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Sodium Salicylate Inhibits Macrophage TNF-α Production and Alters MAPK Activation[1]

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Introduction. **Transcriptional activation of the TNF-α gene in LPS-stimulated macrophages is dependent upon nuclear factor kappa-B (NF-κB) activity. Salicylates may interfere with NF-κB activity through a MAPK (mitogen-activated protein kinase)-dependent process. These studies investigate the effects of sodium salicylate (NaSal) on TNF-α production and MAPK activation in macrophages.**

Methods. **Rat peritoneal macrophages were pretreated or not with sodium salicylate or ibuprofen for 1 h and then stimulated with 100 ng/ml LPS. Six hours following stimulation, cell viability was assessed by MTT assay. At specified time intervals after LPS stimulation, supernatant TNF-α was measured by ELISA. Western blots of cell lysates were performed for analysis of total and activated (phosphorylated) MAPKs.**

Results. **Salicylate and LPS, alone or combined, did not significantly alter macrophage viability. Salicylate, but not ibuprofen, significantly reduced TNF-α production in LPS-stimulated macrophages. LPS-stimulated activation of ERK and SAPK/JNK was inhibited by NaSal pretreatment. NaSal treatment of macrophages activated p38 MAPK independent of LPS stimulation. Pretreatment of samples with the specific p38 MAPK inhibitor, SB203580, did not significantly alter TNF-α production in either LPS or NaSal and LPS-treated samples.**

Conclusions. **Salicylates alter MAPK signaling and suppress TNF-α production in LPS-stimulated macrophages. Salicylate-induced control of inflammatory mediator production in macrophages may, in part, underlie the clinically significant anti-inflammatory effects of these compounds.**

Key Words: **sepsis; signal transduction; salicylates.**

INTRODUCTION

Bacterial endotoxin (LPS) can cause shock and tissue injury by provoking excessive release of inflammatory mediators from host immune cells [1]. LPS-mediated activation of macrophages leads to the production of TNF-α, IL-1, and arachidonic acid metabolites [2]. These cytokines, in turn, amplify themselves locally and systemically and attract the cellular effectors of inflammation. Specific intracellular signaling pathways that modulate cytokine gene expression probably exist in macrophages and may represent novel targets for tomorrow's antisepsis therapies.

Tumor necrosis factor-α (TNF-α), which is produced by LPS-activated macrophages, is the principal mediator of LPS's effects [3]. Depending on the amount, TNF-α can be either damaging or protective [3]. Transcriptional activation of the TNF-α gene in LPS-stimulated macrophages is dependent on nuclear factor kappa-B (NF-κB) activity [4]. Altering NF-κB activation, therefore, could affect TNF-α production.

One potential site for altering NF-κB activation may be the upstream mitogen-activated protein kinases (MAPKs). MAPKs are proline-directed serine-threonine kinases that have important functions as mediators of cellular responses to a variety of extracellular stimuli. LPS is a potent stimulator of MAPK pathways in macrophages and monocytes [5–8] via CD14-dependent tyrosine phosphorylation [9]. There are three main subfamilies of MAPKs in mammalian cells: ERKs (extracellular signal-regulated kinases), p38 kinases, and SAPK/JNKs (c-jun N-terminal kinases) [10]. Several key transcriptional factors have

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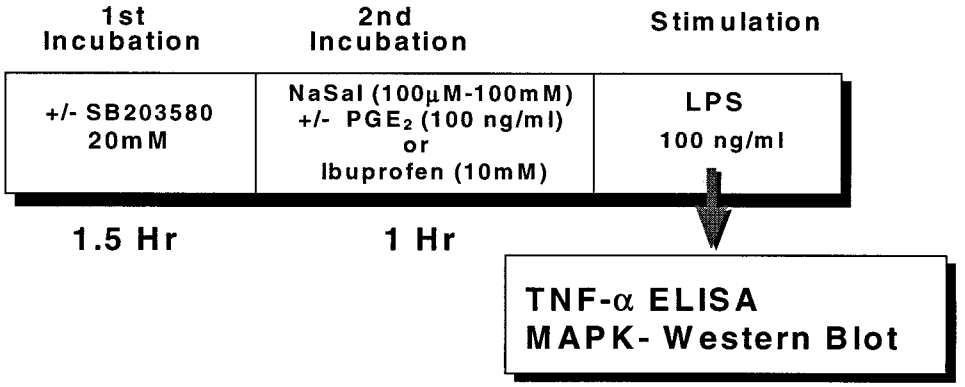

FIG. 1. Schematic of experimental design.

been identified as downstream targets for phosphorylation by MAPKs [11–15].

Sodium salicylate is a nonsteroidal anti-inflammatory drug (NSAID) that inhibits TNF-α production in human monocytes by inhibiting NF-κB [16]. Additionally, salicylates have particular effects on MAPK activation in some cell types. For example, salicylates activate p38 MAPK in COS-1 African green monkey kidney cells [17]. Salicylates' activation of p38 MAPK, in turn, can inhibit NF-κB activation in certain cell types by inhibiting TNF-α-induced IκB-α phosphorylation and degradation [11, 18, 19]. Additionally, salicylates have inhibitory effects on ERK1/2 activation [20].

In this present study, we examined whether salicylates inhibit LPS-stimulated production of TNF-α in rat peritoneal macrophages. Additionally, we studied the effects of sodium salicylate on MAPK activation in macrophages.

METHODS

Materials. RPMI 1640 tissue culture medium (GibcoBRL, Grand Island, NY) was supplemented with L-glutamine and 2% v/v penicillin/streptomycin (Sigma, St. Louis, MO) and 5% v/v heat-inactivated fetal calf serum (Sigma).

Lipopolysaccharide (LPS) from *Eschericia coli* (0111:B4) (Sigma) was reconstituted in distilled, LPS-free water to a concentration of 1 mg/ml. For stimulation studies, this stock solution of LPS was thawed, vortexed, and diluted in RPMI to the final concentration specified. Sodium salicylate (NaSal, Fisher, Springfield, NJ) and ibuprofen (α-methyl-4 [isobutyl] phenylacetic acid, Sigma) were dissolved in LPS-tested Geys balanced salt solution (GibcoBRL). Prior to stimulation studies, sodium salicylate was diluted in RPMI to the desired concentrations. Prostaglandin-E_2 (Sigma) and SB203580 (Calbiochem, San Diego, CA), a specific inhibitor of p38 MAPK, were dissolved in endotoxin-free sterile water and stored in aliquots at −70°C.

Macrophage isolation. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 200–225 g were cared for in accordance with NIH guidelines and acclimatized for 1 week prior to use. All animal manipulations were carried out with the approval of the IACUC of the University of Massachusetts Medical School.

Peritoneal macrophages were isolated from male Sprague-Dawley rats by lavage as previously described [21]. Briefly, animals were placed under general anesthesia with methoxyflurane (Metofane) (Schering-Plough, Union, NJ). Next, the animals were prepped with 70% alcohol, and an anterior abdominal incision was made to the level of the rectus abdominis muscles. A 14-gauge needle was inserted into the peritoneal cavity, and 25 cc of sterile PBS at 37°C was injected. After 5 min of gentle manipulation of the animal, a midline incision was made into the peritoneal cavity. Lavage fluid was recovered in a sterile petri dish. Next, macrophages were spun at 500*g* for 15 min at 4°C. Cells were washed once with PBS and centrifuged again. Contaminating erythrocytes were lysed by treatment with hypotonic Tris-NH_4Cl for 5 min at 37°C. After an equal volume of PBS was added to the centrifuge tube, cells were then spun at 500*g* at 4°C for 15 min. Cells were next counted and plated for experiments.

Macrophage stimulation. Our Experimental Design is outlined in Fig. 1. Briefly, macrophage samples were pretreated with NaSal, or not, and then stimulated with LPS (100 ng/ml). Variations on this basic experimental design included the following: varying the dose of NaSal, pretreating with ibuprofen (10 mM) instead of NaSal, preincubating samples with the SB203580 prior to NaSal pretreatment, and adding PGE_2 (100 ng/ml) to the NaSal pretreatment. The endpoints for these experiments were MTT viability assay, TNF-α ELISA, and Western Blot analysis for total and phosphorylated MAPKs (ERK, SAPK/JNK, and p38).

MTT viability assay. To determine whether LPS and/or NaSal altered macrophage cell viability, macrophages were plated at a concentration of 1×10^5 cells/well in a 96-well plate. Following serum starvation for 14 h, cells were exposed to varying doses of NaSal for 1 h. Next cells were exposed to LPS (100) ng/ml. Cells were incubated at 37°C, 5% CO_2 for 6 h, and the media were replaced by growth media (Complete RPMI with 10% fetal calf serum) with 0.5 mg/ml of MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, Sigma}. Cells were incubated at 37°C, 5% CO_2 for 1 h. The media were then removed and formazan crystals were dissolved in 200 μl DMSO. Absorbance at 570 nm, with a reference value of 750 nm, was determined by an MRX microplate reader. Viability was defined in relation to control cells, which had not been treated {viability (% control) = 100 × (absorbance treated sample)/(absorbance control)}.

TNF-α ELISA. For analysis of TNF-α in supernatants, 1×10^6 macrophages were plated onto 48-well plates. NaSal pretreatment and LPS stimulation were as described above. TNF-α levels were assessed in supernatants using a commercially available murine TNF-α ELISA assay (Genzyme, Cambridge, MA) which is known to be cross-reactive with rat TNF-α. Known TNF-α standards were measured simultaneously. TNF-α content was assessed by measuring absorbance at 450 nm and extrapolating from a standard curve.

Western blotting. For Western blotting experiments, 5×10^6 macrophages were plated onto 6-well plates. For detection of the three

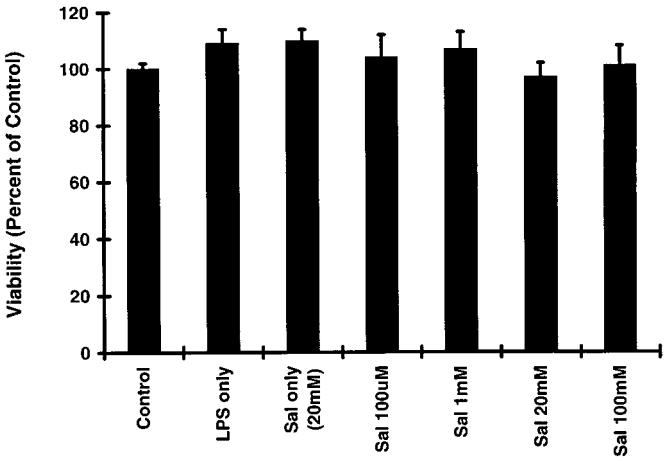

FIG. 2. Effects of salicylate and LPS on macrophage viability. Controls treated with no treatment, LPS only, and salicylate (20 mM) are on the left. Samples were pretreated (1 h) with varying doses of sodium salicylate and then exposed to 100 ng/ml LPS for 6 h (right). MTT uptake was measured against known standards. Results are expressed as average % viability vs control ± SEM.

MAPK subtypes (ERK, SAPK/JNK, and p38), macrophages were washed twice with ice-cold PBS and lysed with ice-cold RIPA buffer (50 mM Tris, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EGTA, 1 mM NaF, 150 mM NaCl) containing protease inhibitor cocktail (Sigma). Lysates were incubated for 20 min on ice and then centrifuged at 2000*g* for 20 min. Supernatants were stored at −20°C.

Total protein content in lysates was measured by the Lowry technique (Bio-Rad, Hercules, CA). Ten micrograms of total cellular protein was mixed with an equal volume of 2X sample buffer and incubated at 95°C for 4 min. Samples were electrophoresed on sodium dodecyl sulfate-10% polyacrylamide (SDS-10% PAGE) gels and transferred to Immobilon membranes (Millipore, Bedford, MA) for immunoblotting with specific antibodies (New England Biolabs, Beverly, MA). Membranes were blocked by incubation for 1 to 3 h in a Tris-buffered saline solution containing 0.05% Tween 20 (0.05% TTBS) and 5% milk. After washing in 0.05% TTBS three times for 5 min, membranes were incubated overnight at 4°C in primary antibody dilution buffer (5% bovine serum albumin in 0.05% TTBS, with a 1:1000 dilution of primary antibody). After washing three times in 0.05% TTBS, membranes were incubated in horseradish peroxidase-linked secondary antibody for 1 h at room temperature. Membranes were again washed three times with 0.05% TTBS and developed using enhanced chemiluminescence reagents (New England Biolabs, Beverly, MA). Membranes were exposed to film (Hyperfilm ECL, Amersham, Arlington Heights, IL).

Statistical analysis. For cytokine analysis, data were compared by two-tailed unpaired Student's *t* test. Data are expressed as means ± SEM. In all cases, significance is defined as $P < 0.05$. Results are representative of at least three separate experiments.

RESULTS

Sodium Salicylate Does Not Effect Macrophage Viability

As measured by MTT assay, cell viability remained comparable between controls and samples treated for 1 h with up to 100 mM sodium salicylate followed by LPS (100 ng/ml) stimulation for 6 h (Fig. 2). This is contrary to viability findings in human monocytes, where sodium salicylate concentrations of 1, 5, and 10 mM caused decreased viabilities, as measured by flow cytometery, of 5, 11, and 13% at 5 h [16].

LPS-Stimulated TNF-α Production Is Inhibited by Sodium Salicylate but Not by Ibuprofen

Stimulation of peritoneal macrophages with 100 ng/ml LPS induced rapid TNF-α production (Fig. 3), which peaked at 4 h and remained stable until the 24 h, when we discontinued measuring. Pretreatment with NaSal (20 mM) for 1 h significantly inhibited this TNF-α production for the entire 24-h duration.

In order to understand better the inhibition of TNF-α production, we examined pretreatment with varying doses of NaSal (Fig. 4). Samples pretreated with a 100 μM dose trended toward decreased TNF-α production, but the decrease in TNF-α production did not reach statistical significance. At doses of 1 and 10 mM, TNF-α production was significantly inhibited ($P < 0.05$). Salicylate pretreatment alone did not result in TNF-α production. Pretreatment with ibuprofen (10 mM), a structurally dissimilar NSAID, as an alternative to NaSal, did not significantly inhibit TNF-α production upon LPS-stimulation compared to LPS-stimulation alone (Fig. 5).

Pretreatment of samples with the specific p38 inhibitor, SB203580, prior to NaSal and LPS treatment did not affect TNF-α production (Fig. 6) either in LPS only stimulated samples or in samples pretreated with NaSal. Previously, SB203580 has been reported to inhibit inflammatory cytokine production in human monocytes and monocytic cell lines [22].

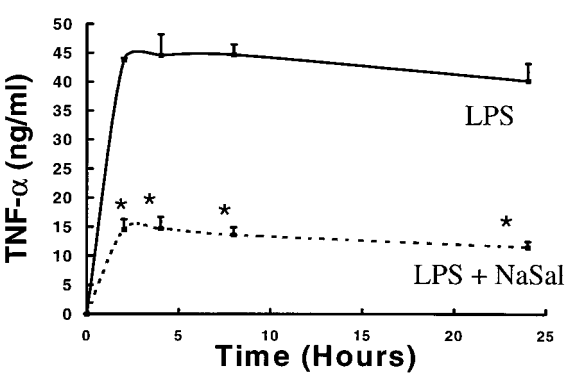

FIG. 3. Effect of salicylate on LPS-induced TNF-α production: Time course. Macrophages were either stimulated with 100 ng/ml LPS (—) or pretreated with NaSal (20 mM, 1 h) and then stimulated with 100 ng/ml LPS (– – –). LPS-induced TNF-α production, as measured by ELISA, was inhibited by NaSal pretreatment at all time points measured over 24 h after stimulation. Data are expressed as means ± SEM. *$P < 0.05$ relative to samples without NaSal treatment.

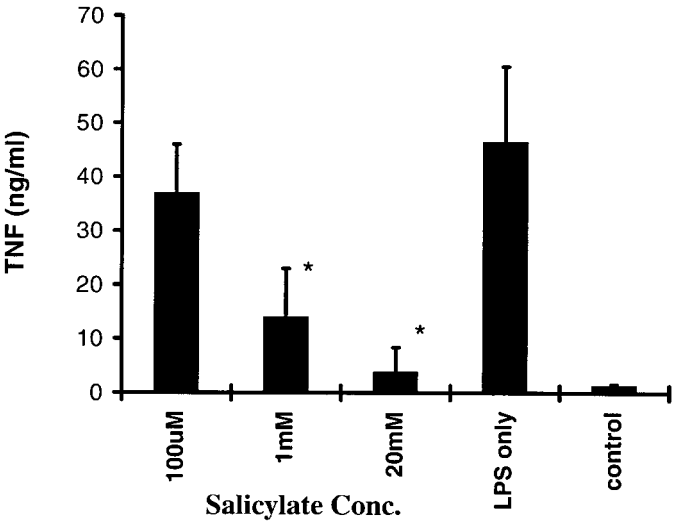

FIG. 4. Effect of salicylate on TNF-α production: Dose response. Macrophages were pretreated with varying doses of NaSal and then stimulated with 100 ng/ml LPS or not. Supernatants were harvested at 6 h after LPS stimulation. TNF-α production, as measured by ELISA, was significantly (*$P < 0.05$ relative to samples without NaSal treatment) inhibited at 1 and 20 mM doses. Data are expressed as means ± SEM.

Prostaglandin E_2 Supplementation Does Not Affect TNF-α Production

Although TNF-α production is inhibited by NaSal in LPS-stimulated rat peritoneal macrophages, whether this is prostaglandin-dependent needed clarification.

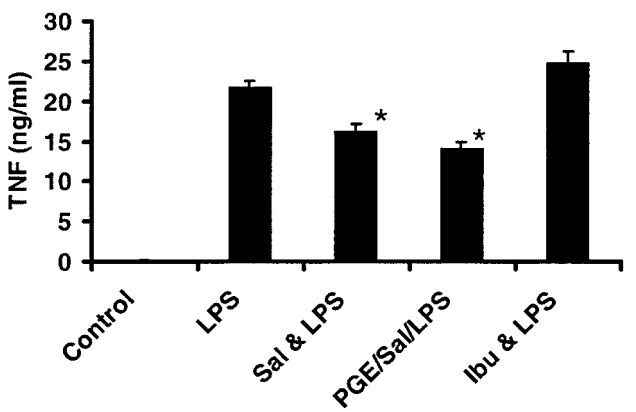

FIG. 5. TNF-α production at 6 h following stimulation with LPS (100 ng/ml). Unstimulated (Control) samples produced no TNF-α. Stimulation with LPS (LPS) resulted in strong TNF production; 1-h pretreatment with sodium salicylate (20 mM) followed by LPS stimulation (SAL & LPS) significantly reduced TNF-α production. (*$P <$ 0.05 vs LPS stimulated alone). The addition of PGE_2 (100 ng/ml) to sodium salicylate (20 mM) pretreatment and LPS stimulation (PGE/Sal/LPS) resulted in TNF-α production which was reduced compared to LPS alone (*$P < 0.05$ vs LPS stimulated alone) with no significant change compared to SAL & LPS. Pretreatment with ibuprofen (10 mM) for 1 h followed by LPS stimulation (Ibu & LPS) resulted in TNF-α production levels which were not significantly altered from LPS alone.

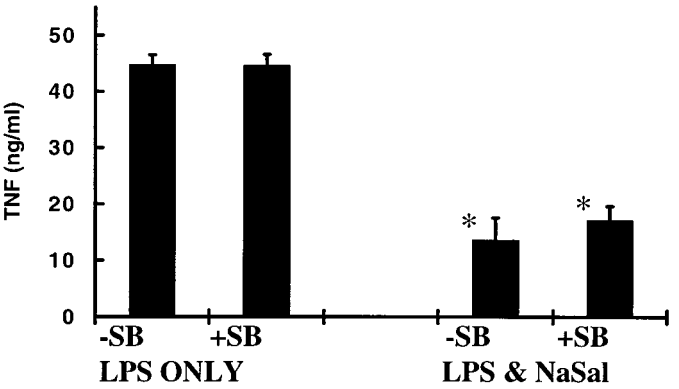

FIG. 6. Lack of effect of the specific p38 inhibitor, SB203580, on TNF-α production. Samples treated with LPS only or NaSal (1 h) & LPS were pretreated with ± SB203580 (20 μM) for 1.5 h. Supernatants were measured at 6 h after LPS stimulation. Results are averages ± SEM for three independent experiments performed in duplicate. (*$P < 0.05$ vs LPS stimulated control.)

Pretreatment of samples with both PGE_2 (100 ng/ml) and NaSal did not significantly alter TNF-α production compared with NaSal pretreatment alone (Fig. 5). This suggests that inhibition of prostaglandin synthesis alone is not responsible for the observed effects of NaSal on TNF-α production in macrophages.

ERK1/ERK2 and SAPK/JNK Phosphorylation Are Inhibited by NaSal

Treatment of macrophage samples with sodium salicylate (20 mM) alone did not activate ERK or SAPK/JNK activation independent of LPS stimulation. Over the 60-min time frame after NaSal treatment, no increases in either ERK or SAPK/JNK activation were noted (data not shown).

As expected, stimulation with LPS alone (100 ng/ml) (Fig. 7, top) activates both forms of ERK. The onset of this activation is within the first 5 min. Both ERK1 and ERK2 remain activated through the first 60 min following stimulation. When 1-h salicylate pretreatment (20 mM) is followed by LPS stimulation, activation of both ERK1 and ERK2 is markedly inhibited. In the NaSal- and LPS-treated samples, some ERK activation is noted at 20 and 60 min. However, this activation is clearly delayed and reduced compared to LPS-stimulated samples. Stimulation of macrophage samples with LPS alone (100 ng/ml) activated SAPK/JNK (Fig. 7, bottom). Phosphorylation of SAPK/JNK appears by 5 minutes, but is short-lived compared to ERK activation. Pretreatment with NaSal inhibited the activation of SAPK/JNK. The increased phosphorylation that is seen in LPS-stimulated samples after 5 min is not seen with NaSal-pretreated samples.

Total ERK and SAPK expression, as determined by Western blot, remained constant throughout the time points measured in all of the samples (data not shown).

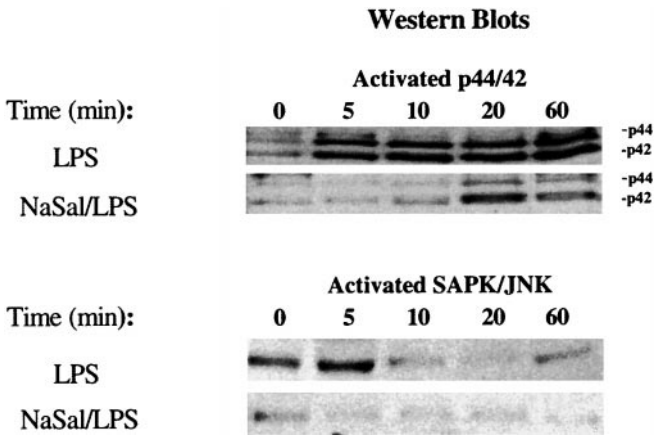

FIG. 7. Effects of NaSal on macrophage ERK1/2 and SAPK/JNK activation. Western blot analysis of peritoneal macrophages treated with LPS (100 ng/ml) stimulation alone or salicylate pretreatment (1 h, 20 mM) followed by LPS (100 ng/ml) stimulation. After stimulation, incubations were stopped at selected intervals over the next 60 min. ERK1/2 blots are on top. ERK 1 (p44) appears as the top band on each blot. ERK2 (p42) appears below. SAPK/JNK blots are at bottom. Equal expression of total ERK and SAPK/JNK expression were confirmed by Western blot (data not shown).

p38 MAPK Is Activated by NaSal

Stimulation of samples with LPS resulted in a gradual phosphorylation of p38 over a 60-min time period (Fig. 8). Pretreatment with NaSal resulted in the activation of p38 MAPK prior to stimulation with LPS. After stimulation with LPS, p38 remained activated for the first 5 min and then decreased. Total p38 present remained stable throughout the period measured.

DISCUSSION

These experiments demonstrate that 20 mM sodium salicylate can significantly inhibit TNF-α production. While a dose of 20 mM is likely unattainable *in vivo* without significant toxicity [24], it inhibited TNF-α production *in vitro* without differentially affecting macrophage viability. A 1 mM NaSal concentration, which can be achieved with systemic administration of salicylates during anti-inflammatory therapy [25], still significantly reduces TNF-α production in macrophages.

The gene for TNF-α is considered to be one of the "immediate-early genes" which can be induced in macrophages by inflammatory stimuli such as LPS and IL-1 [4]. The immediate-early genes are regulated by nuclear translocation of factor NF-κB, a family of transcriptional regulators [26]. Aspirin and sodium salicylate have been shown to inhibit translocation of NF-κB in lymphocytic cell lines [18] and human monocytes [16]. One mechanism of sodium salicylate's actions is the inhibition of IκB-α phosphorylation, which subsequently inhibits IκB degradation and release of NF-κB. Besides NF-κB, other nuclear factors have also been implicated [27]. However, the upstream regulation is unclear.

One possible site of upstream mediation is the MAPKs. MAPKs are felt to be central to LPS signal transduction. LPS is a potent stimulator of MAPK pathways in macrophages and monocytes [5–8, 10] via CD14-dependent tyrosine phosphorylation [9]. These pathways are likely to be a major conduit for LPS signaling in monocytes and macrophages.

These experiments demonstrate that MAPK signaling in macrophages is altered by sodium salicylate. Sodium salicylate activates p38 MAPK and inhibits both ERK1/ERK2 and SAPK/JNK MAPKs in LPS-stimulated macrophages. MAPKs have previously been shown to be targets of salicylates in other cells. Salicylates have been shown to increase p38 activation in COS-1 African green monkey kidney cells. Additionally, it has been shown that p38 activation is required for IκB-mediated inhibition of NF-κB. Similarly, ERK is inhibited by salicylates in these cells in response to TNF stimulation, but not to EGF stimulated ERK under the same conditions [17].

The relationship between salicylates' effects on MAPK signaling and its anti-inflammatory effects is unclear. We have shown that salicylate-induced activation of p38 MAPK cannot simply explain the decreased TNF-α production; selective inhibition of p38 does not reverse salicylate-induced decreases in TNF-α production. The fact that p38 inhibitor does not reverse this effect is not surprising because SB203580, by itself, inhibits TNF-α production in human monocytes [28].

In this model, supplementation of NaSal-treated macrophages with prostaglandin E_2 did not significantly affect TNF-α production. While inhibition of prostaglandin synthesis by NSAIDs has been widely reported, it is not thought to be of primary importance in sodium salicylate's anti-inflammatory activities in macrophages. First, sodium salicylate, unlike aspirin,

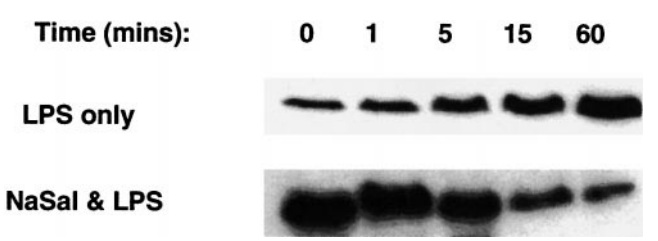

FIG. 8. Activation of p38 by NaSal. Activated p38 MAPK Western blot analysis of peritoneal macrophages treated with (top) LPS alone (100 ng/ml); (bottom) NaSal pretreatment (20 mM, 1 h) followed by LPS stimulation (100 ng/ml). After LPS stimulation, incubations were stopped at selected intervals over the next 60 min. Western blots of lysates were probed with antibodies for the dual phosphorylated form of p38 MAPK. Equal expression of total p38 was confirmed by Western blot (data not shown).

does not inhibit COX-1 [33] and is only a weak inhibitor COX-2 [34]. Sodium salicylate has been shown to inhibit PGE_2 production in human monocytes [16]. However, PGE_2 is a negative feedback effector of TNF-α synthesis [35–37]. Additionally, salicylates have been shown to inhibit nitric oxide production in RAW 264.7 macrophages, and this effect is not reversible with the addition of PGE_2 [29].

Sodium salicylate may have multiple sites of activity in this system. Salicylates affect RAW 264.7 macrophage's production of nitric oxide at multiple sites with different thresholds of response [29, 30]. Additionally, salicylates have been reported to nonspecifically inhibit cellular kinases at high doses [31]. However, sodium salicylate at a dose of 20 mM has been shown to have selective inhibition of ERK and SAPK/JNK in TNF-induced signaling, while having less effect in response to other cytokines (EGF, PDGF, IL-1) [17]. This suggests a selective inhibition of the TNF-α signaling pathway, and similar selectivity may be present in LPS-stimulated signaling. The inability of ibuprofen to inhibit TNF-α production in this model suggests some degree of specificity of sodium salicylate.

Decreased ERK and SAPK/JNK activation has been associated with decreased TNF-α production. LPS-pretreated mouse peritoneal macrophages exhibit decreased ERK and SAPK/JNK activation upon LPS stimulation, with no alteration of p38, as well as decreased TNF-α production [32]. PMA treatment restored ERK activation and TNF-α production, but not p38 or SAPK/JNK activation, in that model. However, in our model a causal role has yet to be established between sodium salicylate's inhibition of ERK and SAPK/JNK, the activation of p38, and the ultimate production of TNF-α.

These data suggest that salicylate-induced control of inflammatory mediator production in macrophages may, in part, underlie the clinically significant anti-inflammatory effects of these compounds. Further research is needed to identify fully the specific intracellular targets of salicylates in macrophages. Increased knowledge of the mechanisms of salicylates' activities may direct us toward therapies that modulate inflammatory gene expression.

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