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Advances in Brief

Growth Inhibition of Human Colon Cancer Cells by Nitric Oxide (NO)-Donating Aspirin Is Associated with Cyclooxygenase-2 Induction and β-Catenin/T-Cell Factor Signaling, Nuclear Factor-κB, and NO Synthase 2 Inhibition: Implications for Chemoprevention

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Abstract

Nitric oxide (NO)-releasing aspirin (ASA), consisting of a traditional ASA molecule to which a NO-donating moiety is covalently bound, is a promising colon cancer chemopreventive agent. NO-ASA inhibits colon cancer cell growth more potently than ASA by inhibiting cell proliferation and enhancing cell killing. We examined in cultured human colon cancer cells the effect of NO-ASA on the β-catenin/T-cell factor signaling pathway, nuclear factor-κB, and NO synthase 2 and on cyclooxygenase (COX) expression, all presumed to participate in colon carcinogenesis. Besides inhibiting cell growth, NO-ASA inhibited the β-catenin/T-cell factor signaling pathway (IC_{50}, 1.1 μM), nuclear factor-κB DNA binding (IC_{50}, 7.5 μM), and NO synthase 2 expression (IC_{50}, 2 μM). Interestingly, NO-ASA induced COX-2 expression, although it had no effect on COX-1. COX-2 induction was accompanied by increased prostaglandin E_2 production. These effects occurred at NO-ASA concentrations below or near its IC_{50} for cell growth (IC_{50}, 2–50 μM). The metabolism of NO-ASA by these cells is characterized by a rapid deacetylation step and the formation of a conjugate with glutathione. NO-ASA had no effect on intracellular cyclic GMP concentrations. We propose a model incorporating the pleiotropic effects of NO-ASA on cell signaling and postulate that collectively these effects may contribute to its strong chemopreventive effect.

Introduction

NO[5]-NSAIDs represent a novel and promising class of compounds. They consist of a traditional NSAID to which a group donating NO has been covalently attached via an aromatic or aliphatic spacer (Fig. 1). Emerging data indicate that these compounds combine the chemopreventive properties of traditional NSAIDs against cancer with enhanced safety and efficacy (1, 2). For example, NO-ASA is between 2540-fold and >5000-fold more potent than traditional ASA in suppressing colon cancer cell growth (3). Moreover, several NO-NSAIDs have shown a strong chemopreventive effect in animal models of colon cancer (4, 5). NO-NSAIDs, including NO-ASA, profoundly affect cancer cell renewal and death (3, 6). However, very little is known about the molecular targets in the cancer cell that are responsible for this effect.

To address this question, we evaluated the effect of NO-ASA on the following four molecular targets implicated in colon carcinogenesis: (*a*) Wnt signaling pathway; (*b*) NF-κB; (*c*) NO synthase; and (*d*) COX. The Wnt signaling pathway likely plays a major role in human colon carcinogenesis, as evidenced by altered levels and/or distribution of β-catenin in colon cancer (reviewed in Ref. 7). For example, nuclear β-catenin expression is closely related to growth of colorectal carcinoma (8), selective nuclear accumulation of β-catenin at the invasion front of colon cancer is correlated with Dukes' stage and predicts tumor recurrence and extremely unfavorable clinical outcomes (9), and β-catenin expression is increased in aberrant crypt foci according to their degree of dysplasia (10). ASA inhibits β-catenin/TCF signaling in colon cancer cell lines (11). The NF-κB family of transcription factors, central to several cellular responses, augments or inhibits apoptosis, depending on cell type and conditions (12). Several NO-NSAIDs inhibited the induction of NOS2 by bacterial lipopolysaccharide in the J774 murine macrophage cell line without directly affecting enzyme activity. This effect on NOS2 expression may be important for the action of NO-NSAIDs because (*a*) NOS2 expression is increased in tumor cells, (*b*) NOS2 is involved in the regulation of COX-2, (*c*) NOS2 activity is correlated with p53 mutations, and (*d*) NOS2 inhibitors prevent colon cancer (summarized in Ref. 3). COX is considered important in colon carcinogenesis (13). Although current data are limited and somewhat conflicting, they indicate an interaction between NO-NSAIDs and the eicosanoid pathway (summarized in Ref. 1).

Furthermore, to gain a better understanding of the interactions of NO-ASA with potentially important signaling pathways, we determined two important aspects of the interactions of NO-ASA with colonocytes. First, we assessed the metabolism of NO-ASA by HT-29 colon cancer cells, and, second, we determined its effect on intracellular levels of cGMP, the product of guanylyl cyclase, a proven receptor of the gaseous ligand NO. In some systems, *e.g.*, monocytes, NO-ASA is known to increase cGMP levels (14), although cGMP inhibitors prevent most of its activity (15). On the other hand, cGMP mediates apoptosis induced by sulindac derivatives via activation of c-Jun NH_2-terminal kinase 1 (16).

Here, we present our results showing that NO-ASA interacts significantly with the four pathways we studied and discuss the potential integration of these effects into the mechanism of action of this novel compound.

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[5] The abbreviations used are: NO, nitric oxide; NSAID, nonsteroidal anti-inflammatory drug; ASA, aspirin; NO-ASA, NO-donating ASA; TCF, T-cell factor; NF-κB, nuclear factor-κB; NOS2, NO synthase 2; COX, cyclooxygenase; cGMP, cyclic GMP; IL, interleukin; SNAP, *S*-nitroso-*N*-acetyl-D-L-penicillamine; HPLC, high-performance liquid chromatography; EMSA, electrophoretic mobility shift assay; PGE_2, prostaglandin E_1; 4-HMP, 4-(hydroxymethyl)phenol; GSH, glutathione; IBMX, 3-isobutyl-1-methylxanthine; APC, adenomatous polyposis coli; dn, denitrated derivative; SA, salicylic acid.

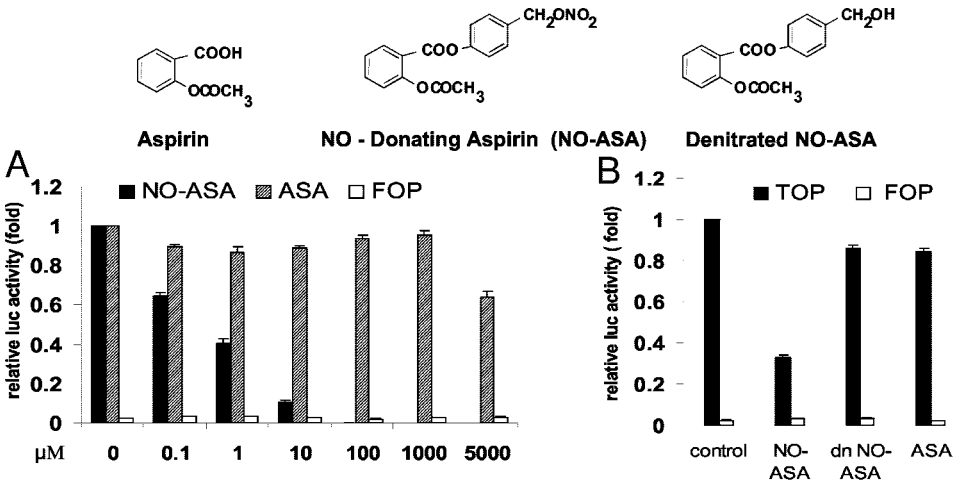

Fig. 1. The structure of NO-ASA and its effect on the β-catenin/TCF signaling pathway in SW480 cells. *Top*, the $-NO_2$ group that releases NO is covalently bound to traditional ASA (*left*) via an aromatic spacer molecule. dn-NO-ASA (*right*) is devoid of the $-NO_2$ group. *A*, SW480 cells co-transfected with luciferase reporter plasmids that have TCF-4-binding sites (TOP) and the pSV-βgal expression plasmid were treated with NO-ASA or ASA for 18 h, as described in "Materials and Methods." Relative transcriptional activity was expressed as the fold activation or inhibition compared with the transfected-untreated cells. FOP is a control plasmid containing mutated TCF-4 binding sites. *B*, dn-NO-ASA poorly inhibits TCF activity in contrast to NO-ASA. The concentration of all compounds is 1 μM.

Materials and Methods

Reagents. NO-ASA [*NCX4040*, 2-(acetyloxy)benzoic acid 4-(nitrooxymethyl)phenyl ester] as 100 mM stock solution (NicOx SA, Sophia Antipolis, France) and traditional ASA (Sigma, St. Louis MO) were prepared in DMSO; the final DMSO concentration in all media was adjusted to 1%. Recombinant human IFN-γ, IL-1β, and IL-6 were obtained from Roche (Indianapolis, IN). SNAP was obtained from Sigma.

Phosphoric acid, BSA, ASA, SA, 5,5′-dithiobis(2-nitrobenoic acid), 2-hydroxybenzyl alcohol, 3-hydroxybenzyl alcohol, 4-hydroxybenzyl alcohol, and *N*-ethyl-maleimide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All general solvents and reagents were of HPLC grade or the highest grade commercially available.

Cell Culture. HT-29, SW480, and DLD-1 human colon and MIA PaCa-2 and BxPc-3 human pancreatic adenocarcinoma cell lines (American Type Culture Collection, Manassas, VA) were grown as described previously (3, 6). Viability was determined by the trypan blue dye exclusion method after harvesting cells by trypsinization (0.05% trypsin/EDTA). Viable cell numbers were also measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay according to the manufacturer's instruction (Boehringer Mannheim).

Transfection and Reporter Gene Assay. Transient transfection was performed using Fugene 6 (Roche) according to the manufacturer's instruction. Briefly, 10^5 SW480 cells were transfected transiently with 400 ng of luciferase reporter gene constructs TOPflash, containing three copies of TCF-4 binding sites, or FOPflash, containing mutated TCF-4 binding sites (Upstate Biotechnology, Lake Placid, NY) and 200 ng of pSV-βgal vector as internal control. Two h after transfection, cells were treated with drugs for 18 h. β-Galactosidase activities were measured using standard protocols, and luciferase assays (Promega) were performed as per the manufacturer's instruction.

EMSA. Nuclear extracts were prepared 3 h after exposing the cells to NO-ASA. Binding reactions were performed using 20 μg of nuclear extract and 33 nmol of a ^{32}P-end-labeled 22-mer double-stranded NF-κB oligonucleotide (AGTTGAGGGGAC TTTCCCAGGC; Promega Corp.) corresponding to the binding site of the p65 subunit; the complexes was separated on a 5% gel following standard protocols.

Western Blot Analysis. Proteins were extracted and separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes, as described previously (17). Probing with monoclonal antibodies against COX-1, COX-2, NOS2, actin, or β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) was done for 1 h; membranes were developed by the enhanced chemiluminescence system (ECL). Bands detected were quantified using UVP LABWORKS Imaging Analysis software (UVP LABWORKS, Upland, CA). The density measurement was correlated to protein expression and normalized to that of a housekeeping gene.

PGE_2 Assay. PGE_2 levels in culture media were determined by immunoassay (Cayman Chemical) following the instructions of the manufacturer.

HPLC Analysis. Cells were seeded in 6-well plates at a density of 1.1×10^5 cells/cm^2 in 4 ml of culture medium and allowed to attach for 24 h. NO-ASA was added to the medium (100 μM, final concentration). At the indicated times, 0.5 ml of medium was removed, extracted with CH_3CN, and fractionated by HPLC as described above. To extract NO-ASA and its metabolites from cells, the cells were washed three times with cold PBS and lysed with 0.5 ml of cold 5% metaphosphoric acid. The cell lysate was kept at −20°C until analysis; 50-μl samples were injected to HPLC and fractionated exactly as described above.

Our HPLC system consists of two Waters 501 pumps with an automated gradient solvent delivery controller, a Waters 990 UV/VIS detector, and a LiChrospher C_8 reverse-phase column (250 × 4.6 mm; particle size, 5 μ; Alltech Inc., Beerfied, IL) with a manual sample injector. The column is maintained at room temperature. Buffer A consists of water-acetonitrile-phosphoric acid (9:1:0.01, v/v/v); buffer B consists of acetonitrile. The flow rate is 1 ml/min. We applied gradient elution from 100% buffer A to 60% buffer B from 0–30 min; it was maintained at 60% buffer B until 43 min.

Isolation and Mass Spectra Analysis of HPLC Fractions. The HPLC peaks corresponding to the GSH conjugates with 4-HMP were collected, concentrated under vacuum, and submitted to mass spectrometry analyses.

cGMP Assay. HT-29 cells were incubated with the phosphodiesterase inhibitor IBMX (500 μM) for 20 min before the addition of SNAP or NO-ASA. At the indicated time intervals, the treated cells and their corresponding IBMX control were lysed in 0.1 M HCl and centrifuged, and cGMP was assayed using a cGMP enzyme immunoassay kit (Cayman Chemical Co.) according to the manufacturer's instructions.

Results

NO-ASA Inhibits the β-Catenin/TCF Signaling Pathway in SW480 Cells. To investigate whether NO-ASA inhibits this pathway, we used SW480 cells, which have a truncating APC mutation, leading to elevated levels of free β-catenin. Fig. 1 shows that NO-ASA strongly inhibits TCF activity in these cells in a concentration-dependent manner. After 18 h of treatment, 10 μM NO-ASA reduced TCF activity to 10% of control, although concentrations as low as 0.1 μM also inhibited TCF activity; the IC_{50} for this effect was 1.1 μM. In contrast, 5 mM ASA decreased TCF activity only to 65% of control. Thus NO-ASA is a much more potent down-regulator of β-catenin/TCF-4 signaling. Of note, the growth inhibitory IC_{50} of NO-ASA in SW480 cells is 50 μM. dn-ASA attenuates this signaling pathway only to a limited degree (88% of control), suggesting an important role of the $-NO_2$ moiety.

NO-ASA Inhibits the Expression of NOS2 in HT-29 Cells. HT-29 cells were seeded at a density of 0.6×10^5 cells/cm^2 and grown for 72 h, followed by cytokine treatment for 18 h with IFN-γ (100 units/ml), IL-1β (100 units/ml), and IL-6 (0.5 ng/ml; Ref. 18). This was followed by treatment with active drug or vehicle in the continued presence of cytokines for an additional 24 h. NO-ASA

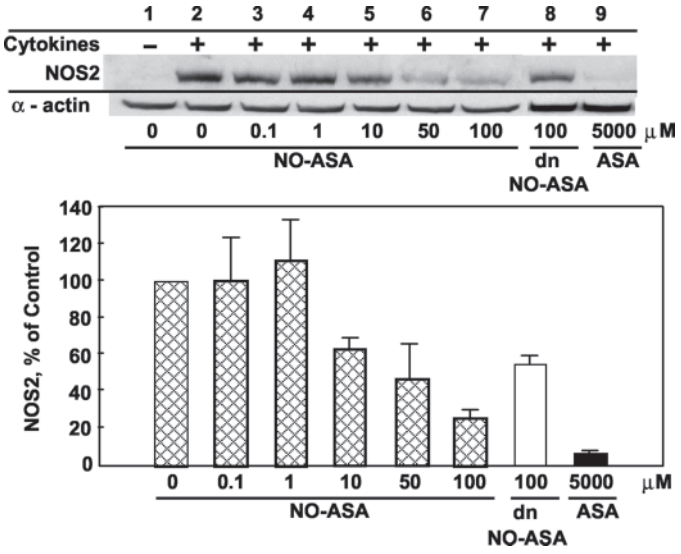

Fig. 2. NO-ASA inhibits cytokine-mediated NOS2 induction. *Top,* HT-29 cells were treated with human recombinant IFN-γ, IL-1β, and IL-6, as described in "Materials and Methods," in the absence or presence of 0, 0.1, 1, 10, 50, and 100 μM NO-ASA (*Lanes 1–7*), 100 μM dn-NO-ASA (*Lane 8*), and 5 mM ASA (*Lane 9*). *Bottom,* cell lysates were analyzed for NOS2 levels and normalized to corresponding α-actin levels.

inhibited the growth of HT-29 cells (IC_{50}, 2 μM). Fig. 2 shows the induction of NOS2 expression in these cells by cytokines; NOS2 was not detectable in the absence of cytokines (nor was NOS1; data not shown). We observed that NO-ASA inhibited the expression of NOS2 in a dose-dependent manner (IC_{50}, 48 μM). Treatment of the cells with dn-NO-ASA (100 μM) reduced the expression of NOS2 by about half, being about half as effective as its parent NO-ASA molecule. The IC_{50} of dn-NO-ASA for HT-29 cell growth inhibition was at least 250-fold greater than that of NO-ASA (data not shown). Of note, a concentration of 5000 μM ASA was extremely effective in inhibiting the expression of NOS2, virtually blocking it completely (95% reduction).

NO-ASA Inhibits the Interaction of NF-κB with DNA in the Nucleus. Activation of the transcription factor NF-κB involves its translocation into the nucleus, where it binds to the appropriate DNA regulatory sequences. We determined whether NO-ASA affects the NF-κB–DNA interaction by performing EMSAs on nuclear extracts from HT-29 colon cancer cells. Cells were seeded at 2.8×10^4 cells/cm^2, grown overnight, and then treated for 3 h with NO-ASA (0–60 μM). NO-ASA decreased the baseline binding of NF-κB to a ^{32}P-end-labeled 22-mer double-stranded NF-κB-binding oligonucleotide in a concentration-dependent manner. Such binding became undetectable at 60 μM NO-ASA; the IC_{50} for this effect was 7.5 μM (Fig. 3*A*). In contrast, traditional ASA had no significant effect at 5,000 μM and only a modest effect at 10,000 μM. The dn-NO-ASA reduced NF-κB binding to 58% of control. Of note, similar results were obtained with the MIA PaCa-2 pancreatic cancer cell line (Fig. 3*C*).

NO-ASA Induces the Expression of COX-2 in HT-29 Cells. HT-29 cells were seeded at a density of 2.8×10^5 cells/cm^2 and grown overnight. Cells were then serum deprived for 24 h in the presence of either the study compound or the vehicle. After treatment, cells and media were harvested and assayed for PGE_2. Treatment of the HT-29 cells with NO-ASA inhibited their growth with $IC_{50} = 28$ μM; this IC_{50} is higher than the one reported previously because of the different treatment protocol.

As shown in Fig. 3*B*, NO-ASA dose-dependently increased the expression of COX-2 from 4.3-fold over control (10 μM NO-ASA) and increased it further to 8.9-fold (20 μM NO-ASA). NO-ASA treatment along with serum stimulation induced COX-2 to a greater degree compared with serum-deprived cells (data not shown). In contrast, ASA had no appreciable effect on COX-2 expression. Neither compound affected the expression of COX-1 (data not shown). Increased levels of PGE_2, the major eicosanoid of this cell line (19), accompanied the up-regulation of COX-2 expression. PGE_2 levels were increased 3.5-fold by 10 μM NO-ASA and 5.1-fold by 20 μM NO-ASA, paralleling to some degree the enhanced expression of COX-2. Interestingly, 200 μM ASA, added to the cells simultaneously with 20 μM NO-ASA, inhibited the production of PGE_2 by slightly over half (56%; data not shown). dn-NO-ASA affected neither the expression of COX-2 nor the production of PGE_2 (data not shown). We have obtained similar induction of COX-2 with both the DLD-1 colon cancer cell line (data not shown) and the BxPc-3 pancreatic cancer cell line (Fig. 3*C*); no induction was seen in the MIA PaCa-2 pancreatic cancer cells, which are COX null (6).

Metabolism of NO-ASA by Colon Cells. To assess the metabolism of NO-ASA by colon cancer cells, we incubated HT-29 human colon cancer cells with 100 μM NO-ASA for up to 24 h and determined the profile of NO-ASA metabolites in both the culture medium and in the cells.

As shown in the *top panel* of Fig. 4, HT-29 cells metabolize NO-ASA. The HPLC chromatogram revealed the formation of several metabolic products. A product for which authentic standard was not available was identified by mass spectroscopy (*bottom panel*). The identity of this peak was confirmed on the basis of its quasi-molecular ion peak at *m/z* 414 $[M + H]^+$ as the conjugate of 4-HMP and GSH (4-HMP-GSH). Two prominent species extracted from the cells at this time point are SA and 4-HMP-GSH. No detectable amounts of intact NO-ASA are seen at 4 h. The culture medium contains SA and deacetylated NO-ASA but no 4-HMP-GSH (Fig. 4, *middle panel*), suggesting the possibility that, at least in part, the metabolites we observed in the medium are not secreted from the cells but are generated before NO-ASA enters the cells.

Effect of NO-ASA on Intracellular cGMP. To assess the effect of NO-ASA on guanylyl cyclase, we determined cGMP levels in HT-29 cells treated with NO-ASA. In parallel, we also treated cells with the NO donor SNAP. As can be seen in Fig. 5, NO-ASA did not affect the levels of cGMP. In contrast, SNAP increased them nearly 3-fold over control at 30 min with a progressive decline to baseline levels over 120 min, in agreement with previous reports (14). No changes in cGMP concentrations were noted for up to 24 h (data not shown).

Discussion

Our results document a pleiotropic effect of NO-ASA on a network of signaling pathways and effector molecules considered important to colon carcinogenesis. In human colon cancer cells, NO-ASA inhibits the β-catenin/TCF signaling pathway, NOS2 expression, and NF-κB binding to DNA regulatory sequences. Significantly, however, NO-ASA induces the expression of COX-2 concomitant with increased transformation of arachidonic acid.

NO-ASA has a profound effect on signaling through the β-catenin/TCF-4 complex. In SW480 colon cancer cells, the inhibition of this pathway took place at concentrations that were much lower than its IC_{50} for growth inhibition (50 μM). In fact, the IC_{50} for inhibition of β-catenin/TCF signaling is 1.1 μM, which represents 2.2% of its IC_{50} for growth inhibition. If such an effect occurs in humans, it may explain much of its chemopreventive effect against colon cancer. All available evidence indicates that inhibition of this pathway may play a pivotal role in colon cancer prevention: (*a*) *APC* mutations are one of the earliest recognized molecular events that initiate a series of

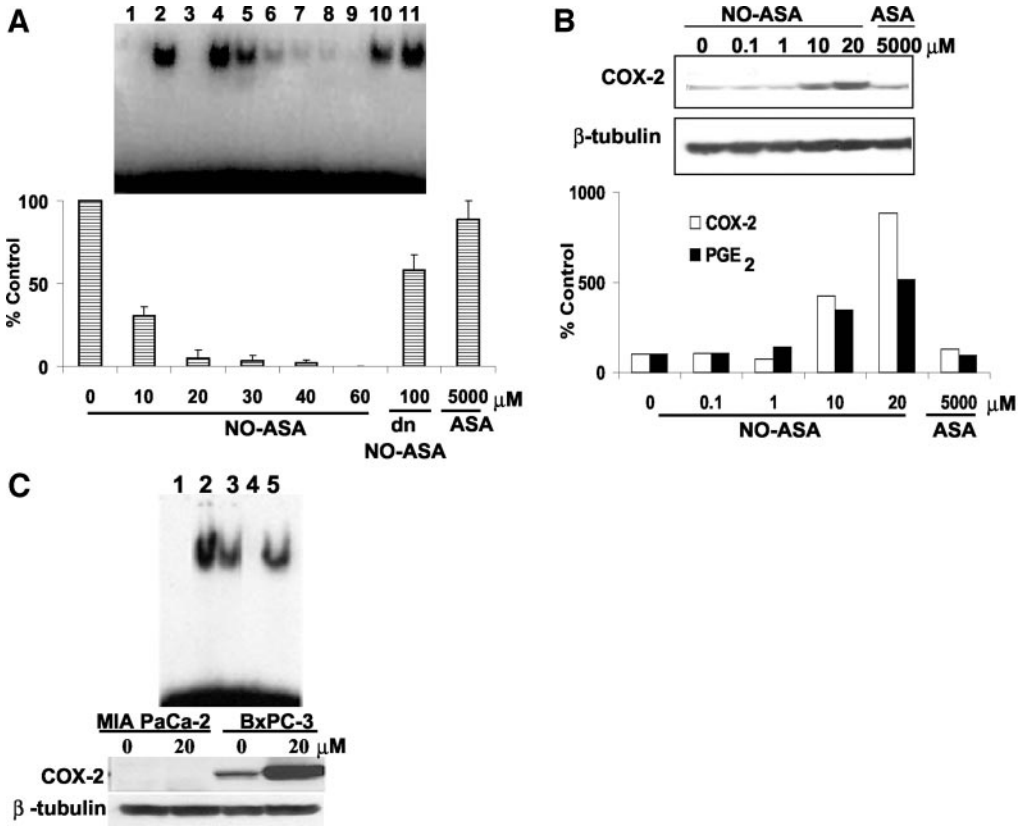

Fig. 3. NO-ASA inhibits NF-κB–DNA binding and induces COX-2 expression. *A*, HT-29 cells were treated with ASA or NO-ASA for 3 h. Nuclear proteins were extracted and combined in EMSA buffer with a double-stranded 22-oligomer bearing the NF-κB binding site. *Lane 1*, no extract added; *Lane 2*, untreated control; *Lane 3*, control plus specific-competitive oligonucleotide; *Lane 4*, control plus nonspecific-competitive oligonucleotide; *Lanes 5–9*, cells treated with 10, 20, 30, 40, or 60 μM NO-ASA, respectively; *Lane 10*, 100 μM dn-NO-ASA; *Lane 11*, 5 mM ASA. *B*, HT-29 cells were serum deprived and treated with 0.1, 1, 10, and 20 μM NO-ASA or 5 mM ASA. COX-2 expression was analyzed by Western blot. Levels were normalized to β-tubulin. Production of PGE_2 in the culture supernatant was determined, as described in "Materials and Methods." *C, top panel*, MIA PaCa-2 pancreatic cancer cells we treated with NO-ASA and subjected to EMSA. *Lane 1*, no extract added; *Lane 2*, untreated control; *Lane 3*, cells treated with 10 μM NO-ASA; *Lane 4*, control plus specific competitive oligonucleotide; *Lane 5*, control plus nonspecific competitive oligonucleotide. In *C, bottom panel*, Western blot shows induction of COX-2 expression by NO-ASA in BxPc-3 but not MIA PaCa-2 pancreatic cancer cells.

gene alterations that culminate in the malignant phenotype; when *APC* mutations lead to a truncated APC protein, as is often the case, the scaffolding provided by APC and axin collapses, leading to high cytoplasmic levels of β-catenin, which translocates to the nucleus, where it complexes with TCF and activates gene transcription; (*b*) mutations in the β-catenin gene, associated with colon cancer, can also activate the Wnt pathway; and (*c*) the transcription of multiple genes, many of which are related to carcinogenesis, is dependent on this pathway. Because Wnt signaling is induced in cancer, its inhibition by NO-ASA may inhibit colon carcinogenesis.

NO-ASA inhibits the induction of NOS2 in a concentration-dependent manner. HT-29 cells have no detectable NOS2 activity when not stimulated by cytokines. Inhibition of NOS by NO-ASA is in agreement with reports that NOS inhibitors prevent colon cancer development in animal models (*e.g.*, Ref. 20).

NO-ASA also inhibits NF-κB binding to DNA with an IC_{50} of 7.5 μM. Traditional NSAIDs, including ASA, facilitate the translocation of NF-κB into the nucleus by degrading IκBα (12). The NF-κB family of transcription factors is central to several cellular responses, and its role in cancer is increasingly recognized. In colon cancer cells, NF-κB was observed to augment apoptosis. Our finding that NO-ASA inhibits NF-κB activation in HT-29 cells lies within the spectrum of the variable effect of ASA and other salicylates on NF-κB [increased (17) or decreased (21) activation]; such responses are likely cell type specific (22).

With regard to the effect of NO-ASA on COX isozymes that transform arachidonic and other fatty acids into eicosanoids, only the expression of COX-2 was induced by 24 h, whereas that of COX-1 was not affected. Of note, NO-ASA treatment along with serum stimulation induced COX-2 even more. Interestingly, the newly synthesized COX-2 was metabolically active, as judged by the increased production of PGE_2, and inhibited by ASA, its classic inhibitor.

There are several additional interesting issues associated with these data. First, the four effects of NO-ASA noted had differential relationships to their respective IC_{50} for inhibition of cell growth. The most sensitive was the inhibition of β-catenin/TCF signaling occurring at 2.2% of the IC_{50} for growth inhibition. The IC_{50} for NOS2 inhibition is 48 μM, 24 times greater than the IC_{50} for growth inhibition. Inhibition of NF-κB binding to DNA occurs at 7.5 μM, before any significant change in cell number is seen. Finally, the increase in COX-2 expression and PGE_2 production occurs between 1 and 10 μM NO-ASA, which is estimated to be around 20% of the IC_{50} for cell growth inhibition. Thus, it appears that (*a*) inhibition of β-catenin/TCF signaling pathway is exquisitely sensitive to NO-ASA and (*b*) all four effects occur at very low NO-ASA concentrations, well below or at concentrations affecting cell growth.

Second, there are potential interactions among these four pathways. Recent literature is replete with examples of "cross-talk" or interactions between endogenous NO and COX-2, between NF-κB and NOS2 or COX-2, and between Wnt and COX-2 (23). The fact that no single cell line was suitable for the study of all four pathways hampered our efforts to directly assess putative interactions among them. On the other hand, this allowed a different set of conclusions. For example, the fact that unstimulated HT-29 cells express no NOS

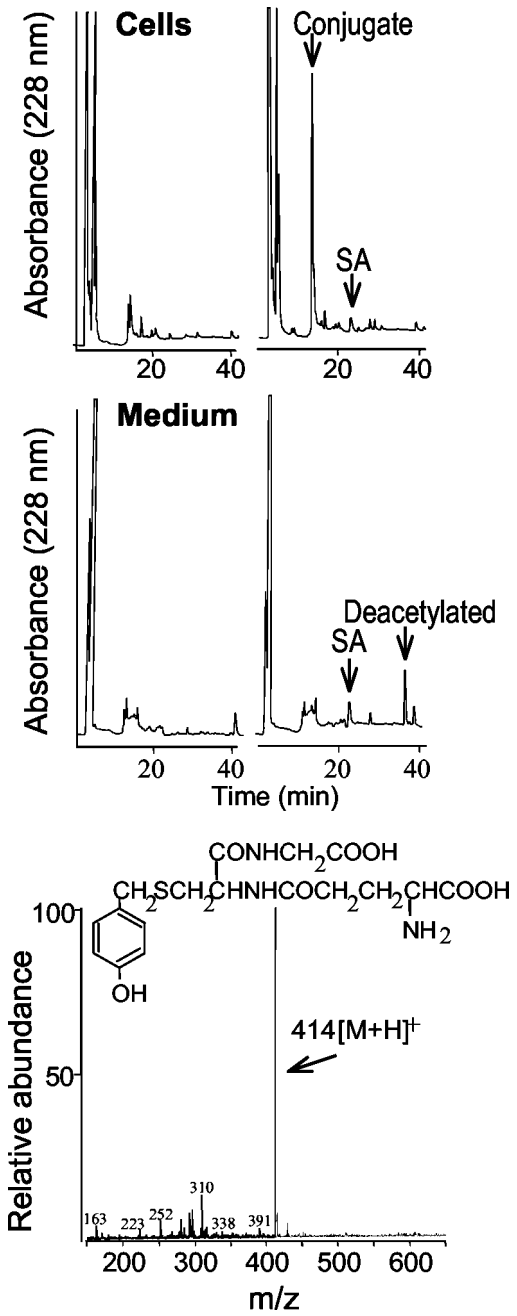

Fig. 4. Metabolism of NO-ASA by human colon cancer cells. NO-ASA (100 μM) was incubated for 4 h with HT-29 human colon cancer cells, and metabolites were extracted from either cells (*top left panel*, untreated cells; *top right panel*, NO-ASA-treated cells) or the culture medium (*middle left panel*, untreated cells; *middle right panel*, NO-ASA-treated cells) and fractionated by HPLC, as described in "Materials and Methods." *Conjugate*, conjugate of 4-HMP and GSH (4HMP-GSH), the structure and mass spectrum of which are shown (*bottom panel*). *Deacetylated*, NO-ASA minus the acetyl group on the ASA moiety.

suggests that the induction of COX-2 by NO-ASA that we observed in these cells is likely independent of NOS.

The third issue relates to contradictions between currently accepted models and our own findings. For example, the induction of COX-2 by NO-ASA is inconsistent with the accepted model of its regulation by the β-catenin/TCF pathway and previous work indicating that in circulating rat monocytes, NO-ASA blunted COX-2 mRNA (24). Unfortunately, SW480, a cell line suitable for the study of β-catenin/TCF signaling (APC mutated, β-catenin overexpression) does not express COX proteins, and thus it is not possible to directly ascertain the role of β-catenin/TCF signaling on COX-2 expression. A more important observation is the induction of COX-2 by NO-ASA, which is accompanied by substantially increased production of PGE_2; PGE_2 enhances proliferation in colon colonocytes and may induce apoptosis (25). COX-2 is generally considered to contribute to colon carcinogenesis, at least in its later stages. Our finding can be interpreted either as indicating that this change is either cell type restricted (*e.g.*, to colon and pancreatic cells) or restricted to cell culture systems or that COX-2 may play a far more complex role in colon carcinogenesis than currently understood.

In our studies, the IC_{50} of NO-ASA for the inhibition of cell growth varied within a relatively narrow range, depending on the cell line and the experimental protocol followed in each case. However, in all instances, it has been remarkably low, being hundreds of times lower than that for traditional ASA. Even more impressive has been the similarly huge difference between the potency of ASA and NO-ASA in bringing about the biological effects that we observed. In all but one case, ASA was either totally ineffective or only partially effective. The notable exception was its inhibitory effect on NOS2, which was stronger than that of NO-ASA (although ASA was used at a 50-fold molar excess over NO-ASA).

The exact structure-activity relationship between NO-ASAs and these biological effects is not clear. The spacer part of the molecule and the $-NO_2$ moiety, which is expected to release NO, must contribute to its enhanced potency. In all instances, dn-NO-ASA was significantly less potent than the full molecule. Thus, a large proportion of the effect of NO-ASA can be accounted for by the contribution of the $-NO_2$ moiety. Of interest, endogenous NO enhances the expression and prevents the degradation of IκBα, the cytoplasmic NF-κB inhibitor (26), thereby inhibiting the activation of NF-κB. This action might augment the inhibitory effect of ASA on NF-κB.

The metabolism of NO-ASA by colon cells bears significantly on the issue of structure-activity relationships. It is apparent that NO-ASA proceeds via a rapid deacetylation step and reacts with GSH, the levels of which are being progressively depleted long after the intact molecule disappears from either the culture medium or the intracellular compartment. Consequently, the effect of NO must be a rapid one because no $-NO_2$-bearing derivative of NO-ASA is detectable by 1 h (data not shown).

It was interesting to note that NO-ASA failed to alter the levels of cGMP, although the exogenous NO donor SNAP increased them 3-fold, thus serving as an important methodological control. This result contrasts with results obtained with NO-ASA in monocytes (14) and serves as a reminder that the type of the target tissue can restrict

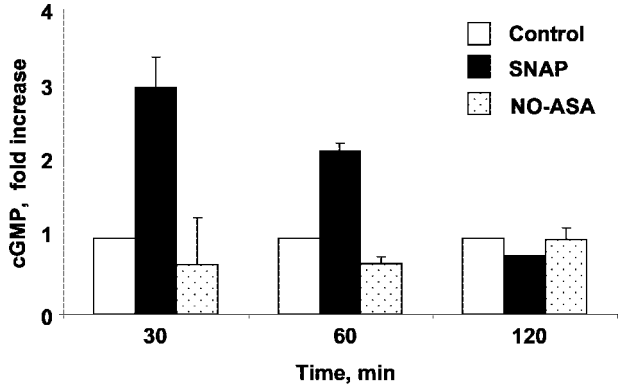

Fig. 5. The effect of NO-ASA on intracellular cGMP levels. HT-29 cells were incubated with a phosphodiesterase inhibitor (500 μM IBMX) for 20 min before treatment with SNAP or NO-ASA (each at 100 μM). At the indicated time points, cells were assayed for cGMP levels. Values are mean ± SE (n = 3).

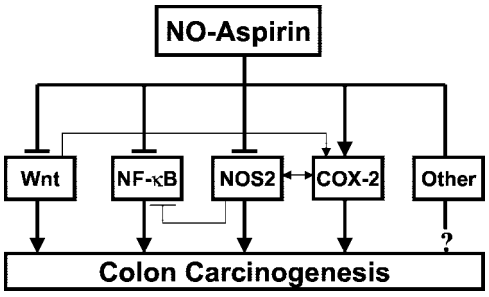

Fig. 6. Working model of the pleiotropic effect of NO-ASA on molecular targets contributing to colon carcinogenesis. The redundancy in effect by NO-ASA is apparent. *Other* indicates our expectation that additional targets will be discovered in the future.

cellular responses. Therefore, it is unlikely that NO-ASA exerts any major metabolic effect on these cells via the cGMP signaling system.

Of necessity, our experimental system did not allow us to examine the effects of NO-ASA on the four pathways that we studied in a single cell line. Nevertheless, our findings can be incorporated into a working model of the effect of NO-ASA on colon carcinogenesis (Fig. 6). Inhibition of β-catenin/TCF signaling, NOS2, and NF-κB is consistent with background knowledge that all three pathways can potentially contribute to colon carcinogenesis and that their inhibition can mitigate the development of colon cancer. The induction of COX-2, however, represents an anomaly, when considered in the light of our current understanding of its role in carcinogenesis. In fact, its increased expression in response to NO-ASA appears to be counterintuitive. At this point, we do not know the role, if any, of the induced expression of COX-2 in the chemopreventive activity of NO-ASA against colon cancer; further study is required to reconcile the possibility of chemoprevention in the face of induced COX-2 expression. Regardless of specific details, this model emphasizes two important aspects of the action of NO-ASA on colon cancer cells. First, there is mechanistic redundancy in the inhibition of colon cancer cell growth by NO-ASA, a feature that may predict a favorable effect against colon cancer. The more mechanisms a compound uses to inhibit cancer cell growth, the more efficacious it is expected to be against a tumor that continuously undergoes clonal expansion. Second, we anticipate that additional mechanisms of action will be discovered in the future, and the box labeled *Other* in the diagram in Fig. 6 indicates this. This expectation is based on our ongoing work and also on the mode of action of traditional ASA (27).

In conclusion, our findings document that NO-ASA induces an array of potentially important molecular changes in the malignant colonocyte that may be relevant to its pharmacological actions in cancer and perhaps elsewhere. The ultimate relevance of these findings to the chemoprevention of human colon cancer, however, requires their testing in animal tumor models and human subjects.

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