NO-donating aspirin induces phase II enzymes \textit{in vitro} and \textit{in vivo}

Jianjun Gao, Khosrow Kashfi\textsuperscript{1}, Xiaoping Liu and Basil Rigas\textsuperscript{*}

Division of Cancer Prevention, Department of Medicine, SUNY at Stony Brook, NY 11794, USA and \textsuperscript{*}Department of Physiology and Pharmacology, City University of New York Medical School, New York, NY 10031, USA

\textsuperscript{1}To whom correspondence should be addressed at: Division of Cancer Prevention, Department of Medicine, Life Sciences Building 06, SUNY at Stony Brook, Stony Brook, NY 11794-5200, USA. Tel: +1 631 632 9035; Fax: +1 631 632 9922; Email: basil.rigas@sunysb.edu

Modulation of drug metabolizing enzymes, leading to facilitated elimination of carcinogens represents a successful strategy for cancer chemoprevention. Nitric oxide-donating aspirin (NO-ASA) is a promising agent for the prevention of colon and other cancers. We studied the effect of NO-ASA on drug metabolizing enzymes in HT-29 human colon adenocarcinoma and Hepa 1c1c7 mouse liver adenocarcinoma cells and in \textit{Min} mice treated with NO-ASA for 3 weeks. In these cell lines, NO-ASA induced the activity and expression of NAD(P)H:quinone oxidoreductase (NQO) and glutathione \textit{S}-transferase (GST). Compared with untreated \textit{Min} mice, NO-ASA increased in the liver the activity (nmol/min/mg; mean \pm SEM for all) of NQO (85 \pm 6 versus 128 \pm 11, \textit{P}<0.05) and GST (2560 \pm 233 versus 4254 \pm 608, \textit{P}<0.005) and also in the intestine but not in the kidney; the expression of NQO1 and GST P1-1 was also increased. NO-ASA had only a marginal effect on P450 1A1 and P450 2E1, two phase I enzymes. The release of NO from NO-ASA, determined with a selective microelectrode was paralleled by the induction of NQO1 and abrogated by NO scavengers; an exogenous NO donor also induced the expression of NQO1. NO-ASA induced concentration-dependently the translocation of Nrf2 into the nucleus as documented by immunofluorescence and immunoblotting; this paralleled the induction of NQO1 and GST P1-1. Thus NO-ASA induces phase II enzymes, at least in part, through the action of NO that it releases and by modulating the Keap1–Nrf2 pathway; this effect may be part of its mechanism of action against colon and other cancers.

Introduction

Nitric oxide-donating non-steroidal anti-inflammatory drugs (NO-NSAIDs) are an emerging novel class of compounds with significant potential for the control of cancer (reviewed in (1)). Each consists of a traditional NSAID that bears covalently attached to it an NO-releasing moiety. NO-donating aspirin (NO-ASA), the most extensively studied NO-NSAID, inhibits the growth of various cancer cell lines (2), inhibits colon carcinogenesis in preclinical animal models (3,4) and a clinical trial for colon cancer prevention is underway. Animal and early human studies suggest that NO-ASA is much safer than conventional ASA (5,6). Thus NO-ASA is a promising agent for the prevention of colon and other cancers.

We have been exploring the important question of the mechanism of NO-ASA’s apparently strong chemopreventive effect. NO-ASA has pleiotropic effects encompassing a strong cell kinetic effect and modulation of several cell-signaling pathways, including the eicosanoid, nitric oxide, Wnt, NF-\kappaB and MAPK pathways (7–10). The relative contribution of each of these effects of NO-ASA to its chemopreventive efficacy remains unclear and is the subject of intense study.

The xenobiotic metabolizing enzymes are classified as phase I (oxidation and reduction reactions) and phase II (conjugation reactions) enzymes; the balance between the phase I carcinogen-activating enzymes and the phase II detoxifying enzymes is critical to determining an individual’s risk for cancer (reviewed in (11)). Phase I enzymes primarily consist of the cytochrome P450 (CYP) superfamily. In humans, four CYP gene families (CYP1–CYP4) are considered the most important (12). Phase II enzymes comprise many enzyme superfamilies including the NAD(P)H:quinone oxidoreductase (NQO), glutathione \textit{S}-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs). NQO protects cells against the toxicity of quinones and their metabolic precursors by promoting the obligatory two-electron reduction of quinones to semiquinones. The human GSTs consist of four main classes, \( \alpha \) (A), \( \mu \) (M), \( \theta \) (T) and \( \pi \) (P), each of which is divided into one or more isoforms (13). GSTs detoxify a number of carcinogenic electrophiles by transforming them into their conjugates with reduced glutathione. A major consequence of the inactivation of chemical carcinogens by GST is thought to be blocking the formation of DNA adducts (reviewed in Ref. 14).

Modulation of drug metabolizing enzymes, leading to facilitated elimination of endogenous and environmental carcinogens represents a successful strategy for cancer chemoprevention (15). This notion is exemplified by dithiolethiones, which induce phase II enzymes. These compounds inhibit tumorigenesis of environmental carcinogens in various animal models and, as shown in human clinical trials, modulate the metabolism of the carcinogen aflatoxin B1 (15). In general, the induction of phase II enzymes is a sufficient strategy for protecting mammals and their cells against carcinogens and other forms of electrophile and oxidant toxicity.

Several chemopreventive agents induce the expression of phase II genes through their effects on the Keap1–Nrf2 complex (16,17). In the nucleus, the transcription factor Nrf2, a member of the NF-E2 family, dimerizes with the small Maf protein and binds to the antioxidant response element (ARE),
a cis-acting regulatory element in the promoter region of phase II enzymes. A cytoplasmic actin-binding protein, Keap1, is an inhibitor of Nrf2 that sequesters it in the cytoplasm. Inducers dissociate this complex, allowing Nrf2 to translocate to the nucleus.

In the present studies, we evaluated the effect of NO-ASA on xenobiotic metabolizing enzymes both in vitro and vivo. We studied its effect on phase I and II enzymes in liver and colon cell lines as well as in the liver and colon of Min mice, a mouse model system of intestinal cancer that displays many of the molecular and histological features of human colon cancer. Our data demonstrate that NO-ASA is a significant inducer of phase II enzymes that probably acts through the Keap1–Nrf2 complex.

Materials and methods

Reagents

Stock (100 mM) solutions of NO-ASA [NCX4016, 2-(ace tolyoxy)benzoic acid 3-nitrooxy methyl]phenyl ester, Nicot, SA, Sophia Antipolis, France] and ASA and DMSO (Sigma Chemical, St Louis, MO) were prepared in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ); the final DMSO concentration was adjusted in all media to 1%. S-nitroso-N-acetyl-penicillamine (SNAP), 2-phenyl-1,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) were from Sigma.

Cell culture

HT-29 human colon adenocarcinoma and Hepa 1c1c7 mouse liver adenocarcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in 5% CO₂ at 37°C in aMEM (Hepa1c1c7) or McCoy 5A (HT-29) medium supplemented with 10% fetal bovine serum and 2% glutamine (v/v). The medium was changed daily, and cells were subcultured at confluence.

Animal treatments

Six-week-old female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). After acclimation, the animals were housed and maintained according to the approved standards of Institutional Animal Care and Use Committee. NO-ASA was suspended (35 mg/ml, wt/v) in a solution of 0.5% carboxy methylcellulose (Sigma Chemical) before treatment. Mice were divided into two groups of four animals each and treated via gavage either with vehicle or with NO-ASA 100 mg/kg/day. Mice were then exposed to DMSO or NO-ASA 24 h, washed with PBS and fixed with methanol for 30 min. After blocking in 2% BSA for 15 min, cells were treated for 2 h with anti-Nrf2 antibody (1 mg/ml). After washing, cells were treated with fluorescein isothiocyanate (FITC) second antibody for 1 h at the room temperature. Slides were mounted and examined under a fluorescence microscope at 100 × magnification.

Results

NO-ASA induces phase II enzyme activity in HT-29 human colon cancer and Hepa 1c1c7 murine hepatoma cells

We examined the effect of NO-ASA on the activity of NQO and GST in mouse hepatoma Hepa 1c1c7 cells, which have been the model cell line for the study of phase II enzymes (25) and in HT-29 human colon cancer cells, which also express GST. The results were expressed as mean ± SEM and differences were compared using Student’s t-test.

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Results

NO-ASA induces phase II enzyme activity in HT-29 human colon cancer and Hepa 1c1c7 murine hepatoma cells

We examined the effect of NO-ASA on the activity of NQO and GST in mouse hepatoma Hepa 1c1c7 cells, which have been the model cell line for the study of phase II enzymes (25) and in HT-29 human colon cancer cells, which also express both NQO and GST. As shown in Figure 1, NO-ASA induces the activity of NQO and GST in...
these two cell lines in a concentration-dependent manner. In both instances, the induction of NQO is quantitatively more pronounced than that of GST. For example, treatment of Hepa 1c1c7 cells with 100 μM NO-ASA resulted in 4.2-fold induction of NQO activity but only 1.3-fold induction of GST activity. Of note, at each concentration the effect of NO-ASA on Hepa 1c1c7 cells was more pronounced than that on HT-29 cells. ASA has no effect on either NQO or GST activity, even at the highest concentration studied (400 μM, not shown).

Treatment of HT-29 cells with 50 μM NO-ASA for 24 h increased significantly (2-fold) the intracellular GSH levels, but there was no change at NO-ASA concentrations of ≤5 μM. This finding suggests that NO-ASA, at the higher concentration, may have induced γ-glutamylcystein synthetase, the rate-limiting enzyme of GSH biosynthesis (28). Conventional ASA at equimolar concentrations had no effect on GSH levels under our experimental protocol.

Induction of phase II enzyme activity is accompanied by an increase of their protein levels

In order to determine whether changes in NQO and GST levels were responsible for their increased enzymatic activity, we determined by immunoblotting the cellular levels of NQO1 and GST P1-1 in response to NO-ASA. These two isoforms are expressed in both cell lines and are easily assayable with existing antibodies. NO-ASA increased the levels of NQO1 and GST P1-1 in both HT-29 and Hepa1c1c7 cells in a concentration-dependent manner. In contrast, ASA did not affect the levels of these two proteins even at 400 μM (data not shown). It is of interest that the degree of increase in protein expression reflects the respective changes in catalytic activity. For example, NQO1 is induced more in liver than colon cells, whereas the difference in induction of GST between these two cell lines is less pronounced; as already mentioned, NQO (but not GST) is more active in the liver cells compared with colon cells.

Many phase II enzyme inducers also induce phase I enzymes (16,17). Thus we examined the effect of NO-ASA on the levels of P450 1A1, an important phase I enzyme in mammalian livers. As shown in Figure 2, NO-ASA induced the expression of P450 1A1 only slightly in Hepa 1c1c7 cells.

Induction of phase II enzymes by NO-ASA is related to NO release

To evaluate whether induction of phase II enzymes by NO-ASA is dependent on NO, we determined the amount of
NO released from NO-ASA. In HT-29 cells treated with NO-
ASA for 18 h, the release of NO was paralleled by the induc-
tion of NQO1; conventional ASA did not release any NO (as
expected) and the expression of NQO1 was not altered
(Figure 3A). We next determined whether NO is responsible
for NQO1’s induction. Pretreatment with PTIO, a specific NO
scavenger, essentially abrogated the induction of NQO activity
by NO-ASA (Figure 3B). Another NO scavenger, hemoglobin,
also decreased the induction of NQO by NO-ASA (data not
shown). These results indicate that the NO released from NO-
ASA is, at least in part, responsible for the induction of this
phase II enzyme.

To further explore this observation, we determined the effect
of SNAP, a widely used NO donor on NQO1 expression.
Treatment of Hepa 1c1c7 cells with various concentrations of
SNAP for 24 h increased the activity of NQO in a concentra-
tion-dependent manner (Figure 3C), with SNAP 400 μM
increasing the activity of NQO by 3-fold over control. Induction
of phase II enzymes by NO in other cell lines has been recently
reported (29). We treated Hepa 1c1c7 cells either with NO-
ASA 100 μM or ASA 100 μM, or combinations of 100 μM
ASA with SNAP (50–400 μM). As shown in Figure 3C, the
presence of ASA did not alter the effect of SNAP, suggesting
that the ASA moiety of NO-ASA had very little effect on the
induction of phase II enzymes by NO-ASA.

**Induction of phase II enzymes by NO-ASA employs the
Nrf2-dependent pathway**

The Nrf2 transcription factor is normally largely bound in the
cytoplasm to Keap1. When inducers disrupt its complex with
Keap1, Nrf2 translocates to the nucleus where it accelerates
the transcription of phase II genes. To determine whether NO-
ASA indeed induces phase II enzymes by modulating Nrf2, we
assessed the cellular distribution of Nrf2 after treatment with
NO-ASA. Immunofluorescence analysis revealed that control
cells showed both cytoplasmic and nuclear staining of Nrf2,
whereas NO-ASA-treated cells showed increased nuclear
staining, which increased progressively with increasing NO-
ASA concentrations (Figure 4A). Immunoblotting also indic-
ated that the nuclear level of Nrf2 was elevated in NO-ASA-
treated cells. ASA had no effect on the cellular distribution
of Nrf2.

**NO-ASA induces the activity of NQO and GSTs in Min mice**

To determine whether NO-ASA induces phase II enzymes in vivo, we measured its effect on both the activity and expression
of NQO and GST in Min mice. Compared with controls, after 3 weeks of treatment NO-ASA significantly induced the activity of GST (66% increase; \(P < 0.005\)) and NQO (50% increase; \(P < 0.05\)) in the liver (Table I). Similarly, NO-ASA increased significantly (but to a lesser extent) the activity of GST (36% increase; \(P < 0.01\)) and NQO (30% increase; \(P < 0.005\)) in the small intestine. In contrast, the increases in the activity of these two enzymes in the kidney did not reach statistical significance.

Immunoblot analysis (Figure 5) demonstrated that NO-ASA significantly increased the liver cytosolic GST P1-1 and NQO1 levels. The levels of two phase I enzymes, P450 1A1 and cytochrome P450 2E1, did not differ between the untreated and treated groups. This finding indicates that in vivo NO-ASA is a monofunctional phase II inducer.

**Discussion**

Our findings establish that NO-ASA induces phase II enzymes both in cultured cells and in an animal model of colon cancer.

This induction is probably brought about, at least in part, through its effect on the Keap1–Nrf2 complex and parallels the release of NO by NO-ASA (Figure 6). Conventional ASA at equimolar concentrations failed to generate similar effects. Of the two classes of xenobiotic metabolizing enzymes, the effect of NO-ASA appears restricted to phase II enzymes; in cultured cells and in mice, NO-ASA had only a marginal effect on P450 1A1 and P450 2E1, two phase I enzymes.

We studied the effect of NO-ASA in cultured Hepa 1c1c7 murine hepatoma cells and in HT-29 human colon cancer cells; they have been used extensively to study the biology of drug metabolizing enzymes and of colon cancer, respectively. These two cell lines also represent two organs relevant to the chemoprevention of colon cancer: the liver, the major organ for drug metabolizing activities where carcinogens can be (in)activated; and the colon, the target organ of NO-ASA's effect. Of the many phase II enzymes, we focused on NQO and GST, two biologically significant members of the phase II enzyme superfamily; each is present as several isoforms and both are potentially important in mediating the effects of chemoprevention agents.

Our data establish that NO-ASA has a dual effect on these phase II enzymes: it induces in a concentration-dependent manner both their intracellular levels and also their catalytic activity. Although the effect on these two enzymes in cultured cells was quantitatively more pronounced for NQO, compared with GST, the differences between the two are generally small (and just the reverse in the liver and intestine of Min mice). The degree of induction of protein levels and of enhancement of catalytic activities is similar for both NQO and GST. However, it is difficult to ascribe the enhanced activity exclusively to the increase in the amount of the enzyme, particularly since...
our antibody was specific for enzyme isoforms and the assay for enzymatic activity was not isoform-specific.

That our findings with cultured cells may be biologically relevant is suggested by the observation that Min mice showed similar changes in NQO and GST. Interestingly, this was not an ‘acute’ effect, as it was observed following a 3 week administration of the drug. Of note, NO-ASA had no effect on the kidneys of these mice, suggesting a degree of tissue specificity, although the size of our study groups precludes a firm conclusion on this.

Three aspects of the mechanism by which NO-ASA induces phase II enzymes merit discussion. First, NO, the molecule that defines this drug, appears involved in this effect. There are two supporting lines of evidence: (i) the release of NO from NO-ASA was paralleled by the induction of NQO1 and this effect was abrogated by NO scavengers; and (ii) an exogenous NO donor (SNAP) also induced the expression of NQO1, consistent with findings by others indicating the ability of NO to induce phase II enzymes (29). Second, NO-ASA appears to induce the expression of phase II genes by modulating the Keap1–Nrf2 complex. This is evidenced by the concentration-dependent translocation of Nrf2 into the nucleus following treatment with NO-ASA. Of note, the translocation of Nrf2 parallels the induction of NQO1 and GST P1-1. Although our data do not conclusively prove that this is the mechanism of action of NO-ASA, they are, nevertheless, consistent with it (15). We can only speculate on the modification of Keap1 that leads to the dissociation of Nrf2; S-nitrosylation of Keap1 by NO-ASA is a distinct possibility (30). And, third, equimolar concentrations of conventional ASA, the prototypical colon cancer chemoprevention agent (31), failed to induce phase II enzymes. Our findings do not, however, preclude such a possibility at high drug concentrations. In general, ASA is known to differ in terms of pharmacological potency from

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**Fig. 5.** Effect of NO-ASA on the expression of murine hepatic detoxification enzymes. Upper panels: Liver cytosol or microsome fractions from Min mice treated with NO-ASA for 21 days were subjected to immunoblotting for the proteins shown here. Each lane represents one animal. Lower panel: The expression of each protein (fold over control) for each group of animals (mean ± SEM, N = 4). *, P < 0.05; **, P < 0.01; ***, P < 0.005, compared with the corresponding control; all other differences are not statistically significant.

**Fig. 6.** Proposed mechanism for the induction of phase II enzymes by NO-ASA. Biotransformation of NO-ASA by the cell ultimately generates NO (38), which may modify Keap1 (via S-nitrosylation?) thus disrupting the Keap1–Nrf2 complex. This is followed by nuclear translocation of the transcription factor Nrf2, which in turn interacts with the ARE in the promoter region of phase II detoxifying enzymes inducing their expression.
NO-ASA, often requiring much higher concentrations to achieve the same effect as NO-ASA (1).

The biological significance of our findings cannot be deduced with certainty from the present data. In general, there is a dichotomy in the activity of these enzymes; they can mediate cancer chemoprevention, or they can activate carcinogens or even be part of the mechanism of drug resistance (12,15,32). NQO1 detoxifies carcinogenic compounds and prevents the generation of oxygen radicals. For example, it catalyzes two-electron transfer reactions to quinones (33,34), thus bypassing the formation of semiquinones and, subsequently, of superoxide anion radicals (35). In general, NQO enzymes provide a cellular detoxifying system and are considered protective. It should be kept in mind, however, that NQO1 can activate important mutagens and carcinogens (36) and may accelerate the formation of reactive oxygen species through the semiquinone pathway. The end result often depends on biological context, such as tissue type or route of administration. A similar argument can be made for GST (13,14). Given the abundant evidence that NO-ASA is chemoprotective against colon cancer (3,4,37), it is reasonable to speculate that the induction of phase II enzymes by NO-ASA is part of its mechanism of chemoprevention.

In conclusion, our data establish that NO-ASA induces phase II enzymes, probably, at least in part, through the action of the NO that it releases and by modulating the Keap1–Nrf2 pathway. It is possible that this effect is part of its mechanism of action against colon and other cancers. Further work is required to provide additional details on the mechanism of these effects and an assessment of the relative contribution of the individual changes that we have described.

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Conflict of Interest Statement: None declared.

References


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