Induction of spermidine/spermine N1-acetyltransferase (SSAT) by aspirin in Caco-2 colon cancer cells

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Epidemiological, experimental and clinical results suggest that aspirin and other NSAIDs (non-steroidal anti-inflammatory drugs) inhibit the development of colon cancer. It has been shown that the NSAID sulindac induces apoptosis and suppresses carcinogenesis, in part, by a mechanism leading to the transcriptional activation of the gene encoding SSAT (spermidine/spermine N1-acetyltransferase), a rate-limiting enzyme in polyamine metabolism. In the present study, we show that a variety of NSAIDs, including aspirin, sulindac, ibuprofen and indomethacin, can induce SSAT gene expression in Caco-2 cells. Aspirin, at physiological concentrations, can induce SSAT mRNA via transcriptional and post-transcriptional mechanisms. This induction leads to increased SSAT protein levels and enzyme activity. Promoter deletion analysis of the 5′ SSAT promoter-flanking region led to the identification of two NF-κB (nuclear factor κB) response elements. Electrophoretic mobility-shift assays showed binding of NF-κB complexes at these sequences after aspirin treatment. Aspirin treatment led to the activation of NF-κB signalling and increased binding at these NF-κB sites in the SSAT promoter, hence providing a potential mechanism for the induction of SSAT by aspirin in these cells. Aspirin-induced SSAT ultimately leads to a decrease in cellular polyamine content, which has been associated with decreased carcinogenesis. These results suggest that activation of SSAT by aspirin and different NSAIDs may be a common property of NSAIDs that plays an important role in their chemopreventive actions in colorectal cancer.

Key words: aspirin, Caco-2 colon cancer cell, nuclear factor κB (NF-κB), non-steroidal anti-inflammatory drugs (NSAIDs), polyamine, spermidine/spermine N1-acetyltransferase.

INTRODUCTION

Many epidemiological studies have revealed that the use of aspirin or other NSAIDs (non-steroidal anti-inflammatory drugs) can reduce the risk of colon cancer. The mechanism of action that defines NSAIDs as a class is their ability to inhibit the COX (cyclooxygenase) activity of the enzyme prostaglandin G/H synthase (also known as COX) and thereby block the biosynthesis of prostaglandins [1]. NSAIDs structurally consist of an acidic moiety (carboxylic acid, enols) attached to a planar, aromatic functionality. Some analgesics also contain a polar linking group, which attaches the planar moiety to an additional lipophilic group. The acidic group in these compounds serves as a major binding group (ionic binding) for plasma proteins. Thus all NSAIDs are highly bound by plasma proteins. The NSAIDs can be subclassified on the basis of chemical structure into salicylates (aspirin), propionic acid derivatives (ibuprofen), aroyl and heteroarylacetic acids (sulindac and indomethacin), oxicasms (piroxicam), anilides (acetylsalicylic) and COX-2 selective inhibitors (celecoxib). Since the well-documented pharmacological action of NSAIDs is the inhibition of COX enzymes, it could be inferred that the beneficial effect of NSAIDs may be mediated through the inhibition of prostaglandin biosynthesis. However, NSAIDs such as sodium salicylate, aspirin, sulindac, ibuprofen and indomethacin cause anti-inflammatory and anti-proliferative effects independent of COX activity and prostaglandin inhibition [2–7].

We have shown that aspirin use decreased the risk of adenoma recurrence by activating catabolism of colonic mucosal polyamines [7]. The polyamines putrescine, spermidine and spermine are abundant polyamines in eukaryotic cells, which are often elevated in neoplastic cells when compared with normal cells and tissues [8]. The polyamine levels are tightly regulated by the biosynthetic enzyme, ODC (ornithine decarboxylase), and the catabolic enzyme, SSAT, spermidine/spermine N1-acetyltransferase, in cells. High levels of polyamines are associated with cellular proliferation [9], while lower polyamine levels have been shown to promote apoptosis [10,11] and inhibit cell growth [12]. It has been shown that many other NSAIDs, besides aspirin, can regulate polyamine metabolism [7,13,14]. Indomethacin impaired the growth of human colon cancer cells, resulting in decreased ODC activity, increased intracellular SSAT enzyme activity and enhanced polyamine acetylation and efflux from colon cancer cells [13]. Aspirin, on the other hand, had no effects on ODC but induced SSAT expression and decreased polyamine levels at physiological concentrations in HT-29 colon cancer cells [7]. Sulindac sulphone can lead to the activation of PPARγ (peroxisome proliferator-activated receptor γ) in colon cancer cells, which can then induce SSAT expression, thereby leading to decreased intracellular polyamines and increased cell death [14]. Further, it was shown that the toxicity induced by sulindac, salicylate and naproxen can be prevented by polyamines, suggesting an integral role.
of polyamines in the chemopreventive action of the NSAIDs [15].

Based on the evidence above, our interest was in examining the mechanism of aspirin- and other NSAID-induced SSAT expression in Caco-2 colon cancer cells.

EXPERIMENTAL

Materials

All cell culture reagents, DNA modifying enzymes, TRIZol® reagent and Lipofectamine™ reagent were purchased from Life Technologies (Rockville, MD, U.S.A.). All primers and oligonucleotides were custom made by Invitrogen (Carlsbad, CA, U.S.A.). Indomethacin and (S)-ibuprofen were purchased from Biomol Research Laboratories (Plymouth, PA, U.S.A.), while sulindac and sulindac sulphone were purchased from ICN Biomedicals (Solon, OH, U.S.A.). Acetylsalicylic acid (aspirin) was purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

Cell culture

The Caco-2 colon cancer cell line was purchased from A.T.C.C. (Rockville, MD, U.S.A.) at passage 12 and was maintained in MEM (minimum essential α-medium; Life Technologies) supplemented with 10% (v/v) FBS (fetal bovine serum) and 50 units/ml each of penicillin and streptomycin. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2.

Plasmids

Full-SSAT-luc was made by cloning 3.493 kb of SSAT sequence 5′ of the transcription start site into a promoterless pGL2-basic vector (Promega, Madison, WI, U.S.A.) as reported previously [16]. A series of smaller SSAT promoter elements was made from Full-SSAT-luc, using PCR. 197-SSAT-luc having SSAT sequences from +83 to −197, 358-SSAT-luc having SSAT sequences from +83 to −335, 659-SSAT-luc having SSAT sequences from +83 to −659 and 791-SSAT-luc having SSAT sequences from +83 to −791 were made from Full-SSAT-luc and subcloned into pGL2-basic vector (Figure 1B). PPRE-tk-luc reporter construct, having three tandem repeats of PPREs (PPAR response elements) 5′ to the luciferase gene, was a gift from Dr Ronald Evans (The Salk Institute, La Jolla, CA, U.S.A.). NF-κB-2-luc reporter, having two NF-κB (nuclear factor κB) response elements, and ΔNF-κB-luc reporter, in which both the NF-κB response elements have been deleted, were obtained from Dr Nancy Davidson (Johns Hopkins University, Baltimore, MD, U.S.A.).

Transient transfections

Transient transfections were performed using Lipofectamine™ reagent according to the manufacturer’s instructions. Briefly, 5 × 104 cells were seeded in a 6-well plate and cultured in normal medium [MEM with 10% FBS and 1% (v/v) penicillin/streptomycin] for 24 h. Each well was transfected with 1 µg of firefly luciferase reporter construct along with 0.2 µg of pCMV-β-galactosidase expression plasmid, used as a transfection efficiency control. After a 6 h incubation with Lipofectamine™–DNA complex, cells were supplemented with complete medium containing 20% FBS and 2% penicillin/streptomycin and grown overnight. On the following day, the transfection media were removed and cells were given fresh media with the indicated concentrations of aspirin or vehicle and treated for 48 h. All transfections were performed in triplicate. Transfected cells were washed once with PBS and lysed, and luciferase activities were measured using 10 µl of cell extract and 50 µl of luciferase reagent (Promega). β-Galactosidase activity was measured using the β-gal assay kit (Invitrogen) according to the manufacturer’s instructions.

RNA isolation and analysis

Total cellular RNA was extracted using TRIZol® reagent and RNA isolation according to the method developed by Chomczynski and Sacchi [17]. Total RNA (20 µg) was separated on 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond-N; Amersham Biosciences). The cDNA probes for Northern blots were prepared using [α-32P]dCTP and the RTS RadPrime DNA labelling system (Life Technologies). The probe was purified with G-50 Sepharose columns (Boehringer Mannheim, Roche, Indianapolis, IN, U.S.A.) and quantified using a scintillation counter. Membranes were hybridized overnight at 42°C with 32P-labelled cDNA encoding human SSAT (EcoRI–EcoRI fragment), and then washed twice in 2× SSC/0.1% SDS at room temperature (25°C), for 5 and 20 min respectively, and once in 0.5× SSC/0.1% SDS for 20 min at 65°C (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7). GAPDH (glyceraldehyde-3-phosphate dehydrogenase; 0.75 kb PstI–XbaI fragment) was used as a control for RNA loading. Northern-blot autoradiograms were quantified by densitometric analysis (ImageQuant; Molecular Dynamics, Sunnyvale, CA, U.S.A.). Results are expressed as the ratio of the integrated densities of 32P-labelled hybridization bands for the SSAT and GAPDH mRNAs.

Gel electrophoretic mobility-shift assays

Nuclear extracts were prepared from Caco-2 cells as described previously [18]. To study the binding of NF-κB complexes to the putative NF-κB sites, double-stranded oligonucleotides for each of the three putative NF-κB sites in the SSAT 5′ sequence were 32P-labelled with polynucleotide kinase (Promega). The oligonucleotide containing the first putative NF-κB site [NF-κB-wild-1 (w)] spanned from −304 to −280 of the SSAT 5′ sequence and had the sequence 5′-GCTGCAGAGGG AAATACCTTCTT-3′, whereas the corresponding mutant NF-κB-mut-1 (m) had
ssAT enzyme activity determination

For enzyme activities, 1 × 10⁶ cells were grown overnight and then treated with various concentrations of aspirin or its vehicle. Cells were harvested after 48 h of treatment and washed in cold PBS. The radiochemical assay of the ssAT activity was performed by estimation of labelled AcSpd (N³-acetyl spermidine) synthesized from [¹⁴C]acetyl-CoA and unlabelled spermidine, as described elsewhere [20]. Fold change was calculated by dividing the enzyme activity for the sample by that for the vehicle. The enzyme assays were performed in triplicate.

Polyamine analysis

Cell extracts were prepared in 0.1 M HCl (4 × 10⁷ cells/900 µl). After sonication, the preparation was adjusted to 0.2 M HClO₄ and the supernatant was analysed by reverse-phase HPLC with 1,7-diaminoheptane as an internal standard [21]. Protein was determined by BCA (bicinchoninic acid) assay [22].

Statistical analysis

Northern and Western blot analyses were performed at least three times and a representative blot is shown. All transient transfection experiments were performed in triplicate and were repeated at least three times. Representative experiments or means ± S.D. are shown. Statistical differences were determined by Student’s t test. A P value of < 0.05 was considered significant.

RESULTS

Regulation of ssAT expression by various NSAIDs in colon cancer cells

Regulation of ssAT was studied with an array of NSAIDs in the Caco-2 colon cancer cells. NSAIDs used in the study were aspirin (acetylsalicylic acid), ibuprofen, indomethacin and sulindac, all of which have been shown to have some chemopreventive effects on colon cancer [6,13,23,24]. All the NSAIDs were used at concentrations that reduced intracellular PGE₂ (prostaglandin E₂) levels and have been shown previously to exert chemopreventive actions in various colon cancer cells [23,25,26]. Expression of ssAT mRNA was determined after 48 h of treatment, except in the case of 600 µM indomethacin where treatment was for 72 h in the Caco-2 cells. This was used because indomethacin has been shown to induce ssAT mRNA in these cells after 72 h of treatment [13]. As shown in Figure 2, most of the NSAIDs were able to induce ssAT mRNA in the Caco-2 cells. Aspirin was able to induce ssAT mRNA at a lower dose of 500 µM but not at 1 mM. In order to determine whether NSAIDs are working at the level of transcription, transient transfections with the Full-ssAT-luc reporter–promoter construct were performed in the Caco-2 cells. The transfected cells were treated with the same array of NSAIDs at the same concentrations. The NSAID, which induced ssAT mRNA also induced the ssAT full-length promoter construct in the Caco-2 cells (results not shown).

Aspirin induces ssAT expression and decreases polyamines in colon cancer cells

Most of the in vitro research with aspirin in colon cancer has been with concentrations ranging from 1 to 10 mM, which are relatively high as compared with the in vivo concentrations achievable in humans. Initial experiments on ssAT expression were with doses that cannot be achieved in vivo. Therefore, to determine whether physiologically relevant doses of aspirin have any effects on the ssAT expression in colon cancer cells, lower concentrations were chosen. Transient transfections using the Full-ssAT-luc...
ODC mRNA expression at 100 μM led to a significant induction in the SSAT promoter activity in Caco-2 cells (Figure 3A). The same doses of aspirin were able to induce SSAT mRNA in the Caco-2 cells after 48 h incubation (Figure 3B), as measured by quantitative RT–PCR.

Next, it was important to determine whether this induction of SSAT promoter activity and SSAT mRNA had any effect on SSAT protein levels and resulting enzyme activity. Since SSAT is a rate-limiting enzyme in polyamine catabolism, polyamine analysis was done to measure any effects on cellular polyamine levels in aspirin-treated cells. As shown in Figure 3(C), 100 μM aspirin, but not 1 mM aspirin, increased SSAT protein levels. Aspirin at 20 μM led to approx. 3.6 ± 0.9-fold induction in the SSAT enzyme activity in Caco-2 cells, while aspirin at 100 μM only induced SSAT enzyme activity by approx. 2 ± 0.5-fold (Figure 3D). Based on the effects of aspirin on SSAT expression, we hypothesized that aspirin treatment would lead to a decrease in the intracellular polyamine levels in these cells. We found that in Caco-2 cells, aspirin at 20 μM reduced all the intracellular polyamine levels but aspirin at 100 μM had no significant effect on the intracellular polyamine levels (Table 1). Intracellular polyamine contents can also be regulated by the expression of the biosynthetic enzyme, ODC. Hence, the reduction in intracellular polyamine content observed at 20 μM but not at 100 μM could be due to an inhibition of ODC in these cells. Aspirin treatment had no effect on ODC expression at 20 μM, but led to a slight increase in ODC mRNA expression at 100 μM (results not shown).

Mapping of aspirin-responsive elements in the SSAT promoter

Based on the result that SSAT transcription is being modulated by aspirin in the Caco-2 cells, an attempt was made to map the aspirin responsive sequence(s) in the SSAT 5′ promoter flanking sequences. In the first set of experiments, transient transfection experiments were done using two SSAT reporter–promoter constructs, Full-SSAT-luc and 197-SSAT-luc, in the Caco-2 cells. Transfected Caco-2 cells were treated with 10, 50 or 100 μM aspirin for 48 h. Aspirin at 50 and 100 μM was able to induce Full-SSAT-luc in the Caco-2 cells but these concentrations of aspirin had no effect on the 197-SSAT-luc reporter–promoter construct (Figure 4A).
Table 1  Effect of aspirin treatment on intracellular polyamine content in human colorectal cancer cells

<table>
<thead>
<tr>
<th>Intracellular polyamine content (nmol/mg of protein)</th>
<th>Putrescine</th>
<th>Spermidine</th>
<th>Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>1.24 ± 0.2</td>
<td>3.78 ± 0.1</td>
<td>4.45 ± 0.33</td>
</tr>
<tr>
<td>Aspirin (20 µM)</td>
<td>0.35 ± 0.1*</td>
<td>1.31 ± 0.1*</td>
<td>2.16 ± 0.08*</td>
</tr>
<tr>
<td>Aspirin (100 µM)</td>
<td>0.90 ± 0.15</td>
<td>3.67 ± 0.25</td>
<td>4.12 ± 0.2</td>
</tr>
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Figure 4  Mapping of a putative aspirin responsive element in the SSAT promoter

(A) Caco-2 cells were grown overnight, transfected with either Full-SSAT-luc or 197-SSAT-luc and then treated with either vehicle (V; open bars) or various concentrations of aspirin (ASA; all closed bars) for 48 h. (B) Caco-2 cells were grown overnight, transfected with a SSAT deletion reporter construct and then treated for 48 h with either vehicle (V; open bars) or 100 µM aspirin (ASA; closed bars). Relative luciferase units (RLU) were calculated after normalizing to the protein and β-galactosidase activities, and fold induction was calculated. Normalized luciferase activities are shown as mean ± S.D. (n = 3) and are expressed as fold inductions relative to the activity in the presence of vehicle alone; *P < 0.05 compared with vehicle.

Aspirin can induce NF-κB, but not PPARs, in Caco-2 colon cancer cells

In order to find potential aspirin responsive elements, a TESS (transcription element search system) database analysis on the SSAT 5′ promoter flanking sequence was done. It was found that there are many putative AP-1 (activator protein-1), Sp-1 and NF-κB sites in the SSAT promoter, in addition to the known sites, PRE (polyamine response element) and PPRE (Figure 1A). Aspirin has been shown to inhibit NF-κB, but it has also been shown to induce NF-κB in colon cancer cells. Sulindac sulphone induces SSAT by activating PPARγ, which can bind to one of the PPREs in the SSAT promoter [14]. Aspirin can act as a ligand for PPARs and can also regulate the synthesis of prostaglandins, which can also act as ligands for PPARs [27]. Based on the presence of putative NF-κB and PPAR sites in the SSAT promoter, we wanted to determine the mechanism of induction of SSAT by aspirin. Transient transfection experiments using the PPRE3-tk-luc plasmid, which has three PPREs cloned in front of a luciferase gene, were performed in the Caco-2 cells. The transfected cells were treated with either 20 or 100 µM aspirin for 48 h. Aspirin treatment did not lead to an increased expression of this plasmid, indicating that aspirin is not acting via PPARs in Caco-2 cells to induce SSAT expression (results not shown).

To test for the role of NF-κB in induction of SSAT by aspirin, Caco-2 cells were transfected with either the NF-κB β2-luc reporter plasmid or its control ΔNF-κB β2-luc reporter construct. The transfected cells were treated with 100 µM aspirin for 48 h. Aspirin at 100 µM was able to induce the NF-κB β2-luc reporter but not the control vector (Figure 5), indicating that NF-κB is functional and that aspirin is influencing the activity/binding of NF-κB protein complexes in the Caco-2 cells. This result is in contrast with the results that aspirin mostly inhibits NF-κB activity, but more recently, it has been shown that in HCT-116, another colon cancer cell line, aspirin can induce NF-κB and lead to apoptosis [28].
DISCUSSION

A number of independent lines of evidence suggest that NSAIDs can prevent colorectal cancer. Aspirin is the most widely studied NSAID for the chemoprevention of colorectal cancer [29–34] and several epidemiological studies have reported an inverse association between colorectal cancer risk and aspirin intake. Recent clinical trials have established the use of lower doses of aspirin in chemopreventive effects on adenoma recurrence [33]. In our previous work, we showed that aspirin was able to induce SSAT expression and decrease intracellular polyamine levels in HT-29 colon adenocarcinoma cells [7]. Because polyamines are essential for neoplastic cell growth, the aim of the present paper was to evaluate the effects of various NSAIDs, in particular aspirin, on polyamine metabolism in Caco-2 colon cancer cells and to determine the mechanism by which aspirin is able to induce SSAT in these cells.

It was found that the NSAIDs aspirin, ibuprofen, sulindac and indomethacin are all able to induce SSAT expression in Caco-2 cells at concentrations which are sufficient to decrease intracellular PGE2 levels. Most of the in vitro research with aspirin in colon cancer has been with concentrations ranging from 1 to 10 mM [3,23,26]. These concentrations are relatively high as compared with the in vivo concentrations achievable in humans. We used lower doses of aspirin that are more physiologically relevant and then studied their effects on SSAT expression. We found that aspirin, at these concentrations, is able to induce SSAT promoter activity and SSAT mRNA levels in Caco-2 cells. Aspirin led to a higher fold induction in the Full-length-SSAT promoter construct (7-fold) as compared with the SSAT mRNA (2-fold) in Caco-2 cells. This difference could be due to the presence of other cis- and trans-acting elements in the SSAT gene, which are not included in the promoter expression studies, and can be influenced by aspirin treatment. Further, aspirin at 20 µM led to a 1.3 ± 0.1-fold induction in SSAT mRNA but a 3.6 ± 0.9-fold induction in the SSAT enzyme activity, whereas aspirin at 100 µM induced both the SSAT mRNA and enzyme activity to approx. 2-fold. This 2-fold difference between the SSAT mRNA and enzyme activity obtained at lower concentrations of aspirin suggests a role for aspirin in the post-transcriptional regulation of SSAT. However, any post-transcriptional role for aspirin in SSAT regulation appears to be considerably less than that observed with the polyamine analogues.

SSAT leads to the production of acetylated spermidine and spermine, which can then be either excreted from the cells, or with the use of p50 antibody, suggesting the presence of a p50 protein in the NF-κB complex binding at the w probe. Using the same amount of the p50 antibody, we found a stronger intensity supershift with 100 µM and 1 mM aspirin, but not with 5 mM aspirin, indicating that aspirin is working predominantly at lower concentrations to induce NF-κB binding to the NF-κB-wild-1 site in the SSAT promoter.

Using other putative NF-κB sequences, we found no significant binding of the aspirin-treated Caco-2 nuclear extract with the w2 NF-κB sequence (results not shown), but found that there was binding of NF-κB complexes to the w3 sequence (Figure 6B). This binding was increased by 100 µM aspirin treatment (1.3-fold as compared with the vehicle treatment), but not by 1 mM aspirin treatment. This binding was specific, as it could be abolished by NF-κB-mutant-3 (m3) sequence and could be competed by unlabelled wild probe (w3). Further, there is generation of a supershifted band with the use of p50 antibody, suggesting the presence of a p50 protein in the NF-κB complex binding to the w3 probe.
Aspirin effects on SSAT in Caco-2 colon cancer cells

by the action of another enzyme, polyamine oxidase, can be converted back into putrescine in the cells. Therefore an induction in the SSAT activity can lead to lower intracellular levels of spermidine and spermine, but an increase or no effect on the intracellular putrescine levels. Our results indicate that aspirin at 20 µM led to a decrease in all three polyamines after 48 h of treatment as has been shown previously with many drugs like aspirin [7], sulindac [14], indomethacin [13] and polyamine analogues [35,36], all of which induce SSAT, and led to lower levels of all the polyamines, including putrescine, in colon and other cancer cells. One possible explanation could be that the reduction in putrescine levels could be due to an inhibition of ODC expression by aspirin in Caco-2 cells. Aspirin slightly increases ODC mRNA at 100 µM, but has no effect on ODC mRNA at 20 µM in the Caco-2 cells. Further, reduction in polyamines could be explained by the fact that aspirin (acetylsalicylic acid) may compete with acetyl-CoA (natural co-substrate) for SSAT enzyme activity or may act as a direct inhibitor of SSAT enzyme, leading to inhibition of enzyme activity at higher aspirin concentrations. Additional experiments are required to characterize the exact role of aspirin as an inhibitor of SSAT enzyme activity. However, it is important to note that patients given a lower dose of aspirin had a significantly greater reduction in adenoma recurrence as compared with patients given a higher dose of aspirin [37]. Alternatively, 100 µM aspirin could lead to the inhibition of the diamine exporter [38] in these cells, which could explain the lack of change in the intracellular polyamine levels.

Due to the effects of aspirin on SSAT mRNA and promoter activation, we propose that aspirin is inducing SSAT by its effect on transcription. We identified two regions, one between 0.28 and 0.44 kb and the other between 0.88 and 3.53 kb in the SSAT promoter flanking sequence, which were responsive to aspirin treatment. TESS analysis identified several potential aspirin-responsive elements in this region, including three NF-κB sites at −286, −594 and −1735 respective to the transcription start site. Aspirin has been shown to inhibit NF-κB [39–41], but it has also been shown to induce NF-κB in HCT-116 cells [28,42]. Further, aspirin can lead to the inhibition of AP-1 activity [43,44], and can act as a ligand for PPARs [45]. Transient transfection experiments using the NF-κB reporter constructs demonstrated that aspirin is able to induce this promoter in Caco-2 colon cancer cells. The involvement of NF-κB in the aspirin-induced expression of SSAT was further implicated by electrophoretic mobility-shift assays, demonstrating that nuclear extracts from Caco-2 cells treated with 100 µM aspirin result in an increased binding at two of the three potential NF-κB response elements in the SSAT promoter. NF-κB complexes are present as homo- or hetero-dimers of p50 and p65 proteins. Supershift experiments demonstrated the presence of the p50 protein in the nuclear proteins bound to both the NF-κB-wild-1 and wild-3 sequences in the SSAT promoter. In preliminary experiments, treatment of Caco-2 cells with TNFα (tumour necrosis factor α), which is known to exert its biological actions by inducing NF-κB, led to an activation of NF-κB signalling in the Caco-2 cells with a concomitant increase in the SSAT mRNA expression. Although observations with TNFα do not directly confirm the relationship between aspirin induction of SSAT by NF-κB, they do strengthen the point that NF-κB activation can lead to the induction of SSAT by increased binding at the NF-κB response elements in the SSAT promoter flanking sequence. Interestingly, unlike sulindac sulphone, which induces SSAT by activating PPARγ, which can then bind to the PPRE in the SSAT promoter flanking region, aspirin does not appear to induce SSAT by this pathway as it was not able to induce the PPRE reporter constructs.

These results appear to contradict published reports indicating that aspirin and its metabolites can bind to and directly inhibit IKKβ (inhibitor of κB kinase β), the kinase responsible for phosphorylating IκBα (inhibitory κB), thereby inhibiting translocation of p65 to the nucleus [46,47] and preventing apoptosis. However, more recent reports indicate that aspirin can induce the NF-κB signalling pathway and this can lead to apoptosis in colon cancer cells [28]. Further, this apoptosis is independent of p53 status [48]. The differential effects of NF-κB may be explained by the DNA-binding specificity and selective transcriptional activation of downstream NF-κB target genes, which is in turn determined by the dimeric composition of the NF-κB complex and by the transcriptional cofactors recruited to the complex [49]. Results presented here demonstrate that one of the target genes induced by NF-κB with aspirin treatment in Caco-2 colon cancer cells is SSAT. Induction of SSAT can lead to lower intracellular polyamine levels in these cells with aspirin treatment. A decrease in polyamines has been shown to lead to increased cell death [14] and/or increased apoptosis [10,11].

In summary, the experimental results presented here demonstrate that induction of polyamine catabolism leading to a reduction in intracellular polyamines could be one of the general mechanisms for the chemopreventive actions of different NSAIDs in colon cancer. Further, the mechanism of induction of SSAT by physiologically relevant doses of aspirin involves NF-κB, but not PPAR, transcription factor complexes, which can bind to their response elements in the SSAT promoter region in Caco-2 colon cancer cells.

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