Free Radical Research, 2002 Vol. 36 (4), pp. 413–419

Taylor & Francis healthsciences

Effect of Dicumarol, a NAD(P)H: Quinone Acceptor Oxidoreductase 1 (DT-diaphorase) Inhibitor on Ubiquinone Redox Cycling in Cultured Rat Hepatocytes

TAKEO KISHI*, TAKAYUKI TAKAHASHI, SHINYA MIZOBUCHI, KOICHI MORI and TADASHI OKAMOTO

Faculty of Pharmaceutical Sciences and High Technology Research Center, Kobe Gakuin University, Nishi-ku, Kobe 651-2180, Japan

Accepted by Professor H. Sies

(Received 26 April 2001; In revised form 12 December 2001)

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

Ubiquinol is considered to serve as an endogenous antioxidant. However, the mechanism by which the redox state of intracellular ubiquinone (UQ) is maintained is not well established. The effect of dicumarol, an inhibitor of NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1 = DT-diaphorase, EC 1.6.99.2), on the reduction of UQ in cultured rat hepatocytes was investigated in order to clarify whether or not NQO1 is involved in reducing intracellular UQ. A concentration of 5 μM dicumarol, which does not inhibit cytosolic NADPH-dependent UQ reductase *in vitro*, was observed to almost completely inhibit NQO1 and thereby to stimulate cytotoxicity of 2-methyl-1,4-naphthoquinone (menadione) in cultured rat hepatocytes. However, 5 μM dicumarol did not inhibit reduction of endogenous UQ-9, as well as exogenous UQ-10 added to the hepatocytes. In addition, it did not stimulate the formation of thiobarbituric acid reactive substances (TBARS) in the hepatocytes. These results suggested that NQO1 is not involved in maintaining UQ in the reduced state in the intact liver cells.

Keywords: Ubiquinone; Rat hepatocytes; Dicumarol; Hydrogen peroxide; Quinone reductase; NQO1

INTRODUCTION

Ubiquinone (UQ), an essential electron carrier in the mitochondrial respiratory chain,[1] is synthesized not only in mitochondria[2] but also in the endoplasmic reticulum–Golgi membrane system,[3–5] and accordingly, present in cytoplasmic and other subcellular membranes.[6] Biological roles of UQ in these non-mitochondrial membranes are not well established, but some UQ is always present in ubiquinol (UQH_2) of reduced form, active as an antioxidant.[6,7] The antioxidant effect of UQH_2 has been demonstrated *in vitro* and *in vivo* in various biological systems.[8–11] Therefore, it has been hypothesized that UQ serves as an endogenous, recycling antioxidant,[12,13] as well as a cellular, lipophilic energy transporter. This hypothesis is attractive, considering that many of the endogenous antioxidants in the soluble fraction of cells; glutathione, ascorbic acid, thioredoxin and so on, are regenerated by intracellular enzymatic systems but α-tocopherol, a main antioxidant in the lipid membrane fractions is considered to be regenerated only by chemical redox reaction with hydrophilic reductants such as ascorbic acid. As UQH_2 resides with α-tocopherol in the membrane fractions, UQH_2 is expected to co-operate with α-tocopherol in preventing lipid peroxidation. For the purpose, however, it is necessary that UQ resulting from the antioxidant action is reduced constantly by some enzyme system(s) in cells.[14]

Reportedly, some enzymes serve as UQ reductases in animal cells. NADH-cytochrome b_5 reductase[15,16] and NADH/NADPH dehydrogenases are found in microsomes,[17] outer mitochondrial membrane,[18] Golgi complex[19] and plasma membranes,[20] and NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1 = DT-diaphorase, EC 1.6.99.2) in cytosol,[21] as well as mitochondrial respiratory UQ reductases

*Corresponding author. Tel.: +81-78-974-1551. Fax: +81-78-974-5689. E-mail: kishi@pharm.kobegakuin.ac.jp

ISSN 1071-5762 print/ISSN 1029-2470 online © 2002 Taylor & Francis Ltd
DOI: 10.1080/10715760290021261

RIGHTSLINK

have been suggested to be the quinone reductases involved in the reduction of cellular UQ. However, one electron transferring enzymes such as NADH-cytochrome b_5 reductase are considered unsuitable because they reduce UQ to UQH_2 via ubisemiquinone radicals as an intermediate which may work as a pro-oxidant in cells.[22] From this view point, NQO1, which is a two electron transferring enzyme mainly located in cytosol but also present in microsomes and mitochondria, seems to be a proper candidate for the enzyme responsible for maintaining cellular UQH_2 as an endogenous antioxidant. In fact, Beyer *et al.*[23] and Landi *et al.*[24] have shown that NQO1 is able to generate and to maintain the reduced UQ in uni- and multi-lamellar lipid vesicles and protected the vesicles from lipid peroxidation induced by 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), a thermolabile radical inducer, and that 20 μM dicumarol, a potent inhibitor of NQO1, made rat hepatocytes more susceptible to doxorubicin-induced lipid peroxidation.

In previous studies,[25–27] however, we found that the cytosol fraction of rat liver contains a novel NADPH-dependent UQ reductase (NADPH-UQ reductase) which has a Km of 12.4 μM for NADPH and 584 μM for NADH, can reduce UQ-10 in lecithin liposomes and subcellular membranes, and is resistant to dicumarol and rotenone, an inhibitor of mitochondrial NADH-UQ reductase (complex I). Furthermore, we observed that prolonged supplementation with UQ-10 caused a significant increase in the NADPH-UQ reductase activity in the liver, and protected the rats partially from carbon tetrachloride-induced hepatotoxicity.[28] In addition, rat hepatocytes having enhanced levels of NADPH-UQ reductase and UQH_2 were more resistant to H_2O_2-induced cytotoxicity than normal rat hepatocytes.[29] The findings as described led us to the notion that UQ with NADPH-UQ reductase constitutes a fundamental defense system against oxidative stress in cellular membranes.

In the present study, therefore, we investigated the effect of dicumarol on NQO1, NADPH-UQ reductase and the reduction of UQ in hepatocytes in an attempt to clarify whether NQO1 or NADPH-UQ reductase is involved in the reduction of UQ and its antioxidant action in cells.

MATERIALS AND METHODS

Chemicals and Animals

Ubiquinone-9 (UQ-9) and ubiquinone-10 (UQ-10) were kindly donated by Eisai (Bunkyo-ku, Tokyo, Japan). Ubiquinol-9 (UQH_2-9) and ubiquinol-10 (UQH_2-10) were prepared by reducing the corresponding UQ homologs with 0.25% sodium borohydride in ethanol.[6] For experiments using the cultured hepatocytes, a formulated UQ-10 solution (2.5 mg UQ-10/ml, Lot no. E0216-Z051, donated by Eisai) was employed in order to dissolve homogeneously in the medium. 3,3′-Methylene-bis(4-hydroxy-coumarin) (dicumarol) and 2-methyl-1,4-naphthoquinone (menadione) were dissolved in dimethylsulfoxide and ethanol, respectively. These vehicles were added to the control medium. 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, Shizuoka, Japan, and fed on Lavo MR Stock, a commercial feed (SLC) until use. The animals were handled according to the Guiding Principles for the Care and Use of Laboratory Animals (that were adhered to the Declaration of Helsinki Ethical Principles for Medical Research), approved by the Japanese Pharmacological Society.

Preparation of Rat Hepatocytes and Liver Cytosol Fraction

The rats were anesthetized with diethylether, injected with 0.2 ml of heparin (1000 U/ml) through the femoral vein, and then dissected. The liver was perfused with 20 ml of chilled isotonic saline through the hepatic portal vein to the inferior *vena cava* and removed as previously described.[6] Then, the liver was homogenized immediately with four volumes of chilled 50 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose and 1 mM ethylenediaminetetraacetic acid using a Potter-Elvehjem glass-Tefron homogenizer and fractionated stepwise by differential centrifugation at 600*g* for 10 min, 6500*g* for 20 min, 10,000*g* for 15 min, and 105,000*g* for 60 min.[30] The supernatant at the centrifugation of 105,000*g* was then used as the cytosol fraction.

Isolated hepatocytes were freshly prepared from rats by collagenase perfusion according to the method of Moldéus *et al.*[31] A rat was anesthetized with i.p. injection of pentobarbital (nembutal injection, from Abbott lab, II.), and dissected. Then, the liver was washed free of blood *in situ* with a buffer (pH 7.2) consisting of 5.4 mM KCl, 0.5 mM NaH_2PO_4, 137 mM NaCl, 0.42 mM Na_2HPO_4, 0.5 mM EGTA, 10 mM HEPES, 4.17 mM $NaHCO_3$ and 5.6 mM glucose through the hepatic portal vein to the inferior *vena cava* at a flow rate of 20 ml/min for 7 min at 38°C and after ligation of the superior *vena cava*, perfused with a collagenase solution (pH 7.5) consisting of 0.05% collagenase, 0.005% trypsin inhibitor, 5.4 mM KCl, 0.5 mM NaH_2PO_4, 137 mM NaCl, 4.17 mM $NaHCO_3$, 0.42 mM Na_2HPO_4, 0.95 mM $CaCl_2$, 10 mM HEPES and 5.6 mM glucose at the same flow rate for 7 min at 38°C. The digested liver was dissected and dispersed in 40 ml of Hanks' balanced salt solution to prepare isolated cells. The

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

RIGHTSLINK

isolated cells were filtrated through two layers of gauze and washed with the Hanks' solution three times. After viability was confirmed by the trypan blue exclusion test to be greater than 80%, the cells obtained were cultivated. The isolation of hepatocytes from rats did not have any striking effect on the cellular levels of antioxidants and related enzymes as described.[29] The hepatocytes were plated at a density of 10^6 cells in 2 ml of William's medium E (Gibco BRL, Life Technologies, Rockville, MD) containing 10 μg/ml streptomycin, 10 IU/ml penicillin, 0.02 U/ml insulin, 10^{-5} M dexamethasone and 10% fetal bovine serum, and maintained for 24 h at 37°C under a flow of 5% CO_2 in air. The primary culture obtained was used for the experiments.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

Cell Viability

The viability of the hepatocytes was determined by the trypan blue exclusion test (mixing of equivolumes of cell suspension and 3% trypan blue solution) and the release of lactate dehydrogenase (LDH, EC 1.1.1.27) from cells as described later.

Determination of UQ Metabolites

The UQH_2-9 and UQH_2-10 in cells were extracted with ethanol/*n*-hexane (2:5, v/v) and measured by high performance liquid chromatography (HPLC) with electrochemical detection (ECD), as described by Okamoto *et al.*[32] UQ-9 and UQ-10 were reduced to the corresponding UQH_2 homologs with 0.25% sodium borohydride before HPLC.

Enzyme Assays

NADPH-UQ reductase activity (pmol/min/mg protein) was determined as previously described.[27] A 240 μl volume of the reaction mixture in 50 mM Tris–HCl buffer (pH 7.4) contained 5 μl of 25 mM 2-mercaptoethanol, 5 μl of 0.5 $MgCl_2$, 5 μl of 10 mM NADPH, 5 μl of 4% Triton X-100 and 5 μl of 2.5 mM UQ-10. The enzyme reaction was started by adding 10 μl of enzyme sample, and after incubation for 10 min at 37°C, stopped with 3.5 ml of ethanol/*n*-hexane (2:5, v/v). Thereafter, the UQH_2-10 that was formed in the reaction mixture was extracted with *n*-hexane and measured by means of the HPLC method described above.

NQO1 activity (nmol/min/mg protein) was evaluated by measuring the reduction of cytochrome c as described by Ernster.[33] A 475 μl volume of the reaction mixture in 50 mM Tris–HCl buffer (pH 7.4) contained 10 μl of 4% Triton X-100, 5 μl of 1 mM menadione, 10 μl of 3.85 mM cytochrome c and 10 μl of 25 mM NADH. The enzyme reaction was started by adding 25 μl of enzyme sample, and an increase in optical density at a wavelength of 550 nm was observed at 37°C. The activity was estimated with molecular extinction coefficient, $\varepsilon = 1.85 \times 10^4\ M^{-1}\ cm^{-1}$.

Lactate dehydrogenase (LDH, EC 1.1.1.27) activity was measured by the method of Moldéus *et al.*[31] A 100 μl volume of the culture medium was added to 1100 μl of Hanks' medium containing 2% bovine serum albumin and 150 μl of 2 mM NADH, and stood for 10 min at 30°C. Then, the enzyme reaction was started with 150 μl of 14 mM pyruvate and a decrease in optical density at a wavelength of 340 nm was observed. Protein content was determined by the method of Lowry *et al.*[34]

Determination of Thiobarbituric Acid-reacting Substances (TBARS)

TBARS was determined by the method of Buege and Aust.[35] The culture was separated into the medium and the cells attached to the dish. The cells were washed once with 10 ml of phosphate buffered saline (consisting of 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4 and 7.8 mM Na_2HPO_4) and then scratched off from the dish by a rubber policeman with 0.5 ml of 0.1 M NaOH and after being neutralized with 0.5 ml of 0.1 M HCl, used for the TBARS measurement. The medium was used directly for the TBARS measurement. The samples were mixed with 3 ml of distilled water and 1 ml of a thiobarbituric acid reagent consisting of 8.8 mM acetic acid, 0.335% (w/v) 2-thiobarbituric acid and 0.04% (w/v) 2,6-di-*tert*-butyl-*p*-cresol and then incubated for 60 min in boiling water. After the mixture had cooled down to room temperature, TBARS fluorescence was extracted with 3 ml of *n*-butanol and fluorescent intensity measured at 515 nm (excitation) and 553 nm (emission). A solution of 1,1,3,3-tetraethoxypropane was used as the standard for the determination of TBARS.

RESULTS AND DISCUSSION

Effect of Dicumarol on NADPH-UQ Reductase and NQO1 Activities

Dicumarol is a potent inhibitor specific to NQO1.[21] In our previous study,[25] NADPH-UQ reductase of rat liver cytosol was 2–3 orders of magnitude more resistant to dicumarol than NQO1. First, the inhibitory effect of various concentrations of dicumarol on NADPH-UQ reductase and NQO1 activities in a cytosol fraction prepared from rat liver was determined (see Fig. 1). As expected, NQO1 was almost three orders of magnitude more sensitive to dicumarol than NADPH-UQ reductase. At concentrations of 1–5 μM, dicumarol almost completely inhibited the reduction of menadione by cytosol, that

RIGHTSLINK

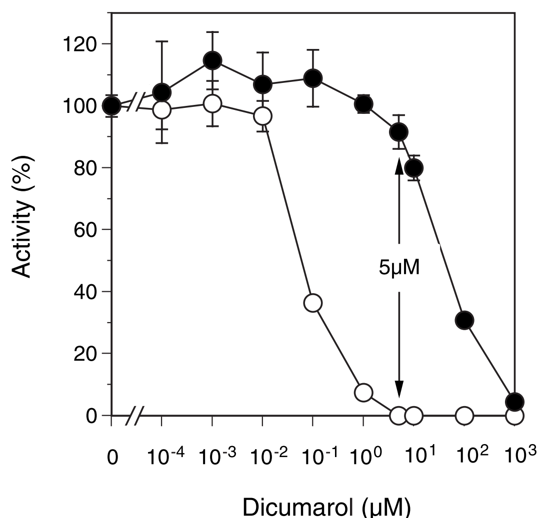

FIGURE 1 Effect of dicumarol on NQO1 and NADPH-UQ reductase activities in rat liver cytosol. NQO1 and NADPH-UQ reductase activities were determined as described in the text. Indicated concentrations of dicumarol were dissolved in dimethylsulfoxide and added to the reaction mixtures prior to addition of rat liver cytosol (enzymes). Open and closed circles show NQO1 and NADPH-UQ reductase activities, respectively. The values are the means ± SD of four experiments (expressed as % of the control without dicumarol).

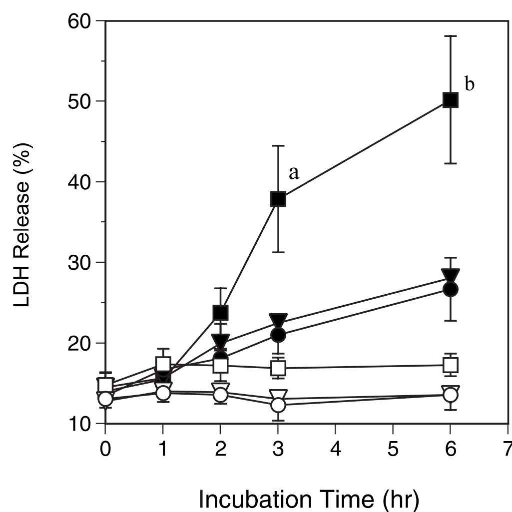

FIGURE 2 Cytotoxicity of menadione against cultured hepatocytes in the presence or absence of dicumarol. Cultured rat hepatocytes were prepared as described in the text, and incubated at 37°C for the indicated periods. Dicumarol and menadione were dissolved in dimethylsulfoxide and added to the culture medium at the onset of the incubation, and their effects were followed with the release of lactate dehydrogenase (LDH) from the cells to the medium. Open circle, no addition; open triangle, 0.5 μM dicumarol; open rectangle, 5 μM dicumarol; closed circle, 30 μM menadione; closed triangle, 30 μM menadione and 0.5 μM dicumarol; closed rectangle, 30 μM menadione and 5 μM dicumarol. The values are the means ± SD of three experiments (expressed as % of the control with no addition). $^{a}p < 0.05$,, $^{b}p < 0.01$ versus 30 μM menadione.

is, NQO1 activity, but hardly inhibited reduction of UQ-10 by cytosol, that is, NADPH-UQ reductase activity. However, dicumarol at over 10 μM was inhibitory to NADPH-UQ reductase as well as NQO1. Reportedly, dicumarol is not cytotoxic to isolated rat hepatocytes at concentrations up to 20 μM,[36] though dicumarol also inhibits some flavoenzymes such as aldehyde oxidase (EC 1.2.3.1) and uncouples mitochondrial oxidative phosphorylation. Therefore, 5 μM dicumarol appeared to be a suitable concentration with which to demonstrate whether NQO1 or NADPH-UQ reductase reduces intracellular UQ-10, provided that the dicumarol was easily incorporated into the cells.

Toxicity of Dicumarol in Cultured Hepatocytes

Menadione incorporated into hepatocytes undergoes one electron reduction by enzymes such as NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and NADH-cytochrome b_5 reductase (EC 1.6.2.2) to yield the labile semi-naphthoquinone radical or otherwise two electron reduction by NQO1 to yield the stable hydroquinone form. The former semiquinone radical can react with molecular oxygen to produce various reactive oxygen species such as superoxide anion, which results in the initiation of radical-induced oxidative alterations in intracellular thiols and Ca^{2+} homeostasis, DNA and cell membrane.[37,38] Therefore, the toxicity of menadione is considered to be influenced by the relative contributions of one electron reduction and two electron reduction in hepatocytes. The physiological role of NQO1 is to protect the cells from such radical-induced cytotoxicity by reducing menadione to the hydroquinone.[39–41] Consequentially, inhibition of intracellular NQO1 by dicumarol is expected to potentiate the cytotoxicity of menadione.

Thus, in order to examine whether or not 5 μM of dicumarol is enough to inhibit NQO1 activity in cultured hepatocytes, the toxicity of menadione to hepatocytes was compared in the presence or absence of 5 μM dicumarol. As shown in Fig. 2, 30 μM menadione alone little inhibited the cultured hepatocytes as reported[36] but the concomitant addition of 5 μM dicumarol remarkably potentiated the cytotoxicity of menadione. This is shown clearly by the release of lactate dehydrogenase from the cells.

Reduction of UQ-10 Added to Hepatocytes in the Presence of Dicumarol

Addition of 5 μM dicumarol to the medium was confirmed to be enough to inhibit NQO1 in cultured cells. Next, to examine whether or not 5 μM dicumarol inhibits UQ-reductase activity in the hepatocytes, the uptake and reduction of UQ-10 by the hepatocytes was compared in the presence or absence of 5 μM dicumarol. The results are shown in Fig. 3.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

RIGHTSLINK

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

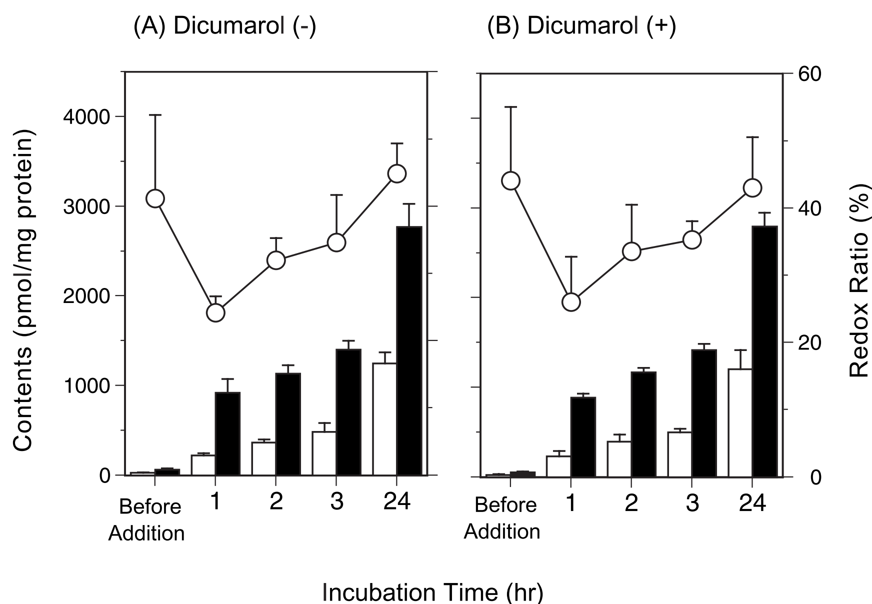

FIGURE 3 UQ-10 uptake and its reduction to UQH_2-10 by cultured hepatocytes in the presence or absence of dicumarol. The cultured rat hepatocytes were prepared as described in Fig. 2. Open and closed bars show the amounts of reduced and total UQ-10 extracted from the cells, respectively. Open circle shows the redox state (%) of UQ-10 which was defined as UQH_2-10 per sum of UQH_2-10 and UQ-10 found in the cells. A and B were performed in the absence and presence of dicumarol, respectively. The values are the means ± SD of three experiments.

Incorporation of the UQ-10 added to the culture medium into hepatocytes and rates of its reduction during incubation for up to 24 h were neither stimulated nor inhibited in the presence of 5 μM dicumarol. Furthermore, the addition of both UQ-10 and dicumarol to the medium did not produce any appreciable change in the amount and redox state of endogenous UQ-9 (Fig. 4). These results showed clearly that inhibition of intracellular NQO1 by dicumarol did not disturb redox states of UQ and UQ-reductase, probably cytosolic NADPH-UQ reductase,[26] activity in hepatocytes.

Effect of Dicumarol on H_2O_2-induced Lipid Peroxidation in Hepatocytes

UQH_2 is considered to be an endogenous antioxidant.[13] The results shown in Figs. 3 and 4

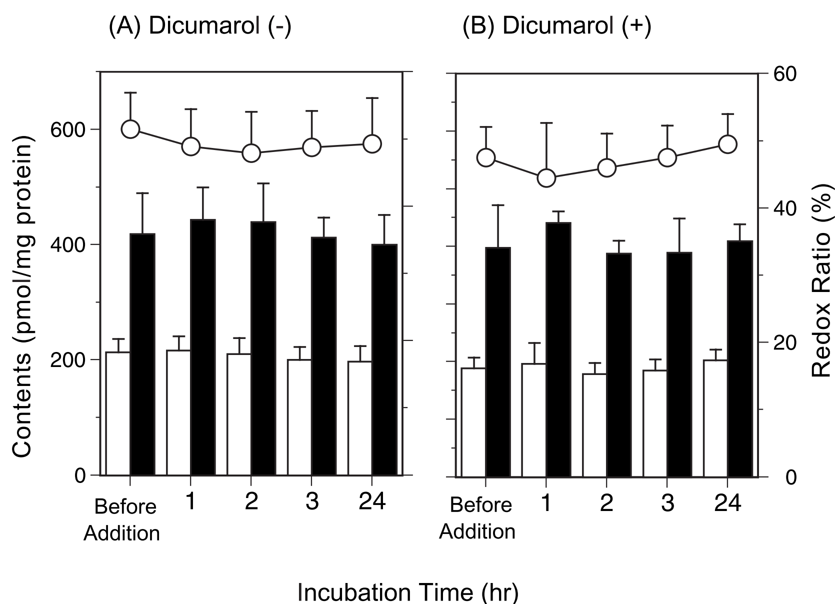

FIGURE 4 Effect of dicumarol on the amounts of endogenous UQ-9 and UQH_2-9 in rat hepatocytes. The cultured rat hepatocytes were prepared as described in Fig. 2. Open and closed bars show the amounts of reduced and total UQ-9 extracted from the cells, respectively. Open circle shows the redox state (%) of UQ-9 which was defined as UQH_2-9 per sum of UQH_2-9 and UQ-9 found in the cells. A and B were performed in the absence and presence of dicumarol, respectively. The values are the means ± SD of three experiments.

RIGHTSLINK

TABLE I Effects of dicumarol and UQ-10 on hydrogen peroxide-induced lipid peroxidation of cultured hepatocytes

Additions	TBARS formation ($nmol/10^6$ cells)
No addition	0.24 ± 0.02
$10\,\mu M$ UQ-10	0.21 ± 0.04
$5\,\mu M$ Dicumarol	0.19 ± 0.03
$5\,\mu M$ Dicumarol + $10\,\mu M$ UQ-10	0.23 ± 0.01
5 mM H_2O_2	1.59 ± 0.12
5 mM H_2O_2 + $5\,\mu M$ Dicumarol	1.74 ± 0.13
5 mM H_2O_2 + $10\,\mu M$ UQ-10	1.35 ± 0.04*
5 mM H_2O_2 + $5\,\mu M$ Dicumarol + $10\,\mu M$ UQ-10	1.26 ± 0.11**

The cultured rat hepatocytes were prepared as described in Fig. 2, and incubated with $5\,\mu M$ dicumarol at 37°C for 3 h and further with $10\,\mu M$ UQ-10 at 37°C for 3 h. Then, the cells were washed with phosphate buffered salts (consisting of 137 mM NaCl, 2.68 mM KCl, 1.47 mM KH_2PO_4, 7.8 mM Na_2HPO_4, 0.9 mM Ca^{2+}, and 0.33 mM Mg^{2+}) and placed in Williams E medium. The amounts of TBARS formed in the cells were determined as described in the text. The values are the means ± SD of three experiments. Statistical significance: $*p < 0.05$ versus 5 mM H_2O_2, $**p < 0.01$ versus 5 mM H_2O_2 + $5\,\mu M$ dicumarol.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

suggested that $5\,\mu M$ dicumarol did not inhibit reduction of externally added UQ-10 and endogenous UQ-9 in cultured hepatocytes. If NQO1 was involved in the cellular reduction of UQ to UQH_2, that is, redox recycling of cellular UQ and consequently cellular antioxidant action, dicumarol is expected to inhibit the antioxidant action of the cells. Then, $5\,\mu M$ dicumarol was added to the culture medium in order to examine whether or not it potentiates hydrogen peroxide-induced lipid peroxidation in cultured hepatocytes. The results are shown in Table I. Addition of 5 mM H_2O_2 produced a considerable increase in TBARS in cultured hepatocytes. On the other hand, addition of $10\,\mu M$ UQ-10 suppressed significantly the H_2O_2-induced formation of TBARS in the cells. The addition of $5\,\mu M$ dicumarol did not have any appreciable effect on either normal or H_2O_2-induced levels of TBARS in the cultured cells, even when $10\,\mu M$ UQ-10 was added to the medium.

NQO1 in rat liver is well known to be induced remarkably by polycyclic hydrocarbons such as 3-methylcoranthrene.[22] In a previous study, however, UQ-10 given to rats did not increase NQO1 activity in the liver.[29] Furthermore, UQ homologs with a long isoprenoid chain are not good substrates for NQO1[33] as compared to low molecular quinones such as menadione and 2,3-dimethyl-6-methyl-1,4-benzoquinone. Considering these points, NQO1 is unlikely to be the enzyme responsible for maintaining the redox state of cellular UQ. However, if low molecular quinones such as menadione and 1,4-naphthoquinone were to co-exist with UQ-10 in cells, reduction of UQ-10 by NQO1 would be stimulated markedly.[21] Therefore, if such a small quinone was present in cells, NQO1 may bear the responsibility for maintaining the redox state of cellular UQ.

The study described here showed that $5\,\mu M$ dicumarol, which inhibited NQO1 but not NADPH-UQ reductase in cultured rat hepatocytes, did not inhibit reduction of externally added UQ-10 or endogenous UQ-9 and did not increase the level of cellular TBARS. In other words, it is clear that NQO1 does not contribute to the regeneration of cellular UQH_2 and its antioxidant action. The present results do not demonstrate directly that NADPH-UQ reductase in cytosol is the enzyme responsible for reduction of cellular UQ. In a previous study, however, UQ-reducing activity was located mainly in the cytosol fraction and that preferred NADPH to NADH as a hydrogen donor,[26] similar to cytosolic antioxidant enzymes such as glutathione reductase (EC 1.6.4.2) and thioredoxin reductase (EC 1.6.4.5). Further, this NADPH-UQ reductase in rat liver was enhanced when UQ-10 was given to the animal,[28] and the animal with the enhanced enzyme activity was more resistant to carbon tetrachloride-induced hepatotoxicity. In addition, hepatocytes prepared from such animals were more resistant against H_2O_2-induced cytotoxicity than the normal hepatocytes.[29] Considering these findings, it is strongly suggested that not NQO1 but NADPH-UQ reductase in cytosol is the enzyme responsible for the reduction of cellular UQ and its antioxidant action.

Acknowledgements

The study was supported in part by a Grant-in-Aid for Science Research (08457617) from the Japanese Ministry of Education, Science, Sports and Culture of Japan and a grant from the Science Research Fund of the Japan Private School Promotion Foundation.

References

[1] Lenaz, G., De Santis, A. and Bertoli, E. (1985) "A survey of the function and specificity of ubiquinone in the mitochondrial respiratory chain", In: Lenaz, G., ed, *Coenzyme Q: Biochemistry, Bioenergeticus and Clinical Applications of Ubiquinone* (Wiley, New York), pp 165–199.

[2] Momose, K. and Rudney, H. (1972) "3-Polyprenyl-4-hydroxybenzoate synthesis in the inner membrane of mitochondria from *p*-hydroxybenzoate and isopentenylpyrophosphate", *J. Biol. Chem.* **247**, 3930–3940.

[3] Kalén, A., Appelkvist, E.L., Chojnacki, T. and Dallner, G. (1990) "Nonaprenyl-4-hydroxybenzoate transferase, an enzyme involved in ubiquinone biosynthesis, in the endoplasmic reticulum–Golgi system of rat liver", *J. Biol. Chem.* **265**, 1158–1164.

[4] Teclebrhan, H., Jakobsson-Borin, Å., Brunk, U. and Dallner, G. (1995) "Relationship between the endoplasmic reticulum–Golgi membrane system and ubiquinone biosynthesis", *Biochim. Biophys. Acta* **1256**, 157–165.

[5] Kalén, A., Norling, B., Appelkvist, E.L. and Dallner, G. (1987) "Ubiquinone biosynthesis by the microsomal fraction from rat liver", *Biochim. Biophys. Acta* **926**, 70–78.

[6] Takahashi, T., Okamoto, T., Mori, K., Sayo, H. and Kishi, T. (1993) "Distribution of ubiquinone and ubiquinol homologues in rat tissues and subcellular fractions", *Lipids* **28**, 803–809.

RIGHTSLINK

[7] Åberg, F., Appelkvist, E.L., Dallner, G. and Ernster, L. (1992) "Distribution and redox state of ubiquinones in rat and human tissues", *Arch. Biochem. Biophys.* **295**, 230–234.
[8] Yamamoto, Y., Komuro, E. and Niki, E. (1990) "Antioxidant activity of ubiquinol in solution and phosphatidylcholine liposome", *J. Nutr. Sci. Vitaminol. (Tokyo)* **36**, 505–511.
[9] Stocker, R., Bowry, V.W. and Frei, B. (1991) "Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α-tocopherol", *Proc. Natl Acad. Sci. USA* **88**, 1646–1650.
[10] Mohr, D., Bowry, V.W. and Stocker, R. (1992) "Dietary supplementation with coenzyme Q10 results in increased levels of ubiquinol-10 within circulating lipoproteins and increased resistance of human low-density lipoprotein to the initiation of lipid peroxidation", *Biochim. Biophys. Acta* **1126**, 247–254.
[11] Beyer, R.E. (1988) "Inhibition by coenzyme Q of ethanol- and carbon tetrachloride-stimulated lipid peroxidation *in vivo* and catalyzed by microsomal and mitochondrial systems", *Free Radic. Biol. Med.* **5**, 297–303.
[12] Ernster, L. and Dallner, G. (1995) "Biochemical, physiological and medical aspects of ubiquinone function", *Biochim. Biophys. Acta* **1272**, 195–204.
[13] Ernster, L. and Forsmark-Andrée, P. (1993) "Ubiquinol: an endogenous antioxidant in aerobic organisms", *Clin. Invest.* **71**, S60–S65.
[14] Kishi, T., Takahashi, T., Usui, A. and Okamoto, T. (1999) "Ubiquinone redox cycle as a cellular antioxidant defense system", *Biofactors* **10**, 131–138.
[15] Navarro, F., Villalba, J.M., Crane, F.L., Mackellar, W.C. and Navas, P. (1995) "A phospholipid-dependent NADH-coenzyme Q reductase from liver plasma membrane", *Biochem. Biophys. Res. Commun.* **212**, 138–143.
[16] Villalba, J.M., Navarro, F., Gomez-Diaz, C., Arroyo, A., Bello, R.I. and Navas, P. (1997) "Role of cytochrome b5 reductase on the antioxidant function of coenzyme Q in the plasma membrane", *Mol. Asp. Med.* **18**, S7–S13.
[17] Shigemura, T., Kang, D., Nagata-Kuno, K., Takeshige, K. and Hamasaki, N. (1993) "Characterization of NAD(P)H-dependent ubiquinone reductase activities in rat liver microsomes", *Biochim. Biophys. Acta* **1141**, 213–220.
[18] Shimada, H., Hirai, K., Simamura, E. and Pan, J. (1998) "Mitochondrial NADH-quinone oxidoreductase of the outer membrane is responsible for paraquat cytotoxicity in rat livers", *Arch. Biochem. Biophys.* **351**, 75–81.
[19] Crane, F. and Morré, D.J. (1977) "Evidence for coenzyme Q function in Golgi membranes", In: Folkers, K. and Yamamura, Y., eds, *Biomedical and Clinical Aspects of Coenzyme Q* (Elsevier, Amsterdam), pp 3–14.
[20] Villalba, J.M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F.L. and Navas, P. (1995) "Coenzyme Q reductase from liver plasma membrane: purification and role in *trans*-plasma-membrane electron transport", *Proc. Natl Acad. Sci. USA* **92**, 4887–4891.
[21] Ernster, L., Danielson, L. and Ljunggren, M. (1962) "DT diaphorase 1. Purification from the soluble fraction of rat-liver cytoplasm, and properties", *Biochim. Biophys. Acta* **58**, 171–188.
[22] Cadenas, E., Hochstein, P. and Ernster, L. (1992) "Pro- and antioxidant functions of quinones and quinone reductases in mammalian cells", *Adv. Enzymol. Relat. Areas Mol. Biol.* **65**, 97–146.
[23] Beyer, R.E., Segura-Aguilar, J.S., Di Bernardo, S., Cavazzoni, M., Feto, R., Fiorentini, D., Galli, M.C., Setti, M., Landi, L. and Lenaz, G. (1996) "The role of DT-diaphorase in the maintenance of the reduced antioxidant form of coenzyme Q in membrane systems", *Proc. Natl Acad. Sci. USA* **93**, 2528–2532.
[24] Landi, L., Fiorentini, D., Galli, M.C., Segura-Aguilar, J. and Beyer, R.E. (1997) "DT-diaphorase maintains the reduced state of ubiquinones in lipid vesicles thereby promoting their antioxidant function", *Free Radic. Biol. Med.* **22**, 329–335.
[25] Takahashi, T., Shitashige, M., Okamoto, T., Kishi, T. and Goshima, K. (1992) "A novel ubiquinone reductase activity in rat cytosol", *FEBS Lett.* **314**, 331–334.
[26] Takahashi, T., Yamaguchi, T., Shitashige, M., Okamoto, T. and Kishi, T. (1995) "Reduction of ubiquinone in membrane lipids by rat liver cytosol and its involvement in the cellular defense system against lipid peroxidation", *Biochem. J.* **309**, 883–890.
[27] Takahashi, T., Okamoto, T. and Kishi, T. (1996) "Characterization of NADPH-dependent ubiquinone reductase activity in rat liver cytosol: effect of various factors on ubiquinone-reducing activity and discrimination from other quinone reductases", *J. Biochem. (Tokyo)* **119**, 256–263.
[28] Takahashi, T., Sugimoto, N., Takahata, K., Okamoto, T. and Kishi, T. (1996) "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", *Biol. Pharm. Bull.* **19**, 1005–1012.
[29] Takahashi, T., Hohda, T., Sugimoto, N., Mizobuchi, S., Okamoto, T., Mori, K. and Kishi, T. (1999) "Antioxidant roles of cellular ubiquinone and related redox cycles: potentiated resistance of rat hepatocytes having stimulated NADPH-dependent ubiquinone reductase against hydrogen peroxide toxicity", *Biol. Pharm. Bull.* **22**, 1226–1233.
[30] Hogeboom, G.H. (1955) "Fractionation of cell components of animal tissues", *Methods Enzymol.* **1**, 16–19.
[31] Moldéus, P., Högberg, J. and Orrenius, S. (1978) "Isolation and use of liver cells", *Methods Enzymol.* **52**, 60–71.
[32] Okamoto, T., Fukunaga, Y., Ida, Y. and Kishi, T. (1988) "Determination of reduced and total ubiquinones in biological materials by liquid chromatography with electrochemical detection", *J. Chromatogr. Biomed. Appl.* **430**, 11–19.
[33] Ernster, L. (1967) "DT-diaphorase", *Methods Enzymol.* **10**, 309–317.
[34] Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) "Protein measurement with the folin phenol reagent", *J. Biol. Chem.* **193**, 265–275.
[35] Buege, J.A. and Aust, S.D. (1978) "Microsomal lipid peroxidation", *Methods Enzymol.* **52**, 302–310.
[36] Silva, J.M., Ramakrishna Rao, D.N. and O'Brien, P.J. (1992) "Modulation of trenimon-induced cytotoxicity by DT-diaphorase in isolated rat hepatocytes under aerobic versus hypoxic conditions", *Cancer Res.* **52**, 3015–3021.
[37] Thor, H., Smith, M.T., Hartzell, P., Bellomo, G., Jewell, P. and Orrenius, S. (1982) "The metabolism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes. A study of the implications of oxidative stress in intact cells", *J. Biol. Chem.* **257**, 12419–12425.
[38] Morrison, H., Jernström, B., Nordenskjöld, M., Thor, H. and Orrenius, S. (1984) "Induction of DNA damage by menadione(2-methyl-1,4–naphthoquinone) in primary cultures of rat hepatocytes", *Biochem. Pharmacol.* **33**, 1763–1769.
[39] Lind, C., Hochstein, P. and Ernster, L. (1982) "DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation", *Arch. Biochem. Biophys.* **216**, 178–185.
[40] Munday, R., Smith, B.L. and Munday, C.M. (1998) "Effects of butylated hydroxyanisole and dicumarol on the toxicity of menadione to rats", *Chem.-Biol. Interact.* **108**, 155–170.
[41] Radjendirane, V., Joseph, P., Lee, Y.-H., Kimura, S., Klein-Szanto, A.J.P., Gonzalez, F.J. and Jaiswal, A.K. (1998) "Disruption of the DT diaphorase (NQO1) gene in mice leads to increased menadione toxicity", *J. Biol. Chem.* **273**, 7382–7389.

Free Radic Res Downloaded from informahealthcare.com by Library of Health Sci-Univ of Il on 11/24/11
For personal use only.

RIGHTSLINK