Transcriptional regulation of the COX-2 expression by nitric oxide in colon cancer cell lines

QIANG LIU¹, HIROYASU INOUE² and RATHA MAHENDRAN¹

¹Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 119260, Republic of Singapore; ²Department of Food Science and Nutrition, Nara Women’s University, Nara, Japan

Received July 4, 2007; Accepted August 22, 2007

Abstract. To determine the effect of nitric oxide (NO) on the cyclooxygenase-2 (COX-2) regulation in colon cancer cell lines we used the physiological NO donor GSNO. The proximal 6.6 kb of the COX-2 promoter was cloned into the pGL3 basic vector and the sequential deletion of the 6.6 kb COX-2 promoter generated promoter constructs of 4, 2.6, 1.9 and 0.9 kb. These constructs clearly show that the main regulatory region lies within 0.9 kb of the transcription start site. Therefore, constructs of the main transcription binding sites within this region namely CRE, NF-IL6 and NF-κB and mutations of these sites were used to monitor the transcriptional activation of COX-2. This study was performed on the colon cancer cell lines HCA7 and HCT116 which have a differential expression of COX-2. There was no evidence that the luciferase activity is negatively affected by NO as was previously reported. The CRE and NF-IL6 binding sites within this region were responsible for the constitutive and physiological NO-induced COX-2 transcriptional activity in the HCA7 and HCT116 cells. While NF-κB involvement was only observed in the HCT116 cells, the cell lines displayed increased NF-κB transcriptional activity after exposure to NO.

Introduction

Cyclooxygenase (COX) catalyzes the rate-limiting step in the synthesis of prostaglandins (PGs). There are two COX isozymes: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and cells under normal physiological conditions and is responsible for the maintenance of the integrity of the gastric mucosa, regulation of renal blood flow and platelet aggregation. In contrast, COX-2 is undetectable in normal tissues but can be rapidly and transiently induced by cytokines, oncogenes and growth factors.

Constitutive COX-2 expression and activity is found in colon adenomas/polyps and colon cancer tissues (1,2). This expression can be regulated at the transcriptional level (3,4). Most studies have focused on the cis-acting elements within 250 bases of the transcriptional start site which include the cyclic-AMP response element binding protein (CREB), the nuclear factor κB (NF-κB) and the CCAAT-enhancer binding protein (C/EBP), PEA3 and NFAT (Nuclear factor activating T cells) binding sites (5-7). However, a peroxisome proliferator-activated receptor (PPAR) site at -3900 bp can regulate enhanced COX-2 production by fatty acids and prostaglandins and the TBE site at -1007 up-regulates COX-2 expression when bound by a TCF4 and β-catenin complex (8,9).

The inducible nitric oxide synthase (iNOS) protein can correlate with the COX-2 expression in colon cancer (10). Cell line studies also confirmed that nitric oxide (NO) up-regulates the COX-2 protein and activity (11,12). The effect of NO on a COX-2 transcription have been studied using several NO donors namely SNAP, SIN-1, NOR-1, SNP, Deta/NO and NOR4 (13-15) and the results obtained were as varied as the cell lines studied. These NO donors have different effects on vasodilation and platelet aggregation which are common activities of NO (16). Some of these NO donors namely SIN-1, SNP and SNAP are also peroxynitrite generators (17,18) while Deta/NO induces oxidative stress (19). Therefore, to determine the effect of NO on COX-2 regulation we used S-nitrosothiol, GSNO, which is a physiological NO donor and a potential storage and transport vehicle for NO in the body.

We previously showed that GSNO treatment up-regulates PGE2 production and COX-2 in colon cancer cell lines (20). The proximal 6.6 kb of the COX-2 promoter was cloned into the pGL3 basic vector and the sequential deletion of the 6.6 kb COX-2 promoter generated promoter constructs of 4, 2.6, 1.9 and 0.9 kb. These constructs together with those of the -250 bp region generated by Inoue et al (5) were used to monitor the transcriptional activation of COX-2 by NO generated from GSNO. This study was performed on the colon cancer cell lines HCA7 and HCT116 which have a differential expression of COX-2.
Materials and methods

Cell culture. The human colonic adenocarcinoma cell lines, HCA7 (a gift from Susan C. Kirkland, The Royal Postgraduate Medical School, City, UK) and HCT116 (from the American Type Culture Collection, MD, USA) 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., MO, USA) supplemented with 10% fetal bovine serum (Trace Scientific Ltd, Melbourne, Australia), penicillin (50 units/ml) and streptomycin (50 μg/ml; Sigma Chemical Co.). Cells were routinely sub-cultured using trypsin (0.5% trypsin/0.2% EDTA, Sigma Chemical Co.).

Chemicals. S-Nitrosoglutathione (GSNO, Sigma Chemical Co.) was freshly dissolved in PBS immediately before adding it into the medium.

Isolation of the 6.6 kb COX-2 promoter. Genomic DNA was extracted from the FHs 74 Int human small intestinal normal cells (ATCC). The proximal 6.6 kb COX-2 promoter was amplified using the Expand long template PCR system (Roche Diagnostics, Mannheim, Germany) and the following primers: Primer -6481: 5’-CGTAACGCGTGGTGTGTCTTATGGTGCAATGC-3’ and Primer +100: 5’-CGTACTCGAGTTTGCTGTCTGAGGGCGTCTGG-3’ containing an MluI and XhoI site, respectively (underlined), to facilitate cloning into the pGL3 basic vector [containing Photinus pyralis (firefly) luciferase, Promega, WI, USA]. The -49 bp fragment of the promoter minus all cis elements but containing the TATA box was constructed by using the following primer: Primer -49: 5’-CATAAACGCGTGGCTGTTTATGAT-3’ containing an MluI and Xhol site, respectively, (underlined), to facilitate cloning into the pGL3 basic vector [containing Photinus pyralis (firefly) luciferase, Promega, WI, USA]. The -49 bp fragment of the promoter minus all cis elements but containing the TATA box was constructed by using the following primer: Primer -49: 5’-CATAAACGCGTGGCTGTTTATGAT-3’ containing an MluI and Xhol site, respectively, (underlined), to facilitate cloning into the pGL3 basic vector [containing Photinus pyralis (firefly) luciferase, Promega, WI, USA]. The -49 bp fragment of the promoter minus all cis elements but containing the TATA box was constructed by using the following primer: Primer -49: 5’-CATAAACGCGTGGCTGTTTATGAT-3’ containing an MluI and Xhol site, respectively, (underlined), to facilitate cloning into the pGL3 basic vector [containing Photinus pyralis (firefly) luciferase, Promega, WI, USA].

Reporter constructs. After restricting enzyme digestion of the 6.6 kb COX-2 pGL3 plasmid with SacI; Scal and HindIII; HincII and HindIII; Stul and HindIII, the isolated fragments were self-ligated or ligated with pGL3 which was digested with SmaI and HindIII to produce the 0.9, 1.9, 2.6 and 4 kb promoter constructs. The six COX-2 promoter constructs namely: -327/+59, CRM, ILM, KBM, CRM+ILM, -52/+59 were previously described (5). The pNF-κB-luc and its control vector were from Clonetech, CA, USA.

Transfection of colon cancer cells. Cells (1x10^5) were plated per well in 24-well culture plates. After 24 h, the cells were transfected with the mixture of 0.4 μg DNA/4 μl lipofectamine Plus/3 μl lipofectamine (Invitrogen, NY, USA) or 0.2 μg DNA/2 μl lipofectamine Plus/3 μl lipofectamine for 3 h. To normalize the transfection efficiency the pRL-TK plasmid [containing Renilla reniformis (sea pansy) luciferase] was included in the total DNA used for transfection. The ratio of the COX-2 promoter constructs to pRL-TK plasmid DNA was 50:1. Thirty hours after transfection, the cells were treated with GSNO 500 μM or spent GSNO control for 15 h. Then the cells were lysed and the cell extracts were assayed for the luciferase activities using the Dual-Luciferase Reporter Assay System (Promega). The spent control for GSNO treatment was produced by pre-incubation of the GSNO (500 μM) in a blank medium at 37˚C for 24 h.

To compare the transfection activity between the plasmids, equal molar concentrations of the different plasmids (equivalent to 0.392 μg of the 6.6 kb promoter construct) were used for transfection. To ensure that the total amount of DNA used for transfection was constant, the difference was made up with the pGL3 basic vector that had no significant luciferase activity (Table I). The total amount of DNA used in the transfection was 0.4 μg (0.392 μg plasmid DNA+0.008 μg pRL-TK) and this was mixed with 4 μl lipofectamine Plus and 3 μl lipofectamine.

Statistical analysis. A one-way ANOVA, with a Bonferroni test was used to compare the relative luciferase activity and determine whether there was a significant difference. Statistical significance was set at P<0.05.

Results

Optimal induction of the COX-2 promoter activity by the GSNO. HCA7 cells were co-transfected with the 6.6 kb insert and the pRL-TK plasmid and then treated with GSNO 500 μM for different lengths of time. This dose was chosen as in a previous study there was maximal stimulation of the PGE2 production at this dose (20). Fig. 1 shows that the COX-2
promoter activity measured as relative light units (RLU) increased with the time of exposure to GSNO, reaching a maximum at 15 h before declining. This reduction correlates with the depletion of GSNO in solution which occurs within 10 h in media in the presence of serum (21). Therefore, for all subsequent work the cells were exposed to the GSNO for 15 h and control cells were treated with the spent GSNO. GSNO breaks down to produce GSH and nitrates and nitrite. Therefore, the spent control confirms that the effects observed are due to NO and not its breakdown products.

GSNO did not have an inhibitory effect on the firefly and renilla luciferase activity. Fan et al (15) reported that three NO donors: sodium nitroprusside (SNP), Deta/NO and NOR4, inhibited the firefly luciferase activity in a dose-dependent manner by decreasing luciferase mRNA stability. To determine if the GSNO would also have this effect, the activity of the firefly and renilla luciferase were monitored after GSNO treatment. HCA7 cells were transfected with the following plasmids: 6.6 kb insert, pGL3 basic, -327/+59 and -52/+59 and then treated with GSNO. Spent GSNO (500 μM) was used as a control. After normalization with the protein content, there was no significant difference in the RLU produced by renilla luciferase per μg protein between the control and GSNO-treated HCA7 cells transfected with the 6.6 kb, pGL3 basic, -327/+59 and -52/+59 plasmids (Table II). However, GSNO treatment significantly increased the RLU produced by the firefly luciferase per μg protein in HCA7 cells which were transfected with the 6.6 kb and -327/+59 promoter plasmids but not those transfected with the pGL3 basic and -52/+59 control plasmids. This indicates that NO did not have an inhibitory effect on the firefly luciferase.

**Induction of the COX-2 promoter activity by NO.** The GSNO significantly increased the transcriptional activity of the 6.6, 4.2.6, 1.9 and 0.9 kb COX-2 promoter plasmids (1.5-fold increase over unstimulated cells). However, there was no significant difference among the plasmids in the control and GSNO-treated groups. The transcriptional activity in the TATA box insert was <90% and the GSNO did not stimulate it. This suggested that the 0.9 kb proximal promoter sequence is critical for COX-2 transcription in the control and GSNO-treated cells. The activity of the pGL3 basic vector was only 20% of the TATA box insert (Fig. 2).

**The proximal 327 bp of the COX-2 promoter constructs.** We examined the activity of the proximal 327 bp of the COX-2 promoter and found it to be similar to the 0.9 kb promoter plasmid. This region contains binding sites for several transcription factors including NF-κB, NF-IL6 and CREB. Six plasmids containing wild-type or mutations in the binding sites for these 3 transcription factors were transfected into HCA7 and HCT116 to analyze the transcriptional activity of the proximal part of the COX-2 promoter and its regulation by NO. The design and transcriptional activity of the six plasmids were shown in Fig. 3 (A-C). Mutation of the CRE (CRM) or NF-IL6 binding site (ILM) decreased the constitutive transcriptional activity. **Figure 1.** Optimization of the duration of the GSNO treatment required to induce increased COX-2 promoter activity is shown. HCA7 cells were plated at 1x10^5 cells per well in 24-well plates 24 h prior to transfection. The cells were co-transfected with 0.196 μg, 6.6 kb insert plasmid and 0.004 μg pRL-TK plasmid (ratio 49:1, total 0.2 μg DNA) using 3 μl lipofectamine and 2 μl Plus reagent. After being treated with GSNO 500 μM for different lengths of time, the cells were lysed at 48 h after transfection. The relative luciferase activities were determined using the Dual-luciferase reporter assay system. The experiments were done in triplicate (n=3) and repeated twice.

| Table II. The effect of GSNO on firefly (F) and Renilla (R) luciferase activity. |
|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| Plasmid                  | 6.6 kb insert | pGL3 basic | -327/+59 | -52/+59 |
| Spent RLU (F)/μg          | 12295±838     | 124±9       | 6076±163 | 211±23 |
| GSNO RLU (F)/μg           | 5117±315      | 4072±106    | 5597±208 | 5591±250|
| Ratio                    | 2.40±0.07     | 0.03±0.002  | 1.09±0.06| 0.04±0.003 |
| GSNO RLU (F)/μg           | 20828±731     | 159±19      | 8959±567| 304±65 |
| 500 μM RLU (R)/μg         | 5872±349      | 4145±357    | 5723±170| 5917±227 |
| Ratio                    | 3.56±0.12     | 0.04±0.006  | 1.57±0.08| 0.05±0.009 |

*P<0.05 when the GSNO-treated group was compared with its spent GSNO control.
activity of COX-2 in HCA7 cells by >80 and 70%, respectively (Fig. 3B). Mutation of the CRE and NF-IL6 binding sites (CRM+ILM) further decreased the transcriptional activity by >90%. However, the mutation of the NF-κB binding site (KBM) had no significant effect on the transcriptional activity of the COX-2 promoter. The GSNO significantly increased the transcriptional activity in cells transfected with the -327/+59 or KBM plasmids, but failed to do so in the CRM, ILM, CRM+ILM transfected cells.

Although HCT116 has a low constitutional COX-2 activity (3), as shown in Fig. 3C, it had a similar pattern of transcriptional regulation as HCA7 except that the mutation of the NF-κB binding site (KBM) significantly decreased the transcriptional activity of COX-2. This decrease was much smaller than that observed with CRM and ILM. This suggests that the binding sites of CRE and NF-IL6 in the proximal human COX-2 promoter are critical for the transcriptional activity of COX-2 in the control and GSNO-treated colon cancer cells but the NF-κB can modulate this transcriptional activity.
The inhibitory effect of NO donors on the firefly luciferase activity previously reported by Fan et al (15) was not observed. This may be due to the fact that GSNO is a physiological NO donor. Its breakdown products include GSH and nitrate/nitrite and these may have fewer side effects than the breakdown products of other NO donors. The short exposure time to the GSNO in our experiment compared to the 20 h used by Fan et al (15) and the kinetics of the GSNO decomposition which occurs within 10 h in the media may be the reason why we did not observe a NO-induced decrease in luciferase activity (21).

By using GSNO, we found that there was no significant difference among the relative luciferase activities of the COX-2 promoter constructs from the 6.6 to 0.9 kb insert either in the presence of GSNO or spent GSNO. This suggested that the sequences between -6481 and -829 may not be critical for COX-2 transcriptional activation by NO. However, the deletion of the COX-2 promoter sequence between -829 to -49 significantly decreased promoter activity by 90% and completely abolished the response to NO.

NF-κB, NF-IL6/C/EBP, PEA3, NFAT and CRE (5-7) are transcription factors with binding sites within -250 kb of the start site. Mutation of the CRE (CRM), the NF-IL6 binding site (ILM) or both sites, resulted in ~80, 70 or 90% decrease, respectively, in the constitutive transcriptional activity of COX-2 in HCA7 cells. This is consistent with previous studies on the role of CRE and NF-IL6 sites on the transcriptional regulation of COX-2 in various cell types (22). NO failed to significantly stimulate the transcriptional activities of CRM, ILM and CRM+ILM, indicating that CRE and NF-IL6 are also critical for the transcriptional activation by NO. The spent GSNO data indicate that these are NO stimulated events.

The role of NF-κB in the regulation of COX-2 is controversial. There are studies that suggest that NF-κB activation may play an important role in promoting COX-2 transcription in cancers associated with inflammation (23-26) and a study that indicates that NF-κB may not be involved in COX-2 transcription (27). This could be due to the p53 status of the cell lines used as NF-κB activation has been linked to p53 expression (28). HCA7 has a mutant p53 and therefore increased basal and NO-activated NF-κB activity. However, mutation of the NF-κB site does not affect the COX-2 expression. HCT116 which has a wild-type p53 has a lower NF-κB activity but the expression of COX-2 appears to be modulated by the NF-κB, though a functional NF-κB site alone is not able to induce the COX-2 expression in HCT116 cells. These results are consistent with the ability of NO to induce a p53 nuclear accumulation at low doses such as that released by 0.5 μM GSNO (29) and the ability of a wild-type p53 to modulate NF-κB regulation of the COX-2 (30).

In contrast to our results, Liu et al (14) working with murine and human colon cell lines found that the NO donors SIN-1, NOR-1 and SNAP induced the β-catenin expression and increased the PEA3 expression which resulted in a COX-2 activation via the ETS sites and NF-IL6 site. Park et al (13) found, using a head and neck squamous cell carcinoma cell line that SNAP induced soluble guanylate cyclase, p38 and c-Jun NH2-terminal kinase and this up-regulated CREB, ATF-2 and c-jun resulting in a COX-2 expression via the CRE but that the NF-IL6 mutation had no effect on the COX-2 expression. A study by Chen et al (31) working with gastric cell lines, indicated that reactive oxygen species activated COX-2 via the NF-IL6 and CRE sites by enhancing the binding of C/EBPβ to these sites, as did studies by Inoue et al and Tamura et al (32,33) in vascular endothelial and endometrial stromal cells. These studies indicate that the transcription factors involved in NO-mediated COX-2 expression vary with the cell lines and drugs used to generate NO.

Our data show that NO generated from GSNO induces the COX-2 expression via NF-IL6 and CRE in the HCA7 and HCT116 cells. These sites also regulate the basal COX-2 function in these cell lines.

Discussion

The inhibitory effect of NO donors on the firefly luciferase activity previously reported by Fan et al (15) was not observed. This may be due to the fact that GSNO is a physiological NO donor. Its breakdown products include GSH and nitrate/nitrite and these may have fewer side effects than the breakdown products of other NO donors. The short exposure time to the GSNO in our experiment compared to the 20 h used by Fan et al (15) and the kinetics of the GSNO decomposition which occurs within 10 h in the media may be the reason why we did not observe a NO-induced decrease in luciferase activity (21).
expression in the colon cancer cell lines. The role of NF-kB in a NO-regulated COX-2 expression, however, appears to be dependent on the p53 status of the cell line.

Acknowledgements

This study was supported by a grant from the National Medical Research Council of Singapore (NMRC/0315/1998).

References


