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SHORT COMMUNICATIONS

Inhibition of phylloquinone epoxide reductase by BHT quinone methide, salicylic acid and α-tocopherolquinone

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Butylated hydroxytoluene (BHT) is a phenolic antioxidant widely used in food products and in rubber and petroleum chemicals. Feeding of a large dose of BHT can cause haemorrhagic death in rats [1]; this animal is the most susceptible to BHT haemorrhage among many species [2]. Its metabolic activation by hepatic drug-metabolizing enzymes may be necessary for the haemorrhagic effect of BHT, and BHT quinone methide (2,6-di-*tert*-butyl-4-methylene-2,5-cyclohexadienone) has been identified as one of the active metabolites [3, 4]. The only place BHT quinone methide is known to be present is in the liver of rats [2]. The haemorrhagic effect of BHT can be prevented by vitamin K, and it has been indicated that critical to this preventive action may be the lowering of plasma concentrations of blood coagulation factors II (prothrombin), VII, IX and X due to the depression of hepatic synthesis of these factors [5, 6]. The direct ingestion of BHT quinone methide causes an even greater reduction in factors II, VII, IX and X than in BHT itself [unpublished results]. BHT quinone methide also inhibits phylloquinone (vitamin K_1) epoxide-dependent protein carboxylation (post-translational protein modification of blood coagulation factors II, VII, IX and X) but not phylloquinone- and vitamin K_1 hydroquinone-dependent protein carboxylation in liver microsomes *in vitro* [7]. These earlier findings suggest that BHT quinone methide may inhibit phylloquinone epoxide reductase, which is considered one of the key enzymes of redox cycling of vitamin K [8].

Vitamin E (α-tocopherol), a naturally occurring antioxidant, is also used as a food additive. Many biological effects of α-tocopherol resemble those of BHT and the haemorrhagic effect of hypervitaminosis E may be one of them [9]. The haemorrhagic effect on rats of α-tocopherol may be less strong than that of BHT, and an active metabolite α-tocopherolquinone as well as BHT quinone methide may play an antivitamin K role in the bleeding [10]. Acetylsalicylic acid is widely used as an analgesic, anti-inflammatory and antipyretic drug and is also well known to cause hypoprothrombinaemia, bleeding and haemorrhagic death [11]. There are no differences between acetylsalicylic acid and BHT in effective dosages, haemorrhage-occurring times, bleeding organs and susceptible species [8]. An active metabolite of acetylsalicylic acid is thought to be salicylic acid, which also causes bleeding and has antivitamin K activity [13]. The mechanism of salicylic acid-induced haemorrhage, however, is not yet known.

In the present report, the inhibitory effects of BHT quinone methide, α-tocopherolquinone and salicylic acid on phylloquinone epoxide reductase were examined.

Materials and methods

Preparation of microsomes. Liver from male Sprague–Dawley rats (300–400 g) were perfused and homogenized with 10 mM phosphate buffer (pH 7.4) containing 1.15% KCl. The 25% homogenates were centrifuged at 10,000 *g* for 15 min followed by 105,000 *g* for 60 min at 4°. The resulting microsomes suspended in 0.1 M phosphate buffer (pH 7.4) were used. Protein content was estimated by the method of Lowry *et al.* [14].

Assay methods. Microsomal phylloquinone 2,3-epoxide reductase activity was determined according the methods of Fasco and Principe [15] and Hildebrandt *et al.* [16] with slight modifications. Reaction mixtures were 2 ml and containing 0.1 M Tris–HCl buffer (pH 7.4), 0.6 ml of microsomes, 0.1 ml ethanol or each inhibitor in 0.1 ml ethanol and 63 nmoles of phylloquinone epoxide in 0.01 ml of 2% (v/v) Emulgen 911. To learn the influence of cytochrome P-450 and/or the presence of NADPH, another assay system was used, in which mixtures were also 2 ml and contained 0.05 M phosphate buffer (pH 7.4), 0.6 ml microsomes, 1.0 mM NADP, 10 mM glucose-6-phosphate, 3.0 units of glucose-6-phosphate dehydrogenase, 7.5 mM magnesium chloride, 0.1 ml ethanol or inhibitors in 0.1 ml ethanol and 63 nmoles phylloquinone epoxide. Both mixtures were incubated at 25° for 5 min and 0.04 ml of dithiothreitol (100 mM) was added with mixing. After 5 additional min at 25°, reaction was terminated with isopropanol/hexane and concentrations of vitamin metabolites were determined by HPLC using a Varian VISTA 54 liquid chromatograph system. Separation was achieved on a Varian MCH-5 (ODS) analytical column (30 cm × 4 mm id) at 1 ml/min with methanol/water (200:5.13 v/v). Retention times of phylloquinone epoxide and phylloquinone were 8.5 and 12.3 min, respectively. External standard quantitation was based on the integrated absorbance at 254 nm. Many peaks containing that of large inhibitor appeared near the peak of vitamin K_1 hydroquinone. Therefore, only a phylloquinone peak was accepted as a product by reductase.

Chemicals. BHT and α-tocopherolquinone were purchase from Tokyo Kasei Kogyo Co. (Tokyo) and ICN Nutritional Biochemicals (Cleveland, OH). Salicylic acid and phylloquinone were from Wako Pure Chem. Ind. (Osaka). BHT quinone methide was synthesized [17, 3]. Phylloquinone epoxide and vitamin K_1 hydroquinone were also synthesized [18–20]. β-NADP was from Oriental Yeast Co. (Tokyo) and glucose-6-phosphate, glucose-6-phosphate dehydrogenase and dithiothreitol (DTT) were from Sigma Chem. Co. (St. Louis, MO).

Results and discussion

The effects of BHT and BHT quinone methide on phylloquinone epoxide reductase activity are shown in Table 1. BHT quinone methide inhibited this activity but BHT did not. The inhibition by BHT quinone methide was also observed when DTT was added even at a quantity of 100 mM; inhibition curves are shown in Fig. 1. BHT quinone methide could inhibit the reductase both in the presence and absence of NADPH, and the half inhibitory concentrations were 2.5×10^{-4} or 3.1×10^{-4} M, respectively.

No kinetics study could be made of this inhibition, probably because the substrate, enzyme and inhibitor were also hydrophobic. However, this inhibitory effect would not be simply due to the decrease in DTT following the formation of a complex with BHT quinone methide, because high concentrations of DTT did not prevent the inhibition.

As previously reported, BHT quinone methide can also inhibit phylloquinone epoxide-dependent microsomal protein carboxylation [7]. The concentrations of BHT quinone methide which inhibited the reductase and carboxylation coincide with the levels of this substance in the liver of

Table 1. Effect of BHT or BHT quinone methide on phylloquinone-epoxide reductase activities

Condition	Inhibitor (mM)	DTT (mM)	Phylloquinone formed (nmoles/mg protein)
Complete	—	2	12.73
Complete	0.45 BHT	2	15.48
Complete	4.5 BHT	2	14.85
Complete	—	100	17.16
Complete	0.45 BHT	100	16.72
Complete	—	100	12.31
Complete	0.48 BHT-QM	100	0
NADPH−	—	100	7.75
NADPH−	0.48 NHT-QM	100	0

Conditions the same as in Fig. 1. NADPH− indicates that NADPH-generating system was not added.

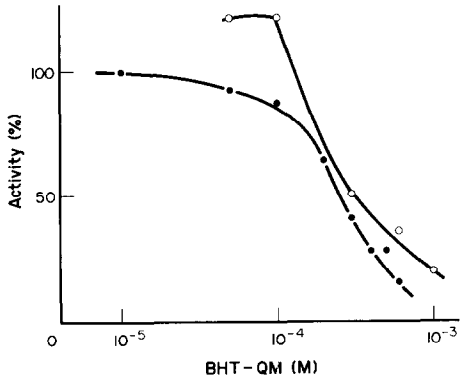

Fig. 1. Inhibition of phylloquinone epoxide reductase by BHT quinone methide. Reaction mixtures (2.0 ml) contained 0.1 M Tris–HCl buffer (pH 7.4); 0.6 ml of microsomes; 0.1 ml BHT quinone methide in ethanol; 63 nmoles of phylloquinone epoxide in 0.01 ml of 2% (v/v) Emulgen 911 (—○—), or mixtures (2.0 ml) contained 0.05 M phosphate buffer (pH 7.4); 0.6 ml microsomes; 1.0 mM NADP; 10 mM glucose-6-phosphate; 3.0 units glucose-6-phosphate dehydrogenase; 7.5 mM magnesium chloride; 63 nmoles phylloquinone epoxide added in 0.01 ml of 2% (v/v) Emulgen 911 and BHT quinone methide in ethanol (an equal volume of ethanol was added in the case of control) (—●—), respectively. After the addition of 2 mM DTT, mixtures were incubated for 5 min at 25° and phylloquinone was analyzed by HPLC. Activity is expressed as % control of the formation of phylloquinone.

haemorrhagic rats given BHT [2]. The results in the present report may reveal the critical mechanism of BHT-induced haemorrhage. That is to say, when BHT is administered to rats, it is metabolised to the corresponding quinone methide in the liver and this active metabolite inhibits phylloquinone epoxide reductase, therefore post-translational modifications of blood coagulation factors II, VII, IX and X cannot take place. The deficiency of those factors in the blood allows bleeding and eventually haemorrhagic death in rats.

The inhibitory effect of salicylic acid on phylloquinone epoxide reductase is shown in Fig. 2. The half inhibition concentrations of salicylic acid are 2.1×10^{-4} or 3.3×10^{-4} M in the presence or absence of NADPH. These results contradict those reported by Hildebrandt and Suttie [20]. Those researchers did not find inhibition by salicylate of this reductase and they explained the mechanism of salicylate-induced bleeding as being due to the inhibition of DT-diaphorase. However, the present results suggest that the inhibition of phylloquinone epoxide reductase may be the most important factor in salicylate-induced haemorrhage. Salicylic acid is thought to be an active metabolite of acetylsalicylic acid in its anticoagulant effect [13], and this compound can inhibit phylloquinone epoxide-dependent protein carboxylation *in vitro* [21]. Inhibition activities of BHT quinone methide and salicylic acid are almost equal. This similarity of *in vitro* results is in agreement with the *in vivo* data of the identical haemorrhagic potencies of acetylsalicylic acid and BHT to rats.

The inhibition of the reductase by α-tocopherolquinone was less strong than those of BHT quinone methide and salicylic acid (Fig. 2). It has been reported that α-toco-

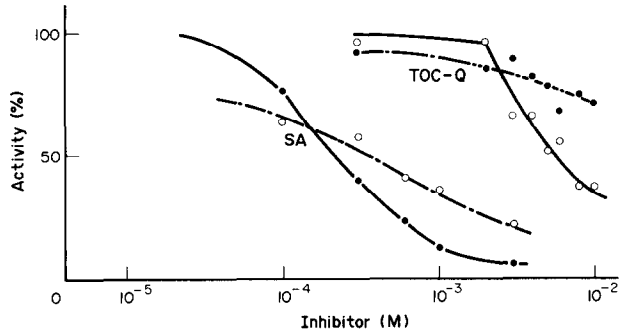

Fig. 2. Inhibition of phylloquinone epoxide reductase by salicylic acid and α-tocopherolquinone. Conditions were the same as in Fig. 1. Salicylic acid and α-tocopherolquinone were added with ethanol. Inhibition curves by salicylic acid with or without NADPH-generating system (—●— or - - -○- - -) and those by α-tocopherolquinone with or without NADPH-generating system (—○— or - - -●- - -).

pherolquinone inhibits vitamin K_1 hydroquinone-dependent protein carboxylation and that this inhibition might be involved in the mechanism of bleeding by hypervitaminosis of α-tocopherol [22].

Generally speaking, NADPH is not necessary for the inhibition of phylloquinone epoxide reductase by BHT quinone methide, salicylic acid or α-tocopherolquinone. Therefore, cytochrome P-450 and/or NADPH-cytochrome P-450 reductase do not participate in those inhibitions.

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Hepatic sulphate conjugation of triiodothyronine (T_3)

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Thyroxine (3,3′,5,5′tetraiodothyronine, T_4) is the main iodothyronine secreted by the thyroid gland. It then undergoes outer ring deiodination in peripheral tissues to produce the active hormone, 3,3′5 triiodothyronine (T_3). Besides deiodination, these iodothyronines undergo conjugation with glucuronic acid or with sulphate [1]. Though these processes have been viewed as independent of each other, sulphation of T_3 as well as T_2 appears to predispose these compounds to deiodination [2]. The sulphate esters of T_3, T_2 (3,3′-diiodothyronine) and T_1 (3′iodothyronine) could be formed *in vitro* from PAP^{35}S by sulphotransferase extracted from rat liver or monkey hepatocarcinoma cells [3]. In this paper, we present evidence that a partially purified extract of rat liver homogenate is capable of sulphate conjugation of T_3 from sodium ^{35}S-sulphate and ATP. The optimum conditions for the formation of T_3 sulphate and kinetics of this sulphate conjugation reaction were examined.

Materials and methods

Chemicals. Sodium ^{35}S-sulphate (specific radioactivity of 621.9 mCi/mmol) was purchased from New England Nuclear Corporation. The other biochemicals were from the usual commercial sources.