Nitric oxide induces cyclooxygenase expression and inhibits cell growth in colon cancer cell lines

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The role of nitric oxide (NO) in colon cancer remains controversial. Inducible nitric oxide synthase (iNOS) has been reported to be up regulated and down regulated in colorectal cancer in both animal models and patient tissue samples. Cyclooxygenase-2 (COX-2) is important in colorectal carcinogenesis but its relationship with NO has never been studied in colon cancer. Three colon cancer cell lines (HCA7, HT29 and HCT116) with different COX-2 expression and activities were used to study the effect of the NO donor, S-nitrosoglutathione (GSNO). The effects of GSNO (10–500 μM) on cell growth, PGE2 production, COX-1/COX-2 protein expression and cell-cycle distribution were evaluated. GSNO increased PGE2 production and induced COX-1 and COX-2 protein expression in a dose- and time-dependent manner. Higher concentrations of GSNO also inhibited cell growth and induced apoptosis in all three cell lines, regardless of their COX-2 expression/activities. Inhibition of PGE2 production did not further improve the inhibitory effect of GSNO.

Introduction

Nitric oxide (NO) is an important signaling molecule in numerous physiological and pathological conditions. This free diatomic radical is produced when nitric oxide synthases (NOSs) catalyze the conversion of L-arginine to L-citrulline. There are three isoforms of NOS. Two of them, endothelial NOS and neuronal NOS, are calcium-dependent, constitutively expressed and responsible for low levels of NO production (pico molar to nano molar) for short periods (minutes). The third one, inducible NOS (iNOS), is calcium-independent, not expressed in most tissues under normal conditions, but can be induced by lipopolysaccharide and various cytokines. It can produce large quantities of NO (μM) over extended periods (days to weeks) (1). NO is reported to have antitumor activities as well as pro-tumor properties. Its effect may depend on the timing, concentration and tissue type (2). Low concentrations of NO can stimulate cell growth and protect many cell types from apoptosis, whereas high concentrations of NO can inhibit cell growth and induce apoptosis (3).

Abbreviations: COX, cyclooxygenase; GSNO, S-nitrosoglutathione; iNOS, inducible nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NSAID, non-steroidal anti-inflammatory drug; SEM, standard error of the mean.

Studies on colorectal cancer show contradictory results. Some clinical studies have shown that iNOS increased significantly in colon adenoma and carcinoma with little or no expression in normal colon tissue (4–6), whereas other studies report iNOS expression is decreased in colon cancer compared with high expression in normal colon tissue (7–10). In animal models, induction of iNOS is correlated with colorectal cancer regression (both in situ and metastatic) (11,12), whereas NOS inhibitors are reported to prevent colonic aberrant crypt foci formation (13–15). NO is also shown to either promote or inhibit tumor growth of colorectal cancer cells (16–18) and the effect was suggested to be concentration-dependent (18). Using knockout mice, two separate groups recently reported opposite effects of iNOS gene knockouts on intestinal carcinogenesis (19,20).

Cyclooxygenase (COX)-2, the inducible form of COX that catalyzes the conversion of arachidonic acid to prostaglandins, is increased in colorectal cancer and non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX activities, can inhibit colorectal carcinogenesis (21,22). Both COX-2 and iNOS are important genes involved in inflammation as well as carcinogenesis. Numerous studies have been done to investigate the interaction between COX-2 and iNOS in many cell systems, especially inflammatory models. In these systems, NO is shown to have a regulatory effect, either stimulatory or inhibitory, on the COX-2 expression and activity (23,24). However, their relationship in colon cancer has not been studied yet.

In this study, HCA7, HT29 and HCT116, three colon cancer cell lines with different COX-2 expression and activities, were treated with different concentrations of S-nitrosoglutathione (GSNO, 10–500 μM) to evaluate the in vitro effect of NO on colon cancer cells and its relationship with COX expression/activity. GSNO is a major biological carrier of NO and serves as a good source of nitric oxide, which is produced by the cleavage of the S–N bond (25).

Materials and methods

Cell culture

Three human colonic adenocarcinoma cell lines, HCA7 (a gift from Susan C.Kirkland, Royal Postgraduate Medical School, England), HT29 and HCT116 (both from the American Type Culture Collection, Rockville, MD), were used. All cell lines were grown at 37°C in a humidified atmosphere of 5% CO2 in McCoy’s 5A Medium (Sigma Chemical Co., St Louis, MO) supplemented with 10% fetal bovine serum (Trace Scientific Ltd, Melbourne, Australia), penicillin (50 U/ml) and streptomycin (50 mg/ml; Sigma Chemical Co.). Cells were routinely subcultured using trypsinization (0.5% trypsin/0.2% EDTA, Sigma Chemical Co.).

Reagents

GSNO (Sigma Chemical Co.) was fresh dissolved in phosphate-buffered saline (PBS) immediately before adding into medium. Stock solutions of NS398 (20 mM) and aspirin (1 M) (Cayman Chemical Co., Ann Arbor, MI) were dissolved in DMSO (Sigma Chemical Co.) and stored at −20°C before use.

Cell growth

Cells were plated at a density of 5 × 10⁴ (HT29 and HCT116) or 1 × 10⁵ (HCA7) cells per well in 6-well plates. After 24 h, the medium was replaced
with fresh warm medium with GSNO (10–500 μM) or GSNO (50, 500 μM) with NSAIDs (NS398 10 μM or aspirin 500 μM), and then incubated with cells up to 72 h. The spent control for GSNO treatment was pre-incubation of GSNO (500 μM) in blank medium at 37°C for 24 h. After 24, 48 and 72 h incubation with/without drugs, the culture medium was centrifuged at 10 000 g for 10 min and the supernatants were stored at −80°C and later assayed for PGE2 and nitrate/nitrite levels. Adherent cells were harvested by trypsinization and counted using a hemocytometer. Experiments were performed in duplicate and repeated at least three times. Results are expressed as the fold increase in the mean cell number (±standard error of the mean; SEM) relative to the number of cells originally plated.

Cell-cycle analysis
After 24, 48 and 72 h incubation with/without drugs, both floating and adherent cells were collected and fixed in ice-cold 70% ethanol overnight at 4°C. The cells were centrifuged down and resuspended in propidium iodide (PI) staining solution (50 μg/ml PI, 100 U/ml RNase A, 1 mg/ml glucose in PBS). After >30 min incubation, the DNA content of cells was measured using flow cytometry. A minimum of 10 000 events was counted for each sample. Data were analyzed using WINMDI (version 2.8, The Scripps Research Institute, La Jolla, CA). The cells with subdiploid DNA content represent apoptotic cells (26). Experiments were done in duplicate and repeated at least twice. Results are expressed as the mean (±SEM) percentage of cells with a sub-diploid DNA content.

PGE2 assay
A PGE2 competitive enzyme immunoassay kit (Cayman Chemical Co.) was used to determine PGE2 levels in cell culture supernatants. The immunoassay was performed according to the manufacturer’s protocol. The assay was sensitive to 10 pg/ml. PGE2 production was normalized with respect to the number of adherent cells in the culture at the time of sampling and the results are expressed as ng/10⁶ cells.

Nitrate/nitrite assay
The supernatants were treated with nitrate reductase (0.1 U/ml), FAD (5 μM) and NADPH (30 μM) at 37°C for 60 min, resulting in all nitrates being converted to nitrite. Then LDH (0.1 kU/ml) and sodium pyruvate (0.3 mM) were added for 5 min to oxidize the unused NADPH to alleviate the interference caused by NADPH (27). Greiss reagents (1% sulfanilamide and 0.1% naphthylethylenediamine in 2.5% phosphoric acid) were added successively and incubated for 10 min. The absorbance was measured at a wavelength of 540 nm. Nitrate/nitrite concentrations were calculated using a nitrate standard solution (0–50 μM) (27).

Western blotting
After 48 h incubation with/without GSNO (500 μM), the medium was removed and the cells were washed with ice-cold PBS twice. The cells were scraped off and collected by centrifugation at 2000 g for 5 min. The pellets were resuspended in pre-heated lysis buffer (10 mM Tris±HCl, pH 7.4, 1 mM sodium orthovanadate, 1% SDS), heated at 95°C for 10 min and incubated at 37°C for 5 min and 95°C for 10 min. The lysates were centrifuged at 10 000 g for 15 min. The supernatants were saved for electrophoresis. Protein concentrations were determined using the BCA Protein Assay kit (Pierce, Rockford, IL). Fifty micrograms of proteins were diluted in SDS–PAGE loading buffer and boiled for 5 min and loaded onto 7.5% SDS–PAGE gel. Proteins were transferred to nitrocellulose membrane. After blocking with 5% non-fat milk, the membranes were incubated with anti-COX1 (Cayman Chemical Co.) or anti-COX2 monoclonal primary antibody (BD Bioscience, Franklin Lakes, NJ), followed by horseradish peroxidase-labeled goat anti-mouse antibody (Pierce), then developed by the supersignal chemiluminescent substrate (Pierce) and exposed to X-ray film. Membranes were routinely stripped and reprobed with anti-actin monoclonal antibody (Chemicon International Inc., Mississauga, ON) to serve as controls. Bands on the X-ray film were scanned using Deskcan and the intensity of the signals was quantified using the Analytical Imaging Station (Imaging Research, Ontario, Canada) and expressed as the ratio of COX-1 or COX-2 relative to actin.

Statistical analysis
One-way ANOVA with a Bonferroni test was used to determine whether there was a significant difference. Statistical significance was assumed if the P value was <0.05.

Results

GSNO inhibited cell growth in colon cancer cell lines
GSNO inhibited cell growth of all three colon cancer cell lines in a concentration-dependent manner, regardless of their COX-2 expression level/activity. GSNO had no effect on cell growth at lower concentrations (10, 20 μM) but significantly inhibited cell growth at higher concentrations (50 μM and above for HT29 and HCT116, 100 μM and above for HCA7). At the highest concentration tested (500 μM), GSNO almost completely blocked cell growth in all three cell lines (Figure 1).

Nitrate/nitrite levels in the culture medium of cells remained at very low levels (5 μM) under normal growing conditions. More than 90% of the nitroso groups of GSNO could be recovered as nitrate/nitrite within 24 h of incubation (data not shown). As a control, GSNO (500 μM) was added into media and incubated at 37°C for 24 h prior to incubation with cells. This spent control has similar levels of nitrate/nitrite but none of the effects observed with GSNO, suggesting it is NO.

Fig. 1. The effect of GSNO (10–500 μM) on HCA7, HT29 and HCT116 colon cancer cell growth. PBS and spent GSNO (500 μM) were used as controls. Data are expressed as the mean (±SEM) fold increase in cell number relative to the originally plated cell number (n = 6, experiments were done in duplicate and repeated three times).
and not any of its stable metabolic breakdown products which produced these effects.

**GSNO induced apoptosis in colon cancer cell lines**

GSNO caused a time- and concentration-dependent increase in the percentage of apoptotic cells in all three cell lines with the maximum effect at the concentration of 300 and 500 μM for 72 h (Table I). The increase in the percentage of apoptotic cells was accompanied by the decrease in the percentage of G1 cells, whereas other cell-cycle parameters showed no significant change.

**GSNO increased PGE2 production and induced COX-1 and COX-2 protein expression**

PGE2 production and the COX-1 and COX-2 protein expression were analyzed in all three cell lines. The PGE2 secreted by untreated HT29 and HCT116 cells were hundreds times lower than that secreted by HCA7 cells (Figure 2). Both HCA7 and HT29 have high COX-2 protein expression (Figure 3) whereas the COX-2 protein in HCT116 was only detectable after a longer exposure time (1 h) (data not shown).

GSNO increased PGE2 production (Figure 2) in all three cell lines, in a time- and concentration-dependent manner. Figure 3 shows that both COX-1 and COX-2 protein expression increased after GSNO treatment (500 μM) for 48 h. However, the increased PGE2 production in all three cell lines was inhibited by NS398 at 10 μM (which inhibits COX-2 but not COX-1), suggesting that COX-2 is the main source for the increased PGE2 production in all three cell lines.

**NSAIDs did not further improve the inhibition effect of GSNO on colon cancer cell lines**

Aspirin (0.5 mM) or NS398 (10 μM) can reduce PGE2 production by HCA7, HT29 and HCT116 to very low levels (a hundred times lower for HCA7 compared with control, and at about the level of PGE2 in medium alone for HT29 and HCT116) (Table II). Aspirin and NS398 had no effect on cell growth at the concentration used.

In order to see if NO and NSAIDs have synergistic effect in inhibiting colon cancer cell growth, HCA7, HT29 and HCT116 were treated with GSNO (50 and 500 μM) plus aspirin (500 μM, non-specific COX inhibitor) or NS398 (10 μM, specific COX-2 inhibitor). Although aspirin and NS398

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**Table I. The effect of GSNO on apoptosis in colon cancer cell lines**

<table>
<thead>
<tr>
<th></th>
<th>HCA7</th>
<th>HT29</th>
<th>HCT116</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
<td>48 h</td>
<td>72 h</td>
</tr>
<tr>
<td>Control</td>
<td>4.6 ± 0.9</td>
<td>6.5 ± 1.0</td>
<td>8.5 ± 0.6</td>
</tr>
<tr>
<td>Spent GSNO control</td>
<td>4.2 ± 0.8</td>
<td>6.5 ± 0.6</td>
<td>8.1 ± 0.4</td>
</tr>
<tr>
<td>GSNO 10 μM</td>
<td>3.3 ± 0.2</td>
<td>6.2 ± 0.3</td>
<td>8.2 ± 0.4</td>
</tr>
<tr>
<td>GSNO 20 μM</td>
<td>3.6 ± 0.8</td>
<td>6.9 ± 0.8</td>
<td>9.4 ± 0.7</td>
</tr>
<tr>
<td>GSNO 50 μM</td>
<td>4.4 ± 0.7</td>
<td>6.2 ± 0.6</td>
<td>9.2 ± 0.7</td>
</tr>
<tr>
<td>GSNO 100 μM</td>
<td>3.3 ± 0.3</td>
<td>7.1 ± 0.7</td>
<td>10.3 ± 1.4</td>
</tr>
<tr>
<td>GSNO 300 μM</td>
<td>13.0 ± 0.9</td>
<td>160 ± 1.5</td>
<td>227 ± 1.7</td>
</tr>
<tr>
<td>GSNO 500 μM</td>
<td>16.8 ± 0.8</td>
<td>24.6 ± 1.4</td>
<td>34.5 ± 4.0</td>
</tr>
</tbody>
</table>

Data are presented as the mean (±SEM) percentage of cells with sub-diploid DNA content at 72 h incubation time with/without drugs (n = 4, experiments were done in duplicate and repeated twice).

*P < 0.05 when compared with control at the same time point.
**Discussion**

This study evaluates the *in vitro* effect of different concentrations of GSNO on three colon cancer cell lines with differing COX-2 expression and activities. Our data demonstrate that GSNO can inhibit colon cancer cell growth at higher concentrations but has no effect at lower concentrations. Although HCA7, the cell line with highest COX-2 activity among the three, needs a higher concentration of NO than HT29 and HCT116 to produce the inhibitory effect on cell growth, this was not related to its COX-2 activity because inhibition of COX-2 did not affect the effect of GSNO on its growth.

At low doses, NO has been reported previously to cause cytostasis by inhibition of ornithine decarboxylase activity in Caco-2 when cells were exposed to NO for 8 days with drug replenishment every other day (28). In our experiment, cells exposed to low doses of GSNO for 72 h did not show any cytostatic response. This was probably due to the short exposure time to GSNO in our experiment and the kinetics of GSNO decomposition which occurs within 10 h in media in the presence of serum whether cells were present or not (29). Our data indicate that apoptosis may be one of the main mechanisms of the inhibition of cell growth by GSNO.

Although Clancy *et al.* reported that NO activated COX-1 but inhibited COX-2 in macrophages (30), we found that GSNO induced both COX-1 and COX-2 protein expression and stimulated PGE2 production in a dose- and time-dependent manner in all three colon cancer cell lines. The increased PGE2 production can be completely demolished by NS398 at 10 μM, indicating that COX-2 is the main source of the increased PGE2 production. In colon cells that were non-tumorigenic and non-transformed, it was found that NO donors, SNAP and NOR-1, increased both mRNA transcription and protein synthesis of the COX-2 gene (31). These data indicate that the presence of iNOS expression may correlate with increased COX-2 expression in cancer cells, which is similar to the observation in inflammatory conditions.

Maximal PGE2 production was obtained from HCA7 and this was in the range of 0.2 μM. This amount of PGE2 is still far below that reported to induce colon cancer cell proliferation by trans-activating EGFR (32). Thus, despite the increase in PGE2 levels, no effect on cell growth was observed. Although COX-2 has been reported previously to block apoptosis we did not observe a change in the effect of GSNO on cell growth when COX-2 activity was inhibited. The more important effect of increased PGE2 expression may only be observed *in vivo* where COX-2 expression has been correlated with angiogenesis and immunosuppression rather than *in vitro* (2).

Unfortunately, animal models and knockout mouse data on the role of iNOS in colon cancer are confusing. This might be due to the fact that the dosage of NO released in these experiments may not have been consistent. Jenkins *et al.* reported that NO slowed down the growth of DLD-1, a colon adenocarcinoma cell line, *in vitro* but accelerated its cell growth *in vivo* (17). However, microencapsulated iNOS-expressing cells could inhibit DLD-1 cell growth *in vivo* and this was ascribed to the difference in iNOS activity in these cells (18).
NSAIDs have been shown to be very effective in the chemoprevention of colon cancer, but their clinical use is restricted by their significant side effects (21,22). NO-NSAIDs—a category of NSAIDs derivatives whose production was aimed at reducing the side effects of NSAIDs—were shown to be much more effective in inhibiting the proliferation of colon cancer cells than their parent NSAIDs, both in vitro (33,34) and in animal models (35). This implies NSNO and NO donor may have synergistic effects. However, our study shows aspirin and NS389, at concentrations that inhibit COX-2 activity and PGE2 production, do not further improve the inhibitory effect of NO on colon cancer cell growth, suggesting the significantly improved effect of NO-NSAIDs may be attributable to the induction of novel pathways.

In conclusion, GSNO increases PGE2 production and induces COX-1 and COX-2 protein expressions while it inhibits in vitro cell growth in HCA7, HT29 and HCT116 colon adenocarcinoma cell lines in a dose- and time-dependent manner, regardless of their different COX-2 expression/activity. Apoptosis may be one of the main killing mechanisms. Inhibition of PGE2 production does not further improve the inhibitory effect of GSNO on colon cancer cell growth. The in vivo effect of NO on colon cancer still needs to be further investigated.

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References


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