Inhibition of Interleukin-1-stimulated NF-κB RelA/p65 Phosphorylation by Mesalamine Is Accompanied by Decreased Transcriptional Activity*

Laurence J. Egan‡§¶, Dennis C. Mays§, Catherine J. Huntoon‡, Michael P. Bell, M. Gennett Pike§, William J. Sandborn‡, James J. Lipsky§, and David J. McKeen

From the ‡Division of Gastroenterology and Hepatology, the ¶Clinical Pharmacology Unit, and the §Department of Immunology, Mayo Clinic, Rochester, Minnesota 55905

The NF-κB family of proteins are important transcriptional regulators of genes involved in immunity and inflammation. The activity of NF-κB is highly regulated: transcriptionally active NF-κB proteins are sequestered in the cytoplasm by inhibitory proteins, IκB. A variety of extracellular signals, including interleukin-1 (IL-1), activate NF-κB by inducing phosphorylation and degradation of IκB, allowing nuclear translocation and DNA binding of NF-κB. Many of the stimuli that activate NF-κB by inducing IκB degradation also cause phosphorylation of the NF-κB RelA (p65) polypeptide. The transactivating capacity of RelA is positively regulated by phosphorylation, suggesting that in addition to cytosolic sequestration by IκB, phosphorylation represents another mechanism for control of NF-κB activity. In this report, we demonstrate that mesalamine, an anti-inflammatory aminosalicylate, dose-dependently inhibits IL-1-stimulated NF-κB-dependent transcription without preventing IκB degradation or nuclear translocation and DNA binding of the transcriptionally active NF-κB proteins, RelA, c-Rel, or RelB. Mesalamine was found to inhibit IL-1-stimulated RelA phosphorylation. These data suggest that pharmacologic modulation of the phosphorylation status of RelA regulates the transcriptional activity of NF-κB, independent of nuclear translocation and DNA binding. These findings highlight the importance of inducible phosphorylation of RelA in the control of NF-κB activity.

The NF-κB family of proteins is an inducible transcription factor that regulates genes important in immunity and inflammation. The activity of NF-κB is highly regulated: transcriptionally active NF-κB proteins are sequestered in the cytoplasm by inhibitory proteins, IκB. A variety of extracellular signals, including interleukin-1 (IL-1), activate NF-κB by inducing phosphorylation and degradation of IκB, allowing nuclear translocation and DNA binding of NF-κB. Many of the stimuli that activate NF-κB by inducing IκB degradation also cause phosphorylation of the NF-κB RelA (p65) polypeptide. The transactivating capacity of RelA is positively regulated by phosphorylation, suggesting that in addition to cytosolic sequestration by IκB, phosphorylation represents another mechanism for control of NF-κB activity. In this report, we demonstrate that mesalamine, an anti-inflammatory aminosalicylate, dose-dependently inhibits IL-1-stimulated NF-κB-dependent transcription without preventing IκB degradation or nuclear translocation and DNA binding of the transcriptionally active NF-κB proteins, RelA, c-Rel, or RelB. Mesalamine was found to inhibit IL-1-stimulated RelA phosphorylation. These data suggest that pharmacologic modulation of the phosphorylation status of RelA regulates the transcriptional activity of NF-κB, independent of nuclear translocation and DNA binding. These findings highlight the importance of inducible phosphorylation of RelA in the control of NF-κB activity.

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¶ To whom correspondence should be addressed. Tel.: 507-284-2511; Fax: 507-284-0538; E-mail: egan.laurence@mayo.edu.

The abbreviations used are: NF-κB, nuclear factor κB; IL, interleukin; TNF-α, tumor necrosis factor α; IκB, inhibitory κB; TRAF-6, TNF receptor associated factor 6; IKK, IκB kinase; NIK, NF-κB inducing kinase; PAGE, polyacrylamide gel electrophoresis.

Saliicylate drugs are in widespread use as inhibitors of inflammation in a variety of disease states. Sodium salicylate and acetyl salicylic acid inhibit NF-κB by preventing inducible degradation of IκB, and this action may underlie the in vivo anti-inflammatory effects of these agents (14). Aminosalicylates such as sulfasalazine (an azo-conjugated aminosalicylate) and mesalamine (5-aminosalicylic acid, a free aminosalicylate) inhibit gut inflammation in inflammatory bowel disease (15) (Fig. 1). Like conventional salicylates, sulfasalazine has been reported to inhibit NF-κB activity by preventing inducible IκBα degradation (16). In this report we show that mesalamine is an inhibitor of inducible NF-κB-dependent transcription in intestinal epithelial cells and T lymphocytes. However, unlike conventional salicylates and sulfasalazine, mesalamine does not prevent IL-1-induced IκBα or β degradation. The transcriptionally active NF-κB proteins RelA, RelB, and c-Rel translocate to the nucleus and bind to κB sites on DNA but are unable to initiate transcription in the presence of mesalamine. Further experiments demonstrated that mesalamine inhibits IL-1-induced phosphorylation of RelA. This suggests that mesalamine regulates NF-κB activity by modulating the phosphorylation of one of its transcriptionally active proteins and that the path-

26448 This paper is available on line at http://www.jbc.org
Inhibition of NF-κB RelA Phosphorylation by Mesalamine

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise stated, materials were purchased from Sigma. Affinity purified rabbit antibody to IκBα has been previously described (17), and affinity purified antibodies to RelA (sc-372), c-Rel (sc-70), and RelB (sc-226) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human IL-1β (R&D 201-LB) was obtained from R & D Systems (Minneapolis, MN).

Cell Culture—A Caco-2 cell line from American Type Culture Collection (HTB 37) was maintained in Dulbecco’s modified Eagle’s medium containing 50 nmol folic acid, supplemented with 10% fetal bovine serum and 1% nonessential amino acids (Life Technologies, Inc.). Jurkat T cells expressing the type I II-1 receptor (Ju.1 cells) (17) were maintained in RPMI 1640, supplemented with 10% calf serum, 2 mM t-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES, pH 7.3.

Transient Transfections and NF-κB Reporter Gene Assay—An NF-κB reporter construct, consisting of the firefly luciferase gene under control of three copies of the consensus NF-κB site from the IgG promoter was used to quantify NF-κB transcriptional activity (18). A Renilla luciferase reporter under control of the herpes simplex virus thymidine kinase promoter, pRLTK (Promega, Madison, WI), was used to normalize the NF-κB reporter gene activity, to prevent nonspecific drug effects such as cytotoxicity confounding the results. Caco-2 cells were first brought into suspension by trypsinization. Caco-2 or Ju.1 cells (10^6) were mixed with 10 μg NF-κB reporter gene, 20 ng of pRLTK, and 20 μg of filler DNA and pulsed once with 325 V for 10 ms using a square wave electroporator (BTX, San Diego, CA). For experiments, 10^6 transfected Caco-2 cells were distributed into 30 wells of a 48-well tissue culture plate, and 10^6 transfected Jurkat cells were distributed into 6 wells of a 6-well tissue culture plate. Experiments were performed 12–48 h after transfection. For luciferase assays, cell lysates were prepared, and luciferase activities were read according to the manufacturer’s instructions (Dual luciferase, Promega), using a model LB 9501/16 Lumat luminometer (Berthold Systems, Aliquippa, PA). Results shown are representative of at least three independent experiments.

Analysis of Protein Synthesis—Caco-2 cells (2.5 × 10^5) were plated into 6-well tissue culture plates and allowed to adhere overnight. Cells were depleted of leucine by replacing the medium with leucine free RPMI supplemented with 2% dialyzed fetal calf serum for 4 h, which was changed once. After addition of mesalamine or control, 25 μCi of [3H]leucine (t-4,5,6-[3H]leucine, 138 Ci/mmol, Amersham Pharmacia Biotech) was added to each well. After various durations, [3H]leucine incorporation into precipitable protein was quantified. Medium was aspirated, and the monolayers were washed twice with ice-cold calcium and magnesium-free phosphate-buffered saline containing 1 mM EDTA. Cells were disrupted by incubating the monolayer for 4 min in 1 ml of ice-cold buffer consisting of 10 mM HEPES, pH 7.9, 1.5 mM MgCl_2, and 10 mM NaCl, supplemented immediately before use with 0.4 mM phenylmethylsulfonyl fluoride, 2 mM diithiothreitol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin, 150 μM o-sulfer, 500 μM spectamine, and 0.4% Nonidet P-40. The disrupted monolayer was mixed in a pipette, transferred to microtubes, and centrifuged at 1500 rpm for 5 min at 4 °C, and the pellet was washed with buffer lacking Nonidet P-40 to remove contaminating cytosolic proteins. Nuclear extracts were prepared by vortexing the cell pellets for 30 min at 4 °C in 50 μl of ice-cold buffer consisting of 20 mM HEPES, pH 7.9, 20% v/v glycerol, 0.4 mM EDTA, and 0.2 mM MgCl_2. isolated nuclei were incubated with 200 mM phenylmethylsulfonyl fluoride, 2 mM diithiothreitol, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 150 μM o-sulfer, 500 μM spectamine, and 0.4% Nonidet P-40. After centrifugation at 10,000 rpm for 10 min at 4 °C, the cell pellets were washed with buffer lacking Nonidet P-40 to remove contaminating cytosolic lysates.

Electrophoretic Mobility Shift Assay—Nuclear lysates were prepared as for immunoblots, and the protein concentration was determined using the Coomassie Plus kit (Pierce). Mobility shift reactions were carried out using 6 μg of protein from each nuclear extract incubated with 32P-labeled IL-2 NF-κB oligonucleotide probe as described previously (18). The DNA sequence of the probe used in this study is 5’-CCCGAACAGAGGGATTTCACCTAAATCCATT-3’ (coding strand only).

Phosphoprotein Analysis—Ju.1 and Caco-2 cells were labeled with 32P (ICN Radiochemicals, Irvine, CA), as described previously (17). Labeled cells were divided into aliquots (10^5/sample) and, after various experimental conditions, were washed with ice-cold phosphate-buffered saline containing 400 μM Na3VO4, 5 mM EDTA, and 10 mM NaF, pH 7.4. After centrifugation, the cells were lysed and immunoprecipitated for c-Rel, RelB, and RelA as described above. The precipitated phosphoproteins were separated by 10% SDS-PAGE and analyzed by autoradiography at 70 °C.

RESULTS

Mesalamine Inhibits the Transcriptional Activity of NF-κB—A variety of salicylates and aminosalicylates have been shown to inhibit NF-κB-dependent transcription (14, 15). To determine whether mesalamine is an inhibitor of NF-κB activity in intestinal epithelial cells, Caco-2 cells were stimulated with IL-1 or phorbol myristate acetate in the presence and absence of varying concentrations of mesalamine. NF-κB transcriptional activity was measured with a transiently transfected NF-κB luciferase reporter gene, and results were normalized to a cotransfected reporter under control of a minimal promoter. Mesalamine inhibited IL-1 and phorbol myristate acetate stim-
Inhibition of NF-κB RelA Phosphorylation by Mesalamine

**Figure 2.** Sulfasalazine and mesalamine dose-dependently inhibit NF-κB transcriptional activity. *a,* Caco-2 cells were co-transfected with an NF-κB firefly luciferase reporter gene and a Renilla luciferase reporter gene under control of a minimal promoter. After a 30-min preincubation with vehicle (phosphate-buffered saline) or sulfasalazine or mesalamine at varying concentrations, cells were stimulated with 0.025 ng/ml IL-1. 4 h later, cells were lysed, and firefly and Renilla luciferase activities were sequentially read. NF-κB firefly luciferase activity was normalized to the control Renilla luciferase activity. Results are expressed as a percentage of NF-κB activity in vehicle-treated cells. Under these conditions, IL-1 stimulated NF-κB reporter gene activity by 15–20-fold in vehicle-treated cells. *b,* parallel experiments were carried out in phorbol myristate acetate (20 ng/ml)-stimulated Ju.1 cells. The means and standard deviations of triplicate samples are shown.

**Figure 3.** Mesalamine does not inhibit protein synthesis in Caco-2 cells. Leucine-depleted Caco-2 cells were treated with 40 mM mesalamine (open symbols) or vehicle (phosphate-buffered saline, closed symbols) before addition of 25 μCi of [3H]leucine to each well. 1, 2, and 4 h after addition of [3H]leucine, cellular proteins were precipitated by the addition of trichloroacetic acid, and the precipitates were washed. Precipitated proteins were resolubilized, and the amount of incorporated tracer was determined by liquid scintillation counting. The means and standard errors of triplicate samples are shown.

Cells were stimulated with IL-1 in the presence and absence mesalamine or sulfasalazine. After stimulation, the kinetics of IκBα degradation were determined by analysis of quantitative immunoblots of cytosolic lysates. Consistent with previous reports (20), stimulation of control cells with IL-1 or phorbol myristate acetate caused rapid depletion of cytosolic IκBα pools, followed by its gradual reappearance after 1 h (Fig. 4). Mesalamine treatment of Caco-2 cells did not prevent IL-1-induced IκBα degradation. However, reappearance of IκBα in the cytosol was delayed by over 2 h in mesalamine-treated cells but had reached base-line levels within 4 h. In contrast to mesalamine, sulfasalazine completely inhibited IL-1-stimulated degradation of IκBα. Mesalamine also did not prevent IL-1-stimulated degradation of IκBβ (data not shown).

**Mesalamine Does Not Inhibit Nuclear Translocation of Transcriptionally Active NF-κB Proteins**—Because mesalamine inhibited NF-κB transactivation but did not prevent degradation of its cytosolic inhibitor, we hypothesized that this drug might be inhibiting nuclear translocation of transcriptionally active NF-κB proteins. To test this hypothesis, we assessed the effect of mesalamine on the kinetics of IL-1-inducible RelA, c-Rel, and RelB nuclear translocation in Caco-2 cells by quantitative immunoblotting from nuclear extracts. Consistent with previous reports, IL-1 caused rapid but transient nuclear translocation of RelA, c-Rel, and RelB, followed later by a decrease in abundance of these proteins in the nuclear extracts (Fig. 5). Mesalamine did not prevent IL-1-induced nuclear localization of RelA, c-Rel, or RelB in Caco-2 cells. Interestingly, nuclear extracts of Caco-2 cells treated with only mesalamine contained RelB, whereas untreated cells displayed no detectable amounts of nuclear RelB.

**Mesalamine Does Not Prevent NF-κB DNA Binding**—Mesalamine inhibits NF-κB transcriptional activity without preventing degradation of IκBα or nuclear translocation of the transcriptionally active NF-κB proteins. To determine whether mesalamine interferes with the ability of NF-κB to bind to DNA κB sites, electrophoretic mobility shift analysis was performed. Caco-2 cells were treated with mesalamine or vehicle and then stimulated with IL-1 for varying durations. Analysis of DNA binding activity in Caco-2 nuclear extracts revealed that IL-1 stimulated NF-κB DNA binding in the presence and absence of mesalamine (Fig. 6). Identical results were obtained using the IL-2 and HIV NF-κB oligonucleotide probes (data not shown). Because mesalamine is absent from the nuclear extracts at the time of incubation with NF-κB oligonucleotide probes, a direct
Inhibition of NF-κB RelA Phosphorylation by Mesalamine

In this study, we explored the effects of mesalamine on the inducible phosphorylation of RelA, c-Rel, and RelB in Caco-2 cells. Mesalamine, an anti-inflammatory drug, inhibited the phosphorylation of RelA but not of c-Rel or RelB. This inhibition was observed in both control and IL-1-stimulated cells. The mechanism by which mesalamine inhibits RelA phosphorylation was investigated, and it was found to be independent of NF-κB degradation. The inhibitory effect of mesalamine on NF-κB DNA binding in vivo cannot be excluded.

**FIG. 4.** Sulfasalazine does not inhibit IL-1 stimulated IkB degradation. Caco-2 cells were preincubated with vehicle (phosphate-buffered saline) or 40 mM sulfasalazine before stimulation with 0.25 ng/ml IL-1. At the time points indicated, nuclear extracts were prepared and analyzed by immunoblotting using a specific antibody to NF-κB.

**FIG. 5.** Mesalamine does not prevent nuclear translocation of RelA, RelB, or c-Rel. Caco-2 cells were preincubated with vehicle (phosphate-buffered saline) or 40 mM mesalamine before stimulation with 0.25 ng/ml IL-1. At the time points indicated, the cells were lysed, and immunoblots were prepared using an antibody to NF-κB and visualized with enhanced chemiluminescence reagents.

**FIG. 6.** Mesalamine does not prevent NF-κB DNA binding. Caco-2 cells were preincubated with vehicle (phosphate-buffered saline) or 40 mM mesalamine before stimulation with 0.25 ng/ml IL-1. At the time points indicated, nuclear extracts were prepared and subjected to electrophoretic mobility shift analysis for NF-κB DNA binding using a 32P-labeled NF-κB probe. The cold probe lane contained the 1 h post stimulation nuclear extract incubated with 32P-labeled NF-κB probe plus 100-fold excess unlabeled probe. The arrow indicates shifted bands.

**FIG. 7.** Mesalamine prevents IL-1 inducible phosphorylation of RelA. Caco-2 cells were labeled with 32P and preincubated with vehicle (phosphate-buffered saline) or 40 mM mesalamine before stimulation with 0.25 ng/ml IL-1. At various time points after stimulation, RelA was immunoprecipitated from cell lysates, separated by SDS-PAGE, and detected by autoradiography.

Discussion

The nuclear localization and transcription regulation activity of NF-κB is stimulated by pro-inflammatory cytokines, IL-1. After exposure to IL-1, cells rapidly degrade cytosolic IkB, allowing NF-κB to translocate to the nucleus and initiate specific gene transcription. Inhibition of the signaling pathways that lead to IkB degradation prevents NF-κB-dependent gene transcription, for example by the oxygen radical scavenger pyrrolidine dithiocarbamate and by anti-inflammatory drugs, such as salicylates (7). In this report, we describe a novel pharmacologic mechanism for control of NF-κB activity. Pre-treatment of cells with mesalamine, an anti-inflammatory aminosalicylate, prevented IL-1-stimulated NF-κB-dependent transcription without affecting IkB degradation, NF-κB nuclear translocation, or DNA binding. IL-1 stimulation of colonic epithelial cells and T lymphocytes inducibly phosphorylated RelA. Mesalamine-mediated inhibition of NF-κB transcriptional activity was accompanied by inhibition of IL-1-stimulated RelA phosphorylation. This suggests that the inducible phosphorylation of RelA positively regulates gene transcription by NF-κB and that RelA phosphorylation constitutes an independent mechanism for the control of NF-κB activity.

The activity of transcription factors is highly regulated to control the timely and co-ordinated expression of different genes. Increased transcriptional activity of NF-κB has been identified in a number of different disease states, including chronic inflammatory disorders (2), cancers (21), and the antiviral state (1). Because of the need for fine control of transcription factor activity, multiple levels of regulation have evolved. Regulation can result from subcellular localization or post-translational modifications by phosphorylation on tyrosine, threonine, or serine residues. The control of the Rel/NF-κB family of transcription factors has been extensively studied, notably with regard to the cytoplasmic sequestration of NF-κB proteins by their natural inhibitors, IkB. Like many other transcription factors, including AP-1 and CREB, NF-κB activity can also be regulated by the phosphorylation state of its transcriptionally active components, especially RelA. Using a variety of experimental conditions and stimuli, it has been demonstrated that the DNA binding of NF-κB (9, 10) and NF-κB-dependent transcription (11–13) are positively regulated by RelA phosphorylation.

Identification of the sites of inducible RelA phosphorylation and the responsible signaling pathways and kinases are currently active fields of investigation. Zhong et al. (12) showed that lipopolysaccharide treatment of cells stimulated protein kinase A-dependent phosphorylation of RelA on serine 276. In a cell-free system, it appeared that protein kinase A could...
directly phosphorylate RelA. Phosphorylation of this serine residue did not affect DNA binding but greatly increased RelA transcriptional activity by promoting interaction with the co-activator, cAMP response element binding protein/p300 (22). Wang and Baldwin demonstrated that TNF-α stimulates phosphorylation of RelA on serine 529 (13). Inducible NF-κB transcription but not DNA binding was dependent upon this phosphorylation. It has recently been shown that IKK α and β, in addition to phosphorylating IκB proteins, can directly phosphorylate RelA (23). Thus, different NF-κB-inducing stimuli appear to cause phosphorylation of RelA at unique sites, at least two distinct kinases can directly phosphorylate RelA, and phosphorylation of RelA appears to promote transactivation. Our experimental results are consistent with the hypothesis that phosphorylation of RelA can positively regulate NF-κB-dependent transcription, because inhibition of this process with mesalamine prevented transactivation without affecting DNA binding.

What is the likely mechanism for the mesalamine-mediated inhibition of IL-1-stimulated RelA phosphorylation? The pathways that transduce the signal from the activated IL-1 receptor that culminate in phosphorylation and degradation of IκBα are incompletely characterized. After ligand binding, the activated IL-1 receptor associates with the transmembrane protein, IL-1 receptor-accessory protein. The IL-1 receptor-accessory protein complex recruits IL-1 receptor-activated kinase (24), a serine threonine kinase, and TRAF-6 (25). TRAF-6 transduces the activation signal to a complex consisting of a mitogen-activated protein kinase kinase kinase called NIK. TRAF-6-mediated activation of NIK initiates the phosphorylation and activation of IKKα and β. Once activated, the IKK α/β heterodimer directly phosphorylates IκBα and IκBβ on N-terminal serine residues. The kinases responsible for IL-1-stimulated phosphorylation of RelA, and the target for inhibition of this process by mesalamine are not known. However, because mesalamine inhibits IL-1-stimulated RelA phosphorylation without preventing IκB degradation, IL-1 receptor-activated kinase, TRAF-6, NIK, and IKK should not be inhibited by mesalamine. Rather, a kinase with specificity for RelA and not IκBα/β is implicated. It has been shown that aspirin and sodium salicylate inhibit IκBβ but not IκBα by competing with ATP for enzyme binding (26). Similarly, recent studies from IKKα−/− and IKKβ−/− mice show that TNF-α and IL-1 activate NF-κB through IκBβ and not IKKα (27–29). Interestingly, although IκBβ−/− mice did not respond to TNF-α by degrading IκBα proteins and activating NF-κB, the IL-1-initiated NF-κB response was only partially inhibited (29). Thus, IL-1 may activate an IκKα/β independent kinase that phosphorylates IκBα polypeptides. Such redundant IL-1-inducible kinases could exhibit overlapping but preferential interactions with IκB and RelA substrates. Mesalamine may selectively inhibit a kinase that preferentially interacts with RelA without inhibiting another kinase that preferentially interacts with IκB polypeptides. Mesalamine should be a valuable reagent to further characterize IL-1-inducible signaling events that regulate NF-κB transcriptional activity.

Uncontrolled overactivity of NF-κB may be prevented by positive transcriptional regulation of the IκBα gene by NF-κB proteins. Newly synthesized IκBα has been reported to downregulate NFκB activity by directly interacting with transcriptionally active NF-κB proteins in the nucleus and cytosol (30). Although mesalamine completely inhibited NF-κB reporter gene activity, the late (4 hr post stimulation) cytosolic reappearance of IκBα was not blocked (Fig. 4). This suggests that not all NF-κB-regulated gene transcription is blocked by mesalamine. RelB is not inductively phosphorylated, so mesalamine may be unable to prevent activation of genes responsive to this transcription factor. In contrast to its effects on RelA and c-Rel, mesalamine caused nuclear translocation of RelB in the absence of stimulation (Fig. 5), further supporting a differential sensitivity to mesalamine among the NF-κB family members. Alternatively, it is also possible that the late reappearance of IκBα in mesalamine-treated cells is due to activity of a transcription factor other than NF-κB. The IκBα promoter is known to contain Sp-1 binding sequences (31), and this ubiquitous transcription factor may be responsible for late appearing IκBα in mesalamine-treated cells.

These data demonstrate that aminosalicylates can inhibit NF-κB activity by two different mechanisms. Sulfasalazine, the aza-conjugated aminosalicylate studied here, is considered a pro-drug, which is activated when bacterial azo-reductase enzymes in the colon split the molecule to release the active component mesalamine and sulfapyridine (32). If inhibition of NF-κB is the true anti-inflammatory mechanism of action of aminosalicylates in vivo, sulfasalazine may in fact have dual anti-NF-κB actions, as a pro-drug of mesalamine and as an active compound itself. It has recently been demonstrated that acetyl salicylic acid inhibits IKKβ but not IKKα (26). Currently it is unknown whether any of the aminosalicylates inhibit IKKα or β or other NF-κB regulatory kinases. A more detailed understanding of the biochemical interactions of the structurally related aminosalicylates with the proteins of the NF-κB system is necessary.

These findings suggest that a novel, pharmacologically maniupulable mechanism for regulation of NF-κB activity exists. Identification of the IL-1-stimulated signaling pathway responsible for the selective phosphorylation of RelA that is inhibited by mesalamine will be an important extension of this work. This pathway is an attractive potential target for therapeutic inhibition in inflammatory diseases.

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