α-Tocopherol Status and Altered Expression of α-Tocopherol-Related Proteins in Streptozotocin-Induced Type 1 Diabetes in Rat Models

Kimitaka TAKITANI1, Keisuke INOUE2, Maki KOH1, Hiroshi MIYAZAKI1, Kanta KISHI1, Akiko INOUE1 and Hiroshi TAMAI1

1 Department of Pediatrics, Osaka Medical College, Takatsuki, Osaka 569–8686, Japan
2 Department of Pediatrics, Hirakata City Hospital, Hirakata, Osaka 573–1013, Japan
3 Department of Pediatrics, Osaka Rosai Hospital, Sakai, Osaka 591–8025, Japan

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Summary Vitamin E plays a critical role as an antioxidant in several pathological conditions, including diabetes, cancer, cardiovascular diseases, and neurodegenerative disorders. Diabetes is a metabolic disorder of glucose due to the lack of adequate insulin production (type 1) or peripheral insulin resistance (type 2). Oxidative stress plays a major role in the pathogenesis of diabetes and its complications. The purpose of the present study was to determine α-tocopherol status and the expression of α-tocopherol-related proteins, including binding proteins and metabolizing enzymes, under streptozotocin (STZ)-induced type 1 diabetes in rat models. In STZ rats, plasma α-tocopherol levels decreased compared to the control rats, whereas hepatic α-tocopherol levels in the STZ rats were significantly increased. CuZn-superoxide dismutase (SOD) gene expression in the liver of STZ rats was markedly decreased, whereas Mn-SOD gene expression remained unaltered. Accelerated lipid peroxidation in the liver of STZ rats was observed and the hepatic expression of α-tocopherol transfer protein (α-TTP) in STZ rats decreased compared to that in the controls. The hepatic expression of cytochrome P450 4F2 (CYP4F2) and CYP3A2 genes in STZ rats also decreased. The reduced expression of hepatic α-TTP and CYP4F2 genes probably leads to decreased plasma α-tocopherol levels and elevated α-tocopherol levels in the liver of STZ rats. The altered expression of hepatic α-tocopherol-related proteins might regulate α-tocopherol status in type 1 diabetes. Determining the mechanism of modulating α-tocopherol status may be helpful in promoting antioxidant therapy in diabetes.

Key Words α-tocopherol, type 1 diabetes, oxidative stress, α-tocopherol transfer protein

Vitamin E is a fat-soluble antioxidant that comprises 4 tocopherols (α-, β-, γ-, and δ-) and 4 tocotrienols (α-, β-, γ-, and δ-). Of all these vitamin E subtypes, α-tocopherol is the most well-known for its antioxidant activity. Lipid-soluble vitamin E has a critical role as an antioxidant protecting the lipids of the plasma membranes and lipoproteins from peroxidation. Dietary vitamin E, including tocopherols and tocotrienols, which are components of bile salt micelles, is absorbed in the intestine. Vitamin E, along with lipids, is incorporated into nascent chylomicrons within the enterocyte, and these chylomicrons are then secreted into the lymphatics and blood vessels. After transporting dietary lipids to other locations in the body, chylomicrons are returned to and taken up by the liver. In the liver, α-tocopherol selectively binds to α-tocopherol transfer protein (α-TTP) and is incorporated into very low-density lipoproteins (VLDL). α-TTP is a hepatic cytosolic protein, which facilitates the transport of α-tocopherol to the plasma membrane for the secretion of α-tocopherol into VLDL. α-TTP has a critical role in maintaining circulatory α-tocopherol levels. α-TTP gene disrupted mice reveal markedly reduced plasma α-tocopherol levels; moreover, the mutation of the human α-TTP gene is responsible for ataxia with vitamin E deficiency. The ATP-binding cassette transporter-1 (ABCA-1), which transports cholesterol and phospholipids, facilitates the secretion of α-tocopherol in the plasma membrane. VLDL, including α-tocopherol, in circulation is lipolysed into low-density lipoprotein (LDL) by lipoprotein lipase, and this LDL is taken up into the peripheral tissues via the action of the LDL receptor. The mechanism of α-tocopherol metabolism has been recently determined. The catabolism of tocopherols and tocotrienols is mediated by cytochrome P450 4F2 (CYP4F2), and the resulting metabolite, carboxylethyl hydroxycroman (CEHC), is excreted in urine as glucuronic conjugates.

Diabetes is a metabolic disorder pertaining to glucose uptake due to a lack of insulin production by pancreatic β-cells (type 1 diabetes), or it manifests as peripheral insulin resistance, including adipose tissue and muscles (type 2 diabetes). The production of reactive oxygen species (ROS) is the major pathogenesis of diabetes and its complications. Hyperglycemia leads to an increased release of ROS in peripheral tissues. In experimental studies, vitamin E supplementation improved the
diabetic state and delayed the development of complications in diabetes rodent models (3). Azzi et al. demonstrated that α-tocopherol has beneficial effects on vascular complications of diabetes (4). This effect of α-tocopherol depends on the inhibition of particular kinase C (PKC) activity in smooth muscle cells, and not on α-tocopherol’s antioxidant properties.

The status of vitamin E in several pathological conditions, including cancer, cardiovascular diseases, metabolic disorders, and neurodegenerative diseases, has been elucidated (3). In intervention trials for diabetic patients, vitamin E supplementation has been shown to have beneficial effects on biological markers, including those involved in lipid peroxidation, vascular function, and inflammation. Clinical trials of vitamin E supplementation have been performed worldwide; however, the trials failed to reveal the benefits of vitamin E in reducing the risk of cancer, cardiovascular disease, and neurodegenerative disorders. Thus, the beneficial effects of vitamin E supplementation on the clinical end-points of cardiovascular disease in diabetic patients have not been proven (3).

We previously assessed the α-tocopherol status and hepatic α-TTP gene expression in Goto-Kakizaki (GK) rat models with type 2 diabetes (5). In this non-insulin dependent rat model, both α-tocopherol levels and hepatic α-TTP expression were markedly increased, and we believe that α-TTP expression may lead to elevated plasma α-tocopherol levels. Here we analyzed the α-tocopherol status, as well as the levels of α-tocopherol-related proteins, including binding proteins and metabolic enzymes, in insulin-dependent type 1 diabetes induced using streptozotocin (STZ) in rat models. STZ causes β-cell toxicity and leads to a hypoinsulinemic-hyperglycemic state (6). STZ has been widely used to induce experimental insulin-dependent diabetes in rodents.

MATERIALS AND METHODS

Animal experiments. Wistar rats (male; 4-wk-old) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The control and STZ groups (n=6, respectively) were fed regular chow and had free access to tap water. The rats were housed in a temperature-controlled, humidity-controlled, and light-controlled environment. The animal food was purchased from Funahashi Farm (Chiba, Japan), and every meal contained α-tocopherol (20 mg/kg). In accordance with previously described reports, the rats of the STZ group were intraperitoneally injected with a single dose of STZ (70 mg/kg in 0.1 ml acetate buffer at pH 4.4), while the control rats received an injection of acetate buffer (7). After 4 wk, the rats were sacrificed by exsanguination under isoflurane anesthesia after overnight fasting. The blood was collected in heparinized tubes, and the plasma was separated and stored at −80˚C. The liver was removed, immediately frozen in liquid nitrogen, and stored at −80˚C. A portion of the liver tissue was processed for paraffin sections. Care and handling of the experimental animals was in accordance with the Osaka Medical College guidelines for the ethical treatment of laboratory animals.

Biochemical examinations. The plasma concentrations of glucose, cholesterol, and triglyceride were measured using the enzymatic colorimetric method (5). Tocopherol concentrations in the plasma and liver homogenate were assayed using high-performance liquid chromatography, as previously described (8). Plasma and liver tissue were homogenized and saponified with one-twentieth of the volume of 60% potassium hydroxide in distilled water. The saponified liver samples were then extracted with hexane, and the protein content was measured using the Bradford method (9). Lipid extraction from the liver samples was performed using the Folich method (10). The levels of thiobarbituric acid-reactive substances (TBARS) in the liver were measured using a previously described method (11).

Immunoblotting. Anti-rat α-TTP IgG was provided by Dr. Hiroyuki Arai, at the University of Tokyo, and antirat CuZn-superoxide dismutase (SOD) and Mn-SOD were provided by Dr. Keiichiro Suzuki at the Hyogo College of Medicine. Anti-β actin antibody (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan) and anti-tocopherol-associated protein (TAP)/supernatant protein factor (SPF) antibody (Santa Cruz Biotecchnology Inc., Santa Cruz, CA) were the primary antibodies that were purchased. The cytosolic fraction of the liver was prepared from the tissue homogenate by ultracentrifugation at 100,000 ×g for 60 min, and its protein content was measured using the Bradford method (9). α-TTP, Mn-SOD, CuZn-SOD, TAP/SPF, and β-actin antibodies were used at the final dilutions of 1:1,000, 1:10,000, 1:10,000, 1:1,000 and 1:1,000, respectively, in Tris-buffered saline containing Tween-20 (TBS-T). Horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA) was used as the secondary antibody, and the target bands were detected using the ECL Western blotting Detection System (GE Healthcare UK Ltd., Little Chalfont, England). The relative intensities of the proteins were determined using Image J 1.46r software (NIH, Bethesda, MD).

Real-time PCR. Total RNA from the rat livers was extracted using ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer’s instructions. Quantitative real-time PCR was performed to determine the gene expression in the RNA samples. Reverse transcription (RT) reactions were performed using the Omniscript kit (Qiagen, Hilden, Germany). Subsequently, each RT reaction mixture was amplified using LightCycler PCR (F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland) and a LightCycler FastStart DNA Master Hybridization Probe Kit or FastStart DNA Master SYBR Green I Kit (Roche), according to the manufacturer’s instructions. Sequences of the oligonucleotide primers and the accession numbers of the genes used are listed in Table 1. α-TTP, cytochrome P450 4F2 (CYP4F2), and β-actin genes were analyzed using the Master Hybridization Probe Kit, and the Master SYBR Green I Kit was used for analyzing afamin and CYP3A2 genes. An RT-PCR product of each gene was verified by performing DNA
sequencing and was used as an external PCR standard. Serial 10-fold dilutions of these RT-PCR products, corresponding to $1 \times 10^{-1}$ to $1 \times 10^{6}$ copies/μL, were amplified in parallel with the experimental samples, by the method described above. Using the LightCycler software, amplification curves of the experimental samples were plotted against the standard curves to determine the gene-specific mRNA copy numbers. To compensate for differences in the RT efficiency of the different samples, values for each gene were normalized relative to the β-actin copy number (12).

Statistical analysis. The results were expressed as mean ± standard deviation (SD). Welch’s t-test was used to determine the significance of the observed differences. Differences between the groups were considered significant at a p-value of <0.05.

RESULTS

**Tocopherol levels, lipid peroxidation, and antioxidant status**

The plasma glucose, cholesterol, and triglyceride levels in the STZ group were significantly increased compared to the control group (Table 2). The plasma α-tocopherol levels of the STZ group were significantly lower than

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

<table>
<thead>
<tr>
<th>Gene (Accession number)</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afamin (NM_172320)</td>
<td>5′-CACTTGTATCTGGTTGCG-3′</td>
<td>5′-AGGGCGAACTTGTGTA-3′</td>
<td>149</td>
</tr>
<tr>
<td>CYP 3A2 (NM_153312)</td>
<td>5′-GGGATTAATGGACTCTCT-3′</td>
<td>5′-GATGGAATACGCAAAGGT-3′</td>
<td>147</td>
</tr>
<tr>
<td>α-TTP (NM_013048)</td>
<td>5′-ATTGAAATAAGCCGGTC-3′</td>
<td>5′-TCATTTGGTGCTCAGAAA-3′</td>
<td>254</td>
</tr>
<tr>
<td>CYP4F2 (NM_019623)</td>
<td>5′-TAATACGCTACTTCGACTCCC-3′-FITC</td>
<td>5′-CACCAACCCAGACATTT-3′</td>
<td>141</td>
</tr>
<tr>
<td>β-Actin (V01217 J00691)</td>
<td>5′-TAAATCAAGCCTACTTCAGCACTTC-3′</td>
<td>5′-TCGTATGAGTAACATTCGTAAG-3′</td>
<td>260</td>
</tr>
</tbody>
</table>

α-TTP, α-tocopherol transfer protein.

**Table 2. Vitamin E and biochemical data for control and STZ rats.**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>STZ rats (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>123.6±19.7</td>
<td>38.9±127.6*</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>52.7±6.2</td>
<td>80.4±10.0**</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>26.6±3.2</td>
<td>40.9±5.6*</td>
</tr>
<tr>
<td>Plasma α-toc/chol (μg/mg)</td>
<td>12.4±0.55</td>
<td>3.1±0.5***</td>
</tr>
<tr>
<td>Hepatic triglyceride (mg/g)</td>
<td>16.4±2.4</td>
<td>17.3±3.1</td>
</tr>
<tr>
<td>Hepatic α-tocopherol (μg/mg protein)</td>
<td>9.3±1.7</td>
<td>15.7±2.3**</td>
</tr>
<tr>
<td>Hepatic TBARS (nmol/mg protein)</td>
<td>3.6±0.57</td>
<td>8.3±2.9*</td>
</tr>
</tbody>
</table>

Values are mean±SD; *p<0.05, **p<0.01, ***p<0.001.

α-toc/chol, α-tocopherol/cholesterol.
Expression of SOD expression in the STZ group remained unaltered. The TBARS levels in the liver of the STZ group were higher than those of the control group. CuZn-SOD expression in the liver of STZ group was significantly lower than that of the control group, and hepatic Mn-SOD expression in the liver of STZ rats with type 1 diabetes was lower than that in the controls (20). However, Uusitalo et al. reported that serum levels of \( \alpha \)-tocopherol are not associated with reduced risk of advanced \( \beta \)-cell autoimmunity in young children (21). To identify the association of dietary vitamin E intake with the development of type 1 diabetes, further investigation that involves a larger number of subjects, the stage of the disease, and the disease duration will be required. In the current study, plasma \( \alpha \)-tocopherol levels decreased in the STZ rat models with type 1 diabetes. The results on the circulatory \( \alpha \)-tocopherol levels in rodent models with type 1 diabetes are conflicting, indicating that \( \alpha \)-tocopherol levels increase, remain unchanged, or decrease in type 1 diabetes (22–24). This discrepancy among the reports can be explained on the basis that \( \alpha \)-tocopherol levels can probably be influenced by the experimental conditions, including the duration and stage of diabetes and the diet of diabetes patients/study models (25).

SODs are the major antioxidant systems that play a critical role in scavenging superoxide anions. SODs consist of three isoforms: cytoplasmic CuZn-SOD (SOD1), mitochondrial Mn-SOD (SOD2), and extracellular SOD (ECOD) (26). The altered expression of SOD is associated with the pathogenesis, duration, and developmental stage of diseases derived from various causes (27). The activity of SODs is altered under several conditions, including aging, diabetes, cancer, and ischemia, which promote oxidative stress (26). In the present study, lipid peroxidation was observed to occur in the liver of STZ rats, which was similar to the results of previous studies (28). Moreover, CuZn-SOD expression in the liver of STZ rats with type 1 diabetes was lower than that in the controls, whereas Mn-SOD expression in the STZ rats with type 1 diabetes remained unaltered. This result is similar to that of a previous study on type 2 diabetes in Goto-Kakizaki (GK) rat models (5). SOD activity in STZ-
induced diabetic rodents was reported to vary with the development of diabetes (28). Oikawara et al. observed that CuZn-SOD undergoes glycation under a hyperglycemic state, which induces the impairment of CuZn-SOD (29). In animal experiments, diabetic rats showed that impaired copper homeostasis led to reduced CuZn-SOD activity (27). Thus, hyperglycemia may reduce the hepatic expression of CuZn-SOD.

Vitamin E has antioxidant activities, and the alteration in hepatic α-TTP expression due to oxidative stress has been investigated in cell culture and animal experiments. α-TTP expression was observed to be upregulated during oxidative stress in studies on cell culture systems, including those on immortalized human hepatocytes and human choriocarcinoma cells (30, 31). However, the experiments on rodents reveal that there are conflicting results on α-TTP expression. Hepatic α-TTP expression decreased under hypoxia (>95% O₂ for 48 h), thus inducing oxidative stress (32). Moreover, α-TTP expression is reduced in the liver of methionine-choline deficiency (MCD) rats, which are known as a model of non-alcoholic fatty liver disease, and presents increased lipid peroxidation of the liver and hepatic dysfunction (33). Bella et al. reported that exposure to environmental tobacco smoke does not alter hepatic expression (34). We have previously reported that hepatic α-TTP expression and plasma α-tocopherol levels were elevated in rat models with type 2 diabetes showing lipid peroxidation (5). On the other hand, in the current study, hepatic α-TTP expression and plasma α-tocopherol levels in STZ rats with type 1 diabetes were decreased, although lipid peroxidation was also observed. This discrepancy is yet to be analyzed; however, insulin signaling rather than oxidative stress probably affects α-TTP expression. As has been mentioned previously, circulatory α-tocopherol levels in STZ rats show variations, which may be due to the developmental stage of diabetes or the diet during diabetes (25); either way, altered α-TTP expression in the liver of diabetic rats may affect plasma α-tocopherol levels as well. Further studies will be required to elucidate the mechanism of the regulation of α-tocopherol in diabetes.

TAP is a 46-kDa cytosolic protein, a member of the CRAL-TRIO family, which is a family of lipid-binding proteins, including cellular retinaldehyde-binding protein (CRALBP), α-TTP, and yeast phosphatidylinositol transfer protein (Sec14p) (35). Shibata et al. demonstrated that SPF, which was identical to TAP, promoted the conversion of squalene to lanosterol and regulated cholesterol biosynthesis (36). TAP/SPF has a weak affinity for α-tocopherol when compared with the other proteins of the CRAL-TRIO family, including CRALBP and Seq14p (37). However, TAP is reported to act as an α-tocopherol dependent transcriptional factor (38). The role of TAP/SPF in the relationship with vitamin E is poorly understood. In addition, in the current study, TAP/SPF expression remained unaltered in the liver of STZ rats. These findings showed that the diabetic condition did not affect hepatic TAP/SPF expression. Moreover, TAP/SPF has low affinity for α-tocopherol (39), and the change in circulatory α-tocopherol levels is not considered to be related to TAP/SPF expression.

Afamin, alpha-albumin, is a member of the albumin gene family that includes albumin, α-fetoprotein (AFP), AFP-related protein, and vitamin D-binding protein. Afamin is a 75-kDa glycoprotein with vitamin E-binding properties, and is abundant in the extravascular fluids including follicular, seminal, and cerebrospinal fluids (40). The role of afamin remains to be determined; however, afamin is considered to influence the uptake and transport of α-tocopherol at the blood-brain barrier, and is thought to have neuroprotective activity (41, 42). In the current study, hepatic afamin expression decreased in STZ rats, and therefore the levels of α-tocopherol in distributed fluids are considered to be low in type 1 diabetes.

Sontag and Parker identified CYP4F2 as a human vitamin E α-hydroxylase, which is involved in the first step of the α-oxidation pathway of vitamin E metabolism (43). Regulation of CYP4F2 expression was elucidated by performing several rodent experiments (44). Hepatic CYP4F2 expression was not altered by α-tocopherol administration. In the present study, hepatic CYP4F2 expression decreased in the STZ rats. It has been reported that reduced expression of both α-TTP and CYP4F2 genes in the liver of MCD rats treated with vitamin E may lead to increased α-tocopherol levels (33), which suggested that the excretion and catabolism of α-tocopherol may be reduced. The triglyceride levels in the liver of the STZ rats were not altered, which suggests that α-tocopherol levels were not affected by the lipid contents of the liver. Thus, reduced hepatic expression of both α-TTP and CYP4F2 may lead to elevated α-tocopherol levels in the liver of STZ rats.

Rat CYP3A2 is a member of the CYP3A superfamily, and is approximately 70% identical to human CYP3A4 (45). The CYP3A superfamily comprises multiple members with multiple functions. Approximately 50% of drugs currently in use are the substrates and/or inhibitors of CYP3A enzymes. The administration of a high-dose of vitamin E increased hepatic expression of CYP3A2 (44, 46). The activities of CYP3A enzymes are influenced during the catabolism of hormones, bile acids, and environmental pollutants. In the current study, hepatic CYP3A2 expression decreased in the STZ rats. This finding suggests that the metabolism of therapeutic drugs, hormones, and bile acids may be altered in type 1 diabetes.

There are however some limitations to the present study. First, as mentioned above, α-tocopherol status in the STZ rats is influenced by different conditions (25). Hence, the α-TTP expression of STZ rats may be altered under any or several of these conditions. Second, the nutritional status of STZ rats may affect circulatory α-tocopherol levels and hepatic α-TTP expression. Hepatic mRNA α-TTP expression is reduced by protein insufficiency (47). Third, we cannot rule out the possibility that the toxicity of STZ affects the hepatic gene expression. In conclusion, we assessed α-tocopherol status and the expression of α-tocopherol-related proteins.
in rat models with type 1 diabetes, and observed that the altered expression of α-TTP and CYP4F2 may affect α-tocopherol status. However, in the current study, we could not elucidate the relationship between α-tocopherol related proteins, so further investigations will be required to define this relationship. Complications of diabetes are aggravated by developed oxidative stress, and hence, clarifying the mechanism for modulating α-tocopherol status may be helpful in promoting antioxidant therapy for diabetes.

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