Protein nitration and nitrosylation by NO-donating aspirin in colon cancer cells: Relevance to its mechanism of action

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Abstract

Nitric oxide-donating aspirin (NO-ASA) is a promising agent for cancer prevention. Although studied extensively, its molecular targets and mechanism of action are still unclear. S-nitrosylation of signaling proteins is emerging as an important regulatory mechanism by NO. Here, we examined whether S-nitrosylation of the NF-κB, p53, and Wnt signaling proteins by NO-ASA might explain, in part, its mechanism of action in colon cancer. NO-ASA releases significant amounts of NO detected intracellularly in HCT116 and HT-29 colon cells. Using a modified biotin-switch assay we demonstrated that NO-ASA S-nitrosylates the signaling proteins p53, β-catenin, and NF-κB, in colon cancer cells in a time- and concentration-dependent manner. NO-ASA suppresses NF-κB binding to its cognate DNA oligonucleotide, which occurs without changes in the nuclear levels of the NF-κB subunits p65 and p50 and is reversed by dithiothreitol that reduces –S-NO to –SH. In addition to S-nitrosylation, we documented both in vitro and in vivo widespread nitration of tyrosine residues of cellular proteins in response to NO-ASA. Our results suggest that the increased intracellular NO levels following treatment with NO-ASA modulate cell signaling by chemically modifying key protein members of signaling cascades. We speculate that S-nitrosylation and tyrosine nitration are responsible, at least in part, for the inhibitory growth effect of NO-ASA on cancer cell growth and that this may represent a general mechanism of action of NO-releasing agents.

INTRODUCTION

Colon carcinogenesis is often a prolonged process requiring years between the initial cellular change and the development of clinical disease [1]. This process is influenced by diet, environment and genetic factors. Inhibition of the initiation phases and delaying progression could best be achieved through effective cancer prevention approaches [2]. Non-steroidal anti-inflammatory drugs (NSAIDs) show promise as chemopreventive agents against colon cancer [3]. Their use, however, is associated with a wide spectrum of side effects, some of which are life-threatening [1, 4–5]. The need for safer and more effective NSAIDs has led to the synthesis of NO-donating NSAIDs (NO-NSAIDs), which have emerged as safer and highly effective chemopreventive agents [6]. We and others have shown that several NO-NSAIDs are much more effective in reducing the growth of colon

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cancer cells than the corresponding parent NSAIDs [7–9]. Inhibition of cell growth is linked to inhibition of cell proliferation, induction of apoptosis and a significant block in cell cycle transitions [8]. One of the most promising NO-NSAID to date is NO-aspirin (NO-ASA), which displays a remarkable chemopreventive ability both in vitro and in animal models of colon cancer [10–12]. The mechanism responsible for the enhanced potency of NO-ASA is complex and appears to include effects on cell signaling mediated by the β-catenin/T-cell factor (TCF) [13] and the NF-κB pathways [14].

S-nitrosylation, the formation of S-nitrosothiols by the covalent addition of NO to cysteine residues, has been shown to regulate the function of several cellular proteins [15]. We have previously suggested that S-nitrosylation of β-catenin in response to NO-ASA may explain the inhibitory effect of NO-ASA on the Wnt pathway [16]. In particular, we have proposed that S-nitrosylation of cysteine residues in the binding domains of β-catenin and TCF prevents their association, which is required for the formation of an effective transcriptional outcome. This effect may be due, in part, to steric hindrance or conformational changes, either one being a consequence of S-nitrosylation. A similar mechanism may account for the inhibitory effect of NO-ASA on NF-κB signaling [14].

Given these considerations, we undertook a direct evaluation of S-nitrosylation in the β-catenin/TCF and NF-κB signaling pathways in HT-29 and HCT116 human colon cancer cells. In addition, we evaluated in HCT116 cells whether NO-ASA S-nitrosylates p53, a tetrameric protein that plays a pivotal role in about half of all human cancers, including colon cancer [17–18]. Furthermore, we evaluated both in vitro and in vivo levels of nitrotyrosine, a stable end-product of the nitration of a tyrosine residue. The nitrate moiety for the formation of nitrotyrosine is provided by the reactive nitrating intermediate peroxynitrite ($ONOO^-$), which in turn can result from the reaction of NO and superoxide anion ($O_2^{\cdot}$) [19–21]. Nitrotyrosine, a marker for peroxynitrite and other nitrating species [22], provides a complementary assessment of the effect of NO on cellular proteins.

MATERIALS AND METHODS

Reagents

NO-ASA [*NCX4040*, 2-(acetyloxy) benzoic acid 4-(nitrooxymethyl) phenyl ester] was synthesized by us [23] and conventional ASA was obtained from Sigma, St. Louis MO. For both we prepared 100 mM stock solutions in DMSO; the final DMSO concentration in all media did not exceed 1%. All general solvents and reagents were of HPLC grade or the highest grade commercially available. All antibodies were from Calbiochem (San Diego, CA), with the exception of the anti-nitro-tyrosine antibody, which was from Chemicon (Temecula, CA).

Cell Culture

We used two human colon cell lines HT-29 and HCT116 (American Type Culture Collection (ATCC), Manassas, VA). HT-29 is mutant for apc and p53 and wt for cox-2 and β-catenin. HCT116 is wt for apc and p53 and mutant for cox-2 and β-catenin [14, 24–25]. Both cell lines were grown as monolayers in the medium suggested by ATCC and supplemented with 10% FCS (Mediatech, Herndon, VA), penicillin (50 units/ml), and streptomycin (50 μg/ml; Life Technologies, Inc., Grand Island, NY). Cells were incubated at 37°C in 5% CO_2. Cell viability/growth was measured using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay according to the manufacturer's instructions (Roche Diagnostics, Nutley, NJ).

Assay of Intracellular NO Levels

NO levels were determined using the DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate) molecular probe according to the manufacturer's instructions (Molecular Probes, Eugene OR). Briefly, after treatment, cells were loaded with 5 μM of DAF-FM DA for 1 hr at room temperature. Excess probe was removed by washing with buffer followed by centrifugation. Fresh buffer was added and de-esterification of the probe was allowed to proceed for 30 min at room temperature. NO detection was achieved with a fluorescent microplate reader (excitation/emission of 495/515 nm).

Confocal Microscopy

Control and NO-ASA-treated colon cancers cells were incubated for 1 hr at 37°C with DAF-FM DA. Excess probe was removed by washing with buffer and cells were scanned using confocal laser scanning microscopy (2 μm sections from the bottom).

Protein Extraction

Whole cell extracts were obtained by lysing cells in RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% NP-40, 0,25% sodium deoxycholate and Protease Inhibitor Cocktail 2 (Sigma-Aldrich, St. Louis, MO)). Cytoplasmic and nuclear extracts were prepared following a standard protocol [26]; trypsinized cells were suspended in lysis buffer to which Nonidet P-40 was added at a subsequent step (the supernatant fraction represented the cytoplasmic extract); nuclei were washed and centrifuged, followed by resuspension in extraction buffer and pelleting. Protein extracts were stored at −80°C until further analysis.

S-nitrosylation

We used a modification of the biotin-switch method by Jaffrey *et al.* [27]. After treatment with NO-ASA, cells were harvested using a rubber policeman and centrifuged at 1000 rpm for 5 minutes at 4°C. Cells were then washed once with cold PBS (containing 5 mM EDTA, 0.1 mM Neocuprine and 1 mM methyl methanethiosulfonate (MMTS)). Nuclear and whole cell extracts were prepared as described above. The supernatant typically contained 0.8 μg protein per μl assayed with the Bio-Rad protein Assay (BIO-RAD Laboratories, Inc. Hercules, CA). Four volumes of blocking buffer (9 volumes of HEN buffer (250 mM HEPES pH 7.7, 1 mM EDTA, and 0.1 μM neocuproine) plus 1 volume 25% SDS (adjusted to 20 mM MMTS with a 2 M stock prepared in dimethylformamide (DMF)) were added to the supernatant which was then incubated for 20 min at 50°C with frequent vortexing. MMTS was removed by precipitation with ice-cold acetone (4 volumes of acetone were added to one volume of supernatant, mixed gently and stored at −20°C for 30 min followed by centrifugation at 3000× g for 10 min at 4°C). Pellets were resuspended in 0.5 ml HENS buffer (HEN buffer plus 1% SDS) and proteins were biotinylated using biotin–HPD; following the instructions of the manufacturer (Thermo Scientific, Rockford, IL). Biotinylated proteins were isolated by acetone precipitation and centrifugation. The pellet was resuspended in HENS buffer as above. To this point, all steps were carried out in the dark. Two volumes of neutralization buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 0.5% Triton X-100) were added and any unresolved particulates were pelleted by centrifugation and discarded. Streptavidin–agarose (Sigma, St. Louis, MO) was added to the solution and the mixture was allowed to incubate for 1 h at room temperature. The mixture was subsequently washed 5 times with neutralization buffer with its NaCl concentration adjusted to 600 mM. To recover the bound protein, the mixture was incubated for 20 min at room temperature in elution buffer (20 mM HEPES pH 7.7, 100 mM NaCl, 1 mM EDTA and 100 mM 2-mercaptoethanol). SDS-PAGE sample buffer was added and proteins were

fractionated by gel electrophoresis and the presence of S-nitrosylated proteins was accessed by western blot analysis.

Electrophoretic Mobility Shift Assays (EMSAs)

The NF-κB-DNA binding activity was assessed by reacting extracts from control and cells treated with NO-ASA with 33 nmol of a ^{32}P-end-labeled double-stranded consensus oligonucleotides for NF-κB (AGTTGAGGGGACTTTCCCAGGC; Promega Corp., San Luis Obispo, CA) or p53 (GAACATGTCTAAGCATGCTG; Santa Cruz Biotechnology Inc., Santa Cruz CA). The specificity of the binding was tested by competition reactions, using 50-fold molar excess of unlabeled NF-κB or p53 (specific competition). The complexes were separated on a 6% gel following standard protocols.

Xenograft Tumor Model

Male athymic nu/nu mice aged 5 weeks (Harlan Bioproducts, Indianapolis, IN) were maintained in a maximum isolation environment, according to an institutionally approved animal protocol, and given food and water ad libitum. After one week of acclimatization, 10^6 HT-29 cells suspended in 100 μl of sterile Dulbecco's phosphate-buffered saline (Mediatech) were implanted subcutaneously into the right flank of each mouse. After 3 weeks, when tumors had reached an average volume of ~300 mm^3, intratumoral injections were conducted twice a week for 3 weeks with 200 μl vehicle or NO-ASA 300 μM. One additional control group received no injections. At the end of the study, mice were euthanized and the tumors, cut sagittally, were fixed in 10% buffered formalin. The area of necrosis in each tumor was determined as reported [12].

Immunohistochemistry

Paraffin sections were deparaffinized, rehydrated and antigen retrieval obtained by heating in a microwave for 15 min in 0.01 M citrate buffer (pH 6.0). After 15 min of blocking with serum, the primary antibody nitro-tyrosine () at 1:100 or the control isotype IgG was applied and incubated overnight at 4°C. Slides were washed three times with phosphate buffered saline for 5 min. The biotinylated secondary antibody and the streptavidin biotin complex (Invitrogen Corporation, Carlsbad, CA) were applied, each for 60 min at room temperature with interval washings. The slides were rinsed with distilled water, dehydrated and a coverslip was applied. Two independent evaluators, blinded to sample identity, scored at least 5 randomly selected fields ($\times$ 200 magnification) and *scored nitro-tyrosine expression* as follows: 0 = negative staining for nitro-tyrosine; 1 = weakly positive; 2 = moderately positive; 3 = more than moderate and less than strong; 4= strongly positive (maximal). The average value was calculated for each sample.

Statistical Methods

Data were expressed as mean ± SEM and differences between groups were analyzed by the t-test. $P< 0.05$ denotes significant differences.

RESULTS

NO-ASA inhibits the growth of human cancer cell lines and releases NO

As expected [8], NO-ASA inhibited the growth of both HT-29 and HCT116 human colon cancer cell lines. The 24-hr IC_{50}s for HT-29 and HCT116 cells were 28 ± 2.2 μM and 92 ± 4.7 μM, respectively. For both cell lines the IC_{50} of aspirin, the parent compound of NO-ASA, was greater than 5 mM. The reason for the nearly 3-fold difference in potency between these two cell lines is not readily apparent.

The intracellular levels of NO were quantified in HT-29 and HCT116 cells treated with and without NO-ASA for 1 or 3 hr. NO levels were determined in cells preloaded with the NO molecular probe in three ways: a) spectrophotometrically; b) by flow cytometry analysis; and c) by confocal microscopy. As an additional control, we treated these cells with deoxycholate, reported to stimulate the production of NO [28]. After 1 hr of incubation with various concentrations of NO-ASA or 500 μM deoxycholate, we observed a modest elevation of NO levels in both cell lines (data not shown). At 3 hr there was a quantitatively more pronounced increase in NO levels, as detected by all three methods (Fig. 1). The changes in NO levels were concentration-dependent. As can be seen in Fig 1, at 1 and 3 hr of incubation the increase in NO levels ranged between 1.4 and 1.8 fold over control (average of 3 methods), respectively. Incidentally, there was marked increase in NO levels in detached cells (floating in the culture medium), compared to those attached to the culture plate. At the time of the assay, all detached cells were viable (as determined by the trypan blue exclusion method) but the process of cell death had been initiated. This finding is consistent with our previous report on the redox changes in cells dying in response to NO-ASA and related compounds [29].

NO-ASA nitrosylates cysteine and nitrates tyrosine residues in proteins

Initially, we assessed the general effect of NO-ASA on S-nitrosylation of proteins in these two cell lines. Cells were treated with various concentrations of NO-ASA for 3 hr, subjected to a modified biotin switch assay and evaluated by Western blot analysis using a primary antibody against biotin. As shown in Fig. 2A, NO-ASA induced S-nitrosylation of several proteins in colon cancer cells. Of interest, among the modified proteins were proteins with sizes similar to those of β-catenin, the p65 and p50 NF-κB subunits, β-catenin and p53. S-nitrosylation occurred in both cytoplasmic and nuclear proteins (data not shown) and was not time-dependent (1 and 3 hr, Fig. 2). A similar effect was demonstrated in BxPC-3 human pancreatic cancer cells treated with NO-ASA (data not shown). A striking change occurs between 1 and 3 hr in the expression of a S-nitrosylated protein of apparent molecular weight slightly larger than 50 kD in the untreated control. The significance of this change is uncertain, especially since this protein has not been identified.

The cellular production of highly reactive nitrogen species derived from NO, such as peroxynitrite, leads to the nitration of tyrosine residues in tissue proteins [30–31]. Peroxynitrite forms through the reaction of NO with superoxide anion, a reaction considered to mediate cytotoxic effects of NO in the cell [32]. The degree of protein nitrotyrosine formation is considered an indication of the production of reactive nitrogen species and of potential cell damage [21, 33]. Of significance to the present work is the fact that tyrosine nitration of nuclear p65 induces its dissociation from p50 and subsequent association with IκBα and export into the cytoplasm [34]. Consequently, we examined the potential nitration of tyrosine residues of proteins in response to NO-ASA.

HCT116 and HT-29 cells were treated for 3 hr with DMSO or various concentrations of NO-ASA. Cytoplasmic and nuclear fractions were subjected to Western blot analysis using a primary antibody that specifically recognizes proteins with nitrated tyrosine residues. As shown in Figure 2B, NO-ASA caused the formation of nitrotyrosines in several proteins, amongst which members of the NF-κB family have been tentatively identified based on their size.

To evaluate the possibility of tyrosine nitration in vivo, we studied by IHC the levels of tyrosine nitration in HT-29 colon cancer xenografts in nude mice treated with NO-ASA [12]. Mice were injected intratumorally twice a week for 3 weeks with vehicle or NO-ASA, while a third group received no injections. At sacrifice, the necrotic area of tumors, expressed as percentage of total area, was similar in the non-injected and vehicle-injected

groups (data not shown). The necrotic area of tumors was higher in the NO-ASA-treated group (65.8±2.4%; mean±SEM for this and all subsequent values), compared with the vehicle group (52.2 ±4.1%; P < 0.001). NO-ASA more than doubled the amount of tyrosine nitration in the xenograft tissues, as shown in Fig. 2C; the tyrosine nitration scores in the two groups were: vehicle = 0.73±0.25 and NO-ASA = 1.82±0.41 (p=0.03).

NO-ASA S-nitrosylates β-catenin, the p65 NF-κB subunit and p53 in HCT116 cells

To assess S-nitrosylation of β-catenin, of the p65 subunit of NF-κB and of p53 in whole cell lysates as well as in cytoplasmic and nuclear factions, HCT116 cells were treated with various concentrations of NO-ASA. As shown in Fig. 3, NO-ASA induced S-nitrosylation of all these three proteins. This effect was clear, regardless of the origin of the proteins (cytoplasmic vs. nuclear). In all instances this effect was concentration-dependent although a plateau seemed to be reached rather quickly. Time dependence was also evident, at least in the case of β–catenin, the only protein for which it was evaluated.

NO-ASA inhibits the binding of nuclear NF-κB to its cognate oligonucleotide

HT-29 and HCT 116 cells were treated for 3 hr with NO-ASA and NF-κB activation was evaluated using EMSAs. As expected [14], NO-ASA inhibited NF-κB activation in a concentration-dependent manner in both cell lines, whereas aspirin, the parent compound, exhibited only a marginal inhibitory effect at a concentration of 5 mM. To further evaluate the role of S-nitrosylation in the inhibition of NF-κB-DNA binding, we treated, for 1 hr, the nuclear extracts of these cells with 20 mM dithiotreitol (DTT); an agent that effectively removes the thiol-bound NO groups from proteins [35]. As shown in Fig 4, DTT restored NF-κB-DNA binding. This was also true of untreated HT-29 and HCT 116 cell extracts, demonstrating the baseline formation of nitrosothiols. However, it is important to note that we cannot rule out the possibility that the reversal by DTT is due to disruption of disulfide bonds that may have formed. In addition, HT-29 and HCT 116 cells were treated for 3 hr with NO-ASA and p53 activation was evaluated using EMSAs. Although we have demonstrated S-nitrosylation of p53, NO-ASA did not inhibit the binding of p53 to its cognate oligonucleotide, indicating the specificity of the overall effect. Interestingly, the role of oxidative modifications of p53 for its biological function remains unclear; p53 being subject to more than one level of conformational modulation through oxidation-reduction of cysteines at or near the p53-DNA interface [36–38].

DISCUSSION

Modified NSAIDs, such as NO-ASA, hold promise for the control of cancer. Although basic parameters of the mechanism of action of NO-ASA have been defined by us and others [8], very little is known about the underlying molecular mechanism responsible for its enhanced potency and efficacy. The structural hallmark of NO-ASA is its moiety that donates NO, a highly reactive radical modulating various cellular responses. Thiols are a well-recognized target of NO, a reaction that leads to the formation of S-nitrosothiols. Our data demonstrate extensive S-nitrosylation of cellular proteins, some of which play critical roles as signaling molecules. S-nitrosylation of signaling proteins emerges as an important regulatory mechanism [39–40].

Treatment of colon cancer cells with NO-ASA increases significantly their NO levels. Of note, in addition to releasing NO, NO-ASA stimulates the catalytic activity of nitric oxide synthase [41]. The increased intracellular NO levels are accompanied by S-nitrosylation and tyrosine nitration of multiple cellular proteins. Both effects vary in degree with respect to target proteins, reflecting perhaps variations in the number of cysteine and tyrosine residues in each protein. Tyrosine nitration results from the reaction of $NO_2^{\cdot}$ (nitrogen dioxide) with

tyrosine; $NO_2^{\cdot}$ is derived either from peroxynitrite or nitrite, both of which reflect the presence of NO [19, 42]. Tyrosine nitration by NO-ASA occurs also in vivo (>2 fold over controls).

Our work focused on three signaling molecules, NF-κB, p53 and β–catenin that are frequently dysregulated in many cancers. All three interact with other proteins and these interactions either sequester them into an inactive state or are required for their biological activity. For example, p53 is bound to mdm2, preventing its action and transporting it from the nucleus to the cytosol; phosphorylation of p53 disrupts mdm2-binding and releases p53 [43–44]. NF-κB, itself a dimer, is also sequestered in the cytoplasm by binding to IκB, whereas β-catenin interacts with cytoplasmic proteins and, released from them, reaches the nucleus and becomes transcriptionally active only when it binds to other proteins [45].

We have speculated that S-nitrosylation may induce steric hindrance in Wnt signaling proteins [16] and the same is conceivable for NF-κB and p53 proteins. NF-κB, electrophoretic mobility shift assays demonstrated that S-nitrosylation was responsible of its reduced binding to DNA; chemical reduction of NF-κB thiols restored the binding of NF-κB to DNA. It is likely that the S-nitrosylation of NF-κB and perhaps of other proteins affects their signaling functions. Thus S-nitrosylation may mediate part of the anticancer effect of NO-ASA. It should be kept in mind, however, that our experimental method did not allow quantification of the extent of S-nitrosylation of a given protein(s), e.g. NF-κB. It is likely that only a fraction of the molecules are S-nitrosylated. Apparently, in our studies the extent of S-nitrosylation exceeds a threshold that allows a functional effect.

Our findings also establish widespread modification of cellular proteins through nitration, another result of increased NO levels in the cell. This finding suggests that NO may affect proteins beyond those that are S-nitrosylated and that nitration of tyrosines may further modulate cell signaling in response to NO-ASA. Indeed, tyrosine nitration of p65 has been recognized to rapidly inactivate NF-κB [34, 46].

Fig. 5 offers a plausible mechanism of action of NO-ASA that integrates S-nitrosylation and tyrosine nitration. Specifically, NO-ASA increases the intracellular levels of NO, both directly and through its stimulatory effect on NOS. The first result is S-nitrosylation of proteins. NO-ASA also increases the production of superoxide anion from mitochondria [47], which can react with NO to form peroxynitrite, which can then be converted to $NO_2^{\cdot}$ (which can also be produced by the reaction of NO with O_2 [19]). $NO_2^{\cdot}$ nitrates protein tyrosine residues. This overall mechanism of action may be applicable to other NO-releasing compounds.

In conclusion, our findings establish extensive and functionally significant S-nitrosylation and nitration of protein tyrosine residues in response to NO-ASA, an agent whose cardinal feature is the release of NO. These findings suggest a mechanism of action that may also apply other members of this class of promising anti-cancer and anti-inflammatory compounds.

Acknowledgments

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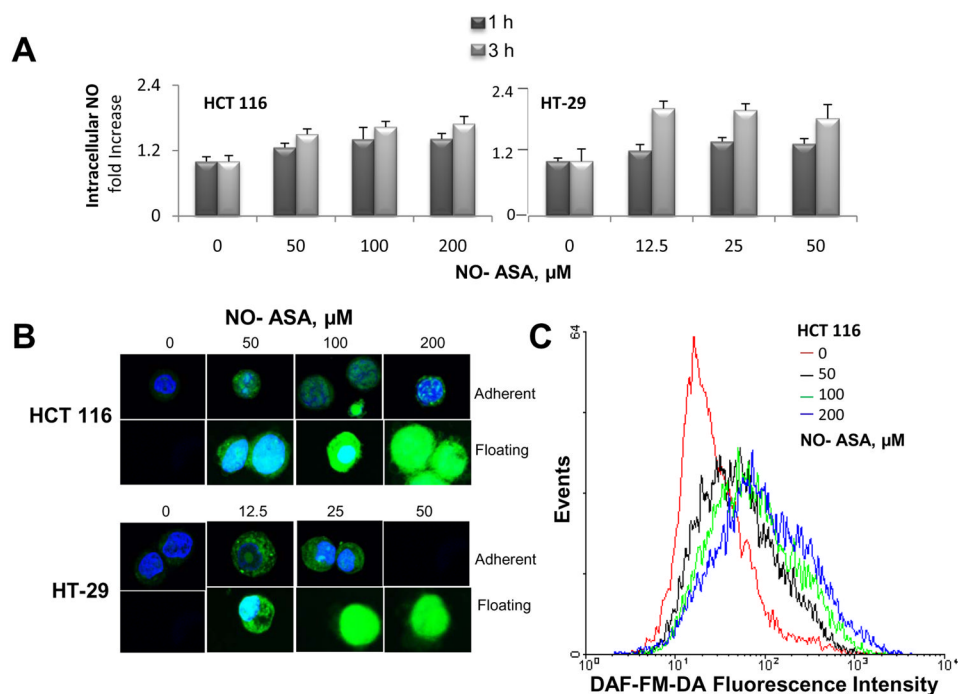
Fig. 1. NO-ASA increases intracellular NO levels in colon cancer cell lines
HT-29 and HCT116 colon cancer cells were treated with various concentrations of NO-ASA for 3 hr. Floating cells were harvested from the gently aspirated culture media by low speed centrifugation, whereas adherent cells were harvested following trypsinization and centrifugation. Both non-adherent and adherent cells were kept on ice and washed two times with cold PBS. Cells were then loaded with the molecular probe DCFDA and fluorescence was assayed using a microplate reader (*top*), imaged by confocal microscopy (*bottom left*) or quantified by flow cytometry (*bottom right*). No adherent cells were apparent on the cell culture plate after a 3 hr incubation with 50 μM NO-ASA.
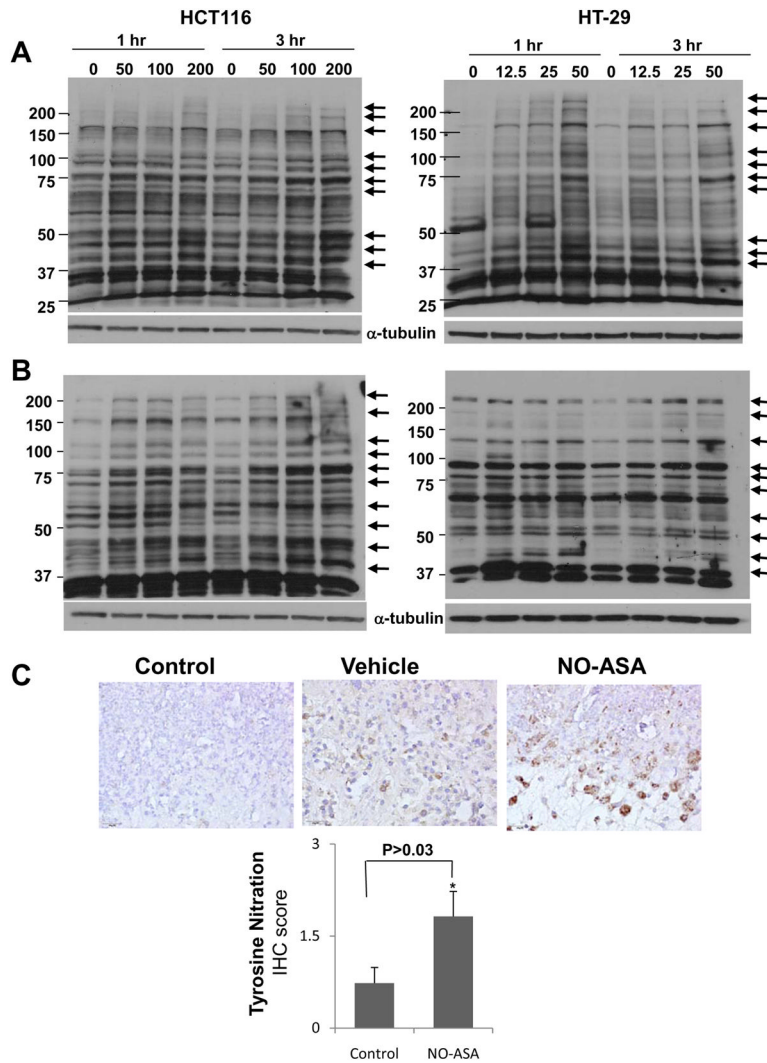
Fig. 2. NO-ASA S-nitrosylates proteins and nitrates protein tyrosine residues in human colon cancer cells
A and B: HCT116 and HT-29 cells were treated with the indicated concentrations of NO-ASA for 1 or 3 hr. Whole cell lysates were isolated and subjected to the modified biotin switch assay (A) or to immunoblot analysis using an anti-nitrotyrosine primary antibody (B). The arrows on the right identify S-nitrosylated proteins or proteins whose nitrotyrosine content changed as a result of treatment with NO-ASA. *Loading control:* α-tubulin. **C:** HT-29 human colon cancer xenografts treated with NO-ASA were evaluated by IHC for their nitrotyrosine content as in Methods. Three representative photomicrographs (magnification 200×) are shown on the top. Control, a section treated with non-specific antibody that was isotypic to that specific for nitrotyrosine used in the other two sections. Vehicle, sample from mice treated with vehicle (n=10), showing limited staining for nitrotyrosine; NO-ASA, sample from mice treated with NO-ASA (n=8), strongly positive for nitrotyrosine. The graph on the bottom depicts the mean ± SEM of the nitrotyrosine score for the two study groups.
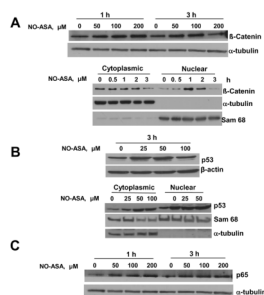
Fig. 3. NO-ASA S-nitrosylates β-catenin, p53 and the NF-κB subunit p65 in HCT 116 cells
Cells were treated as indicated with NO-ASA for 1 or 3 hr and whole cell, cytoplasmic or
nuclear protein extracts, as indicated, were analyzed using the biotin switch method that
detects S-nitrosylated proteins. **A***: Top panel*: S-nitrosylated β-catenin levels in protein
extracts from HCT 116 cells. *Bottom panel:* S-nitrosylated β-catenin levels from the
cytoplasm or nucleus of cells treated with NO-ASA 80 μM. **B**: S-nitrosylated p53 levels in
protein extracts from whole cells (*top panel)* or the cytoplasm and nucleus *(bottom panel)*.
C: S-nitrosylated p65 levels in protein extracts from whole cells. *Loading controls:* α-
tubulin or β-actin for cytoplasmic or whole cell protein extracts; Sam 68, a nucleus-
predominantly expressed protein, for nuclear protein extracts.
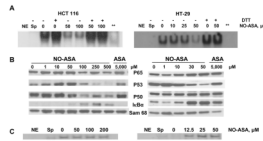
Fig. 4. NO-ASA inhibits DNA binding of NF-κB but not of p53

Top panel: Nuclear extracts of HT-29 (*right*) and HCT 116 (*left*) colon cancer cells treated for 3 hr with NO-ASA at the indicated concentrations were analyzed by EMSA for NF-κB binding. The indicated nuclear extracts were treated with DTT 20 mM for 15 min prior to their reaction with the NF-κB oligonucleotide to determine the effect of the reducing agent on DNA binding. *Middle panel:* Western blot analysis of the nuclear levels of individual NF-κB signaling proteins and of p53 from cells treated with NO-ASA or ASA as indicated. Loading control: Sam 68. *Bottom panel:* Nuclear extracts of HT-29 (*right*) and HCT 116 (*left*) colon cancer cells treated for 3 hr with NO-ASA at the indicated concentrations were analyzed by EMSA for p53 binding. *NE*, no extract; *Sp*, specific competition (unlabeled oligonucleotide); **, sample from cells treated with NO-ASA at a concentration corresponding to IC_{50} for growth.
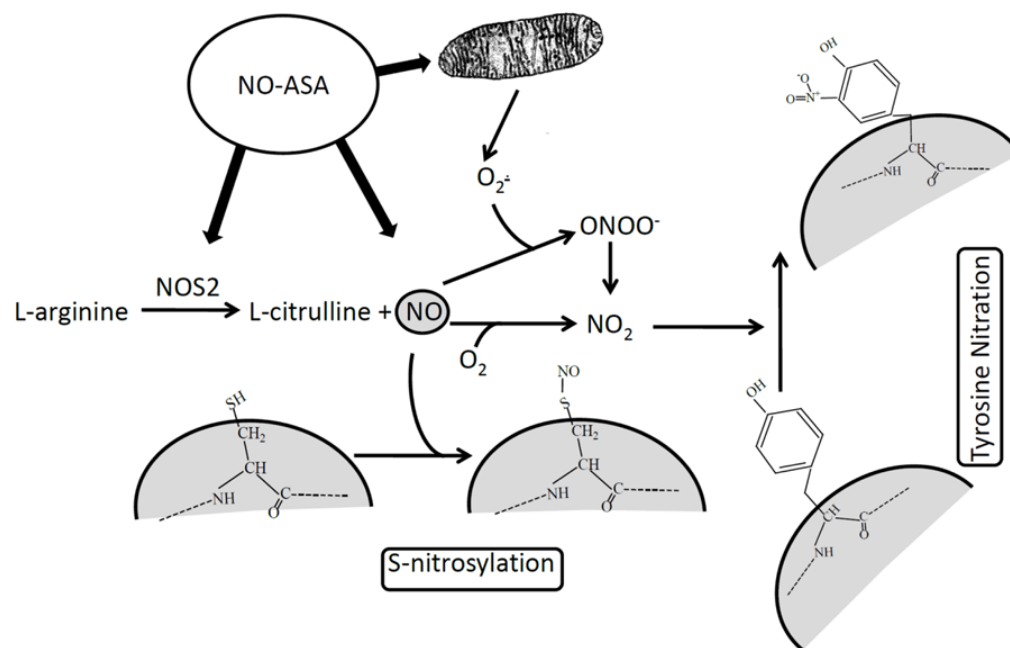
Fig. 5. Proposed model of protein S-nitrosylation and tyrosine nitration by NO-ASA
NO-ASA a) stimulates NOS2 activity increasing NO production form L-arginine, b) releases
NO, and c) stimulates $O_2^.$ production by the mitochondria. NO is shown here to nitrosylate a
cysteine residue of a protein. $NO_2^.$, forming a) from peroxynitrite (ONOO−) or b) from the
reaction of NO with O_2, is shown to nitrate a protein tyrosine residue.