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RELATIONSHIPS BETWEEN ASCORBIC ACID AND α-TOCOPHEROL DURING DIQUAT-INDUCED REDOX CYCLING IN ISOLATED RAT HEPATOCYTES*

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Abstract—The effects of diquat-induced redox cycling on the levels of cellular ascorbic acid and α-tocopherol were investigated in isolated rat hepatocytes. In untreated hepatocytes, the metabolism of 1 or 2 mM diquat resulted in the depletion of cellular ascorbic acid and glutathione, but not of α-tocopherol, in association with the induction of cell death during the experimental period. In 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) pretreated cells, 1 mM diquat induced cell death accompanied by loss of glutathione, ascorbic acid and α-tocopherol, and the initiation of lipid peroxidation. The loss of glutathione was rapid (to 9% of controls by 15 min) and cell ascorbate was completely consumed by 2 hr of incubation. In contrast, cellular α-tocopherol levels were stable for the first 30 min, but were depleted in association with the onset of lipid peroxidation. Supplementation of 0.1 or 1.0 mM ascorbic acid in the incubation medium delayed the onset of diquat-induced α-tocopherol loss, lipid peroxidation and cytotoxicity. When the concentration of exogenous cellular ascorbic acid was consumed to below that of endogenous ascorbic acid, α-tocopherol loss and lipid peroxidation were initiated. The results indicate that untreated hepatocytes have an effective multicomponent antioxidant system against diquat-induced oxidative stress. However, when glutathione is depleted from hepatocytes by treatment with BCNU and diquat, ascorbic acid plays a vital role in maintaining cellular α-tocopherol levels and survival of the cell.

It is well established that α-tocopherol is located in biological membranes and plays an important antioxidant role against oxidative damage of the membranes. Similarly, ascorbic acid is one of the major water soluble antioxidants in the aqueous compartments of cells. Additionally, both vitamins may play a prominent part in the protection of extracellular fluids such as blood plasma [1] and bronchial epithelial lining fluid [2]. Although the antioxidative interaction, or synergistic antioxidant effect, between these vitamins is well studied in *in vitro* systems such as liposomes [3], micelles [4] and cellular organelles [5], little information is available on the relationship between these compartmentalized vitamins in intact hepatocytes.

Diquat, a bipyridilium herbicide, induces the formation of reactive oxygen species by intracellular redox cycling [6, 7] and causes acute hepatocellular toxicity accompanied by glutathione (GSH) depletion and lipid peroxidation [8]. In isolated hepatocytes these effects are critically dependent upon the inhibition of GSH reductase activity by 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) [7, 9]. Other studies have shown that some soluble antioxidants protect hepatocytes against the cytotoxicity of diquat [10–12]. Despite this, although lipid peroxidation is inhibited with tocopherol analogues and some antioxidants, the onset of cytotoxicity is not delayed [7]. Others have suggested that the oxidation of protein thiols is of primary importance for diquat toxicity, since the loss of protein thiols and cytotoxicity were prevented by the thiol reducing agent dithiothreitol (DTT) [13]. The mechanism of diquat-induced hepatotoxicity is, however, still not completely understood.

In this study, we report the comparative disposition of hepatocellular glutathione, ascorbic acid and α-tocopherol during diquat-induced redox cycling. In addition, we have investigated whether or not the supplementation of exogenous ascorbic acid can prevent the cytotoxicity induced by diquat in hepatocytes. The results reveal the complexity of the interrelationships between hepatocellular low molecular weight antioxidants and confirm a central role for cell ascorbate in controlling the consequences of cellular oxidative stress.

MATERIALS AND METHODS

Chemicals. Diquat dibromide (purity >99%) was the kind gift of Dr L. L. Smith of Imperial Chemical Industries PLC (Millbank, London, U.K.). BCNU was purchased from Bristol-Meyers Pharmaceuticals (Stockholm, Sweden). Ascorbic acid, α-tocopherol and all other reagents were commercial products of the highest available grade of purity.

Preparation of hepatocytes. Male Wistar rats (230–300 g) were used in all experiments. Hepatocytes were isolated by the method of Moldéus *et al.* [14]. Cell viability was assayed by counting the percentage of the hepatocytes which excluded 0.16% trypan

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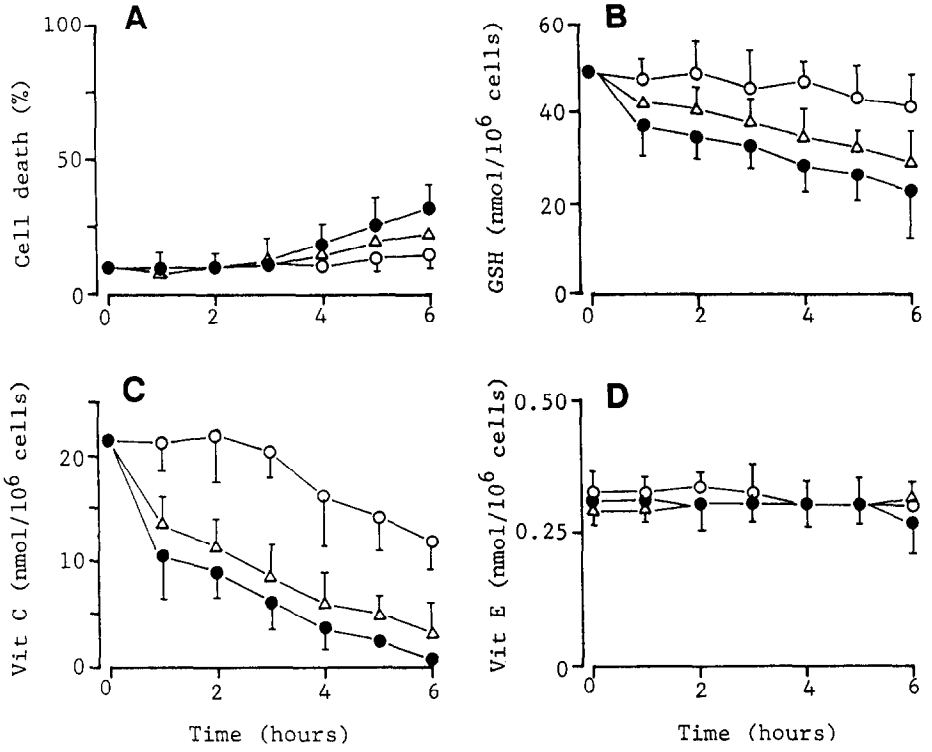

Fig. 1. Effects of 1 or 2 mM diquat on cell viability (A) and levels of GSH (B), ascorbic acid (Vit C) (C) and α-tocopherol (Vit E) (D) of isolated hepatocytes. Hepatocytes were isolated from untreated rats and were incubated at 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 1.0 mM diquat (△) and 2.0 mM diquat (●), as described in Materials and Methods. Results are expressed by the means ± SD from three experiments.

blue and approximately >90% of the freshly hepatocytes routinely excluded trypan blue [14].

Incubation of hepatocytes. Hepatocytes (10^6 cells/mL) were suspended in Krebs–Henseleit buffer (pH 7.4) containing 12.5 mM *N*-(2-hydroxyethyl)piperazone-*N'*-(2-ethanesulfonic acid) (Hepes). All incubations were performed in rotating, round-bottom flasks at 37° under an atmosphere of 95% O_2 and 5% CO_2. In some experiments, the isolated hepatocytes were pretreated with 50 μM BCNU for 25 min and then allowed to recover their reduced GSH in an amino acid medium containing 0.2 mM methionine and 0.2 mM cysteine for 90 min, essentially as described previously [7, 9]. After BCNU treatment, the activity of GSH reductase was inhibited >90% as assayed according to Eklöw *et al.* [9] and cell viability was decreased to approximately 80% post recovery. The recovery treatment restored GSH in the cells to normal level, i.e. 50–60 nmol GSH/10^6 cells. Reactions were started by the addition of diquat dissolved in Krebs–Henseleit buffer. In ascorbic acid supplementation experiments, the compound, dissolved in Krebs–Henseleit buffer, was added into the cell medium 5 min prior to diquat treatment. Aliquots of incubation mixture were taken at various times for the analyses of cell viability, GSH, ascorbic acid, α-tocopherol and malondialdehyde (MDA).

Biochemical assays. Cellular GSH was determined by HPLC essentially as described by Reed *et al.* [15]. Extraction and determination of ascorbic acid and α-tocopherol from hepatocytes were based on the methods of Honegger *et al.* [16] and Lang *et al.* [17], respectively. Both vitamins were analysed by HPLC with electrochemical detection; Antec model CU-03 (Antec Instruments, Leiden, Netherlands) in the oxidative mode (at 0.5–0.7 V). The analytical column used for separation of both vitamins was 150 mm × 4.6 mm i.d. containing Supelcosil L-18, 3 μm average particle size (Supelco, Bellefonte, PA, U.S.A.). The recoveries of ascorbic acid and α-tocopherol were checked for the compounds of interest by addition of known amounts of the working standards to hepatocytes and both recoveries were approximately 95% using these methods. Reproducibility of the procedure was tested by analysing representative samples in triplicate and coefficients of variation of assays of ascorbic acid and α-tocopherol on the same day were 3.4% and 4.2%, respectively. The lower limits of ascorbic acid and α-tocopherol assays were more than 5 and 2 pmol at 0.5 V, respectively. Malondialdehyde (MDA) was measured as thiobarbituric acid-reactive products, as described previously [7]. The amount of reactive products formed was calculated by using an extinction coefficient of 156 mM^{-1} cm^{-1} [18].

RESULTS

Diquat induced the loss of GSH and ascorbic acid

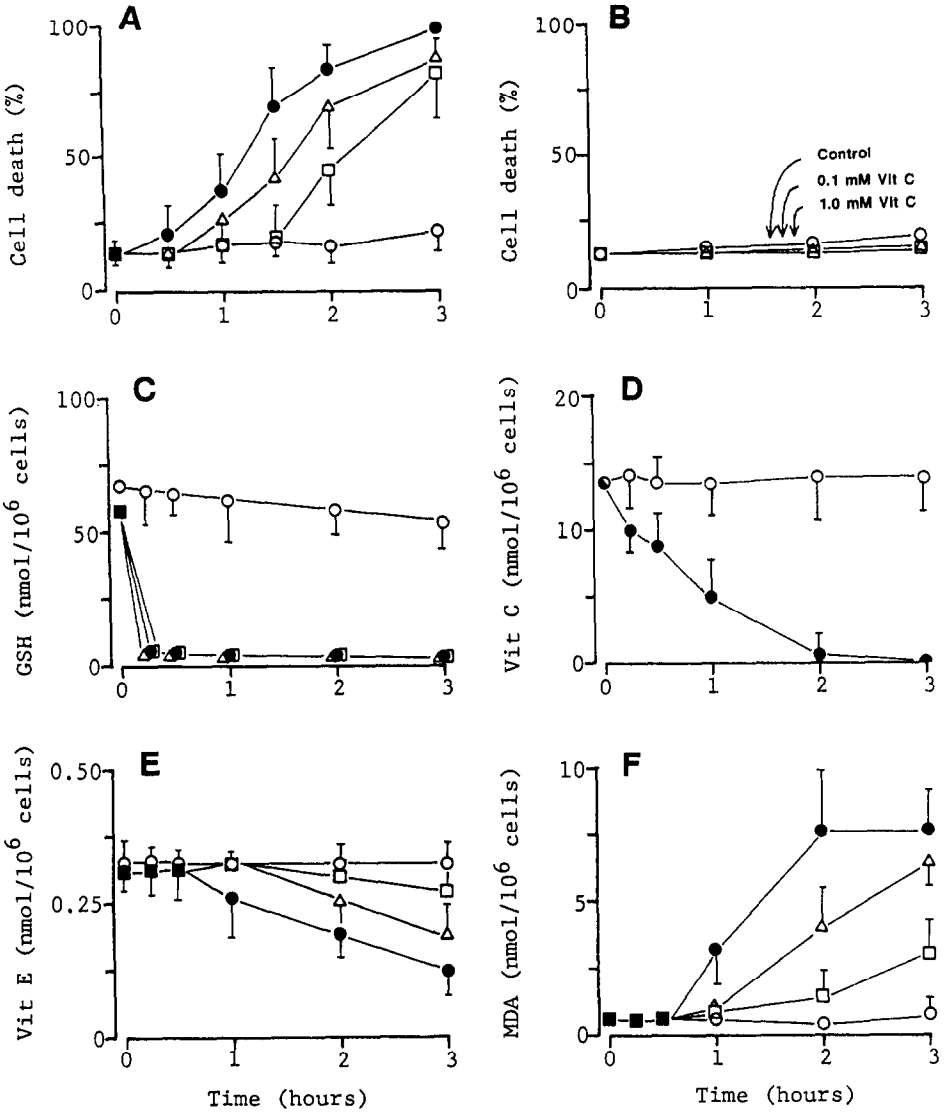

Fig. 2. Effects of diquat and/or ascorbic acid (Vit C) supplementation on BCNU-pretreated hepatocytes; cell viability (A), cell viability with vitamin C alone (B), and levels of GSH (C), vitamin C (D), α-tocopherol (Vit E) (E) and malondialdehyde (MDA) (F). BCNU-pretreated hepatocytes were prepared as described in Materials and Methods and were incubated at 10^6 cells/mL in Krebs–Henseleit buffer, pH 7.4, with no addition (○), 1 mM diquat alone (●), 1 mM diquat plus 0.1 mM vitamin C (△), 1 mM diquat plus 1.0 mM vitamin C (□). Results are expressed by the means ± SD from three experiments.

in untreated (normal) hepatocytes as shown in Fig. 1B and C, respectively. The loss of ascorbic acid was dose-dependent and was more rapid than that of GSH. By 6 hr, ascorbic acid was completely undetectable in 2 mM diquat-treated cells. At the same time, however, GSH levels were approximately 50% of the initial value. α-Tocopherol levels were not affected by diquat (1 and 2 mM) throughout the experimental period (Fig. 1D). Cell viability was slightly decreased by 6 hr after diquat treatment (Fig. 1A) and the changes were not dependent on cellular α-tocopherol level. The results indicate that normal hepatocytes have an effective antioxidant system against diquat-induced cytotoxicity, especially within the aqueous milieu of the cell.

In contrast in BCNU-pretreated hepatocytes 1 mM diquat induced a rapid decline in cell viability accompanied by the consumption of GSH, ascorbic acid, α-tocopherol and by the accumulation of MDA (Fig. 2). The loss of GSH was rapid (to approximately 9% of controls by 15 min) (Fig. 2C) as described previously [11]. Ascorbic acid levels were completely undetectable by 2 hr of incubation (Fig. 2D). In contrast, α-tocopherol levels were stable for the first 30 min but began to decline in association with the onset of MDA accumulation (Fig. 2E and F). When ascorbic acid was consumed by more than 50% of initial levels by diquat, the loss of α-tocopherol proceeded (Fig. 2D and E). Thus, the results suggest that ascorbic acid plays a role as an important

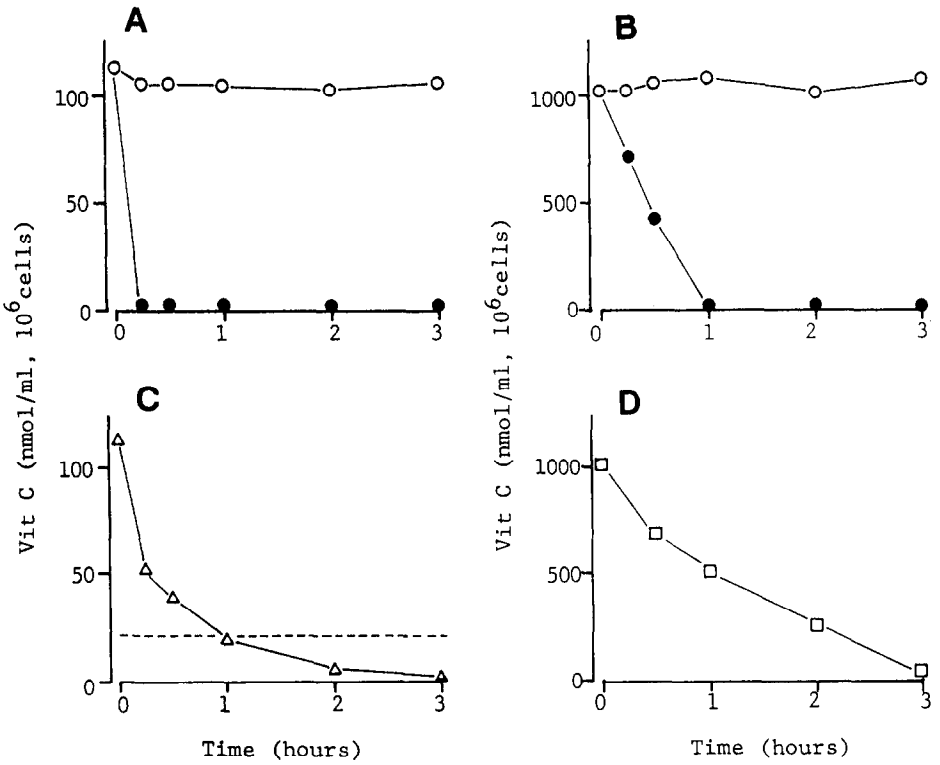

Fig. 3. Changes in total (endogenous and supplemented) ascorbic acid (Vit C) levels in BCNU-pretreated hepatocytes; (A) 0.1 mM vitamin C was added in the cell medium (○) or cell free Krebs–Henseleit buffer (●), (B) 1.0 mM vitamin C was added in the cell medium (○) or cell free Krebs–Henseleit buffer (●), (C) 0.1 mM vitamin C and 1 mM diquat were added in the cell medium (△) and (D) 1.0 mM vitamin C and 1 mM diquat were added in the cell medium (□). A dotted line means normal level of endogenous vitamin C in hepatocytes. Results shown are from one experiment typical of three.

antioxidant in aqueous compartments against the depletion of α-tocopherol, when cellular GSH levels are depleted by BCNU and diquat cotreatment.

To investigate the effect of ascorbic acid on diquat-induced cytotoxicity, ascorbic acid was added to the incubation mixture. Supplementation of 0.1 or 1.0 mM ascorbic acid delayed the onset of cytotoxicity caused by diquat (Fig. 2A). Ascorbic acid alone (0.1 or 1.0 mM) did not affect the cell viability during the incubation (Fig. 2B). Although the depletion of GSH levels induced by diquat was not inhibited by supplementation of ascorbic acid (Fig. 2C), the supplementation did delay the onset of α-tocopherol depletion and MDA accumulation (Fig. 2E and F). The effects caused by added ascorbic acid were dose-dependent and the changes in α-tocopherol levels and MDA levels exhibited an inverse relationship. Thus, supplementation of ascorbic acid merely delayed the development of cytotoxicity and loss of α-tocopherol caused by diquat.

Figure 3 shows the changes in total incubation ascorbic acid levels (endogenous plus exogenous) after the supplementation of ascorbic acid in BCNU and diquat-cotreated hepatocytes. After addition of 0.1 mM exogenous ascorbic acid, total ascorbic acid levels were decreased rapidly by diquat metabolism and fell below normal endogenous levels by 2 hr (Fig. 3C). In comparison, the depletion of ascorbic acid levels after 1.0 mM ascorbic acid supplementation was relatively slow, falling below endogenous levels by 3 hr (Fig. 3D). In contrast, no significant depletion of total ascorbic acid levels was induced in diquat-free hepatocytes (Fig. 3A and B). The results indicate that when total ascorbic acid is consumed to below that of normal endogenous ascorbic acid, the depletion of α-tocopherol and production of MDA are initiated. It was interesting to note that although ascorbic acid was rapidly depleted in Krebs–Henseleit buffer, the compound added into cell medium (hepatocytes plus Krebs–Henseleit buffer) was stable for the experimental period (Fig. 3A and B). This indicates that hepatocytes have an effective maintenance (reducing) system for exogenous ascorbic acid.

DISCUSSION

Protection of the intracellular environment from oxidative stress is dependent on a network of cellular reductants or antioxidants and on the activity of various enzymes [19–21]. Diquat generates superoxide anion radical and hydrogen peroxide in rat liver microsomes and in isolated rat hepatocytes by redox cycling with molecular oxygen [6, 7]. The

present study using rat hepatocytes demonstrates that ascorbic acid and GSH function in a coordinated manner as water soluble antioxidants against the oxidative stress induced by diquat. Although normal (untreated) hepatocytes, which contained sufficient GSH and ascorbic acid, resisted the metabolism of diquat (Fig. 1), diquat treatment of BCNU-pretreated cells caused rapid depletion of GSH and ascorbic acid and subsequently the loss of α-tocopherol and the induction of cytotoxicity and of lipid peroxidation (Fig. 2). In addition, it is apparent that GSH plays a very important part in defense system against diquat-induced cytotoxicity. The lipid peroxidation did not proceed until α-tocopherol was substantially depleted, and the consumption of α-tocopherol did not begin until ascorbic acid level was depleted by more than about 50% of the initial level. These results indicate a cascade of inter-relationships between these redox couples.

The consumption of α-tocopherol could be prevented temporarily by the presence of sufficient ascorbic acid, since the supplementation of exogenous ascorbic acid delayed the onset of diquat-induced depletion of α-tocopherol even if GSH was almost (>90%) consumed (Fig. 2). Ascorbic acid and/or GSH have been observed to maintain α-tocopherol levels in both chemical systems and liposomal systems exposed to the oxidative stress [3, 4, 22–25]. α-Tocopherol is regenerated from the tocopheryl radical by ascorbic acid even in the absence of GSH [26, 27]. In addition, it is known that ascorbic acid is regenerated from ascorbic acid free radicals or dehydroascorbic acid by glutathione or NADH by enzymatic and/or nonenzymatic reactions [28–30]. When cellular GSH levels were irreversibly depleted by the cooperation of BCNU and diquat, the loss of ascorbic acid in hepatocytes was rapid as compared within untreated hepatocytes (Figs 1 and 2). Additionally, the gradual consumption of α-tocopherol followed by ascorbic acid loss was related to the accumulation of lipid peroxidation (Fig. 2). Thus, our results and previous findings support the premise that ascorbic acid plays a vital role in maintaining cellular α-tocopherol levels in hepatocytes during oxidative stress.

Ascorbic acid can alternate between prooxidant and antioxidant activities depending on its concentration and the presence of free transition metal ions [31]. In this study using BCNU-pretreated hepatocytes, ascorbic acid (0.1 or 1.0 mM) added extracellularly acted as an antioxidant in hepatocytes treated with diquat, since the onset of lipid peroxidation and consumption of α-tocopherol following diquat treatment was significantly delayed by the supplementation (Fig. 2E and F). In addition, it is known that ascorbic acid can act as a scavenger of superoxide radical, hydroxy radical and singlet oxygen [30, 32, 33]. In preliminary experiments, we found that the levels of cellular ascorbic acid were increased by the addition of exogenous ascorbic acid and the levels reached a maximum, which was about four times of control level by the addition of 0.1 mM ascorbic acid, 30 min later. Therefore, it appears that intra- and extra-cellular ascorbic acid may directly scavenge reactive oxygen species derived from redox cycling by diquat metabolism and that this reaction may be involved in the protective effect of supplemented ascorbic acid on cellular antioxidant defense and cell survival. Actually, after total ascorbic acid was consumed to below that of normal endogenous ascorbic acid, the depletion of α-tocopherol and accumulation of MDA were initiated (Figs 2 and 3).

The mechanism of diquat-induced cytotoxicity is still not completely understood. For instance, there is debate as to the relative roles of lipid peroxidation and protein thiol oxidation. Previous *in vitro* studies in isolated hepatocytes have demonstrated that oxidation of protein thiols, rather than lipid peroxidation, was more closely associated with the onset of diquat-induced cytotoxicity, since the thiol reductant DTT prevented the oxidation of soluble and protein thiols and protected cells against the cytotoxicity [13]. Despite this, it is clear that agents which scavenge reactive oxygen metabolites clearly protect cells from toxicity. For instance the glutathione peroxidase mimetic ebselen, together with *N*-acetylcysteine, produced a significant delay on the cytotoxicity [11]. Similarly, diquat-induced lipid peroxidation was inhibited by addition of antioxidants such as Trolox C, promethazine and *N*,*N*-diphenyl-*p*-phenylenediamine (DPPD). Despite this, all antioxidant treatments merely delayed the onset of the toxicity [7, 11]. In the present study, the supplementation of ascorbic acid (0.1 or 1.0 mM) significantly delayed the onset of diquat-induced cytotoxicity, lipid peroxidation and α-tocopherol loss (Fig. 2). However, the supplementation of ascorbic acid did not prevent the induction of cytotoxicity even though exogenous ascorbic acid was added repeatedly into the incubation mixture (data not shown). As the concentration of diquat in hepatocytes is maximal 10 min after the addition, Sandy [34] has suggested that the short-term redox cycling of diquat is sufficient to initiate the process leading to cellular injury and death. When GSH reductase is markedly inhibited by BCNU pretreatment, rapid depletion of GSH induced by diquat is accompanied by the increment of oxidized GSH (GSSG) and consequently the formation of protein mixed disulphides [34]. Therefore, an initial irreversible damage of target sites, which perhaps contain sensitive protein thiols may be induced by diquat redox cycling, may not be protected by ascorbic acid and α-tocopherol.

In conclusion, this study has demonstrated that ascorbic acid, together with GSH, plays an important antioxidant role against oxidative stress induced by diquat in hepatocytes. The supplementation of ascorbic acid into BCNU- and diquat-cotreated hepatocytes delayed the onset of cytotoxicity, the depletion of α-tocopherol and the induction of lipid peroxidation.

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