Aspirin and Salicylate Suppress Polymorphonuclear Apoptosis Delay Mediated by Proinflammatory Stimuli

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ABSTRACT

During inflammation, polymorphonuclear leukocyte (PMN) apoptosis can be delayed by different proinflammatory mediators. Classically, it has been accepted that the widely used anti-inflammatory drug acetyl salicylic acid (ASA) exerts its action through inhibition of cyclooxygenases and subsequent prostaglandin synthesis. We hypothesized that another anti-inflammatory action of ASA could be the shortening of PMN survival. We found that at therapeutic concentrations (1–3 mM), ASA and its metabolite salicylate (NaSal), but not indomethacin or ibuprofen, counteracted the prolonged PMN survival mediated by lipopolysaccharide (LPS) through inhibition of nuclear factor-κB (NF-κB) activation. Both salicylates also inhibited interleukin (IL)-1α or acidic conditions antiapoptotic activity. Higher concentrations of both drugs had a direct apoptotic effect. Salicylates were not effective when PMN apoptosis delay was induced by granulocyte macrophage–colony-stimulating factor (GM-CSF), a NF-κB-independent cytokine. Promotion of PMN survival by the combination of IL-1α and LPS was also reversed by salicylates, but higher concentrations were required. ASA concentrations that did not trigger PMN death increase the zymosan- or tumor necrosis factor-α-mediated proapoptotic effect. The LPS- and IL-1α- but not GM-CSF-mediated antiapoptotic effect was markedly reduced in PMNs from donors who had ingested ASA. Using a thioglycolate-induced peritonitis model, we showed that in ASA- or NaSal-treated mice there was not only a decrease in the number of cells recruited but also an increase in the percentage of apoptotic PMNs as well as an enhancement of phagocytosis compared with controls. Our findings demonstrate that acceleration of PMN apoptosis by turning off the NF-κB-mediated survival signals elicited by proinflammatory stimuli is another anti-inflammatory action of ASA and NaSal.

Polymorphonuclear leukocyte (PMNs) are short-lived phagocytic leukocytes that are rapidly recruited from the bloodstream to the site of tissue damage or infection. PMNs dominate the early response against invading microbes. During inflammation, and to optimize PMN bactericidal function, a range of inflammatory mediators, including interleukin (IL)-1α, granulocyte-colony stimulating factor, granulocyte macrophage–colony-stimulating factor (GM-CSF), lipopolysaccharide (LPS), or even acidic conditions function to prolong PMN life span (Trevani et al., 1999; Akgul et al., 2001). After killing the phagocytosed microbe, PMNs die by apoptosis. Both in vitro and in vivo evidence suggest that PMN apoptosis and PMN clearance by macrophages are the major mechanisms for promoting resolution of inflammation (Savill and Fadok, 2000). In fact, dysregulation of apoptosis will lead to persistence of cell exudates at inflammatory sites and has been implicated in the pathogenesis of chronic inflammatory diseases, including glomerulonephritis and systemic lupus erythematosus (Taylor et al., 2000).

Acetyl salicylic acid (ASA), also named aspirin, is a widely used nonsteroidal anti-inflammatory drug. Although suppression of proinflammatory prostaglandins synthesis by inhibition of cyclooxygenase (COX) is the major effect of ASA and its metabolite, salicylate (Mitchell et al., 1993; Xu et al., 1999), other mechanisms, including the synthesis of 15-epi-lipoxin A4 and the release of adenosine (Cronstein et al., 1999) or nitric oxide (Paul-Clark et al., 2004) also seem to be involved in the anti-inflammatory action of ASA.

ABBREVIATIONS: PMN, polymorphonuclear leukocyte; IL, interleukin; GM-CSF, granulocyte macrophage–colony-stimulating factor; LPS, lipopolysaccharide; ASA, acetyl salicylic acid; COX, cyclooxygenase; NaSal, sodium salicylate; PI, propidium iodide; FITC, fluorescein isothiocyanate; NF-κB, nuclear factor-κB; TNF, tumor necrosis factor.

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Nuclear factor-κB (NF-κB) is a critical regulator of the innate early pathogen response, playing an important role in promoting inflammation, in the control of cell proliferation and survival of many cell types. NF-κB normally exists as an inactive cytoplasmic complex whose predominant form is a heterodimer composed of p50 and p65 subunits tightly bound to inhibitory proteins of the IκB family (Baldwin, 1996). NF-κB is activated by numerous diverse agents, including cytokines, viral infection, UV radiation, and free radicals. Upon activation, the inhibitors are phosphorylated and rapidly degraded allowing NF-κB to translocate into the nucleus to activate target genes (Birbach et al., 2002). In PMNs, activation of NF-κB controls the expression of many anti-apoptotic genes, including members of the Bcl-2 family (Whatman Schleicher and Schuell, Keene, NH). We therefore hypothesize that salicylates also exert their anti-inflammatory action by interfering with PMN survival.

Materials and Methods

Isolation of Human PMNs. PMN were isolated from peripheral blood drawn from healthy donors who had provided informed written consent subsequent to approval from institutional ethics committees. PMNs were isolated by Ficoll Hypaque (Glycogen Products, Buenos Aires, Argentina), and dextran sedimentation, as described previously (Trevani et al., 1999). Cell suspensions contained >96% neutrophils, as determined by May Grunwald-Giemsa-stained cytops, and the levels of monocyte contamination were always <0.2%, as evaluated by CD14 staining and flow cytometry. The cells were suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal calf serum (Invitrogen) at a concentration of 2.5 × 10^6/ml.

Cell Cultures. PMN suspensions (100–μl aliquots) were placed in 96-well flat-bottom microwells in a humidified incubator at 37°C and 5% CO2. After a 30-min incubation without (controls) or with nonsteroidal anti-inflammatory drugs (ASA, NaSal, indomethacin, and ibuprofen; all from Sigma-Aldrich, St. Louis, MO), cells were stimulated with LPS, IL-1α, and GM-CSF (Sigma-Aldrich), or the pH was adjusted to 6.5 by addition of isotonic HCl. All drugs were freshly prepared for each experiment and dissolved in RPMI 1640 medium with exception of indomethacin that was dissolved in ethanol. The final ethanol concentrations [0.1% (v/v)] did not induce PMN cytotoxic effects.

Quantitation of PMN Apoptosis and Viability by Fluorescence Microscopy. Cells were analyzed for changes in morphology and viability by labeling cells with a mixture of the fluorescent DNA binding dyes: 100 μg/ml acridine orange to determine the percentage of cells that had undergone apoptosis and 100 μg/ml ethidium bromide to differentiate between viable and nonviable cells (Pozner et al., 2005). Necrotic cells never exceeded 1%.

Quantitation of PMN Apoptosis by Propidium Iodide Staining and Flow Cytometry. Hypodiploid cells were detected by flow cytometry as described previously (Pozner et al., 2005). PMN were suspended in 1 ml of ice-cold 70% ethanol for 30 min, washed three times, and then resuspended in a solution containing 1 mg/ml RNAse (Sigma-Aldrich) and 2 μg/ml propidium iodide (Sigma-Aldrich). After 15 min, cells were analyzed by flow cytometry. The red fluorescence of individual nuclei was measured using a FACScanLibr flow cytometer (BD Biosciences, Franklin Lakes, NJ).

Annexin-V Binding Assay. Phosphatidylserine expression was detected by Annexin-V binding and PI staining using a commercial kit (Sigma-Aldrich). In brief, 20 h after different treatments, 1.5 × 10^6 PMNs were washed and incubated for 30 min with Annexin-V- FITC at 37°C. Cells were then resuspended in 400 μl of binding buffer containing PI and immediately analyzed by flow cytometry.

Degradation of iIkB by Western Blot Analysis. Whole-cell lysates were prepared using a 3% SDS lysis buffer (Roche Diagnostics, Mannheim, Germany). Lysates were boiled for 5 min, and protein was quantified using the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Samples were then frozen at −80°C until use. Equal amounts of proteins (40 μg/sample) were separated by electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were then electrotransferred to polyvinylidene difluoride membranes (Whatman Schleicher and Schuell, Keene, NH). After blocking, membranes were incubated overnight at 4°C with a rabbit polyclonal anti-iIkB antibody (BD Biosciences) followed by a goat anti-rabbit-horseradish peroxidase secondary antibody (Cell Signaling Technology Inc., Beverly, MA). Protein bands were visualized by enhanced chemiluminescence (ECL Plus; GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Animals and Drug Dosing. Male BALB/c mice (18–22 g; between 8 and 10 weeks of age) were housed and bred in the animal facility at the Hematological Research Institute under the Argentine Home Office regulations for the care and use of animals. ASA (200 mg/kg), 200 mg/kg NaSal, or vehicle was dosed orally 24 and 1 h before initiation of peritonitis or blood collection.

Isolation of Murine PMNs. Blood samples were obtained by puncture of the retro-orbital plexus. PMNs were isolated by Ficoll-Hypaque (d = 1.077) gradient centrifugation (Pozner et al., 2005). Monocyte contamination determined by May-Grunwald-Giemsa-stained blood smears was always <10%.

Animal Model of Inflammation. Mice were injected intraperitoneally with 3% thioglycollate in sterile phosphate-buffered saline as described previously (Potter et al., 2003). Exudates were collected 4 h after stimulation by lavaging the cavity with 5 ml of PBS/EDTA (5 mM), and then samples were kept on ice. Lavage fluids were stained with Turk’s solution (0.01% crystal violet in 3% acetic acid), and cell counts were performed by light microscopy using a Neubauer hemocytometer (BOECO, Hamburg, Germany). Phagocytosis was scored on May-Grunwald stained cytospins. Between 300 and 400 macrophages were counted and the phagocytosis was expressed as the percentage of macrophages that contained >50% of an apoptotic body within its body (Fadok et al., 1992). PMN apoptosis was quantified by flow cytometry, containing the cells with Annexin-V-FITC (Sigma-Aldrich) and biotinylated anti-Gr-1 antibody (which specifically recognizes PMNs) followed by streptavidin-phycocerythrin (BD Biosciences PharmMingen, San Diego, CA).

Statistical Analysis. Data are expressed as mean ± S.E.M. and were analyzed by one-way analysis of variance followed by Bonferroni multiple comparison test to determine significant differences between groups. p values < than 0.05 were considered statistically significant.

Results

Aspirin and NaSal Inhibit Delay of PMN Apoptosis Induced by Several Proinflammatory Stimuli. In the first experiments, we analyzed the effect of ASA and its metabolite, NaSal, on PMN life span. When apoptosis was revealed by fluorescence microscopy using the fluorescent DNA binding dyes acridine orange and ethidium bromide, we found that both drugs alone significantly triggered PMN-programmed cellular death only at the highest concentration used (necrosis was always <1%) (Fig. 1A). Similar results were obtained when apoptosis was analyzed by Annexin-V-FITC/PI staining (Fig. 1B). In agreement with previous re-
ports (Colotta et al., 1992), we observed that stimulation of PMNs with 0.5 μg/ml LPS promotes cell survival. However, preincubation with ASA or NaSal significantly suppressed the LPS-mediated increase in PMN survival in a concentration-dependent manner (Fig. 2, A and B).

Because, in addition to LPS, cytokines and the acidic conditions of the inflammatory milieu can also modulate PMN death rate (Colotta et al., 1992; Trevani et al., 1999), we also studied the effect of salicylates on the prolonged PMN survival mediated by three different stimuli: IL-1α, GM-CSF, and acidic conditions. Table 1 summarizes the results obtained by detection of hypodiploid nuclei in permeabilized cells stained with PI and flow cytometry. Either ASA or NaSal at low concentration (1 mM) almost completely prevented the 10 ng/ml IL-1α or pH 6.5 antiapoptotic effect. In contrast, the cellular death protection mediated by 10 ng/ml GM-CSF was not changed by PMN incubation with ASA or NaSal. Although at higher concentrations of both drugs the GM-CSF cytoprotection was slightly reduced, it was still significantly different compared with salicylates alone (Fig. 3).

When the delay in PMN apoptosis was induced by the simultaneous addition of 0.5 μg/ml LPS and 10 ng/ml IL-1α, ASA and NaSal (3 mM) were also effective (control, 45 ± 7%; LPS + IL-1α, 10 ± 4%; ASA + LPS + IL-1α, 29 ± 7%; NaSal + LPS + IL-1α, 38 ± 6% of apoptotic cells; n = 4; *p < 0.05 versus LPS + IL-1α).

**ASA and NaSal Synergized with Proapoptotic Stimuli.** During the course of inflammation, macrophages, lymphocytes, and/or mast cells produce TNF-α (Vassalli, 1992; Tracey and Cerami, 1993). It has been shown that although prolonged incubations (>12 h) of human neutrophils with TNF-α can cause a decrease in apoptosis, they can also induce programmed cell death in a subpopulation of cells at earlier times of incubation (<8 h) (Murray et al., 1997). Thus, we examined the effect of ASA on apoptosis induced by a 3-h exposure to TNF-α and other proapoptotic stimuli, such as zymosan. Although at these early times ASA was unable to induce PMN apoptosis per se, it did enhance programmed cell death mediated by 10 ng/ml TNF-α or 150 μg/ml zymosan (Fig. 4). Similar results were obtained with NaSal (data not shown).

**Salicylates Prolong PMN Survival by a Cyclooxygenase-Independent Pathway.** Whereas ASA inhibits COX-1 and -2 activities by acetylating the enzyme (Mitchell et al., 1993), salicylic acid interferes with COX-2 transcription (Xu et al., 1999). Although the involvement of COX-2 in the LPS-mediated delay of PMN apoptosis has not been described, it is known that LPS triggers COX-2 transcription in several cell types, including neutrophils (Maloney et al.,
Thus, in the next experiments, we analyzed whether other nonsteroidal anti-inflammatory drugs not structurally related with salicylates, but acting through COX inhibition, were able to accelerate PMN death. We found that in contrast to salicylates, preincubation of PMN with 100 μM indomethacin or 100 μM ibuprofen did not modify the antiapoptotic activity exerted by 0.5 μg/ml LPS (control, 55 ± 11; LPS, 27 ± 4; indomethacin + LPS, 24 ± 6; and ibuprofen + LPS, 27 ± 5% of apoptotic cells; n = 5), suggesting that salicylates effect was independent of COX.

It has been previously described that inhibition of NF-κB activation might be another mechanism by which ASA or NaSal exerts its anti-inflammatory action (Kopp and Ghosh, 1994). The involvement of NF-κB pathway was evaluated directly by Western blot analysis of its inhibitor IκB. As shown in Fig. 5, although 0.5 μg/ml LPS degraded intracellular IκBα, pretreatment of PMNs with ASA or NaSal markedly inhibited this effect. In contrast, indomethacin or ibuprofen had no effect.

**LPS and IL-1α, but Not GM-CSF, Antia apoptotic Effect Is Inhibited by ASA Ingestion.** To take these experiments further, we examined the antiapoptotic effect of 0.5

### Table 1

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<td>Control</td>
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<tr>
<td>None</td>
<td>40 ± 3</td>
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<tr>
<td>pH 6.5</td>
<td>28 ± 3*a</td>
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<td>IL-1α, 10 ng/ml</td>
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<td>GM-CSF, 10 ng/ml</td>
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*a p < 0.05 versus none/control.

*b p < 0.05 versus pH 6.5 or IL-1α without ASA or NaSal/control (n = 10).

Fig. 2. PMNs with or without ASA or NaSal were treated or not with 0.5 μg/ml LPS. Apoptosis was evaluated 20 h later by fluorescence microscopy (mean ± S.E.M.; n = 10) (A) or propidium iodide staining and flow cytometry (B). One representative experiment from five independent experiments performed in duplicate is shown in B. ***, p < 0.001 versus ASA or NaSal (0 mM) (open columns); ###, p < 0.01 versus ASA or NaSal (0 mM) (closed columns).
we examined the effect of both salicylates in the removal of PMNs using the thioglycolate-elicited peritonitis experimental model. In ASA- or NaSal-treated animals, we observed a dramatic reduction in the total cell number recruited 4 h after thioglycolate injection (Fig. 7A). Since nuclear morphological changes alone did not allow the accurate identification of the cell type that was undergoing apoptosis, we determined the presence of apoptotic PMN by staining the cells with Annexin-V-FITC and biotinylated anti-Gr-1 antibody (which specifically recognizes PMNs) followed by Streptavidin-phycocyanin (Fadok et al., 1992). Figure 7B demonstrates that in ASA- or NaSal-treated animals, there was a significant increase in the percentage of PMNs showing Annexin-V-positive cells. This was consistent with a 2.5-fold increase in the percentage of phagocytes (Figs. 7C and 8).

These data strongly suggest that both ASA and NaSal accelerate resolution of inflammation by shortening PMN life span and promoting engulfment of PMNs by resident macrophages. Finally, we analyzed whether the apoptosis of PMN from ASA- or NaSal-treated mice was also increased ex vivo after LPS- or IL-1α-stimulation. As observed in human donors, the apoptosis delay mediated by both compounds was significantly reduced in PMN from ASA-treated donors (Fig. 6).

ASA and NaSal Promote Resolution of Inflammation by Accelerating PMN Apoptosis. In the next experiments,

µg/ml LPS, 10 ng/ml IL-1α, or 10 ng/ml GM-CSF on PMNs obtained from individuals who had taken 2.0 g of ASA the day before sampling. Remarkably, although GM-CSF effect was not modify, the LPS- or IL-1α-mediated delay of PMN death was significantly reduced in PMN from ASA-treated donors (Fig. 6).

Fig. 3. PMNs with or without ASA or NaSal were treated or not with 10 ng/ml GM-CSF. Apoptosis (mean ± S.E.M.; n = 4) was evaluated 20 h later by propidium iodide staining and flow cytometry. *** p < 0.001 versus ASA or NaSal (0 mM) (open column); #, p < 0.05 versus ASA or NaSal (0 mM) (closed column); Δ, p < 0.05 or ΔΔ, p < 0.01 versus the same concentration of ASA or NaSal without GM-CSF.

**Discussion**

Our observations revealed that both in vitro and ex vivo, ASA and NaSal abrogated the survival signals elicited in
human PMNs by several different proinflammatory stimuli. Considering that the observed salicylate effect was obtained using similar concentrations to those attained in plasma during anti-inflammatory therapy (1–3 mM) (Weissmann, 1991), our data strongly suggest that interference with PMN survival represents an additional mechanism by which both salicylates exert anti-inflammatory action. It is noteworthy that higher ASA or NaSal concentrations showed a direct proapoptotic effect. Although these are not common therapeutic doses, because salicylates accumulate at the mildly acidic environment (Brooks and Day, 1991; Weissmann, 1991), it cannot be rule out that such salicylate levels might be reached at sites of inflammation. Interestingly, we demonstrated that not only cytokine- or LPS-mediated prolongation of PMN life span was abrogated by ASA or salicylate but also the prevention mediated by extracellular acidosis, a condition frequently associated not only with a variety of inflammatory conditions (Edlow and Sheldon, 1971) but also with tumor microenvironments (Tannock and Rotin, 1989).

Although COX activity or transcription is inhibited by ASA and NaSal, respectively (Vane, 1971; Xu et al., 1999), this pathway seemed not to be involved in acceleration of PMN death, because indomethacin or ibuprofen, structurally unrelated inhibitors of COX, were unable to modify the antiapoptotic activity exerted by LPS. The observation that LPS-mediated degradation of the NF-κB inhibitor IκBα was completely suppressed by pretreatment with ASA or NaSal revealed that blockade of NF-κB activation is at least one of the signaling pathways mediating the salicylate-induced shortening of PMN half-life. Moreover, our data showing that indomethacin or ibuprofen did not block LPS-mediated degradation of IκBα and that prolongation of PMN survival mediated by GM-CSF, a cytokine that exerts its effect independently of NF-κB translocation (McDonald et al., 1997), was not modified by salicylates further support the involvement of this transcription factor in ASA or NaSal proapoptotic activity. It is noteworthy that under conditions where apoptotic nuclei were not yet observed (3 h post-ASA or salicylate treatment) both drugs were capable to potentiate the proapoptotic effect of TNF-α or zymosan. These results are in agreement with previous reports showing that NF-κB inhibitors induced PMN apoptosis or augmented the early proapoptotic effects of TNF-α (Ward et al., 1999).

It was previously demonstrated that in rat primary neuronal cultures and hippocampal slices, activation of NF-κB also accounted for the protective action of salicylates against neurotoxicity elicited by glutamate (Grilli et al., 1996). Therefore, it is quite possible that, depending on whether the stimulus for NF-κB activation triggers antiapoptotic proteins or proapoptotic proteins, salicylates could selectively promote cell survival or accelerate programmed cell death, respectively.

During the preparation of this article, Derouet et al. (2006) reported that NaSal greatly enhanced neutrophil apoptosis by modification in the rate of myeloid cell leukemia 1 turnover over modulation of the activities of members of the mitogen-activated protein kinase family. Thus, it seems that several pathways might be involved in NaSal-mediated regulation of PMN survival. More experiments are required to...
determine whether mitogen-activated protein kinase transduction pathway is also regulated by ASA.

Recent data obtained from in vivo inflammation models indicate that the reduction of leukocyte accumulation within the inflamed areas is another relevant mechanism by which salicylates exert their anti-inflammatory action. Whereas in the in-pouch model both ASA and salicylate-mediated inhibition of PMN influx was associated with the release of adenosine (Cronstein et al., 1999), in thioglycolate-induced peritonitis ASA-mediated induction of nitric oxide release potently inhibits leukocyte/endothelium interaction preventing leukocyte migration (Paul-Clark et al., 2004). This latter effect, which was not shared by NaSal, was related to the acetylation of the active site of inducible COX and generation of a family of lipid mediators called the epi-lipoxins, mainly 15-epi-lipoxin A4. Using the same model, we not only found that both ASA- and NaSal-treated animals showed a marked decrease in leukocyte influx but also we demonstrated that removal of cells from the inflamed peritoneal cavity was associated with an increased percentage of apoptotic PMNs that correlated with an enhanced macrophage phagocytosis. Although we did not directly address the mediator involved in these in vivo salicylates phenomena, our data and previous findings showing that ASA inhibits the production of several inflammatory mediators through the inhibition of NF-κB activation (Pernsterorfer et al., 1999; Tegeder et al., 2001; Yoo et al., 2001), suggests that the reduced accumulation of peritoneal exudates cells and the augmentation of PMN apoptosis in ASA- or NaSal-treated mice could be related to NF-κB inhibition resulting in both, a reduced transduction of PMN antiapoptotic signals and suppression of proinflammatory substances responsible for the delay in PMN survival. In the same line of evidence, it was demonstrated that administration of a membrane-permeable IκBα repressor resulted in an increased infiltration of cells together with an increased apoptosis of leukocytes at sites of inflammation (Blackwell et al., 2004).

Finally, we examined the effect of ASA intake in human ex vivo experiments and we found that the antiapoptotic activity of LPS or IL-1α, but not of GM-CSF, was significantly reduced in PMN from normal individuals who had ingested ASA. The fact that the increased PMN apoptosis was only observed with those agonists acting through NF-κB strengthens the involvement of this signaling pathway in the ASA effect. It is well known that cyclooxygenase inhibition by aspirin is dose-dependent, rapid, and irreversible and that the length of its effect depends on cell capability to synthesize new protein (Higgs, 1983). In contrast, the kinetics of NF-κB inhibition by aspirin is less known. Considering that it was demonstrated that ASA binding to IκB kinase complex β kinase, although not covalent, is very slowly reversible (Yin, 1998), it is possible to speculate that a similar de novo protein synthesis-dependent mechanism also regulates the extent of NF-κB inhibition mediated by aspirin. In this regard, it would be conceivable that although a single dose of aspirin would transiently block NF-κB pathway, a regular ingestion (e.g., treatment for chronic inflammatory diseases) would result in a permanent suppression. However, more studies are required to determine the precise pharmacokinetics of NF-κB inhibition by salicylates.

In conclusion, our results provide another mechanism by which salicylates reduce leukocyte accumulation and promote resolution of inflammation: turning off the PMN NF-κB-mediated survival signals elicited by proinflammatory stimuli, acceleration of PMN apoptosis, and enhancement of PMN removal from the inflammatory area.

Acknowledgments

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References


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