ACCELERATED COMMUNICATION

Sodium Salicylate Inhibits Cyclo-Oxygenase-2 Activity Independently of Transcription Factor (Nuclear Factor κB) Activation: Role of Arachidonic Acid

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SUMMARY

Acetylsalicylic acid (aspirin) is the drug most commonly self-administered to reduce inflammation, swelling, and pain. The established mechanism of action of aspirin is inhibition of the enzyme cyclo-oxygenase (COX). Once taken, aspirin is rapidly deacetylated to form salicylic acid, which may account, at least in part, for the therapeutic actions of aspirin. However, where tested, salicylic acid has been found to be a relatively inactive inhibitor of COX activity *in vitro*, despite being an effective inhibitor of prostanoids formed at the site of inflammation *in vivo*. Recently, the identification of a cytokine-inducible isoform of COX, COX-2, has led to the suggestion that salicylate produces its anti-inflammatory actions by inhibiting COX-2 induction through actions on nuclear factor κB (NF-κB). We have used interleukin 1β–induced COX-2 in human A549 cells to investigate the mechanism of action of salicylate on COX-2 activity. Sodium salicylate inhibited prostaglandin E_2 release when added together with interleukin 1β for 24 hr with an IC_{50} value of 5 μg/ml, an effect that was independent of NF-κB activation or COX-2 transcription or translation. Sodium salicylate acutely (30 min) also caused a concentration-dependent inhibition of COX-2 activity measured in the presence of 0, 1, or 10 μM exogenous arachidonic acid. In contrast, when exogenous arachidonic acid was increased to 30 μM, sodium salicylate was a very weak inhibitor of COX-2 activity with an IC_{50} of >100 μg/ml. Thus, sodium salicylate is an effective inhibitor of COX-2 activity at concentrations far below those required to inhibit NF-κB (20 mg/ml) activation and is easily displaced by arachidonic acid.

The anti-inflammatory properties of extracts from willow trees have been documented for almost 2000 years, and the active ingredient has been identified as salicylate. To improve the original preparations, salicylate was acetylated, yielding acetylsalicylic acid, also known as aspirin (1). Acetylsalicylic acid is better tolerated than salicylic acid but has comparable anti-inflammatory properties (2). Both salicylic acid and acetylsalicylic acid are members of a larger group of chemically diverse drugs known as NSAIDs. In 1971, Vane (3) demonstrated that a range of NSAIDs, including acetylsalicylic acid and sodium salicylate, inhibited the enzyme COX. COX is the first enzyme in the generation of prostanoids from arachidonic acid and has recently been demonstrated to exist in two distinct isoforms. COX-1 is present constitutively and is thought to be responsible for the "housekeeping" functions of the enzyme (4), whereas COX-2 is induced by pro-inflammatory agents *in vitro* (5, 6) and predominates at the site of inflammation *in vivo* (7–9). Since this time, most NSAIDs have been tested and demonstrated to inhibit both COX-1 and COX-2 (10–15). Thus, inhibition of COX and the subsequent reduction in the generation of pro-inflammatory prostanoids is the most established mechanism of action of NSAIDs. Nevertheless, recent reports have suggested that several NSAIDs, particularly the salicylates, exert their therapeutic benefits by inhibiting the "inflammatory" transcription factor NF-κB (16). Indeed, the mechanisms by which different salicylates inhibit COX activity are unresolved. Acetylsalicylic acid inhibits COX by acetylation of an essential serine at the active site of the enzyme (17). However, as salicylic acid lacks an acetyl group, its mechanism of inhibition is unclear. Moreover, in several *in vitro*

ABBREVIATIONS: NSAIDs, nonsteroidal anti-inflammatory drugs; COX, cyclo-oxygenase; PGE_2, prostaglandin E_2; NF-κB, nuclear factor κB; SDS, sodium dodecyl sulfate; IL-1β, interleukin 1β.

systems, salicylic acid seems to be an ineffective inhibitor of COX activity (3, 10) despite being an effective inhibitor of COX at the site of inflammation *in vivo* (18). Thus, the mode of action of salicylic acid remains unclear. However, the tertiary structure of COX-1 contains a channel within the active site with a predicted affinity for arachidonic acid and also for salicylate (19). Such evidence suggests that salicylate may compete with arachidonic acid for the active site of the COX enzyme. We have therefore used a well characterized model of COX-2 induction in human cells (20) to establish the role of NF-κB and exogenous arachidonic acid in the ability of salicylic acid to effectively inhibit COX-2 activity *in vitro*.

Materials and Methods

All compounds used were obtained from Sigma Chemical (Poole, UK) unless otherwise stated. Data were analyzed using the appropriate statistical tests and are described accordingly. A *p* value of less than 0.05 was taken as significant.

Culture of A549 cells. The human pulmonary epithelial cell line A549 was purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in either 96- or 6-well culture plates, as indicated, with DMEM (Gibco, Paisley, UK) containing 10% fetal calf serum, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 u/ml penicillin. When A549 cells were confluent, they were washed and incubated in DMEM with 10% fetal calf serum together with IL-1β (10 ng/ml; Genzyme, West Malling, UK) to induce COX-2, as we have previously reported (20). Under these conditions, A549 cells release PGE$_2$ in a time-dependent manner, which first becomes significant at 6–12 hr (20).

Protocols used to assess the effects of sodium salicylate on COX-2 activity in A549 cells. To assess the effects of drugs on all aspects (i.e., transcription, translation, and activation) of the COX-2 pathway as well as on NF-κB activation, A549 cells were treated with sodium salicylate together with IL-1β for 1, 6, or 24 hr, and the release of PGE$_2$ was measured by radioimmunoassay (10). Antibodies to PGE$_2$ were obtained from Sigma (Poole, Dorset, UK). Tritiated PGE$_2$ was obtained from Amersham International (Amersham, Bucks, UK).

To assess the direct effect of sodium salicylate on COX-2 activity after induction had occurred, cells were first treated with IL-1β for 24 hr, and the culture medium was replaced with DMEM containing different concentrations of the drug. Cells were incubated at 37° for 30 min. Arachidonic acid (1–30 μM) was then added for 15 min, and the medium was removed for the measurement of PGE$_2$.

Cell viability. Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, as previously described (10). Sodium salicylate had no effect on cell viability at concentrations that completely blocked PGE$_2$ formation (up to 1 mg/ml) in any of the protocols used.

Measurement of NF-κB activation. NFκB activation was measured by electrophoretic mobility shift assays, as described previously (21). For experiments designed to assess the effects of sodium salicylate on NF-κB activation, A549 cells were cultured in 6-well culture plates. sodium salicylate (160 μg/ml or 20 mg/ml) was added to the cells together with IL-1β (10 ng/ml) for 1 hr. Cells were harvested and nuclear proteins prepared according to the method described by Osborn *et al.* (22). NFκB was measured by electrophoretic mobility shift assays, as described previously (21). Binding reactions (25 μl) contained nuclear protein, 4% glycerol, 1 mM MgCl$_2$, 0.5 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl, 10 mM Tris (pH 7.5), and 80 μg/ml microwaved salmon sperm DNA. After incubation on ice for 10 min, 17.5 fmol of [^{32}P] kinase-labeled double-stranded oligonucleotide probe was added. The consensus oligonucleotide (Promega, Madison, WI) for NF-κB was 5′-AGT TGA GGG GAC TTT CCC AGG-3′ (sense strand). Binding reactions were carried out on ice for 40 min. The specificity of binding was determined by the prior addition of 100-fold excess of unlabeled competitor consensus oligonucleotide. Bound and unbound probe were separated on 7% nondenaturing acrylamide gels. After vacuum drying, retarded bands were detected by autoradiography.

Measurement of COX-2 mRNA expression by reverse-transcription PCR. A549 cells were cultured in 6-well plates and treated with IL-1β (10 ng/ml) or IL-1β together with sodium salicylate for 6 hr before total RNA was extracted, and reverse-transcription PCR was carried out, as previously described (21). Primers for PCR amplification of GAPDH were as described (21). Primers for COX-2 were for sense primers, TTC AAA TGA GAT TGT GGG AAA ATT GCT (bases 574–600), and for anti-sense primers, AGA TCA TCT CTG CCT GAG TAT CTT T (bases 878–855). Cycling parameters were: 94°, 30 sec; specific annealing temperature, 1 min; 72°, 1 min. Annealing temperatures were 58° for both GAPDH and for COX-2. In each case, the exponential phase of amplification, at which time the starting material is proportional to the product formed, was determined by performing cycle profiles on average samples, as previously described (21). For GAPDH and COX-2, 23 and 24 cycles were found to be within the exponential phase of amplification and were used for PCR analysis in duplicate. Amplification products were size-fractionated on 1.5% agarose gels before Southern blotting and hybridization to the appropriate cloned cDNA to confirm the identify of the products, and because all primer pairs cross at least one intron, the possibility of genomic contamination was excluded (23). In addition, the amplification products (5 μl) were dot-blotted onto Hybond-N membranes (Amersham, Bucks, UK) and hybridized with appropriate cDNA probe. After washing at high stringency (0.1% standard saline citrate at 45°), dot blots were excised, and radioactivity was measured by Cerenkov counting (23). The amount of COX-2 mRNA detected is expressed as the percentage of that for GAPDH present in each sample.

Western blot analysis. Western blot analysis was performed as described previously (20). A549 cells were treated for 24 hr with either IL-1β (10 ng/ml) or IL-1β plus sodium salicylate at a concentration that completely blocked PGE$_2$ release, 100 μg/ml. After 24 hr, cells were washed with phosphate buffered saline (pH 7.4) and incubated (10 min) with 2–3 ml of extraction buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 μM pepstatin A, and 0.2 mM leupeptin) while being gently shaken. The cell extract was then boiled (10 min) with gel loading buffer (50 mM Tris, 10% SDS, 10% glycerol, 10% 2-mercapthethanol, 2 mg/ml bromphenol blue) in a ratio of 1:1. The samples were loaded onto 2.5% SDS stacking gels and separated on 7.5% SDS gels by electrophoresis. After transfer to nitrocellulose, the blot was primed with a specific antibody raised in rabbits to murine COX-2 (Cayman Chemical, Ann Arbor, MI). The blot was then incubated with an anti-rabbit IgG developed in sheep, linked to alkaline phosphatase conjugate, and the blot was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Purified enzymes. Human purified COX-2 were obtained from Cayman Chemical (Ann Arbor, MI). Pure COX-2 and the cofactors glutathione (5 mM), adrenaline (5 mM), and hematin (1 μM) were dissolved in 50 mM Tris buffer (pH 7.5). Hematin was first dissolved in a concentrated stock of 100 mM in 1 M NaOH before being further diluted in Tris buffer. Enzyme reactions were carried out in individual wells of 96-well plates with a final reaction volume of 200 μl. Different concentrations of sodium salicylate were added to the plate, followed by the addition of 10 units of enzyme (180 μl). The plates were incubated at 37° for 30 min before arachidonic acid (10 nM to 30 μMl) was added for a further 15 min. The reaction was stopped by heating the plate to 100° for 5 min. The 96-well plate was then centrifuged at 10,000 × g for 10 min, and appropriated samples were removed and added into the radioimmunoassay.

Results

Characterization of the effects of IL-1β on NF-κB and COX-2 activities in A549 cells. IL-1β stimulated A549 cells to release PGE_2, activated NF-κB, and stimulated the expression of COX-2 mRNA and protein. The basal release of PGE_2 from A549 cells over 24 hr was 0.11 ± 0.001 ng/ml ($n = 24$). This was increased to 12.9 ± 0.5 ng/ml when IL-1β was included in the culture medium. In addition, IL-1β–stimulated A549 cells continued to produce elevated levels of PGE_2 for at least 48 hr (data not shown). The IL-1β–induced activation of NF-κB and expression of COX-2 mRNA was maximal at earlier times (1–8 hr for NF-κB (22) and 2–6 hr for COX-2 mRNA(23)) than for prostanoid release. However, the expression of COX-2 protein was stable for at least 48 hr (data not shown).

Effects of chronic exposure to sodium salicylate on COX-2 activity, NF-κB activation, and COX-2 transcription and translation in IL-1β–treated A549 cells. When added together with IL-1β for 24 hr, sodium salicylate caused a concentration-dependent inhibition of PGE_2 release with an apparent IC_{50} value of approximately 5 μg/ml (Fig. 1). As we have previously shown (20), IL-1β–stimulated A549 cells released a lower level of the other COX metabolites, 6-keto $PGF_{1\alpha}$ (unstimulated, 0; IL-1β–stimulated, 0.17 ± 0.08 ng/ml), $PGF_{2\alpha}$ (unstimulated, 0; IL-1β–stimulated, 0.09 ± 0.01 ng/ml) and thromboxane B_2 (unstimulated, 0; IL-1β–stimulated 0.09 ± 0.06 ng/ml). However, similar to its effects on PGE_2, sodium salicylate (10 μg/ml) blocked the IL-1β–induced release of 6-keto $PGF_{1\alpha}$ (0 ng/ml), $PGF_{2\alpha}$ (0.013 ± 0.013 ng/ml), and thromboxane B_2 (0 ng/ml). In contrast, at concentrations in excess of those required to block prostanoid release (100 μg/ml), sodium salicylate had no effect on the IL-1β–induced expression of COX-2 mRNA (Fig. 2; one-way analysis of variance), protein (Fig. 3), or on NF-κB activation (Fig. 4). However, in agreement with others (16), at a concentration (20 mg/ml) that was at least 100-fold higher than that needed to completely block PGE_2 release (Fig. 1), sodium salicylate inhibited NF-κB activation (Fig. 4).

Effects exogenous arachidonic acid on the ability of sodium salicylate to directly inhibit COX-2 activity in
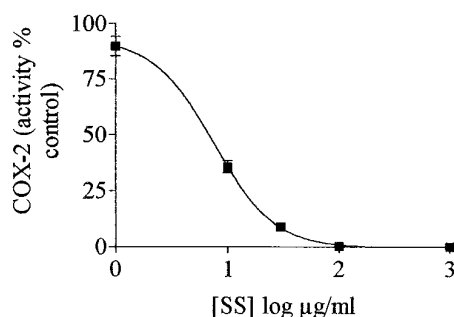
Fig. 1. Effect of sodium salicylate (*SS*), added together with IL-1β, on COX-2 activity induced in A549 cells. A549 cells were treated with different concentrations of sodium salicylate together with IL-1β (10 ng/ml) for 24 hr, and COX-2 activity was measured as the amount of PGE_2 secreted from endogenous stores of arachidonic acid. The amount of PGE_2 released in the presence of each concentration of sodium salicylate was divided by that released in control wells treated with IL-1β and the relevant vehicle control. Thus, the data are represented as the percentage of control COX-2 activity. The data represent the mean $\pm$ standard error for 10 determinations from at least 4 experimental days.
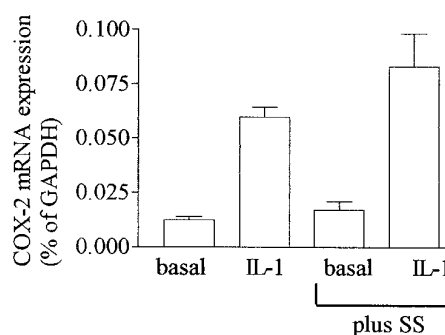

Fig. 2. Effect of sodium salicylate (*SS*) on the expression of COX-2 mRNA in A549 cells treated with IL-1β. The induction of COX-2 mRNA by IL-1β in A549 cells is maximal at 6 hr (23) Thus, cells were either left untreated (basal) or treated with IL-1β (10 ng/ml; IL-1) in the presence or absence of 100 μg/ml sodium salicylate (plus sodium salicylate) for 6 hr before DNA extraction and preparation, as detailed in Materials and Methods. The data were calculated as the ratio of GAPDH present in each sample and represent the mean $\pm$ standard error for four separate experiments.
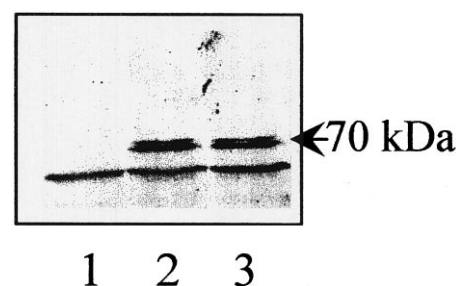
Fig. 3. Effect of sodium salicylate on the synthesis of COX-2 protein in A549 cells treated with IL-1β. A549 cells were either left untreated (*lane 1*) or treated with IL-1β (10 ng/ml) in the absence (*lane 2*) or presence (*lane 3*) of sodium salicylate (100 μg/ml) for 24 hr before cellular proteins were extracted, separated, and probed with anti–COX-2 antibodies, as described in Materials and Methods. The Western blot shows two major bands, one at 70 kDa (COX-2), and a lower molecular protein, which was detected in similar levels in untreated and IL-1β–stimulated cells. Treatment of A549 cells with sodium salicylate had no effect on the intensity of either band detected. The figure shows a representative blot, and similar observations were made in two other separate experiments.

A549 cells. The ability of sodium salicylate to directly inhibit COX-2 activity in A549 cells was tested after a 30-min exposure period, followed by the addition of different concentrations of exogenous arachidonic acid (1, 10, and 30 μM). Sodium salicylate caused a concentration-dependent inhibition of COX-2 activity in the absence of added arachidonic acid or in the presence of 1 or 10 μM exogenous substrate with an apparent IC_{50} value of approximately 5 μg/ml (Fig. 5). However, when the same experiments were performed using 30 μM arachidonic acid, sodium salicylate was an ineffective inhibitor of COX-2 activity, with an apparent IC_{50} value of more than 100 μg/ml, and achieved a maximal inhibition of less than 50%. By contrast, indomethacin (IC_{50}, 0.27 μg/ml; $n = 12$), flurbiprofen (IC_{50}, 0.220 μg/ml; $n = 12$), or aspirin (IC_{50}, 1.67 μg/ml; $n = 12$) were effective inhibitors of COX-2 activity, as measured in the presence of 30 μM arachidonic acid in intact A549 cells. In addition, unlike that for sodium salicylate, the IC_{50} value for aspirin was comparable when measured in the presence of 10 μM (3.4 ± 1 μg/ml) or 30 μM (2.5 ± 1 μg/ml) arachidonic acid ($n = 3$).
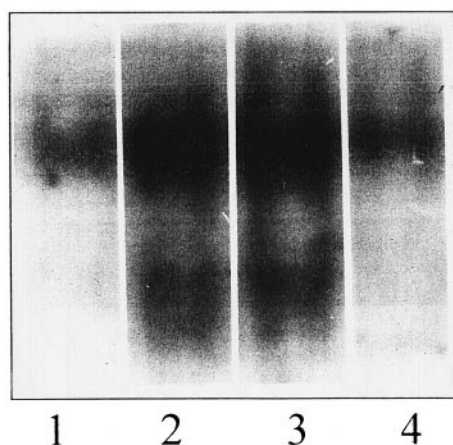
Fig. 4. Effect of sodium salicylate on NF-κB activation stimulated in A549 cells by IL-1β. The activation of NF-κB by IL-1β in A549 cells is maximal at 1 hr (23). Thus, cells were either left untreated (*lane 1*) or treated with IL-1β (10 ng/ml) in the absence (*lane 2*) or presence of sodium salicylate at 160 μg/ml (*lane 3*) or 20 mg/ml (*lane 4*) for 1 hr. The cells were then extracted, and NF-κB was measured by gel electrophoretic mobility assays, as described in Materials and Methods. NFκB activation is represented by the appearance of a lower band in *lanes 2 and 3*. No lower band is present in unstimulated cells (*lane 1*) or in cells treated with IL-1β together with a high concentration of sodium salicylate (20 mg/ml). The figure shows a representative gel, and similar observations were made in two other separate experiments. Only the shifted bands are shown.

Effects of sodium salicylate on human purified COX-2 protein. Purified COX-2 converted arachidonic acid (0.01 to 30 μM) to PGE$_2$ with maximal production occurring at 10 μM arachidonic acid of 589.8 $\pm$ 120.6 ng of PGE$_2$/unit/15 min. COX-2 activity was not significantly (two-way analysis of variance) inhibited by sodium salicylate (1, 3, 10, or 100 μg/ml) measured in the presence of any of the various concentrations of arachidonic acid (Table 1; $n = 6$). In contrast to the effects of sodium salicylate, aspirin (10 μg/ml) inhibited COX-2 activity (measured in the presence of 30 μM arachidonic acid) by 82 $\pm$ 8% ($n = 6$).

Discussion

Salicylate may have been the first anti-inflammatory preparation used by modern-day humans. However, the mechanism of action of salicylates and related drugs is still the subject of debate. Several suggestions have been made to describe how salicylates exert their anti-inflammatory and side effects. In 1971, Vane (3) showed that sodium salicylate, aspirin, and indomethacin inhibited COX and hypothesized that this is how NSAIDs exert their therapeutic effects. For the majority of the NSAIDs, this hypothesis is well supported by numerous studies from different groups. However, unlike aspirin and other NSAIDs, sodium salicylate does not inhibit COX activity in every experimental system. In particular, sodium salicylate is a very weak inhibitor of COX-1 or COX-2 (10) activity *in vitro*. However, sodium salicylate produces all of the classical effects of other NSAIDs in humans (2). Thus, various groups have looked for other mechanisms by which salicylates can modulate inflammatory responses and have cast doubt on the COX hypothesis as an explanation for how these drugs work.

In agreement with others, we found that sodium salicylate is an ineffective inhibitor of COX-2 activity preinduced in
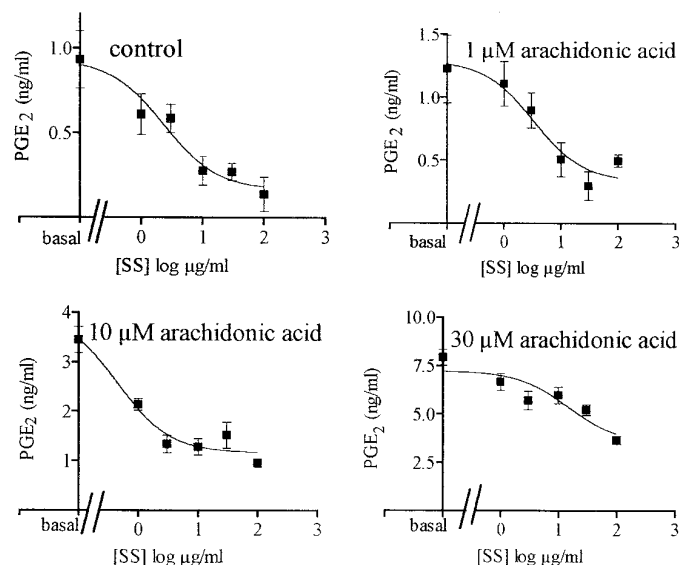
Fig. 5. Effect of different concentrations of exogenous arachidonic acid on the ability of sodium salicylate (*SS*) to inhibit COX-2 activity. A549 cells were treated with IL-1β for 24 hr to induce COX-2. Culture medium was then replaced with fresh medium containing different concentrations of sodium salicylate for 30 min. Arachidonic acid was then added for 15 min, and COX-2 activity was determined from the level of PGE$_2$ formed. The data are shown in nanogram per milliliter of PGE$_2$. The data represent the mean $\pm$ standard error for 10–16 determinations from at least 4 experimental days (10).

intact cells and measured in the presence of 30 μM exogenous arachidonic acid. However, we found that sodium salicylate was an effective inhibitor of COX-2 activity when added together with the inducing agent. Moreover, the IC$_{50}$ for sodium salicylate in these experiments was approximately 5 μg/ml, which, assuming the average person has 5 liters of blood, would provide a predicted therapeutic dose of approximately 25–50 mg. When allowances are made for metabolism *in vivo*, the potency of sodium salicylate under these conditions are in keeping with those required to produce anti-inflammatory effects in humans.

Our observations suggest to us that sodium salicylate could exert its effects on prostanoid release at the level of gene regulation. Indeed, Wu *et al.* (24) showed that sodium salicylate could inhibit the induction of COX in human umbilical endothelial cells stimulated by IL-1β. In contrast, we found that at concentrations that caused maximal inhibition of PGE$_2$ release, no effect of sodium salicylate was seen on either COX-2 mRNA expression or COX-2 protein levels. Thus, an action on COX-2 gene expression could not explain the inhibitory effects of sodium salicylate on COX-2 activity.

The transcription factor NF-κB regulates the expression of many genes, which are induced during the inflammatory response. Thus, agents that interfere with the activity of NF-κB are likely to be important anti-inflammatory agents. In fact, inhibition of NF-κB has recently been proposed as a mechanism by which salicylates exert their effects (16). However, in these studies, relatively high concentrations (1 mg/ml and greater) were required before significant effects were observed. Indeed, the inhibitory effects of NSAIDs on NF-κB have not previously been studied in parallel with their ability to block prostanoids production. We have shown that at concentrations causing maximal inhibition of COX-2, salicylate had no effect on NF-κB activation. Thus, we sug-

TABLE 1

Effects of sodium salicylate on the production of PGE$_2$ produced by purified COX-2 measured in the presence of different concentrations of arachidonic acid.

The table shows the values of prostaglandin E$_2$ (ng/unit/15 min) (mean $\pm$ standard error) for six determinations obtained in three separate experiments using three separate batches of purified COX-2

Arachidonic Acid	Sodium Salicylate (mg/ml)				
	Control	1	3	10	100
0	6 ± 3	5 ± 3	8 ± 6	12 ± 7	9 ± 6
0.01	6 ± 3	6 ± 3	11 ± 6	7 ± 2	8 ± 3
0.1	16 ± 4	13 ± 4	25 ± 6	21 ± 3	21 ± 5
1	61 ± 7	44 ± 9	45 ± 10	51 ± 5	47 ± 7
3	262 ± 58	206 ± 19	205 ± 40	219 ± 5	201 ± 12
10	729 ± 61	629 ± 85	581 ± 45	627 ± 48	597 ± 42
30	825 ± 53	954 ± 92	885 ± 36	899 ± 59	952 ± 91

gest that the primary action of sodium salicylate is to inhibit the production of pro-inflammatory prostanoids independently of any effects on NF-κB. Indeed, agents that inhibit the inflammatory process at a level as high as the induction of immediate early genes may be predicted to have a clinical profile more closely related to that of steroids than to that of NSAIDs.

How, then, does sodium salicylate inhibit the synthesis of COX-2 metabolites? We found that sodium salicylate produced effective concentration-dependent inhibitions of COX-2 when activity was supported by 1 or 10 but not by 30 μM arachidonic acid. Furthermore, under these conditions (i.e., in the presence of 1 or 10 μM arachidonic acid), the IC$_{50}$ value for sodium salicylate was comparable to that achieved when added together with IL-1β for 24 hr. Thus, we hypothesize that sodium salicylate is a weak competitive inhibitor with arachidonic acid of COX-2. This hypothesis is supported by evidence provided after the elucidation of the tertiary structure of COX-1 (19). Arachidonic acid enters the COX molecule via a channel situated at the base of the active site. Loll and Picot (19) predicted that salicylate would also have an affinity for this site and be easily dislodged by excess substrate (19). This prediction would explain our findings in intact cells. With purified enzyme, however, sodium salicylate had no inhibitory effect, even when measured at very low levels of arachidonic acid. Thus, it was not possible to perform comprehensive biochemical characterizations of the actions of sodium salicylate on COX-2. The reasons behind the discrepancy between the action of sodium salicylates on COX activity in intact cells versus purified enzyme are unclear. However, our data illustrate fundamental differences in COX-2 activity in these two systems that are worthy of further investigation. There may be, for example, cooperative elements (e.g., proteins) in intact cells that facilitate the binding of salicylate and/or arachidonic acid to COX-2. Nevertheless, these observations substantiate the rational for using whole-cell assays to study COX-2 functions and in the search for new NSAIDs.

How, then, do our observations help to explain previous studies addressing the action of sodium salicylate? In 1971, using broken cell preparations of guinea pig lung (COX-1), Vane (3) found salicylate to be a weak inhibitor of COX with an apparent IC$_{50}$ value of more than 100 μg/ml, which is in keeping with the potency we describe in our assays in which 30-μM substrates were used. The preparations of guinea pig lung used by Vane were homogenized and, therefore, likely to contain high levels of arachidonic acid. We suggest that the

relatively weak action of sodium salicylate described in Vane's study is in keeping with our observations and reflects the level of arachidonic acid present. In contrast, Higgs *et al.* (25) demonstrated that sodium salicylate and aspirin were both effective inhibitors of COX activity in inflamed sites *ex vivo*. In this study, the NSAIDs were added to the sites, which were then placed in culture for 24 hr. This approach most closely resembles our protocol in which sodium salicylate was added together with IL-1β for 24 hr. Thus, in the study by Higgs *et al.*, no exogenous arachidonic acid was added, and sodium salicylate was able to effectively inhibit COX activity. Interestingly, Whittle *et al.* (18) showed that both sodium salicylate and aspirin were able to inhibit the production of prostanoids at the site of inflammation *in vivo*. In contrast, Whittle *et al.* found that aspirin but not sodium salicylate inhibited the production of prostanoids in the gastric mucosa. In this study, COX activity at the site of inflammation (COX-2) was indexed by the concentration of prostanoids present in the implanted sponge. In contrast, activity in the gastric mucosa (COX-1) was measured by the prostanoids formed *in vitro* by minced mucosal tissue after vigorous mechanical mixing. Thus, in the implanted sponge, prostanoids will be formed by intact cells stimulated by cytokines in a similar manner to that of our A549 cells treated with IL-1β for 24 hr without the addition of high levels of exogenous arachidonic acid. However, the mincing and mixing of gastric mucosa is likely to release large amounts of arachidonic acid from tissue phospholipids and reflect the conditions we found in our A549 cells treated with high levels of exogenous arachidonic acid. Thus, we suggest that a disparity in the amount of arachidonic acid available to cells in the implanted sponge *in vivo* and the minced tissue *in vitro* is the reason why sodium salicylate seemed to have preferential effects.

These findings show that sodium salicylate is an effective inhibitor of COX activity in human cells and substantiate the hypothesis that NSAIDs exert their anti-inflammatory effects by inhibiting the enzyme COX (3). Moreover, we clearly show that the recently demonstrated action of salicylates (and other NSAIDs) on NF-κB is not responsible for their action on inflammatory prostanoid production.

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