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Supplementation of α-tocopherol improves cardiovascular risk factors via the insulin signalling pathway and reduction of mitochondrial reactive oxygen species in type II diabetic rats

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Abstract

This study determined the effects of α- and γ-tocopherol supplementation on metabolic control and oxidative stress in type 2 diabetic Otsuka Long–Evans Tokushima Fatty (OLETF) rats. Blood glucose, haemoglobin A1c (HbA1c), urinary protein, plasma free fatty acid, triacylglycerol and plasminogen activator inhibitor-1 (PAI-1) levels in OLETF rats were significantly higher than in non-diabetic control Long–Evans Tokushima Otsuka (LETO) rats. α-Tocopherol inhibited the increase in urinary protein, blood glucose, HbA1c and PAI-1 levels, but γ-tocopherol did not. Plasma and hepatic lipid peroxidation and hepatic steatosis were increased in OLETF rats. α-Tocopherol decreased lipid peroxidation. Mitochondrial reactive oxygen species production and uncoupling protein 2 (UCP2) expression were significantly increased in the heart and aorta of OLETF rats compared with LETO rats. Endothelial NO synthase and aortic nitrotyrosine were increased in OLETF rats. In contrast, the expression of phosphorylated vasodilator-stimulated phosphoprotein and glucose transporter 4 in the aorta was significantly decreased in OLETF rats. These abnormalities were reversed by α-tocopherol. These findings suggest that α-tocopherol may prevent cardiovascular tissues from oxidative stress and insulin signalling disorder resulting from diabetes mellitus.

Keywords: *OLETF rat, reactive oxygen species, α-tocopherol, γ-tocopherol, type II diabetes*

Abbreviations: *COX, cytochrome oxidase; eNOS, endothelial NO synthase; FFA, free fatty acid; GLUT, glucose transporter; LETO, Long-Evans Tokushima Otsuka; OLETF, Otsuka-Long Evans Tokushima Fatty; pVASP, phosphorylated vasodilator-stimulated phosphoprotein; PAI-1, plasminogen activator inhibitor-1; ROS, reactive oxygen species; SOD, superoxide dismutase; UCP2, Uncoupling protein 2.*

Introduction

Type 2 diabetes is associated with high blood glucose level, altered insulin signalling, reactive oxygen species (ROS) production, inflammation and protein kinase C activation, which may lead to a decrease in nitric oxide (NO) bioavailability [1]. High glucose

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concentration, which contributes to the pathogenesis of atherosclerosis in patients with diabetes, leads to intracellular oxidative stress [2,3]. It is known that hyperglycemia can induce damage through overproduction of superoxide radicals in the mitochondria [4]. Calorie restriction suppresses ROS/reactive nitrogen species (RNS) in plasma and the aorta [5] and we have reported that calorie restriction after the development of diabetes can improve diabetic complications in cardiac tissues [6]. Dietary antioxidants have been hypothesized to have a protective effect against the development of diabetes by inhibiting peroxidation chain reactions. It has been postulated that α-tocopherol supplementation has beneficial effects on metabolic control of diabetes due to its antioxidant activity, which influences lipid peroxidation, protein glycation and insulin sensitivity [7]. However, findings regarding the benefits of α-tocopherol supplementation are conflicting and there are quantitative discrepancies of the efficacy between epidemiological data and intervention studies [8–12]. On the other hand, it has been demonstrated that the pharmacokinetics of γ-tocopherol are quite different from those of α-tocopherol [13,14]. Furthermore, γ-tocopherol has several pharmacological properties such as anti-inflammatory effects [15]. γ-Tocopherol potently inhibited the neointimal formation induced by vascular injury in insulin-resistant rats [16].

The Otsuka Long–Evans Tokushima Fatty (OLETF) rat, a model of spontaneous type 2 diabetes, develops hyperglycemic obesity with hyperinsulinemia and insulin resistance after the age of 25 weeks. Insulin resistance and sensitivity are susceptible to changes in whole-body redox balance [17–19]. The aim of this study was to investigate the potential of α- and γ-tocopherol for treating diabetic complications in OLETF rats.

Materials and methods

Chemicals

Reduced-type MitoTracker Red (MTR) CM-H2XRos (M-7513), MitoTracker Green (MTG) FM (M-7514) and anti-cytochrome oxidase (COX) II antibody were purchased from Molecular Probes (Eugene, OR). Anti-endothelial nitric oxide synthase (eNOS/NOS-III) and anti-plasminogen activator inhibitor-I (PAI-1) antibodies were purchased from BD Biosciences (San Jose, CA). Anti-uncoupling protein 2 (UCP2) antibody was purchased from Biolegend (San Diego, CA) and phosphorylated-vasodilator-stimulated phosphoprotein (pVASP) (Ser239) and VASP antibodies were from Calbiochem (Darmstadt, Germany). Anti-nitrotyrosine antibody and anti-(glucose transporter 4) GLUT4 antibody were purchased from Sigma (St Louis, MO) and R&D Systems (Minneapolis, MN), respectively.

Animals

Male OLETF rats (aged 4 weeks) and age-matched Long–Evans Tokushima Otsuka (LETO) rats were obtained from the Animal Centre of Tokushima Research Institute (Otsuka Pharmaceutical, Tokushima, Japan) and maintained until they reached an appropriate age for the experiment. All rats had free access to standard laboratory chow (MF; Oriental Yeast, Tokyo, Japan) and tap water and were cared for under the specifications outlined in the *Guiding Principles for the Care and Use of Laboratory Animals—Approved by the Authorities of the Local Committee on Experimental Animal Research*. LETO ($n = 15$) and OLETF ($n = 17$) rats were fed control chow and divided into three groups each (control: $n = 5$ (LETO), $n = 7$ (OLETF), α-tocopherol: $n = 5$, and γ-tocopherol: $n = 5$) at 29 weeks after checking body weight, blood glucose and HbA1c of all rats. Blood was collected via a tail vein with a heparinized syringe. Ten rats in each LETO or OLETF group were given 0.05% RRR-α-tocopherol ($n = 5$) or RRR-γ-tocopherol ($n = 5$) between 29–33 weeks of age as a dietary mixture. α-Tocopherol concentration in the control diet was 0.007%. α-Tocopherol and γ-tocopherol were generously donated by Eisai (Tokyo, Japan).

Animals were sacrificed at 33 weeks of age under anaesthesia with urethane (5 g/kg, ip). Blood was collected with heparinized syringes and heart, thoracic aorta and other tissues were dissected out and frozen in liquid nitrogen. Plasma samples were obtained by centrifugation at 12 000 *g* for 5 min. Urine was collected by bladder puncture. A small piece of tissue was removed for morphometric, immunohistochemical or biochemical analysis.

Biochemical measurements

Blood glucose levels were measured immediately after sampling with a glucose test meter (Glutest Ace; Sanwa Kagaku Kenkyusyo, Nagoya, Japan). Total cholesterol, triglyceride and free fatty acid (FFA) levels were determined with commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). As an index of atherosclerosis, plasma PAI-1 (ZYMUTEST Rat-PAI-1; Hyphen BioMed, Neuville-sur-Oise, France) was measured with commercially available ELISA kits.

Immunoblot analysis for UCP2/COXII, eNOS, pVASP and VASP and PAI-1

The heart and aortic tissues (100 mg) were homogenized and sonicated in 0.3 ml 50 mM HEPES, pH 7.5, containing 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 200 mM NaCl, 10% glycerol, 0.1% Tween-20, 0.1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF and a protease inhibitor cocktail tablet (Roche Diagnostics,

Mannheim, Germany). Homogenates were centrifuged at 12 000 *g* for 20 min. The supernatants (30 μg) were used for detection of UCP2 and COXII proteins and were separated on 15% SDS–polyacrylamide gels and transferred to PVDF membranes (Immobilon-PSQ; Millipore, Billerica, MA). Membranes were blocked overnight in buffer containing 1 × Tris-buffered saline (TBS), 0.1% Tween-20 and 5% (w/v) powdered milk. Membranes were cut at the 50-kDa marker and incubated with anti-UCP2 (33 kDa) or anti-COXII (70 kDa) overnight at 4°C. The membranes were washed in TBS-Tween-20 (TBS-T) and incubated with each secondary antibody conjugated with horseradish peroxidase (Amersham Biosciences, Buckinghamshire, UK) for 1.5 h at room temperature. After washing again in TBS-T, the membranes were exposed to enhanced chemiluminescence (Amersham Biosciences) on the film. Protein bands were quantified by densitometry, with results expressed as relative proportions to the mean value for control blots. Since COXII is one of the mitochondrial respiratory chain enzymes, UCP2 levels in the mitochondria were expressed as the ratio of intensity of UCP2/COXII. Antibodies to UCP2 and COXII were diluted with Can Get Signal solution (TOYOBO, Osaka, Japan).

The supernatants (2–10 μg) were analysed by immunoblotting for pVASP and VASP using 7.5% gels as described above. Commercial VASP (20 ng) was used as an internal standard for Western blotting. Anti-VASP phosphoserine 239 antibody was used for analysis of phosphorylation status of VASP at Ser239 (pVASP), a reliable biochemical marker of vascular cGMP-dependent protein kinase-I activity. Applied fractions were separated by SDS-PAGE and transferred electrophoretically to PVDF membranes. The supernatants (5 μg) were also analysed for eNOS and PAI-1, using 7.5% gels as described above. Western blots were probed with a specific antibody and quantified using Scion Image version 1.63 (Scion, Frederick, MD). Band intensities are presented as percentages of the mean value for each group. Activation of VASP is indicated by the intensity ratio pVASP/VASP.

Lipid peroxidation of plasma and liver tissues

Lipid peroxidation of plasma and liver tissues was assayed using a Bioxytech LPO-586 kit (Oxis International, Portland, OR) that measured malondialdehyde (MDA), together with 4-hydroxyalkanals.

In situ *determination of mitochondrial ROS generation*

Loading of sections with MTG or MTR, which are mitochondrial fluorescence probes, showed that mitochondria were predominantly situated in the perigranular, subplasmalemmal and perinuclear regions. MTR (CM-H2XRos, reduced form of the probe) is non-fluorescent. When the probe is oxidized by ROS, it becomes fluorescent. The oxidized product binds to thiol groups and proteins within mitochondria. MTR is excited at 579 nm with an emission spectrum of 599 nm [20]. MTG (excitation, 490 nm; emission, 516 nm) is a mitochondrion-selective fluorescent label [21] and covalently binds to the inner mitochondrial membrane and fluoresces independently of membrane potential ψ and $[Ca^{2+}]_{mt}$. Unfixed frozen tissues were cut into 10-μm-thick sections and placed on glass slides. MTR (2 μM) was applied to each tissue section and the slides were incubated in a light-protected, humidified chamber at 37°C for 15 min, with or without 500 μM NADH, and 200 nM MTG added. After incubation with Mito Tracker, sections were washed twice with Mito Tracker-free PBS. Images were obtained with an LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) coupled to an upright microscope (Zeiss) with a PlanFluor objective (× 63, N.A. 1.4) at the Central Research Laboratory, Okayama University Medical School (Japan). Laser settings were identical to those used for the acquisition of images, as described in our previous report [6].

Images from MTG (green) and CM-H2XRos (red) fluorescence patterns of the cells were processed as one-colour images or two-colour overlays, as indicated. Neither signal exhibited significant photobleaching during the time frame required for analysis. The findings reported here were replicated in three independent experiments. Examination of the intracellular distribution of ROS generation was performed by comparison with the staining pattern of the mitochondrion-specific probe.

Histology and immunohistochemistry

Tissue samples were fixed immediately in 10% buffered formalin. All tissues were embedded in paraffin within 48 h of formalin fixation and were cut to a thickness of 4 μm just before staining. Histological staining was performed using hematoxylin and eosin or anti-nitrotyrosine. Other portions of the tissues were frozen in OCT compound and sectioned to a thickness of 10 μm for GLUT4 staining.

Immunohistochemistry was performed using rabbit or mouse streptavidin–biotin peroxidase kits (Vectastain Universal Elite ABC Kit; Vector Laboratories, Burlingame, CA). Antibodies for immunohistochemical staining were directed at nitrotyrosine and GLUT4. Negative control sections were processed in an identical manner by substitution of normal mouse immunoglobulin 1 for primary antibody.

Statistical analysis

Unless otherwise stated, results are presented as means ± se. Results were considered significant at

$p < 0.05$. Statistical analysis was performed by analysis of variance (ANOVA).

Results

Body weight, urinary protein and blood chemistry

Body weight increased in both the satiated LETO and OLETF rats, with less increase in the LETO rats. Tocopherol supplementation did not change the body weight of OLETF rats (Figure 1). There were no differences in consumption of diet among groups of LETO or among groups of OLETF (data not shown).

Figure 2 shows urinary protein levels after correction for urinary creatinine. OLETF rats exhibited significantly increased urinary protein, which reflected diabetic nephropathy. α-Tocopherol, but not γ-tocopherol, inhibited the increase in urinary protein level.

Haemoglobin A1c (HbA1c), blood glucose, plasma triglyceride, total cholesterol and LDL levels were significantly higher in OLETF rats than in age-matched LETO rats (Figures 3 and 4). α-Tocopherol, but not γ-tocopherol, inhibited the increase in HbA1c and blood glucose. However, no lipid profiles differed between control and supplemented groups of OLETF rats, except for the increase in FFA induced by γ-tocopherol.

Liver histology

α-Tocopherol, not γ-tocopherol, supplementation improved steatosis seen in OLETF rats on non-supplemented food (Figure 5).

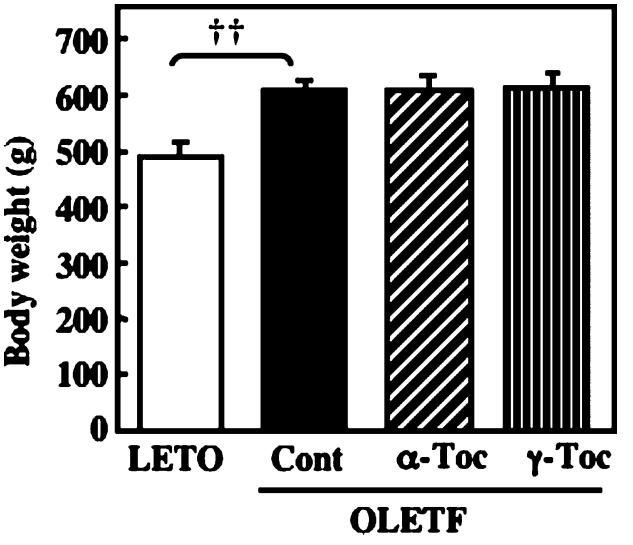

Figure 1. Effect of tocopherol supplementation on body weight. Body weights of LETO and OLETF rats are shown at age 33 weeks. Some OLETF rats were subjected to 0.05% α-tocopherol (α-Toc) or γ-tocopherol (γ-Toc) diets between 29–33 weeks of age. Values are means ± SEM ($n = 5$–7).

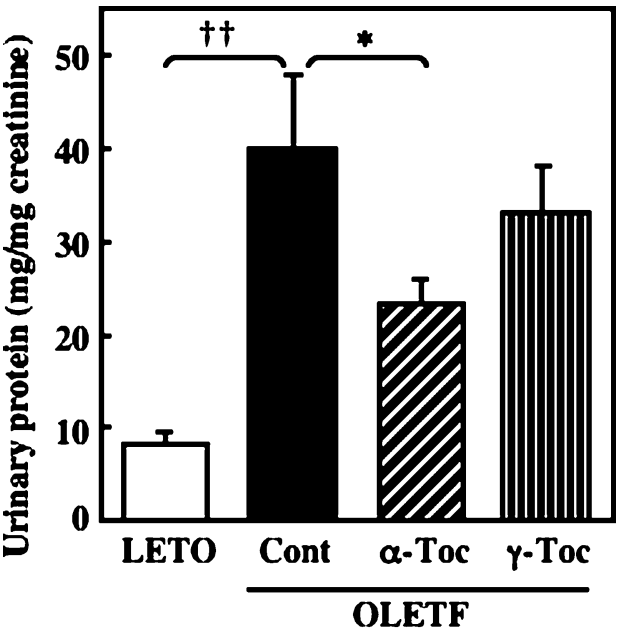

Figure 2. Effect of tocopherol supplementation on urinary protein. Animals were treated as described in Figure 1. Urinary protein and creatinine were measured in the collected urine. Values are means ± SEM ($n = 5$–7). †† $p < 0.01$ compared with LETO rats; *$p < 0.05$ compared with control diet group of OLETF rats.

Plasma and liver lipid peroxidation

Experiments were conducted to test the hypothesis that oxidative stress is heightened in diabetes (Figure 6). Lipid peroxidation in plasma and liver was elevated in OLETF rats. α-Tocopherol returned the level of LPOs to the levels observed in LETO rats. γ-Tocopherol did not have this effect.

Effects of α-tocopherol on cardiovascular ROS generation

Cardiovascular superoxide production was assessed by Mito Tracker fluorescence in heart (Figure 7 upper) and aorta (Figure 7 lower) tissue sections ($n = 4$ per group). MTR that accumulated in response to the membrane potential formed a covalent complex with mitochondrial constituents, which prevented subsequent release of the fluorochrome. MTR was almost completely co-localized with MTG. Superoxide production (MTR positive) increased in the cardiac muscle, coronary endothelial cells and aortic vessel walls in OLETF rats. Mitochondrial ROS generation was inhibited in the α-tocopherol group.

Effects of α-tocopherol on UCP2 expression in the heart

Cardiac expression of UCP2 was significantly increased in the OLETF rats compared with LETO rats (Figure 8). α-Tocopherol significantly decreased UCP2 expression in OLETF rats.

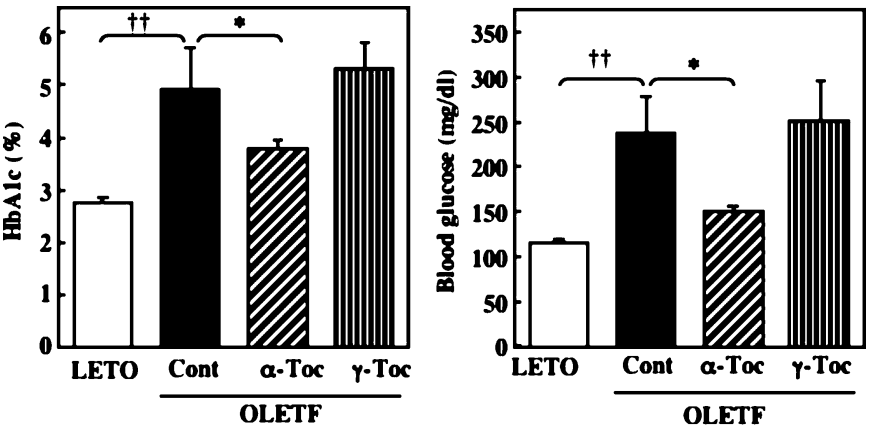

Figure 3. Levels of HbA1c and blood glucose. Animals were treated as described in Figure 1. Values are means ± SEM ($n = 5$–7). ††$p < 0.01$ compared with LETO rats; *$p < 0.05$ compared with control diet group of OLETF rats.

eNOS expression and deposition of 3-nitrotyrosine in the aorta

Expression of aortic eNOS in OLETF rats was elevated 2-fold compared with that in LETO rats (Figure 9). α-Tocopherol further enhanced eNOS expression in both LETO and OLETF rats.

Elevated superoxide is a prerequisite for increased peroxynitrite formation. Accordingly, we investigated whether the increase in eNOS expression, together with enhanced superoxide production in the aortic tissue in OLETF rats, was associated with peroxynitrite formation and nitration of tyrosine residues. The staining intensity of immunoreactive nitrotyrosine in the aortic vessel walls of OLETF rats (Figure 10) was much stronger than that in LETO rats. α-Tocopherol decreased this immunoreactivity in OLETF rats. These findings are consistent with the hypothesis that over-production of superoxide during diabetes results in the formation of peroxynitrite at the expense of NO. The latter oxidant is merely responsible for the nitration of aortic smooth muscle cells.

pVASP and total VASP in the aorta

To determine NO bioavailability, we measured pVASP and total VASP in the aorta. Total VASP in OLETF rats was elevated about 2-fold compared with that in LETO rats (Figure 11). α-Tocopherol returned total VASP level to normal in OLETF rats. In contrast, pVASP (the ratio of pVASP/VASP) was

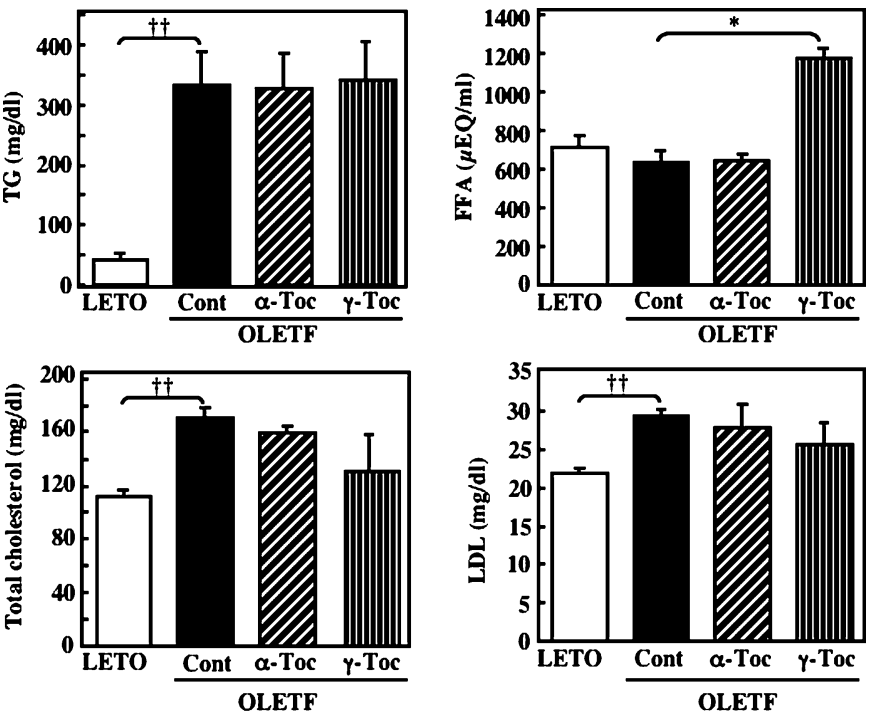

Figure 4. Plasma lipid profiles. Animals were treated as described in Figure 1. Values are means ± SEM ($n = 5$–7). ††$p < 0.01$ compared with LETO rats; *$p < 0.05$ compared with control diet group of OLETF rats.

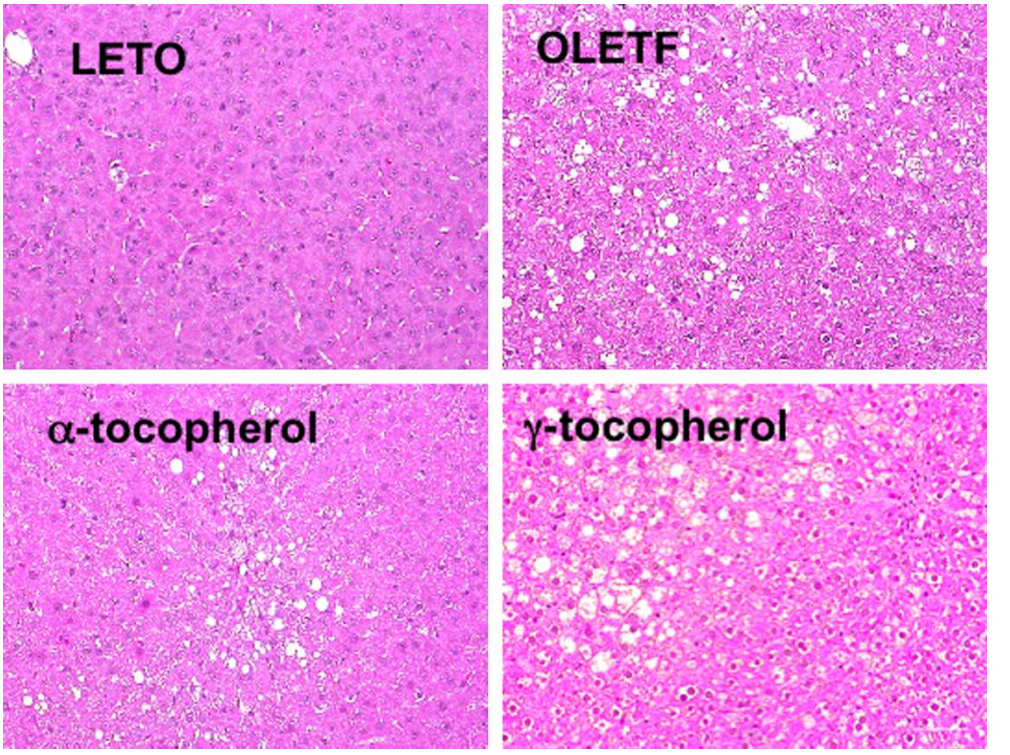

Figure 5. Liver histology. Representative images of HE staining of the liver. Original magnification ×20.

decreased in OLETF rats and α-tocopherol increased pVASP (Figure 11).

Plasma PAI-1 levels

Plasma PAI-1 level was significantly higher in OLETF rats. α-Tocopherol, but not γ-tocopherol, returned PAI-1 to normal in age-matched LETO rats (Figure 12).

GLUT4 and pAKT expression in the aorta

GLUT4 protein staining was present in the aortic endothelial cells and vessel walls of LETO rats. In age-matched OLETF rats, aortic endothelial cells became thickened and GLUT4 staining was abolished. However, in the α-tocopherol group, GLUT4 protein was observed in the endothelial cells (Figure 13). Basal levels of pAkt were reduced in OLETF rats and α-tocopherol induced partial recovery of pAkt protein level (Figure 14).

Discussion

This study revealed that α-tocopherol, but not γ-tocopherol, improved complications of diabetes such as excess mitochondrial ROS generation, which causes vascular NO/cGMP dysfunction and low expression of aortic GLUT4 in OLETF rats. Vitamin

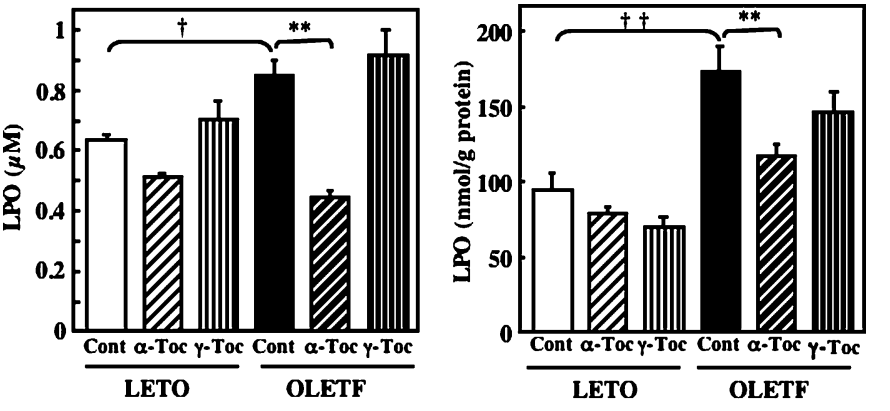

Figure 6. Effects of α- and γ-tocopherol on lipid peroxidation in plasma and liver. Values are means ± SEM ($n = 5$–7). †$p < 0.05$, ††$p < 0.01$ compared with LETO rats; **$p < 0.01$ compared with control diet group of OLETF rats.

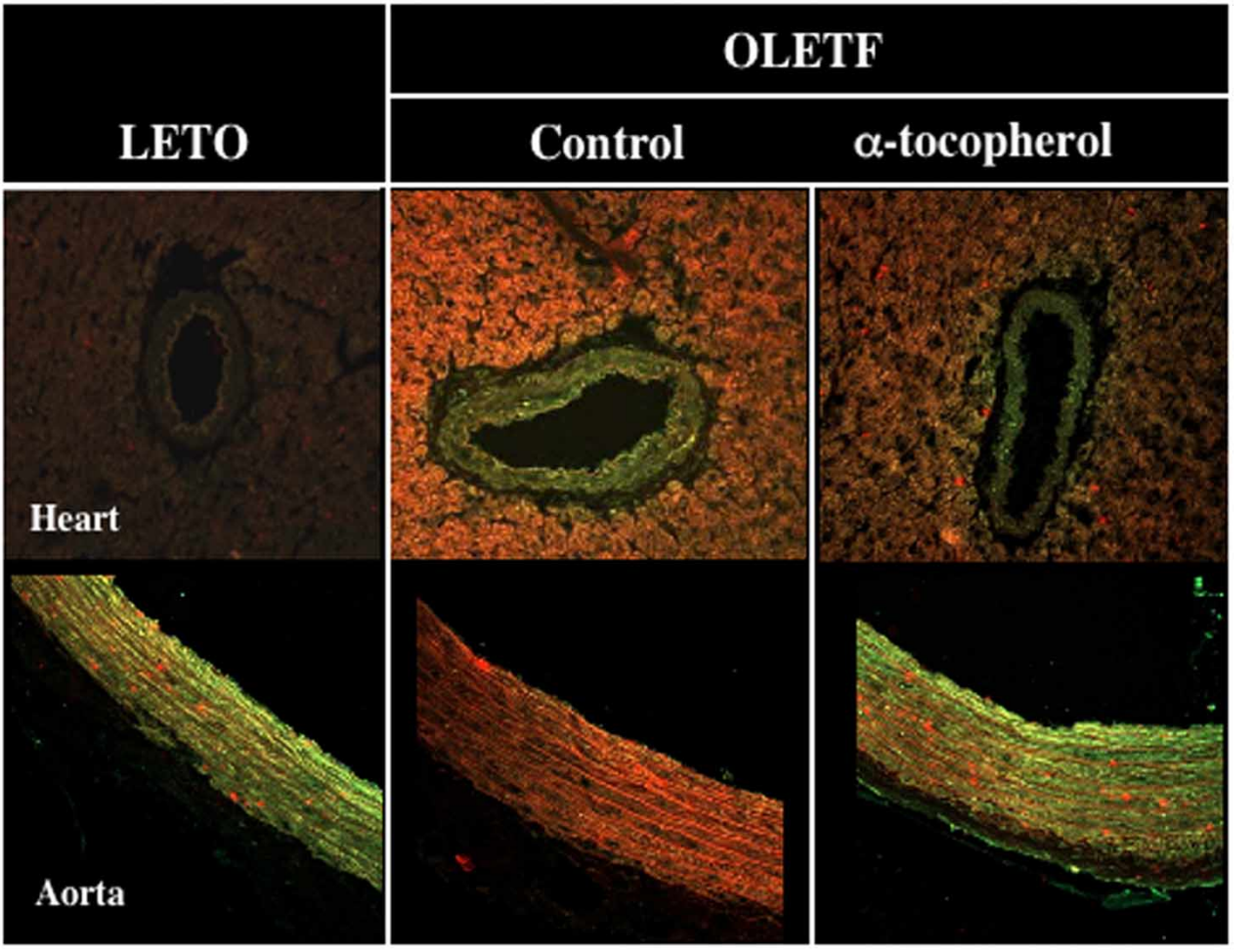

Figure 7. ROS production in heart and aorta. Representative images of confocal microscopy of frozen sections from 33-week-old rats heart (upper panels) and aorta (lower panels). Co-localization of MTG and MTR (ROS generation) fluorescence is shown as shades of orange. Original magnification ×20.

E is a lipid-soluble, chain-breaking antioxidant of lipid peroxidation and antioxidant activity of vitamin E against ROS has been reported [22].

γ-Tocopherol is a major dietary form of vitamin E and may have protective properties different from those of α-tocopherol. γ-Tocopherol provides different antioxidant activities in food and *in vitro* studies and exhibits greater activity in trapping the lipophilic electrophiles, RNS and ROS [23,24]. Equimolar doses of α-tocopherol and γ-tocopherol (1 g/kg body weight, i.m.) were found to have the same protective effect against metal-induced nephrotoxicity in rats [25]. In that study, the maximum tocopherol concentrations in serum and kidney were ∼18 μg/ml for α- and ∼5 μg/ml for γ-tocopherol and ∼26 μg/g tissue for α- and ∼0.5 μg/g tissue for γ-tocopherol, respectively. Furthermore, the half life of α-tocopherol in serum is about eight times longer than that of γ-tocopherol [25]. Therefore, the lower plasma levels of γ- compared to α-tocopherol might be discussed in the light of different bioavailability. Nevertheless, in the present study, γ-tocopherol administration at the same dose as α-tocopherol did not improve diabetic complications in OLETF rats. There have been few studies on the effects of pure γ-tocopherol in type 2 diabetic rats. The effect may reflect the systemic concentrations of antioxidants since diabetes is a systemic illness associated with oxidative stress, even if metabolites of γ-tocopherol, such as 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman are more potent than α-tocopherol. Contradictory reports exist concerning the relative efficacies of α- and γ-tocopherol [15,16,23,26,27] Among these studies, Takahashi et al. [16] have reported that 100 mg/kg/day of γ-tocopherol, but not the same dose of α-tocopherol, inhibits neointimal formation induced by balloon injury of the carotid artery in high-fructose-supplemented rats, with no impact on biochemical parameters such as glucose and FFA levels. They interpreted their results as suggesting that it may be of importance that γ-tocopherol preferentially accumulates in injured arteries (3.8–29.0 pmol/mg tissue), while α-tocopherol supplementation does not result in such preferential accumulation in the injured vascular wall (126.7–107.7 pmol/mg tissue) [16]. They note

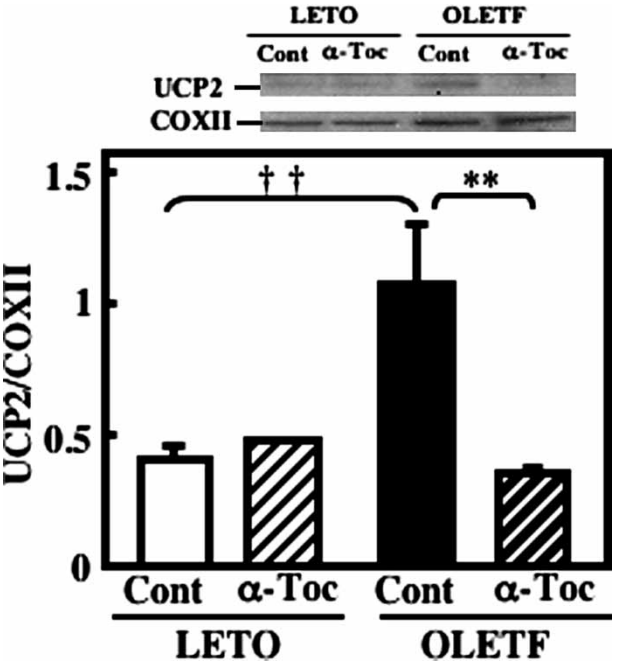

Figure 8. Effects of α-tocopherol on cardiac UCP2 expression. Values are relative to expression of UCP2/COXII. ††$p<0.01$ compared with LETO rats; **$p<0.01$ compared with control diet group of OLETF rats.

that the mechanism of γ-tocopherol accumulation in injured vessels is unclear. In OLETF rats, because there is no blood vessel injury, γ-tocopherol might not have such an effect. Furthermore, Takahashi et al. [16] also provided evidence pointing to α-tocopherol

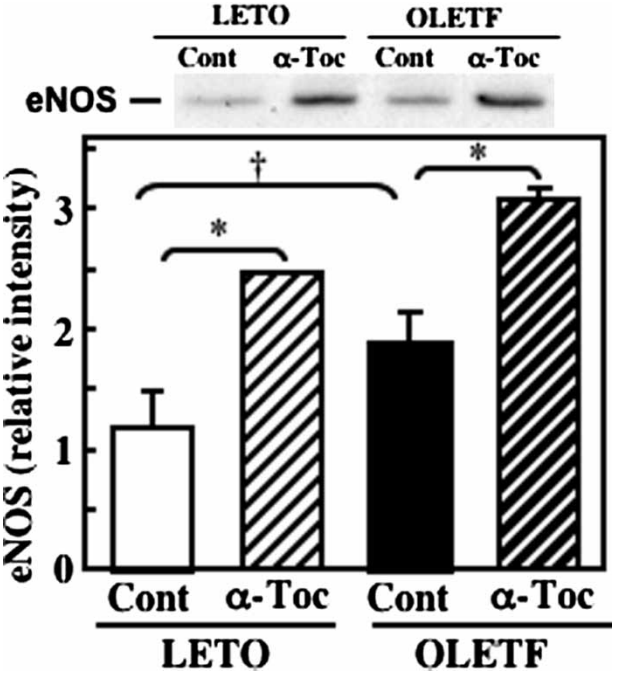

Figure 9. Effects of α-tocopherol on eNOS expression in the aorta. Aortic lysates were resolved by SDS-PAGE. Densitometry data are presented as means±SEM ($n=5$). †$p<0.05$ compared with LETO rats; *$p<0.05$ compared with control diet group of OLETF rats.

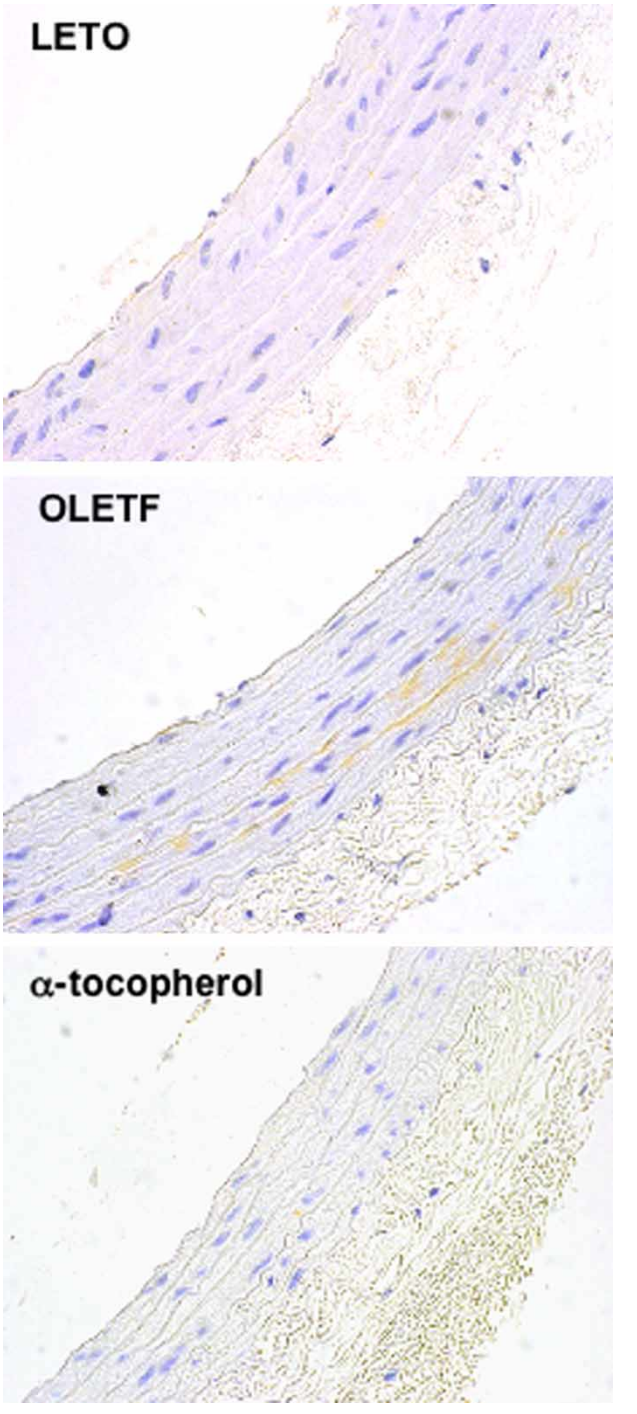

Figure 10. Effects of α-tocopherol on aortic nitrotyrosine immunohistochemistry. Isolated aorta sections stained for nitrotyrosine from age-matched LETO, satiated OLETF and α-tocopherol-supplemented OLETF rats aged 33 weeks (original magnification, ×40).

accumulation in uninjured vessels rather than injured vessels. On the other hand, endothelial NADH oxidase in the aorta of OLETF rats is enhanced after the onset of hyperglycemia, resulting in increased vascular production of superoxide [28].

α-Tocopherol amplifies phosphorylation of eNOS at serine 1177 [29]. α-Tocopherol also increases eNOS protein and mRNA levels [30]. We also found

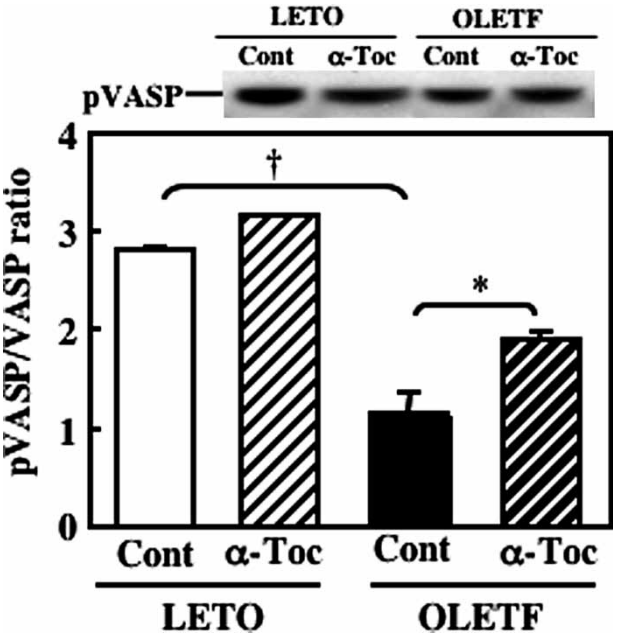

Figure 11. Effects of α-tocopherol on aortic pVASP/total VASP expression. To examine NO/cGMP signal activation, the ratio of phosphorylated (Ser239)-VASP expression/total VASP was assessed. Aortic lysates were resolved by SDS-PAGE. Densitometry data are presented as means ± SEM ($n = 5$). †$p < 0.05$ compared with LETO rats; ⋆$p < 0.05$ compared with control diet group of OLETF rats.

that α-tocopherol increased eNOS protein in OLETF rats. eNOS over-expression in OLETF rats may be induced by ROS generation and UCP2 over-expression [31,32]. In diabetes, activity of the NO-cGMP pathway (VASP phosphorylation) is decreased through NO quenching by superoxide to form peroxynitrite, resulting in nitrotyrosine formation despite of eNOS over-expression.

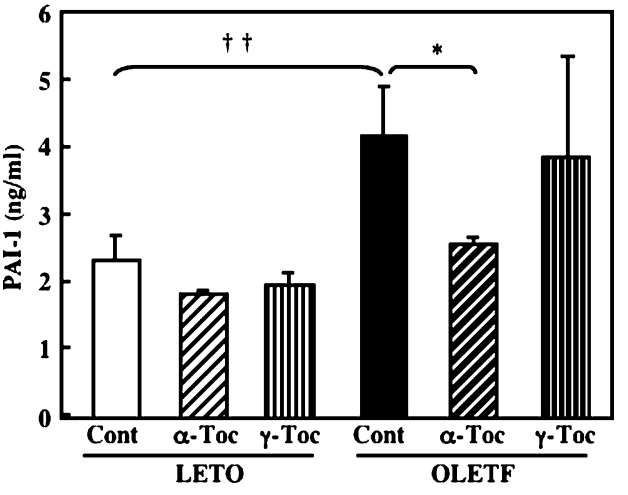

Figure 12. Effect of tocopherol supplementation on plasma PAI-1 level. Values are means ± SEM ($n = 5$–7). ††$p < 0.01$ compared with LETO rats; ⋆$p < 0.05$ compared with control diet group of OLETF rats.

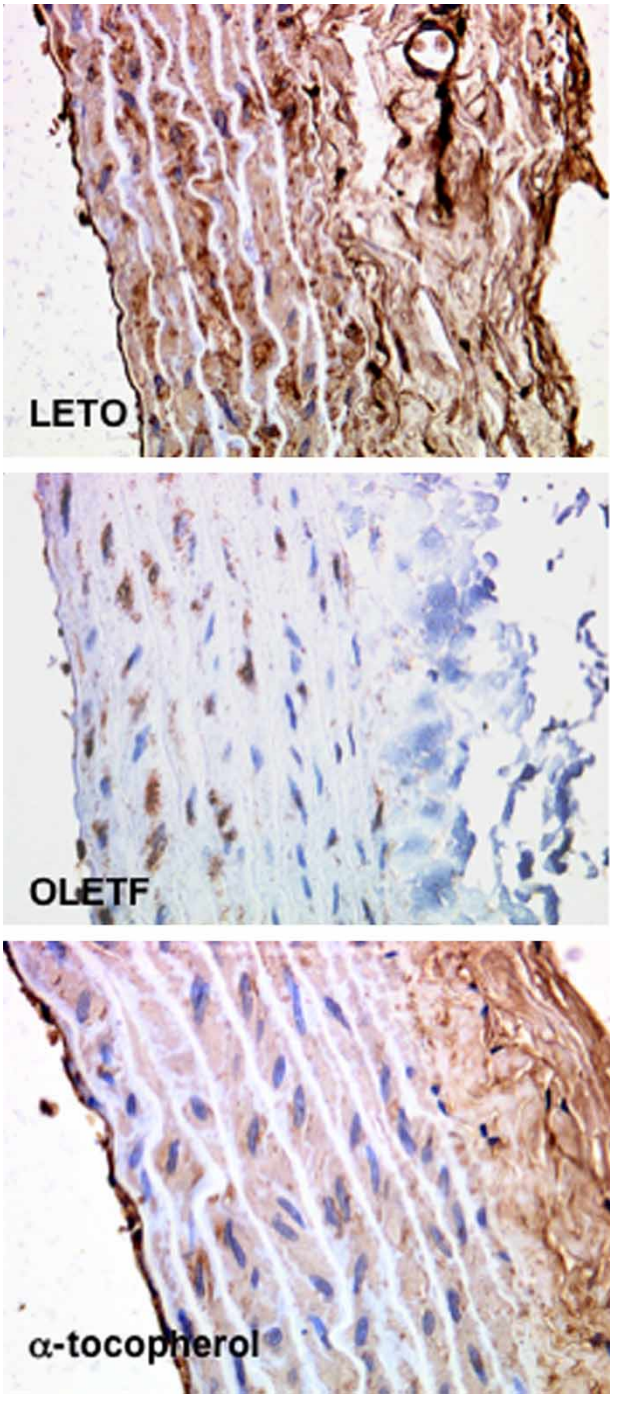

Figure 13. GLUT4 expression in the aorta. Immunohistochemistry of GLUT4 in the aorta. Age-matched LETO, satiated OLETF and α-tocopherol-supplemented OLETF rats aged 33 weeks (original magnification, ×40).

α-Tocopherol restores UCP2 levels to normal. These findings suggest that ROS production is inhibited in OLETF rats.

The increase in GLUT4 in the aorta induced by α-tocopherol may partially improve hyperglycemia and insulin signalling via AKT (Ser473) phosphorylation.In conclusion, α-tocopherol prevented diabetic complications, including ROS production and endothelial dysfunction.

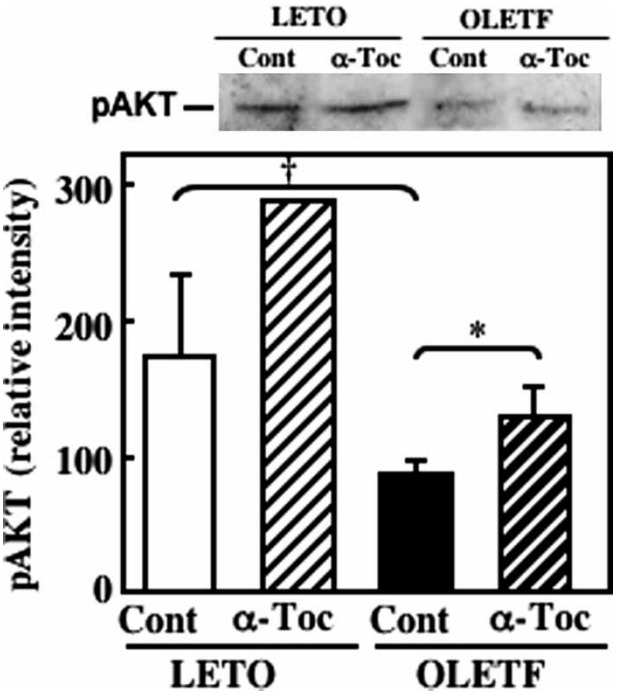

Figure 14. Effect of α-tocopherol on insulin signal protein pAkt expression in the aorta. Aortic expression of immunoreactive pAkt. Aortic lysates were resolved by SDS-PAGE. Densitometry data are presented as means ± SEM ($n = 5$). †$p < 0.05$ compared with LETO rats; *$p < 0.05$ compared with control diet group of OLETF rats.

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