Vitamin E Depletion Enhances Liver Oxidative Damage in Rats with Water-Immersion Restraint Stress

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Summary We examined the effect of vitamin E depletion on liver oxidative damage in rats with water-immersion restraint stress (WIRS). Male Wistar rats were fed a normal diet (N) or vitamin E-depleted diet (VE-D) for 4 wk. N- and VE-D-fed rats were exposed to WIRS for 6 h. The activities of serum transaminases and lactate dehydrogenase and serum ascorbic acid concentration were similar in both diet groups. WIRS exposure increased these serum enzyme activities and the serum ascorbic acid concentration in both diet groups but the ratios of these increases were higher in VE-D-fed rats than in N-fed rats. Serum and liver α-tocopherol concentrations in VE-D-rats were approximately 50% and 30% of those in N-fed rats, respectively. WIRS exposure reduced liver α-tocopherol concentration in VE-D-fed rats, but not in N-fed rats. Liver ascorbic acid and reduced glutathione concentrations were higher in the VE-D-fed group than in the N-fed group. WIRS exposure reduced liver ascorbic acid and reduced glutathione concentrations in both diet groups. There were no differences in liver concentrations of coenzyme Q9 or coenzyme Q10 in the reduced form between the N- and VE-D-fed groups. WIRS exposure reduced liver concentrations of coenzyme Q9 and coenzyme Q10 in the reduced form in both diet groups. Liver lipid peroxide concentration was higher in the VE-D-fed group than in the N-fed group. WIRS exposure raised liver lipid peroxide concentration more in the VE-D-fed group than in the N-fed group. These results indicate that vitamin E depletion enhances liver oxidative damage in rats with WIRS.

Key Words water-immersion restraint stress, rat liver, vitamin E depletion, antioxidants, oxidative damage
present, however, there is no information on whether vitamin E deficiency affects hepatic oxidative damage in rats with WIRS.

In the present study, therefore, we examined whether 4-wk dietary vitamin E depletion affects hepatic oxidative damage in rats with 6 h of WIRS when compared with normal diet feeding. It is known that exposure of rats to 6 h of WIRS increases not only serum ACTH, corticosterone, and glucose levels but also serum ascorbic acid and LPO levels in response to the stress (2–5). Therefore, we examined the effect of dietary vitamin E depletion on serum corticosterone, glucose, ascorbic acid, and LPO concentrations in rats with WIRS in comparison with that of normal diet feeding.

**MATERIALS AND METHODS**

**Materials.** L-Ascorbic acid, corticosterone, α,α'-dipiridyl, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), ethylenediaminetetraacetic acid (EDTA), GSH, 2-thiobarbituric acid, α-tocopherol and δ-tocopherol used for vitamin E determination, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All chemicals used were of reagent grade and were not further purified.

**Animals.** Male Wistar rats aged 3 wk were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animals were housed in cages in a ventilated animal room with controlled temperature (23±2°C) and relative humidity (55±5%) with 12 h of light (7:00 to 19:00). The animals were maintained with free access to rat chow, ANA-76A diet (Research Diets Inc., New Brunswick, NJ, USA), and tap water for 1 wk. All animals received humane care in compliance with the Guidelines of the Management of Laboratory Animals in Fujita Health University. The animal experiment was approved by Institutional Animal Care and Use Committee.

**Feeding of normal diet, vitamin E-depleted diet, and vitamin E-supplemented diet.** Four-week-old rats were administered a normal diet (N) or vitamin E-depleted diet (VE-D) for 4 wk. The above-described ANA-76A diet was used as the normal diet and the composition of the normal diet was as follows: casein, 20.0%; cornstarch, 15.0%; sucrose, 50.0%; cellulose, 5.0%; corn oil, 5.0%; mineral mixture, 3.5%; vitamin mixture, 1.0%; dl-methionine, 0.3%. This normal diet contained vitamin E acetate (50 IU/kg diet). The vitamin mixture did not contain vitamin C. The vitamin E-depleted diet was prepared by removing vitamin E acetate from the normal diet. This diet contained 0.4 IU vitamin E per kg diet. The mean amount of each diet consumed per day was 15 g/animal.

**Induction of WIRS.** According to our previous reports (3–5), WIRS was induced in 7 wk-old rats which were starved for 24 h prior to experiments, but were allowed free access to water. The fasted rats were restrained in wire cages and immersed up to the depth of the xiphoid process in a 23°C water bath for 6 h.

**Determinations of serum and liver components.** All rats were weighed and then sacrificed under ether anesthesia, at which time blood was collected from the infe-rior vena cava. Serum was obtained from the collected blood by centrifugation. Immediately after sacrifice, each liver was perfused with ice-cold 0.9% NaCl through the portal vein and isolated. The isolated liver was washed in ice-cold 0.9% NaCl, wiped on a paper filter, and then weighed. The collected serum and livers were stored at -80°C until use. Serum corticosterone was measured by the fluorometric method (12) using authentic corticosterone as a standard. Serum glucose was assayed using a commercial kit, Glucose-CII Test Wako. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using a commercial kit, Transaminase C II-Test Wako. Serum lactate dehydrogenase (LDH) was assayed using a commercial kit. LDH-Test Wako. These assay kits were obtained from Wako Pure Chemical Industries, Ltd. Serum LPO was assayed by the fluorometric thiobarbituric acid method (13) using tetramethoxypropane as a standard. The concentration of serum LPO is expressed in malondialdehyde (MDA) equivalents. The liver tissue was homogenized in 9 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA to prepare 10% homogenate. The liver homogenate was used for the determinations of ascorbic acid, GSH, α-tocopherol, and LPO. Ascorbic acid in the serum and liver homogenate was determined by the α,α'-dipiridyl method (14) using L-ascorbic acid as a standard. GSH in the liver homogenate was assayed by the DTNB method (15) using GSH as a standard. α-Tocopherol in the serum and liver homogenate was assayed by the high-performance liquid chromatographic method with electrochemical detection using δ-tocopherol as an internal standard as described in our previous report (16). CoQ9 red and CoQ10 red in the liver homogenate were assayed using the high-performance liquid chromatographic method (11, 17). LPO in the liver homogenate was assayed by the colorimetric thiobarbituric acid method (18) using tetramethoxypropane as a standard except that the reaction mixture contained 1 mM EDTA. The amount of liver LPO is expressed in MDA equivalents. Liver protein was assayed using a commercial kit, the Rapid Protein Assay kit (Wako Pure Chemical Industries, Ltd.). Bovine serum albumin was used as a standard in this protein assay.

**Statistical analysis.** All results obtained are expressed as means±SD. The statistical analyses of the results were performed using a computerized statistical package (StatView). Each mean value was compared by two-way analysis of variance (ANOVA) and Fisher’s protected least significance (PLSD) for multiple comparisons as the post-hoc test. The significance level was set at p<0.05.

**RESULTS**

There was no significant difference in serum corticosterone, glucose, or ascorbic acid concentration between N- and VE-D-fed rats (Fig. 1). Exposure to 6 h of WIRS caused significant increases in serum corticosterone, glucose, and ascorbic acid concentrations in the N- and VE-fed groups but the increased serum ascorbic acid concentration was significantly higher in the VE-D-fed group than in the N-fed group (Fig. 1).
There was no significant difference in serum ALT, AST, or LDH activity between N- and VE-D-fed rats (Fig. 2). Exposure to 6 h of WIRS caused significant increases in serum ALT, AST, and LDH activities in the N- and VE-D-fed groups but the increased ALT, AST, and LDH activities were significantly higher in the VE-D-fed group than in the N-fed group (Fig. 2).

There was no significant difference in body weight or liver weight between N- and VE-D-fed rats (Fig. 3). Exposure to 6 h of WIRS had no effect on body weight or liver weight in the N- and VE-D-fed groups (Fig. 3).

Serum α-tocopherol concentration was significantly lower in VE-D-fed rats than in N-fed rats; VE-D-fed rats had approximately 50% of the serum VE concentration of N-fed rats (Fig. 4A). Exposure to 6 h of WIRS had no significant effect on serum α-tocopherol concentrations in N- or VE-D-fed rats (Fig. 4A). There was no significant difference in serum LPO concentration between the N- and VE-D-fed groups (Fig. 4B). Exposure of N-fed rats or VE-D-fed rats to 6 h of WIRS caused a significant increase in serum LPO concentration, but the increased amount of serum LPO was significantly larger in VE-D-fed rats than in N-fed rats (p<0.05) (Fig. 4B). Liver α-tocopherol concentration was significantly lower in VE-D-fed rats than in N-fed rats; the VE-D-fed group had approximately 30% of the liver α-tocopherol concentration of the N-fed group (Fig. 4C). Exposure to 6 h of WIRS caused a significant reduction in liver α-tocopherol concentration in VE-D-fed rats, but not in N-fed rats (Fig. 4C). Liver LPO concentration was significantly higher in VE-D-fed rats than in N-fed rats (Fig. 4D). Exposure of N-fed rats or VE-D-fed rats to 6 h of WIRS caused a significant increase in liver LPO concentration, although the increased amount of liver LPO was significantly larger in VE-D-fed rats than in N-fed rats (p<0.05) (Fig. 4D).

There was no significant differences in liver CoQ9 reduced or CoQ10 reduced concentration between N- and VE-D-fed
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rats (Fig. 5A and B). Exposure to 6 h of WIRS caused significant reductions in liver CoQ9 red and CoQ10 red concentrations in N- and VE-D-fed rats although there were no significant differences in the reduced amounts of liver CoQ9 red or CoQ10 red between N- and VE-D-fed rats (Fig. 5A and B). Liver ascorbic acid and GSH concentrations were significantly higher in VE-D-fed rats than in N-fed rats (Fig. 5C and D). Exposure to 6 h of WIRS caused significant reductions in liver ascorbic acid and GSH concentrations in the N- and VE-D-fed groups, although there was no significant difference in the reduced amount of liver ascorbic acid between the two groups and the reduced amount of liver GSH was significantly larger in the VE-D-fed group than in the N-fed group (p<0.05) (Fig. 5C and B).
DISCUSSION

The present study has clearly shown that vitamin E depletion enhances hepatic oxidative damage in rats exposed to WIRS for 6 h without affecting the stress response.

In the present study, N-fed rats with 6 h of WIRS showed increases in the serum levels of corticosterone, glucose, and ascorbic acid, as reported previously (2–5). Dietary vitamin E depletion for the period of 4 wk had no significant effect on the stress-induced increases in serum corticosterone or glucose levels. These results indicate that dietary vitamin E depletion for the period of 4 wk does not affect the stress response via the hypothalamus-pituitary-adrenal axis or the sympathetic-adrenomedullary system in rats with 6 h of WIRS. However, the increased serum ascorbic acid concentration was significantly higher in VE-D-fed rats than in N-fed rats. We have suggested that an increase in ascorbic acid level in the serum of rats with WIRS could be, at least in part, due to the release of ascorbic acid from the liver tissue (5). Nakano and Suzuki (19) also have suggested that, in rats with restraint stress, an increase in ascorbic acid level in the serum is derived from the liver tissue. Nathani and Nath (20) have reported that a single administration of corticotrophin (or ACTH) causes both increases in the hepatic levels of ascorbic acid and dehydroascorbic acid, an oxidized form of ascorbic acid, and a decrease in the hepatic level of diketogulonic acid, a metabolite of dehydroascorbic acid, by inhibiting hepatic ascorbic acid degrading enzymes in normal rats, but not in adrenalectomized rats, resulting in an increase in blood total ascorbic acid level. A similar action of administered ACTH to increase ascorbic acid levels in the blood and liver of rats has been reported by Lahiri and Lloyd (21). Thus, ACTH causes ascorbic acid accumulation in the liver of normal rats and release of the accumulated ascorbic acid from the liver tissue. In the present study, however, the liver of VE-D-fed rats was damaged by WIRS exposure more strongly than that of N-fed rats, as described below. Therefore, it is assumed that the damaged liver of VE-D-fed rats with WIRS releases ascorbic acid accumulated in the liver into the bloodstream more easily than the damaged liver of N-fed rats with WIRS. In addition, it has been shown in rats with a single administration of ACTH that ascorbic acid is released from the adrenal gland into the bloodstream (22). It has also been shown that human adrenal glands secrete ascorbic acid in response to ACTH (23). As described above, there was no difference in the stress response between N-fed and VE-D-fed rats with WIRS. Therefore, it is suggested that vitamin E depletion in rats with WIRS could enhance the release of ascorbic acid from the damaged liver into the bloodstream rather than the ACTH-mediated release of ascorbic acid from the adrenal gland into the bloodstream, resulting in a
more greatly increased ascorbic acid level in the serum. It has been shown that oxidative stress-mediated liver cell damage occurs in rats with 6 h of WIRS (3–5). In the present study, serum ALT, AST, and LDH activities, indices of liver cell damage, in rats fed VE-D for 4 wk were not significantly different from those in rats fed N for the same period. These results indicate that, like normal feeding, 4-wk dietary vitamin E depletion has no effect on the liver tissue integrity of rats. Exposure of N-fed rats to 6 h of WIRS caused increases in serum ALT, AST, and LDH activities, as reported previously (2–5). The stress-induced increases in serum ALT, AST, and LDH activities were enhanced in VE-D-fed rats. These results indicate that dietary vitamin E depletion enhances WIRS-induced liver cell damage in rats. There were no differences in body weight or liver weight between N- and VE-D-fed rats under feeding of each diet at a daily mean dose of 15 g per animal for 4 wk. These results indicate that VE-D-fed rats have normal growth and no apparent malnutrition. As to body weight and liver weight in rats with and without vitamin E depletion, similar results have been reported by Terada et al. (7) and de Cabo et al. (10). The body weight and liver weight in rats fed N and VE-D were not affected by exposure to 6 h of WIRS. Our previous report showed that no apparent histological change concerning necrosis occurred in the liver tissue of rats with 6 h of WIRS, although significant, but not so high, increases in serum ALT and AST activities occurred in the liver tissue, as found in the present study (3). Taking this finding and the increased serum ALT, AST, and LDH levels found in the present study into consideration, it seems that liver damage occurring in N-fed and VE-D-fed rats with 6 h of WIRS is not so severe. Therefore, it can be thought that there was no effect of WIRS exposure on body weight or liver weight in N-fed and VE-D-fed rats. As described above, there was no difference in the stress response itself between N- and VE-D-fed rats with 6 h of WIRS. Therefore, it is suggested that vitamin E depletion leading to defect of the antioxidant defense system in the liver of rats could cause an enhancement of liver damage induced by WIRS.

When serum and liver α-tocopherol concentrations in rats fed VE-D for 4 wk were compared with those in rats fed N for the same period, the serum and liver α-tocopherol concentrations were significantly lower in VE-D-fed rats than in N-fed rats. In addition, VE-D-fed rats showed a more marked change in α-tocopherol concentration in the liver tissue than in the serum. Similar changes in serum and liver α-tocopherol concentrations in rats with vitamin E deficiency have been shown by Duthie et al. (6) and Terada et al. (7). Exposure to 6 h of WIRS had no significant effect on the serum α-tocopherol concentration either in N-fed rats or in VE-D-fed rats. In contrast, exposure to 6 h of WIRS caused further reduction in the liver α-tocopherol concentration in VE-D-fed rats, although the stress exposure had no effect on that concentration in N-fed rats, as shown in our previous reports (3–5). These results suggest that the further reduction in α-tocopherol concentration in the liver of VE-D-fed rats with WIRS could be due to consumption of the vitamin in response to further increase in WIRS-induced oxidative stress in the tissue.

In the present study, there was no difference in serum LPO concentration between rats fed VE-D and N for 4 wk, while liver LPO concentration was significantly higher in the VE-D-fed group than in N-fed group. These results were well consistent with those reported by Duthie et al. (6) and Terada et al. (7). Exposure to WIRS for 6 h increased serum and liver LPO concentrations in VE-D- and N-fed rats. The increased amounts of serum and liver LPO in VE-D-fed rats with WIRS were significantly larger than those in N-fed rats with WIRS. These results indicate that vitamin E depletion enhances the defect of the antioxidant defense system in the liver of rats with WIRS, leading to the above-described further consumption of vitamin E remaining in the tissue.

It is known that the antioxidant role of CoQ involves an interaction with α-tocopherol (24). It is also known that the reduced form of CoQ supports the chain-breaking antioxidant function of α-tocopherol for lipid peroxidation by regeneration of vitamin E from its oxidized form (25, 26). Furthermore, it has been shown that exposure of rats to cold stress increases CoQ concentrations in all cellular membranes of the liver tissue (27). It has also been shown that intraperitoneal administration of cortisone (40 μg IU) to rats for 7 d increases CoQ synthesis in the liver (28). de Cabo et al. (10) have reported that increases in CoQ9red and CoQ10red levels in the plasma membrane of liver cells occur as the adaptable response to vitamin E depletion in rats fed vitamin E-deficient diet for 2 or 6 wk. In contrast, it has been reported that no increases in CoQ9red or CoQ10red concentrations occur in hepatocytes isolated from rats fed vitamin E-deficient diet for 5 wk when compared with hepatocytes isolated from rats fed vitamin E-sufficient diet for the same period (11). It has also been reported that endogenous CoQred acts as a more potent antioxidant than endogenous α-tocopherol in vitamin E-deficient rat hepatocytes (11). In the present study, rats fed VE-D for 4 wk showed no significant increases in CoQ9red or CoQ10red concentrations in the liver tissue, although these concentrations tended to increase in the tissue. Upon exposure to 6 h of WIRS, liver CoQred concentration was reduced to a similar extent in N- and VE-D-fed rats with WIRS, while a reduction of liver CoQ10red concentration tended to be larger in VE-D-fed rats than in N-fed rats. These results suggest that CoQ9red and CoQ10red could not sufficiently contribute to the antioxidant action of α-tocopherol in the liver of N-fed rats with WIRS, resulting in increased lipid peroxidation in the tissue. These results also suggest that CoQ9red and CoQ10red could exert little compensatory action on the reduction of the antioxidant defense system due to vitamin E depletion in the liver of VE-D-fed rats with WIRS, resulting in further increased lipid peroxidation in the tissue.

It is known that ascorbic acid is consumed to support the chain-breaking antioxidant function of vitamin E for lipid peroxidation through regeneration of vitamin E from its radical form (29–32). It is also known that GSH
spares vitamin E by lowering the rate of the initiation of lipid peroxidation in the bilayer phase and the antioxidant action of ascorbic acid through regeneration of ascorbic acid from its oxidized form (33–35). de Cabo et al. (10) have shown that 2 or 6 wk of vitamin E deficiency induces adaptation to oxidative stress in the liver of rats by increasing the level of ascorbic acid in the liver cytosolic fraction in addition to CoQ10 and CoQ10-fed levels in the plasma membrane of the liver cells. In the present study, VE-D-fed rats showed significantly higher liver ascorbic acid and GSH concentrations than N-fed rats. This result suggests that dietary vitamin E-depletion could cause a compensatory action on the reduction of the antioxidant defense system due to vitamin E depletion in the liver of rats through increases in ascorbic acid and GSH concentrations in the tissue. However, the compensatory reaction to maintain the antioxidant defense system in the liver of VE-D-fed rats was not sufficient because lipid peroxidation was increased in the tissue as described above. Nevertheless, VE-D-fed rats had no clear liver damage, as described above, resulting in no release of the increased ascorbic acid from the liver tissue into the bloodstream. Based on this evidence, it can be thought that there was no difference in serum ascorbic acid level between the N-D- and VE-D-fed rats, as described above.

Exposure to 6 h of WIRS caused significant reductions in liver ascorbic acid and GSH concentrations in N- and VE-D-fed rats, although the reduction in liver GSH was greater in VE-D-fed rats than in N-fed rats. Thus, liver ascorbic acid and GSH levels were reduced by WIRS exposure irrespective of vitamin E intake status. A part of the reduction of ascorbic acid concentration in the liver of N- and VE-D-fed rats with WIRS may be due to the release of ascorbic acid from the tissue into the bloodstream, as described above. Taking the above-described change in liver α-tocopherol concentration in N- and VE-D-fed rats with WIRS into consideration, the following suggestion can be drawn: ascorbic acid and GSH exert their antioxidant action to maintain the antioxidant function of vitamin E in the liver of N-fed rats with WIRS, resulting in their consumption in the tissue. In contrast, ascorbic acid and GSH increasing through compensation mediated by vitamin E depletion are consumed to maintain the antioxidant defense system in place of depleted vitamin E rather than the antioxidant function of vitamin E in the liver of VE-D-fed rats with WIRS. We have reported that a single vitamin E pre-administration to rats with 6 h of WIRS protects against liver damage by reducing increased lipid peroxidation through an increase in vitamin E level in the liver tissue (4). Accordingly, both this finding and the results of the present study allow us to think that liver vitamin E status plays an important role in protecting against oxidative damage in the liver of rats with WIRS.

In conclusion, the results obtained from the present study indicate that vitamin E depletion enhances oxidative liver damage in rats with WIRS through reduction of the antioxidant defense system associated with vitamin E without affecting the stress response.

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