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Liver Membrane Calcium Transport in Diquat-Induced Oxidative Stress *In Vivo*

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SUMMARY

Hepatic necrosis is produced rapidly by 0.1 mmol/kg diquat in male Fischer-344 rats but not Sprague-Dawley rats, yet massive oxidant stress is caused by diquat in both strains of rat. Liver plasma membrane calcium uptake was unaltered by diquat treatment in either strain. However, diquat inhibited ATP-dependent calcium sequestration by hepatic microsomes from Fischer rats by 33% (33 ± 2 versus 50 ± 2 nmol/mg/20 min), whereas liver microsomal calcium uptake in Sprague-Dawley rats was not decreased by diquat treatment. Microsomes of diquat-treated Fischer rats showed marked increases in calcium efflux versus controls (k_{efflux} = 0.115 ± 0.027 versus 0.051 ± 0.005 min^{-1}; $p < 0.025$), but microsomes of diquat-treated Sprague-Dawley rats exhibited no significant change in efflux rate. Calcium uptake by the endoplasmic reticulum of saponin-permeabilized isolated hepatocytes was diminished in parallel with diquat cytotoxicity. Significant increases in 11-, 12-, and 15-hydroxy 20:4 fatty acids were found in liver microsomes isolated after diquat treatment *in vivo* and administration of desferrioxamine (0.24 mmol/kg, intraperitoneally) administered before diquat significantly protected against the inhibition of microsomal calcium uptake. These data suggest a possible role for Fenton chemistry and lipid peroxidation in this feature of diquat-generated hepatic damage *in vivo*.

The role of oxidative stress mechanisms in chemical-induced acute cellular injury has been investigated extensively in isolated cell systems (1, 2) and in the isolated perfused liver (3). In these studies, as well as in recent studies *in vivo* (4, 5), production and efflux of GSSG from the liver cells, or from the intact liver into the bile and plasma, provided a useful index of oxidant stress. However, in the studies *in vivo* development of hepatic necrosis in Sprague-Dawley rats did not correlate with the severity of oxidative stress, i.e., hepatotoxins such as CCl_4 and acetaminophen produced necrosis without elevation of plasma or biliary GSSG, whereas compounds such as diquat and paraquat that produced increased GSSG did not produce hepatic necrosis. Of the substances investigated, diquat gave rise to the largest biliary efflux of GSSG. Diquat does not produce hepatic necrosis in the male Sprague-Dawley rat but, as recently reported (6), male Fischer-344 rats readily develop liver necrosis after diquat administration, thus providing an animal model in which to examine the functional alterations produced by an oxidative stress-generating hepatotoxin *in vivo*.

The calcium hypothesis of acute cell injury (7) suggests that the calcium pumping or retention capabilities of plasma membrane (8, 9) and/or the endoplasmic reticulum (10) are compromised during injury and that loss of calcium homeostasis is a primary causative factor in the development of tissue necrosis. The mechanism producing such membrane functional changes has been suggested to be either alteration of thiol-disulfide equilibria (9, 11), or lipid peroxidation (6), caused in either case by reactive oxygen species generated as a result of redox cycling. The present study was designed to examine the calcium transport properties of hepatic plasma membranes and microsomes isolated after diquat administration to the Fischer rat and to investigate further the hypothesis that lipid peroxidation is a factor in diquat-mediated hepatotoxicity *in vivo*.

Methods

Animals. Male Fischer-344 and Sprague-Dawley rats were obtained from Harlan Industries (Houston, TX). The animals were provided tap water and rat chow (Formulab, Purina) *ad libitum*. Diquat dichloride was dissolved in 0.9% saline and administered at a dose of 0.1 or 0.15 mmole/kg intraperitoneally. Control animals received saline alone. Animals were sacrificed 2 hr post-dose.

Preparation of plasma membrane vesicles and Ca^{2+} transport assay. After sacrifice of the animals, livers were excised rapidly, placed in ice-cold buffered sucrose, and finely minced. Two or three control or drug-treated livers were pooled for a given preparation. Homogenization and differential and sucrose gradient centrifugation were carried out as previously described (8). The final plasma membrane fraction is equivalent to the "purified fraction" of Van Amelsvoort *et al.* (12) shown to possess ATP-dependent calcium transport activity (8, 13).

ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

Calcium uptake was measured by liquid scintillation counting using ^{45}Ca with a Millipore filtration method as previously described (8). Filters were placed in Liquiscint (National Diagnostics, Manville, NJ).

Preparation of microsomal fractions and assay of Ca^{2+} uptake and efflux. Microsomes were prepared from individual livers of control or diquat-treated rats according to Moore *et al.* (14). On some occasions, microsomes were prepared from 1-g samples of the same livers used for plasma membrane preparation. ATP-dependent calcium uptake in an oxalate-containing medium was measured using ^{45}Ca and Millipore filtration as described (14). Efflux of calcium was induced by addition of NaEGTA (2 mM final concentration) after 15 min of calcium uptake; further samples were taken until 21.5 min had elapsed.

Estimation of microsomal membrane permeability for K^+ and other ions. The procedure used is based on a method described by Garty *et al.* (15). Microsomal vesicles were equilibrated with 100 mM KCl. Just before assay, samples of vesicles (200 μg of protein) were applied to Dowex 50W-X8 ($Tris^+$ form) columns made in Pasteur pipettes and eluted with 1 ml of 175 mM sucrose under light vacuum to remove extravesicular K^+. Then 1-ml portions of the eluates were added to incubation vials that already contained 1 ml of 175 mM sucrose, 175 mM sucrose plus 4 μM valinomycin or 100 mM KCl at 25°. After 30 sec, $^{86}RbCl$ (approximately 2 μCi) was added and 100-μl samples were removed at intervals up to 10 min. Extravesicular $^{86}Rb^+$ was removed by passage of samples through Dowex 50W-X8 columns as above, eluting with 1 ml of 175 mM sucrose, and the eluted samples were counted in entirety. $^{86}Rb^+$ uptake has been expressed in counts per minute per sample, corrected for differences in initial radioactivity as necessary.

Permeabilized hepatocyte studies. Hepatocytes were isolated after collagenase perfusion essentially as described by Seglen (16). Cells were 88% to 95% viable as assessed by exclusion of 0.2% (w/v) trypan blue. Hepatocytes were incubated in round-bottom flasks in a Krebs-Henseleit-type buffer (16) supplemented with 45 mM HEPES, 45 mM MOPS, pH 7.6, 1.25 mM $CaCl_2$, 0.6 mM $MgCl_2$, 5 mM D-glucose, and 1% bovine serum albumin (dialyzed) and maintained under an atmosphere of 95% O_2/5% CO_2, at 37°. Preincubation with 50 μM BCNU was routinely carried out for 30 min in the above medium to inhibit glutathione reductase, and cells were sedimented before resuspension in fresh medium containing 500 μM diquat. Preincubation with BCNU is necessary in order to observe cytotoxicity with diquat, as shown by Sandy *et al.* (17). Incubation concentration was typically 2×10^6 cells/ml, and samples were removed at intervals for viability assessment by trypan blue exclusion. After washing twice in 140 mM KCl, 10 mM HEPES, pH 7.5, 10 mM NaCl, 2.5 mM $MgCl_2$, the cells were permeabilized as described (18) in an equivalent volume of the same medium, supplemented with 0.005% saponin, by 20 min incubation at 37°. When tested after washing, 90% to 95% of cells were permeable to trypan blue. Cells were resuspended in Ca-loading medium [130 mM KCl, 9.25 mM HEPES, 9.25 mM NaCl, 2.3 mM $MgCl_2$, 1 mM ATP, 3% polyethylene glycol (approximate M_r 8000), 50 μM EGTA, and 1 μCi/ml $^{45}CaCl_2$] and uptake was allowed to occur to equilibrium. Samples were filtered (Whatman GF/C filters), washed, and counted to determine cellular calcium content. For efflux studies, either A23187 (5 μM) or an equivalent volume of solvent was added to the loaded cells after 4 min at 37°; samples were filtered at timed intervals, washed, and counted.

Lipid hydroxy acid analyses. The hydroxylated unsaturated fatty acid esters, 11-, 12-, and 15-hydroxy 20:4, were quantitated in isolated microsomal fractions as an index of membrane lipid peroxidation, as described by Hughes *et al.* (19).

Chemicals. Diquat was the generous gift of Dr. Ian Wyatt of Imperial Chemical Industries, Ltd. (Macclesfield, Chesire, England). $^{45}CaCl_2$ and $^{86}RbCl$ were products of New England Nuclear (Boston, MA). Desferrioxamine was purchased from the college hospital pharmacy. BCNU was provided by Dr. Jim Keller of Bristol Laboratories (Syracuse, NY). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Data are presented as mean ± standard error and analyzed for statistical differences by the unpaired Student's *t* test or the Mann-Whitney nonparametric rank sum test, with significance assigned at $p < 0.05$, as we have described previously (6).

Results

Liver plasma membranes of control and diquat-treated Fischer-344 and Sprague-Dawley rats were isolated and their calcium uptake activity was assayed in the presence and absence of ATP. Fig. 1 shows that diquat administration at a dose (0.1 mmol/kg) that produces markedly increased biliary GSSG excretion (4) and liver necrosis in the Fischer rat (6) had no effect on ATP-dependent plasma membrane calcium uptake. Similarly, no change in calcium uptake activity was observed in liver plasma membranes of the diquat-treated Sprague-Dawley rat (not shown).

Fischer and Sprague-Dawley rat liver microsomes exhibited similar calcium uptake activity. Fig. 2 shows a decrease in calcium sequestration activity of the hepatic microsomes of diquat-treated Fischer rats. Whereas in the Fischer rat 0.1 mmol/kg diquat produced an average 33% inhibition of calcium accumulation (Fig. 2), no significant difference in uptake was seen between the Sprague-Dawley controls and those treated with diquat.

The efflux of calcium was considerably more rapid than in the controls when EGTA was added to the microsomes of diquat-treated Fischer rats (Table 1). In contrast, no significant change in calcium uptake (Fig. 2) or efflux (Table 1) was observed when the same experiment was performed with liver microsomes of Sprague-Dawley rats after diquat administration. In microsomes of diquat-treated Fischer rats, the average

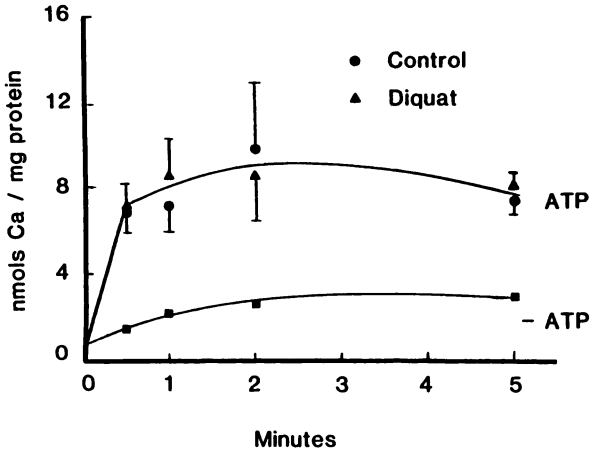

Fig. 1. Calcium uptake by liver plasma membrane vesicles from control and diquat-treated Fischer-344 rats in the presence and absence of ATP. Plasma membrane vesicles were prepared according to Van Amelsvoort *et al.* (12) from male Fischer-344 rats 2 hr after 0 (saline control) or 0.1 mmol/kg diquat intraperitoneally and were incubated as described (8, 13) in a medium containing 100 mM KCl, 20 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 1 mM NaN_3, 20 μM digitoxigenin, and 2 μM ruthenium red. Total Ca was 30 μM; ^{45}Ca was added to give a specific activity of approximately 3×10^4 cpm/nmol. Total volume, 3 ml; temperature 37°; protein concentration, 100 μg/ml. Samples (400 μl) were removed at indicated intervals, filtered (0.22 μm Millipore filters) and washed with 6 ml of 100 mM KCl, 20 mM HEPES, pH 7.5. Filters were counted in Liquiscint (National Diagnostics, Manville, NJ). ●, Control membranes, 1 mM ATP; ▲, membranes from diquat-treated Fischer rats, 1 mM ATP, ■, control and diquat membranes, no added ATP. Data are mean ± standard error from three separate plasma membrane preparations in each group (*+ATP* curves). A single typical *–ATP* curve is shown.

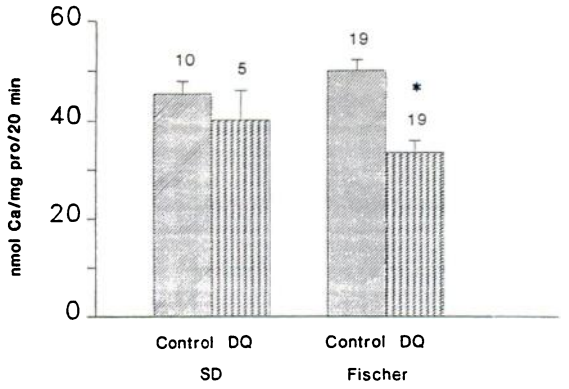

Fig. 2. Calcium uptake by liver microsomes of control and diquat-treated Fischer-344 rats and Sprague-Dawley (*SD*) rats. Fed male Fischer-344 or Sprague-Dawley rats were given 0.1 mmol/kg diquat (*DQ*) intraperitoneally in saline or an equal volume of saline alone (*Control*). Two hours later the animals were killed and hepatic microsomes were prepared and calcium uptake measured by a Millipore filtration method as described by Moore *et al.* (14), in the presence of 5 mM ATP and 7.5 mM oxalate. Total Ca was 30 μM; ^{45}Ca was added to a specific activity of approximately 2×10^4 cpm/nmol. The data represent experiments with separate microsomal preparations from livers, mean ± standard error; numbers of separate preparations (each from one rat liver) are shown in the figure. Calcium sequestration at 20 min of uptake in the presence of ATP was significantly less in hepatic microsomes from diquat-treated Fischer rats compared with controls ($p < 0.0005$) but not in microsomes from Sprague-Dawley rats.

TABLE 1

Efflux of calcium from hepatic microsomes obtained from control and diquat-treated Fischer and Sprague-Dawley rats

First order rate constants of calcium efflux were evaluated by linear regression on semilog plots of vesicle calcium content versus time after EGTA addition. Correlation coefficients were typically >0.98. The mean rate constant for calcium efflux from microsomes of diquat-treated Fischer rats was significantly greater than that of controls ($p < 0.025$), whereas no difference existed between the Sprague-Dawley groups. Sprague-Dawley and Fischer controls were also different ($p < 0.025$) from one another in average rate constant of efflux. Data are mean ± standard error; $n = 6$ for Sprague-Dawley groups, $n = 9$ for Fischer groups.

	Control	Diquat
Fischer-344	0.051 ± 0.005 (9)	0.115 ± 0.027 (9)
Sprague-Dawley	0.019 ± 0.004 (6)	0.028 ± 0.005 (5)

first-order rate constant for calcium efflux was more than doubled (Table 1). It is interesting that the calcium efflux rate of Fischer control microsomes was significantly greater than that of Sprague-Dawley controls, although there was no difference in the calcium uptake rates. The importance of this observation is unclear presently but it could be related to the different susceptibilities of the two strains to diquat-induced oxidative stress and hepatocellular necrosis.

Possible explanations for the enhanced efflux of accumulated calcium from liver microsomes of Fischer rats dosed with diquat are 1) increased activity of an efflux pathway or channel selective for calcium ions or 2) generally increased membrane permeability in these microsomes. To test the latter possibility, microsomes isolated from livers of control and diquat-treated Fischer rats were examined to ascertain whether the ability of microsomes to maintain a K^+ concentration gradient was altered after diquat treatment *in vivo*. After equilibration in 100 mM KCl, vesicles were stripped of external K^+ by cation exchange and diluted into medium containing $^{86}Rb^+$ and 175 mM sucrose, 175 mM sucrose plus 2 μM valinomycin, or 100 mM KCl, and $^{86}Rb^+$ uptake was measured as a function of time. $^{86}RbCl$ enters the vesicles as K^+ leaves, thus maintaining electroneutrality and providing a readily measurable index of K^+ movement out of the vesicles (15).

Table 2 shows that in 175 mM sucrose alone, where K^+ efflux can occur only through endogenous K^+ channels (20) or via nonselective membrane permeation routes, there was a significant amount of $^{86}Rb^+$ uptake, in comparison with 100 mM KCl medium (where no K^+ gradient and no driving force for K^+ efflux exist). However, there was no significant difference in $^{86}Rb^+$ uptake in the control and diquat-treated groups, suggesting that there was no change in selective or nonselective K^+ permeability in the diquat-treated microsomes. Similarly, in the presence of the K^+ ionophore valinomycin (2 μM) $^{86}Rb^+$ uptake was enhanced, reflecting the more rapid K^+ efflux from the vesicles, but again there was no significant difference between control and diquat-treated groups.

These data suggest that the diquat-treated microsomes are no more leaky than controls to K^+ or other ionic constituents of the medium except Ca^{2+} and, furthermore, that no substantive change in the activity of the microsomal K^+-selective channels (20) occurred after diquat treatment.

Finally, the similar levels of intravesicular $^{86}Rb^+$ attained by passive equilibration of the control and diquat-treated membranes in 100 mM KCl medium indicate that the vesicles had similar Rb^+-accessible volumes. Volume estimated on the basis of these measurements was 6.8 μl/mg of protein. This compares well with an estimate of 6.4 μl/mg of protein reported for similar microsomal preparations by Brattin *et al.* (21).

Further evidence for a diquat-associated lesion in calcium regulation at the endoplasmic reticulum membrane was obtained from studies of calcium uptake and efflux in saponin-permeabilized isolated hepatocytes. This experimental approach, used by Joseph and Williamson (22) and Gill and coworkers (18, 23) among others, allows measurement of endoplasmic reticulum calcium metabolism without the need for extensive disruption of the reticular network and prolonged centrifugation.

Microsomes and plasma membrane vesicles isolated from the livers of rats given hepatotoxic doses of acetaminophen and bromobenzene show changes in calcium metabolism (8) that are very distinct from those produced by diquat, despite the comparable extent of hepatic necrosis produced. The changes in calcium metabolism observed therefore are not nonspecific results of cell death and degeneration and are not uniform artifacts introduced during vesicle isolation from damaged livers. Although the effects of diquat on hepatocytes *in vivo* are

TABLE 2

Lack of effect of diquat *in vivo* on microsomal K^+ permeability

Data are $^{86}Rb^+$ counts per minute (corrected for 40 cpm background) in samples containing 6.7 μg of microsomal protein, eluted from Dowex 50W-X8 columns for removal of extravesicular $^{86}Rb^+$. For sucrose, 7 min values representative of the plateau uptake level are given; for sucrose plus valinomycin, peak (1 min) uptake values; for KCl, average plateau values. Data are mean ± standard error except for the KCl data, which are mean ± standard deviation because several time points on the plateau were averaged for each group. Five separate preparations were used except for KCl for which data were obtained from two preparations in each group.

Uptake medium	Control	Diquat
	cpm	
175 mM sucrose	367 ± 32	370 ± 39
175 mM sucrose + 2 μM valinomycin	596 ± 51	686 ± 103
100 mM KCl	204 ± 51	212 ± 43

not necessarily reproduced faithfully in isolated hepatocyte systems, the data presented in Fig. 3 offer additional evidence for diquat-induced changes in calcium metabolism by membranes of the endoplasmic reticulum that are independent of alterations that may be produced through disruption and vesicle isolation.

Diquat (500 μM)-incubated hepatocytes, which had been pretreated with 50 μM BCNU for 30 min to sensitize the cells to diquat cytotoxicity (17, 24) were washed and saponin permeabilized, then $^{45}Ca^{2+}$ uptake was allowed to proceed to equilibrium in a medium buffered to 0.1 μM free calcium (18). Little Ca^{2+} was taken into the mitochondria under these conditions, as indicated by the fact that the presence of ruthenium red (10 μM) had no detectable effect on net cellular Ca^{2+} accumulation (not shown).

Permeabilized control Fischer and Sprague-Dawley hepatocytes and those that had been incubated with diquat for 15 min before permeabilizing with saponin exhibited similar amounts of Ca^{2+} uptake and similar rate and extent of Ca^{2+} efflux in response to addition of ionophore A23187 (see legend to Fig. 3). However, after 2 hr of exposure of Fischer rat hepatocytes to diquat, when the cytotoxic effect of diquat had begun to become manifest, a decrease in the equilibrium value of Ca^{2+} uptake was apparent (Fig. 3). No such decrease in Ca^{2+} uptake was seen with Sprague-Dawley hepatocytes (not shown), which were also much more resistant to diquat cytotoxicity.[1] Thus, the appearance of the flaw in endoplasmic reticulum calcium metabolism appears to correlate with the development of cytotoxicity in isolated hepatocytes incubated with diquat.

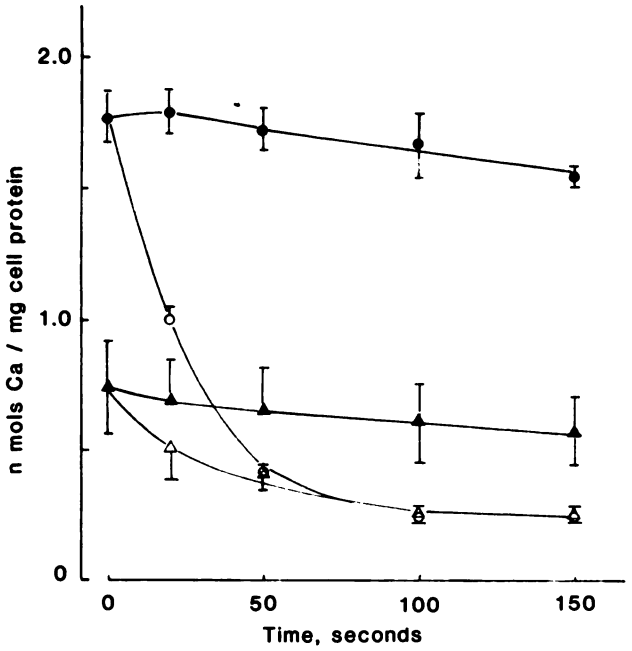

Fig. 3. Efflux of Ca^{2+} after equilibrium ATP-dependent Ca^{2+} uptake into the endoplasmic reticulum compartment of isolated hepatocytes, saponin permeabilized after incubation in the presence and absence of diquat. Freshly isolated hepatocytes were pretreated with BCNU and incubated in the presence or absence of 500 μM diquat, then permeabilized and loaded with $^{45}Ca^{2+}$ as described in Methods. Viability was assessed by trypan blue exclusion periodically during the incubation of the intact cells with or without diquat. Equilibrium $^{45}Ca^{2+}$ content (zero time), and $^{45}Ca^{2+}$ content at timed intervals were determined after filtration of samples of the permeabilized cell suspension. Data are mean ± standard error for three separate preparations in each group. ●, Control cells after 2-hr incubation; ○, 2-hr control cells with ionophore A23187 added immediately after zero time samples were taken; ▲, diquat-treated cells after 2-hr incubation; △, 2-hr diquat-treated cells with ionophore A23187 added. $^{45}Ca^{2+}$ content at zero time and the efflux time course of control cells after 15-min incubation were not different from those after 2-hr incubation. After 15-min incubation in the presence of diquat (before any cytotoxicity was apparent by the measurement of trypan blue exclusion), $^{45}Ca^{2+}$ contents at zero time and subsequent time points were slightly but not significantly lower than the control values with or without A23187 addition (not shown). Viability of 2-hr diquat-incubated cells was 23 ± 9% compared with control value of 70 ± 4%. Viability of 15-min diquat-incubated cells was 69 ± 9% versus control value of 70 ± 2%.

TABLE 3

Lipid hydroxy acid content of liver microsomes of control and diquat-treated Fischer rats

Liver microsomes were prepared as described in Methods 2 hr after injection of 0.1 mmol/kg diquat or an equal volume of saline intraperitoneally. Lipid hydroxy acid content was measured as described by Hughes *et al.* (19). Data are mean ± standard error for separate preparations from five (control) or six (diquat) livers. Nonparametric analysis by rank sum test showed the effects of diquat to be statistically significant on 15-OH 20:4 ($p < 0.01$) and on 12-OH 20:4 ($p < 0.05$); the effects on 11-OH 20:4 were close to, but did not attain, difference at $p < 0.05$. Only the 15-OH 20:4 contents were statistically different by *t* test ($p < 0.01$). The relatively large interanimal variation in lipid hydroxy acid content in the diquat-treated rats may be responsible for the apparent increase in 11-OH 20:4 not attaining statistical significance.

	11-OH 20:4	12-OH 20:4	15-OH 20:4
	pmol/mg of protein		
Control	6.0 ± 0.9	5.8 ± 0.9	8.7 ± 1.2
Diquat	10.0 ± 1.8	12.2 ± 3.0	27.5 ± 6.9

Smith *et al.* (6) reported a significant increase in hepatic lipid peroxidation after diquat in the Fischer rat, as determined by measurement of 11-, 12-, and 15-hydroxyeicosatetraenoic acids (6) and rates of ethane and pentane expiration (24). Table 3 shows that isolated liver microsomal membranes of diquat-treated Fischer rats had significantly increased average content of these lipid hydroxy acids. Lipid peroxidation in the endoplasmic reticulum membranes may play a role in the altered membrane function noted in these studies. Indeed, recently Smith (24) reported evidence supporting a causal role of Fenton chemistry in diquat-generated, reactive oxygen-mediated hepatic injury *in vivo*, such that pretreatments with $FeSO_4$ increased, or conversely with desferrioxamine decreased, diquat hepatic injury and the alkane expiration associated with membrane lipid peroxidation. It was of interest to learn whether the observed alteration in microsomal Ca^{2+} handling was similarly affected by the availability of iron. Male Fischer-344 rats were administered desferrioxamine (0.24 mmol/kg) 30 min before receiving a dose of diquat (0.15 mmol/kg); liver microsomes were isolated and tested in parallel with those of saline-injected control animals and animals receiving saline 30 min before diquat (0.15 mmol/kg). Fig. 4 shows that desferrioxamine offered significant protection against the very marked inhibition of Ca^{2+} accumulation observed after this higher dose of diquat (cf. Fig. 2 and Table 1 where the dose was 0.1 mmol/kg diquat).

Discussion

The observation that diquat, at 0.1 mmol/kg, rapidly produces hepatocellular necrosis in the male Fischer-344 rat (6) provided the first animal model for the study of acute hepatotoxicity *in vivo* apparently produced by oxidative stress mechanisms. Diquat produces severe oxidative stress in the Fischer rat, as reflected by a massive GSSG efflux into the bile, and

[1] J. O. Tsokos-Kuhn, unpublished results.

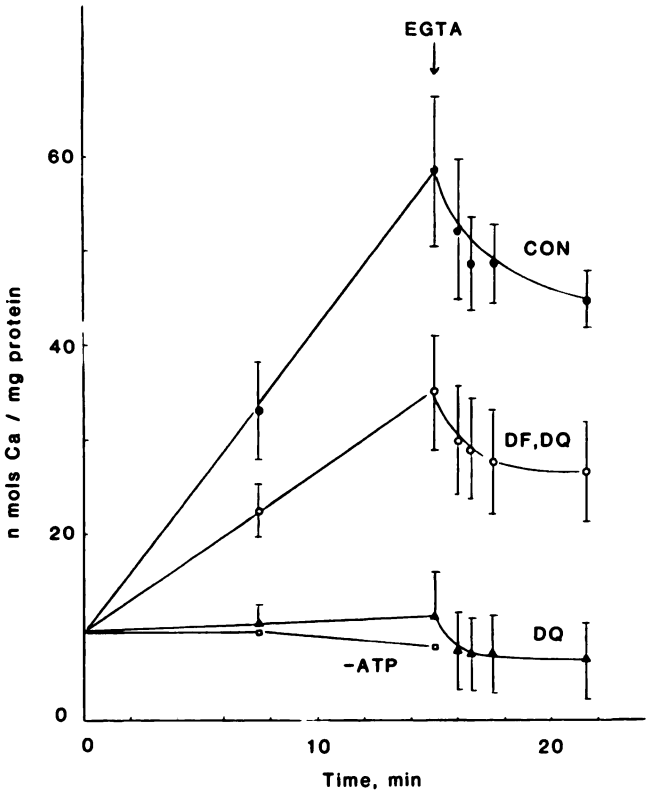

Fig. 4. Effect of desferrioxamine on diquat-induced alteration of liver microsomal Ca^{2+} uptake and efflux. Fischer rat liver microsomes were prepared as described in Methods (except that 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM dithiothreitol were included in the isolation medium) 2 hr after intraperitoneal injection with diquat (0.15 mmol/kg) or an equal volume of saline, preceded 30 min earlier by injection of 0.24 mmol/kg intraperitoneally of desferrioxamine or an equal volume of saline. Calcium uptake in the presence of ATP and efflux after EGTA addition were measured as described for Fig. 2 and Table 2. Data are mean ± standard error for three preparations for each treatment group; average values for uptake in the absence of ATP are shown. ●, Saline, saline control; ▲, saline, diquat; ○ desferrioxamine, diquat; □, control microsomes without ATP.

produces hepatic necrosis and marked increases in plasma transaminases (6).

Studies with isolated hepatocytes have led to the hypothesis that depletion of protein thiols and a resulting impairment of the membrane calcium pumps required for the maintenance of calcium homeostasis (1, 9, 11) are key determinants in the cytotoxicity of the redox cycling quinone compound menadione. However, diquat administered to the Fischer rat *in vivo* does not produce measurable decreases in cellular protein thiols (6) despite the occurrence of oxidative stress leading to GSSG excretion, although alterations of a small number of key protein thiols conceivably could occur. Subsequent studies using administration of ferrous sulfate or desferrioxamine before diquat showed dramatic changes in hepatic damage in the absence of alterations by these pretreatments in the diquat-induced biliary efflux of GSSG (24). The minimal hepatic damage produced by diquat in BCNU-pretreated Sprague-Dawley rats despite enormous increases in GSSG excretion (25) further weakens the apparent correlation between the magnitude of the oxidant stress response and the hepatic injury sustained. In addition, Sandy *et al.* (26) have reported studies in isolated hepatocytes in which desferrioxamine offered significant protection against diquat-induced cytotoxicity but did not alter depletion of acid-soluble thiols. These investigators have shown previously that thiol depletion by diquat in this system is largely a result of GSSG formation (17).

A selective decrease in liver microsomal calcium accumulation and efflux was observed after diquat in the Fischer rat (in which diquat produces hepatocellular necrosis) but not in the Sprague-Dawley (in which hepatic damage does not occur after diquat). However, there seems to be no difference in general cation permeability of the microsomal membranes because no change was found in their ability to maintain a K^+ gradient after diquat.

The present results also indicate that the activity of the plasma membrane calcium pump is unaltered after diquat treatment in the Fischer or Sprague-Dawley rat. These data stand in distinct contrast to those obtained when animals are given hepatotoxic doses of CCl_4, bromobenzene, or acetaminophen, in which ATP-dependent calcium accumulation by liver plasma membranes was decreased 70% or more (8); after CCl_4, the permeability of the plasma membranes to Ca^{2+} was greatly increased as well (27).

The alteration in endoplasmic reticulum Ca^{2+} metabolism observed in isolated microsomal vesicles was borne out by studies with permeabilized isolated hepatocytes previously exposed to cytotoxic concentrations of diquat, suggesting that the lesion is present in the intact cell as well as the isolated membrane preparation. Whether the decreased uptake of calcium is the result of an inhibition of the calcium pump or an activation of Ca^{2+} efflux due to increased membrane permeability or some more specific mechanism is not revealed by these measurements, however.

Numerous studies have suggested lipid peroxidation as a causative factor in acute lethal injury, although little evidence for increased peroxidation of tissue lipids in conjunction with acute hepatic necrosis *in vivo* has appeared. Diquat causes a significant increase in hepatic lipid peroxidation, as determined by measurement of membrane 11-, 12-, and 15-hydroxyeicosatetraenoic acids and by expired ethane and pentane concentrations (6, 19, 24). We have detected significant increases in these metabolites in Fischer rat liver microsomal preparations after diquat as well. Moreover, the manipulation of iron availability with $FeSO_4$ or desferrioxamine altered diquat hepatic damage and alkane expiration in parallel. These correlations were interpreted as indicating that lipid peroxidation may contribute to reactive oxygen-mediated cell death by mechanisms involving degradation of lipid hydroperoxides by iron-catalyzed β-scission mechanisms rather than reduction to the corresponding lipid hydroxy acids (24). The finding reported in the present work that desferrioxamine significantly attenuates the inhibition of liver microsomal Ca^{2+} transport after diquat represents additional support for such as mechanism and, moreover, for the connection between alterations in calcium homeostasis, lipid peroxidation by reactive oxygen, and acute hepatotoxic injury.

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