Cellular Antioxidant Defense by a Ubiquinol-Regenerating System Coupled with Cytosolic NADPH-Dependent Ubiquinone Reductase: Protective Effect against Carbon Tetrachloride-Induced Hepatotoxicity in the Rat

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Received January 22, 1996; accepted May 1, 1996

We found that NADPH-dependent ubiquinone reductase (NADPH-UQ reductase) in rat liver cytosol reduces ubiquinone (UQ) to ubiquinol (UQH₂) in lipid membranes and consequently inhibits lipid peroxidation [Takahashi T., *et al.*, *Biochem. J.*, 309, 883–890 (1995)]. Here we examined whether or not this UQH₂-regenerating system functions as a cellular antioxidant defense in animals. Rats were given UQ-10 for 2 weeks, and were then exposed to carbon tetrachloride (CCl₄). The UQ-10 supplement increased only in the NADPH-UQ reductase and the UQH₂-10 pool of rat liver without any appreciable change in the levels of other antioxidant factors. On the other hand, CCl₄ markedly increased plasma aspartate aminotransferase and alanine aminotransferase, liver weight and thiobarbituric acid reacting substances formation, which are indicators of CCl₄-hepatitis, and it decreased the liver levels of L-ascorbic acid, reduced form of glutathione (GSH), α -tocopherol, NADPH-UQ reductase and glutathione S-transferase. However, all the above indicators of CCl₄-induced hepatitis were significantly improved in rats given UQ-10. Furthermore, α -tocopherol, but neither L-ascorbic acid nor GSH, was significantly saved. UQ-10 supplement also was recovered glutathione S-transferase and NADPH-UQ reductase activities slightly. These results indicated that UQ-10 given to rats increased the cellular UQH₂-10 pool and cytosolic NADPH-UQ reductase activity in their livers, resulting in the inhibition of lipid peroxidation in the biomembranes, and consequently protected the rats from the CCl₄-hepatotoxicity.

Key words carbon tetrachloride; hepatotoxicity; NADPH ubiquinone reductase; lipid peroxidation; ubiquinol

It is known that ubiquinone (UQ) is synthesized by an enzymatic pathway located in mitochondria^{1,2)} and that it functions as an essential electron carrier in the respiratory chain located in the same organelle.³⁾ However, UQ is also found in various biomembranes and plasma lipoproteins, and some biomembranes such as microsomes and Golgi apparatus can synthesize UQ by means of their own enzymes.⁴⁾ Nevertheless, the biochemical role of UQ in these organelles has not been established. Some UQ in these sites is always present as ubiquinol (UQH₂), the reduced form of UQ. In our study of rats,⁵⁾ UQH₂ accounted for 70–80% of the sum of UQ and UQH₂ (tUQ) in the liver and plasma and 10–30% in other tissues. Åberg *et al.*⁶⁾ have obtained much greater ratios of UQH₂/tUQ in rat and human tissues. The antioxidant properties of UQH₂ have received much attention⁷⁾ as these may constitute its physiological role in biomembranes.

The antioxidant effect of UQH₂ in biological systems was first pointed out by Mellors and Tappel⁸⁾ in 1966, who found that UQH₂-6 inhibited the light-induced peroxidation of mitochondrial lipids. Since then, much evidence on the antioxidant action of UQH₂ *in vitro* and *in vivo* has been accumulated. For example, Frei *et al.*⁹⁾ and Yamamoto *et al.*¹⁰⁾ have shown that UQH₂-10, but not UQ-10, is about as effective in scavenging free radicals chemically generated within liposomal membranes as α -tocopherol. Furthermore, Stocker *et al.*¹¹⁾ and Suarna *et al.*¹²⁾ have shown that UQH₂-10 protects human plasma low density lipoprotein more efficiently against lipid peroxidation than α -tocopherol.

In animals, however, UQ administration appears to prevent lipid peroxidation. For example, Leibovitz *et al.*¹³⁾ have shown that the lipid peroxidation induced by *tert*-butyl hydroperoxide in tissue slices from rats was depressed by feeding the rats on diets fortified with UQ-10 before the study. Marubayashi *et al.*¹⁴⁾ reported that in hepatic ischemia and subsequent reperfusion, the levels of the reduced form of glutathione (GSH) were not decreased in rats given UQ-10 injections. Yoshikawa *et al.*¹⁵⁾ and Beyer¹⁶⁾ have also shown that UQ-10 inhibits subsequent carbon tetrachloride (CCl₄)-stimulated lipid peroxidation in rats. Sugino *et al.*¹⁷⁾ have reported that if UQ-10 is injected immediately after the administration of a bacterial endotoxin to mice, it is reduced to UQH₂-10 *in vivo*, and also that it suppressed hepatic damage by toxin-induced lipid peroxidation. Thus, externally administered UQ appears to be converted to UQH₂ in animals and then to act as an antioxidant against lipid peroxidation, as seen in most *in vitro* experimental systems. In fact, UQ supplements given to animals^{18,19)} increases the levels of UQH₂ as well as UQ in their serum and tissues. However, the enzyme responsible for the reduction of UQ to UQH₂ in animal tissues has not been identified, though it is known that UQ is reduced by mitochondrial respiratory enzymes such as NADH dehydrogenase and succinate dehydrogenase, and perhaps by some other reductases such as DT-diaphorase.⁷⁾

We found that the cytosol fraction from rat tissues contained NADPH-dependent UQ reductase (NADPH-UQ reductase) activity^{20–22)} which prefers UQ homologues having a long isoprenoid side-chain as a sub-

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strate,²⁰⁾ in contrast to DT-diaphorase [NAD(P)H: (quinone-acceptor) oxidoreductase, EC 1.6.99.2], and NADPH-UQ reductase was neither inhibited nor stimulated by inhibitors of mitochondrial electron transport such as rotenone and antimycin A.^{20,21)} Furthermore, the enzyme reduces not only UQ in homogenous solution but also UQ in liposomes and microsomes, and subsequently inhibits lipid peroxidation in these membranes.²¹⁾ Thus, we suspected that this cytosolic NADPH-UQ reductase is the enzyme responsible for the reduction of UQ to UQH₂ in biomembranes adjacent to the cytosol. To confirm this notion, we investigated the effects of an expanded cellular UQ pool, prepared after prolonged UQ-10 supplementation to rats, upon cytosolic NADPH-UQ reductase and its effect as a cellular antioxidant defense in their livers. This paper describes that supplementing rats with UQ-10 enhanced the levels of both UQH₂-10 and cytosolic NADPH-UQ reductase in their livers without any change in other antioxidant factors, and thus increased the resistance to CCl₄-induced hepatitis.

MATERIALS AND METHODS

Chemicals and Animals UQ-9 and UQ-10 were donated by Eisai Co. (Tokyo, Japan). UQH₂-9 and UQH₂-10 were prepared by reducing the corresponding UQ homologues with 0.25% sodium borohydride in ethanol. All other chemicals were of the highest grade commercially available. Specific pathogen-free, male Wistar rats (8 weeks old, 170–180 g body weight) were purchased from SLC Co. (Shizuoka, Japan).

UQ-10 and CCl₄ Administration The rats were conditioned by feeding them Lavo MR Stock, a commercial feed (SLC Co.), for a few days after purchase, and they were then divided into UQ- and control groups. The UQ-group (UQ-rats) was intraperitoneally injected with 2 mg of UQ-10 (2 ml of 0.9% NaCl solution containing 1 mg UQ-10 solubilized with 2% HCO-60)/kg body weight daily for 14 d, and the control group (control rats) was sham-treated with the vehicle (0.9% NaCl solution containing 2% HCO-60 only) for the same period. The controls and UQ-groups were further divided into 2, each intraperitoneally injected with either 2 ml of CCl₄/kg body weight or with 2 ml of isotonic saline/kg body weight at 15 h before celiotomy to obtain the livers. The rats were fasted (water was given *ad libitum*) at 21 ± 1°C under air with 55 ± 5% relative humidity from the time of CCl₄ injection until the celiotomy. Each animal was anesthetized with diethylether, injected with 0.2 ml of heparin (1000 U/ml) through the femoral vein, and dissected. First, blood was drawn from the abdominal aorta into a heparinized vacutainer. Next, the liver was perfused *in situ* with 20 ml of chilled isotonic saline through the hepatic portal vein to the inferior *vena cava*, then it was removed for analysis.

Cell Fractionation Intracellular fractions of each rat liver were prepared as described.⁵⁾ In brief, fractions of nuclei, mitochondria, crude lysosomes (containing peroxisomes), crude microsomes (containing Golgi apparatus) and cytosol were prepared from the rat liver homogenate by differential centrifugation at 600 × *g* for 10 min,

6500 × *g* for 20 min, 10000 × *g* for 15 min and 105000 × *g* for 60 min, respectively. The purity of the intracellular fractions was determined by measuring their marker enzyme activities.⁵⁾

Determination of Low Molecular Weight Antioxidants

The amounts of UQH₂-9, UQH₂-10, UQ-9 and UQ-10 were determined by HPLC with electron capture detection (ECD) as described by Okamoto *et al.*²³⁾ L-Ascorbic acid was determined by a modification of Coustard and Sudraud's method,²⁴⁾ with a mixture of 5 mM cetyltrimethyl-ammonium bromide and 0.1 mM KH₂PO₄ in H₂O-methanol (95:5, v/v) as the mobile phase and ECD (Jasco 840-EC) at +0.6 V vs. Ag/AgCl as the detector. The contents of reduced and oxidized (GSSG) forms of glutathione in rat liver homogenates were determined as described by Hissin and Hilf.²⁵⁾ The level of α -tocopherol was determined according to Tamai *et al.*²⁶⁾

Enzyme Assays NADPH-UQ reductase activity was determined by measuring the amount of UQH₂-10 formed in the presence of NADPH as described.²¹⁾ Catalase (EC 1.11.1.6), glutathione S-transferase (EC 2.5.1.18), glutathione reductase (EC 1.6.4.2) and DT-diaphorase were assayed according to Aebi,²⁷⁾ Paglia and Valentina,²⁸⁾ Habig and Jakoby,²⁹⁾ Carlberg and Mannorvik³⁰⁾ and Ernster,³¹⁾ respectively. Superoxide dismutase (SOD, EC 1.15.1.1) [Cu²⁺ and Zn²⁺-containing SOD (Cu²⁺, Zn²⁺-SOD) and Mn²⁺-containing SOD (Mn²⁺-SOD)] activities were determined according to Oyanagui³²⁾ and expressed as nitrite units (NU).³²⁾ Aspartate aminotransferase (GOT, EC 2.6.1.1) and alanine aminotransferase (GPT, EC 2.6.1.2) in rat plasma were assayed using GOT-UV and GPT-UV assay kits (Wako, Osaka, Japan), respectively. The specific activities of plasma GOT and GPT were expressed in Karmen units (KU).³³⁾ One KU was defined as a decrease in absorbance of 0.001 at 340 nm per min per ml plasma.

Determination of Thiobarbituric Acid Reacting Substances (TBARS)

TBARS were determined according to Ohkawa *et al.*³⁴⁾ Twenty milligrams of protein from the intracellular fractions was suspended in 1 ml of 1.15% KCl-50 mM Tris-HCl buffer (pH 7.4). The assay mixture consisted of 50 μ l of the suspension, 0.1 ml of 8.1% SDS, 0.75 μ l of 7.5% acetic acid and 0.75 ml of 0.8% 2-thiobarbituric acid, and the final volume was adjusted to 2 ml with distilled water. The mixture was boiled for 1 h, then quickly cooled in an ice-cold bath. Thereafter, 0.5 ml of distilled water was added, then the red products of the TBARS were extracted by mechanical reciprocal shaking at 80 rpm for 10 min with 2.5 ml of a mixture of *n*-butanol and pyridine (15:1, v/v). The upper layer of *n*-butanol was separated from the mixture by a centrifugation of 1500 × *g* for 10 min and the absorbance was measured at a wavelength of 532 nm. The standard was 1,1,3,3-tetraethoxypropane in the TBARS assay.

Protein Content Protein content was determined as described by Lowry *et al.*³⁵⁾

RESULTS

Effects of UQ-10 Supplementation and CCl₄ upon Rats

UQ- and control rats were exposed to either CCl₄ or saline

Table 1. Effect of CCl₄ on Plasma GOT and GPT Levels and the Liver Weight of Rats

Groups	Treatment	Rats (n)	Plasma GOT (KU)	Plasma GPT (KU)	Liver weight (g)
Control rats	Saline	4	10.0 ± 2.0	4.8 ± 1.0	5.43 ± 0.35
	CCl ₄	3	7080 ± 1740 ^d	4410 ± 840 ^d	7.59 ± 0.37 ^d
UQ-rats	Saline	4	9.3 ± 2.4	4.5 ± 1.2	5.40 ± 0.19
	CCl ₄	4	3200 ± 530 ^{b,d}	1540 ± 250 ^{a,d}	6.42 ± 0.61 ^{a,c}

Control- and UQ-rats were given an intraperitoneal injection of 2 ml/kg body weight of UQ-10 (1 mg UQ-10 and 2% HCO-60 in saline) and the same volume of the vehicle (2% HCO-60 in saline) daily for 14 d, respectively. Two milliliters of CCl₄/kg body weight was injected intraperitoneally to the rats at 15 h before the dissection for drawing blood and obtaining the liver. To the paired controls, 2 ml isotonic saline/kg body weight was injected in the same manner as CCl₄. The values are the means ± S.D. of the number of rats indicated. *a, b*) Significant differences at levels of $p < 0.05$ and $p < 0.01$ (Student's unpaired *t*-test), respectively as compared with the CCl₄-treated control rats. *c, d*) Significant differences at levels of $p < 0.05$ and $p < 0.001$ (Student's unpaired *t*-test), respectively, as compared with the same group of rats given isotonic saline.

Fig. 1. Effects of a UQ-10 Supplement and CCl₄ Treatment on Lipid Peroxidation in Rats

TBARS formation in liver intracellular fractions prepared from the control- and UQ-rats treated with either saline or CCl₄ were measured as described in the text. Open bars, control rats; closed bars, UQ-rats. The values are the means ± S.D. of 6–7 rats. Statistical significances (*p*) between either saline and CCl₄ or the control and UQ-rats were determined using the Student's unpaired *t*-test.

as described in Materials and Methods. The UQ-10 supplementation daily for 14 d did not appreciably affect the body and liver weights of the rats. The body weight at the onset and the body and liver weights at the end of UQ-10 supplementation were 175 ± 4 , 200 ± 9 and 5.40 ± 0.26 g, respectively, and those of the control rats were 175 ± 6 , 201 ± 11 and 5.42 ± 0.61 g, respectively.

The effect of CCl₄ on plasma GOT and GPT activities and the liver weights of the control- and UQ-rats is shown in Table 1. CCl₄ caused marked increases in plasma GOT and GPT activities and wet liver weights, which are indicators of CCl₄-induced hepatitis.³⁶⁾ The elevations of these three indicators in the UQ-rats were, however, significantly smaller than those in the control rats.

The effect of CCl₄ on TBARS generation in rat livers, an indicator of lipid peroxidation, is shown in Fig. 1. Among the subcellular fractions from the control rat liver, CCl₄-induced TBARS were prominent in microsomes, where cytochrome P450 monooxygenases may be involved in the generation of the CCl₄-derived radicals,³⁶⁾ but they were absent in the mitochondria. On the other hand, CCl₄-induced TBARS were not found in the UQ-rats. The low level of TBARS in mitochondria, which was independent of the UQ supplement, may be due to the respiratory chain being located in the inner membrane, since an antioxidant role of the chain has been suggested by Takeshige *et al.*³⁷⁾ These findings verified that the CCl₄

treatment caused CCl₄-induced hepatitis in rats and that a prior supplement of UQ-10 protected the rats to a great extent from the CCl₄-hepatitis.

Effects of UQ-10 Supplement and CCl₄ upon Various Antioxidant Factors in Rat Livers The levels of various antioxidant factors in liver homogenates from the control- and UQ-rats treated with CCl₄ and saline are summarized in Table 2. The UQ-10 supplement affected neither the levels of the low molecular weight antioxidants nor the related antioxidant enzyme activities in the rat livers, except for the UQ-10 content (see Tables 3 and 4). On the other hand, CCl₄ remarkably reduced the levels of GSH, L-ascorbic acid and α -tocopherol, as well as glutathione S-transferase and NADPH-UQ reductase in the livers of both the control- and UQ-rats. However, the decreases in GSH and L-ascorbic acid caused by CCl₄ did not increase the levels of dehydroascorbic acid and GSSG, respectively. Among the antioxidant in the UQ-rats, only the level of α -tocopherol, which is located in the same hydrophobic domain in the cell as UQH₂, was kept significantly high ($p < 0.05$) in comparison with that in the control rats. The above results suggest that UQH₂-10 is not a very active antioxidant in the soluble fractions of the cell. In addition, glutathione S-transferase of the UQ-rats was not depressed by CCl₄. NADPH-UQ reductase activity was also protected slightly in the UQ-rats, though not to a statistically significant extent.

Table 2. Effect of CCl₄ on the Antioxidant Content as Well as the Antioxidant Enzyme and Quinone Reductase Activities in Control and UQ-10 Supplemented Rat Livers

Antioxidant factors	Control rats		UQ-rats	
	+ Saline (n=4)	+ CCl ₄ (n=3)	+ Saline (n=4)	+ CCl ₄ (n=4)
Low Molecular weight antioxidants ^{a)} :				
GSH	6.76 ± 0.31	3.89 ± 0.36 ^{b)}	6.50 ± 0.85	4.01 ± 0.20 ^{a)}
GSSG	0.29 ± 0.01	0.29 ± 0.03	0.30 ± 0.03	0.29 ± 0.03
Ascorbic acid	1.60 ± 0.19	0.49 ± 0.05 ^{b)}	1.53 ± 0.22	0.51 ± 0.16 ^{b)}
Ascorbic acid + dehydroascorbic acid	1.75 ± 0.11	0.53 ± 0.09 ^{b)}	1.81 ± 0.14	0.53 ± 0.16 ^{b)}
α-Tocopherol	0.22 ± 0.01	0.13 ± 0.01 ^{b)}	0.22 ± 0.02	0.16 ± 0.01 ^{a, i)}
Antioxidant enzymes:				
Mn ²⁺ -SOD ^{b)}	72.8 ± 35.5	75.4 ± 23.0	71.7 ± 29.6	65.5 ± 13.6
Cu ²⁺ , Zn ²⁺ -SOD + Mn ²⁺ -SOD ^{b)}	559 ± 218	558 ± 148	566 ± 162	521 ± 172
Catalase ^{c)}	247 ± 63	243 ± 44	251 ± 9	261 ± 73
Glutathione peroxidase ^{d)}	805 ± 91	792 ± 35	832 ± 34	809 ± 87
Glutathione S-transferase ^{d)}	348 ± 37	270 ± 10 ^{f)}	352 ± 31	324 ± 36 ⁱ⁾
Glutathione reductase ^{d)}	31.0 ± 3.3	28.8 ± 1.1	30.9 ± 2.7	30.2 ± 2.6
Quinone reductases:				
DT-diaphorase ^{d)}	36.0 ± 13.1	37.8 ± 12.2	34.6 ± 11.4	35.3 ± 5.2
NADPH-UQ reductase ^{e)}	269 ± 73	141 ± 10 ^{f)}	323 ± 55	154 ± 19 ^{a)}

Control- and UQ-rats were injected intraperitoneally with 2 ml/kg of either saline or CCl₄ at 15 h before dissection. Immediately after dissection, low molecular weight antioxidant contents and antioxidant enzyme and quinone reductase activities in the liver homogenate were measured as described in the text. The values are the means ± S.D. of the number of rats indicated. ^{a)} nmol/mg protein. ^{b)} NU/mg protein. ^{c)} μmol/min/mg protein. ^{d)} nmol/min/mg protein. ^{e)} pmol/min/mg protein. ^{f-h)} Significant differences at levels of $p < 0.05$, $p < 0.005$ and $p < 0.001$ (Student's unpaired *t*-test), respectively, as compared with saline-treated rats in the same group. ⁱ⁾ Significant differences at the level of $p < 0.05$ (Student's unpaired *t*-test) as compared with control rats given the same treatment.

Table 3. Effect of CCl₄ on Ubiquinone and Ubiquinol Homologue Contents in the Livers of the Sham-Treated Control Rats

Intracellular fractions	UQ-9 contents (ng/mg protein)			UQ-10 contents (ng/mg protein)		
	tUQ-9 ^{a)}	UQH ₂ -9	% ^{b)}	tUQ-10 ^{a)}	UQH ₂ -10	% ^{b)}
Saline-treated control rats (n=4):						
Homogenate	347 ± 35	342 ± 32	97 ± 5	34.9 ± 3.8	30.7 ± 2.4	89 ± 12
Nuclei and cell debris	446 ± 28	356 ± 38	80 ± 9	33.5 ± 5.0	27.3 ± 3.9	80 ± 17
Mitochondria	1620 ± 240	1100 ± 65	64 ± 5	214 ± 25	139 ± 16	65 ± 6
Crude lysosomes	696 ± 75	444 ± 25	64 ± 6	104 ± 8	62 ± 3	60 ± 6
Crude microsomes	139 ± 17	80 ± 11	58 ± 5	25.0 ± 3.5	14.5 ± 3.5	58 ± 9
Cytosol	13.1 ± 2.2	9.2 ± 1.2	71 ± 11	2.6 ± 0.7	1.9 ± 0.1	74 ± 17
CCl ₄ -treated control rats (n=3):						
Homogenate	344 ± 20	171 ± 36 ^{e)}	50 ± 9 ^{f)}	35.1 ± 0.8	16.2 ± 3.8 ^{e)}	46 ± 11 ^{e)}
Nuclei and cell debris	462 ± 36	137 ± 7 ^{f)}	28 ± 7 ^{f)}	40.4 ± 6.8	12.0 ± 1.6 ^{e)}	31 ± 8 ^{d)}
Mitochondria	1620 ± 100	630 ± 110 ^{f)}	39 ± 5 ^{e)}	218 ± 19	83 ± 20 ^{d)}	42 ± 10 ^{c)}
Crude lysosomes	638 ± 32	254 ± 36 ^{f)}	40 ± 5 ^{e)}	103 ± 7	32 ± 8 ^{f)}	31 ± 8 ^{e)}
Crude microsomes	146 ± 32	26 ± 11 ^{e)}	18 ± 5 ^{f)}	26.0 ± 7.9	4.8 ± 3.0 ^{e)}	22 ± 19 ^{c)}
Cytosol	16.6 ± 1.7	6.3 ± 1.0 ^{c)}	39 ± 5 ^{c)}	3.0 ± 0.5	1.1 ± 0.4 ^{c)}	36 ± 11 ^{c)}

Control rats were given an intraperitoneal injection of 2 ml/kg of vehicle (2% HCO-60 in saline) daily for 2 weeks, then treated with 2 ml/kg of either saline or CCl₄ at 15 h before dissection. Immediately after dissection, UQ and UQH₂ homologues in the liver were measured as described in the text. The values are the means ± S.D. of the number of rats indicated. ^{a)} The sum of the oxidized and reduced forms of the UQ homologue. ^{b)} (UQH₂/tUQ) × 100. ^{c-f)} Significant differences at levels of $p < 0.05$, $p < 0.01$, $p < 0.005$ and $p < 0.001$ (Student's unpaired *t*-test), respectively, as compared with the saline-treated control rats.

Effects of UQ-10 Supplementation and CCl₄ on UQ and UQH₂ Homologues The levels of reduced and oxidized forms of UQ-9 and UQ-10 in intracellular fractions of livers from the control- and UQ-rats are listed in Tables 3 and 4, respectively. The levels of UQ-10 and UQH₂-10 in the livers of UQ-rats were several times higher than those of the controls, whereas their redox ratios were in the range of 60 to 90% of tUQ-10 and were similar to those of the control rats. On the other hand, the levels of tUQ-9 and UQH₂-9 in the livers were not influenced by UQ-10. These results indicated that supplementation with UQ-10 expanded only the cellular UQH₂-10 pool in the rat liver, without appreciably affecting the levels of other antioxidant factors.

On the other hand, CCl₄ significantly decreased the amounts of both UQH₂-9 and UQH₂-10 in all intracellular fractions of rat livers, particularly in the microsomes, resulting in an increase in almost equivalent amounts of UQ-9 and UQ-10 relative to the decreased amounts of UQH₂-9 and UQH₂-10. Namely, the liver levels of UQH₂-10 in the liver fractions of the control rats and UQ-rats were decreased from 58–89% to 22–46% of tUQ-10 (Table 3) and from 56–87% to 38–70% (Table 4) by CCl₄ treatment, respectively, and those of UQH₂-9 in the same fractions also decreased similarly, whereas the levels of tUQ-9 and tUQ-10 were not changed. These results indicated that UQH₂ was not degraded, but oxidized to the corresponding UQ homologues, which can

Table 4. Effect of CCl₄ on Ubiquinone and Ubiquinol Homologue Contents in the Livers of UQ-Rats

Intracellular fractions	UQ-9 contents (ng/mg protein)			UQ-10 contents (ng/mg protein)		
	tUQ-9 ^{a)}	UQH ₂ -9	% ^{b)}	tUQ-10 ^{a)}	UQH ₂ -10	% ^{b)}
Saline-treated UQ-rats (n=4):						
Homogenate	328 ± 34	313 ± 36	95 ± 3	155 ± 33 ^{f)}	135 ± 31 ^{f)}	87 ± 3
Nuclei and cell debris	441 ± 68	392 ± 70	89 ± 4	216 ± 29 ^{f)}	182 ± 30 ^{f)}	84 ± 6
Mitochondria	1780 ± 430	1120 ± 225	64 ± 12	1250 ± 430 ^{f)}	830 ± 270 ^{f)}	66 ± 5
Crude lysosomes	704 ± 100	453 ± 53	65 ± 12	610 ± 70 ^{f)}	365 ± 59 ^{f)}	60 ± 4
Crude microsomes	140 ± 22	84 ± 20	60 ± 6	88.3 ± 27.7 ^{f)}	49.9 ± 20.3 ^{f)}	56 ± 8
Cytosol	17.5 ± 6.9	11.5 ± 3.7	68 ± 14	7.4 ± 2.1 ^{f)}	5.6 ± 2.3 ^{f)}	72 ± 13
CCl ₄ -treated UQ-rats (n=4):						
Homogenate	320 ± 95	191 ± 22 ⁱ⁾	69 ± 10 ^{c,i)}	193 ± 70 ^{f)}	124 ± 9 ^{f)}	70 ± 11 ^{c,g)}
Nuclei and cell debris	462 ± 36	174 ± 20 ^{c,j)}	38 ± 7 ^{c,j)}	216 ± 9 ^{f)}	109 ± 23 ^{f,g)}	50 ± 8 ^{c,j)}
Mitochondria	1810 ± 220	900 ± 135 ^{c)}	50 ± 4 ^{c)}	1110 ± 110 ^{f)}	640 ± 100 ^{f)}	57 ± 4 ^{c)}
Crude lysosomes	736 ± 129	403 ± 65 ^{c)}	55 ± 3 ^{c)}	663 ± 115 ^{f)}	352 ± 34 ^{f)}	54 ± 6 ^{d)}
Crude microsomes	148 ± 34	54 ± 16 ^{c)}	39 ± 16 ^{c,g)}	96.9 ± 30.8 ^{f)}	40.5 ± 20.6 ^{f)}	42 ± 17 ^{c)}
Cytosol	16.5 ± 3.3	8.4 ± 1.4	52 ± 11	7.5 ± 1.2 ^{f)}	3.0 ± 0.6 ^{f)}	40 ± 10 ^{h)}

UQ-rats were given an intraperitoneal injection of 2 ml/kg body weight of UQ-10 (1 mg UQ-10 and 2% HCO-60 in saline) daily for 2 weeks, then treated with 2 ml/kg of either saline or CCl₄ at 15 h before dissection. Immediately after dissection, UQ and UQH₂ homologues in the liver were measured as described in the text. The values are the means ± S.D. of the number of rats indicated. *a)* The sum of the oxidized and reduced forms of the UQ homologue. *b)* (UQH₂/tUQ) × 100. *c–f)* Significant differences at levels of *p* < 0.05, *p* < 0.01, *p* < 0.005 and *p* < 0.001 (Student's unpaired *t*-test), respectively, as compared with the sham-treated control rats (in Table 3). *g–j)* Significant differences at levels of *p* < 0.05, *p* < 0.01, *p* < 0.005 and *p* < 0.001 (Student's unpaired *t*-test), respectively, as compared with saline-treated UQ-rats.

Fig. 2. Effect of CCl₄ on α -Tocopherol Contents in Control- and UQ-Rat Livers

The α -tocopherol contents in liver intracellular fractions prepared from the control- and UQ-rats treated with either saline or CCl₄ were measured as described in the text. Open bars, control rats; closed bars, UQ-rats. The values are the means ± S.D. of 3–4 rats. Statistical significances (*p*) between either saline and CCl₄ or the control and UQ-rats were determined using the Student's unpaired *t*-test.

be regenerated by NADPH-UQ reductase to the UQH₂ forms again as a consequence of a cellular antioxidant action against CCl₄-induced lipid peroxidation. The results also suggested that a cellular UQH₂-regenerating system in rat liver may be activated by the UQ-10 supplement.

Effects of UQ-10 Supplement and CCl₄ on α -Tocopherol
As described in Table 2, the UQ-10 supplement spared α -tocopherol to be consumed by CCl₄-induced lipid peroxidation in the rat liver homogenates. The levels of α -tocopherol in the intracellular fractions from the rat livers are shown in Fig. 2. The levels of α -tocopherol in both the mitochondria and microsomes were significantly decreased by CCl₄, to about half those in the control rats, but they were significantly saved by prior supplementation with UQ-10.

Effects of UQ-10 Supplement and CCl₄ on NADPH-UQ

Reductase NADPH-UQ reductase activity in the whole liver was inhibited remarkably by CCl₄ in UQ-rats, as well as control rats (Table 2). However, analysis of UQH₂ homologues in these livers shown in Tables 3 and 4 suggested that NADPH-UQ reductase could still regenerate enough UQH₂ to inhibit lipid peroxidation by CCl₄. Thus, the effects of UQ-10 supplementation and CCl₄ on NADPH-UQ reductase activity in liver cytosol are compared in Fig. 3. Pre-supplementation of UQ-10 prevented, to some extent, NADPH-UQ reductase in rat liver cytosol from CCl₄-toxicity. The cytosolic NADPH-UQ reductase activity in the UQ-rats after exposure to CCl₄ was one-and-a-half times as high as those from the paired control rats. Accordingly, we speculated that the cytosolic NADPH-UQ reductase of the UQ-rats after exposure to CCl₄ could still reduce UQ-10 to UQH₂-10 one-and-a-half times more efficiently than that of the

Fig. 3. Effect of CCl₄ Treatment on NADPH-UQ Reductase Activity in Control- and UQ-Rat Livers

NADPH-UQ reductase activity in both the liver homogenate and cytosol prepared from the control- and UQ-rats given either saline or CCl₄ were measured as described in the text. Open bars, control rats; closed bars, UQ-rats. The values are the means $\pm$ S.D. of 3–4 rats. Statistical significances (p) between either saline and CCl₄ or the control and UQ-rats were determined using the Student's unpaired t -test.

control rats which received CCl₄ treatment. From Tables 3 and 4, actually, the total amounts of UQH₂, *i.e.*, UQH₂-9+UQH₂-10 in liver homogenates of the control rats given saline and CCl₄ came to 446 ± 42 and 233 ± 48 pmol/mg protein, respectively, and those of the UQ-rats were 551 ± 74 and 415 ± 56 pmol/mg protein, respectively. Accordingly, the apparent consumptions of liver UQH₂ by the CCl₄ treatment were much higher in the control rats (233 pmol/mg protein) than in the UQ-rats (136 pmol/mg protein). Similar results were obtained in cases of intracellular membrane fractions, such as mitochondria, crude lysosomes and crude microsomes, prepared from these rat livers. Probably, the reductions in the UQH₂ consumption in the UQ-rats could be due to the enhancement of the cyclic reduction of liver UQ to UQH₂ (UQH₂ recycling) by enhanced NADPH-UQ reductase activity.

DISCUSSION

CCl₄ is converted to trichloromethyl, then trichloromethylperoxyl radicals by liver microsomal cytochrome P450-dependent monooxygenases.³⁶⁾ These CCl₄-derived radicals induce lipid peroxidation, which damages liver biomembranes, resulting in the degradation of organelles, the release of cytosolic enzymes into blood circulation, as well as swelling and necrosis of the liver. Therefore, CCl₄-induced hepatitis is apparently a convenient model for investigating lipid radical-induced damage and its prevention in animals. First, Kover *et al.*³⁸⁾ found that CCl₄ caused a decrease in the UQ content of rat liver without an appreciable change in the total tocopherol content. Bertelli *et al.*³⁹⁾ found that UQ-10 significantly reduced subsequent CCl₄-induced lesions in the rat liver dose-dependently and that it did not significantly affect the UQ-9 and UQH₂-9 concentrations of the liver. Yoshikawa *et al.*¹⁵⁾ and Beyer¹⁶⁾ have also shown that UQ-10 inhibits the generation of TBARS in rat liver induced by CCl₄ and consequently protects against CCl₄-induced hepatitis.

The results obtained in this study are in line with these findings. UQ-10 given to rats did not significantly affect the liver content of UQ-9 or UQH₂-9, *i.e.*, native UQ homologues in rats, or that of GSH, L-ascorbic acid, α -tocopherol and various antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, DT-diaphorase, *etc.* However, the UQ-10 supplement specifically stimulated NADPH-UQ reductase in the liver cytosol. This finding supports the notion that NADPH-UQ reductase is responsible for the reduction of UQ to UQH₂ in liver. On the other hand, CCl₄ administered to rats dramatically decreased GSH, L-ascorbic acid and α -tocopherol levels and remarkably increased TBARS in the rat livers. Among them, however, only α -tocopherol was saved, apparently by the prior UQ-10 supplement (Table 2 and Fig. 2). The decreases in GSH and L-ascorbic acid in the livers were not accompanied by increases in GSSG or dehydroascorbic acid. Furthermore, neither antioxidant was saved by a prior UQ-10 supplement. GSH reportedly forms chemical adducts with CCl₄-derived radicals.⁴⁰⁾ The results indicated that L-ascorbic acid regenerating systems coupled with L-ascorbic acid reductase or monoascorbic acid reductase or GSH (and a GSH regenerating system coupled with glutathione reductase) did not function well, at least in inhibiting the CCl₄-induced lipid peroxidation. UQH₂ (and UQ) and α -tocopherol are located mainly in the phospholipid bilayer of intracellular structures, whereas GSH and L-ascorbic acid are found in aqueous fractions. Frei *et al.*⁹⁾ and Yamamoto *et al.*¹⁰⁾ have reported that UQH₂-10 spares α -tocopherol, not only by directly inhibiting lipid peroxidation but also probably by regenerating α -tocopherol from an α -tocopheroxyl radical resulting from its antioxidant action, when both UQH₂ and α -tocopherol were present in the same liposomal membranes, whereas UQH₂-10 did not interact with either GSH or L-ascorbic acid. Mukai *et al.*^{41,42)} have shown that UQH₂-10 regenerates α -tocopherol by donating a hydrogen atom of the 1-OH and/or 4-OH group of UQH₂-10 to the tocopheroxyl radical in a homogenous solution by a

stopped-flow kinetic study. The results presented here are in line with their observations. Furthermore, these findings suggest that the UQH₂ regenerating system coupled with cytosolic NADPH-UQ reductase functions mainly in intracellular lipid membranes as an antioxidant defense in concert with α -tocopherol in protecting the tissue from CCl₄-induced lipid peroxidation. In control rats, the level of TBARS induced by CCl₄ was very high in microsomes but negligible in mitochondria. This may be due to the difference between the metabolic functions of microsomes and mitochondria, as well as the fact that CCl₄-derived radicals formed in microsomes have to diffuse into the mitochondrial membranes to cause lipid peroxidation in mitochondria. The mitochondrial respiratory chain efficiently reduces UQ to UQH₂ which then functions as an antioxidant defense against oxidative damage such as that caused by Fe³⁺-ascorbate-induced lipid peroxidation.^{43,44)}

Among the antioxidant enzymes, glutathione S-transferase and NADPH-UQ reductase were susceptible to CCl₄. It is not clear at present whether either of these enzymes have been released outside the cell, like GOT and GPT, or inactivated by the CCl₄. The molecular weights of these enzymes are smaller than those of GOT and GPT, and NADPH-UQ reductase is very labile.^{20,22)} Danni *et al.*⁴⁵⁾ have also found that CCl₄ causes a decrease in the glutathione S-transferase levels in isolated rat hepatocytes.

There are a number of enzymes which may serve as a UQ reductase in animal cells. The microsomal NADH-cytochrome b₅ and NADPH-cytochrome P-450 reductases, DT-diaphorase and NADH dehydrogenases in mitochondrial and plasma membranes have been suggested as candidates responsible for membranous UQH₂ cycling.⁷⁾ As described above, however, the UQ-10 supplement stimulated only cytosolic NADPH-UQ reductase among the liver enzymes investigated, resulting in a partial but definite protection against CCl₄-induced hepatotoxicity. These results support the notion that the NADPH-UQ reductase in the cytosol is the enzyme responsible for the regeneration of UQH₂ from the UQ, resulting from intracellular antioxidant action.

In conclusion, UQ-10 supplementation causes significant increases in the liver levels of cytosolic NADPH-UQ reductase activity, as well as that of UQH₂-10 without any appreciable change in other antioxidants or in the related enzymes, or the body and liver weights of rats. Furthermore, we speculate that this biochemical change is the reason UQ-10 supplementation was beneficial in protecting the rat liver from CCl₄-induced oxidative damage.

Acknowledgements This work was supported in part by a Grant-in-Aid for Science Research (08457617) from the Japanese Ministry of Education, a grant from the Science Research Fund from the Japan Private School Promotion Foundation and a Grant-in-Aid for Health Science Research from Kobe Gakuin University.

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