Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochemical Pharmacology 70 (2005) 993–1000
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

NO-donating aspirin inhibits both the expression and catalytic activity of inducible nitric oxide synthase in HT-29 human colon cancer cells

Adam Spiegel [a], Thomas R. Hundley [a], Jie Chen [a], Jianjun Gao [a], Nengtai Ouyang [a], Xiaoping Liu [a], Mae F. Go [b], George J. Tsioulias [a], Khosrow Kashfi [c], Basil Rigas [a,*]

[a] *Division of Cancer Prevention, SUNY at Stony Brook, Stony Brook, NY 11794-5200, USA*
[b] *Department of Medicine, VA Medical Center, Salt Lake City, UT 84148, USA*
[c] *Department of Physiology and Pharmacology, City University of New York Medical School, New York, NY 10031, USA*

Received 30 May 2005; accepted 27 June 2005

Abstract

Nitric oxide-releasing aspirin (NO-ASA) is emerging as a potentially important chemopreventive agent against colon cancer. We examined in HT-29 human colon adenocarcinoma cells the effect of NO-ASA on the inducible form of nitric oxide synthase (NOS2), an enzyme implicated in colon carcinogenesis. NO-ASA inhibited in a time- and concentration-dependent manner the expression of NOS2 up to 70% compared to control (IC_{50} for this effect = 46 μM). NO-ASA also decreased the corresponding steady-state mRNA levels and this reduction preceded the reduction of protein levels by at least 6 h. NO-ASA also reduced the enzymatic activity of NOS2, as determined by a direct enzyme assay (maximal reduction = 80%) and by determining the accumulation of NO in the culture medium (IC_{50} for this effect = 36 μM). These effects of NO-ASA on NOS2 were paralleled by inhibition in cell growth (IC_{50} = 8.5 μM). These findings indicate that NO-ASA profoundly inhibits both the expression and enzymatic activity of NOS2 and suggest that these effects may represent an important mechanism for the colon cancer chemopreventive effect of NO-ASA.

Keywords: Nitric oxide synthase; NOS2; Colon cancer; Aspirin; Nitric oxide-releasing aspirin; HT-29 human; Colon adenocarcinoma cells

1. Introduction

The chemoprevention of colon cancer, one of the leading causes of cancer death in both men and women in Western countries [1], has been pursued actively during the last two decades. Ample evidence demonstrates that chemoprevention has the potential to play a large role in the control of colorectal cancer [2]. For example, a large body of data has revealed that aspirin (ASA) and other non-steroidal anti-inflammatory drugs (NSAIDs) have anti-neoplastic properties and can prevent colon cancer [3,4]; two recent human trials demonstrated that ASA prevents colorectal adenomas, thus providing formal proof of the concept [5,6]. The mechanisms responsible for the chemopreventive effect of NSAIDs are not fully understood. Emerging data indicate that NSAIDs target several steps along the pathway toward carcinogenesis [7].

Nitric oxide (NO) is an important messenger that regulates numerous physiological functions and also participates in the pathogenesis of various diseases [8]. NO is produced via the oxidation of the guanido group of L-arginine to L-citrulline; this reaction is catalyzed by nitric oxide synthase (NOS). There are three isoforms of NOS: neural NOS (NOS1 or nNOS), inducible NOS (NOS2 or iNOS), and endothelial NOS (NOS3 or eNOS) [9]. NOS1 and NOS3 are constitutively active in resting cells, while NOS2 is expressed during pathological states. NOS2 produces much larger amounts of NO (μM) compared to NOS1 and NOS3 (pM to nM). NOS2 has been implicated as a factor in colon cancer invasiveness and metastasis [10]. One study suggested that a high expression of NOS2 is associated with aggressive behavior of colorectal adenocarcinomas [11]. Several studies have demonstrated that colonic tumors in laboratory animals, and colonic

* Corresponding author. Tel.: +1 631 632 9035; fax: +1 631 632 1992.
E-mail address: basil.rigas@stonybrook.edu (B. Rigas).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.06.027

adenomas and adenocarcinomas in humans have increased expression of NOS2 when compared with adjacent non-neoplastic tissue [12]. In addition, NOS2 selective inhibitors inhibited the early stages of colon carcinogenesis in rodents [10].

NO-donating NSAIDs (NO-NSAIDs) represent a promising development in the prevention and/or treatment of cancer [13,14]. These novel compounds consist of a traditional NSAID to which an NO-donating group has been covalently attached via an aromatic or aliphatic spacer (Fig. 1). NO-NSAIDs were developed to reduce the gastric toxicity of traditional NSAIDs [15]. Pre-clinical studies have shown that NO-NSAIDs are virtually devoid of gastrointestinal toxicity, while retaining the pharmacological properties of the parent drug [16]. Recently, a double blind placebo-controlled trial on human volunteers confirmed the much greater gastrointestinal safety of NO-aspirin (NO-ASA) compared to traditional ASA [17].

In addition to their improved safety profile, NO-NSAIDs appear effective in the chemoprevention of colon cancer. NO-ASA and other NO-NSAIDs are more potent than their traditional counterparts in inhibiting the growth of cultured colon cancer cells [18–20]; NO-ASA was consistently the most potent NO-NSAID tested. NO-ASA was an effective chemopreventive agent against gastrointestinal cancer in both rats [21] and *Min* mice [22].

In light of the association between NOS2 expression and colon cancer, and the chemopreventive role of NO-ASA, we examined in detail the effect of NO-ASA on NOS2 expression in cultured human colon cancer cells. Here, we present our results documenting that NO-ASA inhibits the expression and activity of NOS2, suggesting a potential mechanism of its chemopreventive activity.

2. Materials and methods

2.1. Reagents

NO-ASA (NCX-4040, 2-(acetyloxy) benzoic acid 4-(nitrooxy methyl) phenyl ester) was a gift of NicOx, SA, Sophia Antipolis, France. We prepared a 100 mM stock solution in DMSO. In all media the final DMSO concentration was adjusted to 1%. MTT labeling reagent and solubilization buffer, IFN-γ, IL-1β, and IL-6 were from Roche Diagnostics, Nutley, NJ. ECL detection reagents were from Amersham Biosciences, Piscataway, NJ. All antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA.

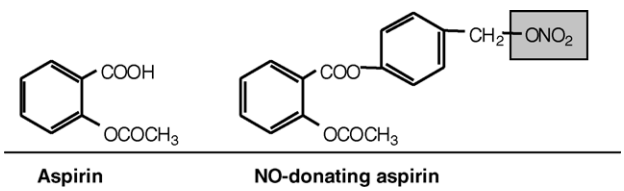

Fig. 1. The chemical structure of NO-aspirin. The three structural components of this novel derivative of aspirin are indicated. The spacer molecule links the traditional aspirin (shown on the left) to $-ONO_2$ (shaded), which can release NO.

2.2. Cell culture

HT-29 cells, obtained from American Type Culture Collection (Manassas, VA), were maintained in McCoy's 5A medium. All media contained 9.5% fetal calf serum, 10,000 IU/mL penicillin, and 10 mg/mL streptomycin. Cells were counted using a hemacytometer and cell viability was determined by the trypan blue dye exclusion method; cells that stained blue were considered non-viable. To induce NOS2 expression in HT-29 cells, which do not express it normally, cells plated at a density of 4.2×10^4 cells/cm^2 were cultured overnight at 37 °C in 5% CO_2 and 90% relative humidity; on the next day, the medium was aspirated and replaced with medium containing 100U/mL IFN-γ, 100U/mL IL-1β, and 0.5 ng/mL IL-6. Cultures were incubated with cytokines overnight before the addition of NO-ASA.

2.3. MTT assay

Cells were plated on 96-well plates at 12,000 cells/well. NOS2 induction and cell treatments were as detailed above. For the MTT assay, we followed the instructions of the manufacturer (Roche Diagnostics, Nutley, NJ).

2.4. Western blot analysis

This was performed following standard protocols that included protein fractionation on 10% polyacrylamide electrophoresis gel and transfer onto nitrocellulose membranes. Proteins were detected using a primary antibody either to NOS2 (*H-174*, Santa Cruz Biotechnology) or to α-actin or β-tubulin. Membranes were washed and incubated with horseradish peroxidase-linked secondary rabbit antibody for 1 h and developed by the enhanced chemiluminescence system. Quantification by densitometry analysis of protein bands was performed using Imaging Analysis Software (UVP Labworks, Upland, CA). Protein density measurements were normalized to those of the housekeeping gene product.

2.5. Nitrite measurement

After 24 h treatment of cultured cells with NO-ASA, the medium was collected and stored at −20° C until assayed. Nitrite levels were quantified using a NO selective microelectrode (amiNO-700, Innovative Instruments, Queen Brooks Court, FL) and an amplifier (inNO meter, Innovative Instruments) following the instructions of the manufacturer. Addition of the sample to a 100 mM H_2SO_4 solution in the presence of NaI converted the nitrite in

medium to NO, which was measured by the electrode using $NaNO_2$ as standard.

2.6. RNA extraction and RT-PCR

After cytokine and NO-ASA treatments as described above, cells were washed with PBS, and then homogenized with RNA-Stat-60 (Tel-Test Inc., Friendswood, TX). RNA was extracted following standard protocols. The primer pair for RT-PCR amplification of human NOS2 was 5′-TCTGCTGGCTTCCTGCTTTCC-3′ and 5′-TCCTGGTA-GATGTGGTCC TCA-3′. PCR amplification consisted of 35 cycles of amplification (94 °C for 45 s, 65 °C for 45 s, and 72 °C for 2 min). PCR products were fractionated on 1% agarose gels and visualized by ethidium bromide staining.

2.7. Immunocytochemistry

Cells treated as above were harvested by trypsinization, washed with phosphate buffered saline (PBS), spun onto coated slides using a cytospin centrifuge (Shandon Thermoelectron, Pittsburgh, PA) and fixed with methanol. Cells were immunostained as described previously [23] using rabbit anti-NOS2 primary antibody in 1:100 dilution.

2.8. Assay of NOS2 enzymatic activity

Cells were plated at a density of $2.5 \times 10^5/cm^2$ and grown according to the instructions of ATTC. Following overnight incubation, the medium was aspirated and fresh medium containing either no cytokines or the combination of 150 μL IFN-γ, 150 μL IL-1β, and 37.5 μL IL-6 was added. Cells were cultured overnight, and then treated with varying concentrations of NO-ASA for 1 h, when the medium was collected, cells were trypsinized off the dishes, and medium and cells were centrifuged for 5 min at 290 × g and 4 °C. Cells were suspended in 100 μL of homogenization buffer using a commercial NOS assay kit (Calbiochem, San Diego, CA). For each sample, we used 400 μg of protein. Thirty microliters of a reaction stock mix was added to each sample, the samples were incubated for 1 h, and then 0.4 mL of stop buffer was added. Next, 0.1 mL of equilibrated resin was added to each sample. The samples were transferred to spin cups and centrifuged for 30 s. The eluate was transferred to scintillation vials, 10 mL of scintillation cocktail was added to each vial, and radioactivity was quantified in a liquid scintillation counter. Enzyme activity was expressed as percent of enzyme inhibition.

2.9. Statistical analyses

Data are presented as means ± S.E.M. for different sets of plates and treatment groups, as indicated. Statistical comparison among the groups was performed using a one-way analysis of variance followed by the least significant difference method.

3. Results

3.1. NOS2 expression and cell growth in HT-29 cells

HT-29 cells, seeded at a density of 4×10^5 cells/cm^2, were cultured for 24 h, and treated with cytokines for 18–24 h. This was followed by treatment with active drug or control in the continued presence of cytokines for 1, 6, 18, or 24 h more.

NO-ASA had no effect on NOS2 expression at 1 h (Fig. 2). At 6 h, there was a very modest but consistent increase in its expression at low NO-ASA concentrations (1–10 μM) and no significant reduction at 50–100 μM NO-ASA. Compared to control, at 18 h, 50 μM NO-ASA reduced NOS2 expression by 15%, and 100 μM of it reduced it by 35%. At 24 h, 50 μM of drug lowered NOS2 expression by 55% and 100 μM by 70%. The IC_{50} of NO-ASA could only be calculated for the 24 h values and it was 46 μM. Cells not treated with cytokines did not express NOS2 (data not shown). Several of the cytokine-free and cytokine-induced samples from each of the four time points that were not treated with NO-ASA were also examined by Western blotting for the expression of NOS1 and NOS3. No expression of either isoform was observed in these samples (data not shown).

NO-ASA inhibited the growth of HT-29 cells, as shown in Fig. 3. The IC_{50} for cell growth was progressively reduced over the 24 h of observation, being >100, 84, 12, and 8.5 μM at 1, 6, 18, and 24 h, respectively. Cell numbers by the trypan blue dye exclusion method were similar to the MTT assay results over 24 h (data not shown). Cells treated with neither cytokines nor NO-ASA (negative control) had slightly improved growth rates in comparison to cells exposed only to cytokines (positive control). ASA 1–3 mM had only a limited effect.

The presence of NOS2 in HT-29 cells was also demonstrated by immunocytochemistry (Fig. 4). Cells induced with cytokines and not treated with NO-ASA demonstrated the strongest expression of NOS2, although such expression was not uniform. Cells were treated for 24 h with NO-ASA or vehicle (control). Cells treated with 10 μM of drug showed reduced NOS2 expression. The highest concentrations of the drug (100 μM) rendered the expression of NOS2 undetectable. Cells that were not induced with cytokines failed to express NOS2.

3.2. NOS2 mRNA expression in HT-29 Cells

HT-29 cells were seeded at a density of 0.4×10^6 cells/cm^2 and following induction of NOS2 with cytokines, were exposed to either active drug or control in the continued presence of cytokines for additional 1, 6, 12, 18, or 24 h.

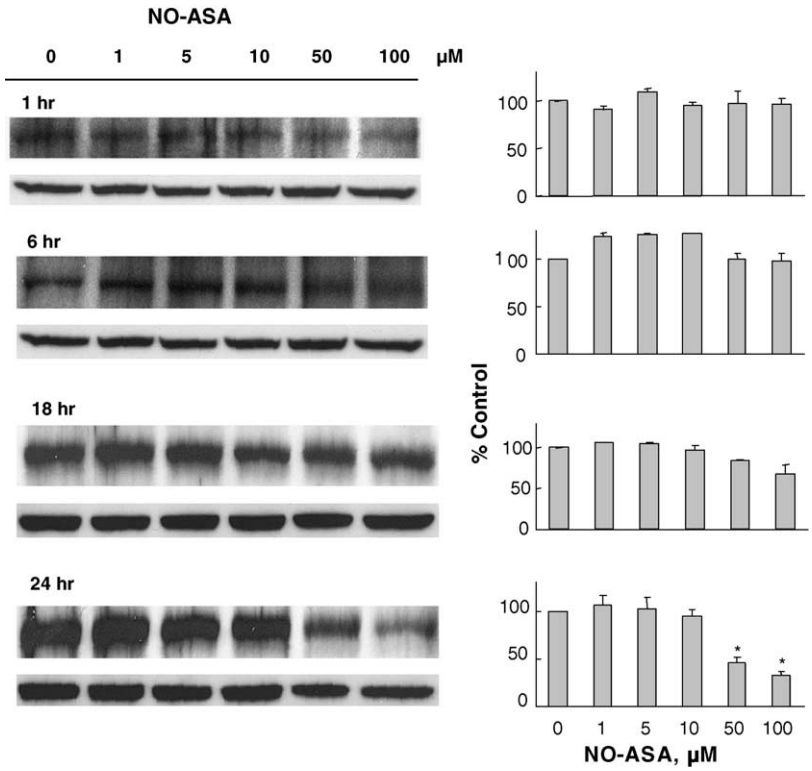

Fig. 2. NOS2 expression in HT-29 cells treated with NO-ASA. Cells were treated with various concentrations of NO-ASA over various treatment periods as in Section 2. The pairs of immunoblots at each time point correspond to NOS2 (*upper panel*) and α-actin (*lower panel*), which was used as a gel loading control. Densitometric analysis provided the values corresponding to each time point (normalized to actin), which are expressed as percent of control. NO-ASA has no effect on NOS2 levels at 1 h. At 6 h, there is a modest increase of NOS2 at low concentrations and no reduction at higher concentrations of NO-ASA. By 18 h, there is noticeable inhibition of NOS2 expression with 50 and 100 μM NO-ASA. The reduction of NOS2 is further appreciated at 24 h, especially with 50 and 100 μM of NO-ASA.

As can be appreciated from Fig. 5, the reduction in steady-state mRNA levels in response to NO-ASA treatment was both concentration- and time-dependent. No effect on NOS2 mRNA transcription was seen over 1 h (data not shown) or 6 h. By 12 h, however, there was a clear reduction in mRNA levels, which increased progressively over the ensuing 12 h.

3.3. NOS2 enzyme activity in HT-29 cells

An important consideration was whether NO-ASA, in addition to affecting the expression of NOS2, also affected its catalytic activity. To assess this possibility, we determined in vitro the catalytic activity of NOS2 extracted from cells treated with or without

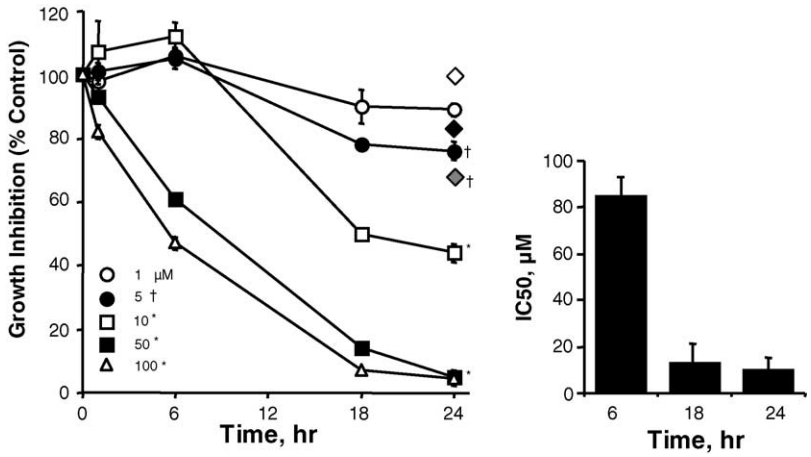

Fig. 3. Growth inhibition of HT-29 cells by NO-ASA. Cells were treated with various concentrations of NO-ASA over 1–24 h. The IC_{50} for cell growth for the 6, 18, and 24 h time points is shown on the right; no IC_{50} could be calculated for 1 h. At 24 h, ASA 1 and 3 mM (superimposed, white diamond) and 5 mM (gray diamond) added after seeding per NOS protocol. $^{\dagger}p < 0.05$; $^{*}p < 0.01$ compared to untreated cells at 24 h.

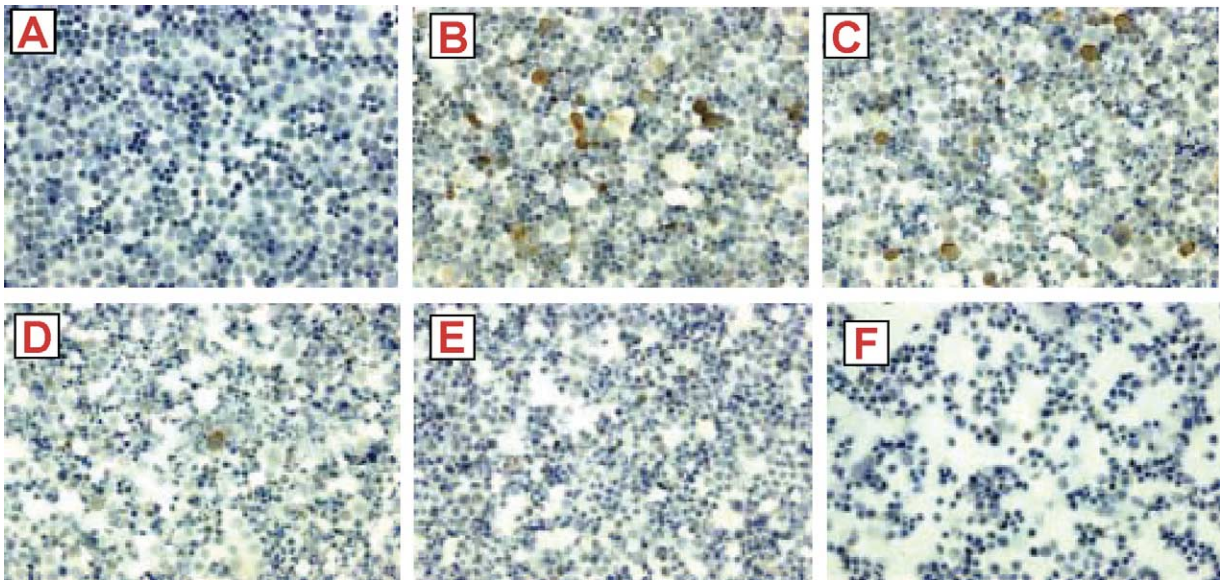

Fig. 4. Immunocytochemical detection of NOS2 in HT-29 cells treated with NO-ASA. Cells induced with cytokines and not treated with NO-ASA demonstrate the strongest presence of NOS2 (B). Cells treated with 10 μM NO-ASA show reduced NOS2 expression (C). Higher concentrations of NO-ASA (D, 25 μM; E, 50 μM; F, 100 μM) reduce further the expression of NOS2 in a concentration-dependent manner. HT-29 cells not induced with cytokines and not treated with NO-ASA do not express NOS2 (A). ICC × 200.

NO-ASA. Cells were plated at a density of 2.0×10^6 cells/cm^2, and following NOS2 induction with cytokines they were treated with varying concentrations of NO-ASA for 1 h. This time point was selected because, as we have already demonstrated, during this period of time cells expressed the same amount of NOS2, regardless of whether they were treated or not with NO-ASA. Thus, we could compare NOS2 activity between our samples; measurements of enzyme activity in samples with varying amounts of NOS2, as would be the case after longer periods of treatment with NO-ASA, would likely be unreliable.

The effect of various concentrations of NO-ASA on the activity of NOS2 was analyzed by employing an assay measuring the conversion of L-arginine to L-citrulline (Fig. 6). The activity of NOS2 was clearly inhibited by NO-ASA. Furthermore, as demonstrated by the double reciprocal plot (inset), this enzyme was 100% inhibitable by NO-ASA [24,25]. Control HT-29 cells that were not exposed to cytokines did not demonstrate appreciable NOS2 activity (data not shown).

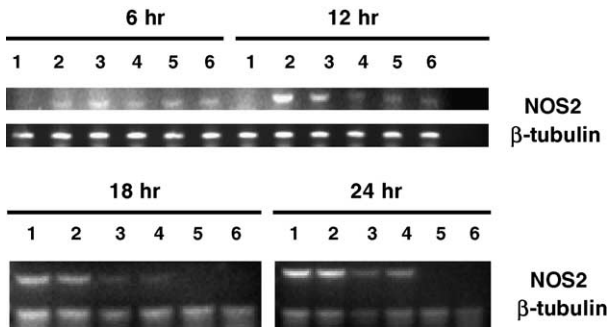

Fig. 5. Effect of NO-ASA on NOS2 mRNA steady-state levels in HT-29 cells. mRNA levels of NOS2 and β-tubulin (control) were determined by RT-PCR at various time points following treatment with NO-ASA. 6 and 12 h: 1, no cytokines, no NO-ASA added; 2, NO-ASA 0 μM; 3, NO-ASA 10 μM; 4, NO-ASA 25 μM; 5, NO-ASA 50 μM; 6, NO-ASA 100 μM. 18 and 24 h: 1, NO-ASA 0 μM; 2, NO-ASA 1 μM; 3, NO-ASA 5 μM; 4, NO-ASA 10 μM; 5, NO-ASA 100 μM; 6, NO-ASA 100 μM.

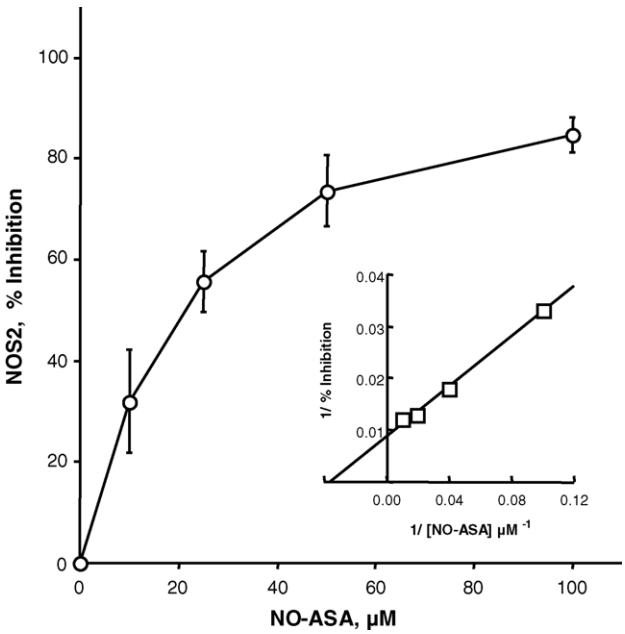

Fig. 6. Effect of NO-ASA on the enzymatic activity of NOS2 in HT-29 cells. Enzyme activity was measured after 1 h of treatment with 10–100 μM NO-ASA. A double reciprocal plot outlining the inhibition of NOS2 by NO-ASA is shown (*inset*).

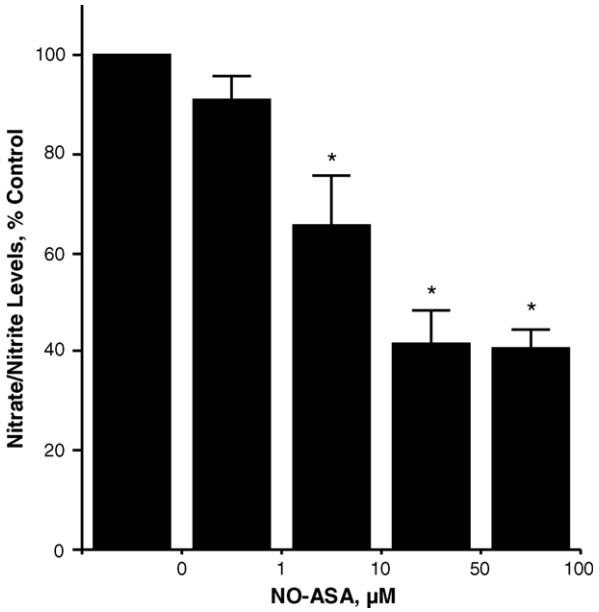

Fig. 7. Nitrite production by HT-29 cells following treatment with NO-ASA. NO levels were determined in the culture medium as nitrite after 24 h of treatment of HT-29 cells with 1–100 μM of NO-ASA. $^{*}P < 0.05$ compared to control.

3.4. Nitrite generation by HT-29 cells

Given both the reduced expression and the diminished catalytic activity of NOS2 in response to NO-ASA, we considered whether treatment of HT-29 cells with NO-ASA leads to diminished production of NO. Thus, following NOS2 induction with cytokines, HT-29 cells were treated with NO-ASA for 24 h and nitrite concentration in the culture medium was measured.

As shown in Fig. 7, nitrite levels in the culture medium, reflecting the production of NO, were markedly reduced compared to control following 24 h of treating the cells with NO-ASA. This reduction was concentration-dependent, reaching 59% at 50 and 100 μM NO-ASA; the IC_{50} for this effect was 36 μM. There was no measurable nitrite production in cells not exposed to cytokines (data not shown).

4. Discussion

Our results document that NO-ASA inhibited significantly both the expression and catalytic activity of NOS2, an enzyme implicated in colon carcinogenesis. In HT-29 human colon cancer cells, NO-ASA's inhibitory effect on NOS2 was paralleled by inhibition of cell growth.

NO-ASA inhibited the growth of HT-29 cells profoundly and with much greater potency than its parent compound, ASA. As we have previously demonstrated this effect is the result of inhibition of proliferation and induction of apoptosis by NO-ASA [18]. The inhibitory effect of NO-ASA on NOS2 cell levels, occurring in a concentration-dependent manner, became significant by 18 h, increasing further at 24 h, when our period of study ended. This effect was preceded by a reduction of the corresponding steady-state mRNA levels, which started decreasing at 12 h and diminished further over the period of observation, becoming nearly undetectable at the highest NO-ASA concentrations. The temporal relationship of the two changes, i.e. the reduction in mRNA levels precedes the reduction in the corresponding protein levels, makes it likely that the change in protein levels reflect reduced translation. While one should keep in mind the possibility that NO-ASA may have affected the stability of NOS2 mRNA, it is, nonetheless, conceivable that NO-ASA inhibited the expression of NOS2 by suppressing the transcription of the *NOS2* gene.

Two additional points require clarification. First, the decreased NOS2 expression was not due to inhibited cell growth by NO-ASA. This is evidenced by the fact that the corresponding mRNA levels were decreased very early durting exposure to NO-ASA, before cell death became evident. Second, the changes in NOS2 levels are specific and do not reflect a generalized suppression of protein synthesis by NO-ASA in these cells. The unchanged expression of the controls in the RT-PCR and Western blots (β-tubulin and α-actin, respectively) argues against such a possibility. Furthermore, as we have reported [22], NO-ASA induces COX-2 expression while it decreases NOS2 levels in HT-29 cells.

Our cytochemical study provided a glimpse into the modulation of NOS2 expression by NO-ASA. Although this method is somewhat limited by its relatively low sensitivity, it provided the interesting finding that the expression of NOS2 in HT-29 cells was not uniform. One explanation for this finding is that NOS2 expression may be cell cycle dependent, and with these cells not being synchronized, their NOS2 expression varied. Regardless of this, there was a clear concentration-dependent reduction in the expression of NOS2 in response to NO-ASA, a finding that is in agreement with the immunoblot results.

The second major effect of NO-ASA on NOS2 was the inhibition of its catalytic activity. Such inhibition was clear-cut in protein extracts obtained from cells treated with NO-ASA, indicating that it indeed takes place in intact cells. The dual inhibitory activity of NO-ASA on NOS2 over 24 h was reflected in the levels of NO, the end product of the reaction it catalyzes, which was reduced significantly in a concentration-dependent manner. One reservation concerning this finding could be the possibility that NO-ASA selectively suppressed NOS2 levels in these cells, and hence the reduced activity reflects simply limited amounts of enzyme. Three lines of evidence, however, argue strongly against such a possibility. First, immunoblots of the same sample revealed no change in protein levels within the 1 h of exposure to NO-ASA. Second, the magnitude of the inhibition was such that even moderate suppression of NOS2, not detectable by immunoblotting,

would not have affected the enzyme activity results to that degree. Third, the time sequence of NOS2 mRNA changes in response to NO-ASA virtually precludes reduction of NOS2 levels through changes of the corresponding message. Although it is unclear how NO-ASA brings about this effect on NOS2 activity, several possibilities can be entertained, such as direct modulation of the enzyme by the NO that is released from NO-ASA; modification of biologically important proteins by NO has been described (e.g., S-nitrosylation of NF-κB [26]).

The profound biochemical changes regarding NOS2 in these cells were accompanied by a significant inhibition of cell growth, a parameter that is central to the anticancer effect of any compound, including NO-ASA. An important question in this context is whether the two effects, inhibition of cell growth and inhibition of NOS2 expression and activity, are etiologically related. Although an unambiguous direct connection between the two is difficult to establish, such a possibility appears intuitively plausible and deserves further evaluation.

A significant body of data indicates that the role of NO in cancer is dichotomous, with both pro- and anti-carcinogenic properties having been reported [27,28]. For example, NO can damage DNA and can interfere with DNA repair, whereas a sustained high level of NO may contribute to tumor initiation and promotion. Furthermore, NOS2 is a part of complex signaling cascades, such as those involving COX-2 and NF-κB, both thought to participate in colon carcinogenesis [29,30]. Several preclinical and clinical lines of evidence emphasize the potentially important role of NOS2 in colon carcinogenesis. Prominent relevant findings include the following: NOS2 expression is significantly increased in colon adenoma and carcinoma with little or no expression in normal colon tissue [12]; NO suppresses apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions [31]; NOS inhibitors prevent colonic aberrant crypt foci formation in animal models [32]; there is strong positive relationship between the presence of NOS2 in the tumors and the frequency of p53 mutations in primary human colon tumors [33] and an increased p53 mutation load in non-cancerous colon tissue from ulcerative colitis, a cancer-prone chronic inflammatory disease [34]. Given that NOS2 can produce large and sustained amounts of NO [9], one could speculate that the inhibition of NOS2 by NO-ASA explains, at least in part, NO-ASA's chemopreventive effect against colon cancer.

Several studies have examined the effect of traditional ASA on NOS2 expression and activity, and nitrite accumulation. In general, millimolar concentrations of ASA were required to obtain detectable effects on NOS2 expression and function in various cell culture systems. For example, treatment of murine macrophages with 5 and 10 mM ASA for 18–20 h inhibited nitrite accumulation by 50–80% [35–37]. Williams et al. found that 24 h of treatment with 5 mM of ASA markedly inhibited cytokine-induced expression of NOS2 in HT-29 cells [22]. Of note, in HT-29 colon cancer cells there was a steep gradient in the cell growth inhibitory effect of traditional ASA versus NO-ASA, with their respective IC_{50} being 3.5 mM and 5 μM [20]. Thus, although traditional ASA does have a clear inhibitory effect on NOS2, this is far weaker than the effect of NO-ASA, as evidenced by the much higher concentration of ASA required to obtain a quantitatively similar effect.

In conclusion, our data establish a quantitatively significant and mechanistically complex inhibitory effect of NO-ASA on NOS2. It remains unclear whether this effect occurs in intact organisms, such as humans, treated with NO-ASA, and whether this effect is critical to NO-ASA's strong chemopreventive effect. Given, however, the plausible role of NO in colon cancer and the clinical potential of NO-ASA as an effective and safe chemopreventive agent against colon cancer, exploring further the mechanism suggested by our study may prove to be a fruitful line of investigation.

Acknowledgements

Grant support: NIH R01-CA92423, R01-CA92423-S2, and the Emmanuel Foundation.

References

[1] Cancer facts and figures 2003. American Cancer Society, Atlanta, GA, 2003.
[2] Krishnan K, Ruffin MT, Brenner DE. Chemoprevention for colorectal cancer. Crit Rev Oncol Hematol 2000;33(3):199–219.
[3] Thun MJ, Henley SJ, Patrono C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst 2002;94(4):252–66.
[4] Shiff SJ, Rigas B. Nonsteroidal anti-inflammatory drugs and colorectal cancer: evolving concepts of their chemopreventive actions. Gastroenterology 1997;113(6):1992–8.
[5] Baron JA, Cole BF, Sandler RS, Haile RW, Ahnen D, Bresalier R, et al. A randomized trial of aspirin to prevent colorectal adenomas. N Engl J Med 2003;348(10):891–9.
[6] Sandler RS, Halabi S, Baron JA, Budinger S, Paskett E, Keresztes R, et al. A randomized trial of aspirin to prevent colorectal adenomas in patients with previous colorectal cancer. N Engl J Med 2003;348(10):883–90.
[7] Rayyan Y, Williams J, Rigas B. The role of NSAIDs in the prevention of colon cancer. Cancer Invest 2002;20(7–8):1002–11.
[8] Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. J Physiol Pharmacol 2002;53(4 Pt 1):503–14.
[9] Griffith OW, Stuehr DJ. Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 1995;57:707–36.
[10] Rao CV. Nitric oxide signaling in colon cancer chemoprevention. Mutat Res 2004;555(1–2):107–19.
[11] Yagihashi N, Kasajima H, Sugai S, Matsumoto K, Ebina Y, Morita T, et al. Increased in situ expression of nitric oxide synthase in human colorectal cancer. Virchows Arch 2000;436(2):109–14.
[12] Ambs S, Merriam WG, Bennett WP, Felley-Bosco E, Ogunfusika MO, Oser SM, et al. Frequent nitric oxide synthase-2 expression in human

colon adenomas: implication for tumor angiogenesis and colon cancer progression. Cancer Res 1998;58(2):334–41.
[13] Rigas B, Kashfi K. Nitric-oxide-donating NSAIDs as agents for cancer prevention. Trends Mol Med 2004;10(7):324–30.
[14] Kashfi K, Borgo S, Williams JL, Chen J, Gao J, Glekas A, et al. Positional isomerism markedly affects the growth inhibition of colon cancer cells by nitric oxide-donating aspirin in vitro and in vivo. J Pharmacol Exp Ther 2005;312(3):978–88.
[15] Del Soldato P, Sorrentino R, Pinto A. NO-aspirins: a class of new anti-inflammatory and antithrombotic agents. Trends Pharmacol Sci 1999; 20(8):319–23.
[16] Wallace JL, Del Soldato P. The therapeutic potential of NO-NSAIDs. Fundam Clin Pharmacol 2003;17(1):11–20.
[17] Fiorucci S, Santucci L, Gresele P, Faccino RM, Del Soldato P, Morelli A. Gastrointestinal safety of NO-aspirin (NCX-4016) in healthy human volunteers: a proof of concept endoscopic study. Gastroenterology 2003;124(3):600–7.
[18] Williams JL, Borgo S, Hasan I, Castillo E, Traganos F, Rigas B. Nitric oxide-releasing nonsteroidal anti-inflammatory drugs (NSAIDs) alter the kinetics of human colon cancer cell lines more effectively than traditional NSAIDs: implications for colon cancer chemoprevention. Cancer Res 2001;61(8):3285–9.
[19] Kashfi K, Ryann Y, Qiao LL, Williams JL, Chen J, Del Soldato P, et al. Nitric oxide-donating nonsteroidal anti-inflammatory drugs inhibit the growth of various cultured human cancer cells: evidence of a tissue type-independent effect. J Pharmacol Exp Ther 2002;303(3):1273–82.
[20] Yeh R, Chen J, Williams JL, Baluch M, Hundley TR, Rosenbaum RE, et al. NO-donating nonsteroidal antiinflammatory drugs (NSAIDs) inhibit colon cancer cell growth more potently than traditional NSAIDs: a general pharmacological property? Biochem Pharmacol 2004;67(12):2197–205.
[21] Bak AW, McKnight W, Li P, Del Soldato P, Calignano A, Cirino G, et al. Cyclooxygenase-independent chemoprevention with an aspirin derivative in a rat model of colonic adenocarcinoma. Life Sci 1998;62(23):L367–73.
[22] Williams JL, Nath N, Chen J, Hundley TR, Gao J, Kopelovich L, et al. Growth inhibition of human colon cancer cells by nitric oxide (NO)-donating aspirin is associated with cyclooxygenase-2 induction and beta-catenin/T-cell factor signaling, nuclear factor-kappaB, and NO synthase 2 inhibition: implications for chemoprevention. Cancer Res 2003;63(22):7613–8.
[23] Kozoni V, Tsioulias G, Shiff S, Rigas B. The effect of lithocholic acid on proliferation and apoptosis during the early stages of colon carcinogenesis: differential effect on apoptosis in the presence of a colon carcinogen. Carcinogenesis 2000;21(5):999–1005.
[24] Kashfi K, Cook GA. Proteinase treatment of intact hepatic mitochondria has differential effects on inhibition of carnitine palmitoyltransferase by different inhibitors. Biochem J 1992;282(Pt 3):909–14.
[25] Cook GA, Lappi MD. Carnitine palmitoyltransferase in the heart is controlled by a different mechanism than the hepatic enzyme. Mol Cell Biochem 1992;116(1–2):39–45.
[26] Marshall HE, Hess DT, Stamler JS. S-nitrosylation: physiological regulation of NF-kappaB. Proc Natl Acad Sci USA 2004;101(24): 8841–2.
[27] Wink DA, Mitchell JB. Nitric oxide and cancer: an introduction. Free Radic Biol Med 2003;34(8):951–4.
[28] Wink DA, Vodovotz Y, Cook JA, Krishna MC, Kim S, Coffin D, et al. The role of nitric oxide chemistry in cancer treatment. Biochemistry (Mosc) 1998;63(7):802–9.
[29] Landino LM, Crews BC, Timmons MD, Morrow JD, Marnett LJ. Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. Proc Natl Acad Sci USA 1996; 93(26):15069–74.
[30] Surh YJ, Chun KS, Cha HH, Han SS, Keum YS, Park KK, et al. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 2001;480–481:243–68.
[31] Wenzel U, Kuntz S, De Sousa UJ, Daniel H. Nitric oxide suppresses apoptosis in human colon cancer cells by scavenging mitochondrial superoxide anions. Int J Cancer 2003;106(5):666–75.
[32] Rao CV, Indranie C, Simi B, Manning PT, Connor JR, Reddy BS. Chemopreventive properties of a selective inducible nitric oxide synthase inhibitor in colon carcinogenesis, administered alone or in combination with celecoxib, a selective cyclooxygenase-2 inhibitor. Cancer Res 2002;62(1):165–70.
[33] Ambs S, Bennett WP, Merriam WG, Ogunfusika MO, Oser SM, Harrington AM, et al. Relationship between p53 mutations and inducible nitric oxide synthase expression in human colorectal cancer. J Natl Cancer Inst 1999;91(1):86–8.
[34] Hussain SP, Amstad P, Raja K, Ambs S, Nagashima M, Bennett WP, et al. Increased p53 mutation load in noncancerous colon tissue from ulcerative colitis: a cancer-prone chronic inflammatory disease. Cancer Res 2000;60(13):3333–7.
[35] Amin AR, Vyas P, Attur M, Leszczynska-Piziak J, Patel IR, Weissmann G, et al. The mode of action of aspirin-like drugs: effect on inducible nitric oxide synthase. Proc Natl Acad Sci USA 1995;92(17): 7926–30.
[36] Farivar RS, Brecher P. Salicylate is a transcriptional inhibitor of the inducible nitric oxide synthase in cultured cardiac fibroblasts. J Biol Chem 1996;271(49):31585–92.
[37] Sanchez de Miguel L, de Frutos T, Gonzalez-Fernandez F, del Pozo V, Lahoz C, Jimenez A, et al. Aspirin inhibits inducible nitric oxide synthase expression and tumour necrosis factor-alpha release by cultured smooth muscle cells. Eur J Clin Invest 1999; 29(2):93–9.