Note

 α -Tocopherol Status and Expression of α -Tocopherol Transfer Protein in Type 2 Diabetic Goto-Kakizaki RatsHiroshi MIYAZAKI, Kimitaka TAKITANI*, Maki KOH, Ryuzo TAKAYA,
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Summary Vitamin E, a critical fat-soluble vitamin antioxidant, is expressed on cell membranes and prevents propagation of lipid peroxidation. α -Tocopherol transfer protein (α -TTP) is a cytosolic protein located in the hepatocytes that acts as the principal regulator of the circulating α -tocopherol levels. Type 2 diabetes is a metabolic disorder characterized by hyperglycemia, caused by insulin resistance. Lipid peroxidation promotes the clinical progression and development of complications in type 2 diabetes. Results of animal and human experiments on the vitamin E status in diabetes are conflicting. The present study was conducted with the objective of investigating the vitamin E status and α -TTP expression in Goto-Kakizaki (GK) rats, a model of type 2 diabetes. In diabetic GK rats, increases of the α -tocopherol levels in the plasma and liver were observed as compared with the levels in the controls. No alternation in the CuZn-superoxide dismutase (SOD) or Mn-SOD gene expression was found in the liver of GK rats as compared with that in the controls. The GK rats showed an increase of the hepatic expression of the α -TTP gene as compared with the level in the controls. It can be suggested that the increased hepatic α -TTP gene expression levels may influence plasma α -tocopherol levels in the diabetic animals. Hence, investigation of the regulatory factors of α -TTP expression may provide important clues to highlighting the antioxidant mechanisms of vitamin E.

Key Words α -tocopherol, α -tocopherol transfer protein, type 2 diabetes

Vitamin E, which has a critical role as a lipid-soluble antioxidant, is expressed on the cell membrane, and prevents lipid peroxidation in a variety of tissues in several pathological conditions, including diabetes, neurodegenerative disorders, cardiovascular diseases, and cancer (1). Vitamin E is present among the lipid components of the cell membranes and lipoproteins and is known to play a critical role in protecting against lipid peroxidation (2). Vitamin E consists of four tocopherols (α , β , δ , γ) and four tocotrienols (α , β , δ , γ). The most abundant vitamin E of all these forms in nature is α -tocopherol, which exhibits a marked antioxidant activity (3).

α -Tocopherol transfer protein (α -TTP) binds selectively α -tocopherol, and regulates the distribution of tocopherol in the plasma and various peripheral tissues (4). In the case of hepatocytes, α -tocopherol bound to α -TTP is transferred to the cell membrane and eluted from the hepatocytes. Circulating α -tocopherol packed in very low density lipoproteins is transferred to peripheral tissues (1). Dysfunction of α -TTP causes familial vitamin E deficiency, which manifests clinically as cerebellar ataxia and retinal degeneration (5, 6). Patients with ataxia with isolated vitamin E deficiency (AVED) are treated by oral administration of vitamin E. Moreover, mice with α -TTP disruption reveal similar pheno-

types to human AVED (7). These mice show pregnancy complications, and the condition proves lethal to the embryo at 12.5 dpc. α -TTP plays an important role in the maintenance of pregnancy and in protecting against lipid peroxidation in the central nervous system (7, 8).

Diabetes is characterized by disturbance of glucose metabolism due to absolute or relative insulin deficiency, and is associated with a number of functional and structural complications. Recent studies have revealed that oxidative stress is strongly involved in the development of insulin resistance and type 2 diabetes (9, 10). Hyperglycemia induces the release of reactive oxygen species (ROS) in several pathways. The hyperglycemia-induced imbalance between ROS production and antioxidant capacity alters cell signaling and gene expression (11). Glucose auto-oxidation and non-enzymatic protein glycation lead to accumulation of advanced glycation end-products (AGEs) and induce the generation of ROS (9, 12). Hyperglycemia-induced oxidative stress has been implicated in the development of diabetic microvascular complications, including nephropathy, retinopathy, and polyneuropathy.

Goto-Kakizaki (GK) rats spontaneously develop type 2 diabetes and are used by a number of investigators to elucidate the pathophysiology of type 2 diabetes. This rat strain was obtained by selective breeding of normal Wistar rats using the glucose tolerance test (13). GK rats exhibit fasting hyperglycemia, an impaired insulin

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response to glucose, and peripheral insulin resistance (14, 15), and genetic analysis has revealed the presence of *Non-insulin dependent diabetes mellitus (Niddm) 1*, the major quantitative trait locus (16).

The vitamin E status has been examined in several pathological conditions, including cancer, cardiovascular diseases, metabolic diseases, and neurological disorders. Vitamin E supplementation has the beneficial effects of protecting against lipid peroxidation, vascular dysfunction, and inflammation in patients with diabetic complications (11, 12). Investigation of the regulation of α -TTP gene expression may contribute to clarification of the antioxidant mechanism through vitamin E metabolism. However, the pattern of α -TTP expression in pathological conditions has not yet been explored. The present study was conducted with the aim of investigating the status of α -tocopherol as well as the α -TTP expression in GK rats, a model of type 2 diabetes.

Materials and Methods

1) *Animal experiments.* Male GK rats ($n=4$) and Wistar rats (control, $n=4$) for this study were obtained at 7 wk of age from Japan SLC, Inc. (Shizuoka, Japan), and fed commercial laboratory chow (MM-3, Funabashi Farm, Chiba, Japan) and water ad libitum. The care and handling of the experimental animals were in accordance with the Osaka Medical College guidelines for the ethical treatment of laboratory animals. At 8 wk of age, the rats were sacrificed by exsanguination under isoflurane anesthesia after overnight fasting. Blood was collected into heparinized tubes, and plasma was stored at -80°C . The liver was removed, immediately frozen in liquid nitrogen, and stored at -80°C .

2) *Biochemical examination.* The plasma concentrations of glucose, cholesterol, and triglyceride were measured by the enzymatic colorimetric method. The tocopherol concentrations in plasma and liver tissues were assayed by high-performance liquid chromatography, as previously described (17). Liver tissues were homogenized and saponified with one-twentieth the volume of 60% potassium hydroxide in distilled water. Then, the saponified liver samples were extracted with hexane. The protein content of liver tissues was measured according to the method of Bradford (18). Thiobarbituric acid-reactive substances (TBARS) in plasma and liver tissues were measured, as previously described (19, 20).

3) *Real-time reverse-transcription polymerase chain reaction (real-time RT-PCR).* Total RNA was isolated by the acid guanidium thiocyanate/phenol/chloroform (AGPC) method (21). Then, quantitative real-time RT-PCR was performed to determine the levels of rat α -TTP and β -actin in the RNA samples. The RT reaction was carried out using Omniscript (Qiagen, CA). Subsequently, one-tenth ($2\ \mu\text{L}$) of each RT reaction mixture was processed in a LightCycler PCR (F. Hoffmann-La Roche Ltd. Diagnostics Division, Basel, Switzerland), as described previously (22). Real-time PCR for α -TTP and β -actin was performed with a Lightcycler HybProbe quantification kit. The oligonucleotide sequences for the primers

were as follows:

5'-ATTTGATAAATGAGCCGGTC-3' (sense), 5'-TCATTGGATGGTCTCAGAAA-3' (antisense), 5'-TAAATCAAGCCTACTTCAGCACTTCCC-3'-FITC (sense-probe), and LC-5'-GACATTCTTCCTCTGGAATATGGTGGT-3' (antisense-probe) for α -TTP; 5'-CCTGTATGCCTCTGGTCGTA-3' (sense), 5'-CCATCTCTTGCTCGAAGTCT-3' (antisense), 5'-CGGGACCTGACAGACTACCTCATG-3'-FITC (sense-probe), and LC-5'-AGATCCTGACCGAGCGTGGCTAC-3' (antisense-probe) for β -actin.

After initial denaturation at 94°C for 2 min, PCR was performed with 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C (β -actin) or 55°C (α -TTP) for 10 s, and extension at 72°C for 12 s. RT-PCR products of 254 bp (α -TTP) and 260 bp (β -actin) were verified by DNA sequencing and used as the external PCR standards. By employing LightCycler software, the amplification curves of the experimental samples were plotted against these standard curves to estimate the number of specific mRNA copies. To compensate for differences in the RT efficiency among the samples, the values of α -TTP were then normalized to the β -actin copy number.

4) *Immunoblotting.* Anti-rat α -TTP IgG was provided by Professor Hiroyuki Arai, the University of Tokyo, and the anti-rat CuZn-superoxide dismutase (SOD) and Mn-SOD were provided by Professor Keiichiro Suzuki, Hyogo College of Medicine. Mouse monoclonal antibody for β -actin (Santa Cruz Biotechnology Inc., CA, USA) was purchased as the primary antibody. The cytosolic fraction was prepared from the homogenate by ultracentrifugation at $100,000 \times g$ for 60 min, and its protein content was measured according to the method of Bradford (18). α -TTP, Mn-SOD, CuZn-SOD, and β -actin antibodies were used at a final dilution of 1 : 1,000, 1 : 10,000, 1 : 10,000, and 1 : 1,000, respectively, in Tris-buffered saline containing Tween-20 (TBS-T). The secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, CA), and the target bands were detected with the ECL Western blotting Detection System (GE Healthcare UK Ltd., England). The relative intensities of proteins were determined using Image J 1.46r software (NIH, MD, USA). The ratio of each protein band intensity to β -actin band intensity was analyzed and the mean and SD of the ratio was calculated.

5) *Statistical analysis.* Results are expressed as the mean $\pm$ SD. To determine the significance of differences, Welch's *t*-test was used. Differences between groups were considered significant at a *p* value of less than 0.05.

Results and Discussion

The plasma lipid profiles of the GK rats revealed dyslipidemia, and α -tocopherol levels in the liver and plasma of GK rats were elevated as compared with those in control rats (Table 1). Previous studies of plasma α -tocopherol levels in diabetic rodents and human subjects have yielded conflicting results (10, 23). In studies of diabetic humans, the plasma levels of α -tocopherol have been

Table 1. Plasma levels of vitamin E and biochemical parameters associated with diabetes in control and diabetic GK rats.

	Control (n=4)	GK rats (n=4)
Glucose (mg/dL)	104.0±12.2	194.8±60.0**
Total lipid (mg/dL)	119.0±13.6	222.0±16.1**
Cholesterol (mg/dL)	42.5±5.4	84.8±8.0**
Triglyceride (mg/dL)	12.7±1.5	17.7±1.5*
Plasma α -tocopherol/triglyceride (μ g/mg)	11.7±0.4	15.9±0.1**
Hepatic α -tocopherol (μ g/mg protein)	16.2±0.522	19.8±0.70**
Plasma TBARS (nmol/mL)	7.78±0.76	11.6±2.61**
Hepatic TBARS (nmol/mg protein)	3.48±0.15	4.67±0.55*

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

Fig. 1. Immunoblotting for rat CuZn-SOD and Mn-SOD in the liver of control and diabetic GK rats. Liver samples from rats ($n=4$ /group) were analyzed by immunoblotting for CuZn-SOD and Mn-SOD gene expression. The extracted protein was subjected to electrophoresis, transferred to a PVDF membrane, and immunoblotted with each primary antibody, as described in "Materials and Methods." Data are expressed as means±SD.

Fig. 2. Real-time PCR and immunoblotting for α -TTP in the liver of control and diabetic GK rats. Liver samples from rats were analyzed by real-time PCR (A, $n=4$ /group) and immunoblotting (B, $n=3$ /group) for α -TTP gene expression, as described in "Materials and Methods." Data are expressed as the mean±SD. Significant difference is shown in each graph.

demonstrated to be unchanged, elevated or reduced. These discrepancies among studies may arise from variations in the duration of diabetes, the presence/absence of diabetes-induced complications, pregnancy, aging, types of diabetes, and daily intake of vitamin E.

The plasma and hepatic levels of TBARS, a marker of lipid peroxidation, were significantly elevated in the diabetic GK rats (Table 1). The diabetic state is usually complicated by increased oxidative stress and reduced antioxidant defenses (10). Several studies have revealed alterations in the levels of oxidative stress biomarkers, such as decreased levels of glutathione (a free-radical scavenger), and increased levels of TBARS (10) in association with diabetes. In STZ-induced type 1 diabetic rats, elevated TBARS levels in several tissues have been reported by several studies, which provide indirect evidence of free-radical production induced in the diabetic state (10, 24). Moreover, tissue TBARS levels in STZ-induced type 1 diabetic rats have been shown to be diminished by treatment with antioxidants (10). In human studies, plasma levels of lipid peroxides, includ-

ing TBARS and isoprostanes, have been shown to be elevated in subjects with type 2 diabetes as compared with those in controls (10). The present results suggest that an acceleration of lipid peroxidation occurs in type 2 diabetic GK rats, as previously described (10).

In the current study, no alteration in the CuZn-SOD or Mn-SOD expression was observed in diabetic GK rats, as shown Fig. 1. The SOD family serves as a defense system against oxygen toxicity and plays a critical role in eliminating superoxide radicals (25). In mammals, three isoforms of SOD have been identified, including CuZn-SOD, Mn-SOD, and EC-SOD, each with a different distribution (26). CuZn-SOD and Mn-SOD are localized in the cytosol and in the matrix of the mitochondria, whereas EC-SOD is localized in the extracellular fluid (27). The reported activity of SOD in the diabetic state varies based on the gender, rodent species, duration of diabetes, and tissues examined. Concerning the liver, no change, reduction or elevation of the SOD activity has been reported in rats with STZ-induced diabetes (10). Altered SOD activity in the diabetic condition is normalized by agents such as

probucol, captopril, dehydroepiandrosterone, and melatonin (10).

The gene expression of α -TTP was increased, at both the protein and mRNA levels, in diabetic GK rats as compared with that in controls (Fig. 2). In the present study, in addition to the hepatic α -TTP expression, the levels of plasma α -tocopherol were also increased in the GK rats. Several studies have explored the gene expression pattern of α -TTP under various conditions (28–32). α -Tocopherol administration increased hepatic α -TTP gene expression in vitamin E-depleted rats (32). On the other hand, hepatic α -TTP gene expression was decreased by a vitamin E-rich diet and increased by a vitamin E-depleted diet (29). The vitamin E status may affect hepatic α -TTP gene expression; however, to clarify the relationship, further investigations will be needed. We previously reported that the hepatic expression of the α -TTP gene in rats was reduced in the hyperoxic state, although no change in the α -tocopherol levels were noted in either the plasma or the liver (30). In this report, rats exposed to >95% oxygen for 48 h showed accelerated lipid peroxidation in the plasma and liver. Thus, it is speculated that oxidative stress may influence the expression of the hepatic α -TTP gene. In the present study, hepatic α -TTP gene expression was up-regulated in diabetic GK rats, while TBARS levels in the plasma and liver were increased. As to the regulation of α -TTP gene expression, this finding in the GK rats is in sharp contrast to that reported in the above-mentioned hyperoxia study (30). The reason is unclear; however, insulin resistance and/or hyperglycemia may also regulate the expression of the α -TTP gene in GK rats, which may result in an increase of the plasma α -tocopherol levels.

In a previous study, α -TTP-deficient mice showed improved glucose tolerance and increased insulin secretion, which could be attributable to an increase in the number of pancreatic islets (33). This report suggests that α -TTP and α -tocopherol may play a very important role in glucose metabolism. However, the relationship between insulin resistance and α -TTP expression is still to be clarified. In a gene profiling study using α -TTP-deficient mice, hepatic-regulated genes related to glucose homeostasis, but not oxidative stress-related genes, were found to be up-regulated (34). The up-regulated genes, including peroxisome proliferator-activated receptor- α , insulin-like growth factor-binding protein 2, deiodinase, type I, and growth factor receptor-bound protein 10, in the liver of α -TTP gene-disrupted mice are known to modulate glucose metabolism (34). Birringer and colleagues speculate that the role of α -TTP is different from that of α -tocopherol under the pathological condition of type 2 diabetes. It has been suggested that the absence of α -TTP may influence glucose metabolism (33). In the present study, we speculate that the pathogenetic mechanisms in type 2 diabetes, including insulin resistance, might be associated with the enhanced expression of α -TTP. Alternatively, increased α -TTP expression might promote the development of type 2 diabetes. Further study is required to clarify the role of α -TTP in glucose metabolism.

In conclusion, it can be suggested that the pathophysiological states in diabetes, including oxidative stress, dyslipidemia, hyperglycemia, insulin resistance, or a combination of these factors, might influence hepatic α -TTP expression, which may result in elevated α -tocopherol levels in the plasma and liver. Tocopherol regulatory proteins, including α -TTP, may affect the vitamin E kinetics in the diabetic state. Further studies are required to clarify the factors regulating α -TTP expression. Investigation of the regulation of α -TTP expression may contribute to further clarification of glucose homeostasis and vitamin E status, which could be expected to lead to a clearer understanding of the antioxidant mechanisms.

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