Red Wine and Black Tea Polyphenols Modulate the Expression of Cyclooxygenase-2, Inducible Nitric Oxide Synthase and Glutathione-Related Enzymes in Azoxymethane-Induced F344 Rat Colon Tumors


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ABSTRACT Polyphenolic compounds extracted from red wine (WE) and black tea (BT), 50 mg/(kg · d) inhibit the promotion phase of the colon carcinogenesis process induced by azoxymethane (AOM) in rodents. To investigate possible mechanisms of this protective activity, we evaluated by RT-PCR the gene expression of cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), γ-glutamylcysteine synthetase (γ-GCS) and two isoforms of glutathione S-transferase (GST), GST-P and GST-M2, in 30 AOM-induced tumors and in the corresponding normal colon mucosa. AOM-induced colon tumors had significantly greater GST-P, GST-M2, COX-2 and iNOS gene expression than the corresponding normal mucosa. However, tumors harvested from rats treated with BT (P < 0.05) and WE (P < 0.01) polyphenols had a lower GST-P mRNA level than tumors from controls. Treatment with WE polyphenols induced a similar inhibitory effect on the colon tumor overexpression of GST-M2 (P < 0.01), COX-2 (P < 0.05) and iNOS (P < 0.05). In the normal mucosa, rats treated with BT polyphenols had greater γ-GCS expression than controls (P < 0.01). Our results provide evidence that WE and BT polyphenols modulate COX-2, iNOS and glutathione-related gene expression in tumors, suggesting that these compounds have possible chemotherapeutic activity. J. Nutr. 132: 1376–1379, 2002.

KEY WORDS: • cyclooxygenase-2 • glutathione S-transferase • inducible nitric oxide synthase • polyphenols • rat colon

Polyphehols from tea and other beverages such as wine, have been considered with interest as possible chemopreventive agents against colon cancer. Recently, we demonstrated that polyphenolic compounds extracted from red wine (WE)4 and black tea (BT) protect against DNA oxidative damage in rat liver and intestine (1) and inhibit colon carcinogenesis induced by azoxymethane (AOM) in rodents (2).

Polyphenols are powerful antioxidants and free radical scavengers (3). They have anti-inflammatory properties (4) and may modulate the activity of phase I and II enzymes, in particular glutathione (GSH)-related enzymes (5).

GSH and its related enzymes have been studied extensively because they are thought to protect against free radicals and xenobiotics. Tumors that express high levels of glutathione S-transferase (GST) (especially GST-P) are associated in humans with failure of cancer chemotherapy and poor patient survival (6). Glutamylcysteine synthetase (γ-GCS) is the rate-limiting step enzyme in GSH synthesis, and cellular GSH levels are substantially regulated by this enzyme (7).

Cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) also play an important role in colon tumor growth and progression. COX-2 is frequently undetectable in normal tissues but is induced by cytokines, growth factors, reactive oxygen species and tumor promoters (8). Gene expression of COX-2 is up-regulated in human colorectal cancers, and COX-2 levels are also elevated in colonic tumors induced by AOM in rodents (9).

Nitric oxide (NO) is endogenously produced by a family of enzymes. It is reported to cause mutagenesis (10) and DNA deamination (11) and is implicated in the inflammatory responses and in the production of vascular endothelial growth factor (12). Some studies have reported that inducible NO synthase (iNOS) is up-regulated in human cancers (13) and in AOM-induced colon tumors in rodents (14).

On the basis of these considerations, we decided to study the expression of COX-2, iNOS, γ-GCS and of two different isoforms of GST (GST-P and GST-M2) in normal mucosa and in AOM-induced colon tumors isolated from a colon carcinogenesis experiment in which we observed that BT and WE polyphenols showed chemopreventive activity, whereas green tea (GT) treatment had no effect.

MATERIALS AND METHODS

Materials. AOM was purchased from Sigma Chemical (Milan, Italy). Dietary components were purchased from Piccioni (Gessate, Milan, Italy). Decaffeinated red wine polyphenolic extract was prepared from vintage 1994 Cabernet Sauvignon, made from Vitis vinifera grapes by standard red wine making procedures at the Arzens Cooperative winery (Arzens, Aude, France). Decaffeinated green and black tea extracts were provided by Unilever (Colworth Laboratory, Florence, Italy). Azoxymethane (AOM) was purchased from Sigma Chemical (Milan, Italy). AOM was purchased from Sigma Chemical (Milan, Italy). AOM was purchased from Sigma Chemical (Milan, Italy). AOM was purchased from Sigma Chemical (Milan, Italy). AOM was purchased from Sigma Chemical (Milan, Italy). AOM was purchased from Sigma Chemical (Milan, Italy).

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During the AOM treatment rats were fed a diet based on the AIN76 EU guidelines (15). Rats were treated subcutaneously with 10 weekly doses of AOM (15 mg/kg body weight) and were then divided randomly to four different groups; the control group was fed a low level of cellulose (2 g/100 g) and a low level of calcium (0.13 g/100 g) (16). One week after the last AOM injection, rats were treated with an intraperitoneal injection of 1 ml/kg body weight of WE polyphenol extract. Rats were killed 16 wk after the last AOM injection, and tumors and samples of apparently normal mucosa were excised and stored at −80°C.

**RT-PCR.** Total RNA was isolated from 30 colorectal cancers (15–25 mg of tissue) and from the matched normal mucosa using the Trizol protocol as suggested by the supplier (Life Technologies, San Giuliano Milanese, Milan, Italy). We analyzed 12 tumors taken from the control group and 6 tumors collected from each of the three dietary treatment groups.

For first-strand cDNA synthesis, 250 ng of RNA from each sample was reverse transcribed using 100 U of RT superscript II (Life Technologies) and 1X random examers (Roche Diagnostics, Monza, Italy). We analyzed 12 tumors taken from the control group and 6 tumors collected from each of the three dietary treatment groups.

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Primers were designed on the basis of the sequences reported by the UNIGENE database for rats (Table 1). Each mRNA was coamplified with β-actin as the control. The PCR reactions were carried out on aliquots of the cDNA preparation, in a 25 µL volume containing 1X PCR buffer, 2 mmol/L MgCl₂, 0.5 mmol/L dNTPs, 1.6 µmol/L of each target gene primer, 0.04 µmol/L of the β-actin primer and 1.25 U of Taq polymerase (Advanced Biotechnologies, Epsom, UK). The PCR conditions were the same for the 5 genes, i.e., 95°C for 7 min and then 30 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 55 s and a final extension at 72°C for 5 min.

The PCR products were separated on 1.6% agarose gel and visualized by ethidium bromide staining. The amplified products were photographed with a digital camera and the band intensities were analyzed using the Quantity-One software (Bio-Rad, Segrate, Milan, Italy). For each target gene, we calculated the relative amount of mRNA in the samples as the ratio of each gene mRNA to β-actin mRNA, as amplified as internal standard. Examples of this analysis are shown in Figure 1.

**Statistical analysis.** Data are expressed as means ± SEM and were analyzed with the Statgraphics program (Manugistics, Rockville, MD). Differences were considered significant at P < 0.05. Statistical analysis of the data was performed by two-way ANOVA to test the main effects on gene expressions and interactions between diet and tissue type (normal vs. tumor). Comparisons within groups (normal vs. tumor) and within tissue type (treated vs. control group) were performed using one-way ANOVA with a two-sided Scheffe’s test, by taking the control group as the reference.

**RESULTS**

The WE and BT polyphenol extract treatment inhibited carcinogenesis by AOM in F344 rats (P < 0.05), whereas GT extract was ineffective, as recently reported by our group (2). The number of intestinal tumors/rat was 2.54 ± 0.34 in control rats, 1.54 ± 0.29 in BT-treated rats, 3.20 ± 0.44 in GT-treated rats and 1.63 ± 0.32 in the WE polyphenol-treated rats.

In the present study, we observed that the expression of GST-P, GST-M2, COX-2 and iNOS was increased in tumors, compared with normal colon mucosa (P < 0.0001) and was significantly affected by the different dietary treatments (P < 0.01 for GST-P and iNOS and P < 0.05 for GST-M2 and COX-2). On the contrary, the mRNA level of the γ-GCS did not change significantly.

**TABLE 1**

Sequence of the oligonucleotide primers used for PCR amplification and the predicted product sizes

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GST-P</td>
<td>5’-TGC CAC CGT ACA CCA TTG TGT-3’</td>
<td>5’-CAT CAG CAG GTC CAG GAA GGA TTA GTA-3’</td>
<td>479 bp</td>
</tr>
<tr>
<td>COX2</td>
<td>5’-TCT GGC ATG TCT TTG GGA GGT-3’</td>
<td>5’-ATG ACA CAG TCT CAG GAA GGA TGA-3’</td>
<td>751 bp</td>
</tr>
<tr>
<td>GST-M2</td>
<td>5’-CAG ACA CAA GGT ATG AGG AG-3’</td>
<td>5’-AAT GAA GAA ATG AGA GGA AGA-3’</td>
<td>768 bp</td>
</tr>
<tr>
<td>INOS</td>
<td>5’-CGG ATA TCT CTT GCA AGT CCA AA-3’</td>
<td>5’-AAT TGT GAA TGT TCT GCA GAT ATG-3’</td>
<td>380 bp</td>
</tr>
<tr>
<td>γ-GCS</td>
<td>5’-GCA TGC CAC TCT TCT CAC GGT-3’</td>
<td>5’-TGC CCA CAG TCA TTA GGT TCT CTA-3’</td>
<td>862 bp</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-ATC CGA TCG CAC TCT AGG AGG-3’</td>
<td>5’-AGG GCC CAG CTT CAG CAG CAG-3’</td>
<td>621 bp</td>
</tr>
</tbody>
</table>

1 Abbreviations used: GST-P, glutathione S-transferase P; COX-2, cyclooxygenase-2; GST-M2, glutathione S-transferase M2; iNOS, inducible nitric oxide synthase; γ-GCS, γ-glutamylcysteine synthetase.
not differ between the types of tissue and was not modulated by the diets (data not shown).

In tumors harvested from rats fed BT (P < 0.05) and WE (P < 0.01) GST-P expression was lower than in tumors of the controls (Fig. 2A). Treatment with WE polyphenols also inhibited GST-M2 (P < 0.01), COX-2 (P < 0.05) and iNOS (P < 0.05) tumor overexpression (Figure 2B, C and D).

In normal colon mucosa, γGCS gene expression was greater in the BT-treated rats (P < 0.01) and tended to be greater (P = 0.051) in the WE-treated rats, compared with the controls (0.63 ± 0.2; 1.97 ± 0.12 and 1.26 ± 0.00, in controls, BT and WE groups, respectively).

In the green tea–treated group GST-P, GST-M2, COX-2, iNOS and γGCS expression in tumors and in the normal mucosa did not differ from controls.

**DISCUSSION**

Polyphenolic compounds have a remarkable variety of biochemical activities that may influence cancer development. These include antioxidant effects, carcinogen inactivation, action on cell cycle proteins and altered expression of enzymes, some of which have been studied in the present experiment.

Most human tumors express large amounts of GST, in particular the GST-P isoform (17), probably as a result of widespread cellular oxidative stress; this high GST-P expression is often associated with a poor prognosis for the patient (18–19).

We observed that in rats, AOM-induced colon tumors overexpressed GST-P and GST-M2 isoforms and that BT and WE, which can inhibit intestinal carcinogenesis (2), depressed this GST-P tumor overexpression. These results suggest that tumor growth inhibition and control of GST up-regulation might be causally connected. Recently, a role for GST-P in carcinogenesis has been suggested because its inhibition is accompanied by a marked reduction in the number of AOM-induced colon adenomas and aberrant crypt foci in mice (20). The same authors reported that human colon cancer cell lines transfected with the GST-P gene are resistant to apoptosis induced by deoxycolic acid (20). GST-P may therefore act as a cytoprotective factor in colon tumors, modifying their apoptotic activity. Our data seem to confirm this hypothesis; previously we reported that tumors harvested from rats treated with BT and WE polyphenols had a markedly increased apoptotic index (2).

Recently we demonstrated that the WE polyphenolic extract exhibited antioxidant and radical scavenging properties in vitro (21). In vivo, WE polyphenolic compounds were protective against chemically induced DNA oxidative damage in rat liver and colon mucosa (1), indicating that these compounds are absorbed.

The reduction in GST overexpression in tumors induced by BT and WE polyphenols may be related to their antioxidant properties. Polyphenols may decrease cellular oxidative stress by modifying the redox status of the tumor cells, consequently regulating the activity of GSH-related genes. On the other hand, GT polyphenols have similar antioxidant properties but, in our study, did not protect against colon carcinogenesis or modulate any of the genes studied. It is possible that WE and BT polyphenolic extracts, rich in polymeric fractions, may scavenge different classes of radicals or scavenge more efficiently than the green tea extract.

The ability of WE and BT polyphenols to reduce the overexpression of GST-P suggests a possible use of polyphenols in GST-mediated multidrug resistance in intestinal tumors. Zhang and Wong reported (22), for instance, that polyphenols inhibiting GST activity of colon cancer cells significantly potentiated clorambucil cytotoxicity.

BT and GT polyphenols have been shown to induce GST enzyme activity in human liver cells (5). On the contrary, green tea polyphenols do not modify GST activity in normal colon mucosa of rats treated with AOM (23). In this study, GST isoenzyme expression in normal colon mucosa of rats treated with polyphenols was not modulated, with the exception of an increase in γ-GCS mRNA levels in rats treated with BT polyphenols. Dietary administration of the synthetic antioxidant butylated hydroxyanisole increases hepatic glutathione and the γ-GCS activity in mice (24). It is therefore possible that BT polyphenols protect cells from chemical stress also by increasing GSH content through up-regulation of γ-GCS expression.

Polyphenols may also participate in cellular defenses by virtue of their anti-inflammatory activity. Reduced production of inflammatory mediators through the inhibition of phospholipase A2, lipoxigenase and cyclooxygenase by polyphenols has been documented (4). Overexpression of iNOS and COX-2 leads to resistance to apoptosis, DNA damage, mutation, increased proliferation, oxidative stress, and increased tumor vascularization and metastatic potential (25). The role of iNOS and COX-2 as enhancers of carcinogenesis in many organs, including the colon, has recently received considerable attention; therefore, the suppression of either the synthesis or activity of these two enzymes is a target for cancer chemoprevention. Several chemopreventive phytochemicals (curcumin, epigallocatechin gallate and resveratrol) have been shown to inhibit COX-2 and iNOS expression by blocking improper nuclear factor-κB activation (26). In our experiments, we
observed that COX-2 and iNOS were expressed at a low level in normal colon mucosa and were increased in colon tumors. In a small subset of tumors and normal colon mucosa, we also measured COX-2 activity by quantifying prostaglandin E2 and observed a very good correlation between COX-2 activity and COX-2 expression (data not shown). In our study, WE polyphenolic extracts significantly suppressed the expression of iNOS and COX-2 in colon tumors. Similarly, other authors have reported that tea polyphenols affect arachidonic acid metabolism in human colon mucosa and colon tumors (27). However, although BT inhibited AOM-induced colon carcinogenesis, it did not modulate COX-2 and iNOS expressions.

Previously we reported that WE and BT polyphenols showed chemopreventive activity in a rat colon carcinogenesis model. These results provide evidence that WE and BT polyphenolic extracts modulate COX-2, iNOS and GSH-related gene expression in tumors, suggesting also a possible chemotherapeutic activity of these compounds.

**LITERATURE CITED**


15. European Community (1986) European Community Regulations on the Care and Use of Laboratory Animals, Law 86/609/EC.


