Antioxidant Roles of Cellular Ubiquinone and Related Redox Cycles: Potentiated Resistance of Rat Hepatocytes Having Stimulated NADPH-Dependent Ubiquinone Reductase against Hydrogen Peroxide Toxicity

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Received March 5, 1999; accepted August 3, 1999

Protective effect of the cellular ubiquinone (UQ) reducing system linked to cytosolic NADPH-dependent ubiquinone reductase (NADPH-UQ reductase) against hydrogen peroxide (H₂O₂)-induced lipid peroxidation was investigated using UQ and control hepatocytes freshly isolated from rats injected with UQ-10 and the vehicles 14 d in advance, respectively. The UQ hepatocytes had higher levels of ubiquinol (UQH₂)10 content and NADPH-UQ reductase activity than the control hepatocytes but did not differ in other antioxidant factors from the latter cells. The UQ hepatocytes exhibited higher cell viability and lower release of lactate dehydrogenase than the control hepatocytes when they were exposed to H₂O₂ of up to 100 μM for 1 h at 37°C. Furthermore, the formation of thiobarbituric acid reactive substances (TBARS) by H₂O₂ was almost completely inhibited in the UQ hepatocytes. Decreases in UQH₂ and α-tocopherol contents and NADPH-UQ reductase activity by H₂O₂ exposure were observed in both types of the hepatocytes, but those levels in the UQ hepatocytes after the exposure were still higher than in the control hepatocytes. The decreases in ascorbic acid, reduced glutathione and protein thiol contents and DT-diaphorase activity by H₂O₂ were not different between in the two types of hepatocytes. Antioxidant enzyme activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione S-transferase and glutathione reductase in the hepatocytes were not inhibited by H₂O₂. From these results, it was concluded that the cellular UQ reducing system linked to cytosolic NADPH-UQ reductase functions mainly as an antioxidant defense for cellular membranes.

Key words antioxidants; rat hepatocytes; hydrogen peroxide; lipid peroxidation; NADPH-quinone reductase; ubiquinone

Ubiquinone (UQ), an essential electron carrier in the mitochondrial respiratory chain, has received much attention as an endogenous antioxidant because it is wide-spread in biological membranes such as microsomes, the Golgi apparatus and cytoplasmic membrane, and some of these membranes have enzymes for UQ biosynthesis. In fact, much evidence of an antioxidant action of UQ and/or ubiquinol (UQH₂) has been obtained by in vitro and in vivo observations in various biological systems.

For example, Stocker et al. showed that UQH₂-10 protects human low density lipoprotein (LDL) more efficiently against lipid peroxidation than does α-tocopherol. Leibovitz et al. showed that tert-butyl hydroperoxide-induced lipid peroxidation in rat tissue slices was depressed by feeding the rats a diet fortified with UQ-10 before the experiment. Similar antioxidant effects of UQ have also been observed in carbon tetrachloride (CCl₄)-induced lipid peroxidation in rats and the endotoxin-induced hepatic damage in mice. These results suggested that UQ is easily reduced to UQH₂, and then acts as an antioxidant in animal tissues. However, Frei and Gaziano reported that UQH₂-10 did not show significant protective effects against the metal ion-dependent or independent oxidation of LDL from human plasma. Zamora et al. also showed that the antioxidant effectiveness of dietary UQ-10 is rather limited in protecting the liver and erythrocytes from uninduced oxidative damage in comparison with α-tocopherol. As described above, the antioxidant action of UQH₂ in the biological systems is still debatable.

In biological systems, some part of UQ is always present as UQH₂. In a previous paper, we showed that UQH₂ accounted for 70–80% of the total amount of UQ and UQH₂ (tUQ) in rat liver and plasma, and for 10–30% in the other tissues. Aberg et al. also found a much greater ratio of UQH₂ in some rat and human tissues. In general, however, the amounts of UQH₂ would be limited as compared with the amounts of lipid peroxides formed in the tissues. Therefore, the effectiveness of UQH₂ as an endogenous antioxidant must be dependent on the reduction rate of UQ resulting from the antioxidant action.

In further studies from this point of view, we found that cytosol fractions from rat tissues have an NADPH-dependent UQ reductase (NADPH-UQ reductase) activity which is resistant to dicumarol, an inhibitor of DT-diaphorase (=NAD(P)H: (quinone-acceptor) oxidereductase, EC 1.6.99.2) and to rotenone and antimycin A, inhibitors of mitochondrial respiratory enzymes. Further, the enzyme reduced not only the UQ incorporated into lecithin liposomes but also the native UQ in microsomes, and subsequently inhibited lipid peroxidation in these membranes. Thus, we suspected that this cytosolic NADPH-UQ reductase may be the enzyme responsible for the reduction of UQ to UQH₂ in intracellular membranes adjacent to the cytosol. In a subsequent study, we observed that a prolonged intraperitoneal supplementation of rats with UQ-10 caused significant increases in the UQH₂-10 content and cytosolic NADPH-UQ reductase activity in the livers without any appreciable change in other endogenous antioxidants and related enzymes. In addition, the rats with the enhanced levels of UQ-10 and NADPH-UQ reductase were resistant to CCl₄-induced hepatitis to some extent, nevertheless the consumption of UQH₂-10 by CCl₄-treatment in their livers was much lower than that in the control rat livers. These results suggested that UQ redox cycling by the cytosolic NADPH-UQ reductase is one of the fundamental defense systems against...
cellular lipid peroxidation.

In the present study, to elucidate how the UQ reducing system linked to cytosolic NADPH-UQ reductase cooperates with other antioxidant factors in cells against oxidative stress, we investigated the effect of hydrogen peroxide (H$_2$O$_2$), a native, reactive oxygen species on hepatocytes having an increased UQH$_2$-10 content and cytosolic NADPH-UQ reductase activity, isolated from the rats described above.\(^7\)

**MATERIALS AND METHODS**

**Materials** UQ-9 and UQ-10 were kindly donated by Eisai Co. (Tokyo, Japan). UQH$_2$-9 and UQH$_2$-10 were prepared by reducing the corresponding UQ homologues with 0.25% sodium borohydride in ethanol. All other chemicals were of the highest grade commercially available.

**Preparation of Hepatocytes** Specific pathogen-free, male Wistar rats (8 weeks old, 170—180 g body weight) were purchased from SLC Co. (Shizuoka, Japan). The animals were conditioned essentially as previously described.\(^7\) Rats were intraperitoneally injected with 2 mg of UQ-10 (2 ml of 0.9% NaCl solution containing 1 mg UQ-10 solubilized with 2% HCO-60) per kilogram body weight daily for 2 weeks. The paired control rats were intraperitoneally injected with an equal volume of the vehicle (0.9% NaCl solution containing 2% HCO-60) for the same period. UQ and control hepatocytes were freshly isolated from the UQ-10-injected and control rat livers, respectively, by collagenase digestion according to the method of Moldes et al.\(^16\) The isolation of hepatocytes from rats did not produce any striking effect on the cellular levels of antioxidants and related enzymes. The viability of cells obtained was always greater than 90%. The hepatocytes were mainly used for the experiments of H$_2$O$_2$ cytotoxicity not as a primary culture but as a freshly prepared cell suspension,\(^7\) because incubating the cells in a culture medium causes modifications of enzyme expression\(^18,19\) and cell structures,\(^19,20\) as well as cellular compositions, such as fatty acids by the ingredients of the medium\(^21\), further, iron added to the medium potentiates cytotoxicity of H$_2$O$_2$ by Fenton’s reaction.\(^22\)

**H$_2$O$_2$ Treatment of Hepatocytes** The freshly isolated hepatocytes were suspended in Krebs-Henseleit buffer containing 12.6 mm HEPES (pH 7.4) and 5 mm glucose as 1×10$^6$ cells/ml, and exposed to final concentrations of 1—100 mm H$_2$O$_2$ in the buffer for 1 h at 37°C under 5% CO$_2$/95% air. Then, various parameters such as the viability of the cells, low molecular weight antioxidant contents, antioxidant enzyme activities and formation of thiobarbituric acid reactive substances (TBARS) in the cells were measured as described below.

**Cell Viability** The viability of the hepatocytes was determined by the trypan blue exclusion test and the release of lactate dehydrogenase (LDH, EC 1.1.1.27) from cells.\(^16\)

**Determination of Low Molecular Weight Antioxidants** The amounts of UQH$_2$-9, UQH$_2$-10, UQ-9 and UQ-10 were determined by high performance liquid chromatography with an electrochemical detector (ECD) according to the method of Okamoto et al.\(^23\) α-Tocopherol content was determined by the method of Tamai et al.\(^24\) Ascorbic acid (AsA) and dehydroascorbic acid (DAS A) contents were determined by a modification of Coustard and Sudraud’s method\(^25\) using a mixture of 5 mm N-hexadecyl-N,N,N-trimethylammonium bromide and 0.1 mm KH$_2$PO$_4$ in H$_2$O—methanol (95:5, v/v) as the mobile phase and ECD (840-EC, Jasco Co., Tokyo) at +0.6 V vs. Ag/AgCl as a detector. This modification allowed the detection of 1 ng of AsA. Reduced glutathione (GSH) and its oxidized dimer (oxidized glutathione, GSSG) in the reaction mixture were extracted by the addition of an equal volume of 13% trichloroacetic acid, followed by centrifugation at 1600 g for 30 min. The GSSG and total glutathione (sum of GSH and GSSG) contents were estimated as measuring acid-soluble thiol by a 5,5’-dithiobis(2-nitrobenzoic acid)-glutathione reductase (GR, EC 1.6.4.2) recycling method.\(^26\) The precipitate obtained by the 13% trichloroacetic acid treatment was dissolved in 0.5 M Tris·HCl buffer (pH 7.6), and was used for the determination of protein thiol content, employing the method of Monte et al.\(^27\)

**Enzyme Assays** The NADPH-UQ reductase activity was determined by measuring the UQH$_2$-10 formed in the presence of NADPH, as previously described.\(^13\) The catalase (EC 1.11.1.6) activity was determined by Aebi’s method.\(^28\) Superoxide dismutase (SOD, EC 1.15.1.1) [Cu$^{2+}$- and Zn$^{2+}$-containing SOD (Cu$^{2+}$, Zn$^{2+}$-SOD) and Mn$^{2+}$-containing SOD (Mn$^{2+}$-SOD)] activities were determined by Oyanagi’s method.\(^29\) The glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was done by Paglia and Valentine’s method.\(^30\) The glutathione S-transferase (GST, EC 2.5.1.18), GR and DT-diaphorase activities were determined by Habig and Jakoby’s method.\(^31\) Carlberg and Mannervik’s method,\(^32\) and Erster’s method,\(^33\) respectively.

**Determination of TBARS and Protein Contents** The TBARS content was determined by the method of Buege and Aust.\(^34\) The protein content was determined by the method of Lowry et al.\(^35\)

**RESULTS**

**Effect of Incubation Media on H$_2$O$_2$, Cytotoxicity** Cytotoxicity of H$_2$O$_2$ is well known to be potentiated by some transition metal ions which stimulate the formation of hydroxyl radical (·OH) from H$_2$O$_2$, by Fenton’s reaction.\(^32\) Therefore, the effect of H$_2$O$_2$ on release of LDH from hepatocytes, i.e., the cell viability, was investigated under 2 conditions of a cell suspension in Krebs-Henseleit buffer with glucose and a primary culture in Williams’ medium E for 24 h at 37°C. The results are shown in Table 1. LDH release from the primary culture by 5 mm H$_2$O$_2$ was more than that from the cell suspension by 100 mm H$_2$O$_2$, and was almost completely protected by the addition of deferoxamine mesylate, an iron chelator. Thus, in order to avoid unexpected effects of extracellular ·OH formed from H$_2$O$_2$ with Fe$^{2+}$ in the

<table>
<thead>
<tr>
<th>Cell suspension</th>
<th>16.6±1.1</th>
<th>34.4±5.4</th>
<th>78.5±4.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture</td>
<td>6.1±4.8</td>
<td>91.2±0.8</td>
<td>96.1±5.7</td>
</tr>
<tr>
<td>Primary culture + 2 mm deferoxamine mesylate</td>
<td>6.9±5.8</td>
<td>9.5±1.5</td>
<td>26.7±10.8</td>
</tr>
</tbody>
</table>

The values are expressed as % of LDH released from hepatocytes treated with indicated amounts of H$_2$O$_2$ for 1 h at 37°C, and are the mean±S.D. of 3 experiments.

Table 1. Effect of H$_2$O$_2$ on Release of LDH from Hepatocyte
medium on cellular antioxidant systems, the cell suspension in the Krebs–Hanseleit buffer was used for the experiments below.

**Biochemical Properties of Freshly Isolated Control and UQ Hepatocytes** The levels of antioxidants and related enzymes in the control and UQ hepatocytes are listed in Table 2. The 2 types of cells had about the same levels of all determined antioxidants and related enzymes, except for NADPH-UQ reductase, as expected from the previous paper.7) The level of cytosolic NADPH-UQ reductase, but not DT-diaphorase in the UQ hepatocytes was significantly higher than that in the control hepatocytes. It is noteworthy that the prior injection with UQ-10 did not induce DT-diaphorase activity in their livers, since it suggests that this enzyme is not necessary for the reduction of UQ to UQH₂.

The levels of UQ and UQH₂ homologues in the hepatocytes are shown in Fig. 1. The UQ-9 was at almost the same level in both hepatocytes, but UQH₂-9 was significantly higher in the UQ hepatocytes than in the control hepatocytes (p<0.001). The UQ-10 and UQH₂-10 contents in the UQ hepatocytes were also 4 to 5 times (p<0.001) as high as those in the control hepatocytes owing to the UQ-10 injected in advance. In addition, the redox ratios (UQH₂/UQ) of UQ-9 and UQ-10 in the UQ hepatocytes were higher than those in the control cells (p<0.001). The increased ratios appear to be due to NADPH-UQ reductase activity which was increased with the injected UQ-10. These results confirmed that freshly isolated hepatocytes preserve almost the same biochemical situation as livers in situ of rats injected with UQ-10 as shown previously.7)

**Cell Toxicity of H₂O₂ to Control and UQ Hepatocytes** Control and UQ hepatocytes were exposed to toxic doses of 10 to 100 mM H₂O₂ for 60 min at 37°C. The cell toxicity of H₂O₂ was evaluated by the 2 parameters of the trypan blue exclusion test (Fig. 2A) and the release of cellular LDH to the medium (Fig. 2B). In all the concentrations from 10 to 100 mM H₂O₂, the number of cells stained by trypan blue and the amounts of LDH released from the cells were significantly lower in the UQ hepatocytes than in the control hepatocytes. However, UQ-10 (which was up to three times the effect magnitude of the UQ hepatocytes) added to the culture of the control hepatocytes did not help to protect the cells from H₂O₂ toxicity at all (data not shown).

**Effect of H₂O₂ on Lipid Peroxidation** Effect of 1 to 100 mM H₂O₂ on the contents of TBARS in hepatocytes is shown in Fig. 3. The level of TBARS in the H₂O₂-exposed control cells increased up to about 5 times that in non-exposed cells, depending on the concentrations of H₂O₂. On the other hand, the level of TBARS in the UQ hepatocytes increased only twice or less even at 100 mM H₂O₂ as much as that in the non-exposed cells, and was much lower than that in the control hepatocytes.

**Effect of H₂O₂ on UQ and UQH₂ Contents** The contents of UQ and UQH₂ homologues in the control and UQ hepatocytes exposed to H₂O₂ are shown in Table 3. UQH₂-9 and UQH₂-10 were rapidly and simultaneously oxidized to...
Table 3. tUQ and UQH₂ Contents in Control and UQ Hepatocytes Exposed to H₂O₂

<table>
<thead>
<tr>
<th>H₂O₂ added</th>
<th>tUQ-9 contents (pmol/mg protein)</th>
<th>UQH₂-9</th>
<th>Redox ratios of UQ-9 (%)</th>
<th>tUQ-10 contents (pmol/mg protein)</th>
<th>UQH₂-10</th>
<th>Redox ratios of UQ-10 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control hepatocytes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>696±17</td>
<td>496±5</td>
<td>71±1</td>
<td>82±5</td>
<td>50±5</td>
<td>61±7</td>
</tr>
<tr>
<td>1 mm</td>
<td>671±63</td>
<td>387±35(e)</td>
<td>58±4(e)</td>
<td>72±9</td>
<td>38±6(e)</td>
<td>52±5</td>
</tr>
<tr>
<td>0 mm</td>
<td>692±19</td>
<td>345±42(e)</td>
<td>50±6(e)</td>
<td>80±6</td>
<td>31±4(e)</td>
<td>39±7(e)</td>
</tr>
<tr>
<td>50 mm</td>
<td>729±49</td>
<td>206±69(e)</td>
<td>28±9(e)</td>
<td>83±9</td>
<td>23±4(e)</td>
<td>28±6(e)</td>
</tr>
<tr>
<td>100 mm</td>
<td>770±94</td>
<td>174±56(e)</td>
<td>22±5(e)</td>
<td>89±9</td>
<td>20±5(e)</td>
<td>22±3(e)</td>
</tr>
<tr>
<td>UQ hepatocytes:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition</td>
<td>726±38</td>
<td>585±10³(1)</td>
<td>81±5³(1)</td>
<td>641±18³(1)</td>
<td>502±18³(1)</td>
<td>78±5³(1)</td>
</tr>
<tr>
<td>1 mm</td>
<td>724±40</td>
<td>512±37³(1)</td>
<td>71±6³(1)</td>
<td>641±27³(1)</td>
<td>458±27³(1)</td>
<td>71±2³(1)</td>
</tr>
<tr>
<td>0 mm</td>
<td>734±45</td>
<td>461±49³(1)</td>
<td>63±3³(1)</td>
<td>632±28³(1)</td>
<td>415±22³(1)</td>
<td>66±2³(1)</td>
</tr>
<tr>
<td>50 mm</td>
<td>714±49</td>
<td>370±58³(1)</td>
<td>52±6³(1)</td>
<td>640±21³(1)</td>
<td>355±21³(1)</td>
<td>55±3³(1)</td>
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<tr>
<td>100 mm</td>
<td>723±32</td>
<td>280±35³(1)</td>
<td>39±3³(1)</td>
<td>633±12³(1)</td>
<td>262±36³(1)</td>
<td>41±5³(1)</td>
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</tbody>
</table>

The values are the mean±S.D. of 3–4 experiments. The indicated amounts of H₂O₂ were added to control and UQ hepatocyte suspensions, and then each cell suspension was incubated at 37°C. After 1 h, the tUQ and UQH₂ homologues in both types of hepatocytes were measured as described in the text. a) (UQH₂/tUQ)×100. b) δ, c) e): Significant differences at levels of p<0.05, p<0.01, p<0.005 and p<0.001, respectively, compared with no addition in the same type of hepatocytes, using Student’s unpaired t-test. j) δ, g) h): Significant differences at levels of p<0.05, p<0.01, p<0.005 and p<0.001, respectively, compared with control hepatocytes, using Student’s unpaired t-test.

Fig. 3. H₂O₂-Induced Lipid Peroxidation of Control and UQ Hepatocytes

The indicated amounts of H₂O₂ were added to control and UQ hepatocyte suspensions, and then each cell suspension was incubated at 37°C. After 1 h, the TBARS content in both types of hepatocytes was assayed as described in the text. Open and solid bars indicate TBARS formations of control and UQ hepatocytes, respectively. The values are the mean±S.D. of 6 experiments. a) and b) Significant differences at levels of p<0.005 and p<0.001, respectively, compared with control hepatocytes, using Student’s unpaired t-test.

Table 4. α-Tocopherol Content of Control and UQ Hepatocytes Exposed to H₂O₂

<table>
<thead>
<tr>
<th>H₂O₂ added</th>
<th>α-Tocopherol content (pmol/mg protein)</th>
<th>Control hepatocytes</th>
<th>UQ hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>148±27</td>
<td>145±18</td>
<td></td>
</tr>
<tr>
<td>1 mm</td>
<td>133±20</td>
<td>142±17</td>
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<tr>
<td>10 mm</td>
<td>126±14</td>
<td>121±14</td>
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<tr>
<td>50 mm</td>
<td>103±29³(1)</td>
<td>112±16³(1)</td>
<td></td>
</tr>
<tr>
<td>100 mm</td>
<td>90±9³(1)</td>
<td>108±13³(1)</td>
<td></td>
</tr>
</tbody>
</table>

The values are the mean±S.D. of 6 experiments. The indicated amounts of H₂O₂ were added to control and UQ hepatocyte suspensions, and then each cell suspension was incubated at 37°C. After 1 h, the α-tocopherol content in both types of hepatocytes were assayed as described in the text. a), b), c) Significant differences at levels of p<0.05, p<0.01 and p<0.001, respectively, compared with no addition of H₂O₂ in the same type of hepatocytes, using Student’s unpaired t-test. j) δ, g) h): Significant difference at level of p<0.05 compared with control hepatocytes, using Student’s unpaired t-test.

Table 5. Disappearance of Protein Thiols in Control and UQ Hepatocytes

<table>
<thead>
<tr>
<th>H₂O₂ added</th>
<th>Protein thiol content (nmol/mg protein)</th>
<th>Control hepatocytes</th>
<th>UQ hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>106±8</td>
<td>102±15</td>
<td></td>
</tr>
<tr>
<td>1 mm</td>
<td>105±7</td>
<td>98±9</td>
<td></td>
</tr>
<tr>
<td>10 mm</td>
<td>99±6</td>
<td>100±14</td>
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<tr>
<td>50 mm</td>
<td>95±4³(1)</td>
<td>97±10</td>
<td></td>
</tr>
<tr>
<td>100 mm</td>
<td>88±4³(1)</td>
<td>85±7³(1)</td>
<td></td>
</tr>
</tbody>
</table>

The values are the mean±S.D. of 3–4 experiments. The indicated amounts of H₂O₂ were added to control and UQ hepatocyte suspensions, and then each cell suspension was incubated at 37°C. After 1 h, the protein thiol contents in both types of hepatocytes were assayed as described in the text. a), b), c) Significant differences at levels of p<0.05 and p<0.01, respectively, compared with no addition of H₂O₂ in the same type of hepatocytes, using Student’s unpaired t-test.

Effect of H₂O₂ on α-Tocopherol Content

Effect of H₂O₂ on the levels of α-tocopherol in the control and UQ hepatocytes is shown in Table 4. The α-tocopherol contents in both types of hepatocytes diminished dose-dependently and significantly by more than 50 mm H₂O₂. In the presence of 100 mm H₂O₂, however, the level of α-tocopherol in the UQ hepatocytes was significantly higher than that in the control hepatocytes (p<0.05). These results demonstrate that α-tocopherol which locate mainly in lipid membranes with UQ is saved or spared in the presence of the increased UQ reducing system.

Effect of H₂O₂ on Cellular Protein Thiols

Effect of H₂O₂ on contents of cellular protein thiols is shown in Table 5. The addition of more than 50 mm H₂O₂ caused a moderate...
loss of cellular protein thiols, but the amounts of protein thiols lost were not significantly different between the control and UQ hepatocytes. This suggests that cellular UQH₂ is not available for protecting the cellular protein thiols, which are assumed to locate mainly in the hydrophilic surface of proteins, from oxidative attack by H₂O₂.

**Effect of H₂O₂ on Water Soluble Antioxidants**  Effect of H₂O₂ on the levels of AsA and GSH in hepatocytes is shown in Fig. 4. The contents of AsA (Figs. 4A and 4B) and GSH (Figs. 4C and 4D) decreased dose-dependently, and there was no significant difference between the decreases in the control and UQ hepatocytes over the tested concentrations of H₂O₂. In addition, the loss of GSH was followed by the formation of almost equivalent amounts of GSSG (Fig. 4D), but the loss of AsA did not produce a concomitant increase in DAsA, the oxidized form of AsA (Fig. 4A). The results suggest that the actions of these hydrophilic antioxidants are independent of UQH₂ in protecting the cells against the toxic effect of H₂O₂. Considering the previous results⁷ that the losses of AsA and GSH in livers of rats injected with CCl₄ did not produce the concomitant increases in their oxidized forms, the present results suggest that DAsA may easily be converted to 2,3-diketo-gulonic acid or further catabolites rather than AsA in the cells. On the other hand, the cellular GSH–GSSG redox cycle appeared to be able to operate as an antioxidant system if GSSG was not released from the cells, namely, as long as the cells were not exposed to such drastic conditions that intracellular proteins like LDH flowed out from the cells.

**Effect of H₂O₂ on DT-diaphorase and NADPH-UQ Reductase**  Effect of H₂O₂ on the levels of DT-diaphorase and NADPH-UQ reductase activities in the hepatocytes is shown in Fig. 5. These two enzymes, of which thiol groups are involved in the enzymatic action, were inhibited strongly by the concentrations of 50 to 100 m mM H₂O₂. DT-diaphorase activity was not significantly different between the control and UQ hepatocytes, but NADPH-UQ reductase activity in the UQ hepatocytes was significantly higher than that in the control over all concentrations of 10 to 100 m mM H₂O₂ tested.

**Effect of H₂O₂ on Antioxidant Enzyme Activities**  Effect of H₂O₂ on the levels of catalase, SOD, GSH-Px, GST and GR in hepatocytes is shown in Fig. 6. The levels of these enzyme activities were not significantly different between the control and UQ hepatocytes and were not inhibited by the exposure to H₂O₂ up to 100 m mM.

Looking over all the results obtained above, there is no doubt that the UQ hepatocytes had a potentiated redox cycle of UQ residing in cellular membranes linked to cytosolic NADPH-UQ reductase, which was significantly high compared to that in the control hepatocytes over all the tested concentrations of H₂O₂ up to 100 m mM. Accordingly, it is deduced that in the UQ hepatocytes, this potentiated UQ redox cycle, but not other antioxidants such as AsA and GSH, depressed lipid peroxidation and/or spared α-tocopherol.

**DISCUSSION**

It is well known that H₂O₂ is always generated by intracellular metabolic systems such as xanthine oxidase, glucose oxidase and mitochondrial respiration. In general, a physiological amount of H₂O₂ must not be cytotoxic because of the existence of 2 intracellular H₂O₂-metabolic enzymes, catalase and GSH-Px. However, an excessive amount of H₂O₂ induces irreversible injuries for many types of cells.⁷⁻⁻^⁻^⁻
Fig. 5. DT-diaphorase and NADPH-UQ Reductase Activities in Control and UQ Hepatocytes Exposed to \( \text{H}_2\text{O}_2 \)

The indicated amounts of \( \text{H}_2\text{O}_2 \) were added to the control (C) and UQ hepatocyte (●) suspensions, and then each cell suspension was incubated at 37 °C. After 1 h, the DT-diaphorase (A) and NADPH-UQ reductase (B) activities in both types of hepatocytes were assayed as described in the text. The values are the mean±S.D. of 6 experiments. a) and b) Significant difference at level of \( p<0.005 \) and \( p<0.001 \), respectively, compared with control hepatocytes, using Student’s unpaired \( t \)-test.

Fig. 6. Antioxidant Enzyme Activities in Control and UQ Hepatocytes Exposed to \( \text{H}_2\text{O}_2 \)

The indicated amounts of \( \text{H}_2\text{O}_2 \) were added to control (C) and UQ hepatocytes (●) suspensions, and then each cell suspension was incubated at 37 °C. After 1 h, the catalase (A), Cu\(^{2+}\), Zn\(^{2+}\)-SOD plus Mn\(^{2+}\)-SOD (B), Mn\(^{2+}\)-SOD (C), GSH-Px (D), GST (E) and GR (F) activities in both types of hepatocytes were assayed as described in the text. The values are the mean±S.D. of 6 experiments.

\( \text{H}_2\text{O}_2 \) can easily pass through the cell membrane,\(^{40} \) and then be quickly converted to 'OH, a very reactive oxygen species by the Fenton's reaction catalyzed by transition metal ions like Fe\(^{3+}\).\(^{22} \) Then, the 'OH causes lipid peroxidation in the cellular membranes\(^{37} \) and other oxidative dysfunctions, such as perturbation of the intracellular Ca\(^{2+}\) homeostasis.\(^{41} \) and finally leads to cell death. Actually, deferoxamine mesylate greatly protected cultured hepatocytes from \( \text{H}_2\text{O}_2 \)-induced lipid peroxidation and cell killing (Table 1). Reportedly, prolonged incubation of over 1 h with \( \text{H}_2\text{O}_2 \) causes the killing of hepatocytes by a mechanism independent of lipid peroxidation.\(^{37} \) In addition, some other ingredients, as well as Fe\(^{3+}\) in the culture medium can also produce some changes in biochemical properties and morphological features of the cells during the incubation.\(^{17}-^{21} \) In the present study, therefore, the hepatocyte suspension was employed and exposed to \( \text{H}_2\text{O}_2 \) for 1 h at 37 °C, in order to know how UQ and related antioxidants in situ in animal cells behave against oxidative stress produced by \( \text{H}_2\text{O}_2 \).

Previously we observed that UQ-10 given to rats produced significant increases in cellular UQH\(_2\)-10 content and NADPH-UQ reductase activity without any appreciable effect in other antioxidants or related enzymes in their livers.\(^{2} \) Such rats were resistant to CCl\(_4\)-induced radical hepatitis to some extent. Furthermore, analyses of intracellular antioxidants revealed that in the UQ hepatocytes \( \alpha \)-tocopherol was
spared, but the water-soluble antioxidants AsA and GSH were not. Interestingly, the oxidation of AsA and GSH did not produce concomitant increases in their oxidized forms. The observations imply that UQH₂ protected lipid membranes and their residents like α-tocopherol, but not watersoluble antioxidants of AsA and GSH from oxidative stress. In addition, the enzymatic regeneration of AsA and GSH seemed not to be operative in the cells.

The hepatocytes employed here exhibited about the same antioxidant properties as the original animals did (see Table 2), that is, the UQ hepatocytes were also resistant to oxidative stress produced by H₂O₂, to some extent. The formation of TBARS and the loss of α-tocopherol by H₂O₂ were apparently depressed in the UQ hepatocytes. A similar result was obtained by Suarna et al.⁵² using freshly isolated rat plasma LDL. Frei et al.⁴³ and Yamamoto et al.⁴⁴ also reported that UQH₂-10 spares α-tocopherol, not only by directly inhibiting lipid peroxidation, but also probably by regenerating it from an α-tocopherol radical resulting from its antioxidant action. The increased UQH₂-10 and NADPH-UQ reductase did not inhibit the dose-dependent decreases in cellular AsA, GSH and protein thiol by H₂O₂. These observations suggest that the cellular UQ redox cycle does not function as an antioxidant in aqueous phase of cells.

Interestingly, the decrease in GSH accompanied a concomitant increase in GSSG, but that in AsA did not accompany the concomitant formation of DAsA. In animals, water-soluble, low molecular antioxidants would easily flow out from the cells to blood circulation due to plasma membrane disruption as shown by the release of LDH, but in cell suspensions, the compounds flowing out can be recovered in the medium. The results obtained from the present and previous studies described above suggest that the enzymatic recycling of GSH is operative in the hepatocytes as long as the soluble antioxidant does not release from the cells, but that of AsA is not operative due to the further degradation, even if the cell membrane is preserved. A number of papers⁴⁵—⁵⁷ have proposed a chemical redox linkage of AsA and α-tocopherol. However, considering that most cellular defense systems against oxidative attacks are supported by enzymes, the chemical linkage of AsA and α-tocopherol may be thought to be an accidental reaction unanticipated by biological systems.

Recently, Landi et al.⁴⁸ reported that DT-diaphorase was able to reduce UQ to UQH₂ and to inhibit lipid peroxidation, as did NAPDH-UQ reductase. However, the DT-diaphorase activity in the UQ hepatocytes was at the same level and declined at the same rate as that in the control hepatocytes by H₂O₂ treatment. Therefore, it is evident that the potentiated redox cycle of UQ in the UQ hepatocytes is not due to DT-diaphorase.

It is well known that NADPH is supplied mainly through the pentose phosphate pathway in cytosol. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) catalyzes oxidation of Glucose-6-phosphate to d-glucono-δ-lactone-6-phosphate in the presence of NADP⁺, i.e., the first reaction of this pathway is reported to be activated by a concentration range of GSSG which can be observed in cells exhibiting lipid peroxidation.⁴⁹ Actually, increased activity of this enzyme has been observed in livers of rats exposed to oxidative stress.⁵⁰ On the other hand, phosphofructokinase (EC 2.7.1.11)⁵¹ and pyruvate kinase (EC 2.7.1.40)⁵² key enzymes of glycolysis which exclusively produce NADH, have been reported to be inactivated by GSSG. A gear-shift of glucose metabolism from the glycolysis to the pentose phosphate cycle by GSSG increased by oxidative stress is assumed to result in the enhanced production of NADPH in cytosol. Then, the enhanced level of NADPH must stimulate the enzymatic systems related to NADPH and GSH of glutaredoxin reductase, thioredoxin reductase, GSH-Px, GST and GR in aqueous phase of the cell and, at the same time, result in the enhanced formation of UQH₂ by the NADPH-UQ reductase in the membrane phase of the cell.

Acknowledgements His work 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.

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