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THE GLUTATHIONE CONJUGATES OF *tert*-BUTYL HYDROQUINONE AS POTENT REDOX CYCLING AGENTS AND POSSIBLE REACTIVE AGENTS UNDERLYING THE TOXICITY OF BUTYLATED HYDROXYANISOLE

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The glutathione conjugates of *tert*-butyl hydroquinone, a metabolite of butylated hydroxyanisole (BHA), possess redox potentials which are much higher as compared to the non-conjugated hydroquinone (0.36 V for the hydroquinone and 1.2-1.4 V for the conjugates). As a result, the redox cycling activity of the conjugates, as measured by oxygen consumption in the presence of a reducing agent, is increased tenfold as compared to the non-conjugated hydroquinone. Since evidence for both oxidative damage and the involvement for glutathione in the toxicity of butylated hydroxyanisole is available, this mechanism may be involved in the toxic action of this compound.

Butylated hydroxyanisole (BHA) is a widely used synthetic food antioxidant. Toxicological data indicate forestomach carcinogenicity in rats, preceded by an increase in cell proliferation (1). Although extensive research efforts have been made to elucidate the mechanism responsible for this mode of action, no conclusive scheme has been accepted sofar.

The metabolism of BHA consists of either direct glucuronidation or sulfatation, or an O-demethylation resulting in *tert*-butyl hydroquinone (TBHQ). This hydroquinone may again be conjugated with sulfate or glucuronic acid, or undergo oxidation towards *tert*-butyl benzoquinone (TBBQ) and subsequent reaction with glutathione. Both *in vitro* and in *in vivo* data support these routes (1, 2). Concerning the mechanism underlying the carcinogenicity of BHA in rodent forestomach, a number of noteworthy observations have been made. 1) Although quinones are formed and *in vivo* evidence for covalent binding to proteins is available, no evidence exists for *in vivo* covalent binding of these compounds to DNA in rat forestomach (3). 2) Glutathione seems to be involved in the process of BHA-induced cell proliferation, since *in vivo* depletion of this agent with diethylmaleate prevents the formation of forestomach hyperplasia (4). This argument is in line with the first argument, since glutathione would decrease the intracellular concentration of quinones both by conjugation and reduction (5). 3) BHA induces the formation of reactive oxygen species both *in vitro* and in the rat forestomach (6-10).

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The above mentioned arguments led us to the hypothesis that the glutathione conjugates of TBHQ (or its benzoquinone form, TBBQ) may be involved in the mechanism of action of BHA through the stimulation of formation of reactive oxygen species.

MATERIALS AND METHODS

Synthesis of glutathione conjugates
TBBQ was obtained by oxidation of TBHQ by potassium bromate in 1 n sulphuric acid. Glutathione conjugates were synthesized by addition of an aqueous solution of 256 mg of glutathione to a solution of 137 mg TBBQ in methanol. This reaction mixture was stirred for 22 h under N_2. The formed conjugates were purified from the reaction mixture by preparative HPLC after reducing all constituents with ascorbic acid, removing the solvents by evaporation and three extractions with ethyl acetate. HPLC conditions were as follows: a Zorbax 22x250 mm RP18 column was eluted with 55% water (containing 0.5% formic acid) and 45% methanol, at a flow rate of 5 ml/min. Detection with a diode array detector (Pharmacia Rapid Spectra 2140) provided information on retention times and spectra of the various reaction products. Structural characterization of the products was performed by Fast Atom Bombardment Mass Spectrometry, positive ion detection (Finigan-Matt HSQ-30, BEQQ geometry), and proton NMR, using a Varian Unity-400.
Determination of redox potentials
Redox potentials of TBHQ and its glutathione conjugates were obtained by repeated injection of the purified compounds onto an LC system equipped with an electrochemical detector (Bioanalytical systems, model BC-l4), set at increasing applied voltages. E½ was calculated from the relation between change in applied potential and change in detector resonse. The buffer used was 0.1 M Tris/HCl (pH 7.4). A platinum electrode was used as reference.
Oxygen consumption measurements
All oxygen consumption measurements were performed in a Clark type electrode cell. The measurements were performed in 0.1 M potassium phospate (pH 7.4) at 25°C in a 1 ml volume. 100 µM ascorbic acid was added as a reducing agent, while the hydroquinones were added to a concentration of 100 µM. All reagents were added as aqueous solution, with the exeption of TBHQ (in methanol, 25 µl).
Enzymatic reactions
The microsomal oxygen consumption was measured in a Clark type cell, using microsomes derived from phenobarbital treated male rats (11). 0.5 mg of microsomal protein was incubated with various concentrations of hydroquinones in a volume of 1 ml of 0.1 M potassium phospate (pH 7.4) buffer. NADPH was added to a concentration of 1 mM. All hydroquinones were added as an aqueous solution.
The enzymatic and non-enzymatic conjugation of TBHQ with glutathione was measured by the formation of the conjugate at 306 nm, using a differential molar absorption coefficient of 2.175 mM^{-1}. cm^{-1}. This value was derived from the spectra of the two monoconjugates, and therefore a slight error may have been incorporated due to simultaneous formation of the diconjugate. All major rat glutathione S-transferases (GST 1-1, 2-2, 3-3 and 4-4) were used at a subunit concentration of 1 µM. The purification of these isoenzymes has been published previously (12). The incubations were performed both at pH 6.5 and pH 7.4, using potassium phosphate as buffer. Substrate concentrations were 1 mM (glutathione) and 4 mM (TBHQ).

RESULTS

The reaction of equimolar amounts of *tert*-butyl hydroquinone and glutathione resulted in the formation of three glutathione conjugates. FAB-MS showed two of these to be monoconjugates (m/z = 472) and the third a diconjugate (m/z = 777). Using proton NMR, indications were obtained for the location of the glutathione residue. One of the two monoconjugates showed a two-proton

resonance at ca. 6.92 ppm, with a typical para-coupling of less than 0.3 Hz. The other monoconjugate resulted in a two-proton resonance (two doublets) at ca. 6.89 ppm, with a metacoupling of ca 2.7 Hz (Figure 1). Therefore, the first conjugate was assigned 5-glutathione-*tert*-butylhydroquinone (5-GS-TBHQ), and the latter 6-glutathione-*tert*-butylhydroquinone (6-GS-TBHQ). The diconjugate showed a one-proton resonance at 7.01 ppm, not revealing the positions of the two conjugates. This conjugate was therefore nominated diGS-TBHQ.

The redox cycling capacity of TBHQ and its three glutathione conjugates were studied by measuring the oxygen consumption in the presence of an equimolar amount of a reducing agent (ascorbic acid). Thus, a redox cycle of oxidation by oxygen and reduction by ascorbic acid was obtained. The non-conjugated hydroquinone showed a moderate redox cycling activity, with an initial rate of oxygen consuption of 6.6 nmol/min. (Table 1). Compared to this compound, the 5-GS-conjugate showed an 8-fold increase, while oxygen consumption of the 6-GS-conjugate was raised 11-fold. The redox cycling activity of the diglutathione conjugate was doubled as compared to the nonconjugated compound. The redox potential, as determined in the same buffer as used for the oxygen consumption measurement, was 1.18-1.42 V for the three conjugates, and 0.36 V for the nonconjugated hydroquinone (Table 1).

TBHQ did not stimulate microsomes derived from phenobarbital treated rats to an increased rate of oxygen consumption in the presence of NADPH, in the concentration range of 0-100 μM. The rate of oxygen consuption in this system was constant at 19 nmol/min/mg protein.

The initial rate of formation of the glutathione conjugates was estimated in incubations with all major rat glutathione S-transferase isoenzymes (GST 1-1, 2-2, 3-3 and 4-4). However, no difference was

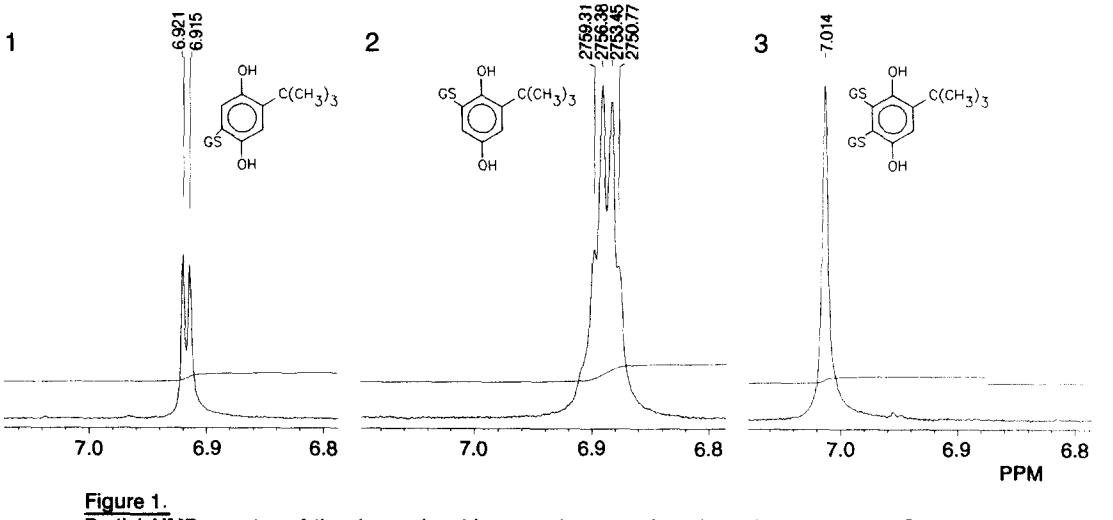

Figure 1.
Partial NMR spectra of the three glutathione conjugates of *tert*-butyl hydroquinone. Spectrum 1 shows the para coupling of less than 0.3 Hz of the two aromatic protons of 6GS-TBHQ. This coupling is ca. 2.7 Hz for the two para-protons of 5GS-TBHQ (spectrum 2), while the diglutathione conjugate shows only one aromatic proton (spectrum 3).

Table 1. Redox potentials and oxygen consumption of *tert*-butyl hydroquinone and its glutathione conjugates

hydroquinone	redox potential ($E_{1/2}$) (V)	oxygen consumption (nmol/min)
TBHQ	0.36	6.6 ± 0.5
5-GS-TBHQ	1.42	53 ± 15
6-GS-TBHQ	1.18	70 ± 0.8
diGS-TBHQ	1.31	15 ± 4

Redox potentials were determined by electrochemical detection at various applied voltages. Oxygen consumption was determined in a Clark type electrode, using 100 μM hydroquinone concentrations in the presence of 100 μM ascorbic acid. The average of five incubations is presented.

observed between incubations with and without enzyme. The chemical rate of conjugation was found 0.34 ± 0.1 nmol/min. Raising the pH from 6.5 to 7.4 resulted in an increase to 0.96 ±0.01 nmol/min.

DISCUSSION

The present study was undertaken to obtain support for the hypothesis that BHA gives rise to the formation of reactive oxygen species via redox cycling of GS-TBHQ conjugates. This mechanism is presented in Figure 2. The existence of redox cycling metabolites from BHA links four important aspects involved in the mechanism of carcinogenicity of BHA: 1) no binding of BHA or metabolites to DNA in rodent forestomach; binding of BHA to protein (3), 2) inhibition of BHA-induced hyperplasia *in vivo* by depletion of GSH with diethylmaleate (4), 3) the existance of sulphur-containing metabolites of BHA (2), and 4) formation of reactive oxygen species in the presence of BHA (6-10).

BHA-protein binding has been reported by many authors, also in rodent forestomach (3, 14). In contrast, in rat forestomach no BHA-DNA binding was found, as no radioactivity was recovered from DNA after administration of radiolabelled BHA, although some BHA-DNA binding was observed in non-target tissues like liver and kidney (3). In addition, no BHA-DNA adducts could be detected in the forestomach by the very sensitive [^{32}P]-postlabelling method (15).

Simultaneous administration of BHA and the glutathione depleting agent diethylmaleate completely inhibits the forestomach hyperplasia induced by treatment with BHA alone, indicating a role for glutathione in the activation of BHA (4). After i.p. administration of BHA or TBHQ to rats, two methylthioconjugates of TBHQ were identified (2). After *in vitro* incubation of TBHQ and GSH in the presence of rat liver microsomes, the two corresponding GS-TBHQ conjugates were found (5GS-TBHQ and 6GS-TBHQ) (2). In the present study we confirm the formation of these two GS-TBHQ conjugates and additionally report the formation of a third diGS-TBHQ conjugate. The formation of GS-TBHQ conjugates is a spontaneous reaction, not catalyzed by any of the purified GSH-S-transferase isozymes or by rat liver cytosol (2).

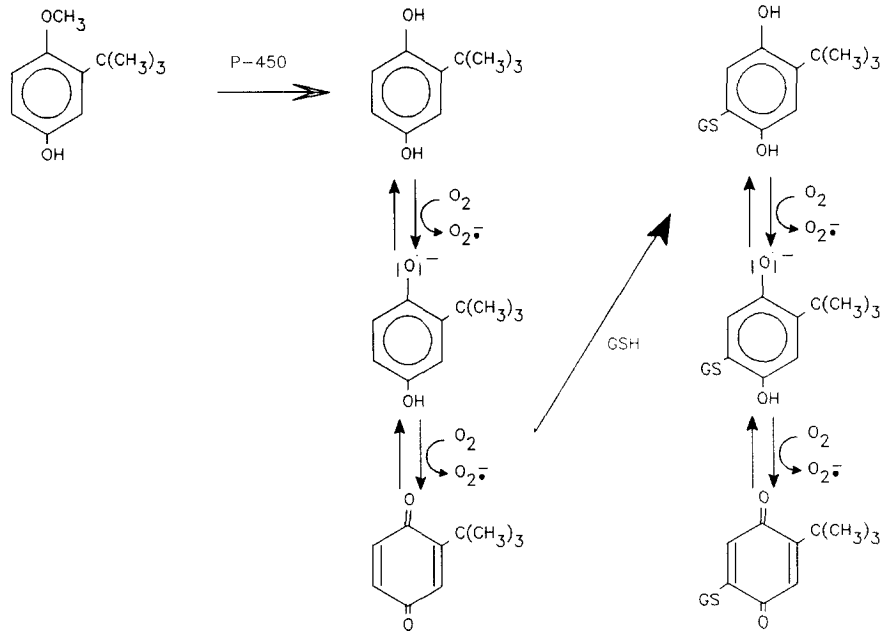

Figure 2.
Proposed pathway of activation of BHA to its reactive glutathione conjugate, resulting in oxidative DNA damage.

There are several indications for the formation of reactive oxygen species in the presence of BHA. Phillips *et al.* (6) found a microsome-mediated induction of chromosomal aberrations in Chinese hamster ovary (CHO) cells by BHA (and TBHQ), while H_2O_2 was being formed and catalase protected against this damage. BHA, TBHQ and TBBQ stimulate superoxide formation in rat liver microsomes and rat forestomach (7). TBHQ and TBBQ, but not BHA, elicit redox cycling in vitro concomitant with the formation of reactive oxygen species. In these studies BHA gave rise to formation of reactive oxygen species only after microsome-mediated metabolism. In addition, BHA stimulated NADPH-oxidase activity and H_2O_2 production in liver microsomes (8-10). Two major pathway leading to this production are the oxidase activity of cytochrome P-450 (17) and redox cycling (13). Since TBHQ did not stimulate oxygen consumption in microsomal incubations with NADPH, the first pathway seem unlikely. Aparently NADPH is needed for the biotransformation of BHA to TBHQ and its oxidation to TBBQ (2), although it is evident from the redox cycling experiments that molecular oxygen is also capable of performing the latter reaction.

Our hypothesis that BHA is a DNA-reactive agent in an indirect way, i.e. by initial metabolism to TBHQ and subsequent conjugation of TBHQ with GSH, followed by extensive redox cycling of the GS-TBHQ conjugates is fully supported by the data obtained in our experiments. TBHQ possesses redox cycling properties (7) but the redox cycling properties of the GS-TBHQ conjugates overwhelms those of TBHQ itself (Table 1). Thus the DNA-reactivity of BHA may be explained by an increase in the cellular burden to reactive oxygen species ('oxidative stress') caused by extensive redox cycling of GS-TBHQ conjugates. BHA is not unique in this respect as an increase in toxicity

by conjugation of quinones with glutathione is not uncommon. For example, the glutathione conjugates of bromobenzoquinone appear to be the reactive species involved in the nephrotoxicity of bromobenzene (reviewed in 16).

The most reactive of reactive oxygen species, hydroxyl radicals, readily react with DNA (18, 19). In this indirect way DNA-adducts (e.g. 80H-2'-deoxyguanosine) may be formed in a process mediated by BHA, leading to DNA-reactive species that have no molecular link with BHA. Misreading of DNA-templates containing 80H-2'deoxyguanosine has been reported by Kuchino et al. (20) indicating that this adduct may stand at the molecular basis of heritable changes in DNA. Production of reactive oxygen species like hydroxylradicals following metabolism of BHA may therefore be considered a genotoxic risk for this 'nongenotoxic' carcinogen. The data obtained in the present study serve to explain the mechanism underlying the carcinogenicity of BHA. Much more work has to be performed in the future to get full insight into this mechanism.

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