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Induction of rat hepatic and intestinal glutathione *S*-transferases by dietary butylated hydroxyanisole

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Abstract—To obtain insight into the protection mechanism of butylated hydroxyanisole (BHA), a widely used food preservative with anticarcinogenic properties, we investigated the effects of dietary BHA on rat hepatic and intestinal glutathione *S*-transferase (GST) enzyme activity, and GST isozyme levels. In the proximal small intestine and liver, BHA supplementation significantly increased GST enzyme activity as compared with controls (2.3- and 1.7-fold, respectively, $P < 0.05$). GST class α and μ contents were significantly higher only in the small intestine (1.6–2.1-fold and 1.3–1.5-fold, respectively, $P < 0.05$), whereas GST class π was significantly induced in liver (4.6-fold, $P < 0.05$).

Humans are exposed daily to complex mixtures of chemical compounds in their food [1]. An important portal of entry for these compounds is the gastrointestinal tract [1, 2]. In this case the intestinal mucosa is the first-line barrier. The capability of mucosal cells to detoxify these substances is essential for good protection against these xenobiotics. The detoxification or biotransformation is the total of biochemical reactions which results in the modification and excretion of the exogenous molecules. Important biotransformation enzymes are glutathione *S*-transferases (GSTs*), consisting of a family of isoenzymes with partly overlapping substrate specificities [3, 4]. Therefore, not only the total enzyme activity but also the levels of the different isoenzymes may determine the risk of damaging effects or even the development of cancer [5, 6].

Colon cancer is a major health problem in Western society. It is the second most frequent malignancy and the incidence is still increasing. Epidemiological studies showed that environmental factors, such as dietary habits, may play a role in colon carcinogenesis [7]. However, food also contains compounds which are considered to be anticarcinogenic [7–9]. Butylated hydroxyanisole (BHA) is a widely used food preservative with anticarcinogenic properties [10–12]. A possible mechanism of the chemopreventive action of BHA may be the induction of detoxification enzymes resulting in lower levels of reactive electrophilic metabolites of many xenobiotics [13–17]. Knowledge of the exact protection mechanisms of anticarcinogenic compounds present in food may be of importance for the reduction or prevention of colon cancer. This paper reveals the induction of GSTs in the liver and intestine by BHA as a possible anticarcinogenic mechanism.

* Abbreviations: BHA, 2(3)-*tert*-butyl-4-hydroxyanisole; GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

Materials and Methods

Treatment of animals. Male Wistar rats (200 ± 10 g), were obtained from the Central Laboratory Animal Center (University of Nijmegen, The Netherlands). The animals were housed individually on wooden shavings in macrolon cages maintained at 20–25° and 30–60% relative humidity. A ventilation rate of seven air changes/hr and a 12 hr light/dark cycle were used.

The rats were randomly assigned into two groups (nine animals each). Both groups were fed powdered RMH-TM lab chow (Hope Farms, Woerden, The Netherlands). After acclimatization for 3 days, the animals were fed either the basal diet (RHM-TM) or the experimental diet, which was prepared by supplementation with 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) at a concentration of 1% (w/w). A food processor was used to obtain a homogeneous mixture of BHA and the powered lab chow. Body weight was recorded daily and freshly prepared food was given every 2–3 days. During the experiment, the rats had free access to diet and tap water. After 2 weeks the animals were killed by decapitation.

Tissue preparation. The intestine and liver were immediately excised and the intestine was dissected into four segments: proximal, middle and distal small intestine, and large intestine. Each segment was slit longitudinally and the contents were removed by washing with chilled buffer A (0.25 M saccharose, 20 mM Tris, 1 mM dithiothreitol, pH 7.4). The organs were frozen in liquid nitrogen and stored at −80°. For preparation of the cytosolic fraction, the tissue was thawed quickly using cold running water. The mucosal surface of the intestine segments was collected by scraping with a scalpel and the mucosal scrapings were homogenized in buffer A (4 mL/g tissue) in a glass/glass Potter–Elvehjem tube. The liver was also homogenized in buffer A (4 mL/g tissue) with 10 strokes at 1000 rpm of a motor-driven Potter–Elvehjem

Teflon Homogenizer (Braun, Germany). The homogenate was centrifuged at 9000 *g* (4°) for 20 min. The resulting supernatant fraction was transferred to an ultracentrifuge tube and spun at 150,000 *g* (4°) for 50 min. Aliquots of the 150,000 *g* supernatant, representing the cytoslic fraction, were frozen in liquid nitrogen and stored at −80° until use. All aforementioned handlings were performed on ice.

Assays. Protein concentration was assayed in duplicate by the method of Lowry *et al.* [18] using bovine serum albumin as the standard. GST activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate was determined according to Habig *et al.* [19]. Cytosolic GST samples were subjected to SDS–PAGE (11% w/v acrylamide) according to Laemmli [20] and, subsequently, western blotting. Western blotting was performed as described before [21], using here a semi-dry blotting system (Novablot II, Pharmacia, Upsala, Sweden). The western blots were treated with monoclonal antibodies against human GST class α [22], π [23] and μ [24]. Class α antibodies react with rat GST subunit 1, class π with rat GST subunit 7 and class μ with rat GST subunits 3 and 4. The specific binding of the monoclonal antibodies to their antigens was detected with 4-chloro-1-naphthol after incubation with peroxidase-conjugated rabbit anti-mouse second antibody (Dakopatts, Glostrup, Denmark). Staining on the immunoblots was quantified by densitometric analysis using a laser densitometer (Ultrascan XL, LKB, Bromma, Sweden). Known amounts of purified GST-α, -π and -μ were run in parallel with the experimental samples and served as standards for the calculation of the absolute amounts of these enzymes.

Materials. BHA, dithiothreitol, glutathione, CDNB and bovine serum albumin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Statistical analysis. Wilcoxon rank-sum test was used to assess statistical significance of differences between experimental and control groups; $P < 0.05$ is considered as significantly different.

Results

At the termination of the experiment, a significantly lower gain in body weight for the BHA group (mean ± SEM, 34.5 ± 3.4 g, $P < 0.05$) versus the control group (74 ± 2.8 g) was noted. The average daily consumption of BHA was about 0.5–1 g/kg body weight. Table 1 shows the effects of dietary BHA on the specific activity of GST in the different parts of the intestine and in the liver. The most profound changes in GST activity were observed in the liver and proximal small intestine where the observed values were significantly higher in the treated animals. Longitudinal distribution of the GST activities measured showed a decline in activity going from the proximal to the distal small intestine, with a rise in the large intestine. GST subclasses were quantified by densitometric analysis of western blots, after immunodetection with specific monoclonal antibodies against class α, π and μ GST. Results are presented in Table 2 and Fig. 1. GST-α was expressed in the small intestine and liver in considerable amounts, but was hardly detectable in the large intestine. In contrast, GST-π was most profoundly expressed in the large intestine, but was hardly detectable in the liver (Fig. 1). BHA supplementation results in an increase in all

Table 1. GST activity in hepatic and intestinal cytosols of control and BHA-supplemented Wistar rats

	GST activity (nmol/min. mg protein)				
	Small intestine				
	Proximal	Middle	Distal	Large intestine	Liver
Control	298 ± 16	137 ± 9	44 ± 4	74 ± 5	1592 ± 57
BHA-supplemented	679 ± 53*	198 ± 20	53 ± 3	80 ± 5	2702 ± 159*

GST activity was assayed as described in Materials and Methods. Values given are means ± SEM for nine animals in each group, with three measurements per rat.

Wilcoxon rank-sum test: *$P < 0.05$; BHA-supplemented vs control.

Table 2. Hepatic and intestinal class α, class μ and class π GST in control and BHA-supplemented Wistar rats

		GST content (ng/mg protein)				
		Small intestine				
		Proximal	Middle	Distal	Large intestine	Liver
GST-α	Control	4130 ± 630	2430 ± 360	650 ± 130	70 ± 30	11580 ± 1610
	BHA-supplemented	6180 ± 690*	4110 ± 680*	1370 ± 400*	60 ± 20	16120 ± 2260
GST-μ	Control	4070 ± 480	3230 ± 310	2590 ± 250	3860 ± 440	47530 ± 2530
	BHA-supplemented	6220 ± 500*	3970 ± 390	3450 ± 310*	4990 ± 640	54890 ± 4220
GST-π	Control	631 ± 95	397 ± 90	168 ± 46	1397 ± 217	90 ± 58
	BHA-supplemented	777 ± 81	557 ± 127	266 ± 93	1477 ± 264	416 ± 146*

GST content was determined as described in Materials and Methods. Values given are means ± SEM for eight animals in each group.

Wilcoxon rank-sum test: *$P < 0.05$; BHA-supplemented vs control.

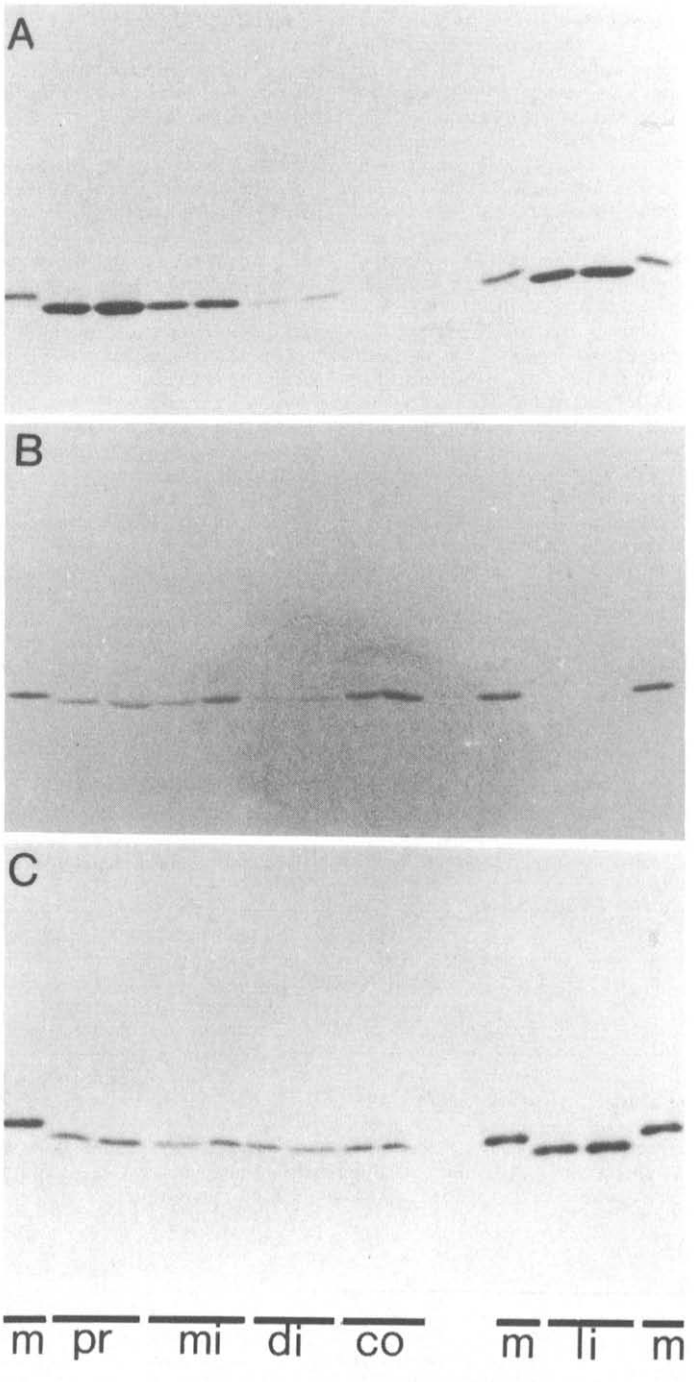

Fig. 1. Effects of dietary BHA on rat hepatic and intestinal class-α, class-π and class-μ GSTs. The expression levels of the three classes of GST in different parts of the intestine and liver from Wistar rats were examined. Hepatic and intestinal 150,000-*g* supernatant fractions were isolated, and subjected to SDS–PAGE and western blotting as described in Materials and Methods. Western blots were treated with monoclonal antibodies against class α (panel A), class π (panel B) and class μ (panel C) GST. Pr, Mi and Di, proximal, middle and distal small intestine, respectively, Co, colon plus coecum; Li, liver. Purified GSTs serve as marker (M). Each lane consists of cytosolic protein from a control (first) and a BHA-treated animal (second). Panel A: Pr, Mi and Di 20 μg; Co 40 μg; Li 2.5 μg and purified GST-α from human liver 62 ng. Panel B: Pr, Mi, Di and Co 80 μg; Li 40 μg and purified GST-π from human placenta 100 ng. Panel C: Pr, Mi, Di and Co 10 μg; Li 2.5 μg and purified GST-μ from human liver 137 ng.

classes of GST. In the small intestine GST-α and -μ were significantly induced, whereas in the liver GST-π was significantly induced. In the large intestine none of the three classes was induced (Table 2).

Discussion

Dietary administration of the antioxidant BHA has been shown to decrease the incidence of carcinogen-induced neoplasia in rodents [25]. Biochemical investigations have revealed that BHA has a profound effect on several hepatic and extrahepatic enzyme activities such as GSTs [17, 26–29]. In addition, GSTs have been shown to protect against the damaging of DNA by metabolites of polycyclic hydrocarbons or aflatoxin B_1, both by catalysing the conjugation of reactive metabolites with glutathione and by covalent binding of reactive species [11, 12]. Thus, it is suggested that the concentration of reactive metabolites of drugs, plant toxins and environmental pollutants will decrease more rapidly when GST levels are higher. Although the protective mechanism(s) of BHA against colon carcinogenesis has been studied extensively, little is known about the effects of BHA on GST enzyme activity at the level of the target organ: the colon. Furthermore, it is well established that GST is a family of isozymes that have different but overlapping substrate specificities and are under the regulation of different genes [3]. To understand the mechanisms of modulation of drug metabolism by dietary factors such as BHA, it is important to study the effects not only on the total enzyme activity, but also to notify the changes in isozyme levels that may occur.

In a previous study by Brouard *et al.* [30], it was shown that a lower food intake had no influence on the inducibility of drug-metabolizing enzymes. Thus, the observed lower gain in body weight for the BHA group as compared with the controls may not influence the effects of BHA on GST. Dietary BHA has been shown to increase hepatic GST activity in rodents [27, 31–33]. Some studies showed an increase in GST activity in the (proximal) small intestine [27, 29, 32, 33]. However, much less is known about the effects in the colon. De Long *et al.* [27] found no differences in GST activity towards CDNB in the colon between BHA-treated mice and controls. Jaeschke and Wendel [32] studied the effects of BHA 1 g/kg/day for 5 days on mouse colon and found also no induction of GST enzyme activity. However, in another study by the same authors [33] mice were treated for 14 days with the same dose and a significant induction ($P < 0.01$) in the colon was seen. In our study on BHA-treated rats GST activity was significantly increased only in the proximal small intestine and liver. This might be explained by the absorption of BHA mainly in the proximal small intestine. As a consequence, only minor amounts of BHA may reach the lower part of the intestine. Nevertheless, the load of toxic or carcinogenic compounds in the colon may be reduced by a more efficient detoxification, due to increased levels of GSTs in the proximal intestine. Therefore, one may speculate that BHA can protect against colon carcinogenesis, although no dramatic increase in GSTs was achieved in the colon itself.

Pearson *et al.* [34] studied GST induction by BHA at the level of mRNAs. Densitometrical analysis of autoradiographs showed an induction in the liver and intestine of BHA-treated mice. Class μ and α GST mRNA levels increased by 15- and 50-fold, respectively, in the liver, and 15- and 100-fold, respectively, in the intestinal mucosa in response to BHA treatment. Class π mRNAs were much less responsive to BHA treatment. Studying GST induction at the enzyme (protein) level, we find much lower responses (0.9–2.1-fold), with a 4.6-fold induction of GST-π in the liver. This indicates that changes at the mRNA level are of another order of magnitude as compared with the changes at the enzyme level. This observation is supported by the data on GST enzyme activity (1.1–2.3-fold induction; see Table 1) and by the studies of Benson *et al.* [35], McLellan and Hayes [36] and Hayes *et al.* [37] who showed that GST isozyme contents in the livers of mice were affected by dietary BHA in a way similar to that described here. In addition to the studies on GST induction by BHA, we also investigated the isozyme patterns at different locations in the intestine. As shown in Table 2, statistically significant differences were observed in the small intestine for class α and μ GSTs, and in the liver for GST π.

The present study demonstrates that a marked induction of hepatic and intestinal GSTs occurs in BHA-fed rats. The ability of dietary BHA to attenuate the carcinogenic effects of certain xenobiotics may be the result of an accelerated elimination of these compounds due to this induction of GSTs. It should be stated that the dose used in our study seems to be of minor relevance to the human situation, especially since the FAO/WHO Joint Expert Committee on Food Additives established an acceptable daily intake of BHA of 0.5 mg/kg body weight [38]. However, a significant hepatic GST induction occurs in mice at a dose of 10 mg BHA/kg body weight/day [33] which suggests that some effect on GSTs may be achieved in humans consuming much lower levels of BHA than those used in our study.

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