites sulindac sulfide and sulfone can inhibit NF-κB-mediated gene expression. Surprisingly, we find that sulindac and particularly its sulfide metabolite are potent inhibitors of the NF-κB pathway. The mechanism of action of these agents is due to inhibition of IKKβ but not IKKα kinase activity. These results suggest that several non-steroidal anti-inflammatory agents are potent inhibitors of the NF-κB pathway and function by inhibiting IKKβ kinase activity.

Aspirin and sulindac, in addition to being anti-inflammatory agents, are inhibitors of cellular proliferation (37, 44, 45, 47, 48). For example, both sulindac and aspirin have been demonstrated to reduce the incidence of developing colon cancer in patients with adenomatous polyps (27, 29–31, 39, 40, 42, 43). Although both the anti-inflammatory and growth inhibitory properties of aspirin and sulindac may be due to their inhibition of cyclooxygenases and subsequent reduction in prostaglandin synthesis (45, 47, 57–59), other regulatory pathways may also be targeted by these agents. Since the NF-κB pathway is involved in both the pathogenesis of the inflammatory response and in cellular growth control (reviewed in Refs. 1 and 2), this pathway is also a potential target for inhibition by aspirin and sulindac. Our data suggest that these agents inhibit IKKβ activity to prevent κBα degradation and thus prevent NF-κB-mediated increases in gene expression. Furthermore, the concentrations of these agents needed to inhibit the NF-κB pathway do not result in cellular toxicity. The serum concentration of aspirin in patients with arthritis treated with chronic aspirin therapy is similar to that used in this study (60). Sulindac and its sulfide metabolite have relatively low concentrations in the serum but substantially higher levels in the colonic epithelium where the sulfide metabolite is concentrated at least 20-fold (32, 33). The concentrations of sulindac and sulindac sulfide used in this study likely reflect the levels seen in the colonic epithelium.

At the present time, we cannot determine whether the effects of sulindac and aspirin on inducing apoptosis in HCT-15 cells are predominantly due to effects on inhibiting the NF-κB pathway or potentially other regulatory pathways. The fact that HCT-15 cells are defective in the generation of prostaglandins (37), yet undergo apoptosis in response to treatment with either sulindac or aspirin, suggests that alternative pathways other than the one mediated by cyclooxygenases likely exist. Both sulindac and sulindac sulfide can reduce the proliferation rate, change the morphology of cells, and cause G0/G1 cell cycle arrest and subsequent apoptosis of colon cancer cell lines (45, 47). Sulindac has also recently been demonstrated to cause microsatellite stability in cells isolated with hereditary nonpolyposis colon cancer syndrome (48). The concentrations of sulindac needed to inhibit the growth of colon cancer cell lines are similar to those that inhibit the NF-κB pathway. Although sulindac-mediated effects on a variety of other cellular regulatory factors have been demonstrated (45, 47), our results are consistent with a role for sulindac inhibition of the NF-κB pathway as a potential mechanism that may be involved in inducing apoptosis. The relationships between the different pathways that are inhibited by sulindac treatment will need to be further investigated to understand better the mechanisms of its growth inhibitory properties.

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