Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 66 (2003) 51–61
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Positive and negative regulation of prostaglandin E_2 biosynthesis in human colorectal carcinoma cells by cancer chemopreventive agents

Philip J. Sherratt*, Lesley I. McLellan, John D. Hayes

Biomedical Research Centre, University of Dundee, Ninewells Hospital and Medical School, Dundee DD1 9SY, Scotland, UK

Received 22 July 2002; accepted 9 January 2003

Abstract

Increased production of prostaglandin E_2 (PGE_2) by the combined activities of cyclooxygenase-2 (COX-2) and microsomal glutathione *S*-transferase 1-like 1 (MGST1-L1) enhances the progression of colorectal cancer. To assess how chemopreventive agents influence colon tumorigenesis, the modulation of PGE_2 production by indolo[3,2-*b*]carbazole (ICZ), β-naphthoflavone (β-NF), and *tert*-butylhydroquinone (*t*BHQ), as well as the nonsteroidal anti-inflammatory drug Piroxicam, has been studied in the human HCA-7 colon carcinoma cell line. We have found that these xenobiotics both down-regulate and up-regulate the expression of COX-2 and MGST1-L1. They can also either inhibit or stimulate PGE_2 synthesis. COX-2 mRNA levels were increased significantly by those compounds that activate transcription through the xenobiotic responsive element (XRE) and/or the antioxidant responsive element (ARE). A possible ARE enhancer was identified in the *COX-2* promoter, and reporter gene experiments demonstrated that *t*BHQ induction of a transgene driven by the 5′-flanking region of COX-2 was increased by co-transfection with an expression vector for the Nrf2 transcription factor. By contrast, only compounds such as ICZ and β-NF which activate the XRE increased the mRNA levels of MGST1-L1. While the ARE-specific inducer *t*BHQ did not modulate the basal expression of MGST1-L1, it was found to act as an antagonist of interleukin-1β-stimulated MGST1-L1 overexpression. Changes in COX-2 and MGST1-L1 expression were not always coincident with a corresponding change in PGE_2 production by human colon carcinoma cells. Importantly, dietary compounds can modulate PGE_2 biosynthesis, and this is likely to influence colon tumorigenesis.

Keywords: Cyclooxygenase; Antioxidant responsive element; Prostaglandin E_2; Indoles; *tert*-Butylhydroquinone; Piroxicam

1. Introduction

Colon cancer is a major cause of death from malignant disease in the Western world [1]. Prevention of colon cancer is a practical proposition because its incidence is influenced significantly by diet and by medication [1–5]. However, the reason why environmental factors influence susceptibility to colorectal cancer is poorly understood. Several lines of evidence, including studies of patients and experimental animals administered NSAIDs, as well as gene knockout mice lacking *cox*, suggest that inhibition of PG biosynthesis represents one mechanism by which colon cancer susceptibility might be altered by diet [6–10].

PGs are formed from AA found in membrane lipids. PGE_2 is quantitatively a major prostanoid, and its concentration is increased in colorectal tumors [11–14]. It is believed to contribute to tumorigenesis by stimulating cell proliferation, inhibiting apoptosis, and supporting angiogenesis and the invasive potential of tumor cells [15–20]. Synthesis of PGE_2 occurs in three steps. First, in common with other PGs, its production involves the liberation of AA from membranes through activation of phospholipase A_2. This is followed by the oxidation of AA to PGH_2, catalyzed by one of three COX enzymes, the constitutive COX-1, the inducible COX-2 [21], or the splice variant COX-3 [22]. Last, in an isomerization step, PGH_2 serves as a substrate for PGE_2 synthase (PGES). Two genetically

* Corresponding author. Current address: K15-C409, Schering-Plough Research Institute, 2015 Galloping Hill Road, Kenilworth, NJ 07033-1300, USA. Tel.: +1-908-740-4813; fax: +1-908-740-7514.
E-mail address: Philip.Sherratt@spcorp.com (P.J. Sherratt).

Abbreviations: AA, arachidonic acid; AhR, arylhydrocarbon receptor; ARE, antioxidant responsive element; *t*BHQ, *tert*-butylhydroquinone; COX, cyclooxygenase; ICZ, indolo[3,2-*b*]carbazole; IL-1β, interleukin-1β; MGST1-L1, microsomal glutathione *S*-transferase 1-like 1; β-NF, β-naphthoflavone; NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; RT–PCR, reverse transcription–polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and XRE, xenobiotic responsive element.

0006-2952/03/$ – see front matter © 2003 Published by Elsevier Science Inc.
doi:10.1016/S0006-2952(03)00206-5

distinct types of glutathione-dependent PGES have been identified. A cytosolic protein called p23 exhibits PGES activity and is coupled functionally to COX-1 [23]. Also, MGST1-L1 has been identified as a PGES [24] that is functionally linked to COX-2 [25].

A strong association exists between COX-2 overexpression and the progression of colorectal cancer. An increase in COX-2 expression is observed in human colonic adenocarcinomas, and evidence suggests that gene induction occurs early during tumorigenesis [26–32]. The PGH_2 formed by COX-2 in colorectal cancers is preferentially metabolized to PGE_2 [33], a reaction that is likely to be catalyzed by MGST1-L1 because this enzyme is also overexpressed in colorectal adenomas and cancer [34]. The presumed co-regulation of COX-2 and MGST1-L1 is physiologically important because overproduction of PGE_2 facilitates the development of cancer [15–20]. Further information about the regulation of the PGE_2 biosynthetic pathway is required in order to understand pathophysiological factors that influence the development of colorectal cancer.

Both COX-2 and MGST1-L1 are induced by inflammatory cytokines, and by the AhR ligand TCDD. The regulation of the expression of the *COX-2* gene is controlled in part at the transcriptional level. A number of *cis*-acting elements have been identified within the human *COX-2* promoter that drive a transcriptional response to inflammatory signals such as IL-1β [31,35–38]. By contrast, although the 5′-flanking region of the human *MGST1-L1* gene has been isolated [39], the molecular basis for its induction by cytokines and TCDD is not known.

The fact that TCDD induces *COX-2* and *MGST1-L1* suggests that the promoters of both genes contain an XRE (5′-GCGTG-3′). This enhancer recruits the AhR, a helix-loop-helix transcription factor with high affinity for TCDD and polycyclic hydrocarbons [40]. In addition to binding environmental carcinogens, the AhR is also activated by chemicals derived from edible plants, including indoles, quercetin, curcumin, and dibenzoylmethane [41–43]. Thus, dietary phytochemicals may influence expression of COX-2 and MGST1-L1 in the GI tract. Besides the XRE, another *cis*-acting element that dietary constituents can stimulate is the ARE, defined by Rushmore *et al*. [44] as 5′-A/GGTGACnnnGC-3′. The basic region leucine zipper transcription factor Nrf2 mediates activation of the ARE, and this enhancer is found in the promoter region of a number of detoxification and antioxidant enzyme genes [45–47].

Compounds like the phenolic antioxidant *t*BHQ, a monofunctional inducer that stimulates ARE-driven transcription, confer cytoprotection on a cell by increasing its capacity to detoxify carcinogens and oxidants.

In an attempt to determine how dietary phytochemicals protect against colorectal cancer, we have examined the effects of model chemopreventive agents on both the regulation of *COX-2* and *MGST1-L1* gene expression and the production of PGE_2. Our results show that these compounds inhibit and stimulate both the constitutive and cytokine-induced expression of PGE_2-producing enzymes, as well as the biosynthesis of the prostanoid in human colon cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM) containing 4500 mg/L of D-glucose, heat-inactivated fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, non-essential amino acids, trypsin, versene, and TRIzol were purchased from Invitrogen Life Technologies. Human colon carcinoma HCA-7 cells were obtained from ICRF Clare Hall Laboratories (Potters Bar), and the human hepatoma Hep-G2 cell line was obtained from the American Type Culture Collection. All chemicals were purchased from the Sigma-Aldrich Chemical Co., except for ICZ, which was provided by Dr. Christine Bonnesen (Biomedical Research Centre, University of Dundee).

2.2. Culture and treatment of HCA-7 cells

Monolayer cultures were maintained in a humidified incubator at 37° with 5% CO_2. Cells were grown in DMEM containing 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 0.1% non-essential amino acids, and penicillin/streptomycin (50 IU/mL and 50 mg/mL, respectively), hereafter referred to as complete medium.

In experiments that entailed treating the HCA-7 cell line with xenobiotics, the cells were seeded in 60 mm culture dishes, and allowed to recover for 24 hr in complete medium. Thereafter, the cells were serum-starved overnight before being exposed to the dietary compound in serum-free medium. Xenobiotics were added to the medium at the following concentrations: 25 or 50 μM *t*BHQ, 1 μM ICZ, 25 or 50 μM β-NF, and 10 or 25 μM Piroxicam; these concentrations gave >90% cell survival in a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay following exposure for 24 hr, performed as described previously [43]. The optimum treatment period for measuring changes in the level of mRNA was determined as 6 hr. Experiments were performed in duplicate on at least two separate occasions.

2.3. Estimation of human COX-2 and MGST1-L1 mRNA levels

The relative levels of mRNA encoding COX-2 and MGST1-L1 in HCA-7 cells were compared by TaqMan® real-time PCR. Total RNA was isolated using TRIzol reagent and then treated with RQ1 DNase (Promega). The cDNA was synthesized from 500 ng of total RNA

using Superscript-RT (Invitrogen Life Technologies). The relative amounts of synthesized cDNA were analyzed subsequently by TaqMan® using an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Primers and probes were designed for human COX-2 and MGST1-L1 using Primer Express Software (Applied Biosystems). Oligonucleotides were ordered from MWG-Biotech AG, and probes were obtained from Applied Biosystems.

For analysis of human COX-2, the forward 5′-GCTCAGCCATACAGCAAATCCT-3′ and reverse 5′-aaaattccggtgttgagcagtt-3′ primers were used along with the TaqMan® probe 5′-[FAM]-CTGTTCCCACCCATGTCAAAACCGAG-[TAMARA]-3′. The forward and reverse primers were designed over the first and second intron/exon boundaries, respectively, of the human *COX-2* gene and gave rise to a 140 bp amplicon. For human MGST1-L1, the forward 5′-ACGCTGCCTCAGGGCC-3′ and reverse 5′-CCCAGAAAGGAGTAGACGAAGC-3′ primers were used along with the TaqMan® probe 5′-[FAM]-AACGACATGGAGACCATCTACCCCTTCCT-[TAMARA]-3′. The forward primer was designed over the second intron/exon boundary, and the primer pair yielded an 81 bp amplicon. To normalize relative mRNA levels between prepared cDNA, a primer/probe set for 18S RNA was purchased from Applied Biosystems.

TaqMan® reactions included a 200 nM concentration of each primer with 100 nM of each probe and a 1× final concentration of TaqMan® Universal Mastermix (Applied Biosystems). All reactions were carried out with duplicate pipetting. TaqMan® software was used to calculate a *Ct* value. The fold increase or decrease in the levels of individual transcripts was calculated as follows: $Ct_{\text{COX-2}}$ or $Ct_{\text{MGST1-L1}} - Ct_{18S} = \Delta Ct$; then $2^{40-\Delta Ct}$ for treated samples was divided by the same calculated value for control samples.

2.4. COX-2-CAT reporter assays

A gene reporter construct was generated by ligating an amplified portion of the human *COX-2* gene promoter into the pCAT3basic vector (Promega). Amplification was achieved using the forward primer 5′-GGAATTCCCTCGAGCGCTGCTGAGGAGTTCCT-3′ and the reverse primer 5′-GGAATTCCGGTACCTATGTACACTGAAGGTAGCTATTTCA-3′. The 733 bp product was ligated into the *Eco*RI site of pBS SK^+, and its identity was confirmed by automated DNA sequencing. The insert was then subsequently cloned into the *Kpn*I and *Bgl*II sites of the pCAT3basic vector, which was designated pCOX2-673CAT.

Transfection of the CAT reporter construct into Hep-G2 cells was achieved by calcium phosphate precipitation. All such transfections included the pcDNAlacZ plasmid (Invitrogen), which allowed the use of β-galactosidase activity as an internal control for transfection efficiency. Measurement of CAT and β-galactosidase activities was performed by standard methods. In certain experiments, co-transfection with a pcDNA3 construct containing the coding sequence for the mouse Nrf2 transcription factor was undertaken; the cDNA for murine Nrf2 is described elsewhere [48].

2.5. Determination of PGE_2 production in HCA-7 cells

Human colon carcinoma cells were seeded in 24-well plates at a density of 2×10^5/mL and grown in complete medium for 24 hr to 80% confluency. Cells were then serum-starved overnight before being exposed to chemopreventive agents in serum-free medium for a further 24 hr. For co-treatment experiments, HCA-7 cells were incubated for 1 hr with the different chemicals before the addition of IL-1β to a final concentration of 5 ng/mL. Thereafter, cells were incubated for a further 23 hr before being washed with PBS and stimulated with 10 μM AA in fresh serum-free medium for 15 min at 37°. The levels of PGE_2 released were measured in the culture medium by enzyme immunoassay using a kit purchased from the Cayman Chemical Co. Results were expressed as nanograms/milliliter of medium. Determinations were carried out on three similarly treated wells of cells, and the medium from each experiment was assayed at two dilutions.

2.6. Computer analysis of the human COX-2 promoter region

Potential *cis*-acting elements within a 7 kb region of the human *COX-2* promoter (Accession Number: AF044206) were identified using MatInspector V2.2 based at the TRANSFAC 4.0 internet site (http://transfac.gbf.de/TRANSFAC/) [49].

2.7. Statistical analysis

Results from the TaqMan® and PGE_2 determination experiments were subjected to statistical analysis. Unpaired two-tailed *t*-tests were carried out using Graphpad Prism® Software (Graphpad Software, Inc.).

3. Results

3.1. Regulation of constitutive COX-2 transcription by chemopreventive agents

Exposure of HCA-7 colon cancer cells to ICZ, *t*BHQ, or β-NF increased the level of COX-2 mRNA (Fig. 1). The indole ICZ was the most potent, increasing the amount of COX-2 mRNA 2.8-fold at a concentration of 1 μM. Another strong inducing agent was β-NF; this flavonoid affected a 2.5-fold increase in COX-2 mRNA at a concentration of 25 μM and a 3.5-fold increase in COX-2

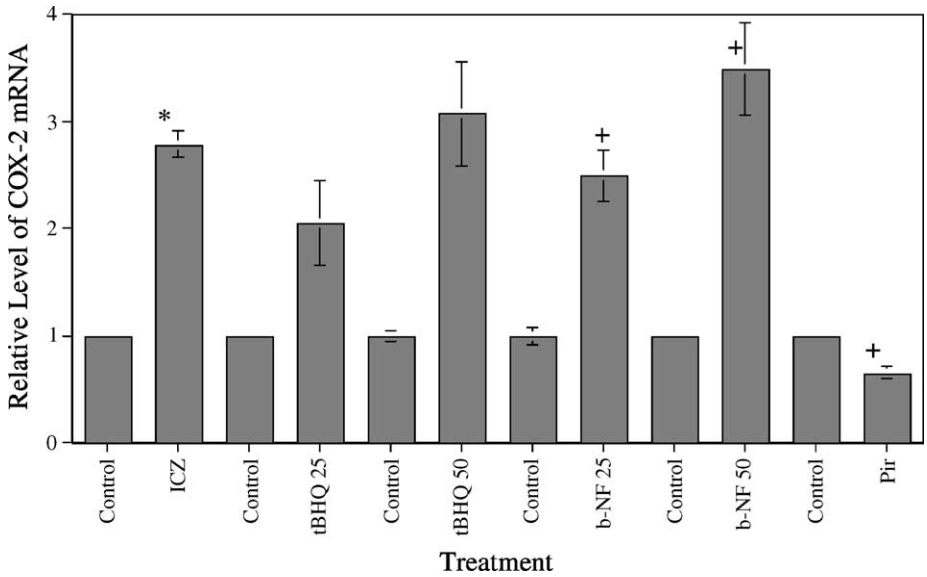

Fig. 1. Regulation of constitutive COX-2 expression by chemopreventive agents. HCA-7 cells were exposed to chemopreventive agents for 6 hr. The cells were harvested, and total RNA was isolated for TaqMan® analysis. The concentrations of compounds employed were: ICZ, 1 μM; *t*BHQ, 25 or 50 μM (designated *t*BHQ 25 and *t*BHQ 50, respectively); β-NF, 25 or 50 μM (designated b-NF 25 and b-NF 50, respectively) and Pir, 25 μM. Each treatment is shown with its appropriate control where cells were cultured in serum-free medium alone. The control values are normalized to equal 1. Values are means ± range (bars) from a typical set of results where N = 2. Key: (+) $P < 0.05$ compared to control values, and (∗) $P < 0.01$ compared to control values.

mRNA at a concentration of 50 μM. Exposure of HCA-7 cells to medium containing 25 or 50 μM *t*BHQ resulted in COX-2 mRNA increasing 2.0- or 3.0-fold, respectively. By contrast, treatment of HCA-7 cells with Piroxicam reduced the relative level of COX-2 mRNA to 66% of that determined in control cells.

3.2. Identification of possible cis-acting elements in the human COX-2 promoter

The induction of COX-2 in HCA-7 cells by ICZ and β-NF is consistent with the presence of functional XRE enhancers in the gene promoter. Furthermore, induction of COX-2 by *t*BHQ suggests that the gene promoter contains an ARE. Computer-aided DNA sequence analysis identified three possible XRE enhancers in the 5′-flanking region of the human *COX-2* gene between nucleotides −3444 and −3440, −958 and −955, and −507 and −504 (Fig. 2). Further analysis showed that there is a single consensus ARE sequence (5′-AGTGACGACGC-3′) in reverse orientation between nucleotides −562 and −572 of the human *COX-2* promoter. To investigate whether this 5′-flanking region of *COX-2* contained a functional ARE enhancer, a portion of the promoter was ligated into the pCAT3basic vector. The construct, designated pCOX2-679CAT, consisted of the region between nucleotides −679 and +59 of the human *COX-2* promoter and contained the putative ARE sequence.

In Hep-G2 cells transfected with pCOX2-673CAT, a concentration-dependent increase in CAT reporter gene activity was observed following treatment with *t*BHQ (Fig. 3). Co-transfection of Hep-G2 cells with pCOX2-679CAT and a construct that expresses murine Nrf2 enhanced *t*BHQ-induced CAT activity. Hep-G2 cells were used for functional analysis of the human *COX-2* promoter since reporter gene activity was undetectable in HCA-7 cells under the conditions used. These findings suggest that the putative ARE located between −562 and −572 of COX-2 may contribute to gene induction by *t*BHQ. No other sequences of the proximal 5′-flanking region of COX-2 were found to be identical to the ARE consensus reported by Rushmore *et al.* [44].

3.3. Effects of chemopreventive agents on the induction of COX-2 by IL-1β

NF-κB mediated induction of COX-2 by inflammatory cytokines can be prevented by xenobiotics that either modulate binding of the transcription factor to DNA or inhibit the upstream NIK/IKK signaling complex [50–54]. To date, no studies have reported on whether COX-2 expression can be altered through interaction between NF-κB-binding sites and other regulatory elements, such as XRE and ARE enhancers, within the gene promoter. Therefore, experiments were carried out with combinations of ICZ or *t*BHQ and IL-1β that stimulate either XRE-, ARE- or NF-κB-driven transcription. Exposure of HCA-7 cells to IL-1β alone typically resulted in an increase of between 2.3- and 3-fold in the amount of COX-2 transcript (Fig. 4). Co-treatment of the cells with the cytokine plus either ICZ or *t*BHQ further increased COX-2 mRNA levels above control cells and cells exposed to IL-1β alone. The

```
                                                        AHR/ARNT
-3488 CATGCCAGAT CAGAAACTGA AAGTCCTAAG CCTCATATTC TGTGCGTGGG ----------
       AHR/ARNT
 -961 ATCCACGCAT CAGGGAGAGA AATGCCTTAA GGCATACGTT TTGGACATTT ----------
                                                          COX2 FP
 -720 AGTATTTCTT CTGTTGAAAG CAACTTAGCT ACAAAGATAA ATTACAGCTA TGTACACTGA

 -660 AGGTAGCTAT TTCATTCCAC AAAATAAGAG TTTTTTAAAA AGCTATGTAT GTATGTCCTG
                                  CONSENSUS ARE
 -600 CATATAGAGC AGATATACAG CCTATTAAGC GTCGTCACTA AAACATAAAA CATGTCAGCC
                                  AHR/ARNT
 -540 TTTCTTAACC TTACTCGCCC CAGTCTGTCC CGACGTGACT TCCTCGACCC TCTAAAGACG
                                  NF-κB
 -480 TACAGACCAG ACACGGCGGC GGCGGCGGGA GAGGGGATTC CCTGCGCCCC CGGACCTCAG

 -420 GGCCGCTCAG ATTCCTGGAG AGGAAGCCAA GTGTCCTTCT GCCCTCCCCC GGTATCCCAT

 -360 CCAAGGCGAT CAGTCCAGAA CTGGCTCTCG GAAGCGCTCG GGCAAAGACT GCGAAGAAGA

 -300 AAAGACATCT GGCGGAAACC TGTGCGCCTG GGGCGGTGGA ACTCGGGGAG GAGAGGGAGG
                      NF-κB
 -240 GATCAGACAG GAGAGTGGGG ACTACCCCCT CTGCTCCCAA ATTGGGGCAG CTTCCTGGGT

 -180 TTCCGATTTT CTCATTTCCG TGGGTAAAAA ACCCTGCCCC CACCGGGCTT ACGCAATTTT

 -120 TTTAAGGGGA GAGGAGGGAA AAATTTGTGG GGGGTACGAA AAGGCGGAAA GAAACAGTCA
                                  TATA BOX
  -60 TTTCGTCACA TGGGCTTGGT TTTCAGTCTT ATAAAAAGGA AGGTTCTCTC GGTTAGCGAC
     +1                                                    COX2 RP
     CAATTGTCAT ACGACTTGCA GTGAGCGTCA GGAGCACGTC CAGGAACTCC TCAGCAGCGC
```

Fig. 2. Identification of *cis*-acting elements in the human *COX-2* promoter. The promoter region of the human *COX-2* gene was analyzed for the existence of potential *cis*-acting XRE and ARE enhancers. Three potential XRE sequences and one ARE consensus sequence were identified (shown in bold and underlined). The XRE-like sequences are between nucleotides −3444 and −3440, −958 and −955, and −507 and −504. The ARE consensus sequence is between nucleotides −562 and −572. Also highlighted are two NF-κB sites (underlined and italicized), the TATA box (bold underlined), the transcriptional start site (+1 bold underlined), and the primers used to construct the CAT reporter construct used in this study (underlined).

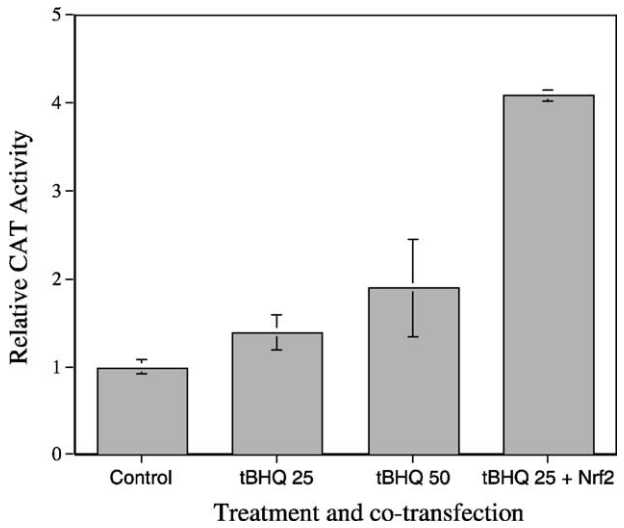

Fig. 3. Regulation of human *COX-2* reporter gene expression by *t*BHQ and Nrf2. Hep-G2 cells were transfected with a *CAT* reporter gene as described in Section 2. The *CAT* reporter gene was also co-transfected with 25 ng of an expression plasmid encoding mouse Nrf2. Following transfection, cells were allowed to recover overnight in serum-free medium, and then treated with either medium alone (control) or medium with 25 or 50 μM *t*BHQ (designated *t*BHQ 25 and *t*BHQ 50, respectively). The control value is normalized to equal 1. Values are means ± SEM (bars) where N = 3.

inclusion of all three inducing agents within the culture medium did not produce any further increase in COX-2 mRNA (data not shown).

In contrast with the other compounds studied, Piroxicam antagonized cytokine induction of *COX-2* gene expression. However, induction of COX-2 mRNA by IL-1β was not reduced completely by Piroxicam and still exceeded that seen under serum-free control conditions.

3.4. Effect of chemopreventive agents on PGE_2 production in HCA-7 cells

Since many of the physiological effects of COX-2 expression in colon cancer are attributed to PGE_2, the effect of xenobiotics on the production of this prostanoid was investigated in HCA-7 cells. Table 1 shows that among the compounds tested only ICZ significantly increased PGE_2 production in HCA-7 cells. A 1.6-fold increase in PGE_2 production was observed in ICZ-treated cells. Although β-NF and *t*BHQ induced levels of *COX-2* gene expression, this was not translated into an increase in prostanoid biosynthesis. Treatment with *t*BHQ reduced prostanoid production in stimulated cells to 44% of that

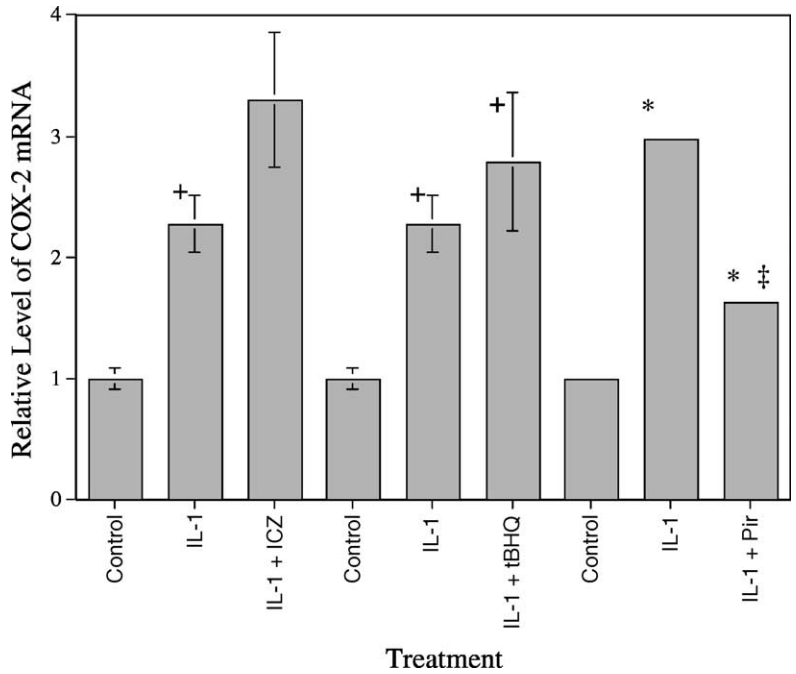

Fig. 4. Effects of chemopreventive agents on the cytokine-induced expression of COX-2. HCA-7 cells were treated with serum-free medium alone or exposed to IL-1β at a final concentration of 5 ng/mL for 5 hr. In co-treatment experiments, the cells were incubated with dietary constituents for 1 hr prior to treatment with IL-1β at the following concentrations: ICZ, 1 μM; *t*BHQ, 25 μM; Pir, 10 μM. Cells were harvested, and total RNA was isolated for TaqMan® analysis of COX-2 transcript levels. The control values are normalized to equal 1. Values are means ± range (bars) from a typical set of results where N = 2. Key: (+) $P < 0.05$ compared to control values, (∗) $P < 0.01$ compared to control values, and (‡) $P < 0.01$ when comparing the IL-1β-treated values with the IL-1β + Pir co-treated values.

in control cells. These results suggest that β-NF and *t*BHQ may be affecting the PGE_2 pathway downstream of COX-2. Piroxicam, which decreased levels of COX-2 mRNA in HCA-7 cells, also reduced PGE_2 production in stimulated cells to 80% of PGE_2 produced in control cells.

In co-treatment experiments, IL-1β-induced PGE_2 production was elevated further by the inclusion of ICZ, thereby underlining the possibility that it can still induce COX-2 activity within cancer cells during cytokine induction. Also consistent with previous data, Piroxicam antagonized IL-1β-induced PGE_2 synthesis, but did not abolish induction by the cytokine completely. Interestingly, treatment with *t*BHQ, reduced the level of PGE_2 synthesis below that of untreated cells.

Table 1
PGE_2 production in HCA-7 cells treated with chemopreventive agents for 24 hr without and with IL-1β

Treatment	Concentration of xenobiotic	PGE_2 produced (ng/mL medium)
With chemopreventive agent alone		
Control		1.9 ± 0.16
*t*BHQ	25 μM	0.91 ± 0.03*
ICZ	1 μM	3.1 ± 0.12*
β-NF	25 μM	1.94 ± 0.06
Piroxicam	25 μM	1.45 ± 0.01
With chemopreventive agent + IL-1β		
Control		3.35 ± 0.3
IL-1β	5 ng/mL	5.45 ± 0.1**
+*t*BHQ	25 μM	1.85 ± 0.2*,***
+ICZ	1 μM	10.1 ± 0.35**,***
+Piroxicam	25 μM	4.45 ± 0.04**,****

Values are means ± SEM where N = 3.

*,**Significantly different from control values: $^{*}P < 0.05$, and $^{**}P < 0.01$.

,*Significantly different from IL-1β-treated values: $^{***}P < 0.01$, and $^{****}P < 0.05$.

3.5. Effects of chemopreventive agents on the expression of MGST1-L1

To explain the lack of correlation between COX-2 mRNA levels and PGE_2 synthesis following treatment of AA-stimulated HCA-7 cells with *t*BHQ and β-NF, the effect of these compounds on the expression of the microsomal PGE_2 synthase MGST1-L1 was investigated (Fig. 5). Treatment of HCA-7 cells showed a reproducible increase in the amount of mRNA encoding MGST1-L1 in response to ICZ and β-NF, with ICZ increasing the level of synthase mRNA about 1.5-fold. However, none of the inductions of MGST1-L1 mRNA reached statistical significance. The monofunctional inducer *t*BHQ failed to affect a reproducible increase or decrease in the constitutive level of MGST1-L1 mRNA. As previously observed with COX-2, the level of MGST1-L1 mRNA was reduced in cells treated with Piroxicam. Thus, COX-2 and MGST1-L1 enzymes are only co-regulated by cytokines and AhR ligands, and are not jointly regulated by monofunctional

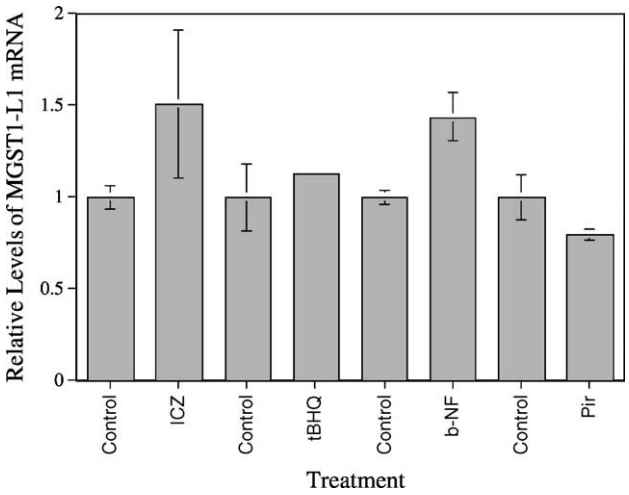

Fig. 5. Regulation of constitutive MGST1-L1 expression by chemopreventive agents. HCA-7 cells were exposed to chemopreventive agents for 6 hr. The cells were harvested, and total RNA was isolated for TaqMan® analysis of MGST1-L1 transcript levels. The concentrations of compounds employed were: ICZ, 1 μM; *t*BHQ, 25 μM; β-NF (designated b-NF), 25 μM; and Pir, 25 μM. Each treatment is shown with its appropriate control where cells were cultured in serum-free medium alone. The control values are normalized to equal 1. Values are means ± range (bars) from a typical set of results where N = 2.

inducers such as *t*BHQ. Most importantly, *t*BHQ was found to antagonize IL-1β-stimulated expression of MGST1-L1 (Fig. 6). This is the first report of differences between the regulation of these two enzymes. Consistent with our previous findings mentioned above, Piroxicam antagonized MGST1-L1 induction by IL-1β.

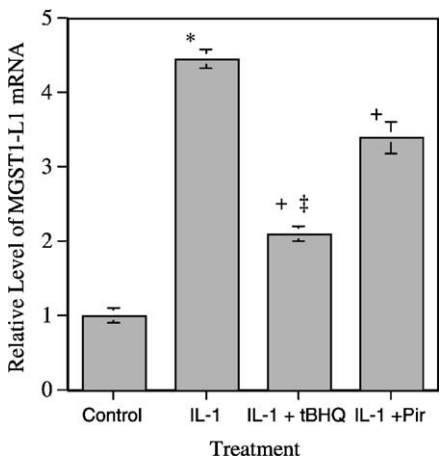

Fig. 6. Effects of chemopreventive agents on the cytokine-induced expression of MGST1-L1. HCA-7 cells were either cultured in serum-free medium alone or treated with medium containing either *t*BHQ to 25 μM or Pir to 25 μM for 1 hr. IL-1β was added, and the cells were incubated for a further 5 hr. Cells were harvested, and total RNA was isolated for TaqMan® analysis of MGST1-L1 transcript levels. The control value is normalized to equal 1. Values are means ± range (bars) from a typical set of results where N = 2. Key: (+) $P < 0.05$ when compared to control values, (∗) $P < 0.01$ compared to control values, and (‡) $P < 0.01$ compared to IL-1β values.

The ability of *t*BHQ to inhibit both constitutive and IL-1β-induced PGE_2 production below that of untreated control cells cannot be explained through the down-regulation of either COX-2 or MGST1-L1. Experiments were performed where *t*BHQ and Piroxicam were used as competitive inhibitors of PG biosynthesis from AA in HCA-7 cells. The production of PGE_2 was efficiently blocked by the addition of *t*BHQ or Piroxicam simultaneously with AA to 18 and 21%, respectively, of that of the control cells stimulated with AA alone (data not shown). It appears, therefore, that *t*BHQ, like Piroxicam, serves as an inhibitor of either COX-2 or MGST1-L1, or both. Interestingly, the NSAID was considerably more effective at inhibiting PGE_2 production directly than at reducing the capacity of cells to synthesize PGE_2 following 24-hr exposure to the drug. This suggests that Piroxicam is short-lived as a competitive inhibitor with AA, and the PGE_2 assays may well be a reflection of its action in reducing levels of *COX-2* and *MGST1-L1* gene expression.

4. Discussion

We have sought to test whether compounds with cancer chemopreventive properties can target the PGE_2 biosynthetic pathway. In this paper, the effects of xenobiotics on the regulation of COX-2 and MGST1-L1 expression, as well as the synthesis of PGE_2, have been examined. To this end, ICZ, β-NF, and *t*BHQ were employed as examples of indoles, flavonoids, and phenolic antioxidants, respectively. These compounds were chosen for study because their abilities to stimulate XRE- and/or ARE-driven gene induction have been well characterized [43,55]. Piroxicam was also selected for study because it is an NSAID [7], and this class of compound may possess cancer chemopreventive properties.

4.1. Mechanisms of regulation of COX-2

The results in Fig. 1 show that chemicals known to stimulate XRE- and/or ARE-driven transcription can induce levels of COX-2 mRNA in HCA-7 cells. The indole derivative ICZ is a potent activator of XRE-driven transcription [43], and in the present study it was found to increase COX-2 mRNA levels significantly. Similarly, the hydroquinone *t*BHQ, which stimulates ARE-driven gene expression, was demonstrated to induce COX-2 expression in human colon cancer cells. Increases in the amount of COX-2 mRNA were also observed following treatment with β-NF (which can stimulate both the XRE and the ARE). Interestingly, these xenobiotics could also augment the level of COX-2 transcript induced by the pro-inflammatory cytokine IL-1β (Fig. 4). Thus, COX-2 can be induced by compounds that stimulate XRE- or ARE-driven transcription, albeit modestly, even when levels are already elevated during inflammation. This is of concern with

respect to colon cancer where such compounds are referred to as chemopreventive agents, but could actually increase levels of COX-2. This may be expected to drive progression of the disease.

The induction of COX-2 by inflammatory cytokines and AhR ligands has been reported previously [15,56]. However, the induction of COX-2 through an ARE enhancer has not been investigated previously. Analysis of the *COX-2* promoter region identified a potential ARE in reverse orientation between nucleotides −572 and −564 that fulfills the 5′-A/GGTGACnnnGC-3′ consensus sequence defined by Rushmore *et al*. [44]. The 3′-flanking sequence of this ARE is A/T rich, indicating that it shares further contextual similarity to the rat *GSTA2* and *NQO1* enhancers. The *COX-2*-CAT reporter gene construct used was responsive to *t*BHQ, and co-transfection with the ARE-specific transcription factor Nrf2 was found to elevate reporter gene activity. It is worth noting that parallels may be drawn between the regulation of human COX-2 and rat inducible nitric oxide synthase gene expression [57]. Both genes contain AREs that are in a reverse orientation and situated 5′ of two functional NF-κB sites. Both enzymes can be induced above cytokine-stimulated levels by compounds acting through the ARE. Further detailed analysis is required to fully characterize this potentially functional ARE within the human *COX-2* gene. The presence of both XRE and ARE enhancers in the human *COX-2* promoter could lead to marked variations between populations, according to their diet, in the level of its expression in both normal and cancerous tissue.

4.2. Differential and co-regulation of human COX-2 and MGST1-L1

The observation that COX-2 and the microsomal PGE_2 synthase are functionally coupled suggests that they may also be co-regulated. This hypothesis appears to be correct insofar as mRNAs encoding the two enzymes are both increased in HCA-7 cells following treatment with the AhR ligand ICZ. However, this generalization does not hold true for the ARE-specific inducer *t*BHQ. In this case, the hydroquinone increased the level of COX-2 mRNA in HCA-7 cells, but did not cause the induction of MGST1-L1. It appears unlikely that MGST1-L1 has a functional ARE sequence within its promoter region. The effect of *t*BHQ on HCA-7 cells is one of the first examples where COX-2 and MGST1-L1 are not co-regulated.

Both COX-2 and MGST1-L1 are induced by the cytokine IL-1β. Treatment of HCA-7 cells with both IL-1β and *t*BHQ caused an increase in COX-2 over-and-above that seen with IL-1β alone. By contrast, treatment of HCA-7 cells with the cytokine and the hydroquinone caused a decrease in the level of MGST1-L1 when compared with IL-1β. It seems from these results that *t*BHQ can also antagonize cytokine-stimulated gene induction. It is possible that this negative regulatory effect of *t*BHQ is due either to its direct antioxidant effects or to its ability to induce antioxidant genes such as *GCSh* that inhibit pro-oxidant signaling processes. We have, for example, found that *t*BHQ treatment of HCA-7 cells significantly increased the cellular level of reduced glutathione (data not shown). It is therefore worth considering that this antagonism of cytokine induction of MGST1-L1 by *t*BHQ could also occur with COX-2. However, in the latter case, the increase of expression through the ARE masks this effect.

4.3. Effects of tBHQ and ICZ on PGE_2 biosynthesis

As described above, ICZ stimulated expression of both COX-2 and MGST1-L1 in HCA-7 cells. Treatment of the colon cell line with this indole caused a significant elevation in the synthesis of PGE_2 (Table 1). Interestingly, ICZ produced an increase of almost 2-fold in PGE_2 synthesis when added to medium alone or in the presence of IL-1β. These results suggest that indoles, which are potent AhR ligands, may not be effective at preventing or inhibiting the progression of colorectal cancer.

The measurement of PGE_2 production by HCA-7 cells revealed that *t*BHQ treatment did not effect an increase in the prostanoid, despite the fact that it can induce COX-2 mRNA. By contrast, the compound significantly decreased the production of PGE_2 by HCA-7 cells. The basis for *t*BHQ antagonism of PGE_2 synthesis appears to be due to a lack of increase in MGST1-L1 expression and the fact that it is also a competitive inhibitor of AA. It has been shown previously that *t*BHQ can be generated from butylated hydroxyanisole (BHA) by a COX-catalyzed reaction [58,59], and it is therefore possible that the quinone metabolite acts by product inhibition to block PGH_2 production. The inhibitory effect of *t*BHQ appears to be prolonged in that cells treated with the compound continue to inhibit the production of PGE_2 from AA after 24 hr in culture. It is also possible that treatment with *t*BHQ stimulates antioxidant mechanisms within the cell, and that it is an alteration in glutathione levels that inhibits production of PGE_2 [60,61]. It would therefore be interesting to determine whether other chemopreventive agents that also stimulate ARE-driven transcription, such as the isothiocyanate sulforaphane and dithiole-3-thione, can also antagonize PGE_2 biosynthesis. The HCA-7 cell line can synthesize substantial amounts of glutathione conjugates of PGJ_2 [62]. This prostanoid is derived from PGD_2 and represents a competing pathway to that responsible for the formation of PGE_2. Hence, it is also possible that the ability of *t*BHQ to antagonize PGE_2 synthesis is a consequence of increased production of the PGJ_2–glutathione conjugate.

The NSAID included in this study was less potent at reducing PGE_2 biosynthesis after 24 hr in culture when measured by a direct competition assay with AA. Piroxicam could be inactivated through metabolism during this time. However, the levels of PGE_2 production did correlate

with the data showing that Piroxicam antagonizes both the basal expression of COX-2 and MGST1-L1 and their induction by cytokines. This was achieved at concentrations in the micromolar range. It appears that Piroxicam in its role as an NSAID has gene regulatory properties that are potentially long lasting. This finding warrants further investigation in terms of its potential therapeutic value.

4.4. Concluding comments

It has been found that chemopreventive agents can regulate COX-2 and MGST1-L1 expression in human colon carcinoma cells. The indole derivative ICZ, which stimulates XRE-driven transcription, was shown to be a potent inducer of COX-2 and stimulated an increase in PGE_2 production. The hydroquinone *t*BHQ, which stimulates ARE-driven transcription, was also shown to induce *COX-2* gene expression. This led to the discovery of an ARE enhancer within the 5′-flanking region of the human *COX-2* gene which, along with the XRE, can modulate gene expression to a broad range of dietary and pharmacological compounds. By contrast with ICZ, *t*BHQ proved to be a particularly effective antagonist of PGE_2 production in HCA-7 cells, a property associated with an ability to directly inhibit PGE_2 production or possibly with an increase in cellular glutathione levels. This, however, does raise concerns that other compounds that stimulate ARE-driven transcription but do not inhibit PGE_2 biosynthesis could, along with ICZ, actually drive colon carcinogenesis by increasing COX-2 expression. Interestingly, Piroxicam is capable of reducing PGE_2 biosynthesis by antagonizing both the constitutive and induced expression of COX-2 and MGST1-L1. This represents an added property to the therapeutic potential of this compound for the prevention of colon cancer.

Acknowledgments

We thank Professor Masayuki Yamamoto and Dr. Ken Itoh for the gift of the mouse Nrf2 cDNA. We also thank Dr. Christine Bonnesen and Dr. Michael McMahon for their helpful comments. This work was funded by the Biotechnology and Biological Sciences Research Council (94/F08200), and we thank them for financial support.

References

[1] Food nutrition and the prevention of cancer: a global perspective. World Cancer Research Fund/American Institute for Cancer Research Expert Panel Report. Washington, DC: American Institute for Cancer Research; 1997. p. 216–51.

[2] Verhoeven DTH, Verhagen H, Goldbohm RA, van den Brandt PA, van Poppel G. A review of mechanisms underlying anticarcinogenicity by brassica vegetables. Chem Biol Interact 1997;103:79–129.

[3] Potter JD. Colorectal cancer: molecules and populations. J Natl Cancer Inst 1999;91:916–32.

[4] Marks F, Fürstenberger G. Cancer chemoprevention through interruption of multistage carcinogenesis: the lessons learnt by comparing mouse skin carcinogenesis and human large bowel cancer. Eur J Cancer 2000;36:314–29.

[5] Bruce WR, Giacca A, Medline A. Possible mechanisms relating diet and risk of colon cancer. Cancer Epidemiol Biomarkers Prev 2000;9:1271–9.

[6] Smalley WE, DuBois RN. Colorectal cancer and nonsteroidal anti-inflammatory drugs. Adv Pharmacol 1997;39:1–20.

[7] Rao CV, Tokumo K, Rigotty J, Zang E, Kelloff G, Reddy BS. Chemoprevention of colon carcinogenesis by dietary administration of Piroxicam, α-difluoromethylornithine, 16α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. Cancer Res 1991;51:4528–34.

[8] Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM. Suppression of intestinal polposis in $Apc^{\Delta 716}$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996;87:803–9.

[9] Kawamori T, Rao CV, Seibert K, Reddy BS. Chemopreventive activity of celecoxib, a specific cyclo-oxygenase-2 inhibitor against colon carcinogenesis. Cancer Res 1998;58:409–12.

[10] Li H, Schut HAJ, Conran P, Kramer PM, Lubet RA, Steele VE, Hawk EE, Kelloff GJ, Pereira MA. Prevention by aspirin and its combination with α-difluoromethylornithine of azoxymethane-induced tumors, aberrant crypt foci and prostaglandin E_2 levels in rat colon. Carcinogenesis 1999;20:425–30.

[11] Bennett A, del Tacca M. Prostaglandins in human colonic carcinoma. Gut 1975;16:409.

[12] Rigas B, Goldman IS, Levine L. Altered eicosanoid levels in human colon cancer. J Lab Clin Med 1993;122:518–23.

[13] Oka M, Inaba A, Uchiyama T, Hazama S, Shimoda K, Suzuki M, Suzuki T. Prostaglandin E_2 levels and lymphocyte subsets in portal venous drainage of colorectal cancers. Am J Surg 1994;167:264–7.

[14] Pugh S, Thomas G. Patients with adenomatous polyps and carcinomas have increased colonic mucosal prostaglandin E_2. Gut 1994;35:675–8.

[15] Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.

[16] Tsujii M, Kawano S, DuBois RN. Cyclo-oxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA 1997;94:3336–40.

[17] Tsujii M, Kawano S, Tsujii S, Sawaoka H, Hori M, DuBois RN. Cyclooxygenase regulates angiogenesis induced by colon cancer cells. Cell 1998;93:705–16.

[18] Sheng H, Shao J, Morrow JD, Beauchamp RD, DuBois RN. Modulation of apoptosis and Bcl-2 expression by prostaglandin E_2 in human colon cancer cells. Cancer Res 1998;58:362–6.

[19] Di Popolo A, Memoli A, Apicella A, Tuccillo C, di Palma A, Ricchi P, Acquaviva AM, Zarrilli R. IGF-II/IGF-I receptor pathway up-regulates COX-2 mRNA expression and PGE_2 synthesis in Caco-2 human colon carcinoma cells. Oncogene 2000;19:5517–24.

[20] Sheng H, Shao J, Washington MK, DuBois RN. Prostaglandin E_2 increases growth and motility of colorectal carcinoma cells. J Biol Chem 2001;276:18075–81.

[21] Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998;38:97–120.

[22] Warner TD, Mitchell JA. Cyclooxygenase-3 (COX-3): filling in the gaps toward a COX continuum? Proc Natl Acad Sci USA 2002;99:13371–3.

[23] Tanioka T, Nakatani Y, Semmyo N, Murakami M, Kudo I. Molecular identification of cytosolic prostaglandin E_2 synthase that is functionally

coupled with cyclooxygenase-1 in immediate prostaglandin E_2 biosynthesis. J Biol Chem 2000;275:32775–82.

[24] Jakobsson PJ, Thoren S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: a microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. Proc Natl Acad Sci USA 1999;96:7220–5.

[25] Murakami M, Naraba H, Tanioka T, Semmyo N, Nakatani Y, Kojima F, Ikeda T, Fueki M, Ueno A, Ohishi S, Kudo I. Regulation of prostaglandin E_2 biosynthesis by inducible membrane-associated prostaglandin E_2 synthase that acts in concert with cyclooxygenase-2. J Biol Chem 2000;275:32783–92.

[26] Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994;107:1183–8.

[27] Kargman SL, O'Neill GP, Vickers PJ, Evans JF, Mancini JA, Jothy S. Expression of prostaglandin G/H synthase-1 and -2 protein in human colon cancer. Cancer Res 1995;55:2556–9.

[28] Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M, Hla T. Expression of cyclooxygenase-1 and cyclooxygenase-2 in human colorectal cancer. Cancer Res 1995;55:3785–9.

[29] Kutchera W, Jones DA, Matsunami N, Groden J, McIntyre TM, Zimmerman GA, White RL, Prescott SM. Prostaglandin H synthase 2 is expressed abnormally in human colon cancer: evidence for a transcriptional effect. Proc Natl Acad Sci USA 1996;93:4816–20.

[30] Fujita T, Matsui M, Takaku K, Uetake H, Ichikawa W, Taketo MM, Sugihara K. Size-dependent and invasion-dependent increase in cyclooxygenase-2 levels in human colorectal carcinomas. Cancer Res 1998;58:4823–6.

[31] Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Vandeputte LBA, Lipsky PE. Cyclooxygenase in biology and disease. FASEB J 1998;12:1063–73.

[32] Hao XP, Bishop AE, Wallace M, Wang H, Willcocks TC, Maclouf J, Polak JM, Knight S, Talbot IC. Early expression of cyclo-oxygenase-2 during sporadic colorectal carcinogenesis. J Pathol 1999;187:295–301.

[33] Brock TG, McNish RW, Peters-Golden M. Arachidonic acid is preferentially metabolized by cyclooxygenase-2 to prostacyclin and prostaglandin E_2. J Biol Chem 1999;274:11660–6.

[34] Yoshimatsu K, Golijanin D, Paty PB, Soslow RA, Jakobsson P-J, DeLellis RA, Subbaramaiah K, Dannenberg AJ. Inducible microsomal prostaglandin E synthase is overexpressed in colorectal adenomas and cancer. Clin Cancer Res 2001;7:3971–6.

[35] Appleby SB, Ristimaki A, Neilson K, Narko K, Hla T. Structure of the human cyclo-oxygenase-2 gene. Biochem J 1994;302:723–7.

[36] Yang X, Hou F, Taylor L, Polgar P. Characterization of human cyclooxygenase-2 gene promoter localization of a TGF-β response element. Biochim Biophys Acta 1997;1350:287–92.

[37] Suh NJ, Wang YP, Honda T, Gribble GW, Dmitrovsky E, Hickey WF, Maue RA, Place AE, Porter DM, Spinella MJ, Williams CR, Wu GF, Dannenberg AJ, Flanders KC, Letterio JJ, Mangelsdorf DJ, Nathan CF, Nguyen L, Porter WW, Ren RF, Roberts AB, Roche NS, Subbaramaiah K, Sporn MB. A novel synthetic oleanane triterpenoid, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid, with potent differentiating, antiproliferative, and anti-inflammatory activity. Cancer Res 1999;59:336–41.

[38] Shao JY, Sheng HM, Inoue H, Morrow JD, DuBois RN. Regulation of constitutive cyclooxygenase-2 expression in colon carcinoma cells. J Biol Chem 2000;275:33951–6.

[39] Forsberg L, Leeb L, Thoren S, Morgenstern R, Jakobsson PJ. Human glutathione dependent prostaglandin E synthase: gene structure and regulation. FEBS Lett 2000;471:78–82.

[40] Mimura J, Fujii-Kuriyama Y. Regulatory roles of AhR. Environ Sci 2002;9:71–81.

[41] Ciolino HP, Daschner PJ, Yeh GC. Dietary flavonols quercetin and kaempferol are ligands of the aryl hydrocarbon receptor that affect CYP1A1 transcription differentially. Biochem J 1999;340:715–22.

[42] MacDonald CJ, Ciolino HP, Yeh GC. Dibenzoylmethane modulates aryl hydrocarbon receptor function and expression of cytochromes P450 1A1, 1A2, and 1B1. Cancer Res 2001;61:3919–24.

[43] Bonnesen C, Eggleston IM, Hayes JD. Dietary indoles and isothiocyanates that are generated from cruciferous vegetables can both stimulate apoptosis and confer protection against DNA damage in human colon cell lines. Cancer Res 2001;61:6120–30.

[44] Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J Biol Chem 1991;266:11632–9.

[45] Itoh K, Chiba T, Takahashi S, Ishii T, Igarashi K, Katoh Y, Oyake T, Hayashi N, Satoh K, Hatayama I, Yamamoto M, Nabeshima Y-I. An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem Biophys Res Commun 1997;236:313–22.

[46] Nguyen T, Sherratt PJ, Pickett CB. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu Rev Pharmacol Toxicol 2003;43:233–60.

[47] Hayes JD, McMahon M. Molecular basis for the contribution of the antioxidant responsive element to cancer chemoprevention. Cancer Lett 2001;174:103–13.

[48] Chui DHK, Tang W, Orkin SH. cDNA cloning of murine Nrf2 gene, coding for a p45 NF-E2 related transcription factor. Biochem Biophys Res Commun 1995;209:40–6.

[49] Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995;23:4878–84.

[50] Plummer SM, Holloway KA, Manson MM, Munks RJL, Kaptein A, Farrow S, Howells L. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-κB activation via the NIK/IKK signalling complex. Oncogene 1999;18:6013–20.

[51] Kapahi P, Takahashi T, Natoli G, Adams SR, Chen Y, Tsien RY, Karin M. Inhibition of NF-κB activation by arsenite through reaction with a critical cysteine in the activation loop of IκB kinase. J Biol Chem 2000;275:36062–6.

[52] Rossi A, Kapahi P, Natoli G, Takahashi T, Chen Y, Karin M, Santoro MG. Anti-inflammatory cyclopentenone prostaglandins are direct inhibitors of IκB kinase. Nature 2000;403:103–8.

[53] Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C. Nuclear factor κB is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 2001;276:32008–15.

[54] Korn SH, Wouters EFM, Vos N, Janssen-Heininger YMW. Cytokine-induced activation of nuclear factor-κB is inhibited by hydrogen peroxide through oxidative inactivation of IκB kinase. J Biol Chem 2001;276:35693–700.

[55] Rushmore TH, Pickett CB. Transcriptional regulation of the rat glutathione *S*-transferase Ya subunit gene. Characterization of a xenobiotic-responsive element controlling inducible expression by phenolic antioxidants. J Biol Chem 1990;265:14648–53.

[56] Wölfle D, Marotzki S, Dartsch D, Schäfer W, Marquardt H. Induction of cyclooxygenase expression and enhancement of malignant cell transformation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Carcinogenesis 2000;21:15–21.

[57] Huo PC, Abe K, Schroeder RA. Superoxide enhances interleukin 1β-mediated transcription of the hepatocyte-inducible nitric oxide synthase gene. Gastroenterology 2000;118:608–18.

[58] Thompson D, Eling T. Mechanism of inhibition of prostaglandin H synthase by eugenol and other phenolic peroxidase substrates. Mol Pharmacol 1989;36:809–17.

[59] Schildermam PA, van Maanen JM, Smeets EJ, ten Hoor F, Kleinjans JC. Oxygen radical formation during prostaglandin H synthase-mediated biotransformation of butylated hydroxyanisole. Carcinogenesis 1993;14:347–53.

[60] Capdevila JH, Morrow JD, Belosludtsev YY, Beauchamp DR, Dubois RN, Falck JR. The catalytic outcomes of the constitutive and the mitogen-inducible isoforms of prostaglandin H_2 synthase are markedly affected by glutathione and glutathione peroxidase(s). Biochemistry 1995;34:3325–37.

[61] Chinery R, Beauchamp RD, Shyr Y, Kirkland SC, Coffey RJ, Morrow JD. Antioxidants reduce cyclooxygenase-2 expression, prostaglandin production, and proliferation in colorectal cancer cells. Cancer Res 1998;58:2323–7.

[62] Cox B, Murphey LJ, Zackert WE, Chinery R, Graves-Deal R, Boutaud O, Oates JA, Coffey RJ, Morrow JD. Human colorectal cancer cells efficiently conjugate the cyclopentenone, prostaglandin J_2, to glutathione. Biochim Biophys Acta 2002;1584:37–45.