Salicylates Inhibit Lipopolysaccharide-Induced Transcriptional Activation of the Tissue Factor Gene in Human Monocytic Cells

By Paul Oeth and Nigel Mackman

Binding of plasma Factor VII/Vila to the tissue factor (TF) receptor initiates the coagulation protease cascades. TF expression by circulating monocytes is associated with thrombotic and inflammatory complications in a variety of diseases. Transcriptional activation of the human TF gene in mononuclear cells exposed to bacterial lipopolysaccharide (LPS) is mediated by binding of c-Rel/p65 heterodimers to a kB site in the TF promoter. Here, we report that a family of anti-inflammatory agents, known as the salicylates, inhibited LPS induction of TF activity and TF gene transcription in human monocytes and monocytic THP-1 cells at clinically relevant doses. Furthermore, sodium salicylate blocked the LPS-induced proteolytic degradation of IxBa, which prevented the nuclear translocation of c-Rel/p65 heterodimers. In contrast, two other nonsteroidal anti-inflammatory drugs, ibuprofen and indomethacin, did not inhibit LPS induction of the TF gene. These results indicated that salicylates inhibited LPS induction of TF gene transcription in monocytic cells by preventing nuclear translocation of c-Rel/p65 heterodimers. The clinical benefits of salicylates in the treatment of several diseases, including atherosclerosis and rheumatoid arthritis, may be related to their ability to reduce monocyte gene expression.

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In this study, we showed that NaSal, salsalate, and aspirin all inhibited LPS induction of TF gene expression in human monocyteic cells in a dose-dependent manner. All three agents blocked LPS-induced nuclear translocation of c-Rel/p65 heterodimers, indicating that they inhibited TF expression by preventing transcriptional activation of the TF gene.

MATERIALS AND METHODS

Materials. LPS (Escherichia coli serotype 011:B4) was purchased from Calbiochem (San Diego, CA). Phorbol 12-myristate 13-acetate (PMA), acetyl salicylic acid, ibuprofen, and indomethacin were obtained from Sigma (St Louis, MO). NaSal and salsalate were purchased from Aldrich Chemical (Milwaukee, WI). A fluorescein isothiocyanate (FITC)-labeled conjugate of MY4 (MY4-FITC), a murine monoclonal antibody to human CD14, was purchased from Coulter Immunology (Hialeah, FL). An FITC-labeled antihuman TF antibody, TF8-5G9, was kindly provided by T.S. Edgington (The Scripps Research Institute, La Jolla, CA).

Cell viability and toxicity. Cell viability was determined using the CellTitre 96 Aqueous Non-Radioactive Cell Proliferation Assay Kit (Promega Corp, Madison, WI). Briefly, 5 x 10^5 cells were treated and transferred to a 96-well plate (Costar, Cambridge, MA) in a volume of 100 μL. A total of 20 μL of the MTS/PlMS solution was added to each sample, and the plate was incubated at 37°C, in 5% CO₂ for 5 hours. The plate was then read at 490 nm on an enzyme-linked immunosorbent assay (ELISA) reader (Molecular Devices, Menlo Park, CA). Cell viability was also assessed using trypan blue (Sigma). 35S-translabeled incorporation was conducted as follows: 2 x 10^6 cells were pelleted at 300g for 15 minutes and then resuspended in RPMI 1640 medium without cysteine or methionine (Bio-Whittaker Inc, Walkersville, MD) and tranzS Metabolic Labeling Reagent (ICN Pharmaceuticals Inc, Costa Mesa, CA) to a final concentration of 1 μCi/mL. After treatment, cells were pelleted at 300g, washed once with phosphate-buffered saline, and reselected in 100 μL of bovine serum albumin (1 mg/mL). Trichloroacetic acid precipitable counts were counted in the presence of 2 mL of liquid scintillant (Ultima Gold) by a Beta counter (Beckman Instruments, Irvine, CA).

Monocyte isolation and cell culture. Heparinized blood was drawn from volunteers, and plasma was removed after centrifugation at 200g. Blood cells were diluted 1:1 (vol:vol) in endotoxin-free RPMI 1640 medium (Bio-Whittaker, Inc) and were overlaid onto Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). After centrifugation at 400g, the peripheral blood mononuclear cells (PBMCs) were removed and washed twice with medium and resuspended in RPMI 1640 media with 2% fetal calf serum (Gemini Bioproducts, Calabasas, CA). Human monocyteic leukemia THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured at a density of 2 x 10^6 cells/mL as described. All reagents were tested by Whittaker Inc (Walkersville, MD) and contained less than 0.05 units of endotoxin contamination.

TF activity. Cell pellets were solubilized at 37°C for 15 minutes using 15 mmol/L octyl β-D-glucopyranoside. TF activity was assayed in cell lysates using a one-stage clotting assay as described. Clotting times were converted to milliunits of TF activity by comparison with a standard curve established with purified human brain TF. For reference, a clotting time of 50 seconds corresponds to 1,000 μU/mL of TF activity.

Fluorescence activated cell sorting (FACS). PBMCs (1 x 10^6 cells/mL) were stimulated for 5 hours with 10 ng/mL LPS before incubation of cells for 30 minutes at 4°C with antihuman TF-FITC or MY4-FITC antibodies. Cells were then fixed in 0.5% paraformaldehyde and were analyzed by flow cytometry using a FACSscan (Becton Dickinson, San Jose, CA). Live gating on the monocyte population was accomplished using near forward angle and orthogonal light scatter as well as staining with MY4-FITC antibody.

Analysis of TF mRNA. Total cellular RNA, purified using TRIzol reagent (GIBCO-BRL, Gaithersburg, MD) was analyzed by Northern blotting as described. A 641-bp human TF cDNA fragment was labeled with [α-32P]deoxyctydine triphosphate (ICN) using the Prime-It Kit (Stratagene, La Jolla, CA) and was used to determine TF mRNA levels. An egr-1 cDNA fragment was kindly provided by E. Adamson (La Jolla Cancer Research Foundation, La Jolla, CA). Blots were rehybridized with the housekeeping genes, glucose-6-phosphate dehydrogenase (a gift from G. Parry, The Scripps Research Institute) or glyceraldehyde 3-phosphate dehydrogenase (G3PDH; Clontech Laboratories, Inc, Palo Alto, CA). Autoradiography was performed at -70°C, using Kodak XAR-5 film with intensifying screens (Eastman-Kodak, Rochester, NY). Band intensities were quantified by densitometric analysis using a Phosphorimage and ImageQuant software (Molecular Dynamics, Sunnyvale, CA) or Personal Densitometer (Molecular Dynamics).

Nuclear run-on. Nuclear run-on assays were performed using a modification of a procedure described previously. Cells were unstimulated or LPS (10 μg/mL)-stimulated (for 1 hour) with or without a 15-minute pretreatment with NaSal (10 μmol/L). Cells (5 x 10^5) were lysed in a homogenizer and nuclei were collected using a sucrose (2.0 mol/L) cushion. Nuclear RNA was labeled using [α-32P] uridine triphosphate (UTP), and samples were treated sequentially with DNase 1 (final concentration, 1 U/L) for 10 minutes at 37°C and with proteinase K (final concentration, 200 μg/mL) for 45
Nasal (mM) cystic cells. pg/mL) for 5 hours with or without a 15-minute pretreatment with aspirin from 0.1 to 10 mmol/L.

Incubation for 5 hours without LPS or Nasal. TF activity (mean ± SD) is shown from three independent experiments.

**Plasmids and transfections.** TF, G3PDH and tumor necrosis factor-α (TNFα) cDNAs present in pGEM3Z (Promega Corp), pSP73 (Promega Corp), and pSP64,1 respectively, were used in the nuclear run-on assays. pTF-(278)LUC contains 278 bp of the human TF promoter cloned upstream of the luciferase reporter gene20 and p(TF),SVLUC contains four copies of the TF κB site cloned upstream of the simian virus 40 promoter encoding the luciferase reporter gene.21 Cells were transfected using diethyl aminoethyl-dextran22 and were cultivated for 46 hours before a 5-hour stimulation with LPS. Luciferase activity was determined using the Luciferase Assay System (Promega Corp) and Monolight 2010 luminometer (Analytical Luminescence Inc, San Diego, CA). A total of 2 μg of pRSVCAT was used as an internal control to assess transfection efficiencies. Chloramphenicol acetyl transferase activity expressed by pRSVCAT was determined using a diffusion-based assay and showed less than a 10% variation between samples (data not shown).

**Electrophoretic mobility shift assay (EMSA).** Nuclear extracts were prepared from 5 × 10⁶ cells as described.23 Protein concentrations in these extracts were 1 to 5 mg/mL, as determined by BCA protein assay (Pierce, Rockford, IL). An oligonucleotide (Operon Technologies Inc, Alameda, CA) containing the TF κB site (underlined), 5'-GTCCCGGAGTTTCTACCGGG-3', was annealed with a complimentary primer and was radiolabeled using [α-³²P]deoxyctydine triphosphate (ICN) as described.24 Similarly, binding of the transcription factor, EGR-1, was performed using a radiolabeled oligonucleotide (Operon Tech) containing a prototypic EGR-1 site (underlined), 5'-CCCCGGCCGGGGGGCCGTTTTTACCGGG-3'.25 Protein-DNA complexes were separated from free DNA probe by electrophoresis through 6% nondenaturing acrylamide gels (Novex, San Diego, CA) in 0.5× Tris borate-EDTA. Band intensities were quantified by densitometric analysis using a Personal Densitometer and ImageQuant software (Molecular Dynamics).

**Western blotting.** Analysis of IκBα protein was performed by Western blotting. THP-1 cells were unstimulated or were LPS-stimulated (1 hour) with or without a 15-minute pretreatment with various concentrations of Nasal. Cytosolic extracts were electrophoresed on 8% to 16% sodium dodecyl sulfate-polyacrylamide gels (Novex) and were transferred to Hybond-enhanced chemiluminescence (ECL; Amersham Corp, Arlington Heights, IL). IκBα protein was detected according to the ECL protocol (Amersham Corp) using a 1:2500 dilution of an IκBα antiserum kindly provided by W.C. Greene (J. David Gladstone Institute, San Francisco, CA).

**RESULTS**

Salicylates inhibit LPS-induced TF expression in human monocytic cells. Nasal and aspirin are able to inhibit NF-κB activation, as was recently observed.26 We have shown that LPS induction of the TF gene requires activation of c-Rel/p65 heterodimers in human monocytic cells.27 Therefore, we examined the ability of various salicylates and other NSAIDs to inhibit LPS-induced TF expression in both PBMCs and monocytic THP-1 cells. Cells were pretreated for 15 minutes with Nasal (10 mmol/L), salsalate (10 mmol/L), aspirin (10 mmol/L), ibuprofen (200 μmol/L), or indomethacin (25 μmol/L) before LPS stimulation for 5 hours. Nasal, salsalate, and aspirin all inhibited LPS induction of TF activity (mean ± SD) is shown from three independent experiments.

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**Fig 2.** NaSal inhibition of LPS-induced TF activity in human monocytic cells. (A) PBMCs were exposed to LPS (10 ng/mL) for 5 hours with or without a 15-minute pretreatment with NaSal at doses ranging from 0.1 to 10 mmol/L. (B) THP-1 cells were exposed to LPS (10 μg/mL) for 5 hours with or without a 15-minute pretreatment with NaSal at doses ranging from 0.1 to 10 mmol/L. Control samples were incubated for 5 hours without LPS or NaSal. TF activity (mean ± SD) is shown from three independent experiments.
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TF activity in PBMCs and monocyteic THP-1 cells, whereas ibuprofen and indomethacin had no significant effect (Fig 1). These results indicated that both nonacetylated and acetylated salicylates inhibited LPS-induced TF expression.

We next examined the effect of various doses of NaSal on LPS-induced TF activity. Both PBMCs and THP-1 cells were pretreated for 15 minutes with NaSal (0.1 to 10 mmol/L) before LPS stimulation for 5 hours to maximally induce TF activity. NaSal inhibited LPS induction of TF activity in PBMCs and monocyteic THP-1 cells in a dose-dependent manner (Figs 2A and B). Similar results were observed using aspirin and salicylic acid (data not shown). Time course experiments analyzed the effect of NaSal (10 mmol/L) at different times (2, 5, 8, and 24 hours) after LPS stimulation. These results showed that NaSal abolished LPS induction of TF activity at all time points examined (data not shown), indicating that NaSal did not act by simply delaying the induction of TF expression.

The level of inhibition after the addition of NaSal at various times before and after LPS stimulation was also examined. LPS induction of TF activity was abolished by preincubating cells with NaSal (10 mmol/L) for between 0 and 120 minutes before LPS stimulation (Fig 3). In addition, longer preincubation times (60 to 120 minutes) with lower concentrations of NaSal (0.5 to 10 mmol/L) did not increase the level of inhibition (data not shown). However, there was a time-dependent loss of inhibitory activity when NaSal was added after LPS stimulation (Fig 3). The inhibitory activity required the continuous presence of NaSal because full LPS-induced TF activity was recovered when NaSal was removed before LPS stimulation (data not shown). The effect of NaSal was not a result of inhibition of procoagulant activity in the one-stage clotting assay, because this inhibitor had no effect on clotting times when added to cells immediately before harvest (data not shown).

LPS induction of cell-surface TF expression in PBMCs was measured to determine if NaSal inhibited increases in TF protein expression (Fig 4). PBMCs were pretreated with NaSal (0.1 and 10 mmol/L) for 15 minutes before a 5-hour incubation with or without LPS (10 ng/mL). LPS induction of TF expression was again inhibited in a dose-dependent manner, with complete inhibition at 10 mmol/L (Fig 4).

To exclude the possibility that inhibition by NaSal was due to toxicity, we examined protein incorporation of LPS in the presence of LPS and various concentrations of NaSal. Incorporation of these radiolabeled amino acids into newly synthesized cellular protein is a general measure of protein synthesis within a population of cells. PBMCs and THP-1 cells pretreated with NaSal and then stimulated with LPS showed no significant decrease in protein synthesis at any concentration of NaSal used in these experiments (data not shown). In addition, measurement of cell viability was assessed by MTS conversion to formazan via dehydrogenase enzymes found in metabolically active cells using a calorimetric assay. No reduction in cell viability was observed using LPS and NaSal (data not shown). Trypan blue cell counts also showed no significant reduction in cell viability (data not shown). Taken together, we observed no significant cell toxicity at the concentrations of NaSal and LPS used during the investigation.

NaSal inhibits LPS-induced TF mRNA expression. We next examined if NaSal inhibited LPS induction of TF mRNA. Before LPS stimulation for 2 hours to maximally induce TF mRNA, monocyteic THP-1 cells were pretreated for 15 minutes with NaSal at doses ranging from 0.1 to 10 mmol/L. LPS induction of TF mRNA was inhibited in a
dose-dependent manner by NaSal (Fig 5A). To exclude the possibility that NaSal was a general transcriptional inhibitor of THP-1 cells, we analyzed PMA induction of another gene, egr-1, which is regulated by a NF-κB-independent mechanism. The egr-1 gene expresses a nuclear phosphoprotein that binds to DNA in a zinc-dependent manner. PMA induction of EGR-1 mRNA expression was not affected by NaSal (10 mmol/L; see Fig 5B), indicating that NaSal was not acting as a general inhibitor of transcriptional activation in these monocytic cells.

NaSal inhibits LPS induction of TF gene transcription and the human TF promoter. To determine if NaSal blocked LPS induction of TF gene transcription, nuclear run-on experiments were performed. LPS stimulation of THP-1 cells transiently increased the rate of TF gene transcription at 1 hour (Fig 6). This increase was abolished by pretreating the cells with NaSal (Fig 6). Similarly, NaSal abolished the LPS-stimulated increase in TNF-α gene transcription (Fig 6), which is also regulated by NF-κB/Rel proteins. In contrast, the rate of transcription of the housekeeping gene, G3PDH, was not altered by LPS stimulation or NaSal and LPS (Fig 6).
To further analyze the NaSal inhibition of TF gene transcription, THP-1 cells were transiently transfected with a plasmid, pTF(-278)LUC, which contains 278 bp of the human TF promoter cloned upstream of a luciferase reporter gene, and a plasmid, (pTF)_4SVLUC, which contains four copies of the TF κB site cloned upstream of the minimal simian virus 40 promoter expressing the luciferase gene. Previous studies have shown that LPS induction of the TF promoter is mediated by an LPS response element (−227 to −172 bp) in the wild-type TF promoter or by tandem copies of the isolated TF κB site. Before a 5-hour LPS stimulation to maximally induce luciferase expression, transfected cells were pretreated for 15 minutes with NaSal in doses ranging from 0.1 to 10 mmol/L. LPS induction of luciferase activity expressed by pTF(-278)LUC and (pTF)_4SVLUC was inhibited in a dose-dependent manner by NaSal (Fig 7).

Salicylates inhibit LPS activation of c-Rel/p65 heterodimers. To examine if NaSal acted by preventing activation of c-Rel/p65 heterodimers, THP-1 cells were pretreated for 15 minutes with NaSal (0.1 to 10 mmol/L) before a 2-hour LPS stimulation. Nuclear extracts were analyzed by EMSA using a radiolabeled oligonucleotide containing the TF κB site. LPS activation of c-Rel/p65 heterodimers in THP-1 cells was inhibited by NaSal in a dose-dependent manner (Fig 8A). NaSal also inhibited the LPS activation of NF-κB(p50/p65) in THP-1 cells (data not shown). In addition, Western blots showed that NaSal prevented the reduction in...
IkBα protein in the cytosol 1 hour after LPS stimulation (Fig 8B). Similar results were observed in cells stimulated with LPS for 2 hours (data not shown). These results indicated that NaSal inhibited LPS induction of TF gene expression in human mononuclear cells by preventing proteolytic degradation of IkBα protein and nuclear translocation of c-Rel/p65 heterodimers.

To exclude the possibility that NaSal nonspecifically inhibited protein-DNA interactions, we analyzed the binding of EGR-1 to a prototypic EGR-1 site using nuclear extracts from THP-1 cells with or without NaSal. THP-1 cells were pretreated for 15 minutes with NaSal (10 mmol/L) before a 2-hour PMA (50 ng/mL) stimulation. EMSA showed that EGR-1 binding activity induced by PMA was not affected by the presence of NaSal (Fig SC).

Other NSAIDs, including salsalate, aspirin, ibuprofen, and indomethacin, were examined to determine if these agents also inhibited the activation of c-Rel/p65 heterodimers. THP-1 cells were pretreated for 15 minutes with aspirin (10 mmol/L), NaSal (10 mmol/L), salsalate (10 mmol/L), ibuprofen (200 μmol/L), or indomethacin (25 μmol/L) and then were stimulated with LPS (10 μg/mL) for 2 hours. Aspirin, NaSal, and salsalate all abolished the nuclear translocation of c-Rel/p65 heterodimers, whereas ibuprofen and indomethacin had no effect (Fig 9), which is consistent with the inability of ibuprofen and indomethacin to inhibit LPS induction of TF activity in monocytic cells (Fig. 1).

NaSal concentrations inhibiting 50% of the LPS-induced TF expression in the different assays (IC50) were 0.656 to 1.030 mmol/L (Table 1). Differences in the IC50 may be caused by the different incubation times of these assays or may reflect variations in the sensitivity of each assay to inhibition by NaSal.

DISCUSSION

This study showed that salicylates inhibited LPS-induced TF expression in human monocytic cells. This inhibition was observed with NaSal, salsalate, and aspirin, indicating that both nonacetylated and acetylated salicylates share a common pathway in their ability to inhibit TF expression. NaSal inhibited LPS induction of TF activity, TF mRNA, and TF gene transcription but did not prevent PMA induction of EGR-1 mRNA expression, indicating that it was not a general inhibitor of transcriptional activation. In addition, NaSal inhibited LPS-induced nuclear translocation of c-Rel/p65 heterodimers. Other NSAIDs, such as ibuprofen or indomethacin, failed to inhibit LPS-induced TF activity or activation of c-Rel/p65 heterodimers. Thus, salicylates suppressed LPS induction of the TF gene in monocytic cells by preventing activation of c-Rel/p65 heterodimers.

LPS stimulation of THP-1 cells has been shown to lead to phosphorylation of IkBα, with a corresponding activation of transcription factors containing c-Rel and p65.41 We have shown that LPS stimulation of THP-1 cells transiently reduces levels of cytosolic IkBα protein45 and that overexpression of IkBα in THP-1 cells prevents LPS induction of TF gene transcription.21 Here, we show that NaSal blocks activation of c-Rel/p65 heterodimers in monocytic cells by preventing the LPS-induced proteolytic degradation of IkBα.

TF is expressed by monocytes/macrophages in rheumatoid arthritis in which increased levels of TF are found in the synovium of patients.5 In addition, TF protein and mRNA are expressed by macrophage-derived foam cells in atherosclerotic plaques.6 Both of these diseases are treated clinically with salicylates; therefore, the ability of salicylates to inhibit TF expression by monocytes may contribute to their efficacy and antithrombotic properties.

Cell surface expression of TF by monocyte/macrophages and secretion of various cytokines, including TNFα, are associated with the pathology of Gram-negative sepsis.11,12,14 An effective clinical inhibitor of transcriptional activation of TF and cytokine genes could be useful in the treatment of sepsis. In this study, we found that NaSal inhibited LPS-induced transcription of both the TF and TNFα genes in THP-1 monocyte cells. Aspirin has previously been examined in rodent and nonhuman primate models of endotoxemia with beneficial results,46-48; however, no follow-up studies assessed its clinical use in sepsis therapy. Another NSAID, ibuprofen, has been extensively studied in relation to sepsis therapy.49 Ibuprofen clearly reduces certain symptoms observed during septic shock, such as increased cardiac output,
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Table 1. Inhibition (%) of c-Rel/p65 Activation and TF Expression by Sodium Salicylate

<table>
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<th>Assay</th>
<th>NaSal Concentration (mmol/L)</th>
<th>IC50 (mmol/L)</th>
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<tr>
<td>Clotting, PBMC (5 h)</td>
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<td>1</td>
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<tr>
<td></td>
<td>0.5</td>
<td>1</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>5</td>
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<td></td>
<td>62 ± 7</td>
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<tr>
<td></td>
<td>93 ± 9</td>
<td>0.656</td>
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<tr>
<td>Clotting, THP-1 (5 h)</td>
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<td>28 ± 19</td>
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<td></td>
<td>59 ± 11</td>
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<td>92 ± 9</td>
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<tr>
<td>Northern (2 h)</td>
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<td>72 ± 7</td>
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<td>93 ± 2</td>
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<tr>
<td>EMSA (2 h)</td>
<td>18 ± 17</td>
<td>20 ± 17</td>
</tr>
<tr>
<td></td>
<td>51 ± 10</td>
<td>85 ± 6</td>
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<tr>
<td></td>
<td>93 ± 2</td>
<td>1.110</td>
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The dose-dependent inhibition (%) (mean ± SD) was calculated from at least two independent experiments. NaSal concentrations inhibiting 50% of the LPS activation of c-Rel/p65 and LPS-induced TF expression (IC50) were determined from linear regression (Microsoft Excel; Microsoft Corp, Bothell, WA).

elevated mean arterial blood pressure, and end products of the cyclooxygenase pathway. In contrast, endotoxin-induced elevation of the NF-κB-regulated genes interleukin-6 and TNFα were not decreased by ibuprofen administration. Indomethacin, another NSAID, which (like ibuprofen) inhibits cyclooxygenase, was unable to inhibit transcription of the human immunodeficiency virus. Our data and those of others show that ibuprofen and indomethacin failed to inhibit activation of NF-κB/Rel proteins, which is consistent with their inability to reduce transcription of NF-κB/Rel-regulated genes. Sepsis is a complex pathological condition that will most likely require a well-defined multiple-drug treatment for effective therapy. Given the new findings in relation to the modes of action observed with salicylates, it seems their use in sepsis therapy should be reexamined.

NF-κB/Rel proteins regulate the activation of many genes that are induced during inflammatory responses, including genes encoding various cytokines and adhesion molecules. LPS induction of these genes is mediated by κB binding sites. Therefore, inhibiting the activation of NF-κB/Rel proteins, especially in monocytes, may account for many of the anti-inflammatory effects of the salicylates.

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