

## Effects of vitamin E and its derivatives on diabetic nephropathy in Rats and identification of diacylglycerol kinase subtype involved in the improvement of diabetic nephropathy

Tomoko Kakehi<sup>1</sup>, Keiko Yagi<sup>2</sup>, Naoaki Saito<sup>1</sup>, and Yasuhito Shirai<sup>3\*</sup>

<sup>1</sup>Laboratory of Molecular Pharmacology, Biosignal Research Center, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan; <sup>2</sup>Department of Clinical Pharmacy Kobe Pharmaceutical University, Kobe 658-8558, Japan; <sup>3</sup>Laboratory of Chemistry and Utilization of Animal Production Resources, Applied Chemistry in Bioscience Division, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501, Japan.

\***Corresponding Author:** Yasuhito Shirai, Ph.D. Laboratory of Chemistry and Utilization of Animal Production Resources, Applied Chemistry in Bioscience Division, Graduate School of Agricultural Science, Kobe University, Rokkodai-cho 1-1, Nada-ku, Kobe 657-8501

**Submission Date:** August 22<sup>nd</sup>, 2017, **Acceptance Date:** October 28<sup>th</sup>, 2017, **Publication Date:** October 31<sup>st</sup>, 2017

**Citation:** Kakehi T., Yagi K., Saito N., Shirai Y., Effects of vitamin E and its derivatives on diabetic nephropathy in Rats and identification of diacylglycerol kinase subtype involved in the improvement of diabetic nephropathy. *Functional Foods in Health and Disease* 2017; 7(10):816-832

### ABSTRACT

**Background:** Diabetes is a significant social issue. Controlling diabetic complications such as nephropathy is very important for QOL of diabetic patients. One of the mechanisms which causes diabetic complications is the abnormal activation of protein kinase C (PKC) by increased diacylglycerol (DG) from hyperglycemia. Diacylglycerol kinase (DGK) can attenuate PKC activity by converting DG to phosphatidic acid. Thus far, d- $\alpha$ -tocopherol (VtE) treatment has been shown to prevent early changes of diabetic renal dysfunctions by activating DGK. However, it is

still unknown whether VtE derivatives improve diabetic nephropathy similarly to VtE, and which DGK subtype is activated by VtE and/or the derivatives.

**Objective:** The purpose of the study was to investigate effects of VtE and its derivatives on diabetic nephropathy in rats, in addition to identifying the DGK subtype involved in the improvement of nephropathy in vivo.

**Methods:** To induce diabetes in rats, six weeks old male Sprague-Dawley rats were intraperitoneally administrated 65 mg/kg streptozocin (STZ) in 20 mM citrate buffer. For two or eight weeks, 40 mg/kg VtE, 44 mg/kg acetate VtE (aVtE) or 49.3 mg/kg succinate VtE (sVtE) was intraperitoneally administrated every other day after STZ administration. The blood glucose level, body weight, and kidney weight, in addition to urinary volume, albumin, and BUN were measured every week after STZ administration. Additionally, in order to identify the DGK subtype activated by VtE and aVtE, the DGK subtype expressed in the rat glomerulus was checked by RT-PCR and western blotting, and the activity in the glomerulus from the rats before and after the VtE and aVtE treatments were measured in the presence or absence of EGTA.

**Results:** Averages of kidney weight and BUN of rats treated with VtE, aVtE and sVtE for 8 weeks were reduced, compared to the control. However, the intraperitoneal administration of sVtE was toxic. Additionally, the amount of urine volume and urinary albumin significantly improved by the two-weeks treatment of VtE and aVtE. mRNA of DGK $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  were detected in the glomerulus, but only the protein of DGK $\alpha$  and DGK $\delta$  were detected as calcium-dependent and independent subtype respectively. Both VtE and aVtE activated DGK in the glomerulus. However, the calcium dependent DGK subtype was mainly activated by aVtE, with not only the calcium-dependent subtype but also the calcium-independent subtype being activated in the case of VtE. In other words, DGK $\alpha$  was activated by aVtE and DGK $\delta$  was additionally enhanced in the case of VtE.

**Conclusion:** These results clearly indicated that aVtE as well as VtE improved diabetic nephropathy, with the activation of DGK $\alpha$  and/or  $\delta$  being potentially involved with this improvement.

**Key words:** diabetic nephropathy, diacylglycerol kinase, vitamin E

## INTRODUCTION

Currently, diabetes is a significant social problem as the number of patients over the world has increased to 4 billion people, with related medical expense reaching to 900 billion dollars in 2015 [1]. This is highly significant for the QOL of diabetic patients and reduction of the expense to not only reduce the number of the patients but to also control diabetic renal dysfunction including neuropathy, retinopathy, and nephropathy. However, the mechanisms of diabetic complications are not clear. One of the mechanisms which causes the diabetic complications is the abnormal activation of protein kinase C (PKC), especially  $\beta$  subtype, by increased diacylglycerol (DG) from hyperglycemia [2-6]. Based on the findings, a PKC $\beta$  specific inhibitor was developed by the pharmaceutical company and applied to the medicines for diabetic complications [7]. Unfortunately, this trial failed at phase III. However, it has been reported that d- $\alpha$ -tocopherol (VtE) treatment prevented early changes of diabetic renal dysfunctions by normalizing both DG levels and PKC activity in glomerular cells [8, 9]. This improvement by VtE was likely due to the activation of DG kinase (DGK).

DGK phosphorylates DG in order to produce phosphatidic acid. In other words, DGK can attenuate PKC activity. At least 10 subtypes of mammalian DGKs have been cloned and divided into five groups based on their structure [10-12]. Generally, all DGKs have cysteine-rich regions homologous to the C1A and C1B motifs of PKCs in the regulatory domain at the N terminus and possess a conserved catalytic domain in the C terminus. Type I DGKs, DGK $\alpha$ ,  $\beta$ , and  $\gamma$ , have EF-hand motifs and two cysteine-rich regions in the regulatory domain [13-16]. Type II DGKs, DGK $\delta$  and  $\eta$ , have a pleckstrin homology domain instead of the EF-hand motif, in addition to two cysteine-rich regions. The catalytic domains of DGK $\delta$  and  $\eta$  are separated [17, 18]. Type III, DGK $\epsilon$ , has only two cysteine-rich regions in the regulatory domain [19]. Type IV, DGK $\zeta$ , and  $\iota$ , have a motif similar to the myristoylated alanine-rich C-kinase substrate phosphorylation site in the regulatory domain and four ankyrin repeats at its C terminus [20-22]. Type V, DGK $\theta$ , has three cysteine-rich regions and a pleckstrin homology domain [23].

To identify the DGK subtype involved in the VtE-induced improvement of nephropathy, we investigated translocation of GFP-tagged DGK $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\epsilon$ , and  $\zeta$  subtype in response to VtE because the translocation is a hall mark of DGK activation and the 5 DGK subtypes were suggested to be expressed in rat glomerulus [24]. With the results, only DGK $\alpha$  was translocated from the cytoplasm to the plasma membrane within 1 min, with elevation of kinase activity [24]. These results suggested that DGK $\alpha$  is involved in the VtE-induced improvement of nephropathy.

However, the previous study was *in vitro* experiment and whether DGK $\alpha$  is indeed involved in the VtE-induced improvement of nephropathy in diabetic rat is still unknown.

In addition to VtE, two non-oxidative VtE derivatives, d- $\alpha$ -tocopheryl succinate (sVtE) and d- $\alpha$ -tocopheryl acetate (aVtE), also induced the translocation of DGK $\alpha$  [24], indicating that antioxidant activity is not necessary for the activation of DGK $\alpha$  itself because the VtE derivatives has been shown to be stable in the medium for at least 24 hours [25]. Interestingly, patterns of the DGK $\alpha$  translocation induced by VtE and its derivatives were different. VtE and aVtE induced very rapid and transient translocation of DGK $\alpha$ . In contrast, sVtE, which demonstrated the strongest effect among the three, induced irreversible translocation. However, it is still unknown whether the VtE derivatives cause similar improvement of diabetic nephropathy to VtE *in vivo*, and whether same DGK subtype is activated by VtE and the derivatives. Therefore, we investigated effects of VtE, sVtE, and aVtE on the nephropathy in diabetic rats and tried to identify DGK subtype involved in the improvement of nephropathy *in vivo*.

## MATERIALS AND METHODS

### Materials

D- $\alpha$ -tocopherol (VtE), d- $\alpha$ -tocopheryl succinate (sVtE), dl- $\alpha$ -tocopheryl acetate (aVtE), and streptozocin (STZ) was purchased from SIGMA-aldrich (St. Louis, Mo.). DGK $\alpha$  and  $\gamma$  antibodies were prepared by our hands [26]. DGK $\delta$  and  $\zeta$  antibodies were kindly given by Dr. Sakane (Chiba Univ.) and Dr. Goto (Yamagata Univ.) respectively. DGK $\epsilon$  antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). All other chemicals were of analytical grade.

### Rats

All animal studies followed the Kobe university ethic committee regulations. Male Sprague-Dawley rats that were six-weeks old (160-180g) were purchased from Japan SLC, Inc. (Shizuoka, Japan) and bred with free access to normal diet and water under 12-hour light-dark cycle at 18~26 °C. The six weeks old male rats were intraperitoneal administrated 65 mg/kg STZ in 20 mM citrate buffer. The same volume of citrate buffer was used for the control (defined as non DM rats). On the next day, the blood glucose level of the STD-treated rats was measured and diabetic rats with more than 300 mg/dl glucose were used for following experiments (DM rats). Considering the molecular weight of the compounds, 40 mg/kg VtE, 44 mg/kg aVtE, or 49.3 mg/kg sVtE was intraperitoneally administrated every other day after STZ administration and blood

glucose and body weight were measured every week after STZ administration.

### ***Urine and plasma analysis***

The urine of each group's rats was collected for 24 hrs in metabolic cages under fasting conditions and its volume was measured by mess cylinder. The collected urine was centrifuged at 3,000 rpm x 10 min and the supernatant was used for urine albumin and creatinine analysis. The urine albumin, creatinine, and BUN analysis were consigned to Oriental Yeast Co., Ltd. The plasma was collected in microtube with very small amounts of novo heparin (Mochida Pharmaceutical Co., LTD) from mice tail after the urine collection. The collected plasma was centrifuged at 3,000 rpm X 10 min and the supernatant was used plasma creatinine analysis. The plasma creatinine was measured by LC-MS/MS.

### ***Measurement of kidney weight***

Rats at 8 weeks after STZ administration or control rat were euthanized by thiopental and perfused with saline via left ventricle puncture and removed kidney. After cortex was eliminated, kidney weight was measured. Kidney weight was normalized with body weight.

### ***Preparation of glomerulus***

After measurement of weight, the kidney was sliced into 3 mm and divided into cortex and medulla. The cortex of kidney was homogenized and filtered using mesh with 180, 106, and 75  $\mu\text{m}$  pore. Finally, the glomerulus on the 75  $\mu\text{m}$  mesh was collected by centrifugation.

### ***Assay for DGK activity***

The glomerulus collected from the VtE-treated or control non-DM and DM rat were homogenized and sonicated at power 5 for 30 sec on ice (TOMY SEIKO Co. Ltd., Tokyo, Japan). The homogenate was centrifuged at 800 x g for 10 min, and the supernatant was used for the DGK assay. The DGK activity was measured by the octyl-glucoside mixed micelle assay as described by Sakane et al [18]. Briefly, the reaction was initiated by the addition of enzyme in a mixture containing 50 mM 3-(N-Morpholino) propanesulfonic acid (MOPS; pH 7.2), 50 mM octyl-glucoside, 100 mM NaCl, 1 mM dithiothreitol (DTT), 20 mM NaF, 2.1 mM  $\text{CaCl}_2$ , 2 mM EGTA, 0.8 mM EDTA, 10 mM  $\text{MgCl}_2$ , 6.7 mM phosphatidylserine, and 1mM  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  (10,000 cpm/mmol, Sigma, St. Louis, Mo.) in the presence of 1mM 1,2-didecanoyl-sn-glycerol (Avanti Polar Lipids, Alabaster, AL) and incubated for 10 min at 30 °C. Lipids were extracted from the

mixture and counted by a liquid scintillation counter.

### **Western blotting**

A hundred micro gram of the protein from the glomerulus of control rats were applied to 7.5% SDS-PAGE and followed by western blotting using ant-DGK $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  antibodies.

### **RT-PCR**

mRNA was prepared from the glomerulus using SV total RNA isolation system (Promega). Two hundred nano gram of the respective mRNA was applied for cDNA synthesis using ThermoScript RT-PCR system (Invitrogen). After measurement of concentration of the synthesized cDNA, PCR was performed using following primers. DGK $\alpha$ ; forward: GATGAGATTGGGTACCTGG, Reverse: ACTGCCATCCCGGTCC, DGK $\beta$ ; forward: CCAGCAAATACGTGCTTCCC, Reverse: TTGATGCAGGAGGGCGG, DGK $\gamma$ ; forward: TGGACAAAGGGGGGCCTA, Reverse: GGGCATGACCTCTAGGTAC, DGK $\delta$ ; forward: GGCATGCTGACCAAACAGAAC, Reverse: CCTCAATGATGATATCC, DGK $\epsilon$ ; forward: ATGGTGTAGTCTGCAGCGTTCCG, Reverse: CACCTGGACGGGATTTAACAGCA, DGK $\iota$ ; forward: GCAGGAGCGAAAATGTAAGCA, Reverse: GCAAAGGCTTCATGAGAGGA, DGK $\eta$ ; forward: TGCCTCCATAGCTGCGAG, Reverse: CTGCGTAGCTTGGGATGTTC, DGK $\theta$ ; forward: ACGATTATGACACGTATCACCACC, Reverse: GGCGAAACTGATTTTTCCTCATGG, DGK $\xi$ ; forward: TCCTTGGCCGCATTGCAG, Reverse: TCCTGCGCCCTGTTCGAT.

### **Measurement of VtE in the plasma**

High-performance liquid chromatography (HPLC) was used to detect the level of VtE. Briefly, plasma from mice was mixed with an equal volume of acetonitrile and vortexed for 2 min and centrifuged (3000 $\times$ g, 10 min). The supernatants were collected to measure. The separation was performed using an STR ODS-II column (Shimadzu, Japan) and UV detection at 280 nm. The mobile phase was acetonitrile. The flow rate was 1.0 ml/min.

## **RESULTS**

### **Effects of 8-weeks treatment of VtE and its derivatives on blood glucose level, kidney weight and BUN in diabetic rats**

First, we treated diabetic and non-diabetic rats with VtE, aVtE, and sVtE (Fig. 1) for 8 weeks,

measuring their body weight, blood glucose level, kidney weight, and BUN. The STZ treatment significantly increased blood glucose level from 146.8 to 403 mg/dl and reduced weight from 434 to 238 g in the vehicle-treated rats, confirming that STZ treatment effectively induced diabetes (Table 1). The treatment with VtE, sVtE, and aVtE did not significantly affect the glucose level and body weight in both diabetic and non-diabetic rats. In contrast, the average kidney weight of rats treated with vtE, aVtE, and sVtE were 10.63, 10.40, and 9.69 mg/g respectively, although the kidney weight of the control was 12.76 mg/g. Additionally, the average of BUN of rats treated with VtE and sVtE were reduced to 37.72 and 29.70 mg/dl, compared to the control 44.69 mg/dl. These results suggested that treatment with aVtE and sVtE also improved diabetic nephropathy similarly to VtE, and that sVtE seemed to be most effective among three. However, intraperitoneal administration of sVtE was toxic: 40% of non-diabetic and 75% of diabetic rats treated with sVtE were died within 8 weeks. Accordingly, sVtE was omitted for following experiments.

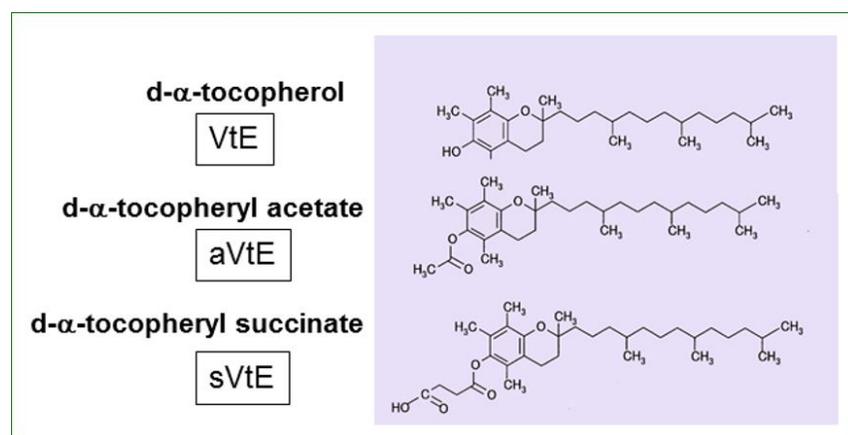


Figure 1. Chemical structure of vitamin E and its derivatives.

Table 1. Effect of 8-weeks treatment with VtE and its derivatives on blood glucose level and kidney weight ad BUN in the diabetic rats.

		survival	weight (g)	blood glucose (mg/dl)	kidney weight (mg/g body weight)	BUN (mg/dl)
non-DM	vehicle	5/5	434.0±31.9	146.8±19.4	6.02±0.37	23.7±3.8
	VtE	5/5	396.0±20.5	146.8±16.2	6.60±0.28	24.1±5.1
	aVtE	4/4	435.0±20.7	141.0±9.5	6.30±0.22	25.9±4.1
	sVtE	3/5	396.7±19.8	139.7±11.8	6.88±0.31	26.5±2.8
DM	vehicle	7/7	**238.0±45.1	**403.0±37.9	**12.76±0.98	*44.7±5.8
	VtE	8/8	**276.3±30.5	**415.1±26.7	**10.63±1.02	*37.7±9.2
	aVtE	4/5	**255.0±26.7	**515.3±41.3	**10.40±0.99	*42.2±6.2
	sVtE	2/8	**240.0±35.0	**460.5±30.9	*9.69±0.82	*29.7±9.4

Number in the survival box shows the rate of the number of rats that survived and were used. 2/8 means that 8 rats were used and 2 rats survived. \* and \*\* mean that provability are P<0.05 and P<0.01 VS non-diabetic rats treated with vehicle by T-test.

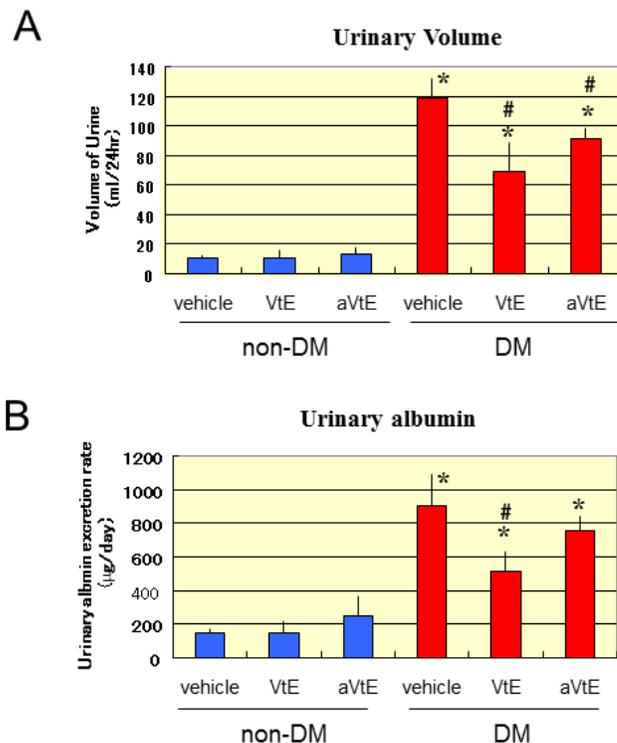
**Effects of 2-weeks treatment of VtE and aVtE on glucose level, kidney weight, urine volume, urinary creatinine and urinary albumin**

To confirm the effect of aVtE on early stage of the diabetic nephropathy, we treated diabetic and non-diabetic rats with VtE and aVtE for 2 weeks and measured glucose level, kidney weight, urine volume, urinary creatinine, and urinary albumin. As shown in Fig. 2 and Table 2, the amount of urine volume and urinary albumin increased remarkably in diabetic rats, with those makers being significantly reduced by both VtE and aVtE treatment. The urine volume of the vehicle-treated diabetic rat was about 118 ml, while those of the VtE- and aVtE-treated diabetic rats were about 69 and 91 ml. The amount of urinary albumin of the vehicle-treated diabetic rat was about 900 µg/day, while those of the VtE and aVtE-treated diabetic rats were about 517 and 759 µg/day. These results clearly indicated how aVtE, in addition to VtE, improved diabetic nephropathy although there were no significant difference in kidney weight and urinary creatinine between the control, VtE, and aVtE-treated rats (Table 2).

**Table 2.** Effect of 2-weeks treatment with VtE and aVtE on blood glucose level, kidney weight, and urinary creatinine.

		survival	weight (g)	glucose level (mg/dl)	kidney weight (mg/body weight g)	Urinary creatinin (g/day)
non-DM	vehicle	5/5	294.2±24.3	157.2±16.2	6.93±0.33	0.10±0.02
	VtE	5/5	273.6±17.8	144.2±21.0	7.08±0.23	0.09±0.03
	aVtE	5/5	295.2±21.7	157.6±13.9	6.74±0.24	0.13±0.03
DM	vehicle	8/8	221.8±23.1	**436.5±43.1	**10.06±0.99	*0.24±0.10
	VtE	7/7	240.0±31.2	**356.7±45.1	**9.86±0.57	*0.22±0.06
	aVtE	8/8	243.1±25.3	**429.6±31.7	**9.67±0.72	*0.28±0.09

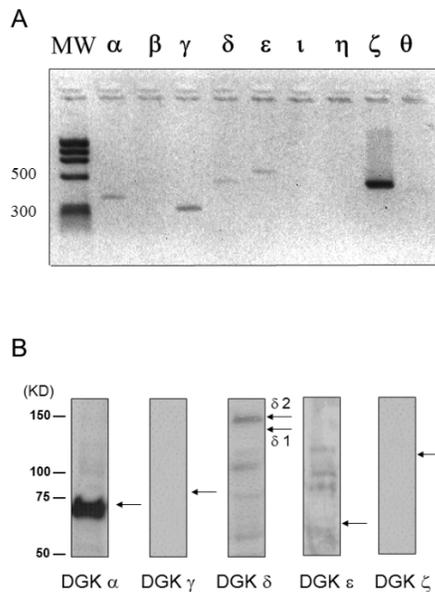
Number in the survival box shows rate of number of survived and used rats. 8/8 means that 8 rats were used and 8 rats survived \* and \*\* mean that provability are P<0.05 and P<0.01 VS non-diabetic rats treated with vehicle by T-test.



**Figure 2.** Improvement of urinary volume and albumin by VtE and aVtE treatment. The graph was made based on the data in Table 2. Each datum points indicate mean±SE. # means P<0.05 VS diabetic rats treated with vehicle by T-test. \* represents P<0.01 VS diabetic rats treated with vehicle by t-test.

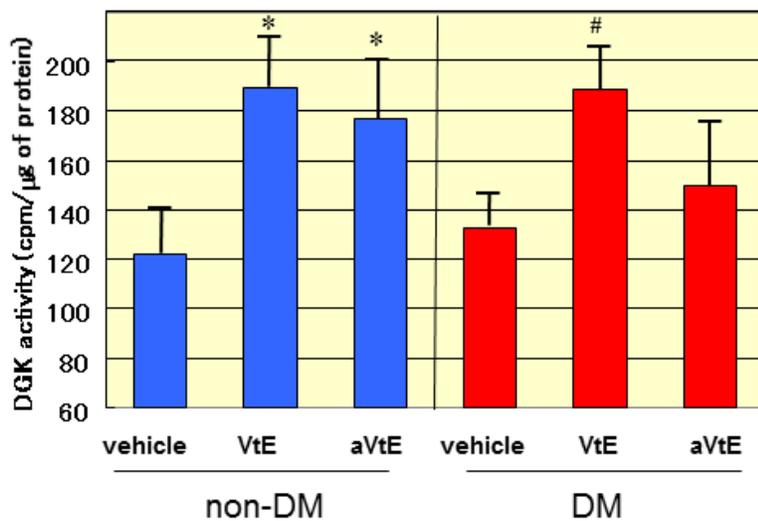
### ***Identification of DGK subtype involved in the VtE and aVtE-induced improvement of diabetic nephropathy***

It has been previously reported how VtE treatment activated DGK activity in glomeruli of diabetic rat [8] and it has been suggested that DGK $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  are expressed in the glomeruli of rats [24]. To confirm the DGK subtypes expressed in the glomerulus of rat kidney, RT-PCR was performed. As shown in Fig. 3, enough amount of mRNA of DGK $\alpha$ ,  $\gamma$  and  $\zeta$  were confirmed and those of DGK $\delta$  and  $\epsilon$  were also detected slightly. However,  $\beta$  subtype was not detected, although it was suggested to be expressed in the rat glomerulus [24]. Then, we checked the protein level of DGK $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$  by western blotting. Among calcium-dependent subtypes, DGK $\alpha$  protein was abundant and DGK $\gamma$  was not detected. Of the calcium-independent subtypes, only DGK $\delta$  protein was detected in the rat glomerulus.



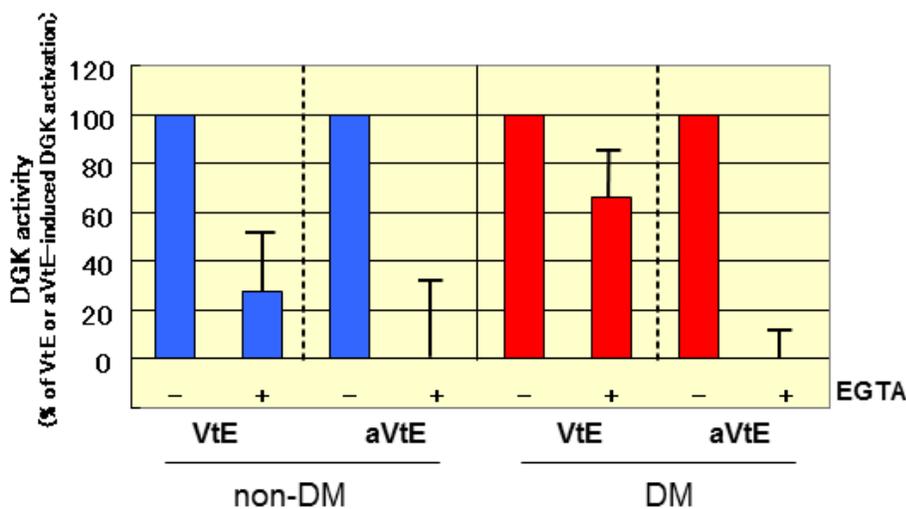
**Figure 3.** Expression of DGK subtype in the rat glomerulus. The expression of DGK subtype at mRNA level (A) and protein level (B). Size of expected PCR product. DGK $\alpha$ , 383 bp;  $\gamma$ , 292 bp;  $\delta$ , 519 bp;  $\epsilon$ , 633 bp;  $\zeta$ , 499 bp. Protein size, DGK $\alpha$ , 80KD;  $\gamma$ , 88KD;  $\delta$ 1, 130KD;  $\delta$ 2, 135KD;  $\epsilon$ , 64KD;  $\zeta$ , 104 KD.

To confirm that VtE and aVtE induced the activation of DGK in the kidney, we first compared the DGK activity in the glomerulus of rats before and after the VtE and aVtE treatments. VtE treatment induced about 1.5 times higher activity of total DGK in the glomerulus of both non-diabetic and diabetic Rats (Fig. 4). Similarly, aVtE enhanced the DGK activity in the glomerulus, although its effect was weaker in the diabetic rats than non-diabetic rats (Fig. 4). Therefore, in order to identify the DGK subtype activated by VtE and/or aVtE, we investigated effect of EGTA, calcium chelator, on the VtE or aVtE-upregulated DGK activity because only one calcium-dependent DGK subtype, DGK $\alpha$ , was expressed in the glomerulus (Fig. 3). In the case of diabetic rats, presence of EGTA inhibited 40% of the DGK activity activated by VtE. In contrast, the EGTA abolished almost all of the DGK activity activated by aVtE (Fig.5). Similar results were obtained in the case of non-diabetic rats (Fig. 5). These results demonstrated that DGK $\alpha$  was mainly activated by aVtE, while calcium-independent subtype was additionally activated in the case of VtE. The additional calcium independent subtype seemed to be DGK $\delta$  because it was only one calcium-independent DGK subtype detected in the glomerulus of Rats (Fig. 3). In other words, DGK $\alpha$  seems to be mainly important for the aVtE-induced improvement of diabetic nephropathy and DGK $\delta$ , in addition to DGK $\alpha$  is involved in the case of VtE treatment.



**Figure 4.** Effects of VtE and aVtE on the DGK activity in the glomerulus.

The glomeruli sample were prepared from the rats treated with VtE or aVtE for two weeks and applied to DGK assay, as described in the Method section. The datum points show the average+S.E. \* means that, among non-diabetic rats,  $P < 0.05$  VS control rats treated with vehicle by T-test. # means that, among diabetic rats,  $P < 0.05$  VS control rats treated with vehicle by t-test.

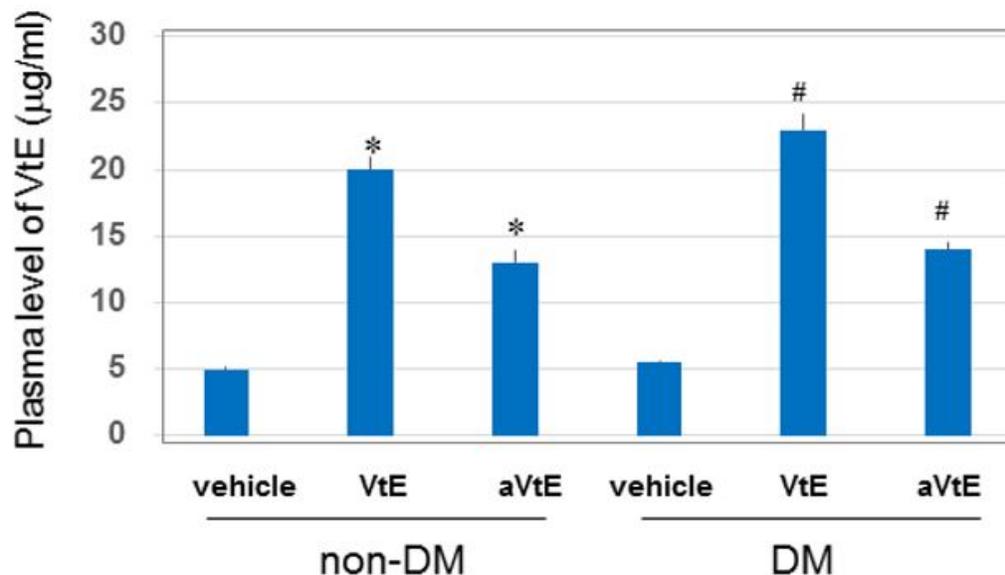


**Figure 5.** Effect of EGTA on the VtE or aVtE-upregulated DGK activity.

We measured the DGK activity with or without EGTA using glomerulus from the rats before and after the VtE and aVtE treatments. The graph shows that the remaining DGK activity in the presence of EGTA as percentage, when the VtE and aVtE-induced DGK activity is 100% respectively.

**Measurement of VtE in the blood before and after VtE and aVtE treatment.**

Finally, we compared the amount of VtE in the blood of rats treated by VtE or aVtE because aVtE is metabolized by reductase into VtE in vivo. VtE level in the blood of rats treated by VtE were significantly higher than the control in both non-diabetic and diabetic condtons (Fig. 6). However, the amount of VtE in the blood of the rats treated with aVtE was less than that in the VtE-treated rats (Fig. 6).



**Figure 6.** Effect of VtE and aVtE on the plasma level of VtE in non-diabetic and diabetic rats. Values represent the mean  $\pm$  S.E (n=5-8). \*P<0.01 VS non-diabetic rats treated with vehicle by Turkey-Kramer test. #P<0.01 VS diabetic rats treated with vehicle by Turkey-Kramer test.

**DISCUSSION**

We showed for the first time that the intraperitoneal administration of non-oxidative VtE derivatives, in addition to VtE, induced improvement of nephropathy in diabetic rats. However, it was predictable because aVtE and sVtE were converted to VtE by reductase in vivo. Instead, the toxic effect of sVtE was very surprising because sVtE is commonly used as food supplement instead of VtE, due to its stability. The toxicity may be related to the characteristic of sVtE. As reported previously, only sVtE induced irreversible translocation of DGK $\alpha$  [24], suggesting that sVtE is relatively difficult to be metabolized compared to VtE and aVtE because the duration of translocation generally depends on the time until the stimulant is removed. In other words, unlike the case of oral administration, direct application of sVtE to abdominal cavity may be harmful.

Indeed, we found a lot of tumors and/or inflammation in the abnormal cavity of nearly all rats died by sVtE treatment.

We detected mRNA of DGK $\alpha$ ,  $\gamma$ ,  $\delta$ ,  $\epsilon$ , and  $\zeta$ . However, there was only expression of DGK $\alpha$  and DGK $\delta$  (Fig. 3), which may be due to the stability of the proteins or the affinity of the antibodies used. However, we can conclude that at least activation of DGK $\alpha$  occurred in the cases of both VtE and aVtE treatment. The results suggested that the DGK $\alpha$  is involved in the improvement of nephropathy. The importance of DGK $\alpha$  in the VtE-induced improvement of nephropathy was supported by the experiment using DGK $\alpha$  KO mice; the protective effect of VtE on the nephropathy disappeared in the diabetic DGK $\alpha$ KO mice [27]. So far, we have discovered that DGK $\alpha$  is localized in the podocytes [28] and the VtE-activated DGK $\alpha$  normalized abnormal PKC $\alpha$  and PKC $\beta$ II activation, resulting in prevention of podocyte loss [27]. Of course, anti-oxidative activity of VtE is important for the VtE-induced improvement of nephropathy because the expression of heme oxygenase-1 was indeed normalized by VtE treatment in mice [27, 28]. However, in addition to oxidative activity, DGK $\alpha$  seems to be activated by non-antioxidant activities of VtE, which has been reported in inhibition of proliferation, PKC activation, and protein expression [29-31]. In light of these results, we believe that DGK $\alpha$  is a key enzyme in the improvement of diabetic nephropathy.

In addition to DGK $\alpha$ , the other DGK subtype DGK $\delta$  seemed to be activated in the case of VtE treatment. Because DGK $\delta$  has been reported to be involved in the insulin resistant [32], activation of DGK $\delta$  would be beneficial in preventing both diabetes and diabetic nephropathy. Among the DGK $\delta$  splice variant, DGK $\delta$ 2 was detected in the glomerulus of rat kidney, which is an interesting finding because this is a first report to show the localization of DGK $\delta$ 2 in the glomerulus. However, the way in which DGK $\delta$ 2 contributes to the improvement of nephropathy is still unknown. Moreover, the activation mechanism of DGK $\delta$ 2 by VtE is a kind of mystery. As described above, aVtE is metabolized into VtE *in vivo*. Indeed, we detected VtE in the blood of rats treated with aVtE (Fig. 6). The amount of VtE in the blood in the rats treated with aVtE was less than that in the VtE-treated Rats, suggesting that the conversion of aVtE to VtE takes time. This means that aVtE, in addition to VtE, exists in the blood of rats treated with aVtE. In other words, there is a possibility that un-metabolized aVtE activates additional DGK subtype. However, our result was contrary, with the additional DGK subtype, DGK $\delta$ 2 being activated by VtE rather than aVtE. One explanation for this may be related to how the concentration of VtE in the blood of rats with treated with VtE was always higher than that in the case of aVtE (Fig. 6). Indeed, VtE improvement of nephropathy stronger than aVtE (Fig. 2). These results suggest that prolonged

and/or strong activation by a high concentration of VtE may induce additional activation of DGK including DGK $\delta$ 2. However, to confirm this hypothesis, further experiments will be needed.

Significantly, our results in this study suggest that VtE and aVtE are useful for functional food to improve diabetic nephropathy. Recently, we discovered oral administration of VtE also induced improvement of nephropathy [28]. Accordingly, we have to investigate the effect of oral administration of aVtE. Furthermore, we are curious about the effect of oral administration of sVtE because sVtE seems to be the strongest effect on the activation of DGK $\alpha$  and the improvement of nephropathy. Although VtE was toxic when it was administrated intraperitoneally, we expect to have a different result in the case of oral administration.

## CONCLUSION

aVtE in addition to VtE improved diabetic nephropathy and activation of DGK $\alpha$  and/or  $\delta$  may be involved in the improvement, suggesting that VtE and its derivatives are attractive as functional food targeting on DGK for diabetic nephropathy.

**Abbreviations:** d- $\alpha$ -tocopherol (VtE), d- $\alpha$ -tocopherol succinate (sVtE), d- $\alpha$ -tocopherol acetate (aVtE), conserved domain 1 (C1), diacylglycerol (DG), diacylglycerol kinase (DGK), blood urea nitrogen (BUN), ethylenediamine tetraacetic acid (EDTA), ethylenediamine bis-tetraacetic acid (EGTA), green fluorescent protein (GFP), high-performance liquid chromatography (HPLC), knock out (KO), liquid chromatography(LC), mass-spectrography (MS), polymerase chain reaction (PCR), protein kinase C (PKC), quality of life (QOL), streptozocin (STZ), sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Competing interests:** Authors have no conflict of interest.

**Author's contribution:** Tomoko Kakehi performed almost all experiments and Dr. Keiko Yagi measured VtE concentration in plasma. Dr. Yasuhito Shirai designed and conducted the experiments and Dr Naoaki Saito helped design and financially support the experiments.

**Acknowledgements:** This work was supported in part by Daiichi Sankyo Foundation of Life Science, and by Grant-in –Aid for scientific research (C) of ministry of Education, Culture, Sports, Science and Technology in Japan.

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