Reduction of ubiquinone in membrane lipids by rat liver cytosol and its involvement in the cellular defence system against lipid peroxidation

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Rat liver homogenates reduced ubiquinone (UQ)-10 to ubiquinol (UQH₂)-10 in the presence of NADPH rather than NADH. This NADPH-dependent UQ reductase (NADPH-UQ reductase) activity that was not inhibited by antimycin A and rotenone, was located mainly in the cytosol fraction and its activity accounted for 68 % of that of the homogenates. Furthermore, the NADPH-UQ reductase from rat liver cytosol efficiently reduced both UQ-10 incorporated into egg yolk lecithin liposomes, and native UQ-9 residing in rat microsomes, to the respective UQH₂ form in the presence of NADPH. The gross redox ratios of UQH₂-9/(UQ-9 + UQH₂-9) in individual tissues of rat correlated positively with the log of their respective cytosolic NADPH-UQ reductase activities, while the redox ratios in every intracellular fraction from liver were at about the same level, irrespective of NADPH-UQ reductase activities in the

respective fractions. The combined addition of rat liver cytosol and NADPH inhibited to a great extent 2,2'-azobis(2,4-dimethylvaleronitrile)-induced lipid peroxidation of UQ-10-fortified lecithin liposomes and completely inhibited such peroxidation in the liposomes in which UQH₂-10 replaced UQ-10. The NADPH-UQ reductase activity was clearly separated from DT-diaphorase (EC 1.6.99.2) activity by means of Cibacron Blue-immobilized Bio-Gel A-5m chromatography. In conclusion, the NADPH-UQ reductase in cytosol, which is a novel enzyme to our knowledge, was presumed to be responsible for maintaining the steady-state redox levels of intracellular UQ and thereby to act as an endogenous antioxidant in protecting intracellular membranes from lipid peroxidation that is inevitably induced in aerobic metabolism.

INTRODUCTION

Ubiquinone (UQ) is an essential electron carrier in the mitochondrial respiratory chain [1]. However, UQ is found not only in mitochondria, but also in almost all intracellular organelles [2,3]. In addition, UQ can be synthesized *de novo* not only in mitochondria but also in some other intracellular organelles [3,4]. Incidentally, a part, usually a greater part, of UQ is present in biological systems as the reduced form, ubiquinol (UQH₂). For example, Åberg et al. [5] reported that the greater portion of UQ in some rat and human tissues was found as UQH₂. We also observed that a considerable amount of UQ was present as UQH₂ in subcellular fractions from rat tissues [6]. Furthermore, UQ-10 administered orally or injected intraperitoneally into rats was recovered mostly as UQH₂-10 from tissues and blood [7–9]. These findings suggest that UQH₂, or oxidation–reduction cycling of UQ, may also play some important roles in intracellular organelles besides that of electron transport in mitochondrial inner membranes.

On the other hand, as UQH₂ is a hydrogen donor, its antioxidant properties in both model and biological systems have also received much interest since an observation by Mellors and Tappel [10] in 1966 that light-induced peroxidation of mitochondrial lipids was effectively prevented by UQH₂-6. At present there is almost no divergent view about the finding that UQH₂ inhibits to some extent lipid peroxidation in intracellular organelles *in vitro* [11], as well as in plasma lipoproteins [12] and phospholipid liposomes [13]. However, these observations do not support the attractive view that UQH₂ participates as an en-

dogenous antioxidant against lipid peroxidation in biological systems, unless a steady-state level of UQH₂ in the biological systems is maintained *in vivo*. In other words, the UQH₂ consumed with lipid peroxidation must be regenerated continuously and efficiently to act as an effective antioxidant in biological systems. Indeed, some enzymes such as NAD(P)H:(quinone-acceptor) oxidoreductase (DT-diaphorase, EC 1.6.99.2), as well as the mitochondrial NADH dehydrogenase complex (complex I, EC 1.6.99.3) and the succinate dehydrogenase complex (complex II, EC 1.3.99.1) are known to be able to reduce UQ to UQH₂ [14]. However, it has not been established what kind of enzymic systems in the cell are responsible for the regeneration of UQH₂ from UQ, because long-chain UQ homologues are not a favourite substrate for the enzymes known to be capable of reducing quinone compounds, except for mitochondrial respiratory enzymes [15].

We recently found that rat tissues had NADPH-dependent UQ reductase (NADPH-UQ reductase) activity, and that the *K_m* values of the enzyme in liver cytosol were 307 μM for NADH and 19.2 μM for NADPH [16]. This enzyme appeared to be different from DT-diaphorase and mitochondrial respiratory enzymes. In addition, we found that UQH₂ concentrations in various intracellular organelles in the rat liver and other tissues were at levels of 70–80 % and 10–30 % respectively of the total amount of UQ and UQH₂ (tUQ), regardless of the biochemical features of individual organelles [6]. The broad distribution of UQH₂ in intracellular organelles and high NADPH-UQ reductase activities in various tissue cytosols as described above suggest that the cytosolic NADPH-UQ re-

Abbreviations used: AMVN, 2,2'-azobis(2,4-dimethylvaleronitrile); DCPIP, 2,6-dichlorophenol-indophenol; TBARS, thiobarbituric acid-reactive substances; UQ, ubiquinone; UQH₂, ubiquinol; tUQ, total amount of UQ and UQH₂; DT-diaphorase, NAD(P)H:(quinone-acceptor) oxidoreductase.

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ductase is involved in the formation of UQH₂ from UQ in intracellular membranes. Thus, we investigated whether or not cytosolic NADPH-UQ reductase can reduce UQ to UQH₂ in phospholipid membranes in the presence of a proper hydrogen donor and consequently prevent the radical-induced lipid peroxidation of the phospholipid membranes.

EXPERIMENTAL

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 tetrahydroborate in ethanol. All other chemicals, including 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN), were of the highest grade commercially available. Specific pathogen-free, male Wistar rats (8 weeks old, 180–200 g body weight) were purchased from SLC Co. (Shizuoka, Japan) and fed on Lavo MR Stock, a commercial feed (SLC Co.), until use.

Cell fractionation

Intracellular fractions of rat liver were prepared as described previously [6]. In brief, fractions of nuclei, mitochondria, crude lysosomes (containing peroxisomes), crude microsomes (containing Golgi complex) and cytosol were prepared from rat liver homogenates 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 [17]. Subfractions of the inner and outer mitochondrial membranes and matrix were prepared from the mitochondrial pellets described above, using the method of Sottocasa et al. [18]. The subfractions of the Golgi complex, namely, light, intermediate and heavy fractions, were prepared directly from rat liver homogenates by sucrose density gradient centrifugation, as described by Ehrenreich et al. [19]. Smooth and rough endoplasmic reticular fractions were also separated by the method of Ikehara and Pitot [20]. The purity of the intracellular fractions was determined by measuring their marker enzyme activities [6].

Enzyme assays

NADPH-UQ reductase activity (pmol/min per mg of protein) was determined by measuring the amount of UQH₂-10 formed after incubating UQ-10 with tissue homogenates or their intracellular fractions in the presence of NADPH at 37 °C. A 250 μ l volume of the reaction mixture contained 0.5 mM 2-mercaptoethanol (to protect the UQH₂ formed against re-oxidation) and 0.08% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.4), as well as 0.05 mM UQ-10 and enzyme preparations. UQ-10 was dissolved in ethanol to a concentration of 2.5 mM and added to the reaction mixture. The enzyme reaction was started by adding 0.2 mM NADPH, and stopped with 3.5 ml of ethanol/n-hexane (2:5, v/v). Thereafter, UQH₂-10 formed in the reaction mixture was extracted with n-hexane and measured by means of HPLC as described [21]. DT-diaphorase activity was determined by measuring the reduction of 2,6-dichlorophenol-indophenol (DCPIP) by enzyme samples in the presence of NADH as described by Ernster [22]. The enzyme activity in fraction E (see Table 6) was also determined by the reduction of menadione to dihydromenadione. Marker enzyme activities in the intracellular fractions were determined as described [6]. The protein content of the intracellular fractions was determined by the method of Lowry et al. [23].

Preparation of liposomes

A mixture of 5 mM egg yolk lecithin, 2.5 μ M UQ-10 or UQH₂-10 and 1 mM AMVN in chloroform (the last two of which were dissolved in ethanol and then added to the mixture) was prepared and the solvents were evaporated under vacuum leaving a lecithin film. When UQ-10, UQH₂-10 or AMVN was not added to the mixture, the same volume of ethanol was added instead. The lecithin film was suspended in 50 mM Tris-HCl buffer (pH 7.4) to make 10 mM lecithin, and dispersed by a vortex mixer and then by a water-bath type sonicator at ice-cold temperature in a nitrogen atmosphere to form liposomes.

Lipid peroxidation assay

The reaction mixture (1 ml) for lipid peroxidation assays consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM lecithin liposomes, 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol and 1.45 mg of protein of rat liver cytosol (310 pmol UQH₂-10 formed/min per mg of protein of NADPH-UQ reductase activity) and was incubated at 37 °C for 2 h. The peroxidation rates of the liposomes were followed by measuring the amount of conjugated dienes produced, as described by Shimasaki et al. [24] and of thiobarbituric acid-reactive substances (TBARS) by the Buege and Aust method [25].

Reduction of endogenous UQ-9 in microsomes by NADPH-UQ reductase

The reaction mixture (250 μ l) for endogenous UQ-9 reduction consisted of 0.25 M sucrose, 50 mM Tris-HCl buffer (pH 7.4), 0.5 mM 2-mercaptoethanol, 0.25 mg of microsomal proteins and 0.375 mg of cytosolic proteins. The reaction was started by adding 0.2 mM NADPH and was carried out at 37 °C. After 15 min, the reaction was stopped with 3.5 ml of ethanol/n-hexane (2:5 v/v), then the amount of UQH₂-9 formed and tUQ-9 were determined by HPLC as described [21].

Cibacron Blue-immobilized Bio-Gel A-5m chromatography

This chromatographic method has been described by Prochaska [26]. Cibacron Blue-immobilized Bio-Gel A-5m was prepared by incubating 100 ml of 50% (v/v) Bio-Gel A-5m (Bio-Rad) in 2% NaCl (adjusted to pH 10 with Na₂CO₃) with 0.2 g of Cibacron Blue F3GA at 37 °C, followed by the addition of 0.2 g of Cibacron Blue F3GA twice every 24 h. Unbound Cibacron Blue F3GA was removed with distilled water followed by 6 M urea in 0.5 M NaOH. A 10 ml volume of the gel, equilibrated with 0.15 M NaCl, was incubated with 10 ml of rat liver cytosol for 1.5 h at 0 °C, then packed into a column (Pharmacia, XK16/20). Proteins bound to the gel were eluted in turn using a 0.15–3.5 M linear NaCl gradient, followed by 3.5 M NaCl in 5 mM Tris-HCl buffer (pH 7.4), 5 mM Tris-HCl buffer (pH 7.4), 20 mM Tris base (pH 10) in 50% ethylene glycol, and finally 50% ethylene glycol containing 1 mM NADH and 20 mM Tris base (pH 10) at a flow rate of 0.5 ml/min. Proteins eluted from the column were monitored at a wavelength of 280 nm.

Reduction of UQ-10 in liposomes by NADPH-UQ reductase and DT-diaphorase

The reaction mixture consisted of 5 mM liposomes, 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM NADPH, 0.5 mM 2-mercapto-

ethanol and either fraction A or E, both of which were obtained by Cibacron Blue-immobilized Bio-Gel A-5m chromatography (see Table 6). The reaction was started by the addition of fraction A or E and was carried out at 37 °C. At the times indicated, an aliquot of the reaction mixture was removed and the amount of UQH₂-10 formed was determined as described above.

RESULTS

Reduction of UQ by rat liver homogenate

There are some enzymes in cells, which may serve as UQ reductases. For example, the microsomal NADH-cytochrome *b₅* reductase (EC 1.6.2.2), NADPH-cytochrome reductase (EC 1.6.2.4), DT-diaphorase and the NADH dehydrogenases associated with the mitochondrial and plasma membranes may perform this function [27]. Thus, we initially investigated whether or not rat liver homogenates can reduce UQ to UQH₂ in the presence of NADH or NADPH as a hydrogen donor. As listed in Table 1, the homogenates showed higher UQ-10 reducing

activity in the presence of NADPH rather than NADH. The reduction by NADH was partly inhibited by rotenone, an inhibitor of the mitochondrial NADH dehydrogenase complex, and stimulated by antimycin A and NaN₃, inhibitors of mitochondrial ubiquinol-cytochrome *c* reductase (complex III, EC 1.10.2.2) and cytochrome *c* oxidase (complex IV, EC 1.9.3.1) respectively.

On the other hand, the reduction by NADPH was not affected by the inhibitors of mitochondrial electron transport. The reduction by both hydrogen donors of NADH and NADPH was also partly inhibited by dicoumarol, an inhibitor of DT-diaphorase, and highly stimulated by KCN, which is an inhibitor of many kinds of oxidative enzymes [28], such as xanthine oxidase (EC 1.1.3.22) and copper and zinc-containing superoxide dismutase (EC 1.15.1.1). The reason why UQ reduction is considerably enhanced by KCN is obscure, although it seemed to be related to the inhibition of some of the oxidative enzymes mentioned. This effect of KCN was not observed when liver cytosol was used instead of the liver homogenate (results not shown). The above results also suggest that DT-diaphorase in the liver homogenate slightly contributes to the reduction of UQ to UQH₂ in the presence of either NADH or NADPH and that mitochondrial respiratory enzymes do not contribute at all to UQ reduction by the homogenate in the presence of NADPH.

Table 1 Effect of electron donors on UQ-10 reduction by rat liver homogenate

The values are the means $\pm$ S.D. of four experiments. *, †, ‡ Significant differences at levels of $P < 0.01$, $P < 0.005$ and $P < 0.001$ respectively compared with no addition, using Student's unpaired *t*-test.

Addition	UQ-10 reduction (pmol/min per mg of protein)		
	None	NADPH	NADH
None	36 $\pm$ 4	312 $\pm$ 7	147 $\pm$ 1
5 μ M Dicoumarol	22 $\pm$ 4†	263 $\pm$ 23*	121 $\pm$ 4‡
10 μ M Rotenone	35 $\pm$ 7	306 $\pm$ 16	118 $\pm$ 9‡
1 μ M Antimycin A	38 $\pm$ 4	310 $\pm$ 15	167 $\pm$ 10*
10 mM NaN ₃	36 $\pm$ 2	305 $\pm$ 14	177 $\pm$ 11†
10 mM KCN	157 $\pm$ 25‡	409 $\pm$ 13‡	230 $\pm$ 5‡
10 μ M Rotenone + 1 μ M antimycin A	34 $\pm$ 4	308 $\pm$ 26	131 $\pm$ 6†
10 μ M Rotenone + 10 mM NaN ₃	37 $\pm$ 3	308 $\pm$ 15	127 $\pm$ 5‡
10 μ M Rotenone + 10 mM KCN	158 $\pm$ 6‡	401 $\pm$ 24‡	214 $\pm$ 17‡

Distribution of NADPH-UQ reductase activities in rat liver intracellular fractions

We found that a set of intracellular fractions obtained from tissues, such as liver and kidney, had about the same UQH₂/tUQ ratios calculated as UQH₂/tUQ $\times$ 100 [6]. Therefore, the results shown above suggest that the NADPH-UQ reductase in the cytosol [16] might play a central role in maintaining the UQH₂/tUQ ratios in most intracellular organelles. Then, the distribution of NADPH-UQ reductase activity in rat liver intracellular fractions was investigated to compare with their respective UQH₂/tUQ ratios. The results are summarized in Table 2. Among the intracellular fractions, cytosol and crude microsomes had the highest and the second highest levels of NADPH-UQ reductase activities per mg of protein respectively. Moreover, based upon the distribution of marker enzyme activities, we estimated that 67.9 $\pm$ 1.9 % of the total NADPH-UQ

Table 2 NADPH-UQ reductase activities and UQH₂-9/tUQ-9 ratios in rat liver intracellular fractions

The values are the means $\pm$ S.D. of the measurements obtained from three rats. N.D., not determined.

Intracellular fraction	NADPH-UQ reductase activity (pmol/min per mg of protein)	Distribution of NADPH-UQ reductase activity (%)	UQH ₂ -9/tUQ-9 ratio (%)
Nuclei	16.3 $\pm$ 20.9	5.8 $\pm$ 6.1	62 $\pm$ 11
Mitochondria	40.7 $\pm$ 11.6	8.2 $\pm$ 4.0	66 $\pm$ 8
Inner membranes	16.7 $\pm$ 3.2	N.D.	N.D.
Outer membranes	8.3 $\pm$ 5.9	N.D.	N.D.
Matrix	20.4 $\pm$ 14.1	N.D.	N.D.
Crude lysosomes	8.7 $\pm$ 9.0	2.6 $\pm$ 0.5	64 $\pm$ 10
Crude microsomes	105 $\pm$ 64	15.0 $\pm$ 4.3	68 $\pm$ 13
Smooth endoplasmic reticulum	50.8 $\pm$ 49.1	N.D.	N.D.
Rough endoplasmic reticulum	60.1 $\pm$ 9.0	N.D.	N.D.
Golgi complex:			
Light fraction	0	N.D.	N.D.
Intermediate fraction	11.9 $\pm$ 8.9	N.D.	N.D.
Heavy fraction	32.8 $\pm$ 21.9	N.D.	N.D.
Cytosol	312 $\pm$ 36	67.9 $\pm$ 1.9	70 $\pm$ 3

Figure 1 The reduction of UQ-10 in egg yolk lecithin liposomes by rat liver intracellular fractions

The reaction mixture consisted of 5 mM lecithin liposomes, 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol and 5.4 mg of protein from intracellular fractions: nuclei (■), mitochondria (△), crude lysosomes (▲), crude microsomes (○) or cytosol (●) per ml of reaction mixture. The reaction was started by adding NADPH and carried out at 37 °C. At the indicated times, an aliquot of the reaction mixture was removed and the amount of UQH₂-10 formed was measured by HPLC as described in the text. The values are the means $\pm$ S.D. of three experiments.

reductase activity in the rat liver homogenate was present in the cytosolic fraction. On the other hand, the total and specific activities of the enzymes in mitochondria were almost one-eighth those of cytosol. However, the UQH₂-9/tUQ-9 ratios in these fractions were not significantly different.

Reduction of UQ-10 in liposomes to UQH₂-10 by rat liver intracellular organelles

The results described above, that the cytosol fraction from liver homogenates had the highest activity of UQ reduction among the intracellular fractions, suggest that cytosolic NADPH-UQ reductase could be responsible for UQ reduction. Thus, UQ-10 embedded in egg yolk lecithin liposomes was incubated with various intracellular fractions from rat liver to determine whether or not cytosol can reduce UQ-10 in membrane lipids. The results are depicted in Figure 1. Rat liver cytosol (5.4 mg of protein) reduced about 55% of UQ-10 in the liposomes in the presence of NADPH, but the other intracellular fractions (5.4 mg of protein each), namely, nuclei, mitochondria, crude lysosomes and crude microsomes, though they can interact with the liposomes through

random collision, were unable to reduce UQ-10 in the liposomes under these conditions. These results indicate that cytosolic NADPH-UQ reductase can contribute to maintaining the redox state of UQ buried in the membranes of intracellular organelles.

Reduction of endogenous UQ-9 in liver microsomes by cytosolic NADPH-UQ reductase

To confirm the reduction of endogenous UQ in intracellular membranes by cytosolic NADPH-UQ reductase, rat liver microsomes were incubated with rat liver cytosol and NAD(P)H. The cytosol and microsomes were added to the reaction mixture according to their intracellular distribution ratios. The UQH₂-9/tUQ-9 ratio in the liver microsomes was depressed to 26.2% following repeated freeze/thaw cycles at room temperature before use. The results are shown in Table 3. During the 15 min incubation, the UQH₂-9/tUQ-9 ratios in the mixture of microsomes and cytosol quickly increased to 62.9 and 54.6% in the presence of NADPH and NADH respectively. The reaction was not inhibited by either dicoumarol or rotenone. In the absence of cytosol, the UQH₂-9/tUQ-9 ratio in the microsomes was decreased significantly by addition of NADPH, but not by NADH. This seems to be due to the lipid peroxidation induced by the activation of NADPH-cytochrome reductase and/or other NADPH-dependent radical-forming enzymes in the microsomes.

Relationship between UQH₂-9/tUQ-9 ratio and NADPH-UQ reductase activity in rat tissues

To determine whether cytosolic NADPH-UQ reductase is responsible for maintaining UQH₂-9/tUQ-9 ratios in tissues, NADPH-UQ reductase activities in the cytosolic fractions were plotted against the UQH₂-9/tUQ-9 ratios in their respective tissues. The results are shown in Figure 2. The UQH₂-9/tUQ-9 ratios correlated linearly with the log of NADPH-UQ reductase activities. The correlation coefficient and regression formula between both parameters were calculated as $r = 0.899$ ($P < 0.01$) and $Y = 3.95 \times 10^{0.0233X}$ (Y , NADPH-UQ reductase activities in pmol of UQH₂-10 formed/min per mg of protein; X , UQH₂-9/tUQ-9 ratios in %) respectively.

Antioxidant action of UQ-10 in liposomes in the presence of NADPH-UQ reductase

The concept that UQH₂ works as a lipid-soluble antioxidant in biomembranes is widely accepted [27]. Therefore, we determined whether or not UQ embedded in liposomes can act as an antioxidant against the lipid peroxidation of liposomes in the

Table 3 Reduction of endogenous UQ-9 in microsomes by cytosolic NADPH-UQ reductase in the presence of NAD(P)H

The values are the means $\pm$ S.D. of three experiments. Incubation was carried out at 37 °C for 15 min. *Significant differences at levels of $P < 0.001$ compared with before incubation, using Student's unpaired t -test.

Reaction mixture	tUQ-9 contents (ng)	UQH ₂ -9/tUQ-9 ratios (%)		
		Before incubation	After incubation	
			NADPH	NADH
Complete	51.8 $\pm$ 1.2	26.8 $\pm$ 3.7	62.9 $\pm$ 1.2*	54.6 $\pm$ 3.6*
— Cytosol	42.5 $\pm$ 2.4	26.2 $\pm$ 5.2	19.1 $\pm$ 2.1*	26.9 $\pm$ 1.4*
— Microsomes	9.0 $\pm$ 1.7	30.1 $\pm$ 6.1	65.1 $\pm$ 7.3*	55.6 $\pm$ 4.8*
+ 5 μ M Dicoumarol	51.8 $\pm$ 3.6	26.6 $\pm$ 8.6	62.4 $\pm$ 3.1*	51.1 $\pm$ 4.1*
+ 10 μ M Rotenone	50.8 $\pm$ 2.8	26.7 $\pm$ 3.0	60.8 $\pm$ 2.4*	53.2 $\pm$ 2.7*

Figure 2 The relationship between UQH₂-9/tUQ-9 ratios and cytosolic NADPH-UQ reductase activities in rat tissues

The amounts of UQH₂ and tUQ in tissue homogenates were determined by HPLC, as described in the text. Tissue cytosols were prepared from individual tissue homogenates by centrifugation at 105 000 *g* for 60 min as described for the liver cytosol. NADPH-UQ reductase activities in the cytosols were assayed as described in the text: 1, liver; 2, small intestine; 3, testis; 4, kidney; 5, lung; 6, brain; 7 spleen; 8, heart; 9, skeletal muscle. The values are the means $\pm$ S.D. of three experiments.

presence of cytosol and NADPH. UQ-10-fortified egg yolk lecithin liposomes were prepared in the presence of AMVN, a lipid-soluble and thermolytic pro-oxidant, and dispersed in a

reaction mixture containing rat liver cytosol with NADPH. AMVN-induced lipid peroxidation was measured by the formation of conjugated dienes and TBARS, which increased linearly during incubation periods of up to 4 h under our conditions, regardless of the presence of UQ-10 (results not shown). The regression formulae of conjugated diene formation (Y_c , nmol/ml reaction mixture) and TBARS formation (Y_t , nmol/ml reaction mixture) in the reaction mixture containing rat liver cytosol and NADPH with respect to reaction time (X , h) were calculated based upon four repeated experiments as $Y_c = 8.2X - 0.4$ ($r = 0.980$, $P < 0.01$) and $Y_t = X + 4.4$ ($r = 0.996$, $P < 0.01$) in the presence of UQ-10, and $Y_c = 16.4X + 2.2$ ($r = 0.994$, $P < 0.01$) and $Y_t = 1.8X - 4.9$ ($r = 0.992$, $P < 0.01$) in the absence of UQ-10 respectively. The effect of each component in the reaction mixture on the peroxidation of liposomes during a 2 h incubation is listed in Table 4. The coexistence of cytosol and NADPH significantly depressed lipid peroxidation of the liposomes induced by AMVN, and tUQ-10 was not degraded during the incubation. The removal of either UQ-10 embedded in the liposomes or NADPH, or the use of heated, instead of native, cytosol, resulted in an increase in the formation of conjugated dienes and TBARS. Furthermore, the antioxidant action of UQ-10 with cytosol and NADPH was not inhibited by dicoumarol. Under these conditions, the removal of AMVN from the liposomes increased UQH₂-10 formation almost 2-fold as compared with that in the complete system. Namely, the balance between the amounts of UQH₂-10 formed in the presence and absence of AMVN was probably used for quenching the AMVN-induced radicals.

Table 4 Antioxidant effect of cytosolic NADPH-UQ reductase on AMVN-induced lipid peroxidation of UQ-10-fortified lecithin liposomes in the presence of NADPH

The complete reaction mixture (1 ml) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, 1.45 mg protein of rat liver cytosol and lecithin liposomes which consisted of 5 mM egg yolk lecithin, 1 mM AMVN and 2.5 μ M UQ-10. Incubation was carried out at 37 $^{\circ}$ C for 2 h. The values are the means $\pm$ S.D. of four experiments. n.d., not detected. *, †, ‡, § Significant differences at levels of $P < 0.05$, $P < 0.01$, $P < 0.005$ and $P < 0.001$ respectively compared with the complete reaction mixture, using Student's unpaired *t*-test. || Cytosol denatured by heating at 95 $^{\circ}$ C for 5 min replaced native cytosol.

Reaction mixtures	UQH ₂ -10 formed (nmol/ml per 2 h)	tUQ-10 recovered (nmol/ml per 2 h)	Conjugated dienes formed (nmol/ml per 2 h)	TBARS formed (nmol/ml per 2 h)
Complete	0.56 $\pm$ 0.01	2.51 $\pm$ 0.17	22.5 $\pm$ 1.4	2.34 $\pm$ 0.25
— NADPH	n.d.	2.15 $\pm$ 0.51	34.5 $\pm$ 5.0†	3.66 $\pm$ 0.84*
— UQ-10	n.d.	n.d.	32.9 $\pm$ 5.2†	3.54 $\pm$ 0.47†
— AMVN	0.96 $\pm$ 0.02§	2.36 $\pm$ 0.47	1.46 $\pm$ 0.40§	0.96 $\pm$ 0.06§
+ 5 μ M Dicoumarol	0.44 $\pm$ 0.02§	2.37 $\pm$ 0.68	23.4 $\pm$ 1.2	2.44 $\pm$ 0.88
Heated cytosol	n.d.	2.31 $\pm$ 0.35	32.6 $\pm$ 5.8†	3.98 $\pm$ 0.89†

Table 5 Effect of cytosolic NADPH-UQ reductase on AMVN-induced lipid peroxidation of UQH₂-10-fortified lecithin liposomes in the presence of NADPH

The standard reaction mixture (1 ml) consisted of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol, 1.45 mg protein of rat liver cytosol and lecithin liposomes which consisted of 5 mM egg yolk lecithin and 1 mM AMVN with or without 2.5 μ M UQH₂-10. Incubation was carried out at 37 $^{\circ}$ C for the indicated time periods. * Control liposomes did not contain UQH₂-10. † Cytosol denatured by heating at 95 $^{\circ}$ C for 5 min replaced native cytosol. The values are the means $\pm$ S.D. of three experiments. ‡ (Versus control liposomes) and § (versus heated cytosol) denote significant difference ($P < 0.001$, Student's unpaired *t*-test) in conjugated dienes formed due to AMVN.

	Conjugated dienes formed (nmol/ml)				
	0 h	1 h	2 h	3 h	4 h
Control liposomes*					
Native cytosol	0	18.1 $\pm$ 2.6	34.4 $\pm$ 8.9	52.7 $\pm$ 8.7	64.4 $\pm$ 8.9
UQH ₂ -10-fortified liposomes					
Native cytosol	0	1.0 $\pm$ 0.1‡	2.5 $\pm$ 0.3‡	1.2 $\pm$ 0.5‡,§	3.4 $\pm$ 0.3‡,§
Heated cytosol†	0	1.2 $\pm$ 0.1‡	3.1 $\pm$ 0.6‡	20.5 $\pm$ 2.3‡	38.6 $\pm$ 5.6‡

Table 6 Fractionation of rat liver cytosol by Cibacron Blue-immobilized Bio-Gel A-5m chromatography

The recoveries of NADPH-UQ reductase and DT-diaphorase activities obtained at each elution step are shown as the percentage of the total enzyme activities eluted from the column.

Eluents	Volume (ml)	NADPH-UQ reductase activity		DT-diaphorase activity	
		Total activity (pmol/min)	Recovery (%)	Total activity (nmol/min)	Recovery (%)
Fraction A: Void volume + linear gradient (0.15–3.5 M NaCl)	66	52400	99.0	128	1.8
Fraction B: 3.5 M NaCl/5 mM Tris-HCl buffer (pH 7.4)	24	139	0.3	80.1	1.1
Fraction C: 5 mM Tris-HCl buffer (pH 7.4)	15	1.5	0.0	2.1	0.0
Fraction D: 20 mM Tris base (pH 10)/50% ethylene glycol	40.5	34.5	0.1	233	3.3
Fraction E: 20 mM Tris base (pH 10)/50% ethylene glycol/1 mM NADH	16.5	330	0.6	6690	93.8

The result of a similar experiment using UQH₂-10-fortified lecithin liposomes instead of UQ-10-fortified liposomes is shown in Table 5. The formation of conjugated dienes in the UQH₂-10-fortified liposomes was completely inhibited for 2 h until the premixed UQH₂-10 was exhausted, and then began to increase in parallel with the control with neither UQ-10 nor UQH₂-10 present, while the formation of conjugated dienes in the UQH₂-10-fortified liposomes was not observed at all in the presence of rat liver cytosol and NADPH during the incubation period up to 4 h. Therefore, UQH₂ is presumed to act as antioxidant at a very early stage of the AMVN-induced peroxidation. In any case, these results support the notion that cytosolic NADPH-UQ reductase is involved in cellular antioxidant systems through generating the UQH₂ that is oxidized in lipid membranes.

Separation of NADPH-UQ reductase and DT-diaphorase activities by Cibacron Blue-immobilized Bio-Gel A-5m chromatography

To confirm that cytosolic NADPH-UQ reductase differs from DT-diaphorase, the supernatant fraction obtained from rat liver homogenates by centrifugation at 105000 *g* for 60 min was applied to Cibacron Blue-immobilized Bio-Gel A-5m chromatography. The eluate was collected as 1.5 ml fractions. The protein content (absorbance at 280 nm) and DT-diaphorase (NADH-dependent DCPIP reductase) and NADPH-UQ reductase activities were measured and are summarized in Table 6. Almost all the NADPH-UQ reductase activity in the supernatant was recovered in the void volume and in the eluate obtained by a 0.15–3.5 M linear NaCl gradient. Both of these fractions were mixed together as fraction A. The NADPH-UQ reductase activity in fraction A was not inhibited by dicoumarol, a specific inhibitor of DT-diaphorase (results not shown). On the other hand, 93.8% of the DT-diaphorase activity recovered from the column was located in fraction E eluted with 50% ethylene glycol containing 1 mM NADH and 20 mM Tris base (pH 10). Furthermore, fraction A had virtually no DT-diaphorase activity. Likewise, fraction E had almost no NADPH-UQ reductase activity.

Figure 3 Reduction of UQ-10 in egg yolk lecithin liposomes by fractions obtained by Cibacron Blue-immobilized Bio-Gel A-5m chromatography

The reaction mixture consisted of 5 mM lecithin liposomes, 50 mM Tris-HCl buffer (pH 7.4), 0.2 mM NADPH, 0.5 mM 2-mercaptoethanol and either fraction A (●, 3.5 nmol UQH₂-10 formed/min NADPH-UQ reductase activity per ml of reaction mixture) or E (○, 2.1 μmol dihydromenadiene formed/min DT-diaphorase activity per ml of reaction mixture). The reaction was started by addition of NADPH and carried out at 37 °C. At the indicated times, an aliquot of the reaction mixture was removed and the amount of UQH₂-10 formed was measured by HPLC as described in the text. The values are the means ± S.D. of three experiments.

Reduction of UQ-10 in liposomes to UQH₂-10 by NADPH-UQ reductase and DT-diaphorase fractions

The results described above indicate that NADPH-UQ reductase and DT-diaphorase are two distinct enzymes and that NADPH-UQ reductase is responsible for UQ reduction by cytosol. Therefore, the reduction rates of UQ-10 to UQH₂-10 by fractions A (NADPH-UQ reductase-rich fraction) and E (DT-diaphorase-rich fraction), obtained by Cibacron Blue-immobilized Bio-Gel A-5m chromatography (see Table 6), were compared using UQ-10 embedded in egg yolk lecithin liposomes and NADPH as the substrates (Figure 3). Aliquots of either fraction A (3.5 nmol

UQH₂-10 formed/min of NADPH-UQ reductase activity) or E (2.1 μ mol dihydromenadione formed/min of DT-diaphorase activity) having activities equivalent to 5.4 mg of the parent cytosolic protein were added to a final volume of 1 ml of the reaction mixtures. Fraction A reduced about 50% of UQ-10 in the liposomes during an 8 h incubation and it had three times the UQ-reducing capability of fraction E.

DISCUSSION

The mechanism of UQ reduction in intracellular fractions is not completely understood. There are some enzymes that are capable of reducing UQ to UQH₂ in liver cells [13,27], but it is unclear which enzyme is responsible for maintaining the steady-state redox ratios of UQH₂/tUQ in intracellular organelles. UQ can be reduced by the mitochondrial respiratory chain, but it is not easy to explain biochemically how the UQH₂ formed in mitochondria is transferred to other subcellular organelles. Microsomes have enzymes, such as NADPH-cytochrome reductase, which may reduce UQ [29]. In general, however, NADPH-cytochrome reductase is believed to stimulate lipid peroxidation in the presence of hydrogen donors such as NADPH [30]. In fact, the addition of NADPH without cytosol induced oxidation of UQH₂ in microsomes (Table 3). DT-diaphorase, which is located mainly in cytosol and to some extent in microsomes and mitochondria [15], potentially produces intracellular UQ reduction [14]. However, UQ, especially that with a long isoprenoid side-chain, is not a preferred substrate for DT-diaphorase [15]. In fact, as shown in Table 6, DT-diaphorase contributed to only 0.6% of the total reductase activity of UQ by rat liver cytosol. Considering these findings, a novel NADPH-UQ reductase activity observed in rat cytosol [16] is worth notice in determining the mechanism of extramitochondrial UQ reduction.

The results presented here show that the reduction of UQ to UQH₂ in rat liver homogenates is stimulated more by NADPH than NADH and that the NADPH-UQ reductase activity is exclusively located in the cytosol (68% of the total enzyme activity in liver homogenates). In addition, the UQH₂-9/tUQ-9 ratios of rat tissues were positively correlated with the log of NADPH-UQ reductase activities in their cytosols.

A positive correlation between the log of NADPH-UQ reductase activities and the UQH₂/tUQ ratios would be reasonable, since the UQH₂ ratios of intracellular membranes resulted from a dynamic equilibrium between the reduction of UQ by this NADPH-UQ reductase and the re-oxidation of UQH₂ to UQ accompanied by lipid peroxidation or quenching radicals. If NADPH-UQ reductase in the cytosol, which fills gaps among all intracellular membranes, was responsible for regulating the intracellular UQH₂/tUQ ratios, the finding that the UQH₂/tUQ ratios of all intracellular fractions in a tissue are similar to each other [6] is not surprising.

These observations suggest that NADPH-UQ reductase in cytosol plays a major role in maintaining the steady-state levels of UQH₂/tUQ in intracellular organelles. In fact, rat liver cytosol also reduced the native UQ-9 buried in rat liver microsomes as well as the UQ-10 incorporated into liposomes in the presence of NADPH. When incubated with NADPH, the UQH₂-9/tUQ-9 ratio of rat microsomes was elevated to more than twice the initial ratio after the addition of cytosol, whereas in the absence of cytosol it decreased to two thirds of the initial level. The antipodal change of the UQH₂-9/tUQ-9 ratio in microsomes in the presence and absence of cytosol suggests that NADPH-UQ reductase in cytosol is essential in protecting the endoplasmic reticulum and probably other intracellular organelles from

oxidative damage by active oxygen radicals induced inevitably by radical-forming enzymes such as microsomal cytochrome *P*-450 [31]. In rat liver cytosol, the level of NADPH reported [32] is $97 \pm 13 \mu$ M, which is much higher than the K_m value of 19.2μ M for NADPH of NADPH-UQ reductase. Furthermore, the ratios of NAD⁺/NADH and NADP⁺/NADPH are also reportedly 1164 and 0.014 respectively [33]. In other words, the molar ratio of NADP⁺/NADPH provides an optimal environment for the reductive reaction. Therefore, NADPH-UQ reductase in rat cytosol is believed to reduce UQ-9 to UQH₂-9 at all times. The reason why the level of NADPH-UQ reductase activity is highest in liver may also be explained from this viewpoint, since the liver contains several radical-forming enzymic systems and is obligated to secrete easily oxidizable lipoproteins, which have a high UQH₂/tUQ ratio comparable to that in the liver, into the blood circulation [8].

NADPH-UQ reductase activity in rat liver cytosol was separated from DT-diaphorase by column chromatography and the NADPH-UQ reductase fraction reduced almost twice as much UQ-10 embedded in egg yolk lecithin liposomes as the DT-diaphorase fraction. Though the enzyme protein was not further purified because of its instability, it appears to be a flavoprotein with a molecular mass of about 30 kDa, according to gel filtration chromatography and inhibition by flavo-enzyme inhibitors (results not shown). From preliminary experiments using rat liver cytosol (results not shown), NADPH-UQ reductase is presumed to prefer long side-chain UQ homologues as substrate, because its UQ-10 reducing activity was not inhibited in the presence of short side-chain UQ homologues. In addition, the enzyme activity was inhibited by *N*-ethylmaleimide, *p*-mercuribenzoate and chlorpromazine, stimulated by FAD and FMN, and neither stimulated nor inhibited by ascorbate, glutathione (reduced form), cysteine, 2-mercaptoethanol and many of the inhibitors of the mitochondrial respiratory chain. Furthermore, it was neither inhibited by cytochrome *c*, which can accept an electron from superoxide anion, nor stimulated by KCN, a strong inhibitor of copper and zinc-containing superoxide dismutase. The characteristics of the NADPH-UQ reductase as mentioned above, as well as those reported previously [16], appear not to be consistent with those of known enzymes capable of reducing UQ.

The manner of UQ reduction by the enzyme, that is to say, whether or not it can reduce the ubisemiquinone radical, is interesting in considering the antioxidant efficacy of UQH₂ in the lipid membrane. The unstable ubisemiquinone radical is known to undergo autoxidation to UQ with concomitant generation of superoxide anion [34,35]. However, UQ reduction by the enzyme was accompanied by neither reduction of acetylated cytochrome *c* nor chemiluminescence from 2-methyl-6-phenyl-3,7-dihydroimidazo(1,2- α)pyrazine-3-one (results not shown), which are often used to detect superoxide anion [36,37]. Therefore, it is likely to convert UQ to UQH₂ by two-electron reduction as does DT-diaphorase.

It is also of great interest how UQ, which is believed to reside mainly in the hydrophobic core of phospholipid membranes [38,39], can be reduced by cytosolic NADPH-UQ reductase suspended in the aqueous medium. The results presented are not unexpected, considering observations in which UQ incorporated into phospholipid liposomes was reduced to UQH₂ by externally added, hydrophilic reagents and enzymes. For example, Ulrich et al. [39] showed that UQ in dipalmitoylphosphatidylcholine liposomes was reduced by tetraborohydrate and that UQ in liposomes mediated the reduction of ferricyanide entrapped in the liposomes by externally added dithionite. Takada et al. [14] also showed that cytosol, microsomes and mitochondria from

guinea pig liver reduced UQ-10 in liposomes. Furthermore, Takeshige et al. [40] reported that lipid peroxidation of liposomes containing UQ-10, induced by externally added submitochondrial particles, was inhibited by externally added succinate, which is a substrate for the mitochondrial respiratory chain. In addition, Stocker and Suarna [41] reported recently that UQ-10 in serum low-density lipoproteins could be reduced to some extent even by hepatocytes and blood cells. Chazotte et al. [42] suggested that UQ in phospholipid liposomes is highly mobile laterally and transversely and its motion is limited by cholesterol and the viscosity of the phospholipid. The reduction of UQ in liposomes by hydrophilic agents as described above, though it is presumed to be dependent on the affinity of the agents and the physical properties of the membranes, might be explained by rapid transmembrane flip-flop motion of UQ in a direction perpendicular to the plane of phospholipid membranes, as postulated in the proton-motive UQ cycle for mitochondrial electron and proton transport [43].

In conclusion, we have revealed that a novel NADPH-UQ reductase in cytosol reduces UQ to UQH₂ in phospholipid membranes and subsequently protects the membranes from lipid peroxidation. These results also support the notion [27] that the oxidation-reduction cycle of intracellular UQ functions as an endogenous, lipid-soluble antioxidant in cellular defence against oxidative damage, as well as an essential electron and proton carrier in the mitochondrial respiratory chain.

This work was supported in part by the Science Research Fund from the Japan Private School Promotion Foundation and a grant-in-aid for health science research from Kobe Gakuin University.

REFERENCES

- Ernster, L. (1977) in *Biomedical and Clinical Aspects of Coenzyme Q* (Folkers, K. and Yamamura, Y., eds.), vol. 1, pp. 15–21, Elsevier, Amsterdam
- Crane, F. L. and Morre, D. J. (1977) in *Biomedical and Clinical Aspects of Coenzyme Q* (Folkers, K. and Yamamura, Y., eds.), vol. 1, pp. 3–14, Elsevier, Amsterdam
- Kalén, A., Norling, B., Appelkvist, E.-L. and Dallner, G. (1987) *Biochim. Biophys. Acta* **926**, 70–78
- Kalén, A., Appelkvist, E.-L., Chojnacki, T. and Dallner, G. (1990) *J. Biol. Chem.* **265**, 1158–1164
- Åberg, F., Appelkvist, E.-L., Dallner, G. and Ernster, L. (1992) *Arch. Biochem. Biophys.* **295**, 230–234
- Takahashi, T., Okamoto, T., Mori, K., Sayo, H. and Kishi, T. (1993) *Lipids* **28**, 803–809
- Takada, M. and Watanabe, J. (1987) *J. Pharmacobio-Dyn.* **10**, 124–127
- Okamoto, T., Matsuya, T., Fukunaga, Y., Kishi, T. and Yamagami, T. (1989) *Int. J. Vit. Nutr. Res.* **59**, 288–292
- Reahal, S. and Wigglesworth, J. (1992) *Drug Metab. Dispos.* **20**, 423–427
- Mellors, A. and Tappel, A. L. (1966) *J. Biol. Chem.* **241**, 4353–4356
- Kagan, V. E., Serbinova, E. A., Koyanova, G. M., Kitanova, S. A., Tyurin, V. A., Stoytchev, T. S., Quinn, P. J. and Packer, L. (1990) *Free Radicals Biol. Med.* **9**, 117–126
- Ingold, K. U., Bowry, V. W., Stocker, R. and Walling, C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 45–49
- Frei, B., Kim, M. C. and Ames, B. N. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4879–4883
- Takada, M., Yuzuriha, T. and Yamato, C. (1985) *J. Nutr. Sci. Vitaminol.* **31**, 147–155
- Ernster, L., Danielson, L. and Ljunggren, M. (1962) *Biochim. Biophys. Acta* **58**, 171–188
- Takahashi, T., Shitashige, M., Okamoto, T., Kishi, T. and Goshima, K. (1992) *FEBS Lett.* **314**, 331–334
- Hogeboom, G. H. (1955) *Methods Enzymol.* **1**, 16–19
- Sottocasa, G. L., Kuylensstierna, B., Ernster, L. and Bergstrand, A. (1967) *J. Cell. Biol.* **32**, 415–438
- Ehrenreich, J. H., Bergeron, J. J. M., Siekevitz, P. and Palade, G. E. (1973) *J. Cell. Biol.* **59**, 45–72
- Ikehara, Y. and Pitot, H. C. (1973) *J. Cell. Biol.* **59**, 28–44
- Okamoto, T., Fukunaga, Y., Ida, Y. and Kishi, T. (1988) *J. Chromatogr.* **430**, 11–19
- Ernster, L. (1967) *Methods Enzymol.* **10**, 309–317
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Shimasaki, H., Privett, O. S. and Hara, I. (1977) *J. Am. Oil Chem. Sci.* **54**, 119–123
- Buege, J. A. and Aust, S. D. (1978) *Methods Enzymol.* **52**, 302–310
- Prochaska, H. J. (1988) *Arch. Biochem. Biophys.* **267**, 529–538
- Ernster, L. and Forsmark-Andrée, P. (1993) *Clin. Invest.* **71**, S60–S65
- Zöllner, H. (1993) in *Handbook of Enzyme Inhibitors* (Zöllner, H., ed.), pp. 656–668, VCH, Weinheim
- Powis, G. and Appel, P. L. (1980) *Biochem. Pharmacol.* **29**, 2567–2572
- Grover, T. A. and Piette, L. H. (1981) *Arch. Biochem. Biophys.* **212**, 105–114
- Bartoli, G. M., Galeotti, T., Palombini, G., Parisi, G. and Azzì, A. (1977) *Arch. Biochem. Biophys.* **184**, 276–281
- Glock, G. E. and McLean, P. (1956) *Exp. Cell Res.* **11**, 234–236
- Krebs, H. A. and Veech, R. L. (1969) in *Advances in Enzyme Regulation* (Weber, G., ed.), vol. 7, pp. 397–413, Pergamon Press, London
- Beyer, R. E. (1990) *Free Radicals Biol. Med.* **8**, 545–565
- Sugioka, K., Nakano, M., Totsune Nakano, H., Minakami, H., Tero Kubota, S. and Ikegami, Y. (1988) *Biochim. Biophys. Acta* **936**, 377–385
- Kakinuma, K. and Minakami, S. (1978) *Biochim. Biophys. Acta* **538**, 50–59
- Goto, T. and Takagi, T. (1980) *Bull. Chem. Soc. Jpn.* **53**, 833–834
- Michaelis, L. and Moore, M. J. (1985) *Biochim. Biophys. Acta* **821**, 121–129
- Ulrich, E. L., Girvin, M. E., Cramer, W. A. and Markley, J. L. (1985) *Biochemistry* **24**, 2501–2508
- Takeshige, K., Takayanagi, R. and Minakami, S. (1980) in *Biomedical and Clinical Aspects of Coenzyme Q* (Yamamura, Y., Folkers, K. and Ito, Y., eds.), vol. 2, pp. 15–26, Elsevier, Amsterdam
- Stocker, R. and Suarna, C. (1993) *Biochim. Biophys. Acta* **1158**, 15–22
- Chazotte, B., Wu, E.-S. and Hackenbrock, C. R. (1991) *Biochim. Biophys. Acta* **1058**, 400–409
- Mitchell, P. (1975) *J. Theor. Biol.* **62**, 327–367