The α-tocopherol status and expression of α-tocopherol-related proteins in methionine-choline deficient rats treated with vitamin E

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Non-alcoholic fatty liver disease is the most common liver disorder in developed countries, and its incidence is increasing in all population groups. As an antioxidant, vitamin E is effective in the treatment of non-alcoholic fatty liver disease, although the mechanism is still unclear. Methionine-choline deficient Wistar rats (n = 5) used as an experimental model of non-alcoholic fatty liver disease were fed a vitamin E-enriched diet (500 mg/kg) for 4 weeks. The effects were assessed by measuring lipid peroxidation, α-tocopherol levels, and the expression of α-tocopherol-related proteins in the liver. In vitamin E-treated methionine-choline deficient rats, lipid peroxidation was reduced, but liver histopathological changes were not improved. Hepatic α-tocopherol levels in these rats were significantly elevated compared to normal rats treated with vitamin E. Expression of liver α-tocopherol transfer protein in vitamin E-treated methionine-choline deficient rats was significantly repressed compared to methionine-choline deficient rats. The expression of liver cytochrome P450 4F2 and ATP-binding cassette transporter protein 1, involved in metabolism and transport of α-tocopherol, respectively, was significantly repressed in vitamin E-treated methionine-choline deficient rats. In methionine-choline deficient rats, vitamin E treatment altered the hepatic α-tocopherol-related protein expression, which may affect α-tocopherol status in the liver, leading to reduced lipid peroxidation.

Key Words: non-alcoholic fatty liver disease, α-tocopherol, α-tocopherol transfer protein, antioxidant

Vitamin E (which includes both tocopherols and tocotrienols) functions as a lipid-soluble antioxidant in plasma lipoproteins and cell membranes. There are four forms of tocopherols and tocotrienols, namely, α, β, γ, and δ. Tocopherols have a saturated side-chain, whereas tocotrienols have an unsaturated side-chain. Of all vitamin subtypes, α-tocopherol is the most potent in inhibiting lipid oxidation. As vitamin E is lipid-soluble, its absorption depends on that of dietary lipids. Esterified forms of vitamin E are hydrolyzed by a pancreatic esterase, and micellization of these vitamin E-containing dietary lipids requires bile acids. The micelles are absorbed by intestinal enterocytes, and vitamin E travels through the lymphatic system into the blood circulation. Although all forms of vitamin E are taken up by the liver, only α-tocopherol is selectively released into the circulation. α-tocopherol transfer protein (α-TTP) plays an essential role in maintaining circulatory α-tocopherol levels. α-TTP is mainly expressed in hepatic cytosol where it selectively binds α-tocopherol. α-tocopherol-bound α-TTP is preferentially incorporated into the nascent very low-density lipoproteins (VLDLs) that are secreted into the circulation. Circulating VLDLs are converted by a lipoprotein lipase into low-density lipoproteins (LDLs), which are taken up by peripheral tissues through the LDL receptor.

Epidemiological investigations revealed that vitamin E deficiency is associated with pathological conditions induced by oxidative stress such as neurodegenerative disorders, diabetes, and cataracts. However, vitamin E supplementation in clinical trials has failed to reveal benefits for reducing the risk of cancer, cardiovascular disease, or neurodegenerative disorders.

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in developed countries, and the incidence is increasing among children as well as adults. NAFLD encompasses a broad spectrum of metabolic fatty liver disorders ranging from simple steatosis to non-alcoholic steatohepatitis (NASH), which can develop into cirrhosis and liver cancer. NAFLD is closely associated with central adiposity, obesity, insulin resistance, metabolic syndrome, and type 2 diabetes, and it is currently considered an important public health issue worldwide. The diagnosis of NASH requires liver histology; the spectrum of pathologic features includes steatosis, lobular inflammation, and fibrosis. Dysfunction of adipose tissues in NAFLD is tightly related to common disorders, including metabolic syndrome, type 2 diabetes, and cardiovascular disease.

Fatty acids released from dysfunctional and insulin-resistant adipocytes cause lipotoxicity, and triglyceride-derived toxic metabolites accumulate in peripheral tissues, including liver, muscle, and pancreatic β cells. The cross-talk between the dysfunctional adipocytes and hepatocytes leads to the development of lipotoxic liver disease. In addition to lipotoxicity, oxidative stress, inflammatory cytokines, and other proinflammatory factors promote the transition from steatosis to NASH.

Regarding the treatment of NAFLD, a special diet (with or without an additional exercise program) is important in overcoming insulin resistance and helps in the prevention of NAFLD development. Oxidative stress, which is generally seen in several liver diseases, is shown to play a role in the pathogenesis of NAFLD. Several clinical trials assessing the efficacy of vitamin E mono- or combination therapy for the treatment of NASH or NAFLD demonstrated that the antioxidant therapy was effective in preventing disease development. In a randomized placebo-controlled prospective double-blind trial, the combination therapy of vitamin E (1,000 IU/day) and vitamin C (1,000 mg/day) for 6 weeks produced a statistically significant improvement in the fibrosis score among NASH patients. Recently, two large randomized controlled trials have been conducted to evaluate the efficacy of vitamin E therapy for adults with NASH and children with NAFLD. In the PIVENS trial, 247 non-diabetic NASH patients were randomized to receive vitamin E (800 IU/day),
pioglitazone (30 mg/day), or placebo for 96 weeks. Vitamin E administration was associated with a significantly higher rate of improvement in patients with NASH compared with that in the placebo group, whereas the effect of pioglitazone therapy did not differ from that of the placebo. In the TONIC trial, 173 children and adolescents with NAFLD were randomized to receive vitamin E (800 IU/day), metformin (1,000 mg/day), or placebo for 96 weeks. Neither vitamin E nor metformin was superior to placebo in reducing alanine transferase levels in children with NAFLD. The results of recent studies indicate that the antioxidant therapy based on vitamin E and/or statins may be useful for treatment of NASH and NAFLD.

We have previously reported changes in the expression of hepatic α-TTP mRNA and α-tocopherol status in methionine-choline deficient (MCD) rats fed vitamin E-enriched diets. The MCD rats that develop hepatic steatosis, necroinflammation, and fibrosis have been used as a model of NAFLD. The absence of methionine and choline inhibits VLDL production and triglyceride transport, resulting in rapid lipid accumulation in the liver. A limitation of the MCD model is the absence of the metabolic syndrome associated with NASH or NAFLD in humans; however, this model has been used in previous studies to suitably reproduce certain features of human NASH. In the present study, we examined whether the expression of hepatic α-tocopherol-related proteins (a binding protein, a metabolic enzyme, and a transporter) might affect α-tocopherol status in the MCD rat model of NAFLD.

**Materials and Methods**

**Animal experiments.** Four-week-old male Wistar rats were purchased from Japan SLC Inc. (Sizuka, Japan). The rats were divided into four groups (n = 5 each) and given free access to the following diets for 4 weeks: (1) standard diet with α-tocopherol (20 mg/kg) (control group), (2) methionine-choline deficient diet (MCD group), (3) standard diet with high dose α-tocopherol (500 mg/kg) (Cont + Vit E group), and (4) a combination of MCD and high-dose α-tocopherol (MCD + Vit E group), as described previously. Rats were sacrificed by exsanguination under isoflurane anesthesia. Blood was collected into heparinized tubes, and plasma was stored at –80°C; liver tissue was immediately frozen in liquid nitrogen and stored at –80°C. A portion of the liver tissue was fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned. All animal experiments were performed in accordance with the Osaka Medical College guidelines for the ethical treatment of laboratory animals.

**Measurement of α-tocopherol, lipids, aminotransferase, and lipid peroxidation.** α-Tocopherol levels in the plasma and liver was measured by high-performance liquid chromatography coupled with electrochemical detection. α-Tocopherol concentration in plasma was normalized against total lipid, and in the liver tissue against total protein levels. Concentrations of plasma lipids and alanine aminotransferase (ALT) were determined by enzymatic assay kits (Wako Pure Chem. Ind., Ltd., Osaka, Japan). To evaluate lipid peroxidation, 4-hydroxyhexenal (4-HNE) levels in the liver were measured using the Oxiselect HNE-His adduct ELISA kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s recommendations.

**Immunoblotting.** Primary antibodies used were anti-rat α-TTP IgG (provided by Dr. Hiroyuki Arai, University of Tokyo, Tokyo, Japan), CuZn-superoxide dismutase (CuZn-SOD) and Mn-SOD antibodies (provided by Dr. Keiichiro Suzuki, Hyogo College of Medicine, Hyogo, Japan), and polyclonal antibodies to β-actin (Santa Cruz Biotechnology Inc., Santa Cruz, CA). A cytosolic fraction was obtained from homogenized tissues by ultracentrifugation at 100,000 x g for 60 min, and the protein content was measured using the Bradford method. The protein extract was subjected to electrophoresis, transferred to a PVDF membrane, and immunoblotted with each of the primary antibodies (β-actin, α-TTP, Mn-SOD, CuZn-SOD, and β-actin, used at final dilutions of 1:1,000, 1:10,000, 1:1,000 and 1:1,000, respectively) in Tris-buffered saline containing Tween-20 (TBS-T).

**Results**

**Immunohistochemistry for α-SMA.** In the liver, α-SMA staining was observed in the portal vein (Fig. 1A–C) and in inflamed and fibrotic areas (Fig. 1C and D). In MCD rats, severe macrovesicular steatosis was associated with foci of mild fibrosis and inflammation (Fig. 1C), and administration of vitamin E did not affect liver fibrosis and the formation of lipid droplets (Fig. 1D).
Expression of TGF-β, TIMP-1, and type I procollagen genes. To investigate the effect of α-tocopherol on fibrogenesis in the rat liver, we assessed the expression of transforming growth factor-β (TGF-β), tissue inhibitor of metalloproteinase-1 (TIMP-1), and type I procollagen mRNA by using real-time PCR (Fig. 2). Compared to the control group, the expression of TGF-β, TIMP-1, and type I procollagen in the liver was not altered in control rats fed vitamin E-enriched diet (Fig. 2, Cont vs Cont + Vit E) but was significantly elevated in the livers of MCD rats (Fig. 2, Cont vs MCD). However, α-tocopherol administration did not alter the expression of TGF-β, TIMP1, and type I procollagen genes in the livers of MCD rats (Fig. 2, MCD vs MCD + Vit E).

Expression of 4-HNE in the liver and ALT in plasma. Immunohistochemical staining for 4-HNE in the liver of a control rat was performed to assess the expression of 4-HNE-reactive proteins in the hepatocyte cytoplasm (Fig. 3A). Hepatic 4-HNE levels were not different between the control and high-dose vitamin E-treated rats (Fig. 3B, Cont vs Cont + Vit E). Although

### Table 1. Sequences of primers for real-time PCR

| Gene (Accession Number) | Forward                                      | Reverse                                      | Product (bp) |
|-------------------------|----------------------------------------------|----------------------------------------------|--------------|
| TGF-β (X52498)          | 5'-TCAAGTCAACTGTGGAGCAA-3'                   | 5'-TTCCGTCTCTCGTTGCTCA-3'                    | 162          |
| Procollagen -type1-α2 (NM_053356) | 5'-GCATGGGACTCCCTGACAG-3'                   | 5'-TGGCAGGGGAGATGGGCTTAT-3'                | 197          |
| TIMP-1 (NM_053819)      | 5'-TCTCTGCTCCTCCGGCATAT-3'                  | 5'-CTGATCTGTCACAAGACATGATG-3'              | 149          |
| ABCA1 (NM_178095)       | 5'-TGGAGGACGGGTTACTAC-3'                    | 5'-ATGGTCAGGCTGTCACTT-3'                    | 170          |
| Gpx (NM_030826)         | 5'-CAATCATGACGGACATCA-3'                    | 5'-CGCACTCTCAAAACGTGA-3'                    | 113          |
| α-TTP (NM_013048)       | 5'-ATTTGAAAATGAGCGCGTC-3'                   | 5'-TAAATAAGCCTATCCAGCCTCC-3'-FITC           | 254          |
| CYP4F2 (NM_019623)      | 5'-TGACCCAGATCTACGGTGC-3'                   | 5'-CCACCAACCGGTTCAATTC-3'                   | 141          |
| β-actin (V01217 J00691) | 5'-CCTGACGGCAGATCTACGGTGC-3'                 | 5'-CCACCATGACGGCAGATCTACGGTG-3'-FITC        | 260          |

Abbreviations: TGF-β, transforming growth factor-β; TIMP-1, tissue inhibitor of metalloproteinase-1; ABCA1, ATP-binding cassette transporter A1; Gpx, glutathione peroxidase; α-TTP, α-tocopherol transfer protein; CYP4F2, cytochrome P450 4F2.

Fig. 1. Immunohistochemistry for α-SMA in the liver. (A) Control, (B) Cont + Vit E, (C) MCD, (D) MCD + Vit E. In the rats of control and Cont + Vit E groups, portal veins were positive for α-SMA (A and B). α-SMA staining (arrows) was observed in the inflamed and fibrotic areas (C and D). Magnification, ×400. PV, portal vein; CV, central vein. Immunostaining was performed as described in Materials and Methods.
Fig. 2. Expression of the hepatic TGF-β, TIMP-1, and type I procollagen genes by real-time PCR. Liver samples (n = 5 in each group) were analyzed as described in Materials and Methods. Data are expressed as means ± SD. Mean values represented with different letters indicate significant differences (p<0.05).

Fig. 3. Expression of 4-HNE in the liver and ALT in plasma. (A) Immunohistochemistry for 4-HNE in the liver of a control rat. Strong signals were observed in the cytoplasm of hepatocytes (arrows). Magnification, ×400. PV, portal vein. (B) Hepatic 4-HNE-His adduct levels (n = 5 in each group). (C) Plasma ALT levels (n = 5 in each group). The procedures were performed as described in Materials and Methods. Data are expressed as means ± SD. Mean values represented with different letters indicate significant differences (p<0.05).
4-HNE expression in MCD rats was significantly elevated compared to the control group (Fig. 3B, Cont vs MCD), the high dose of vitamin E markedly reversed this effect (Fig. 3B, MCD vs MCD + Vit E). Plasma ALT levels were not altered by vitamin E enrichment of standard diet (Fig. 3C, Cont vs Cont + Vit E); however, ALT levels were significantly elevated by MCD diet (Fig. 3C, Cont vs MCD), but not reduced by the high dose of vitamin E (Fig. 3C, MCD vs MCD + Vit E).

Expression of antioxidant enzymes. To investigate the effect of α-tocopherol on the antioxidant status in the rat liver, we assessed Mn-SOD and CuZn-SOD expression by immunoblotting and glutathione peroxidase (GPx) expression by real-time PCR (Fig. 4). Hepatic expression of Mn-SOD and GPx was significantly increased by vitamin E-enriched standard diet (Fig. 4A and B, Cont vs Cont + Vit E) and down-regulated by MCD diet (Fig. 4A and B, Cont vs MCD). In contrast, hepatic expression of CuZn-SOD was not altered by the high dose of vitamin E in standard diet (Fig. 4A and B, Cont vs Cont + Vit E) and decreased by MCD diet (Fig. 4A and B, Cont vs MCD). However, the expression of Mn-SOD, CuZn-SOD, and GPx in the livers of MCD rats was not altered by the high dose of vitamin E (Fig. 4A and B, MCD vs MCD + Vit E).

α-Tocopherol levels in the plasma and liver. Plasma levels of α-tocopherol in MCD group were significantly lower than those in the control group (Fig. 5A, Cont vs MCD), but they were increased by the high dose of vitamin E (Fig. 5A, MCD vs MCD + Vit E). Hepatic levels of α-tocopherol were significantly (10-fold) increased by feeding rats with standard diet enriched with vitamin E (Fig. 5B, Cont vs Cont + Vit E). In the livers of MCD animals, α-tocopherol levels were not altered compared to those in the control group (Fig. 5B, Cont vs MCD), but they were significantly (14.5-fold) increased by addition of the high dose of vitamin E (Fig. 5B, MCD vs MCD + Vit E). The increase in hepatic α-tocopherol caused by vitamin E enrichment was higher in MCD rats than in control rats.

Expression of α-TTP, CYP4F2, and ABCA1 genes in the liver. Furthermore, we investigated the expression of genes associated with α-tocopherol metabolism. Hepatic expression of α-TTP in control rats was not altered by vitamin E (Fig. 6, Cont vs Cont + Vit E). α-TTP levels in the livers of MCD rats were lower than those in control rats (Fig. 6, Cont vs MCD), and further down-regulated by the high dose of vitamin E (Fig. 6, MCD vs MCD + Vit E). However, the expression of these genes was significantly increased by vitamin E-enriched standard diet (Fig. 7, Cont vs Cont + Vit E) and markedly decreased by MCD diet (Fig. 7, Cont vs MCD). Expression of CYP4F2 and ABCA1 in the livers of MCD rats was not altered by the high dose of vitamin E (Fig. 7, MCD vs MCD + Vit E).
Fig. 5. α-Tocopherol levels in the plasma and liver (n = 5 in each group). The procedure was described in Materials and Methods. Data are expressed as means ± SD. Mean values represented with different letters indicate significant differences (p<0.05).

Fig. 6. Immunoblotting for rat hepatic α-TTP. Liver samples (n = 3 in each group) were assayed by immunoblotting as described in Materials and Methods. Data are expressed as mean ± SD. Mean values represented with different letters indicate significant differences (p<0.05).

Fig. 7. Expression of CYP4F2 and ABCA1 genes in the liver. Liver samples (n = 5 in each group) were analyzed by real-time PCR as described in Materials and Methods. Data are expressed as means ± SD. Mean values represented with different letters indicate significant differences (p<0.05).
Discussion

In the present study, we evaluated the effect of vitamin E in a rat model of NAFLD by assessing α-tocopherol status and regulation of α-tocopherol-related proteins in the liver. Administration of vitamin E ameliorated lipid peroxidation in the NAFLD rat model, but it could not prevent infiltration of inflammatory cells or lipid deposition and fibrosis. Several studies revealed that the administration of vitamin E attenuated lipid peroxidation, improved histopathological changes, and decreased the expression of apoptotic mediators in rodent models of obesity. Experiments using MCD mice treated with vitamin E showed reduced lipid peroxidation and ameliorated steatohepatitis. The reason for the lack of improvement in the liver histopathological changes and ALT levels in MCD rats treated with vitamin E in the present study is unclear; it may be caused by the difference in rodent models, and dose and/or duration of vitamin E administration, or by a combination of these factors. Here, we discuss the results of the present study from the standpoint of the α-tocopherol metabolism in MCD rats treated with vitamin E.

Vitamin E as an antioxidant ameliorates the effects of oxidative stress, which has been implicated in the regulation of α-TTP expression in the liver, as shown in several studies. Studies examining the oxidative stress effects on hepatic α-TTP levels have shown conflicting results. While the hepatic α-TTP mRNA was shown to decrease under hypoxic conditions that induce oxidative stress, environmental exposure to tobacco did not alter α-TTP expression in the liver. In the type 2 diabetic Goto-Kakizaki rat model characterized by increased rates of lipid peroxidation, hepatic α-TTP levels were upregulated compared to those in controls. In the present study, the expression of hepatic α-TTP was repressed in MCD rats, which also demonstrated increased lipid peroxidation and reduced expression of antioxidant enzymes such as Mn-SOD, CuZn-SOD, and GPx. The reasons for such conflicting findings are unclear; it can be hypothesized that oxidative stress is induced by different pathological conditions, which in turn may lead to variations in the expression of hepatic α-TTP. The mechanism underlying regulation of α-TTP expression in the liver by oxidative stress remains to be determined.

In the previous study, we suggested that hepatic α-tocopherol levels may be linked to the expression of α-TTP mRNA. In the present study, we examined α-TTP protein expression in the rat livers by immunoblotting and found that it was down-regulated in MCD rats compared to control rats. The reason for the reduced α-TTP expression in MCD rats is unclear. MCD may affect transcriptional or translational regulation of α-TTP gene. Hepatic α-TTP expression is believed to be closely linked to the nutritional status and was shown to be decreased by protein insufficiency. In MCD rats, lipid metabolism is altered, which may affect α-TTP expression. Further investigation is required to clarify the regulation of α-TTP expression in the liver.

Interestingly, vitamin E administration markedly repressed α-TTP expression in MCD rats. Therefore, in the MCD + Vit E group, hepatic levels of α-tocopherol increased, whereas the plasma levels decreased compared to those in Cont + Vit E group rats. These findings suggest that the change in α-TTP expression in MCD + Vit E group rats may affect α-tocopherol status in the liver and blood circulation. The more markedly reduced hepatic expression of α-TTP in MCD + Vit E rats may lead to increases in α-tocopherol levels in the liver, where α-tocopherol protects against lipid peroxidation induced by methionine and choline deficiency. However, the mechanism of transcriptional or translational regulation of the α-TTP expression is unclear. In the liver antioxidant system, regulation of α-TTP expression may also define hepatic α-tocopherol levels. In NAFLD patients treated with vitamin E, the expression of hepatic α-TTP may be reduced in a similar fashion. Further studies are needed to clarify this.

Mustacich et al. reported that daily administration of α-tocopherol in rats increased the expression of ABCA1, multi-drug resistance protein 1, and a subset of hepatic CYP enzymes of CYP3A, 2B, and 2C families, but not that of CYP4F2 or 1A. In contrast, in the present study, the hepatic CYP4F2 expression was found to be elevated in vitamin E-fed control rats. The reason underlying the difference in the results is unclear; the duration of vitamin E administration may be one of the factors. CYP4F2 expression was repressed in the livers of MCD and MCD + Vit E rats. Because of the excessive vitamin E dose, the reduced expression of CYP4F2 in MCD + Vit E rats may be related to an increase in α-tocopherol levels in the liver. In a recent study, vitamin E was shown to alter the expression of vitamin E-related proteins including cytochrome P450 and sulfotransferase. Excess vitamin E has a positive feedback effect on its own metabolism and on the expression of its transporter genes, which are considered to induce secretion of vitamin E and its metabolites.

ABC1A plays a critical role in promoting transport of cholesterol and phospholipids to apolipoproteins. Oram et al. showed that ABCA1 mediated the secretion of cellular α-tocopherol into the HDL pathway. Shihriri et al. demonstrated that α-tocopherol was a direct substrate of ABCA1, which promoted α-tocopherol secretion in hepatocytes. However, there is no evidence of direct interaction between α-TTP and ABCA1 in the regulation of α-tocopherol transport. In the present study, the expression of hepatic ABCA1 was increased in rats fed with vitamin E-enriched standard diet, which led to an increase in plasma α-tocopherol. The increased ABCA1 expression in vitamin E-treated rats was also shown in the earlier study. However, ABCA1 levels in the livers of MCD + Vit E rats were not increased compared to MCD rats. Repressed expression of hepatic ABCA1 in MCD + Vit E rats may affect the hepatic levels of α-tocopherol by inhibiting α-tocopherol secretion in the liver.

Our study indicates that vitamin E reduces lipid peroxidation in the livers of MCD rats, but it does not prevent fibrosis, steatosis, or inflammation. In MCD rats treated with vitamin E, the expression of α-tocopherol-related proteins, including α-TTP, CYP4F2, and ABCA1, may coordinately regulate α-tocopherol levels in the plasma and liver. Further studies are needed to clarify α-tocopherol status in NAFLD. We believe that the findings of this study may contribute to development of antioxidant-based therapeutic approaches for the treatment of NAFLD and other diseases associated with oxidative stress.

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Abbreviations

| Abbreviation | Full Name |
|--------------|-----------|
| ABCA1 | ATP-binding cassette transporter A1 |
| ALT | alanine aminotransferase |
| α-SMA | α-smooth muscle actin |
| α-TTP | α-tocopherol transfer protein |
| CYP4F2 | cytochrome P450 4F2 |
| GPx | glutathione peroxidase |
| 4-HNE | 4-hydroxynonenal |
| MCD | methionine-choline deficient |
| NAFLD | non-alcoholic fatty liver disease |
| NASH | non-alcoholic steatohepatitis |

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Conflict of Interest

No potential conflicts of interest were disclosed.