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Copper Toxicity and Lipid Peroxidation in Isolated Rat Hepatocytes: Effect of Vitamin E[1]

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ABSTRACT. We investigated the role of lipid peroxidation as the mechanism mediating copper toxicity in isolated rat hepatocytes and the modulating effect of vitamin E. Hepatocytes, isolated from rats fed diets containing deficient (E−), sufficient (E+), and excess (E++) amounts of vitamin E, were incubated with $CuCl_2$ (0–2400 μM) for 150 min. Dose and time-dependent decreases in hepatocyte viability (determined by trypan blue exclusion and lactate dehydrogenase release) due to copper toxicity correlated with production of malonyldialdehyde in E− and E+ hepatocytes. However, malonyldialdehyde generation did not accompany copper toxicity in E++ cells. Copper toxicity was enhanced in E− compared to E+ and E++ hepatocytes as assessed by cell viability studies and ultrastructural plasma membrane bleb formation. *In vitro* vitamin E repletion of E− hepatocytes restored resistance to copper and decreased malonyldialdehyde production proportionately. Thus vitamin E deficiency appeared to increase the susceptibility of hepatocytes to copper toxicity. We conclude that lipid peroxidation may not be the mechanism by which copper is toxic to isolated hepatocytes but that the site of injury may be thiol-rich cellular proteins. (*Pediatr Res* 25:55–62, 1989)

Abbreviations

MDA, malonyldialdehyde
E−, vitamin E-deficient
E+, vitamin E-sufficient
E++, vitamin E-excess (overloaded)
LDH, lactate dehydrogenase

Copper is a potent hepatotoxin in man after acute ingestion (1), environmental exposure (2), or chronic accumulation resulting from inherited defects in copper metabolism (3–5). Although the mechanism of copper-induced hepatocyte injury is not fully understood (6), there is accumulating evidence that peroxidation of cell membrane fatty acids may be involved. Copper can catalyze the generation of reactive oxygen species capable of initiating lipid peroxidation by substituting for ferrous iron in the Haber-Weiss reaction (7), by interacting with Hb (8), and by other mechanisms (8). Moreover, emulsions of unsaturated fatty acids undergo lipid peroxidation when incubated with cupric chloride (9). The generation of lipid peroxides in the copper-induced liver injury of rats is manifested by increased production of both pentane and hepatic MDA (10) and by accelerated ethane production from liver homogenate incubated with cupric chloride (11). In addition, Dougherty and Hoekstra (11) showed that pretreatment with the antioxidant vitamin E lessened hepatic lipid peroxidation and decreased mortality in rats injected intraperitoneally with copper sulfate. Although an association between lipid peroxidation and copper toxicity has been clearly demonstrated, it is not clear whether lipid peroxidation actually mediates copper hepatotoxicity or simply occurs in injured hepatocytes after copper toxicity has perturbed metabolic processes by other mechanisms.

The aim of this study was to investigate the role of lipid peroxidation in producing injury to isolated rat hepatocytes during acute copper toxicity. By manipulating the hepatocyte content of vitamin E, the major membrane-localized antioxidant that protects against peroxidation of membrane lipids (12), we attempted to clarify the association between lipid peroxidation and copper hepatocyte injury. Because vitamin E deficiency was likely to enhance oxidant injury to hepatocytes and increase lipid peroxide generation and vitamin E overload to cause the opposite effect, we predicted parallel changes in indices of hepatocyte injury and MDA generation if copper toxicity was indeed mediated by lipid peroxidation. We chose to investigate this issue in isolated hepatocytes because copper overload and vitamin E status could be easily controlled; other variables, such as hepatic blood flow, could be eliminated; and because this model is ideally suited for study of hepatotoxicity (13) and lipid peroxidation (14). In addition, this acute toxicity model would not allow other protective mechanisms (such as induction of metallothionein synthesis) (15) to play a major role, thus permitting the investigation of a limited number of variables related to copper toxicity.

MATERIALS AND METHODS

Animals. Twenty-day-old male, weanling Sprague-Dawley rats (purchased from Sasco, Inc., Omaha, NE) were housed in polyethylene cages with stainless steel wire tops. To achieve three different states of vitamin E nutriture, rats from each shipment were randomly assigned to receive one of three semisynthetic, stripped-lard diets (Bioserve, Inc., Frenchtown, NJ, or Dyets, Inc., Bethlehem, PA), for either an 18-wk (initial experiments)

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or an 8-wk (vitamin E repletion experiments) period. Diet A, an E− (containing less than 10 IU of all racemic-α-tocopheryl acetate/kg of diet); diet B, an E+ diet (50 IU of α-tocopheryl acetate/kg of diet); and diet C, an E++ diet (1000 IU of α-tocopheryl acetate/kg of diet), were nutritionally balanced and identical in composition with the exception of the vitamin E content, which was confirmed by HPLC analysis (courtesy of Lawrence Machlin, Hoffmann-LaRoche, Inc., Nutley, NJ). Diets were obtained every 1–3 mo and stored in the dark at 4°C until used. The diets were designed to produce E−, E+, or E++ rats. The rats were given free access to the specific diets and water. The vitamin E status of each rat was confirmed at the time it was killed by measurement of serum vitamin E concentration by the fluorometric method (16), and in selected animals by measurement of vitamin E content in isolated hepatocytes.

Experimental design. The overall design of our study was to expose hepatocytes isolated from E−, E+, or E++ rats to varying concentrations of $CuCl_2$ for up to 150 min, and to assess changes in lipid peroxidation (generation of MDA) and hepatocyte viability (trypan blue exclusion and LDH release) and morphology over time. In addition, *in vitro* vitamin E repletion of E− hepatocytes was followed by similar $CuCl_2$ incubation studies.

Hepatocyte isolation technique. Hepatocytes were isolated from each rat after the dietary period by a recirculating collagenase perfusion technique (17). Briefly, after intraperitoneal pentobarbital anesthesia (50 mg/kg body weight) and removal of 1.0 ml of blood (for serum vitamin E analysis) from the inferior vena cava, 500 U/kg of heparin sulfate was injected into the inferior vena cava. The rat liver was then perfused *in situ* via the portal vein and inferior vena cava using a Masterflex variable speed drive pump (Cole-Parmer Instrument Co., Chicago, IL) equipped with a Masterflex pumphead, initially with oxygenated Krebs-Henseleit buffer (17) (pH = 7.4, 37°C) until blood-free (~25–50 ml of perfusate), followed by perfusion for 10 min with a 0.05% solution of collagenase type II (Worthington Biochemical Co., Freehold, NJ) in the same oxygenated buffer. The liver was then excised, minced, and filtered twice through nylon mesh (0.3 × 0.5 mm), the resulting cell preparation washed three times, and then suspended in the same buffer at a concentration of approximately 10^6 cells/ml for all experiments. If hepatocyte viability, assessed by trypan blue exclusion (18), exceeded 85%, the remainder of the experiment was conducted. Cells were stored at 4°C and used for study within 2 h of isolation, a time period during which viability was stable. Before incubation with $CuCl_2$, hepatocytes were preincubated in Krebs-Henseleit buffer containing 2% BSA and 1% D-glucose in a 37°C rotary water bath at 80 oscillations/min under an atmosphere of 95% O_2/5% CO_2 for 10 min to "resusitate" cells stored at 4°C (19, 20).

Incubation of hepatocytes with $CuCl_2$. After the preincubation procedure, 10- to 20-ml aliquots of E−, E+, and E++ hepatocytes were incubated with varying concentrations of $CuCl_2$ (0, 200, 400, 800, 1200, 1600, 2400 μM) in Krebs-Henseleit buffer containing 2% BSA and 1% D-glucose in a 37°C rotary water bath at 80 oscillations/min under an atmosphere of 95% O_2/5% CO_2 for 30-min and 150-min periods. In successive experiments, the order of incubation with the progressive concentrations of $CuCl_2$ was reversed. To estimate the degree of copper uptake into the hepatocytes during the 30-min incubation, 5 ml of cell suspension (approximately 5 × 10^6 cells) were removed and washed once in Krebs-Henseleit buffer containing 2% BSA and then twice in buffer without BSA. The resulting hepatocyte pellet was stored at −20°C and analyzed within 3 wk for copper content by atomic absorbance spectroscopy (21) on a Perkin-Elmer 360 Atomic Absorption Spectrophotometer and 2100-HGA Controller and Furnace (Perkin-Elmer Corp., Norwalk, CT). Hepatocyte copper content was expressed as μg/10^6 cells. The effect of $CuCl_2$ incubation on hepatocyte viability was analyzed by trypan blue exclusion and LDH release from hepatocytes (18). LDH release, analyzed in hepatocytes before and after the 30-min incubations, was assessed by comparing the final supernatant LDH to the initial intracellular LDH, and expressed as the percentage of cellular LDH released per 30-min incubation period.

MDA production. Lipid peroxidation in hepatocytes was assessed by measurements of MDA (an end-product of peroxidized fatty acids) by the thiobarbituric acid reaction (22) in fresh aliquots of hepatocytes before and after each of the $CuCl_2$ incubations. After a 0.25-ml aliquot of cells was added to 0.5 ml of 10% trichloroacetic acid in 0.25 N HCl and 5 μl of 2% butylated hydroxytoluene (in ethanol), 1 ml of 0.67% thiobarbituric acid was added, and the mixture was heated at 100°C in a water bath for 15 min, cooled to room temperature, and centrifuged at 1000 × g for 10 min. The supernatant was analyzed for absorbance at 532 nm on a Unicam SP 1700 Ultraviolet Spectrophotometer. MDA bis-(dimethylacetal) (Aldrich Chemical Co., Milwaukee, WI) was used as a standard for construction of separate standard curves for each concentration of $CuCl_2$ used in the hepatocyte incubation experiments.

In vitro vitamin E repletion of hepatocytes. To determine the reversibility of the increased susceptibility to copper toxicity demonstrated in E− hepatocytes (see "Results"), we repeated the $CuCl_2$ incubation experiments at 0, 400, and 800 μM $CuCl_2$ after *in vitro* vitamin E repletion of E− hepatocytes. Vitamin E repletion of hepatocytes was performed by incubating fresh E− hepatocytes with Krebs-Henseleit buffer containing 50 μM D-α-tocopheryl succinate (Sigma Chemical Co., St. Louis, MI) in a rotary waterbath at 37°C under a 95% O_2/5% CO_2 atmosphere for 30 min, conditions which adequately repleted E− hepatocytes during preliminary studies. Vitamin E content of E−, E+, E++, and E-repleted hepatocytes was measured to confirm repletion of deficient cells. After incubation with either the D-α-tocopheryl succinate or control buffer, under subdued light, 4 × 10^6 hepatocytes were washed once in the vitamin E-free incubation buffer containing 2% BSA and then twice in BSA-free, vitamin E-free PBS and stored in a 2:1 mixture of PBS (vitamin E-free) and 1% ascorbic acid (in ethanol) at −70°C protected from light. Aliquots of incubation medium and cells were shipped within 1 wk on Dry Ice to New York University School of Medicine where vitamin E content (ng α-tocopherol and ng total tocopherol/10^6 cells and /μg cholesterol) was measured in duplicate samples by high pressure liquid chromatography with fluorescence detection (23). Vitamin E-repleted cells and incubation medium were analyzed both with and without saponification of the samples in order to detect total (tocopheryl succinate + free tocopherol) and free tocopherol, respectively.

Electron microscopy. Ultrastructural morphology was examined in hepatocytes isolated from E++, E+, and E− rats (two animals from each group) after the 30-min incubations with 0 or 400 μM $CuCl_2$. This latter concentration of $CuCl_2$ was chosen because cell viability was maintained in all groups of hepatocytes, thus avoiding nonspecific morphologic alterations after cell death. Cells were prepared for electron microscopic examination by centrifugation of 2 × 10^6 hepatocytes (1500 rpm × 5 min), resuspension, and centrifugation through a 1:1 mixture of incubation buffer and 3% glutaraldehyde in phosphate buffer (pH 7.4), removal of the supernatant, and immersion of the cell pellet under 3% glutaraldehyde, fixation with 1% osmium tetraoxide, dehydration with ascending concentrations of ethanol, embedding in epoxy resin, sectioning with an LKB Ultramicrotome, (LKB Instruments, Gaithersburg, MD) and counterstaining with uranyl acetate and lead citrate. More than 100 hepatocytes from each rat were examined on a Philips EM 201 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ) without knowledge of the vitamin E or copper status of the cells.

Statistical analysis. Statistical comparisons between experimental groups were conducted by the one-way ANOVA or Student's *t* test using the Prostat (Wadsworth, Inc., Wentworth, NH) computer software. A p value of <0.05 was considered statistically significant. All values are expressed as mean ± SEM.

RESULTS

To investigate the effect of vitamin E status on susceptibility to copper toxicity, we devised a model system using hepatocytes isolated from rats fed diets differing in vitamin E. Growth did not vary significantly among rats from the three vitamin E dietary groups (Fig. 1). Vitamin E status of the rats was confirmed by serum vitamin E concentrations. Serum vitamin E concentrations were less than 3 μg/ml in E− rats, between 5 and 12 μg/ml in E+ rats, and more than 15 μg/ml in E++ rats. After incubation with $CuCl_2$, isolated hepatocyte copper content increased in proportion to $CuCl_2$ concentrations in each dietary group without significant differences between groups (Fig. 2). Copper content was approximately 5- to 20-fold that of control hepatocytes (0 μM $CuCl_2$ incubation concentration) at the lower $CuCl_2$ incubation concentrations (200–800 μM), and 30- to 150-fold that of controls at the higher $CuCl_2$ concentrations (1200–2400 μM).

Initial cell viability assessed by trypan blue exclusion (mean ± SEM) was not significantly different between hepatocytes isolated from rats raised on the three vitamin E diets: 91 ± 0.9% for E++, 87 ± 1.3% for E+, and 89 ± 1.0% for E−. Dose- and time-dependent decreases in cell viability were demonstrated in each group of hepatocytes during $CuCl_2$ incubation experiments (Figs. 3 and 4); however, E− hepatocytes showed significantly decreased ($p < 0.05$, ANOVA) trypan blue exclusion at 30 and 150 min (Fig. 3) and increased LDH release at 30 min compared to E+ and E++ hepatocytes (Fig. 4). There were no significant differences at any $CuCl_2$ concentration between E+ and E++ hepatocytes. The early time course of $CuCl_2$ hepatotoxicity (10-min intervals for 30 min) was examined at concentrations of 0, 1200, and 2400 μM $CuCl_2$ (Fig. 5). E− hepatocytes showed a more rapid decline ($p < 0.05$) in viability in the presence of 1200 μM $CuCl_2$ than did E++ and E+ cells.

Standard curves of MDA production were constructed for each of the $CuCl_2$ concentrations used in the incubation experiments. As $CuCl_2$ concentration increased, the slope of the stan-

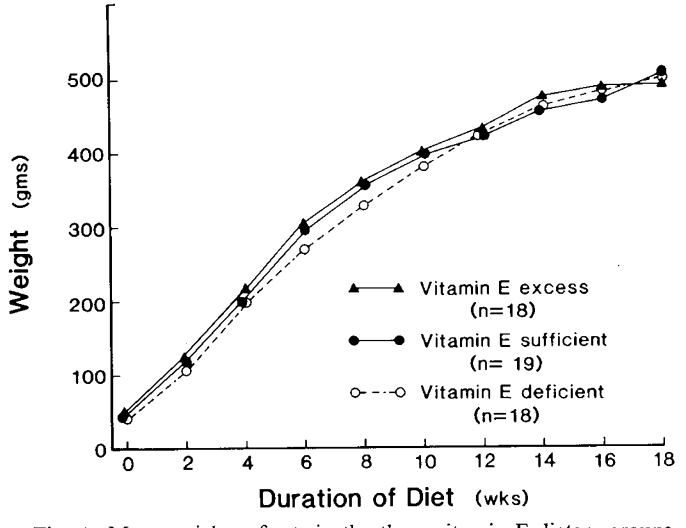

Fig. 1. Mean weights of rats in the three vitamin E dietary groups. Weanling male rats were placed on diets at 3 wk of age and weighed weekly until they were killed at 16 to 18 wk of age. Differences between three groups were not statistically significant by ANOVA.

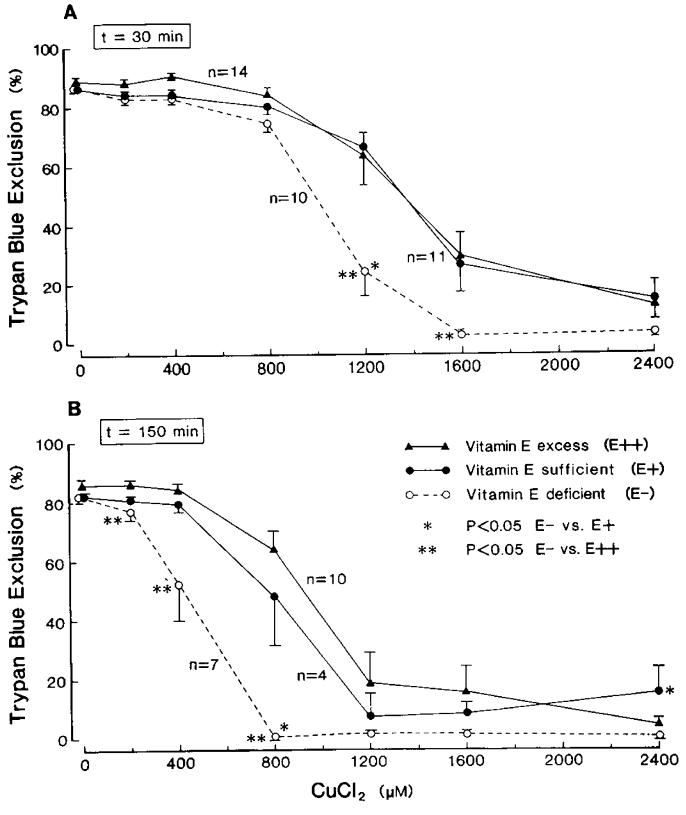

Fig. 3. Trypan blue exclusion of isolated hepatocytes from three vitamin E dietary groups after 30-min (*A*) and 150-min (*B*) incubations with varying concentrations of $CuCl_2$ (0–2400 μM). Statistical analysis by ANOVA.

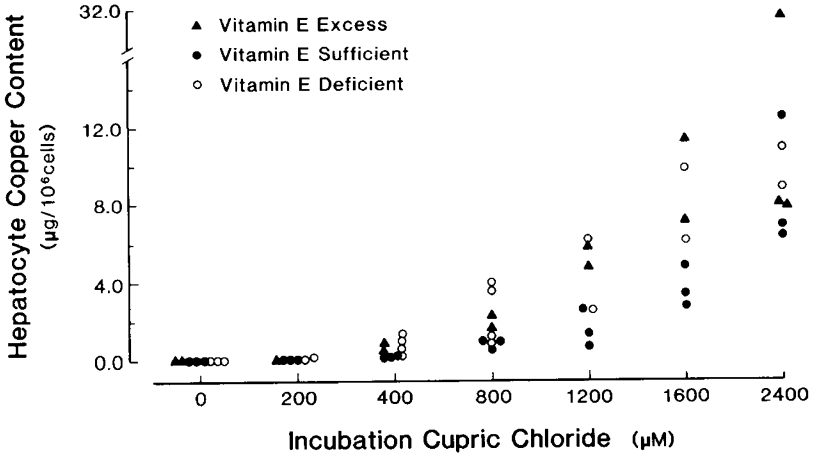

Fig. 2. Copper content of isolated rat hepatocytes from three vitamin E dietary groups after incubation for 30 min with varying concentrations of cupric chloride (0–2400 μM), expressed as μg copper/10^6 cells. Each data point represents one animal studied.

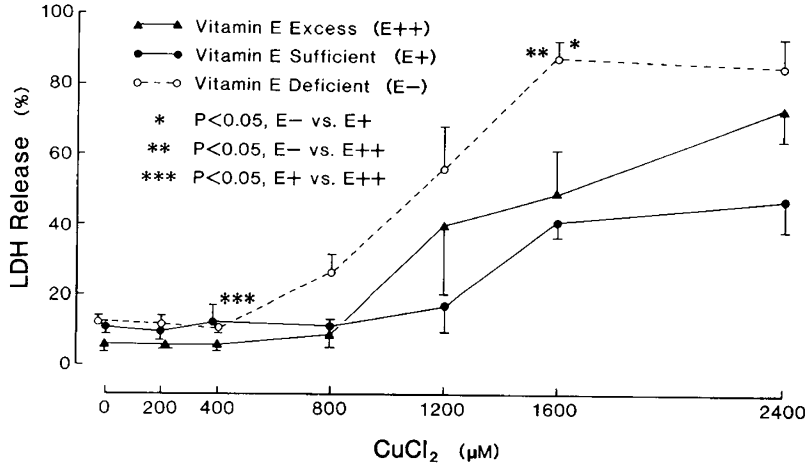

Fig. 4. LDH release from isolated hepatocytes from three vitamin E dietary groups after 30 min incubation with varying concentrations of cupric chloride. Results expressed as percentage of total cellular LDH released into medium over 30 min.

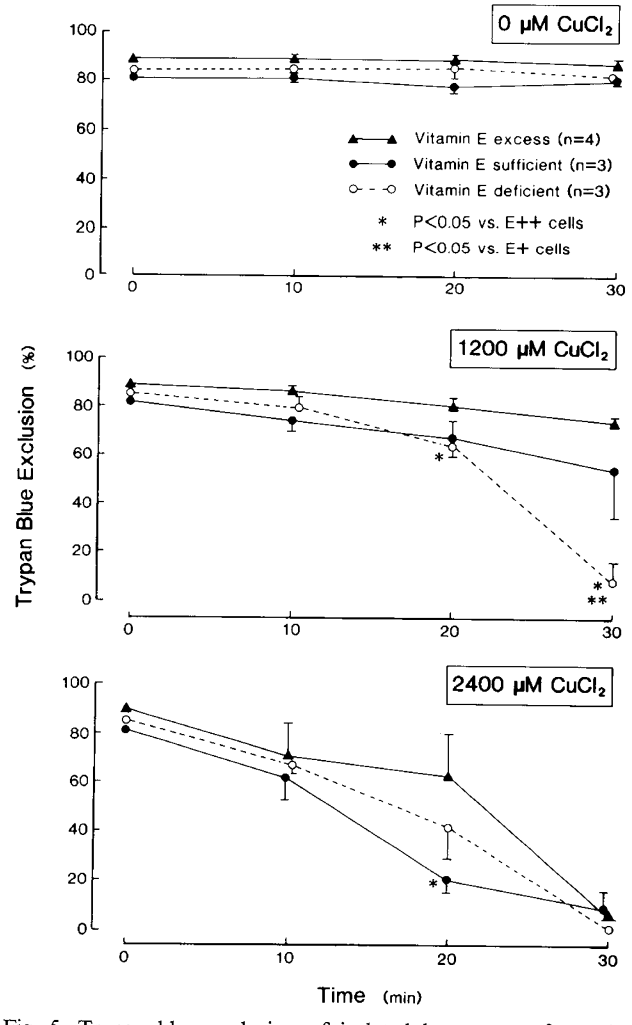

Fig. 5. Trypan blue exclusion of isolated hepatocytes from three vitamin E dietary groups after 10-, 20-, and 30-min incubations with 0, 1200, and 2400 μM $CuCl_2$.

dard curves decreased (Fig. 6). Although the ranges of hepatocyte MDA values in individual experimental groups were broad, certain trends were evident in the E− and E+ cells (Fig. 7). MDA production increased with *1*) longer hepatocyte exposure to $CuCl_2$ (E− and E+ groups), *2*) higher concentrations of $CuCl_2$ (except 2400 μM $CuCl_2$), and *3*) lower hepatocyte vitamin E content after 150 min of exposure. In contrast to the E− and E+ hepatocytes, there were no increases from baseline in MDA production in E++ cells during the 150 min of exposure to any of the $CuCl_2$ concentrations. In addition, MDA values for E+ and E− cells after 30 and 150 min exposures to 2400 μM $CuCl_2$ were lower than MDA values after similar exposures to 1200 and 1600 μM $CuCl_2$. This discrepancy may have been due to more rapid cell injury and death in the 2400 μM $CuCl_2$-exposed hepatocytes leading to inactivation or leakage of enzymes that maintain copper in the reduced state (Cu+) necessary for the initiation of oxidant injury (7). In light of the hepatocyte viability results (Figs. 3 and 4), the MDA data suggest that lipid peroxidation accompanied loss of hepatocyte viability in the E− and E+ cells but not in the E++ cells.

To determine the reversibility of the apparent potentiating effect of vitamin E deficiency on $CuCl_2$ toxicity in our experimental model, *in vitro* vitamin E repletion of hepatocytes was performed. Hepatocytes were incubated for 30 min with vitamin E, as 50 μM α-tocopheryl succinate, because this form of the vitamin has been reported to be taken up relatively rapidly by isolated hepatocytes (24). However, tocopheryl succinate cannot function as an antioxidant because the hydroxyl group necessary for antioxidant function is esterified to succinate. Therefore, both the tocopheryl succinate and the free tocopherol contents of the hepatocytes were measured. Hepatocyte-free α-tocopherol content increased 3–7-fold in the vitamin E-repleted cells and was similar to the tocopherol content of E+ hepatocytes (Table 1). In the vitamin E repletion medium, the free α-tocopherol made up only 0.28% of the total tocopherol (free tocopherol plus tocopheryl succinate). In hepatocytes incubated with tocopheryl succinate for 30 min, free α-tocopherol was 9% of the total tocopherol present in the cells, indicating that tocopheryl succinate was taken up and hydrolyzed by the isolated hepatocytes. After incubation with $CuCl_2$, the vitamin E-repleted hepatocytes showed restoration of resistance to $CuCl_2$ toxicity with a proportional reduction in MDA production (Fig. 8).

Ultrastructural examination was performed on hepatocytes exposed to 0 or 400 μM $CuCl_2$ for 30 min. Normal morphology was present in E−, E+, and E++ hepatocytes not exposed to $CuCl_2$. These cells were round, with intact cell membranes, microvilli circumferentially distributed, and unremarkable nuclei and other organelles. The E++ and E+ cells exposed to 400 μM $CuCl_2$ were generally intact except for the presence of small plasma membrane blebs on less than 10% of the cells, making up 1–2% of the cross-sectional area of the cells. The blebs contained a granular matrix of "cell sap" with scattered free ribosomes and glycogen granules. In contrast, 50–75% of E−

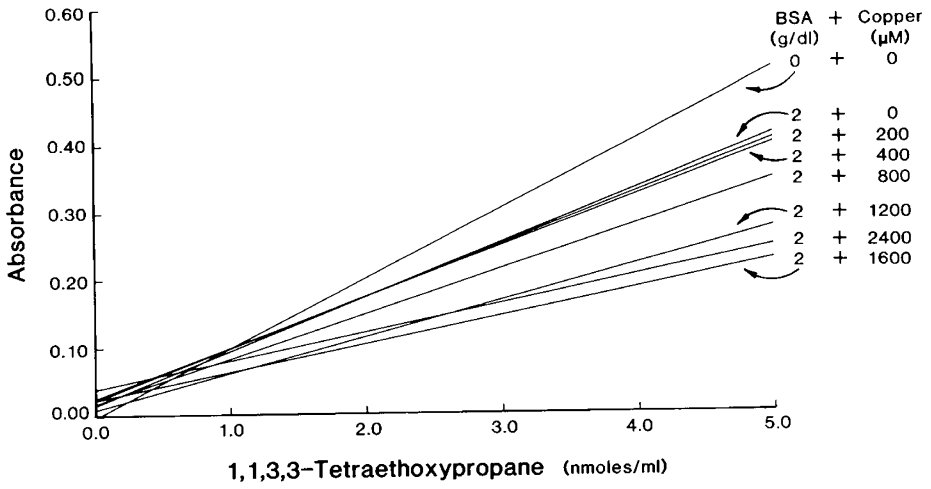

Fig. 6. Standard curves for MDA assay. Absorbance (at 532 nm) plotted against MDA standard (1,1,3,3-tetraethoxypropane; nmol/ml) under different experimental conditions, including the addition of 0 or 2.0 g/dl of BSA and varying concentrations of $CuCl_2$ (0–2400 μM).

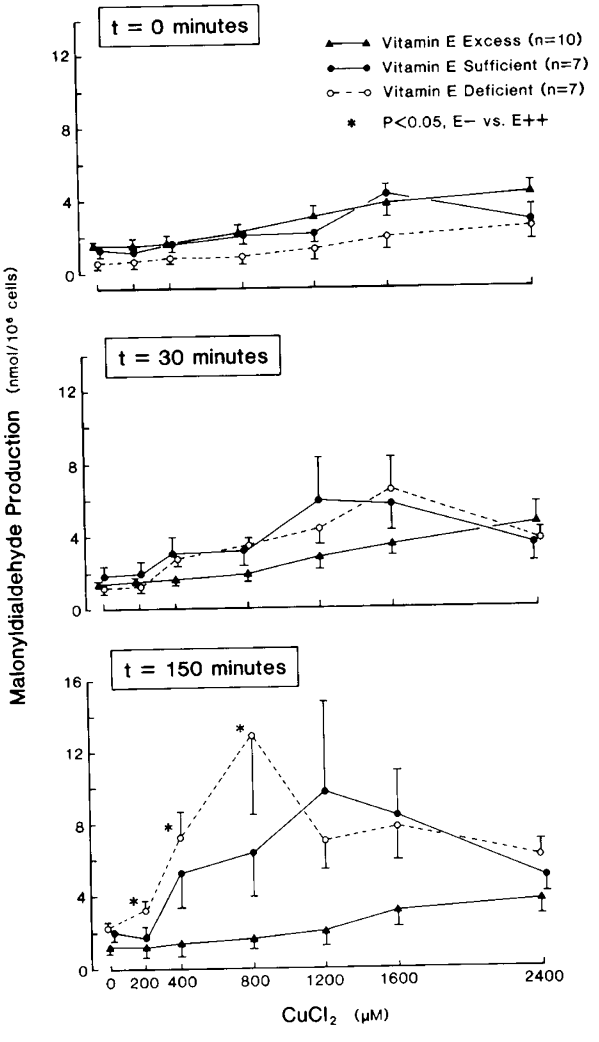

Fig. 7. MDA production (nmol/10^6 cells) by isolated hepatocytes from three vitamin E dietary groups after incubation for 0, 30, or 150 min with varying concentrations of $CuCl_2$ (0–2400 μM). Results for each experimental group are expressed as mean ± SEM. Note that at every concentration of $CuCl_2$, there is no increase with time in MDA production in E++ hepatocytes, in contrast to the progressive increases in MDA production observed in E+ or E− hepatocytes.

hepatocytes exposed to 400 μM $CuCl_2$ had larger plasma membrane blebs, each bleb comprising up to 15% of a cell's cross-sectional area and extending well beyond the cell's otherwise circular conformation (Fig. 9). The bleb membrane was intact, devoid of microvilli, and contained no organelles except for minimal endoplasmic reticulum. In addition, areas in the cell subjacent to the large blebs were also devoid of organelles. These E− cells were otherwise intact with normal-appearing organelles and microvilli on the nonprotruding portions of the plasma membrane.

DISCUSSION

The results of our study demonstrated that acute exposure of isolated rat hepatocytes to $CuCl_2$ resulted in dose- and time-dependent toxicity manifested by changes in trypan blue exclusion, LDH release, and ultrastructural morphology. That lipid peroxidation might mediate this toxicity was suggested by our results. We noted increased MDA generation from E− and E+ hepatocytes that related directly to both the duration of copper exposure and to $CuCl_2$ concentrations. Furthermore, copper toxicity and MDA production were enhanced in E− hepatocytes. In addition, the *in vitro* vitamin E-repletion experiments showed that restoration of E− hepatocyte resistance to copper toxicity after vitamin E repletion was associated with suppression of MDA production (Fig. 8). However, the dissociation between MDA production and copper toxicity in the E++ hepatocyte provided compelling evidence against the role of lipid peroxidation as the mechanism of copper toxicity in our model. In contrast to E+ cells, MDA production did not increase in E++ hepatocytes during incubation with any of the $CuCl_2$ concentrations despite almost identical decreases in cell viability in E++ and E+ cells at all concentrations of $CuCl_2$ (Figs. 3 and 4). Thus, hepatocyte injury occurred in E++ cells in the absence of the generation of lipid peroxides.

MDA was used as the measure of lipid peroxidation in our study. Despite the fact that MDA can be degraded by functioning mitochondria (25), Smith *et al.* (26) have shown that MDA production correlates well with other measures of lipid peroxidation in isolated hepatocytes. Recknagel *et al.* (27) also propose MDA as the method of choice for monitoring *in vitro* lipid peroxidation. Our MDA standard curves for each concentration of incubation $CuCl_2$ demonstrated a marked effect of BSA and $CuCl_2$, which significantly altered final MDA values. Stacy and Klassen (28) did not construct such standard curves, perhaps explaining why they found no increase in MDA production during $CuCl_2$ incubation of isolated hepatocytes.

Table 1. *Tocopherol content of isolated rat hepatocytes**

Hepatocyte group	ng α-tocopherol/10^6 cells		ng α-tocopherol/μg cholesterol	
	Total	Free	Total	Free
E− (n = 6)	3.4 ± 2.7		0.41 ± 0.29	
E+ (n = 3)	47.0 ± 11.8		8.2 ± 3.5	
E++ (n = 1)	757.4		135.5	
Vitamin E-repleted (n = 5)	304 ± 79	27.0 ± 9.0	50.6 ± 32.1	9.4 ± 7.9

* Values (mean ± SD) represent results of tocopherol analysis after saponification (total tocopherol = free + esterified) and nonsaponification (free tocopherol only) of isolated rat hepatocytes. The total tocopherol equals the free tocopherol for groups E−, E+, and E++ because tocopheryl esters are not found *in vivo* in liver. Because tocopheryl succinate was used for *in vitro* vitamin E repletion, only the nonsaponified sample values represent the free tocopherol in this group.

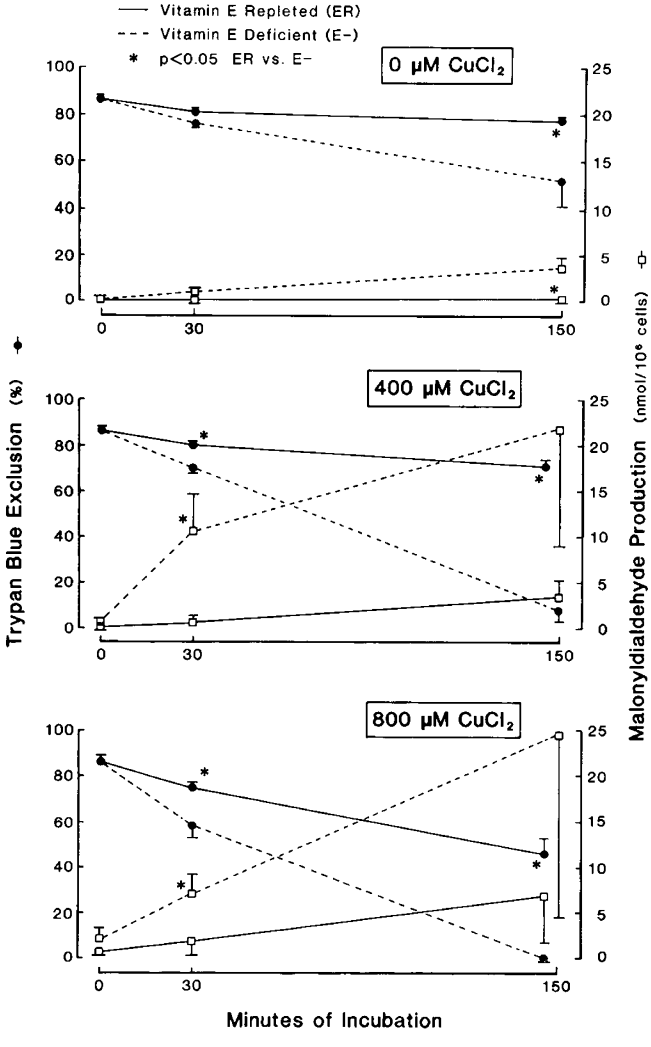

Fig. 8. Results of *in vitro* vitamin E repletion experiments. Trypan blue exclusion and MDA production of isolated hepatocytes, that were either E− or had undergone *in vitro* vitamin E repletion, are plotted against time (min) of incubation with 0, 400, or 800 μM $CuCl_2$. Results are expressed as mean ± SEM.

There are several ways to interpret the disassociation between lipid peroxide production and cell injury in E++ hepatocytes. Our MDA assay may have lacked the sensitivity to detect subtle changes of lipid peroxide formation in the E++ hepatocytes (26). However, because changes in MDA were easily detected in E+ and E− cells under identical experimental conditions, it is unlikely that similar differences in MDA production would not have been detected in E++ hepatocytes if they had been present. It is possible that the MDA generated by hepatocytes after exposure to $CuCl_2$ was a consequence rather than the cause of cell injury (29, 30) and that high levels of vitamin E in the E++ cells suppressed this postinjury lipid peroxidation, whereas the vitamin E content of E− and E+ cells was insufficient for inhibition of this process.

Another possibility is that the site of the copper-induced oxidant injury in the hepatocyte was not the fatty acids but rather nucleic acids (31) or thiol-rich proteins associated with cellular membranes or cytoskeleton. Oxidant stress has been shown to inactivate cellular enzymes by causing polymerization and cross-linking, polypeptide chain scission, and chemical changes in individual amino acids (32). In this respect, copper has been reported to alter the interaction of membrane protein and lipid (33) and to induce polymerization of intracellular proteins such as tubulin (34). Moreover, recent studies (35, 36) suggest that oxidative hepatocyte injury may be mediated through alterations in protein thiol and intracellular calcium homeostasis, resulting in alterations of cytoskeleton structure causing surface blebbing of hepatocytes similar to that observed in our study. Thus, it is plausible that the high vitamin E level in the E++ cells may have been sufficient to protect membrane lipids but not thiol-rich proteins from oxidant injury.

A third possibility is that there may be multiple targets damaged by free radicals generated by the exposure of hepatocytes to $CuCl_2$. The crucial target leading to cell death does not appear to be membrane fatty acids, based on the data in the E++ cells. However, vitamin E deficiency may unveil an additional target of oxidant injury, *i.e.* membrane unsaturated fatty acids, which leads to the generation of more free radicals that may, in turn, further damage the critical target (perhaps thiol-rich proteins). Thus, copper is toxic to all hepatocytes, but even more so when vitamin E deficiency promotes the generation of free radicals through membrane lipid peroxidation.

The results of our study demonstrate an increased susceptibility to acute copper toxicity in hepatocytes from E− rats compared to hepatocytes from E+ or overloaded rats, and that *in vitro* vitamin E repletion of E− hepatocytes restores the resistance to copper toxicity. This potentiating effect of vitamin E deficiency on copper toxicity may have clinical relevance in regard to chronic cholestatic liver diseases, where both secondary hepatic copper overload (37, 38) and vitamin E deficiency (39, 40) are common. Although it is not certain if copper overload during cholestasis is toxic to the liver (41), we have observed that reversing vitamin E deficiency in children with chronic cholestasis and secondary hepatic copper overload was associated with a decrease of fasting serum bile acid concentrations (42), suggesting that vitamin E repletion lessened hepatic injury and improved hepatic function. Although studies in isolated hepatocytes should not be generalized to the intact animal without some reservation, our data suggest that vitamin E deficiency during conditions where hepatic copper content is increased may predispose the liver to additional injury caused by the copper. In support of this hypothesis, a preliminary report has shown that vitamin E deficiency exacerbates copper hepatotoxicity in an

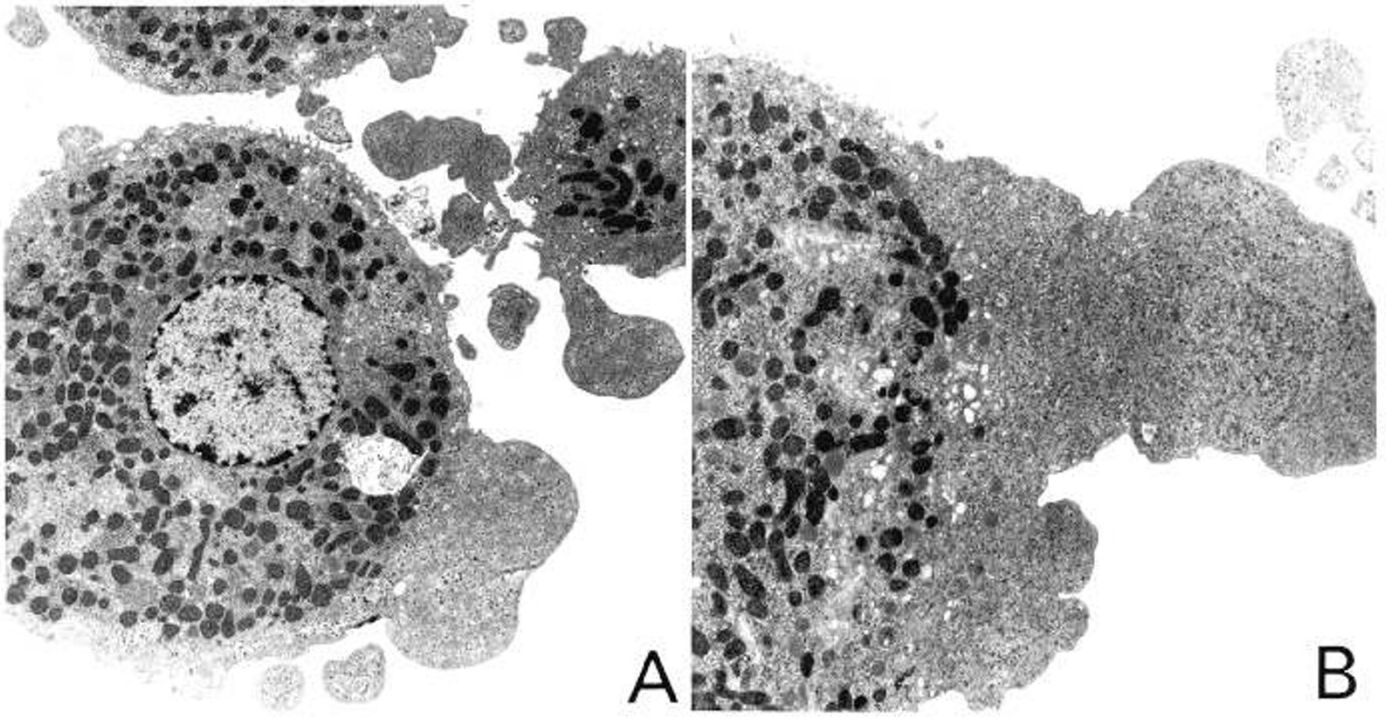

Fig. 9. Ultrastructural appearance of E− hepatocytes after 30-min exposure to 400 μM $CuCl_2$. *A*, numerous plasma membrane "blebs" devoid of microvilli and organelles are subjacent to similar organelle-free areas of cells. Otherwise, the cells are intact, with microvilli distributed on unaffected portions of the cell membrane and a uniform cell substructure (original magnification × 7500). *B*, higher power view of a large bleb protruding from an otherwise intact hepatocyte (original magnification × 9000).

intact rat model of dietary copper overload (43). This interaction between vitamin E and copper in the liver merits further study.

In conclusion, the results of our study suggest that *1*) lipid peroxidation may not directly mediate acute toxicity of copper to isolated rat hepatocytes, and *2*) vitamin E deficiency predisposes isolated hepatocytes to more severe injury upon exposure to copper, whereas excess vitamin E, compared to normal vitamin E levels, offers no additional protection. The possible role of oxidant injury to thiol-rich proteins or nucleic acids in copper-induced cellular injury should be explored.

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