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EFFECTS OF OXIDATIVE STRESS CAUSED BY HYPEROXIA AND DIQUAT. A STUDY IN ISOLATED HEPATOCYTES

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The effects of oxidative stress caused by hyperoxia or administration of the redox active compound diquat were studied in isolated hepatocytes, and the relative contribution of lipid peroxidation, glutathione (GSH) depletion, and NADPH oxidation to the cytotoxicity of active oxygen species was investigated.

The redox cycling of diquat occurred primarily in the microsomal fraction since diquat was found not to penetrate into the mitochondria. Depletion of intracellular GSH by pretreatment of the animals with diethyl maleate promoted lipid peroxidation and sensitized the cells to oxidative stress. Diquat toxicity was also greatly enhanced when glutathione reductase was inhibited by pretreatment of the cells with 1,3-bis(2-chloroethyl)-1-nitrosourea. Despite extensive lipid peroxidation, loss of cell viability was not observed, with either hyperoxia or diquat, until the GSH level had fallen below ~ 6 nmol/10^6 cells.

The iron chelator desferrioxamine provided complete protection against both diquat-induced lipid peroxidation and loss of cell viability. In contrast, the antioxidant α-tocopherol inhibited lipid peroxidation but provided only partial protection from toxicity. The hydroxyl radical scavenger α-keto-γ-methiol butyric acid, finally, also provided partial protection against diquat toxicity but had no effect on lipid peroxidation.

The results indicate that there is a critical GSH level above which cell death due to oxidative stress is not observed. As long as the glutathione peroxidase — glutathione reductase system is unaffected, even relatively low amounts of GSH can protect the cells by supporting glutathione peroxidase-mediated metabolism of H_2O_2 and lipid hydroperoxides.

Key words: Hepatocytes, glutathione, lipid peroxidation, cell toxicity, hyperoxia, diquat

INTRODUCTION

Superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) are active metabolites of dioxygen that can be formed as a result of normal cellular metabolism[1,2]. Their production can be further stimulated by hyperoxia or administration of different redox

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active compounds[3,4,5,6,7]. The production of large amounts of these active oxygen species may result in "oxidative stress".

Hepatocytes and other mammalian cells possess enzymes which can inactivate active oxygen species. Superoxide dismutase catalyzes the dismutation of $O_2^{\cdot-}$ to H_2O_2 which can be further catabolized by either catalase or the selenoprotein, glutathione peroxidase. The metabolism of H_2O_2 and organic hydroperoxides by glutathione peroxidase is associated with glutathione (GSH) oxidation. Most of the glutathione disulfide (GSSG) formed is subsequently re-reduced by glutathione reductase, and GSH is regenerated at the expense of NADPH. However, an efflux of GSSG, which may lead to depletion of intracellular GSH under extreme conditions, is sometimes observed as a consequence of stimulated glutathione peroxidase activity.

$O_2^{\cdot-}$ and H_2O_2 can interact in an iron-catalyzed Haber-Weiss reaction generating the highly reactive hydroxyl radical ($^{\cdot}OH$), which can initiate free radical chain reactions, e.g. the peroxidation of cellular lipids. In isolated hepatocytes, low GSH levels and lipid peroxidation create a "vicious cycle", as low GSH seems to predispose the cells to lipid peroxidation[8], and lipid hydroperoxides formed as a result of this process can consume GSH through glutathione peroxidase activity[9].

In the present study we have produced oxidative stress by either increasing the oxygen concentration in the hepatocyte incubation medium or exposing the cells to diquat (1,1'-ethylene-2,2'-bipyridilium dibromide), a commercially available herbicide which has been proposed to be a pure redox cycling compound. The pulmonary toxicity of both diquat and paraquat, another bipyridilium compound, has been shown to be exacerbated by hyperoxia[10] and in a study of paraquat toxicity in the liver, it was suggested that the hepatotoxicity results from NADPH depletion[11].

It has previously been reported that diquat is metabolized by the cytochrome P-450-linked monooxygenase system[12], and that incubation of microsomes with diquat plus NADPH under anaerobic conditions forms a relatively stable[13], green-colored radical[6]. Under aerobic conditions, the diquat radical reacts with dioxygen resulting in the formation of $O_2^{\cdot-}$ and the regeneration of diquat[7].

In the present study, production of oxidative stress in isolated hepatocytes by either hyperoxia or diquat has allowed us to examine the relative contribution of lipid peroxidation, GSH depletion, and NADPH oxidation to the cytotoxicity of active oxygen species.

MATERIALS AND METHODS

Materials

Collagenase (grade II), superoxide dismutase (SOD), NADH, and NADPH were obtained from Boehringer-Mannheim. Sigma was the source of α-keto-γ-methiol-butyric acid (KMBA).

DL-buthionine-S-R-sulfoximine was purchased from Chemical Dynamics Corporation, DL-α-tocopherol from Merck, and desferrioxamine (Desferal®) from Ciba-Geigy. Diethyl maleate was obtained from Aldrich.

Diquat dibromide, as the monohydrate (analytical grade), was a generous gift of Dr. L.L. Smith of Imperial Chemical Industries PLC, Millbank, London, England. Bristol Laboratories, Stockholm, Sweden kindly supplied the 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). All other reagents were commercial products of the highest available grade of purity.

Experimental Procedures

Male Sprague-Dawley rats (250–300 g), allowed food and water *ad libitum* were used in all experiments; the rats received sodium phenobarbital in the drinking water (1 mg/ml) for one week prior to use.

When required, diethyl maleate as a 20% solution in corn oil, was administered by intraperitoneal injection (3.9 mmol/kg body-weight) one hour before killing, in order to deplete intracellular glutathione[14].

Isolation and Incubation of Hepatocytes

Hepatocytes were isolated as described previously[15] and incubated in rotating, round-bottom flasks at a concentration of 10^6 cells/ml at 37°C in a modified Krebs-Henseleit buffer supplemented with 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (Hepes) (12.5 mM), pH 7.4. When indicated, an amino acid mixture, containing serine, glutamine, cysteine, and methionine (all at 0.2 mM), and other amino acids at the concentrations given in[16], was added to the incubation medium.

Incubations were carried out under atmospheres of 95% O_2, 5% CO_2 (carbogen) or 20% O_2, 5% CO_2, 75% N_2; all experiments with diquat were performed in Krebs medium using carbogen as gas phase. After 20 min of preincubation of the hepatocytes, additions were made to produce the following final concentrations: diquat (2 mM), desferrioxamine (0.25 mM), KMBA (1 mM), and α-tocopherol (1 mM in dimethylsulfoxide).

Inhibition of glutathione synthesis was obtained by administration of the γ-glutamylcysteine synthetase inhibitor DL-buthionine-SR-sulfoximine[17,18]. BCNU treatment of the hepatocytes, to inhibit the activity of glutathione reductase, was performed according to Eklöw *et al.*[3]. Cell viability was determined by the Trypan blue exclusion test[15].

Isolation of Subcellular Fractions

The microsomal and cytosolic fractions of rat liver were isolated as described by Ernster *et al.*[19]. Mitochondria were isolated as in[20]. Mitochondrial fragments were obtained by sonicating the mitochondrial fraction for 5 sec with a Branson sonifier, model B15 (power ~50 watt) in cold 0.25 M sucrose. Protein concentration of the mitochondrial suspension was approximately 20 mg/ml.

Analytical Procedures

The intracellular level of GSH was routinely measured by the colorimetric assay of Saville[21]. In addition, the GSH level was also checked for each set of experiments by high performance liquid chromatography (HPLC) as described by Reed *et al.*[22], and the results obtained by these two methods showed good correlation. GSSG was measured by HPLC[22].

Lipid peroxidation was assayed, as reported in[23], by measuring the amount of malondialdehyde (MDA) present in 0.5 ml sample of incubates by the thiobarbituric acid method, using an extinction coefficient of 156 mM^{-1} cm^{-1}. Intracellular concentrations of $NADP^+$ and NADPH were determined by the spectrophotometric method of Klingenberg[24].

A Clark-type oxygen electrode was used to measure O_2 uptake. The microsomal

fraction or the cytosol was incubated at 25°C in 100 mM Tris-HCl buffer, pH 7.4, containing 50 mM KCl. The final protein concentrations were ~0.25 and ~0.50 mg/ml, respectively. The mitochondrial fraction was incubated in 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA at a final protein concentration of ~2 mg/ml. Protein was determined according to the method of Lowry *et al.*[25].

RESULTS

Effects of Hyperoxia

Freshly isolated hepatocytes from diethyl maleate-treated rats contained about 10 nmol GSH/10^6 cells. Following diethyl maleate treatment, the cells were allowed to resynthesize GSH in an amino acid containing medium. When this incubation was performed under a gas mixture containing 20% O_2, the intracellular GSH level increased from 10 to 100 nmol/10^6 cells over five hours (Fig. 1A). Moreover, under these conditions little lipid peroxidation was detected (Fig. 1B). However, when cells were exposed to 95% O_2 the increase in GSH level was less marked (74 nmol/10^6 cells after five hours) and lipid peroxidation was evident; 11 nmol MDA/10^6 cells after five hours incubation (Fig. 1A and B). Despite the lipid peroxidation, cell viability was not affected.

To investigate the effects of prolonged GSH depletion on hepatocyte viability GSH resynthesis was blocked by DL-buthionine-SR-sulfoximine, a compound which binds irreversibly to γ-glutamylcysteine synthetase. Under these conditions, an increase in MDA accumulation was observed. The GSH concentration remained at ~10 nmol/10^6 cells for three hours of incubation, after which it began to decrease.

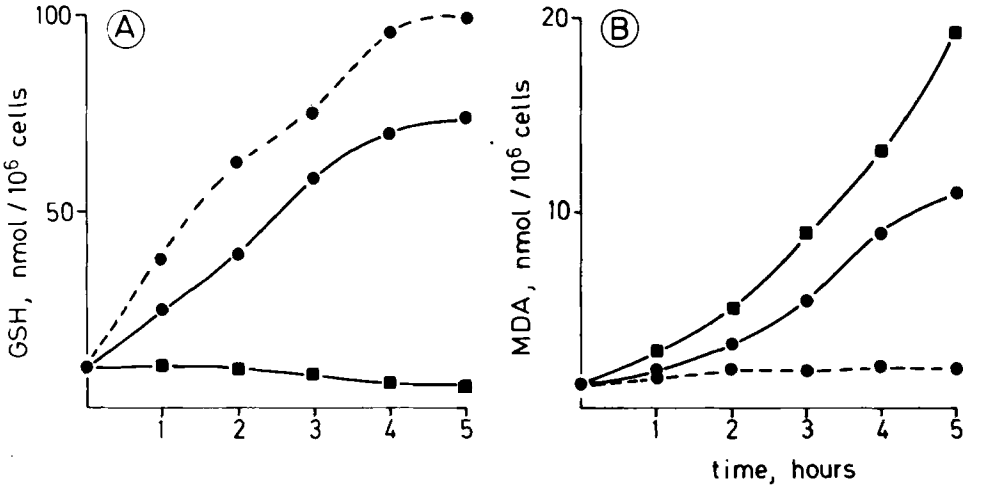

FIGURE 1 *Effects of different O_2 tensions and buthionine sulfoximine on GSH resynthesis and lipid peroxidation in isolated hepatocytes.*

Hepatocytes were isolated from diethyl maleate-treated rats and incubated in an amino acid containing medium as described in the Material and Methods section. (A), GSH content; (B), MDA accumulation. Experimental conditions were: 20% O_2, 5% CO_2, 75% N_2 (●---●), 95% O_2, 5% CO_2 either in the absence (●——●) or in the presence (■——■) of buthionine sulfoximine. One experiment typical of four.

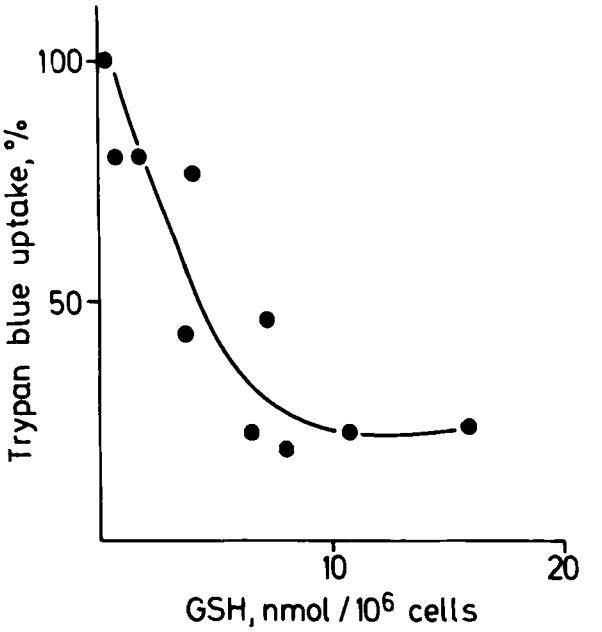

FIGURE 2 *Correlation between decrease in intracellular GSH level and loss of cell viability following the addition of buthionine sulfoximine to isolated hepatocytes.*

Hepatocytes isolated from diethyl maleate-treated rats were incubated for four hours in an amino acid containing medium under carbogen in the presence of buthionine sulfoximine (0.5 mM). Each point represents the four-hour value from separate experiments.

When results from the four hour incubation were compiled by plotting GSH content *vs.* Trypan blue uptake it was found that cell viability decreased only when the GSH content was less than ~6 nmol/10^6 cells (Fig. 2).

To further investigate the role of glutathione peroxidase in the metabolism of endogenous H_2O_2 and lipid hydroperoxides, several experiments were carried out in which catalase was inhibited by azide in order to block its contribution to H_2O_2 metabolism. When diethyl maleate-pretreated hepatocytes were incubated with 20% O_2 and allowed to resynthesize GSH in an amino acid containing medium, also containing 1 mM azide, the intracellular GSH level reached 100 nmol GSH/10^6 cells after five hours of incubation (Fig. 3A). However, in the presence of 95% O_2 replenishment of the GSH pool was inhibited and the level of lipid peroxidation was high (19 nmol MDA/10^6 cells) (Fig. 3B).

Desferrioxamine can scavenge free iron (Fe^{3+}) thereby blocking the formation of $^{\cdot}OH$[26] which, in turn, is known to initiate lipid peroxidation. In order to investigate if the decreased recovery of GSH seen with 95% O_2, was due to GSH consumption linked to the metabolism of lipid hydroperoxides or H_2O_2 by glutathione peroxidase, the cells were incubated in 95% O_2 in presence of 0.25 mM desferrioxamine. As shown in Fig. 3C, with desferrioxamine present intracellular GSH reached a level of 70 nmol/10^6 cells after five hours incubation, as compared to 50 nmol/10^6 cells in hepatocytes incubated with 95% O_2, but in the absence of desferrioxamine.

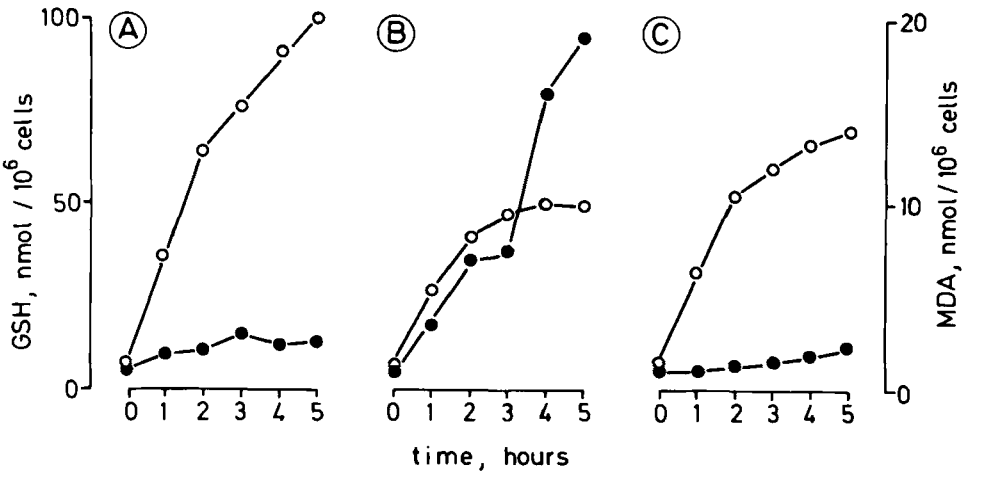

FIGURE 3 *Effects of different O_2 tensions and of desferrioxamine on GSH synthesis and lipid peroxidation in hepatocytes.*

Hepatocytes isolated from diethyl maleate-treated rats were incubated in an amino acid containing medium in the presence of 1 mM azide under the following conditions: 20% O_2, 5% CO_2, 75% N_2 (A); 95% O_2, 5% CO_2 either in the absence (B) or in the presence (C) of desferrioxamine (0.25 mM). (○——○), intracellular GSH level; (●——●), MDA formation. One experiment typical of three.

Effects of Diquat

Diquat metabolism in hepatocytes is associated with O_2 consumption and $O_2^{\cdot-}$ production through a process of redox cycling[7]. The contribution of the various subcellular compartments to this redox cycling was studied using different subcellular fractions. The highest rate of O_2 uptake, using 2 mM diquat, was observed with the microsomal fraction (Table I). This rate differed depending on the electron donor used (129.0 and 41.9 nmol/mg protein per min for NADPH and NADH, respectively). In contrast, negligible O_2 uptake (5.3 nmol/mg protein per min) was seen when diquat was incubated with intact liver mitochondria. However, this rate was markedly stimulated (16.6 nmol/mg protein per min) when the incubation was performed with mitochondrial fragments (Table I). In addition, the cytosol was found not to contribute to the redox cycling of diquat.

TABLE I
Redox cycling of diquat in various hepatic subcellular fractions

Fractions	Additions	O_2 Uptake nmol × min^{-1} × mg $protein^{-1}$	
		Control	+ 2 mM Diquat
Microsomes	NADPH, 0.5 mM	61.4 ± 7.7	129.0 ± 19.5
	NADH, 0.5 mM	0	41.9 ± 8.1
Cytosol	NADH, 0.5 mM	0	0
Mitochondria	Succinate, 5 mM	4.8 ± 1.1	5.3 ± 1.2
Mitochondrial fragments	NADH, 5 mM	5.8 ± 1.8	16.6 ± 3.8

Subcellular fractions were incubated under conditions detailed in materials and Methods.
Values are expressed as mean ± S.D. of three experiments.

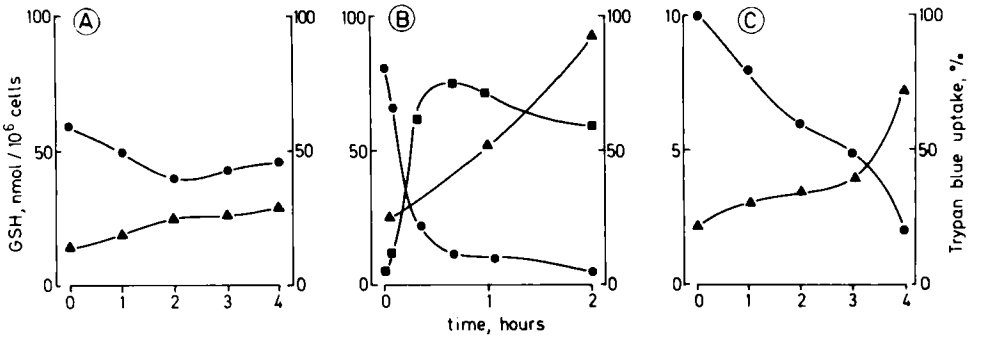

FIGURE 4 *Effect of diquat on GSH and GSSG content in hepatocytes and on cell viability.*
Diquat (2 mM) was added to a suspension of control hepatocytes (A), or to BCNU-treated hepatocytes (B), or to hepatocytes isolated from diethyl maleate-treated rats (C).
At the times indicated, the intracellular GSH concentration (●—●) and the percentage of cells permeable to Trypan blue (▲—▲) were determined. The extracellular level of GSSG is expressed in terms of GSH equivalents (■—■). Results are typical of 3–4 experiments.

In isolated hepatocytes incubated under control conditions, 2 mM diquat produced only a small decrease in GSH concentration and had almost no effect on viability (Fig. 4A). However, when the glutathione reductase had been inhibited by BCNU pretreatment of the cells prior to diquat administration, there was a rapid loss of GSH, a corresponding increase in GSSG and, after two hours, 80% of the cells were permeable to Trypan blue (Fig. 4B).

Another method of compromising cellular defense against oxidative stress is treatment with diethyl maleate (DEM), a compound which readily depletes intracellular GSH content. When cells isolated from DEM-treated rats were incubated with 2 mM diquat, the remaining GSH content was depleted over four hours and, when the GSH level had fallen below ~5 nmol/10^6 cells, the loss of cell viability was markedly increased (Fig. 4C). In addition, 2 mM diquat gave rise to greater malondialdehyde formation in hepatocytes from DEM-treated rats when compared to cells from untreated animals; 19 *vs.* 4 nmol/10^6 cells after four hours of incubation (Fig. 5).

Mechanism(s) of Diquat Toxicity

Microsomes incubated with diquat in presence of NADPH were found to produce $^{\cdot}OH$ as suggested by the enhanced formation of formaldehyde from dimethyl sulfoxide; the diquat-stimulated formaldehyde production was abolished in presence of desferrioxamine[27]. To further investigate the role of $^{\cdot}OH$ formation and lipid peroxidation in diquat toxicity, hepatocytes were incubated with diquat in the absence or presence of desferrioxamine. As shown in Fig. 6, addition of 0.25 mM desferrioxamine together with 2 mM diquat completely protected the cells from diquat toxicity; cell viability was not decreased when compared to control cells incubated in the absence of diquat. Moreover, in the presence of desferrioxamine diquat-induced lipid peroxidation was completely inhibited and intracellular GSH was protected. However, desferrioxamine did not prevent the drop in the NADPH/$NADP^+$ ratio occurring as result of diquat redox cycling (Fig. 7). The results with desferrioxamine demonstrate that inhibition of $^{\cdot}OH$ production completely prevented both the lipid

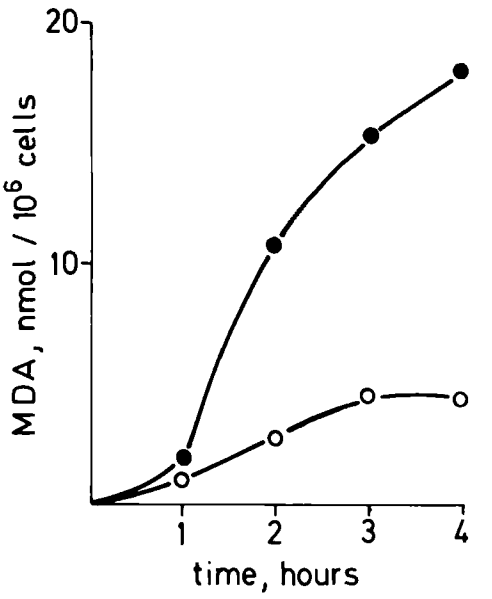

FIGURE 5 *Potentiation of diquat-induced lipid peroxidation in hepatocytes by diethyl maleate pretreatment.*

Diquat-dependent MDA formation was measured in control hepatocytes (○—○) and in hepatocytes isolated from diethyl maleate-treated rats (●—●). Values represent the increase in MDA formation, over that of the control, due to the presence of diquat. Results are typical of four experiments.

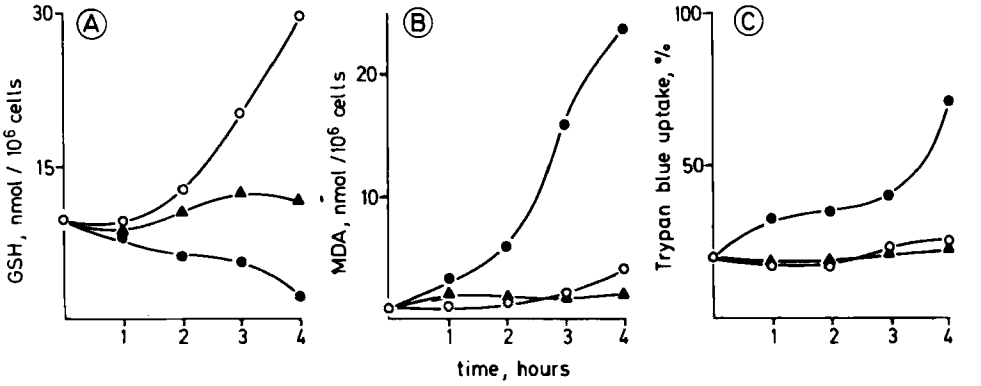

FIGURE 6 *The effect of desferrioxamine on diquat-induced alterations in GSH level, lipid peroxidation, and cell viability.*

Hepatocytes were isolated from diethyl maleate-treated rats and incubated either under control conditions (○—○), or in presence of 2 mM diquat (●—●), or in presence of both 2 mM diquat and 0.25 mM desferrioxamine (▲—▲). (A), GSH concentration; (B), MDA content; (C), Trypan blue uptake. Results of one experiment typical of four are shown.

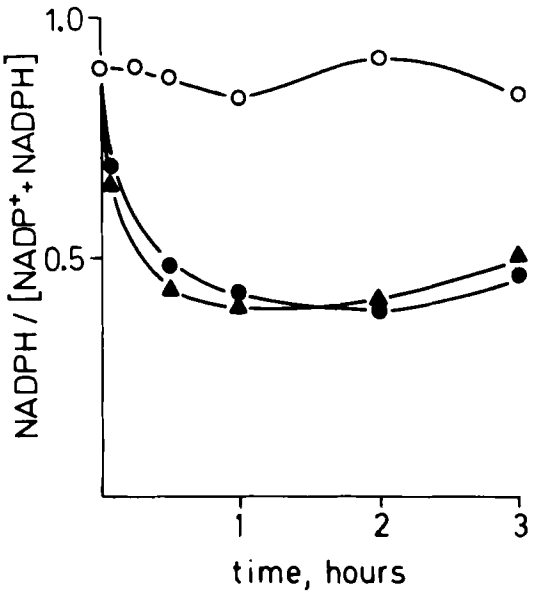

FIGURE 7 *The effect of diquat on the NADPH/NADP$^+$ ratio in hepatocytes.*
Hepatocytes isolated from diethyl maleate-treated rats were incubated under control conditions (○—○), or in the presence of 2 mM diquat (●—●), or in the presence of both 2 mM diquat and 0.25 mM desferrioxamine (▲—▲). Data represent one experiment typical of three.

peroxidation and cytotoxicity induced by diquat. To investigate whether this protection from cytotoxicity was the result of the abolishment of lipid peroxidation by desferrioxamine, the effects of an ·OH scavenger (KMBA)[28] and an antioxidant (α-tocopherol) were studied in this system. Interestingly, KMBA decreased the diquat-induced cytotoxicity without having any effect on the extent of lipid peroxidation whereas α-tocopherol completely inhibited lipid peroxidation with only a partial decrease in cytotoxicity (Figs. 8A and B).

DISCUSSION

In this study we have investigated the effects of diquat-induced oxidative stress in hepatocytes. Formation of the diquat radical was easily detectable by its green colour[29] when either hepatocytes, liver microsomes, or mitochondrial fragments were incubated with diquat under anaerobic conditions; the green colour disappeared rapidly upon oxygenation of the medium. Redox cycling of diquat occurs predominantly in the endoplasmic reticulum, and both NADPH-cytochrome P-450 reductase and NADH-cytochrome b_5 reductase seem to participate in this process. Although mitochondrial fragments could also catalyze the redox cycling of diquat in presence of NADH, our results suggest that this activity may not be important *in vivo*, since diquat does not appear to penetrate into intact mitochondria.

Despite its rapid redox cycling in the microsomal fraction, incubation of hepatocytes with diquat had only minimal effects on intracellular GSH content.

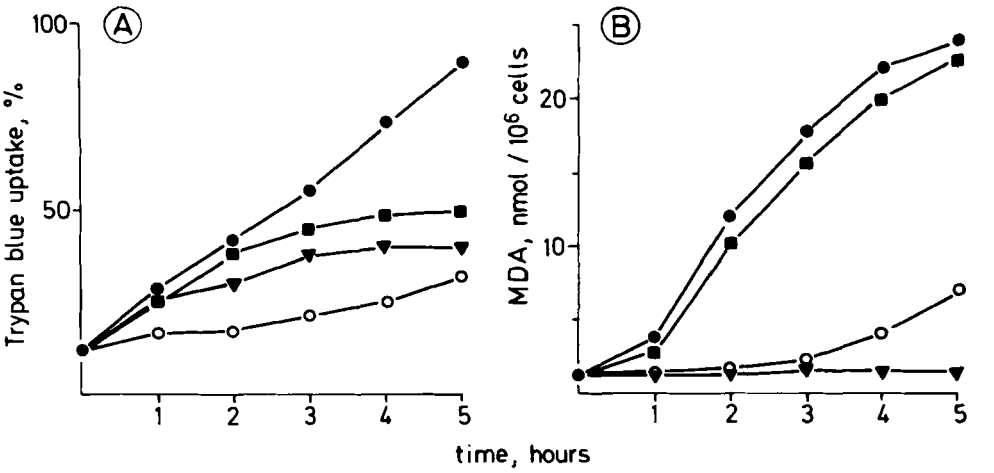

FIGURE 8 *Different effects of KMBA and α-tocopherol on the cytotoxicity, and lipid peroxidation induced by diquat.*

Trypan blue uptake (A) and MDA formation (B) were measured in suspensions of hepatocytes isolated from diethyl maleate-treated rats either under control conditions (○——○), in presence of 2 mM diquat (●——●), or in presence of both 2 mM diquat and 1 mM α-tocopherol (▼——▼) or 1 mM KMBA (■——■). Results are typical of three experiments.

However, when cellular defense against oxidative stress was compromised by pretreatment with either a GSH depleting agent (DEM) or an inhibitor of glutathione reductase (BCNU), both diquat and hyperoxia caused cytotoxicity. Under these conditions loss of cell viability was observed when intracellular GSH content had fallen below ~6 nmol/10^6 cells. Thus, there appears to be a critical level of GSH in hepatocytes above which cell death due to oxidative stress is not observed. It is interesting to note that this critical level of GSH is of similar size as the intramitochondrial GSH pool, which has been proposed to be particularly important for maintenance of cell viability during oxidative stress[30]. Consumption of mitochondrial GSH during diquat metabolism may be the result of glutathione peroxidase-mediated catabolism of extramitochondrially formed H_2O_2. Thus, the results of the present investigation are in accordance with those of previous *in vitro*[4,31,32] and *in vivo*[33] studies, emphasizing the critical role of the glutathione peroxidase — glutathione reductase system in cellular defense against oxidative stress.

In a previous study[11], paraquat toxicity has been linked to the shift in pyridine nucleotide redox state resulting from the redox cycling of the drug. Although incubation of hepatocytes with toxic concentrations of diquat was also associated with pyridine nucleotide oxidation, our results do not support this mechanism since desferrioxamine protected against diquat toxicity without affecting the change in pyridine nucleotide redox ratio. Thus, some other mechanism appears to have been responsible or the oxidative cell damage produced under our experimental conditions.

Although the cytotoxicity associated with oxidative stress is frequently blamed on lipid peroxidation, the evidence supporting this conclusion is often rather weak[34]. To accept lipid peroxidation as the major cause of cell damage, the following criteria should be fullfilled:

a) lipid peroxidation should be extensive,

b) it should precede other signs of cytotoxicity, and

c) antioxidants should protect.

This is rarely the case. In the present study, both hyperoxia and diquat exposure caused rather extensive lipid peroxidation in hepatocytes without any marked change in their parmeability to Trypan blue. Only when intracellular GSH content fell below a critical level was there a loss of cell viability. Administration of antioxidants provided only partial protection against this loss of viability. Thus, our results suggest that even extensive lipid peroxidation does not lead to irreversible cell damage unless the GSH content falls below a critical level.

Our results imply that removal of the increased amounts of H_2O_2 formed during oxidative stress is of primary importance for maintenance of cell viability. When this is no longer possible, toxic effects of H_2O_2 and of other, more aggressive, oxygen species become apparent. The desferrioxamine experiments suggest that formation of $^{\cdot}OH$ is particularly important for the cytotoxicity associated with oxidative stress, and that lipid peroxidation is one of the consequences of $^{\cdot}OH$ formation. It is not yet clear whether diquat-induced $^{\cdot}OH$ formation occurs by a Haber-Weiss reaction ($O_2^{\cdot-} + H_2O_2 \rightarrow O_2 + OH^- + {}^{\cdot}OH$) or by an iron-catalyzed Fenton-type reaction ($DQ^{+\cdot} + H_2O_2 \rightarrow DQ^{++} + OH^- + {}^{\cdot}OH$), a mechanism that has been suggested for the paraquat radical[35]. Further studies are also required to elucidate the contribution of various effects of $^{\cdot}OH$ formation to the cytotoxicity associated with oxidative stress.

Acknowledgement

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ABBREVIATIONS USED: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DEM, diethyl maleate; GSH, glutathione, reduced form; GSSG, glutathione disulfide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; KMBA, α-keto-γ-methiol-butyric acid; MDA, malondialdehyde; SOD, superoxide dismutase.

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