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Original Contribution

INHIBITION BY COENZYME Q OF ETHANOL- AND CARBON TETRACHLORIDE-STIMULATED LIPID PEROXIDATION IN VIVO AND CATALYZED BY MICROSOMAL AND MITOCHONDRIAL SYSTEMS

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Abstract— The ability of coenzyme Q to inhibit lipid peroxidation in intact animals as well as in mitochondrial, submitochondrial, and microsomal systems has been tested. Rats fed coenzyme Q prior to being treated with carbon tetrachloride or while being treated with ethanol excrete less thiobarbituric acid-reacting material in the urine than such rats not fed coenzyme Q. Liver homogenates, mitochondria, and microsomes isolated from rats treated with carbon tetrachloride and ethanol catalyze lipid peroxidation at rates which exceed those from animals also fed coenzyme Q. The rate of lipid peroxidation catalyzed by submitochondrial particles isolated from hearts of young, old, and endurance trained elderly rats was inversely proportional to the coenzyme Q content of the submitochondrial preparation in assays in which succinate was employed to reduce the endogenous coenzyme Q. Reduced, but not oxidized, coenzyme Q inhibited lipid peroxidation catalyzed by rat liver microsomal preparations. These results provide additional evidence in support of an antioxidant role for coenzyme Q.

Keywords—Coenzyme Q, Ubiquinone, Lipid peroxidation, Ethanol, Carbon tetrachloride, Antioxidation, Mitochondria, Microsomes, Free radical

INTRODUCTION

Evolution of organisms to the aerobic life style has required that mechanisms be selected to protect cells from the damaging effects of reactive oxygen free radical species produced during oxidative reactions. Of the many enzymatic and molecular devices which have been demonstrated to provide protection from free radical damage, perhaps the most controversial is the role of coenzyme Q (otherwise known as ubiquinone) in free radical production and as an antioxidant. In addition to its function in the mitochondrial electron transfer chain,[1] reduced coenzyme Q influences the rate of electron flux through the succinate dehydrogenase,[2] NADH dehydrogenase,[3] and cytochrome *b*–c_1[4] complexes. CoQ also appears to be involved in the establishment of an inner mitochondrial transmembrane protonmotive gradient, the "protonmotive Q cycle," resulting in the conservation of energy released by oxidation-reduction reactions of the electron transfer chain.[5] CoQ has been implicated as the electron transfer chain member responsible for superoxide radical production as well as serving as an inhibitor of superoxide radical production during electron transfer chain activity, subjects which have been reviewed.[6–9]

The experiments reported herein were designed to study the effect of CoQ on the extent of lipid peroxidation both in vivo and in vitro employing treatments such as ethanol and carbon tetrachloride known to result in free radical damage.

MATERIALS AND METHODS

Animals

All animals used were male Sprague–Dawley rats obtained from Charles River Breeding Laboratories, Portage, Michigan, except for those used in the experiments on microsomal lipid peroxidation which were from the University of Stockholm Research Animal Quarters. Aged rats were obtained as retired breeders at 9 months of age and rested until 25 months of age except in the case of exercise-trained rats which were run 5 days each week for 21 weeks until 25 months old as described previously.[10] They were housed singly in wire bottom cages in a room segregated from other species and maintained at 22°C on a 12 hour light–dark cycle. All animals received Purina Laboratory Rat chow and water ad libitum. To study the effect of chronic ethanol administration, 2.5% ethanol (w/v in water) was substituted for drinking water for 30 days. CoQ_{10} dissolved in olive oil was

administered orally to rats daily (3 mg/kg/day) for 14 days during the latter portion of the experimental period. Rat urine was collected using a home-made stainless steel screen-funnel device.

The effect of carbon tetrachloride was studied by injecting (i.p.) 1 ml CCl_4/kg body weight as a 25% solution in mineral oil to rats fasted overnight (17 hours). Controls were treated with mineral oil. Rats were killed by decapitation 24 hours after treatment.

Materials

Carbon tetrachloride was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. CoQ_{10} was a generous gift of Eisai Co. Ltd., Tokyo, and in Stockholm, was "borrowed" from Gustav Dallner's cache with the help of Peter Löw. Reduced CoQ solutions were prepared immediately prior to use by reduction in ethanol with $NaBH_4$, extraction with petroleum ether (BR 40–60°), drying in vacuo under N_2 and dissolving the colorless residue in 2% cardiolipin in ethanol. The solution was maintained under N_2 and protected from light. Oxidized CoQ used in microsomal experiments was treated identically except for the reduction by $NaBH_4$. Cardiolipin, NADH, NADPH, ADP, 2-thiobarbituric acid, menadione and ascorbate were obtained from Sigma Chemical Co., St. Louis, Missouri.

Preparations and assays

Rat liver microsomes were prepared according to Ernster et al.[11] Rat liver mitocochondria were isolated according to Johnson and Lardy[12] and heart SMPs prepared essentially as previously described.[13] Lipid peroxidation was assayed in vitro by measuring malondialdehyde formation and oxygen consumption according to Ernster and Nordenbrand.[14] Urinary malondialdehyde was measured according to Draper et al.[15] Protein was determined with a biuret procedure.[16] Rat urine, together with cage washings, were collected over a 24 hour period and lyophilized to dryness. The residue was dissolved in 1 ml of water. One ml of a saturated thiobarbituric acid solution was added, the pH adjusted to 3.0 with HCl, and the mixture heated in a boiling water bath for 30 minutes and cooled. After centrifugation, the absorbtivity of the clear solution was determined at 535 nm. An extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$ was used[14] to calculate the MDA content. CoQ was extracted and its concentration determined spectrophotometrically.[17] NADH-supported lipid peroxidation catalyzed by rat heart SMPs was assayed at 37° by incubating 1 mg of SMP protein in 1 ml of a solution containing 4 nmol rotenone, 2 mM ADP, 0.2 mM $FeCl_3$, 50 mM TrisHCl, pH 7.4 and, when present to reduce endogenous CoQ, 10 mM succinate. The reaction was initiated by the addition of 1 mM NADH and terminated after 10 minutes by the addition of 50 uL of 100% TCA. MDA formation was determined according to Ernster and Nordenbrand.[14] Whenever possible, all assays were performed in triplicate.

RESULTS

Although MDA produced as a product of lipid peroxidation in vivo, to a large extent, is metabolized to CO_2, about 10% of orally administered MDA is excreted in the urine which normally contains MDA in an acid hydrolizable form.[18] MDA, as an indicator of lipid peroxidation in vivo[15,18] was measured in urine from rats pretreated with CoQ and to which CCl_4 or ethanol was administered (Table 1). CoQ administration tended to decrease urinary MDA in control animals. The large increase in urinary MDA which resulted from CCl_4 administration was lowered to a significant degree in animals pretreated with CoQ (Table 1). Although ethanol was a less potent stimulator of lipid peroxidation compared to CCl_4, the increase in urinary MDA as a result of ethanol consumption was also diminished by pretreatment with CoQ (Table 1).

As is well established,[19] treatment of rats with CCl_4 or ethanol results in an increase in products of lipid peroxidation (MDA) in liver and its particulate fractions. The data in Table 2 indicate that treatment of rats with oral administration of CoQ reduced the endogenous control levels of TBA-reacting material, indicative of peroxidized lipids, in the liver homogenate and its mitochondrial and microsomal fractions. Administration of CCl_4 resulted in a large increase in MDA formation after 24 hours in the three tissue fractions studied (Table 2). Ethanol treatment also resulted in an increase in all fractions, but to a lesser degree. Prior treatment with CoQ gave rise to a lower concentration of TBA-reacting material in the homogenate, and mitochondrial and microsomal fractions in both CCl_4- and ethanol-treated rats (Table 2).

Mitochondrial CoQ concentrations have been ma-

Table 1. TBA-Reacting Material in 24 Hour Urine Samples of Rats Treated with CCL_4, Ethanol, and CoQ. Data as Mean ± SE

#	Treatment	*n*	Urine MDA (ug/24 hrs)	Comparison	*P* =
1	Control	6	0.92 ± 0.045		
2	CoQ	6	0.62 ± 0.037	2 vs. 1	NS
3	CCl_4	5	4.88 ± 0.32	3 vs. 1	<0.01
4	CCl_4 + CoQ	6	1.46 ± 0.18	4 vs. 3	<0.01
5	EtOH	7	2.42 ± 0.22	5 vs. 1	<0.05
6	EtOH + CoQ	6	0.81 ± 0.07	6 vs. 5	<0.05

Table 2. TBA-Reacting Material (MDA) in Liver Fractions from Rats Treated with CCl_4, Ethanol, and CoQ. Data as mean ± SE

			Fraction								
#	Group	(n)	Homog.[a]	Comp.[c]	P	Mito.[b]	Comp.[c]	P	Mic.[b]	Comp.[c]	P
1	Control	(8)	86 ± 4	—	-	8.2 ± 0.3	—	-	5.4 ± 0.3	—	-
2	CoQ	(6)	13 ± 1	2 & 1	<.001	3.7 ± 0.3	2 & 1	<.01	3.2 ± 0.1	2 & 1	<.02
3	CCl_4	(8)	766 ± 14	3 & 1	<.001	28.3 ± 2.8	3 & 1	<.05	10.7 ± 0.8	3 & 1	<.05
4	CCl_4 + CoQ	(7)	465 ± 13	4 & 3	<.001	11.1 ± 0.7	4 & 3	<.05	6.5 ± 0.4	4 & 3	<.1
5	EtOH	(6)	188 ± 7	5 & 1	<.001	19.8 ± 2.2	5 & 1	<.05	8.4 ± 0.4	5 & 1	<.05
6	EtOH + CoQ	(5)	15 ± 2	6 & 5	<.001	4.6 ± 0.4	6 & 5	<.02	3.4 ± 0.5	6 & 5	<.01

[a]nmol MDA/gram liver; [b]nmol MDA/mg protein; [c]Comp.; Groups compared statistically.

nipulated by extraction with organic solvents and reincorporated to varying degrees with sonic treatment. However, we have used physiological regulation of tissue CoQ content by endurance training to increase[10,20] and aging to lower[21,22] cardiac CoQ levels in order to study the influence of differing endogenous CoQ levels on lipid peroxidation catalyzed by heart SMPs. The data in Table 3 indicate that IMM CoQ concentrations decreased 30% between 3 and 25 months of age in sedentary animals while a regimen of moderate running 5 days/week for 5 months increased the CoQ concentration 41% compared to elderly sedentary animals. In addition, small differences were observed in the rate of NADH-supported lipid peroxidation catalyzed by SMPs isolated from hearts of these three groups of animals. The differences were in the direction of changes in overall tissue respiratory activity reported under these circumstances previously.[10,23,24] The addition of succinate to reduce endogenous CoQ resulted in inhibition of lipid peroxidation in each case. The extent of inhibition by succinate was in direct proportion to the endogenous CoQ content of the SMPs, being greatest in particles from 25 month old trained and 3 month old sedentary rats and lowest in SMPs prepared from hearts of 25 month old sedentary animals.

As microsomal membranes have been reported to contain appreciable quantities of CoQ,[25,26] the effect of the oxidoreduction state of CoQ added to isolated rat liver microsomal preparations has been studied (Table 4). The addition of CoQ in the fully oxidized state stimulated oxygen consumption from NADPH via ADP-Fe^{2+} but did not affect the extent of MDA formation compared to the EtOH-CL solvent control. Oxidized CoQ addition was observed to have a weak inhibitory effect occasionally. The inclusion of 150 μM and 300 μM $CoQH_2$ inhibited MDA formation to 20% and 4% respectively of the EtOH-CL control level. The presence of oxidized CoQ did not interfere with the antioxidative activity of the reduced form. Inhibition of oxygen consumption and MDA formation by $CoQ_{10}H_2$ over the concentration range 15–300 μM gave an apparent Ki, or IC_{50} of 57 μM. The IC_{50} values determined for two other antioxidant quinones, menadione and vitamin K_5 (4-amino-2-methyl-1-naphthol), were 20 nM for menadione and 37 nM for vitamin K_5. The IC_{50} that we have obtained for menadione is slightly lower than 37 nM reported by Talcott et al.[27]

DISCUSSION

Incomplete understanding of the functional rationale for the relatively high molar ratio of CoQ to other mitochondrial electron transfer chain components,[28] and the role of CoQ found in intracellular membranes in addition to the IMM,[29] has led to speculation[20,21] about other functions for CoQ in addition to those in energy conservation and related reactions. Since the initial studies suggesting an antioxidant role for CoQ,[30–32] evidence has continued to accumulate from

Table 3. The Effect of Endurance Training and Age on CoQ Concentrations in and Lipid Peroxidation Catalyzed by Rat Heart SMPs. Data are mean ± SE

#	SMPs from	n	CoQ[a]	Comp[b]	P	Addn.	MDA formed[a]	Comp[b]	P
1	Sed-25[b]	7	2.4 ± .10				6.48 ± .19	2 & 1	<.001
2	Sed-25					Succinate	2.79 ± .17		
3	Tr-25	8	3.4 ± .10	3 & 1	<.05		7.27 ± .21	4 & 3	<.001
4	Tr-25					Succinate	1.02 ± .11		
5	Sed-3	8	3.6 ± .14	5 & 1	<.05		6.83 ± .18	6 & 5	<.001
6	Sed-3			5 & 3	>.5	Succinate	1.37 ± .26		

[a]nmols/mg protein. [b]Abbreviations: Comp: groups compared statistically; Sed-25, Tr-25, Sed-3: 25 month old sedentary and trained, and 3 month old sedentary rats respectively.

Table 4.[a] Effect of Redox State of Coenzyme Q on NADPH, ADP-Fe^{2+}-supported Lipid Peroxidation Catalyzed by Rat Liver Microsomes

Additions	Concentration (μM)	Oxygen Consumption (natoms/min/mg protein)	Oxygen Consumption % of C[b]	MDA formed (nmol)	MDA formed % of C[b]
EtOH-CL[b]	—	224	100	5.87	100
CoQ_{10}	150	320	143	5.71	97
$CoQ_{10}H_2$	150	59	26	1.19	20
$CoQ_{10}H_2$	300	31	14	0.26	4
CoQ_{10} + $CoQ_{10}H_2$	150 + 75	97	43	2.01	34

[a]Too few experiments (3) performed for meaningful statistical analysis; data are from a typical experiment, each of which was internally consistent. [b]Abbreviations: C, control; CL, cardiolipin.

experiments at levels ranging from intact animals to recontituted systems in vitro supporting the concept of CoQ as a membrane protective device against free radical damage.[6-9] The results reported herein are from experiments designed to test the hypothesis that CoQ may provide such protection, from the intact animal to the subcellular level, under experimental conditions reported by others to result in oxidative stress.

Ethanol has long been known to produce tissue damage resulting from superoxide, hydrogen peroxide, and hydroxyl radical production[33-38] and ensuing lipid peroxidation.[39,40] Ethanol has been shown to alter microsomal metabolism[38] and to depress mitochondrial oxidative phosphorylation,[41-44] possibly via oxygen radical-mediated protein damage[45] to the IMM Fo · F_1 ATP synthetase.[46] In addition, the antioxidant vitamin A has been reported to ameliorate testicular lipid peroxidation and atrophy that occurs with chronic ethanol feeding.[47] In a similar, but more potent manner, carbon tetrachloride administration has been shown to result in pathology[19] resulting from free radical formation[48] and subsequent lipid peroxidation.[49]

The results of the experiments on alcohol and carbon tetrachloride intoxication support the concept that CoQ, administered to the intact animal or added in vitro, is able to diminish the rate of free radical-mediated lipid peroxidation in vivo or in subcellular systems isolated from animals so treated. In addition, the antioxidative capacity of $CoQH_2$ has been demonstrated by its ability to inhibit lipid peroxidation in an isolated microsomal system. These data are consistant with reports that CoQ has a protective role in CCl_4 toxicity,[50,51] and that CoQ is able to protect against adriamycin-induced mitochondrial respiratory chain inactivation and lipid peroxidation.[52]

Our demonstration that physiologically manipulated endogenous mitochondrial CoQ levels are related to the degree of lipid peroxidation catalyzed by SMPs derived therefrom, is consistant with results of similar experiments on lipid peroxidation in mitochondria[52] and SMPs[53] chemically depleted of and reconstituted with CoQ. The data in Table 3 above also confirm those of Takeshige et al.[53] and others [54] on the ability of succinate to decrease the rate of lipid peroxidation in mitochondrial systems and to lower the subsequent membrane damage. The direct relationship between the inhibition by reduced CoQ in microsomal membranes (Table 4) as well as the protective effect of succinate in the submitochondrial system (Table 3) supports the contention that it is the reduced state of CoQ which is able to react as an antioxidant. The strong antioxidant effect of CoQ in the microsomal system would also suggest a role for CoQ in endoplasmic reticular membranes, a question of function which is unresolved.[55]

Discovery of the existence of the semiquinone form of CoQ during electron transfer[56] has led to speculation that CoQ may be the source of free radicals produced during electron transfer chain activity.[57,58] The finding[53] (Table 3) that rotenone, a potent inhibitor of NADH-CoQ reductase which blocks the flow of electrons from NADH to CoQ, does not inhibit free radical production from NADH, strongly suggests that the superoxide radical produced during electron transfer is generated from flavin radicals[59] or nonheme-iron-sulfur protein[60] prior to the reduction of CoQ. Additional support for mitochondrial components other than CoQ as generators of the superoxide radical has appeared.[61,62]

The mechanism by which $CoQH_2$ acts as an antioxidant remains unresolved. Among the possibilities is the reaction of $CoQH_2$ with ADP-perferryl ion as suggested by Svingen et al.[63]:

$$ADP\text{-}Fe^{3+} - O_2^{\cdot -} + CoQH_2 \longrightarrow ADP\text{-}Fe^{2+} + H_2O_2 + CoQ$$

H_2O_2 would be removed by catalase, peroxidase, or glutathione peroxidase. Alternatively, reduced CoQ could react with superoxide directly as a free radical

quencher:

$$2\ O_2^{\cdot-} + CoQH_2 \longrightarrow H_2O_2 + O_2 + CoQ$$

and thus interfere with the initiation of lipid peroxidation. Furthermore, $CoQH_2$ might react with lipid free radicals or lipid peroxide free radicals:

$$2\ L\cdot + CoQH_2 \longrightarrow 2\ LH + CoQ$$

$$2\ LOO\cdot + CoQH_2 \longrightarrow 2\ LOOH + CoQ$$

and thus interfere with the propagation reaction of lipid peroxidation.

The role of free radicals and natural antioxidants in longevity and disease[64–66] is of obvious concern. Tissue CoQ concentrations fall with age[21,22] and, together with alpha-tocopherol,[67] are elevated in physically active individuals.[10,20,21] It would appear reasonable that investigation of the antioxidant role of CoQ in the increase in longevity in exercised rats[68,69] and active humans,[70,71] as well as the low incidence of breast cancer and cancer of the female reproductive system in former college athletes,[72,73] deserves serious attention. The efficacy of CoQ in the treatment and prevention of a number of pathological states has been well documented.[74–79]

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ABBREVIATIONS

CoQ—coenzyme Q
$CoQH_2$—reduced coenzyme Q
SMP—submitochondrial particle
TBA—thiobarbituric acid
IMM—inner mitochondrial membrane
CL—cardiolipin
MDA—malondialdehyde.