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Aspirin Inhibits Tumor Necrosis Factor-α Gene Expression in Murine Tissue Macrophages

RODNEY E. SHACKELFORD, PAUL B. ALFORD, YAN XUE, SHEAU-FUNG THAI, DOLPH O. ADAMS, and SALVATORE PIZZO

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

Aspirin has been reported to inhibit the activation of nuclear factor-κB (NF-κB) through stabilization of inhibitor κB (IκB). This observation led us to investigate the role of aspirin in suppressing the activation of the NF-κB-regulated tumor necrosis factor-α (TNF-α) gene expression in primary macrophages. We now report that therapeutic doses of aspirin suppress lipopolysaccharide-inducible NF-κB binding to an NF-κB binding site in the TNF-α promoter, lipopolysaccharide-induced TNF-α mRNA accumulation, and protein secretion. IκB is also stabilized under these conditions. The aspirin-initiated stabilization of IκB, suppression of induced TNF-α mRNA, and NF-κB binding to the TNF-α promoter are blocked by pretreatment with pertussis toxin. These studies suggest that aspirin may exert significant anti-inflammatory effects by suppressing the production of macrophage-derived inflammatory mediators.

Macrophages are found in all body tissues and constitute a host-wide effector system capable of performing a wide array of different functions, such as antigen presentation, phagocytosis of pathogens, immune surveillance, and defense against tumors. Macrophages also play an important role in both chronic and acute inflammation and are known to secret >100 soluble molecules, many of which are inflammatory mediators (for a review, see Ref. 1).

Aspirin and its analogs are among the most widely used drugs on a worldwide basis (2). Therapeutic doses of aspirin exhibit two types of actions depending on the dose of the drug (2). At low therapeutic doses, aspirin is an effective inhibitor of the cyclooxygenase pathway and, hence, prostaglandin-mediated signaling (2). At higher therapeutic doses, aspirin has anti-inflammatory effects that are independent of the inhibition of prostaglandin synthesis (2, 3). Recently, aspirin has been reported to inhibit the activation of NF-κB through the stabilization of IκB (4). NF-κB is a *rel* family transcription factor found in all cell types examined (for a review, see Ref. 5). In most cell types, NF-κB exists in the cytosol as an inactive heterodimer composed of 50-kDa (p50) and 65-kDa (p65, Rel-A) subunits bound to an IκB inhibitory protein (5). Activation of NF-κB involves the phosphorylation and rapid proteolysis of IκB and the subsequent translocation of NF-κB to the nucleus, in which it acts as a transcriptional activator (5). In macrophages, NF-κB regulates several genes encoding inflammatory mediators, including TNF-α (6–8).

The previous observations that aspirin inhibits NF-κB binding and NF-κB-mediated gene expression (4) led us to hypothesize that aspirin may suppress the activation of NF-κB and NF-κB-regulated gene expression in primary elicited macrophages. Here we report that therapeutic doses of aspirin, but not ibuprofen or acetaminophen, suppress inducible NF-κB binding to NF-κB sites in the TNF-α promoter. In turn, therapeutic doses of aspirin, but not ibuprofen or acetaminophen, also suppress TNF-α mRNA accumulation and secretion of TNF-α protein. Last, we report that IκB stabilization and the suppressive effects of aspirin on p50/p65 NF-κB binding to the TNF-α promoter site are mediated via a pertussis toxin-sensitive mechanism. These observations indicate that aspirin may exert some of its anti-inflammatory effects through the suppression of macrophage-derived inflammatory mediators and macrophage activation.

Experimental Procedures

Materials. Tissue culture media were purchased from MediaTech (Washington, DC) and fetal bovine serum from Hyclone Laboratories (Logan, UT). All tissue culture reagents contained <0.125 ng/ml endotoxin (LPS), as quantified by the *Limulus* amebocyte assay supplied by Associates of Cape Cod (Woods Hole, MA). Pertussis toxin was purchased from Calbiochem (San Diego, CA) and was activated with 40 mM DTT for 30 min at room temperature. DuPont-

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ABBREVIATIONS: NF-κB, nuclear factor-κB; IκB, inhibitor κB; TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; MTT, 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, RPMI, Roswell Park Memorial Institute; ELISA, enzyme-linked immunosorbent assay.

New England Nuclear (Boston, MA) was the source of all radiolabeled chemicals. LPS from *Escherichia coli* 026:B6 was purchased from Difco (Detroit, MI). Antisera to p50 and IκB were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Leupeptin, poly(dI/dC), acetylsalicylic acid (aspirin), 4-acetamidophenol (acetaminophen), ibuprofen, and Na^+ salicylate$^-$ were obtained from Sigma Chemical (St. Louis, MO).

Cell culture. Specific pathogen-free inbred C57B1/6J mice (6–8 weeks old) were purchased from Charles River Breeding Laboratories (Raleigh, NC) or from Harlan Sprague-Dawley (Indianapolis, IN). Thioglycolate-elicited macrophages were obtained as previously reported (9). The cells were suspended in RPMI medium containing 2.5% fetal bovine serum, 2 mM glutamine, 12.5 units/ml penicillin, and 6.25 μg/ml streptomycin. Macrophages were plated in appropriate plastic culture wells, incubated for 0.5–1 hr at 37° in 5% CO_2, washed three times with 5 ml of Hanks' balanced salt solution to remove nonadherent cells, and cultured with fresh medium. The cell monolayers were routinely found to contain >98% macrophages, as determined by Giemsa stain or histochemical assay for nonspecific esterase. After 16–24 hr, plated cultured macrophages were treated with various stimuli as indicated.

EMSA. Nuclear extracts were prepared as previously reported (10) according to a modification of the procedure of Dignam *et al.* (11). Each experimental procedure used 2.5×10^7 macrophages and was performed at 4°. After treatment, each tissue culture plate was washed twice with 5 ml of PBS. The cells were removed by scraping in 5 ml of PBS and pelleted by centrifugation at $600 \times g$ for 5 min. The cells were washed in 5 ml of modified Dignam's solution A (10 mM HEPES, pH 8.0, 2.5 mM $MgCl_2$) and pelleted as previously described. The cells were then resuspended in 1 ml of solution A and lysed with 20 strokes of an A-type pestle in a glass Dounce homogenizer (Wheaton, Millville, NJ). The nuclei were placed into a 5-ml ultracentrifuge tube (Sorvall), pelleted at $1200 \times g$ for 10 min in a swinging bucket rotor, and extracted on 0.05 ml of modified Dignam solution C (100 mM HEPES, pH 8.0, 25% glycerol, 1 mM leupeptin, 400 mM NaCl). The final extracts were obtained by centrifugation at $25{,}000 \times g$ for 7 min, aliquoted into 1.5-ml Eppendorf tubes, and stored at −70°. Protein concentrations were determined according to the Bradford assay (12) using bovine serum albumin as a standard. DNA binding proteins present in the nuclear extracts were analyzed using 3 μg of protein to bind the synthetic nucleotide 5′-AAA-CAGGGGGCTTT-CCCTCCTC-AATATCAT-3′ (TNF-α/κB; Ref. 6). Each assay (0.02 ml) had a final concentration of 20,000 cpm of ^{32}P-labeled DNA (~0.1 ng), 1 μg poly(dI/dC), 100 mM NaCl, 25 mM HEPES, 6.25% glycerol, and 0.25 mM leupeptin, pH 8.0. The binding assays were loaded onto 6% polyacrylamide gels (acrylamide/bisacrylamide, 29:1) in 0.25× TBE buffer (22 mM Tris, 22 mM Na^+ borate, 0.5 mM EDTA, pH 8.0) that had been prerun for 30 min. After electrophoresis at 12 V/cm, the gels were dried and exposed to Kodak X-AR film. Oligonucleotides were labeled with γ-^{32}P-ATP by T4 polynucleotide kinase and then annealed to the complementary DNA. Double-stranded DNAs were isolated by electrophoresis in 3% NuSieve agarose (Keene, NH) onto DEAE-cellulose membrane (Whatman, Rockland, ME). Data represent the results of at least three typical experiments.

Preparation of RNA probes. The cDNAs encoding TNF-α and γ-actin were purified from the vector sequences by agarose gel electrophoresis after digestion with appropriate endonucleases (10, 13, 14). Purified DNA (50 ng) was labeled by the oligolabeling method using random primed hexamers to a specific activity of 1×10^8 cpm/mg.

Northern blot preparations and analysis. Total cellular RNA was prepared according to the guanidine thiocyanate-cesium chloride method as previously described (13). The concentration of RNA and its purity were determined by obtaining absorbance readings at 260 and 280 nm (A_{260nm} and A_{280nm}). RNA (10 μg) was used in each lane of the gel. The RNA was denatured and subjected to electrophoresis in 1% agarose-formaldehyde gels. RNA was then transferred by capillary transfer (13) onto Gene-Screen-Plus membranes (DuPont, Wilmington, DE) and prehybridized and hybridized as previously described (13, 15). After washing, the blots were dried and scanned with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). To ensure that equivalent amounts of RNA were blotted to each lane, the blots were rehybridized with the probe for γ-actin, and the results were normalized to actin. All Northern blots were performed at least in triplicate.

Run-on assay for transcription. Nuclei were isolated from macrophage cultures as previously described (16, 17). The incorporation of radiolabel by transcription of genes in nuclear preparations and the isolation of ^{32}P- labeled RNA and hybridization to filters using equal radioactivity per filter were performed as previously described (16). After washing, the filters were gently blotted and exposed to phosphor screens. The data obtained were analyzed by using the program Image Quant from Molecular Dynamics. The run-on assay was performed in duplicate.

Sandwich ELISA for secreted TNF-α. Secreted TNF-α was measured by a double-sandwich ELISA. Macrophages were plated at 2.0×10^5 cells/well and cultured overnight (16–24 hr) in 96-well tissue culture plates (Costar, Cambridge, MA) using 0.2 ml of fresh medium/well. Six wells were used per treatment. At treatment time, the media was changed, and 0.2 ml of fresh medium was added to each well, with or without LPS plus various concentrations of aspirin or its analogs. After 2 hr, 0.1 ml of supernatant media was removed, and the relative amount of secreted TNF-α protein was quantified in a previously prepared 96-well plate. The "receiver" 96-well plate was prepared by the addition of 200 ng of anti-rMuTNF-α monoclonal antibody (Genzyme, Boston, MA) in 0.1 ml of PBS (three wells per treatment). An additional three wells received 0.1 ml of PBS only to allow us to measure background TNF-α. Anti-CD18 (hamster IgG; American Type Culture Collection, Rockville, MD) was used an as irrelevant antibody control. The anti-recombinant murine TNF-α and anti-CD18 monoclonal antibodies were allowed to adhere overnight at 4°. Uncoupled binding sites in the wells were blocked with 0.2 ml of PBS and 5% Carnation nonfat dry milk (Blotto) for 30 min. All subsequent steps were performed at 4°. Then, 0.1 ml of medium from each treatment was added to each of six wells (three with antibody and three without) and allowed to bind overnight. The wells were washed three times with 0.2 ml of Blotto, and 4 μg of goat anti-murine TNF-α polyclonal antibody (R&D Systems, Minneapolis, MN) was added to each well and allowed to bind for 1 hr. The wells were washed three times with Blotto and peroxidase-conjugated rabbit anti-mouse IgG (Organon Teknika, West Chester, PA) at a 1:150 dilution was added for 1 hr. The wells were washed four times with Blotto and three times with PBS and developed with 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma). The plates were analyzed in a Molecular Devices (Menlo Park, CA) plate reader at a wavelength of 410 nm. Each experimental result is the average of three experiments.

Western blot for IκB. To analyze IκB protein levels, macrophages were treated with aspirin, an aspirin analog, or aspirin and pertussis toxin. After treatment, whole-cell extracts were prepared by scraping the cells in 5 ml of PBS and pelleting by centrifugation at $600 \times g$ for 5 min. The PBS was removed, and 0.3 ml of lysis buffer was added (4% SDS, 20% glycerol, 100 mM Tris, pH 6.8, 1 mM Na_3VO_4, 5 mM DTT), the samples were boiled for 5 min, and the protein concentration was determined with the BioRad D_C Protein Assay kit (Hercules, CA). The samples were subjected to 12% SDS-polyacrylamide gel electrophoresis and transblotted onto nitrocellulose membrane (BioRad) in 25 mM Tris, 20% methanol, and 192 mM glycine; 50 μg of protein was loaded per lane. Each experimental procedure used 2.5×10^7 macrophages. Anti-p50 and anti-IκB anti-sera were used at 1:1000 dilutions. Immunoreactive proteins were detected by enhanced chemiluminescent protocol (Pierce, Rockford, IL) using 1:5000 peroxidase-linked goat anti-rabbit IgG (Boehringer-Mannheim Biochemicals, Indianapolis, IN). Blots were exposed for 2–5 min and developed.

Viability determination using the MTT assay. Viability of cells treated with aspirin or its analogs was determined by assaying the ability of mitochondrial dehydrogenases to convert a soluble tetrazolium salt, MTT, into an insoluble purple formazan by cleavage of the tetrazolium ring (18). Briefly, the cells on a 96-well plate were treated with 500 μM ibuprofen or acetaminophen or with 20 mM aspirin or salicylate for 4 hr. Then, the cells were washed, and medium without phenol red and containing MTT (Sigma) at a concentration of 0.5 μg/ml was added for 3–4 hr. The plate was then flicked to remove the medium, and the water-insoluble purple formazan was solubilized by the addition of 0.04 N HCl in isopropanol. The plate was read at a wavelength of 570 nm with a plate reader (Molecular Devices).

Results

Aspirin suppresses inducible NF-κB binding to an NF-κB site in the TNF-α promoter in murine tissue macrophages. To initiate these studies, we examined the effect of aspirin treatment on LPS-induced NF-κB binding to an NF-κB site in the murine TNF-α promoter. When extracts of LPS-stimulated macrophages were examined in the EMSA against the labeled TNF-α promoter NF-κB-binding oligonucleotide, we observed a distinct retardation band (Fig. 1, *band 2, lane 4*) comprising p50/p65 NF-κB heterodimers consistent with previous reports (6–8, 10). When macrophages were concurrently exposed to aspirin and LPS, a dose-dependent suppression of LPS-inducible NF-κB binding was observed (Fig. 1, *band 2, lanes 4–8*). In all experiments, aspirin exerted a significant suppressive effect on NF-κB binding at concentrations as low as 1 mM. Aspirin had little effect, however, on constitutive NF-κB binding (Fig. 1, *band 1, lanes 2–8*), consistent with previous reports (6–8, 10). Some variation in binding to the lower molecular weight constitutive band (*band 1*) was found within experiments; however, in no case did this variation correlate with the presence of either aspirin or LPS. For example, in Fig. 1, whereas *band 1* binding is lower in *lane 7* than in other lanes (LPS plus 10 mM aspirin), it is not lower in *lane 8* (LPS plus 20 mM aspirin). Similarly, in Fig. 1, *band 1, lane 2* is nearly identical to *band 1, lane 3*, although *lane 2* was treated with 20 mM aspirin and *lane 3* was untreated. Aspirin (20 mM), when added to LPS-treated macrophage nuclear extract/oligonucleotide/binding buffer reaction mix (see Experimental Procedures), had no effect on LPS-inducible or constitutive NF-κB binding in the EMSA (data not shown).

Previous studies suggest that aspirin and salicylate, but not acetaminophen or indomethacin, suppress NF-κB-dependent gene expression in a human T lymphocyte cell line (4). To test the effects of aspirin and other agents on NF-κB binding in primary macrophages, we treated macrophages with LPS with and without aspirin, salicylate, ibuprofen, or acetaminophen. Both aspirin and salicylate suppressed LPS-inducible NF-κB binding to the NF-κB site in the TNF-α promoter oligonucleotide (Fig. 2, compare *lane 3, band 2*, with *lanes 5 and 7, band 2*). In most experiments, high concentrations of ibuprofen (200 μM) somewhat suppressed LPS-inducible binding (Fig. 2, *lane 9, band 2*), whereas acetaminophen at the same concentration had only a slight suppressive effect on LPS-inducible binding (Fig. 2, *lane 11, band 2*) and, in many experiments, did not suppress induced NF-κB binding at all.

Our finding that 200 μM ibuprofen suppressed LPS-induced NF-κB binding to the TNF-α NF-κB site led us to examine the effects of a therapeutic dose of ibuprofen on inducible NF-κB binding. As shown in Fig. 3, a roughly therapeutic dose (50 μM) of ibuprofen failed to suppress LPS-inducible NF-κB binding (Fig. 3, *lane 4, band 2*). Similarly, a 100-μM dose of ibuprofen also failed to suppress induced

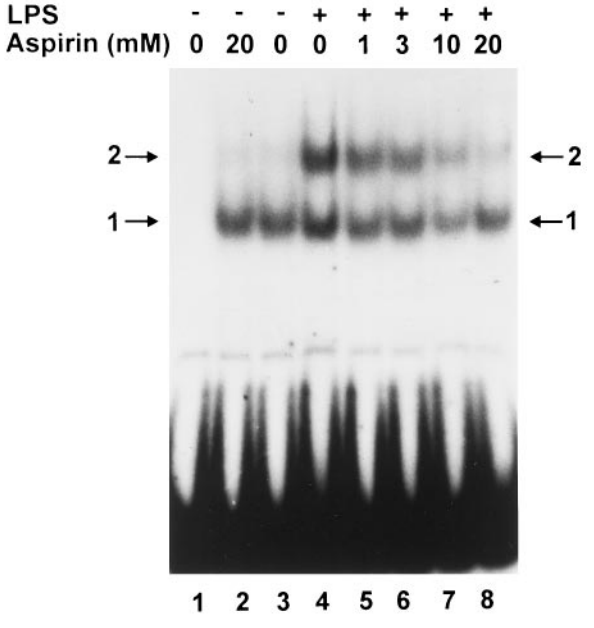

Fig. 1. EMSA of nuclear proteins from macrophages treated with LPS and concentrations of aspirin ranging from 1–20 mM for 1 hr. Nuclear extracts were then prepared and used in the EMSA with the TNF-α NF-κB oligonucleotide as described under Experimental procedures. *Lane 1*, free oligonucleotide. The LPS concentration was 10 ng/ml. *Arrows*, retardation bands 1 and 2.

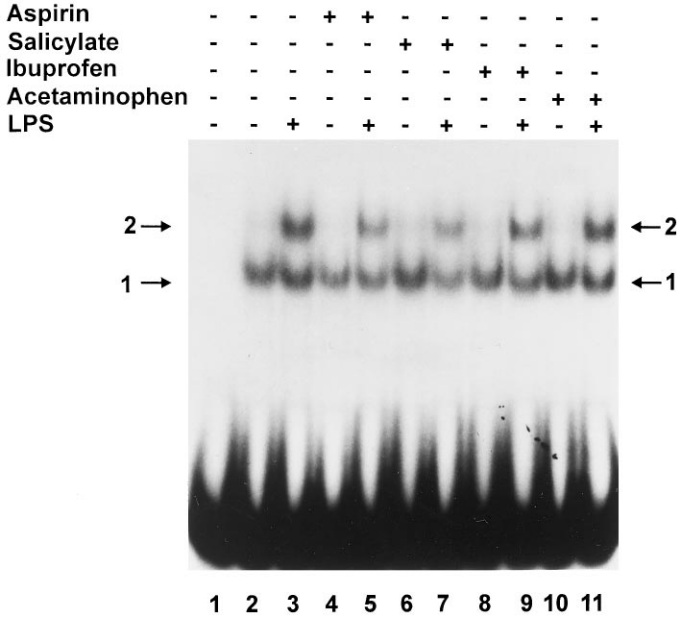

Fig. 2. The effect of aspirin and other agents on LPS-inducible NF-κB binding in macrophages. Macrophages were treated with LPS (10 ng/ml) and aspirin, salicylate, ibuprofen, or acetaminophen for 1 hr. Nuclear extracts were then prepared, and binding to the TNF-α oligonucleotide was analyzed. The concentrations of agents were 20 mM aspirin, 20 mM salicylate, 200 μM ibuprofen, and 200 μM acetaminophen.

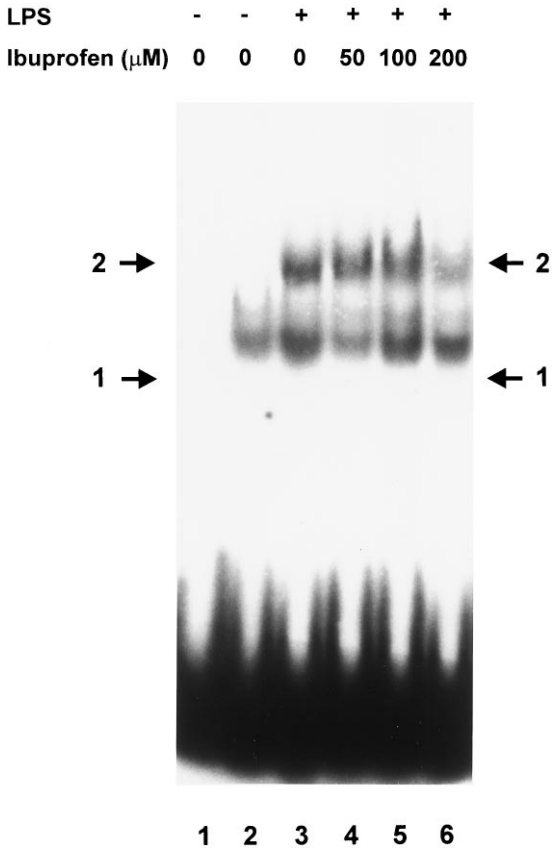

Fig. 3. The effect of different concentrations of ibuprofen on LPS-inducible NF-κB binding in macrophages. Macrophages were treated with LPS (10 ng/ml) and ibuprofen for 1 hr. Nuclear extracts were then prepared, and binding to the TNF-α oligonucleotide was analyzed. The concentrations of agents were 50, 100, and 200 μM ibuprofen.

NF-κB binding (Fig. 3, *lane 5, band 2*), whereas 200 μM ibuprofen partially suppressed binding (Fig. 3, *lane 6, band 2*).

The MTT viability assay demonstrated no significant difference in the ability of control cells and cells treated with 20 mM aspirin or salicylate for 4 hr to cleave the tetrazolium ring of MTT. Similarly, neither ibuprofen (500 μM) nor acetaminophen (500 μM) treatment altered the ability of macrophages to cleave the tetrazolium ring of MTT. These data suggest that there is no loss of macrophage viability under our experimental conditions.

Aspirin suppresses TNF-α mRNA transcription in macrophages. The TNF-α gene is regulated at the transcriptional level in macrophages (14). We hypothesized that the suppressive effects of aspirin on LPS-inducible NF-κB binding to an NF-κB site in the TNF-α promoter should result in suppressed transcription of the gene. As shown in Fig. 4, LPS induced transcription of the TNF-α gene, whereas a high concentration of aspirin (10 mM) suppressed LPS-induced TNF-α transcription. As previously reported, IL-1α transcription was not inducible by LPS (14). No RNA binding to the control pBR322 plasmid was detected.

Aspirin suppresses TNF-α mRNA induction in macrophages. The NF-κB binding site within the oligonucleotide used in the EMSA plays an important role in the induction of TNF-α (8). We also found that aspirin suppressed LPS-induced transcription of this gene. We next used Northern blot analysis to examine the effects of aspirin, salicylate, ibuprofen, and acetaminophen on inducible TNF-α mRNA accumulation. As previously observed, LPS induced TNF-α mRNA accumulation in primary macrophages (10, 14). When the cells were treated simultaneously with LPS and aspirin, we observed a dose-dependent suppression of accumulated TNF-α mRNA with increasing concentrations of aspirin (Fig. 5). Fifty percent suppression of TNF-α mRNA accumulation occurred at <1 mM aspirin, as measured by the ratio of TNF-α to actin RNA. Salicylate (20 mM) strongly suppressed TNF-α mRNA production (Fig. 6), whereas ibuprofen (200 μM) slightly suppressed LPS-induced TNF-α mRNA accumulation. Acetaminophen at the same concentration had no effect.

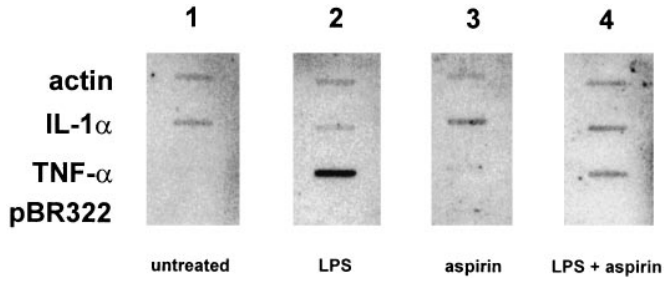

Fig. 4. Nuclear run-on experiment examining the effect of aspirin on induced TNF-α transcription. Macrophages were treated as indicated for 1 hr to examine TNF-α transcription (*lanes 1–4*). The concentrations of agents were 10.0 ng/ml LPS and 10 mM aspirin.

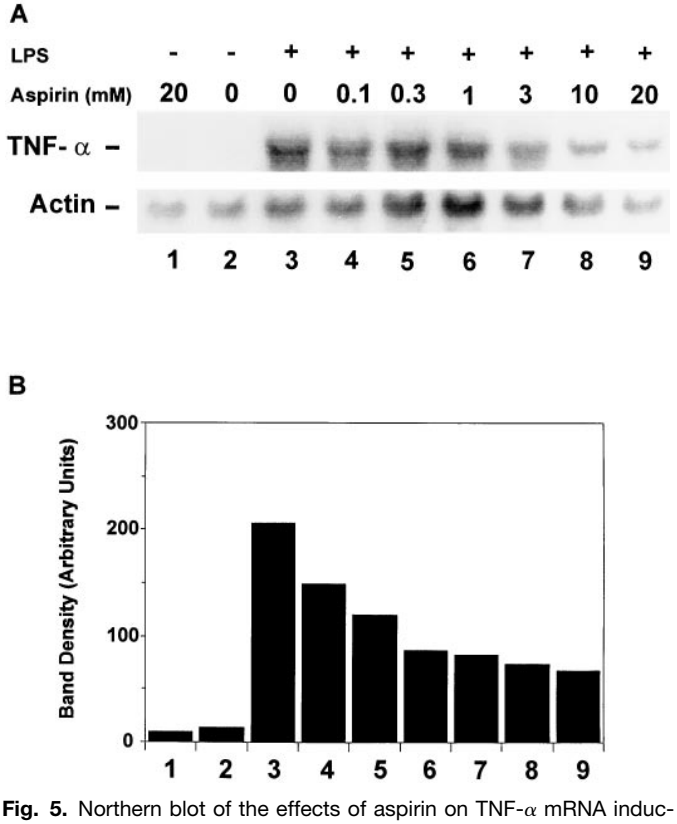

Fig. 5. Northern blot of the effects of aspirin on TNF-α mRNA induction. Macrophages were treated with LPS (10 ng/ml) for 2 hr to induce TNF-α mRNA (A). B, Levels of TNF-α were then normalized to actin. Conditions were 20 mM aspirin without LPS (*lane 1*), buffer control (*lane 2*), and LPS without and with aspirin at the concentrations of 0 (*lane 3*), 0.1 mM (*lane 4*), 0.3 mM (*lane 5*), 1.0 mM (*lane 6*), 3.0 mM (*lane 7*), 10 mM (*lane 8*), and 20 mM (*lane 9*).

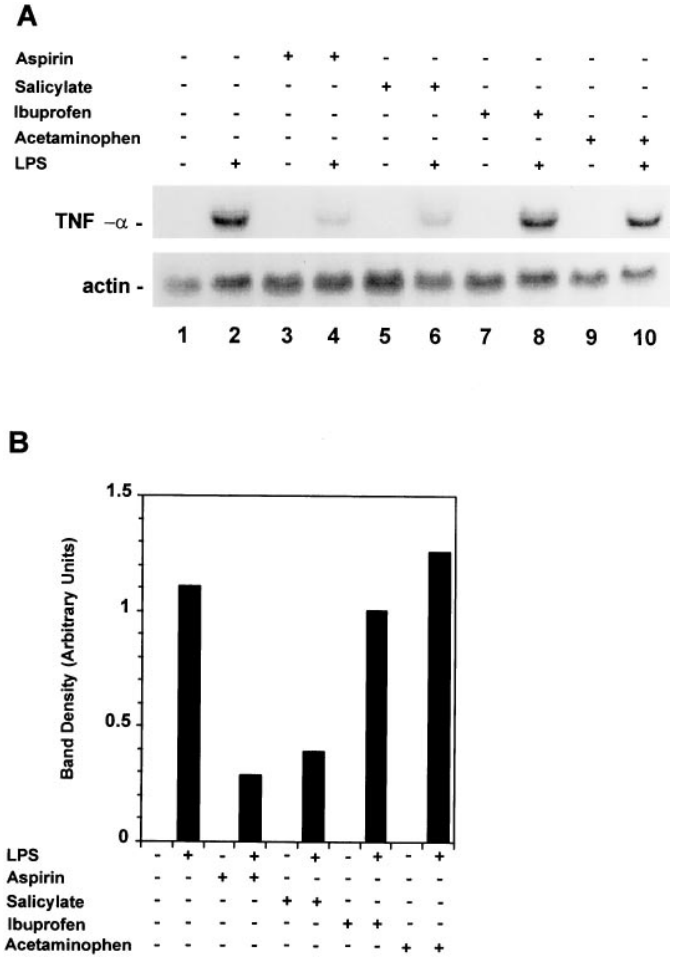

Fig. 6. Northern blot of the effects of aspirin, salicylate, ibuprofen, or acetaminophen on TNF-α mRNA induction. Macrophages were treated with LPS (10 ng/ml) for 2 hr to induce TNF-α mRNA (A). B, Level of TNF-α mRNA normalized to actin. The concentrations of agents were 200 μM ibuprofen, 200 μM acetaminophen, 20 mM aspirin, and 20 mM salicylate.

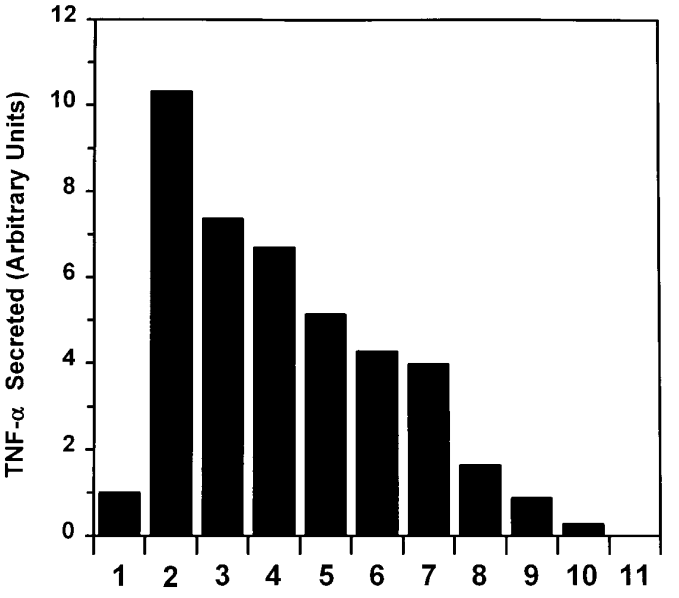

Fig. 7. The effect of increasing concentrations of aspirin on LPS-induced TNF-α secretion. Macrophages were treated with LPS (10 ng/ml) and aspirin concentrations of 0.1–20 mM for 2 hr. Secreted TNF-α protein was analyzed by a double-sandwich ELISA. Condition were untreated control (*lane 1*); LPS without (*lane 2*); LPS with aspirin at the concentrations of 0.1 mM (*lane 3*), 0.3 mM (*lane 4*), 1.0 mM (*lane 5*), 3 mM (*lane 6*), 10 mM (*lane 7*), and 20 mM (*lane 8*); aspirin alone (20 mM) (*lane 9*); and irrelevant antibody control without (*lane 10*) and with LPS (*lane 11*).

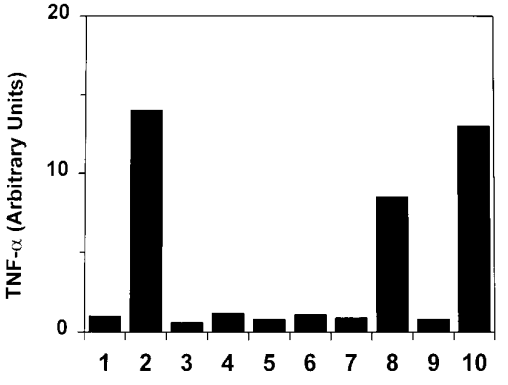

Fig. 8. The effect of aspirin and aspirin analogs on LPS-induced TNF-α secretion. Macrophages were treated with LPS (10 ng/ml) and aspirin or an analog for 2 hr. Secreted TNF-α protein was analyzed by a double-sandwich ELISA. Conditions were untreated control (*lane 1*), LPS (*lane 2*), aspirin (*lane 3*), LPS plus aspirin (*lane 4*), salicylate (*lane 5*), LPS plus salicylate (*lane 6*), ibuprofen (*lane 7*), LPS plus ibuprofen (*lane 8*), acetaminophen (*lane 9*), and LPS plus acetaminophen (*lane 10*). The concentrations of agents were 200 μM ibuprofen, 200 μM acetaminophen, 20 mM aspirin, and 20 mM salicylate.

Aspirin suppresses secretion of TNF-α protein by macrophages. To measure the effect of aspirin exposure on secreted TNF-α protein, we used a double-sandwich ELISA. As previously reported, LPS dramatically increased the secretion of TNF-α by macrophages (Fig. 7) (19). When macrophages were treated simultaneously with both LPS and aspirin, a dose-dependent suppression of secreted TNF-α protein was found that closely paralleled the suppression of TNF-α at the mRNA level. Aspirin (0.1 mM) suppressed TNF-α protein secretion by an average of 28%, with 50% suppression at 1 mM. By itself, 20 mM aspirin slightly suppressed TNF-α protein secretion compared with untreated macrophages. When the ELISA was performed with the irrelevant hamster anti-CD18 antibody as a control, no LPS-inducted secretion of TNF-α protein could be detected; 20 mM salicylate suppressed secreted TNF-α protein as effectively as did 20 mM aspirin. Ibuprofen (200 μM) suppressed secreted TNF-α protein by 41%, whereas acetaminophen (200 μM) suppressed 11% in an average of three experiments (Fig. 8).

Pertussis toxin blocks the suppressive effects of aspirin on LPS-inducible TNF-α mRNA expression and NF-κB binding. Salicylates interfere with processes regulated by pertussis toxin-sensitive G proteins in human neutrophils (20). Recently, we reported that the suppressive effects of oxidized low-density lipoprotein on LPS-induced NF-κB binding and TNF-α mRNA accumulation were blocked by pretreatment of macrophages with pertussis toxin (10). Based on these observations, we hypothesized that pretreatment of macrophages with pertussis toxin might block the suppressive effects of aspirin on NF-κB binding. To test this hypothesis, macrophages were pretreated with DTT-activated pertussis toxin for 2 hr and treated as before with various combinations of LPS and aspirin. LPS-induced NF-κB binding was suppressed by the addition of 10 mM

aspirin (Fig. 9). However, when macrophages were pretreated with pertussis toxin followed by treatment with LPS and aspirin, the suppressive effect of aspirin on inducible NF-κB binding was blocked. Similar results were obtained when a 3 mM aspirin concentration was used (data not shown). Pretreatment with pertussis toxin did not affect the induction of NF-κB binding by LPS, and neither aspirin nor pertussis toxin, nor the two together, affected the constitutive NF-κB binding (Fig. 9, *band 1*). When macrophages were stimulated with LPS, the enhanced levels of TNF-α mRNA were inhibited by simultaneous treatment with a therapeutic dose of aspirin (3 mM) (Fig. 10). Similar results were obtained with a 10 mM concentration of aspirin, with the same concentrations of pertussis toxin and LPS (data not shown). This inhibition was essentially blocked by pretreatment of the cells with pertussis toxin. Pertussis toxin itself did not significantly alter TNF-α mRNA induction by LPS.

Aspirins stabilizes IκB protein in primary macrophages via a pertussis toxin-sensitive mechanism. Treatment of a murine B lymphocyte-like cell lines with aspirin or salicylate stabilized IκB by inhibiting its phosphorylation (21). Based on these findings and our current results, we hypothesized that aspirin and salicylate, but not ibuprofen or acetaminophen, would stabilize IκB protein in macrophages. Furthermore, this stabilization should be blocked by pretreatment of macrophages with pertussis toxin. As shown in Fig. 11A, Western blot analysis of IκB protein levels in whole-macrophage extracts demonstrated that therapeutic doses (3 mM) of aspirin and salicylate stabilized IκB protein while having relatively little effect on p50 NF-κB protein levels (Fig. 11A, *lanes 4 and 5*, respectively). Ibuprofen and acetaminophen at concentrations well above the therapeutic levels (200 μM) failed to stabilize IκB protein levels (Fig. 11A, *lanes 2 and 3*, respectively). The stabilization of IκB by aspirin was also blocked by pretreatment of macrophages with pertussis toxin. This blocking of IκB stabilization was found at an aspirin concentration of 3 mM (Fig. 11B, compare *lanes 2 and 4*) and 10 mM (data not shown). Again, p50 protein levels were unaffected (Fig. 11B).

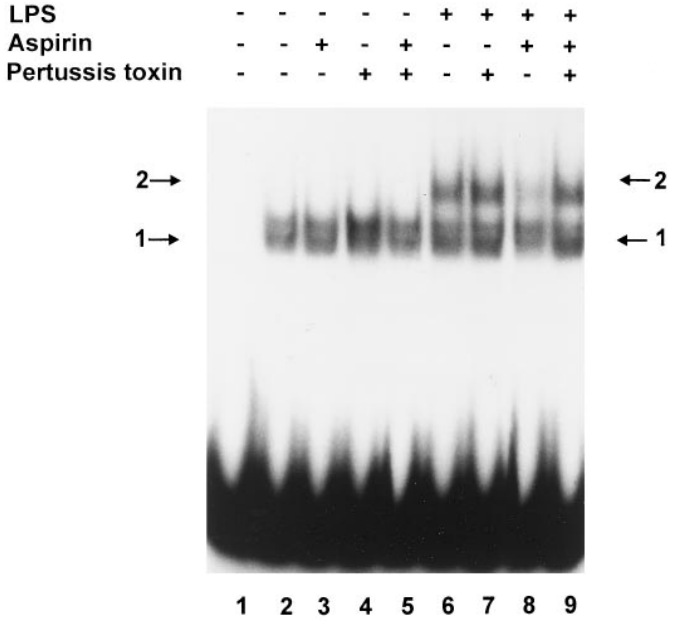

Fig. 9. The effect of pertussis toxin on the suppression of LPS-inducible NF-κB binding by aspirin. Macrophages were pretreated pertussis toxin for 2 hr and then treated with LPS, aspirin, or LPS (10 ng/ml) and aspirin (10 ng/ml) for 1 hr. Nuclear extracts were then prepared, and binding to the TNF-α NF-κB oligonucleotide was analyzed. The concentrations were is 20 mM aspirin and 1 μg/ml pertussis toxin.

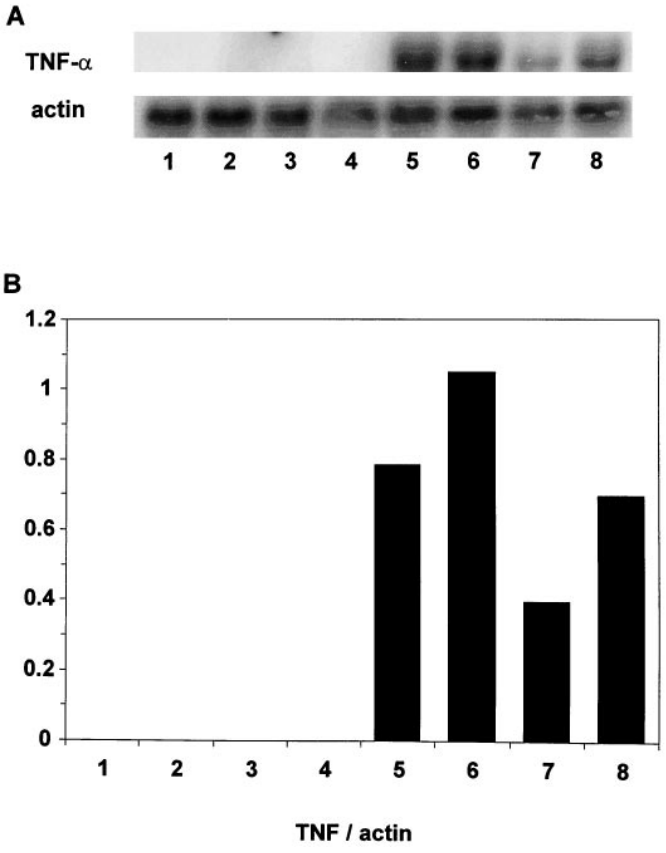

Fig. 10. Northern blot of the effects of pertussis toxin on the suppression of LPS-inducible TNF-α mRNA accumulation by aspirin. Macrophages were pretreated for 2 hr with pertussis toxin and treated an additional 2 hr with LPS (10 ng/ml), aspirin, or LPS and aspirin. Conditions were untreated control (*lane 1*), aspirin (*lane 2*), pertussis toxin (*lane 3*), pertussis toxin plus aspirin (*lane 4*), LPS (*lane 5*), LPS plus pertussis toxin (*lane 6*), LPS plus aspirin (*lane 7*), and LPS plus aspirin plus pertussis toxin (*lane 8*). B, Level of TNF-α mRNA normalized to actin. Concentrations were 3 mM aspirin and 1 μg/ml pertussis toxin.

Discussion

Macrophages are known to secrete several inflammatory genes products regulated by NF-κB (for reviews, see Refs. 1 and 5). In particular, TNF-α is regulated by NF-κB in macrophage-like cell lines (6–8) Previously, aspirin was shown to suppress inducible NF-κB binding and NF-κB-mediated gene expression in human T and murine B lymphocyte-like cell lines (4). Therapeutic concentrations of aspirin also suppress tissue factor production in primary human monocytes (22). Furthermore, aspirin exerts some of its effect on human neutrophils through a membrane-associated, pertussis toxin-sensitive G protein (20). We hypothesized that aspirin could exert some of its anti-inflammatory effects by suppressing NF-κB-regulated inflammatory genes in primary macrophages. To test this hypothesis, we used an EMSA to examine LPS-inducible NF-κB binding to an oligonucleotide containing an NF-κB site present in the TNF-α promoter (6).

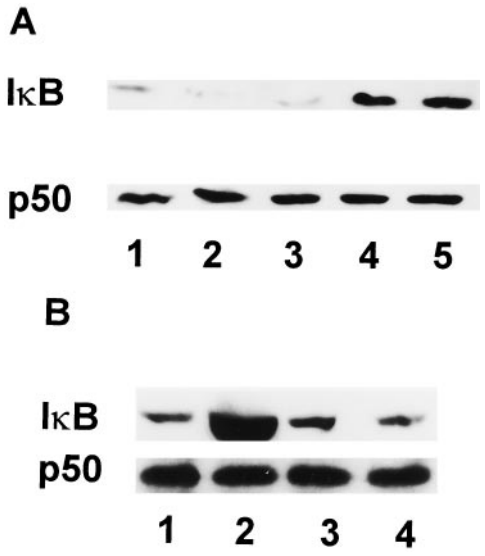

Fig. 11. The effect of aspirin and its analogs on IκB and p50 protein levels macrophage extracts and the effect of pertussis toxin pretreatment on IκB stabilization by aspirin. Macrophages were either treated with aspirin or an aspirin analog for 1 hr (A) or pretreated with pertussis toxin for 2 hr and then treated with aspirin for 1 hr (B). Whole-cell extracts were prepared, and IκB protein levels were analyzed by Western blot. A, Conditions were untreated control (*lane 1*), ibuprofen (*lane 2*) acetaminophen (*lane 3*), aspirin (*lane 4*), and salicylate (*lane 5*). B, Conditions were untreated control (*lane 1*), aspirin (*lane 2*) pertussis toxin (*lane 3*), and aspirin plus pertussis toxin (*lane 4*). Concentrations of agents were 200 μM ibuprofen, 200 μM acetaminophen, 3 mM aspirin, 3 mM salicylate, and 1 μg/ml pertussis toxin.

Aspirin significantly inhibited LPS-inducible NF-κB binding at concentrations as low as 1 mM and suppressed TNF-α mRNA accumulation and secretion of protein at a 0.1 mM concentration. Similarly, 3 mM aspirin effectively stabilized IκB protein. Aspirin is known to exert anti-inflammatory effects at plasma concentrations of 1–3 mM, although there is evidence that aspirin and related compounds may be concentrated significantly above plasma concentrations in certain tissues (24, 25). Thus, LPS-inducible NF-κB binding to the NF-kB site in the TNF-α promoter, induction of TNF-α mRNA and secreted protein, and stabilization of IκB protein occur within known therapeutic concentrations of aspirin.

TNF-α is transcriptionally regulated in murine macrophages under the conditions we used in the current study (14). Thus, suppressed NF-κB binding to the NF-κB site in the TNF-α promoter should result in suppressed transcription. When nuclear run-on experiments were performed, we found that 10 mM aspirin did indeed suppress induced TNF-α transcription. To measure the effects of lower concentrations of aspirin and its analogs on TNF-α expression, we examined the levels of induced TNF-α mRNA, as well as secreted TNF-α protein. Aspirin suppressed TNF-α mRNA accumulation within the 0.1–3 mM range, with ~50% mRNA suppression occurring at 1 mM aspirin, as quantified by TNF-α/actin mRNA levels. LPS-induced TNF-α mRNA accumulation and secreted protein were fairly sensitive to the suppressive effects of aspirin, with as little as 100 μM aspirin suppressing mRNA accumulation and secretion of protein by ~30%. To our knowledge, this is the first report that aspirin suppresses either secretion of TNF-α protein or induction of TNF-α mRNA.

In our study, ibuprofen (200 μM) had a slight, although reproducible, suppressive effect on LPS-inducible NF-κB binding, TNF-α mRNA accumulation, and secretion of TNF-α. It did not, however, stabilize IκB to any significant degree. The therapeutic plasma concentration of ibuprofen is ~44 μM (3). When 50 and 100 μM doses of ibuprofen were used in the EMSA, neither concentration significantly suppressed LPS-induced NF-κB binding to the NF-κB site in the TNF-α promoter. Because the effects of 200 μM ibuprofen were slight for the assays used and because lower doses in the therapeutic range of ibuprofen had no effect on inducible NF-κB binding, we conclude that ibuprofen probably does not significantly affect the macrophage functions tested in this study. This finding is comparable to a previous report in which 200 μM ibuprofen did not significantly suppress NF-κB binding to an NF-κB site in the tissue factor gene promoter in primary human monocytes (22).

Acetaminophen (200 μM) had either no effect or only a very slight suppressive effect on the macrophage functions we tested. Similarly, 200 μM acetaminophen failed to stabilize IκB. Because therapeutic plasma concentrations of acetaminophen occur in the 66–130 μM range (2), we conclude that it exerts its primary anti-inflammatory effects through mechanisms other than the suppression of TNF-α in macrophages. We found that a high (20 mM) concentration of salicylate was as effective a suppresser of macrophage function as was aspirin (20 mM). Also, 3 mM salicylate stabilized IκB protein as effectively as the same concentration of aspirin. These findings are to be expected because aspirin and salicylate share many common pharmacological features, including anti-inflammatory properties in the 1–3 mM range (2).

Previously, we reported that pretreatment of macrophages with pertussis toxin blocked the suppressive effects of oxidized low-density lipoprotein on NF-κB binding to the TNF-α NF-κB oligonucleotide used here (10). Pretreatment with pertussis toxin also blocked the suppressive effects of oxidized LDL on LPS-inducible TNF-α mRNA accumulation. Furthermore, pertussis toxin blocks some of the effects of aspirin on human neutrophils (20). Based on these data, we hypothesized that aspirin exerts some of its suppressive effects via a pertussis toxin-sensitive mechanism. When macrophages were pretreated with pertussis toxin, the suppressive effect of 10 mM aspirin on LPS-induced NF-κB binding to the TNF-α oligonucleotide was largely blocked, as was the suppressive effect of aspirin on TNF-α mRNA accumulation. Similarly, the previously described stabilization of IκB by aspirin (4, 21) was inhibited by pretreatment with pertussis toxin. Because all three of the above experiments involving pertussis toxin were performed with a nonphysiological, high- aspirin dose (10 mM), these experiments were also performed under identical conditions with a therapeutic dose of aspirin (3 mM). In all three experiments, pertussis toxin blocked the effects of aspirin. Thus, pertussis toxin may blocks some of the effects of aspirin in macrophages at therapeutic concentrations.

Collectively, our findings contribute to a growing body of information suggesting that aspirin exerts some of its effects through interactions with G proteins (20). To our knowledge, this is the first report that the stabilization of IκB by aspirin is pertussis toxin sensitive. Although the suppression of IκB stabilization by aspirin was largely blocked by pertussis toxin, the binding of inducible NF-κB to an NF-κB site in the TNF-α promoter and the induction of TNF-α RNA were not

completely blocked. Aspirin therefore probably exerts some suppressive effects that are pertussis toxin insensitive.

Our findings have several implications. First, our finding that the previously described stabilization of IκB by aspirin (4, 20) is sensitive to pretreatment with pertussis toxin, suggests, but does not prove, that aspirin stabilizes IκB by interacting with G proteins, which may in turn impinge on the phosphorylation and/or proteolysis events regulating IκB protein levels (for a review, see Ref. 5). Second, TNF-α plays a role in a wide variety of circumstances, including pregnancy, cancer, rheumatoid arthritis and other autoimmune disorders, infectious disease, transplantation, and septic shock (for reviews, see Refs. 30–33). Our finding that therapeutic concentrations of aspirin (0.1–3 mM) can suppress TNF-α expression in primary macrophages suggests that aspirin may impinge on some of these TNF-α-modulated events. For example, both inducible nitric oxide synthetase and TNF-α are thought to play an important role in the pathogenesis of endotoxic shock (for a review, see Ref. 33). Our findings here that aspirin suppresses TNF-α, combined with the previous observations that aspirin can inhibit inducible nitric oxide synthetase, may partially explain the beneficial effects of aspirin and other salicylates on models of endotoxic shock (28, 29, 34). Similarly, macrophages have been identified as a major source of TNF-α within inflamed synovium (35). In rheumatoid arthritis, TNF-α positive macrophages have been implicated in the development and maintenance of the disease process (for reviews, see Refs. 35 and 36). Our finding that aspirin suppresses TNF-α in primary macrophages may explain why aspirin is such an effective treatment for rheumatoid arthritis. Support for this hypothesis comes from the recent observation that block of TNF-α activity with neutralizing TNF-α antibodies reduced damage to joints in rodent models of rheumatoid arthritis (37).

Last, a number of genes have been demonstrated to be or are good candidates to be regulated by NF-κB in macrophages. Among these genes are macrophages, granulocytes, and granulocyte/macrophage colony-stimulating factors, MCP-1/JE, interleukin-1, interleukin-6, tissue factor, interleukin-1 receptor α-chain, and inducible nitric oxide synthetase (5, 26–28). For genes whose expression in macrophages is dependent on inducible NF-κB, aspirin may act as a suppressor. Support for this hypothesis comes from the recent observation that aspirin and salicylate can suppress the NF-κB-regulated genes, tissue factor gene, and inducible nitric oxide synthetase in primary human monocytes and macrophage-like cell lines (22, 28, 29). These findings, combined with the observations made here, suggest that aspirin may exert some of its anti-inflammatory effects through the suppression of monocyte/macrophage-derived inflammatory mediators.

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Send reprint requests to: Salvatore V. Pizzo, M.D., Ph.D., Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710. E-mail: pizzo0001@mc.duke.edu