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The Crucial Antioxidant Action of Schisandrin B in Protecting Against Carbon Tetrachloride Hepatotoxicity in Mice: A Comparative Study with Butylated Hydroxytoluene

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ABSTRACT. A comparison between the effects of schisandrin B (Sch B) and butylated hydroxytoluene (BHT) treatments on hepatic antioxidant status was made to identify the critical antioxidant action of Sch B involved in hepatoprotection in mice. Whereas Sch B treatment (3 mmol/kg/day × 3, p.o.) increased the hepatic mitochondrial-reduced glutathione (GSH) level, BHT treatment at the same dosage regimen decreased it. However, both Sch B and BHT increased, albeit to a different extent, the activity of mitochondrial glutathione reductase. The differential effect of Sch B and BHT treatment on hepatic mitochondrial glutathione status became more apparent after carbon tetrachloride (CCl_4) challenge. Pretreatment with Sch B could sustain the hepatic mitochondrial GSH level in CCl_4-intoxicated mice and protect against CCl_4 hepatotoxicity. BHT pretreatment did not produce any protective effect on CCl_4-induced GSH depletion in mitochondrion and hepatocellular damage. Although both Sch B and BHT treatments increased hepatic ascorbic acid (VC) level in control animals, only Sch B pretreatment sustained a high hepatic VC level in CCl_4-intoxicated mice. Moreover, Sch B pretreatment prevented the CCl_4-induced decrease in the hepatic α-tocopherol (VE) level. However, Sch B inhibited NADPH oxidation in mouse liver microsomes incubated with CCl_4 *in vitro*, whereas BHT stimulated this oxidation. The ensemble of results suggests that the ability to sustain the hepatic mitochondrial GSH level and the hepatic VC and VE levels may represent the crucial antioxidant action of Sch B in protection against CCl_4 hepatotoxicity. The possible inhibition of CCl_4 metabolism by Sch B may also contribute to its hepatoprotective action. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 52;11:1687–1693, 1996.

KEY WORDS. *Schisandra chinensis*; schisandrin B; butylated hydroxytoluene; glutathione; ascorbic acid; α-tocopherol

FS,† the fruit of *Schisandra chinensis*, has been used in traditional Chinese medicine as a tonic and sedative for centuries. In recent years, it has been clinically used in the treatment of viral and chemical hepatitis [1]. Previous studies in our laboratory have shown that pretreatment with a lignan-enriched extract of FS protected against CCl_4 hepatotoxicity in rats [2]. Hepatoprotection was associated with an enhanced hepatic glutathione status, as reflected by an increase in the GSH level and a decrease in susceptibility of GSH to *in vitro* peroxide-induced depletion [3]. Sch B, a lignoid compound isolated from FS (see Fig. 1a), also protected against CCl_4 hepatotoxicity in mice, presumably by enhancing the hepatic glutathione antioxidant system through increasing the activities of GRD (EC 1.6.4.2), glutathione S-transferases (EC 2.5.1.18) and the hepatic GSH level [3]. CCl_4 is mainly metabolized in the liver by the cytochrome P450 system. The metabolism of CCl_4 produces trichloromethyl radicals and other oxidant species that can inactivate cellular macromolecules and initiate lipid peroxidation reactions [4, 5]. In this regard, the hepatoprotective effect of Sch B on CCl_4-induced toxicity has been attributed to the inhibition of binding of CCl_4 metabolites to liver microsomal lipids and CCl_4-induced lipid peroxidation [6]. Whereas BHT, a synthetic phenolic antioxidant (see Fig. 1b), produced a similar effect on the activity of hepatic glutathione antioxidant enzymes such as Sch B [3, 7], pretreatment with BHT did not protect against CCl_4-induced hepatocellular damage in mice (unpublished data). Given the differential action of Sch B and BHT toward CCl_4 hepatotoxicity, a comparison between their effect on hepatic antioxidant status may help to identify the critical factor(s) involved in the hepatoprotective action of Sch B. In the present study, we examined the effect of Sch

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† *Abbreviations*: ALT, alanine aminotransferase; BHT, butylated hydroxytoluene; CCl_4, carbon tetrachloride; FS, fructus Schisandrae; GRD, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde; Sch B, schisandrin B; Tris, Tris(hydroxymethyl) aminomethane; VC, ascorbic acid; VE, α-tocopherol.
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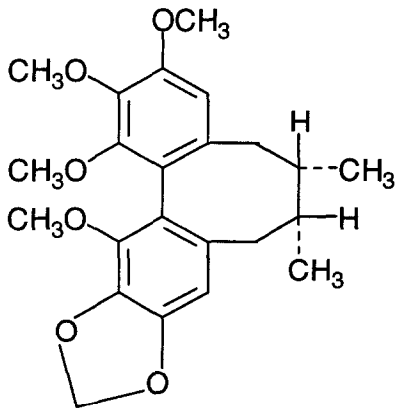
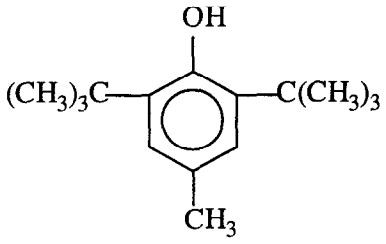


(a)



(b)

FIG. 1. Structures of (a) Sch B and (b) BHT.

B and BHT treatment on hepatic antioxidant status in control and CCl_4-intoxicated mice. The experimental results suggest that the ability to sustain the hepatic mitochondrial GSH level and hepatic VC and VE levels may be a critical factor in the hepatoprotective action of Sch B against CCl_4 toxicity.

MATERIALS AND METHODS

Chemicals

GSH, GSSG, VC and VE were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Solvents used for high-performance liquid chromatography were of HPLC grade; they were filtered and degassed prior to use. Dried fruits of *Schisandra chinensis* were imported from China. Sch B was purified from the petroleum ether extract of *Schisandra chinensis* as described in [3].

Animal Treatment

Female Balb/c mice (24–26 g) were maintained on a 12-hr light/dark cycle at 22°C and allowed food and water *ad libitum*. Animals were randomly assigned to groups of 10 individuals. In the pretreatment groups, animals were treated intragastrically with Sch B or BHT at a daily dose of 3 mmol/kg for 3 days. Twenty-four hours after the last dosing, animals were administered an oral dose of CCl_4 (1%, v/v, in olive oil) at 1.0 mmol/kg. Control animals were given the vehicle (i.e. olive oil, 1 mL/kg). Twenty-four hours after intoxication, heparinized blood samples were drawn from ether-anesthetized mice by cardiac puncture, and the animals were killed by cardiac excision thereafter.

Sample Preparation

Plasma samples were obtained by centrifuging the whole blood at 2000*g* at 4°C. Hepatic tissue samples were excised and rinsed with ice-cold homogenizing buffer (50 mM Tris, 0.1 mM EDTA, pH 7.6). Tissue homogenate was prepared by homogenizing 1 g of hepatic tissue sample in 10 mL ice-cold homogenizing buffer with two 10-sec bursts of a tissue disintegrator (Ika Ultra Turax T25) at 135,000 rpm. Hepatic mitochondrial fraction was prepared by differential centrifugation in isotonic buffer (0.25 mM sucrose, 0.1 mM EDTA, 5 mM Tris, pH 7.4) as described [8].

Biochemical Analysis

Plasma ALT (EC 2.6.1.2) activity was measured by using an assay kit from Sigma Chemical Co. GRD activity was determined as described by Godin and Garnett [9].

GSH and GSSG levels were measured by an HPLC method modified from Reed *et al.* [10]. Samples of hepatic homogenate or mitochondrial fraction were derivatized with iodoacetic acid and 1-fluoro-2,4-dinitrobenzene. A commercially available μ-Bondapak NH_2 C-18 column (200 mm × 3.9 mm I.D., Waters) was used. Solvent A consisted of methanol-water (4:1, v/v) and solvent B was obtained by mixing 109 g anhydrous sodium acetate with 210 mL Milli-Q water, 150 mL acetic acid and 640 mL methanol. An aliquot (20 μL) of the samples was injected through a Waters 717 Autosampler maintained at 4°C. The gradient for HPLC analysis started with a 10-min isocratic period of 20% solvent B at a flow rate of 1 mL/min. Solvent B was then increased linearly to 60% over the next 10 min. After another 5-min isocratic period, the proportion of solvent B was reduced to 20% in 1 min. The separation was monitored by a Waters 996 Photodiode Array Detector and the eluted peaks were extracted at 355 nm. The detection limit for GSH and GSSG was 1 μM.

Plasma and hepatic VC levels were determined by HPLC according to the method of Liau *et al.* [11], with minor modifications. Briefly, 100 μL of plasma or hepatic homogenate were deproteinized by incubating with 100 μL mobile phase (20 mM ammonium dihydrogenphosphate, 0.15%, w/v meta-phosphoric acid, pH 2.95), and the mixture was centrifuged at 3000*g* for 10 min. An aliquot (20 μL) of the supernatants was injected as described above. The sample was analyzed by a μ-Bondapak C-18 column (200 mm × 3.9

TABLE 1. Effect of Sch B and BHT pretreatment on CCl_4-induced hepatotoxicity in mice

	Plasma ALT (U/L)	
	Non-CCl_4	CCl_4
CON	13.5 ± 1.0	11200 ± 300†
Sch B	14.4 ± 1.6	26.3 ± 3.5‡
BHT	40.5 ± 5.9†	21300 ± 690†‡

Values given are the mean ± SEM, n = 10. †Significantly different from the non-CCl_4 CON, control; ‡significantly different from the CCl_4-treated CON.

mm I.D., Waters) using the mobile phase maintained at a flow rate of 1 mL/min at room temperature. The separation was monitored by a Waters 996 Photodiode Array Detector and the eluted peaks were extracted at 245 nm.

Plasma and hepatic VE levels were determined by HPLC as described by Sadrzadeh *et al.* [23], with minor modifications. Briefly, VE from plasma or hepatic homogenate was extracted into hexane and dried under a stream of nitrogen. The dried sample was resuspended in methanol and then injected as described above. The sample was analyzed by a μ-Bondapak C-18 column (200 mm × 3.9 mm I.D., Waters) using methanol as the mobile phase maintained at a flow rate of 1 mL/min at room temperature. The eluant was monitored by a Waters 474 Scanning Fluorescence Detector set at excitation and emission wavelengths of 292 nm and 330 nm, respectively. All chromatographic data were recorded and analyzed by computer software (Millennium 2010 Chromatography Manager, Waters).

In Vitro Metabolism of CCl_4 in Mouse Liver Microsomes

Balb/c mice (24–26 g) were pretreated with phenobarbital at a daily dose of 100 mg/kg (i.p.) for 3 consecutive days before the preparation of hepatic microsomes. Measurement of *in vitro* CCl_4 metabolism was performed according to the method modified from Wolf *et al.* [4]. Microsomal incubations used in the determination of lipid peroxidation contained 2 mg/mL microsomal protein, 1 mM NADPH, 10 mM CCl_4 and 0.1 μM Sch B or BHT in 0.1 M Tris-HCl buffer, pH 7.6. The reaction mixtures were incubated at 37°C for 10 min, and MDA formation, an indirect index of lipid peroxidation, was determined using the HPLC method as described in Young and Trimble [13]. NADPH oxidation of the reaction mixture, containing 1 mg/mL microsomal protein, 0.5 mM NADPH, 10 mM CCl_4 and 0.2 μM Sch B or BHT, was monitored at 340 nm under the same incubation conditions for 5 min.

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test to detect intergroup differences. Significant difference was determined when $P < 0.05$.

RESULTS

BHT treatment (3 mmol/kg/day ×3, p.o.) caused a slight but significant increase in plasma ALT activity, an indication of hepatocellular damage (Table 1). Sch B treatment (3 mmol/kg/day ×3, p.o.) did not produce any detectable change. CCl_4 treatment (1.0 mmol/kg, p.o.) caused hepatocellular damage in mice, as shown by a drastic increase in plasma ALT activity. Pretreating mice with Sch B at the same dosage regimen completely prevented CCl_4-induced hepatotoxicity, as indicated by a precipitous drop in plasma ALT activity compared with that of the unpretreated and CCl_4-intoxicated control. The hepatocellular damaging effect of BHT was aggravated after CCl_4 challenge, as indicated by a onefold increase in plasma ALT activity compared with the unpretreated and CCl_4-intoxicated control.

Sch B treatment caused a significant increase in the mitochondrial GSH level and a reciprocal decrease in the GSSG level, with a resultant elevation in the mitochondrial GSH:GSSG ratio, an index of glutathione redox status (Table 2). BHT treatment decreased both mitochondrial GSH and GSSG levels but did not cause significant alteration in the GSH:GSSG ratio. Following CCl_4 intoxication, hepatic mitochondrial glutathione redox status was greatly impaired, as indicated by a drastic decrease in the mitochondrial GSH level and a large increase in the GSSG level. Whereas the favorable mitochondrial GSH:GSSG

TABLE 2. Effect of Sch B and BHT treatment on hepatic mitochondrial glutathione antioxidant status

	GSH (nmol/mg protein)	GSSG (nmol/mg protein)	GSH/GSSG	GRD (mU/mg protein)
Non-CCl_4				
CON	4.59 ± 0.47	0.425 ± 0.031	11.0 ± 1.44	12.5 ± 0.22
Sch B	6.25 ± 0.66†	0.188 ± 0.034†	35.7 ± 3.33†	26.5 ± 0.98†
BHT	3.27 ± 0.23	0.244 ± 0.026†	14.0 ± 1.80	18.4 ± 0.62†
CCl_4				
CON	1.10 ± 0.51†	1.610 ± 0.230†	0.63 ± 0.23†	8.5 ± 0.52†
Sch B	6.69 ± 0.62†‡	0.313 ± 0.060‡	24.3 ± 4.34‡	22.6 ± 0.31†‡
BHT	ND	ND	ND	12.1 ± 0.49‡

Values given are the mean ± SEM, n = 10. †Significantly different from the non-CCl_4 CON; ‡significantly different from the CCl_4-treated CON; ND, not detectable.

ratio was maintained in Sch B-treated mice after CCl_4 challenge, mitochondrial GSH and GSSG levels became undetectable in BHT-pretreated and CCl_4-intoxicated mice. However, both Sch B and BHT treatments increased mitochondrial GRD activity in control and CCl_4-intoxicated mice, with the stimulatory action of Sch B being more potent (Table 2).

Although both Sch B and BHT treatments caused a significant increase in the hepatic VC level, the plasma VC level was significantly decreased in Sch B-treated animals compared with that of the control (Fig. 2). CCl_4 intoxication decreased both hepatic and plasma VC levels to a large extent. Whereas both Sch B- and BHT-treated mice sustained a higher plasma VC level after CCl_4 intoxication, Sch B treatment also prevented the CCl_4-induced decrease in the hepatic VC level. However, this hepatic VC level was drastically depleted in BHT-treated mice after CCl_4 intoxication. Whereas both Sch B and BHT treatments decreased the hepatic VE level in control mice, the plasma VE level was increased in Sch B-treated mice (Fig. 3). There was no detectable change in plasma VE level in BHT-treated animals. CCl_4 intoxication caused significant decreases in both hepatic and plasma VE levels compared

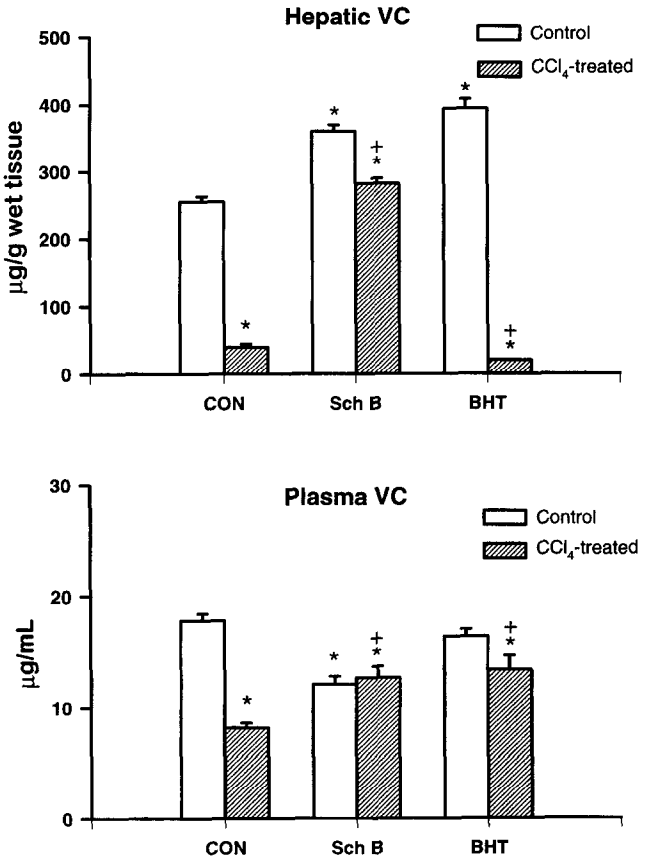


FIG. 2. Effect of Sch B and BHT treatment on hepatic and plasma ascorbic acid levels. Each bar represents the mean ± SEM, n = 10. *Significantly different from the non-CCl_4 CON group; †significantly different from the CCl_4-treated CON group.

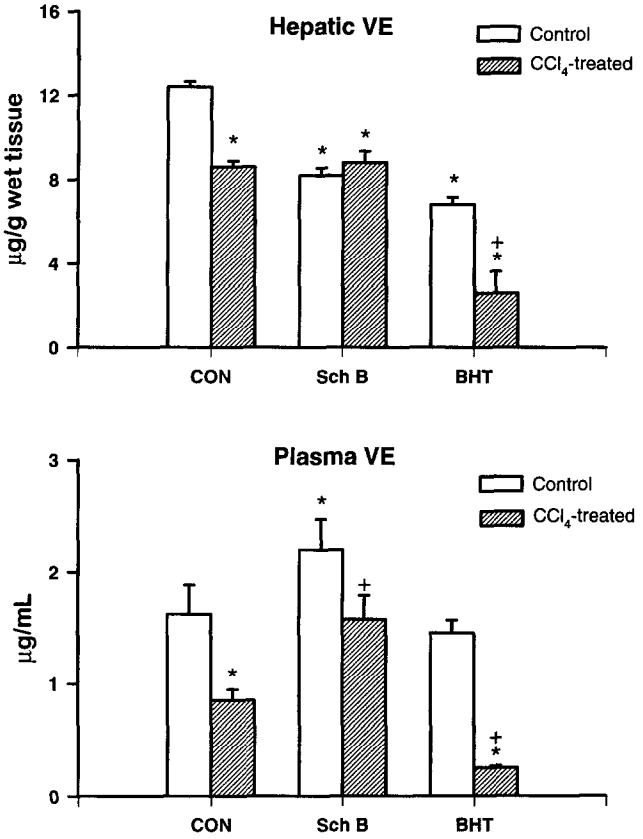


FIG. 3. Effect of Sch B and BHT treatment on hepatic and plasma VE levels. Each bar represents the mean ± SEM, n = 10. *Significantly different from the non-CCl_4 CON group; †significantly different from the CCl_4-treated CON group.

with those of the non-CCl_4 control. The hepatic VE level was not further decreased by CCl_4 intoxication in Sch B-treated mice, but the plasma VE level was reduced back to the value of non-CCl_4 control animals. However, both hepatic and plasma VE levels were largely depleted in BHT-treated animals after CCl_4 intoxication.

As shown in Table 3, the incubation of mouse microsomes with CCl_4 caused a drastic increase in lipid peroxidation *in vitro*, as indicated by a 3.5-fold increase in MDA formation and a 17% increase in the rate of NADPH oxidation. CCl_4-induced lipid peroxidation in mouse liver

TABLE 3. Effect of Sch B and BHT on CCl_4-induced lipid peroxidation and NADPH oxidation in hepatic microsomes from phenobarbital-treated mice

	MDA (nmol/min/mg)	NADPH Oxidized (nmol/min/mg)
CON	0.087 ± 0.001	16.7 ± 0.41
CCl_4-CON	0.394 ± 0.001	19.6 ± 0.44
CCl_4-Sch B	0.129 ± 0.001†	11.8 ± 0.13†
CCl_4-BHT	0.102 ± 0.002†	35.0 ± 1.1†

Values given are the mean ± SEM, n = 5. †Significantly different from the CCl_4-CON. Sch B and BHT were added at a final concentration of 0.1 µM.

microsomes was inhibited by both Sch B and BHT, as shown by the significant decrease in MDA formation. Under the same experimental conditions, Sch B inhibited NADPH oxidation by 40% (compared to CCl_4-control) in microsomes incubated with CCl_4; the resultant rate of NADPH oxidation was lower than that of control microsomal incubation without CCl_4. However, the incubation of microsomes with BHT in the presence of CCl_4 increased the rate of NADPH oxidation by 78%.

DISCUSSION

BHT is widely used as an antioxidant in processed foods, cosmetics and petroleum products. However, the use of BHT is limited by its toxicity. High doses of BHT have been reported to cause hemorrhagic death and liver damage in rats [14], and pulmonary injury in mice [15]. Our observation of the BHT-induced hepatocellular damage in mice is consistent with these findings. Sch B, when administered at the same dosage regimen as BHT, did not cause any detectable hepatic damage (Table 1). Our finding suggests that Sch B may be a more effective *in vivo* antioxidant than BHT. Consistent with this notion, BHT has been found to possess prooxidant activity [16].

The biochemical mechanism involved in the development of CCl_4 hepatotoxicity has long been investigated: it is now generally believed that the formation of reactive trichloromethyl radicals from CCl_4 metabolism is a crucial factor in the pathogenesis of CCl_4 hepatotoxicity [4]. The findings that CCl_4 toxicity could be ameliorated by pretreatment with inhibitors of CCl_4 metabolism [17] and antioxidants [18] support this notion. In this connection, Sch B has been shown to inhibit CCl_4 metabolism in mouse liver microsomes, as assessed by the measurement of carbon monoxide production and cofactor utilization [19]. This result is consistent with our finding that Sch B inhibited the CCl_4-induced NADPH oxidation in mouse liver microsomes (Table 3). In contrast, BHT was found to increase the rate of NADPH oxidation under the same experimental conditions. Because BHT could also stimulate NADPH oxidation in liver microsomes without CCl_4 (data not shown), the increase in NADPH oxidation was likely due to the metabolism of BHT by NADPH-dependent microsomal enzymes [20]. Although both Sch B and BHT acted as free radical scavengers in the inhibition of lipid peroxidation *in vitro* (Table 3), the ability of Sch B to suppress the cytochrome P-450-mediated metabolism of CCl_4 may also contribute to its hepatoprotective action.

The expression of chemically induced cellular toxicity has been correlated with mitochondrial GSH depletion subsequent to the loss of cytosolic GSH [21]. Because the GSH-mediated reduction of protein thiols in the mitochondrion is critical for cell viability [22], the maintenance of mitochondrial glutathione redox status is therefore important to protect against xenobiotic-induced hepatic damage. In this regard, CCl_4 hepatotoxicity has been shown to be strongly related to mitochondrial functional changes secondary to alterations in mitochondrial thiols and calcium ion concentration [23]. Our results indicate that Sch B treatment enhanced hepatic mitochondrial glutathione redox status by increasing the GSH level and decreasing the GSSG level in liver mitochondria prepared from control or CCl_4-intoxicated mice (Table 2). It has been reported that CCl_4 can undergo reductive metabolism in the mitochondrion [24]. The resultant formation of toxic oxidant species can lead to the accumulation of mitochondrial GSSG arising from the GSH-mediated detoxification reactions. Although Sch B-treated mice maintained a favorable mitochondrial glutathione redox status after CCl_4 intoxication, mitochondrial GSH and GSSG levels were reduced to undetectable levels in BHT-pretreated mice (Table 2). The observation of increased mitochondrial GRD activity in BHT-pretreated and CCl_4-challenged animals indicates that the extremely low levels of mitochondrial GSH and GSSG were not likely due to the nonspecific leakage caused by mechanical disruption of mitochondrial structural integrity during the preparative procedure. Owing to the absence of enzymes necessary for GSH synthesis in mitochondria [25], the compartmentalized pool of mitochondrial GSH has to be sequestered from cytosolic GSH through receptor-mediated transport [26]. The increase in mitochondrial GSH, as shown in Sch B-treated mice (Table 2), may be due to the facilitation of GSH import from the cytosolic compartment to the mitochondrion. Similarly, the BHT-induced depletion in mitochondrial GSH may be related to the decrease in cytosolic GSH caused by BHT metabolism [27], thereby reducing the GSH import to the mitochondrion. Because liver mitochondria cannot export GSSG [28], the decrease in the mitochondrial GSSG level is mainly mediated by the GRD-catalyzed reduction of GSSG back into GSH, which is consistent with our finding that the increase in mitochondrial GRD activity was associated with a corresponding decrease in the mitochondrial GSSG level in both Sch B- and BHT-treated animals (Table 2).

Nonenzymatic antioxidants such as GSH, VC and VE may work synergistically in cellular antioxidant defense [29, 30]. GSH and VC are water-soluble antioxidants that serve as the first line of defense in combating free radicals [31]. In this regard, drastic decreases in hepatic and plasma VC levels were observed in mice after CCl_4 intoxication (Fig. 2). It has been reported that GSH can facilitate the regeneration of VC from its oxidized form [32]. The ability of Sch B treatment to enhance the hepatic VC level may therefore be related to the favorable glutathione redox status induced by Sch B [3]. In contrast, the failure of BHT to maintain hepatic VC level may reflect a shortage of GSH supply for the regenerative process in CCl_4-intoxicated mice. The Sch B-induced depletion of plasma VC in control animals may be attributed to the redistribution of VC among tissues, as suggested by the concomitant increase in the hepatic VC

level. In contrast to the CCl_4-induced drastic depletion of hepatic VC, plasma VC was maintained at a higher level in BHT-pretreated mice than that of the CCl_4 control. The complex interaction between BHT and VC metabolism remains to be elucidated. With regard to the lipid-soluble antioxidant VE, CCl_4 intoxication caused a significant depletion in both hepatic and plasma VE levels in control mice (Fig. 3). GSH might maintain tissue VE level either by direct reduction of α-tocopheroxyl radical to VE or via the reductive action of VC [33]. The ability of Sch B treatment to maintain the hepatic VE level under the oxidative stress condition may therefore be an event secondary to the enhanced hepatic glutathione and ascorbate redox status.

In conclusion, the ability of Sch B, in striking contrast to that of BHT, to sustain the hepatic mitochondrial GSH level and hepatic VC and VE levels may represent the crucial antioxidant action of Sch B in the protection against CCl_4 hepatotoxicity. The possible inhibition of CCl_4 metabolism by Sch B may also contribute to its hepatoprotective action.

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