# Prevention of Glomerular Dysfunction in Diabetic Rats by Treatment with $d-\alpha$ -Tocopherol

## DAISUKE KOYA,\* IN-KYU LEE,\* HIDEHIRO ISHII,\* HIDEO KANOH,<sup>†</sup> and GEORGE L. KING\*

\*Research Division, Joslin Diabetes Center and Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts and <sup>†</sup>Department of Biochemistry, Sapporo Medical University School of Medicine, Sapporo, Japan.

Abstract. Because d- $\alpha$ -tocopherol (vitamin E) has been shown to decrease diacylglycerol (DAG) levels and prevent the activation of protein kinase C (PKC), which is associated with retinal and renal dysfunctions in diabetes, the study presented here characterized the effect of d- $\alpha$ -tocopherol treatment to prevent glomerular hyperfiltration and increased albuminuria as well as PKC activities in streptozotocin (STZ)-induced diabetic rats. Two weeks after the induction of diabetes, total DAG content and PKC activity in glomeruli were significantly increased in diabetic rats by 106.4  $\pm$  16.8% and 66.4  $\pm$  8.4%, respectively, compared with control rats. Intraperitoneal injection of d- $\alpha$ -tocopherol (40 mg/kg of body weight) every other day prevented the increases in total DAG content and PKC activity in glomeruli of diabetic rats. Glomerular filtration rate (GFR) and filtration fraction (FF) were significantly elevated to 4.98  $\pm$  0.34 mL/min and 0.36  $\pm$  0.05, respectively, in diabetic rats, compared with 2.90  $\pm$  0.14 mL/min and 0.25  $\pm$ 0.02, respectively, in control rats. These hemodynamic abnormalities in diabetic rats were normalized to 2.98  $\pm$  0.09 mL/ min and 0.24  $\pm$  0.01, respectively, by d- $\alpha$ -tocopherol. Albuminuria in 10-wk diabetic rats was significantly increased to

Early changes in renal functions of diabetic patients and animal models are characterized by glomerular hyperfiltration and microalbuminuria (1–5), which have been associated with subsequent development of diabetic nephropathy (6–8). Among the many metabolic factors and cytokines that have been linked to the development of diabetic nephropathy (9–13), clinical studies have established hyperglycemia and actions of angiotensins as having causal roles (14,15). The results of Diabetes Control and Complication Trial (DCCT) showed that glycemic control can reduce both the onset and progression of diabetic microvascular complications, including nephropathy, establishing the importance of hyperglycemia in the development of diabetic complications (14).

Recently, we and others have suggested that the activation of

9.1  $\pm$  2.2 mg/day compared with 1.2  $\pm$  0.3 mg/day in control rats, whereas d- $\alpha$ -tocopherol treatment improved albumin excretion rate to  $2.4 \pm 0.6$  mg/day in diabetic rats. To clarify the mechanism of d- $\alpha$ -tocopherol's effect on DAG-PKC pathway, the activity and protein levels of DAG kinase  $\alpha$  and  $\gamma$ , which metabolize DAG to phosphatidic acid, were examined. Treatment with d- $\alpha$ -tocopherol increased DAG kinase activity in the glomeruli of both control and diabetic rats, by  $22.6 \pm 3.6\%$  and  $28.5 \pm 2.3\%$  respectively, although no differences were observed in the basal DAG kinase activity between control and diabetic rats. Because immunoblotting studies did not exhibit any difference in the protein levels of DAG kinase  $\alpha$  and  $\gamma$ , the effect of d- $\alpha$ -tocopherol is probably modulating the enzyme kinetics of DAG kinase. These findings suggest that the increases in DAG-PKC pathway play an important role for the development of glomerular hyperfiltration and increased albuminuria in diabetes and that  $d-\alpha$ -tocopherol treatment could be preventing early changes of diabetic renal dysfunctions by normalizing the increases in DAG and PKC levels in glomerular cells. (J Am Soc Nephrol 8: 426-435, 1997)

diacylglycerol (DAG)-protein kinase C (PKC) pathway in vascular tissues from diabetic rats (5,16-19) and cultured cells exposed to high concentrations of glucose (17-28) could be a cellular mechanism by which hyperglycemia causes vascular dysfunction. In vascular tissues, PKC activation has been reported to regulate cell growth, contraction, extracellular matrix synthesis, prostanoid metabolism, and gene expression (10,29,30). Also recently, we have reported that abnormal retinal and renal hemodynamics in diabetic rats can be prevented by PKC inhibitors (5,17), supporting the hypothesis that PKC activation by hyperglycemia may have causal roles. Because we and others have reported that  $d-\alpha$ -tocopherol can prevent the activation of PKC induced either by high glucose or phorbol ester in vascular cells (18,19,31), it is possible that d- $\alpha$ -tocopherol treatment can ameliorate early renal dysfunction's in diabetic rats by normalizing DAG-PKC pathway in the glomeruli.

In study presented here, we have examined the possibility that the treatment of diabetic rats with d- $\alpha$ -tocopherol will prevent the activation of DAG-PKC pathways and the onset of early renal dysfunctions. In addition, we have characterized the

Received August 16, 1996 Accepted October 30, 1996

Correspondence to Dr. George L. King, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

<sup>1046-6673/0803-0426\$03.00/0</sup> 

Journal of the American Society of Nephrology

Copyright © 1997 by the American Society of Nephrology

effect of d- $\alpha$ -tocopherol on the enzymatic activities of DAG kinase (32), which is an important regulator of intracellular DAG levels, to test the possibility that d- $\alpha$ -tocopherol in inhibiting PKC activation by lowering intracellular DAG levels.

#### **Materials and Methods**

#### Animals

Male Sprague-Dawley rats (200 g, Taconic Farms, German Town, NY) were placed in one of four groups: control, control treated with d- $\alpha$ -tocopherol, 2- or 10-wk-old diabetic, and 2- or 10-wk-old diabetic treated with d- $\alpha$ -tocopherol. The control group received 1 mL/kg sterile 20 mM citrate buffer (pH 4.5) by intraperitoneal injection. Both diabetic groups received a single intraperitoneal injection of sterile streptozotocin (STZ) (65 mg/kg) (Sigma, St. Louis, MO) in citrate buffer. The diabetic state was confirmed 48 h later after STZ injections by measurements of blood glucose exceeding 300 mg/dL. Control and diabetic rats were randomly divided into four groups to receive d- $\alpha$ -tocopherol (40 mg/kg) or placebo as vehicle (polyethylene 60-hydrogenated caster oil) (HCO) provided by Eisai Co. Ltd. (Tokyo, Japan), which was injected intraperitoneally every other day for 2 wk or 10 wk after the onset of diabetes. All rats were allowed free access to water and standard chow (PMI Feeds, St. Louis, MO) that contained 49 IU d- $\alpha$ -tocopherol/kg of chow. Blood glucose concentration was determined weekly in all animals. Low doses of insulin were given occasionally to prevent weight loss in diabetic animals. Blood pressure was measured by the tail-cuff method (Ueda UR-5000, Tokyo, Japan) at the end of experiment. In plasma and kidney, d- $\alpha$ -tocopherol levels were determined by the method of Ueda et al. using high-performance liquid chromatography (HPLC) at the end of experiment (33). All experiments were approved by Joslin Diabetes Center Animal Care Committees.

#### Preparation of Glomeruli

After 2 wk or 10 wk of d-a-tocopherol or vehicle treatment, rats were anesthetized with an intraperitoneal injection of thiopental sodium (50 mg/kg) (Gersia, Irvine, CA), and a catheter (Clear Vinyl Tube, Dural Plastics & Engineering, Auburn, Australia) was inserted in the left carotid artery. A 20-mL syringe was attached to the catheter, and kidneys were perfused for 30 s with 20 mL of Dulbecco modified Eagle medium (DMEM) containing 20 mM N-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) as described previously (5). Glomeruli were isolated by graded sieving with nylon meshes. The final suspension was composed of >93% glomeruli as assessed by light microscopy.

#### Determination of Total DAG Contents

Total DAG contents in isolated glomeruli from each 2-wk group were determined with a radio-enzymatic assay kit (Amersham, Arlington Heights, IL) employing DAG kinase (Calbiochem, San Diego, CA), which quantitatively converts DAG to [<sup>32</sup>P]-phosphatidic acid (PA) in the presence of  $[\gamma^{-32}P]$ -ATP (NEN, Boston, MA). In brief, total glomerular lipids were extracted twice according to the methods of Bligh and Dyer (34), and total DAG was measured according to the manufacturer's instructions. The resulting [<sup>32</sup>P]-PA was separated by silica gel G thin layer plates (EM Separations, Gibbstown, NJ) in a chamber containing chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) according to the method of Priess et al. (35). PA was visualized by autoradiography and identified by comigration with radiolabeled PA derived from DAG standard from 31.25 to 2000 pmol. The spots were scraped and radioactivity was counted by liquid

scintillation counter (Beckman CS6500, Fullerton, CA). The values of total DAG contents were normalized by protein content of glomeruli measured as described by Bradford (36).

#### Assay of PKC Activity

PKC activity in glomeruli was determined by a modified method described by Heasley and Johonson (37) and published previously by us (5). Briefly, isolated glomeruli were rinsed twice with 2 mL of RPMI 1640 containing 20 mM HEPES (pH 7.4) and once with 2 mL of a salt solution (137 mM NaCl, 5.4 mM KCl, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose, 10 mM MgCl<sub>2</sub>, 25 mM  $\beta$ -glycerophosphate, 5 mM EGTA, 2.5 mM CaCl<sub>2</sub> and 20 mM HEPES). The glomeruli were preincubated with a salt solution for 10 min at 37°C and incubated for another 15 min in the presence or absence of 100 µM PKC-specific peptide substrate, RKRTLRRL (38), after the addition of 5 mg/mL digitonin (final concentration, 50  $\mu$ g/mL) and 1 mM ATP (final concentration, 100  $\mu$ M) mixed with  $[\gamma^{-32}P]ATP$  (<1500 cpm/pmol). The reaction was terminated with 5% trichloroacetic acid (TCA) (final concentration). Aliquots of the reaction mixture described above were spotted on  $3 \times 3$  cm phosphocellulose papers (Whatman P-81, Maidstone, England) and washed in three changes of 75 mM phosphoric acid and one change of 75 mM sodium phosphate (pH 7.5). The radioactivity of phosphorylated substrate was determined by liquid scintillation counting. Protein content of each samples was measured by Bradford's method (36). The PKC activities measured using this method were validated by two ways. First, the PKC activities in the in situ assays using the renal glomeruli were stimulated by 100 nM phorbol 12-myristate 13- acetate (PMA) from 14.5  $\pm$  0.5 to 42.0  $\pm$  1.3 pmol/mg protein, min (17). The PMA-stimulated activities were inhibited by PKC inhibitor, GF  $109,203 \times$  to 6.6  $\pm$  0.4 pmol/mg protein, min, a reduction of 90% (59). Second, the efficiency of digitonin-induced permeability was determined by measuring PKC activity in the membranous fraction of renal glomeruli treated with 50 µg/mL digitonin using in vitro method (5,30), which was 46.5  $\pm$  2.0 pmol/mg protein min., comparable to the in situ PKC assay of  $42.0 \pm 1.3$  pmol/mg protein, min.

#### Assay of DAG Kinase Activity

Isolated glomeruli were lysed in a buffer containing 25 mM Tris-HCl (pH 7.4), 0.25 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 µg/mL leupeptin, 20 µg/mL aprotinin, and 1 mM polymethylsulfonyl fluoride (PMSF), sonicated, and homogenized by Dounce homogenizer. After a low-speed centrifugation (550 $\times$  g for 10 min), the supernatant was used for the assay of total DAG kinase activity. The DAG kinase activity was measured by the octyl-glucoside mixed micelle assay as described by Sakane F et al. (39). In brief, the reaction was initiated by addition of enzyme  $(10-20 \mu g \text{ total})$ glomerular lysates) in 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 CaCl<sub>2</sub>, 2 mM EGTA, 0.8 mM EDTA, 10 mM MgCl<sub>2</sub>, 6.7 mM phosphatidylserine, and 1 mM [ $\gamma$ -<sup>32</sup>P]-ATP (10.000 cpm/mmol) in the presence of 1 mM 1,2-didecanoyl-sn-glycerol (Avanti Polar Lipids, Alabaster, AL) and continued for 10 min at 30°C. Lipids were extracted from the mixture, and PA separated on thin-layer plates of silica gel was scraped and counted by a liquid scintillation counter. The values of DAG kinase activity were normalized by protein content of glomeruli measured by Bradford's method (36).

#### Western Blot Analysis of DAG Kinase

Isolated glomeruli were solublized with a lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 2 mM EDTA, 2 mM PMSF, 10  $\mu$ g/mL leupeptin, 20  $\mu$ g/mL aprotinin, 1% NP-40, and 10% glycerol, and sonicated and homogenized by Dounce homogenizer. The glomerular lysates (50  $\mu$ g of protein/lane) were separated on 4–12% gradient Tris-Glycine gel (Novex, San Diego, CA) in Tris-Glycine-SDS running buffer. The proteins were transferred to a polyvinylidene fluoride membrane (Schleicher & Schuell, Keene, NH) and blocked at 25°C for 3 h in phosphatebuffered saline (PBS) (pH 7.4) containing 0.1% Tween 20 and 5% bovine serum albumin. The membrane was incubated with the anti-DAG kinase  $\alpha$  and  $\gamma$  polyclonal antibody for 2 h at 25°C (40, 41). The immunoreactive bands were visualized by enhanced chemiluminesence (Amersham ECL system, Arlington Heights, IL) according to the manufacture's instructions. Protein expression was quantified with a phosphor image densitometer (Molecular Dynamics, Sunnyucle, CA).

#### Measurement of GFR, Renal Plasma Flow, and Urinary Albumin Excretion Rate

To determine GFR and renal plasma flow (RPF), rats from each group underwent inulin and para aminohippuric acid (PAH) clearance study after 2 wk of d- $\alpha$ -tocopherol or vehicle treatment. In brief, rats were anesthetized with thiopental sodium (50 mg/kg body wt) and placed on a temperature-regulated plate. Catheters were placed in the left carotid artery for blood sampling; in the left jugular vein for infusion of inulin, PAH solution, and the supplemental administration of anesthesia and surgery; and in the urinary bladder for collection of urine in preweighed containers. Inulin (0.6%) and PAH (1.5%) in normal saline were infused at a rate of 6.0 mL/h for 30 min, followed by a sustained infusion of 2.0 mL/h throughout the remainder of the experiment. After 60 min of equilibration time, urine was collected during two timed 30 min periods, and 300  $\mu$ l of blood was taken at the midpoint of each urine collection. The concentrations of inulin in plasma and urine were measured by a cysteine-tryptophan reaction (42), and PAH was determined by a calorimetric technique (43). GFR was calculated from inulin clearance, RPF from PAH clearance, and FF from a standard formula, GFR/RPF ratio. Urinary and serum albumin were measured by enzyme-linked immunosorbent assay (ELISA), as previously described (5). Intra- and interassay coefficients of variation were 2.4% and 6.5%, respectively, and the detection range of the assay was 3-250 ng/mL. Urine samples every 24 h were collected from control and diabetic rats with or without 10-wk of d- $\alpha$ -tocopherol treatment on two consecutive days. Serum and urinary creatinine were measured with a creatinine assay kit (Sigma, St. Louis, MO).

#### Statistical Analyses

Results were expressed as mean  $\pm$  standard error (SE). Comparisons among each group were performed by one-way analysis of variance (ANOVA) followed by Neuman-Keuls test to evaluate statistical significance between two groups. P values of <0.05 were defined as statistically significant.

#### Results

#### Metabolic Characteristics of Animals

After 2 wk of diabetes, the glucose levels in the whole blood of diabetic rats (376.1  $\pm$  62.4 mg/dL) and diabetic rats treated with d- $\alpha$ -tocopherol (374.1 ± 32.6 mg/dL) were significantly higher than control (101.5  $\pm$  1.6 mg/dL) and control rats treated with d- $\alpha$ tocopherol (104.9  $\pm$  7.6 mg/dL) as shown in Table 1. Diabetic and diabetic rats treated with d- $\alpha$ -tocopherol gained less body weight than control and control rats treated with d- $\alpha$ -tocopherol (Table 1). Intraperitoneal injection of d- $\alpha$ -tocopherol did not affect blood glucose levels and body weight in either control or diabetic rats. Kidney weight normalized by 100 g/body wt in diabetic  $(1.29 \pm 0.03 \text{ g}/100 \text{ g})$ g) and diabetic rats treated with d- $\alpha$ -tocopherol (1.15  $\pm$  0.04 g/100 g) was significantly heavier than control  $(0.87 \pm 0.03 \text{ g}/100 \text{ g})$  and control rats treated with d- $\alpha$ -tocopherol (0.77  $\pm$  0.04 g/100 g). The kidney weight/body weight ratio was less in diabetic rats treated with d- $\alpha$ -tocopherol than that in diabetic rats (Table 1). Parameters of blood glucose, body weight, and kidney weight/body weight in control and diabetic rats after 10 wk of treatment with d- $\alpha$ -tocopherol or vehicle are summarized in Table 2; similar trends were observed in the rats with 2-wk of diabetes.

#### Plasma and Kidney d- $\alpha$ -Tocopherol Levels

Plasma and kidney d- $\alpha$ -tocopherol levels were determined by HPLC at the end of experiment. Treatment with d- $\alpha$ -tocopherol (40 mg/kg every other day) significantly elevated its levels in plasma three- to fourfold and in kidney twofold in both control and diabetic rats (Table 3). No significant differences in the levels of d- $\alpha$ -tocopherol were noted in plasma and kidney of control and diabetic rats without d- $\alpha$ -tocopherol treatment. Mean blood pressure (MBP) measured by the tail-cuff method did not differ between control and diabetic rats with or without d- $\alpha$ -tocopherol treatment (Table 3).

#### Total DAG Contents in Glomeruli

Since we reported that hyperglycemia increased the total DAG levels in the vascular tissues or cells (5,16-19,22), the total DAG levels in the glomeruli were studied. After 2 wk of diabetes, total DAG content in the glomeruli from diabetic rats  $(2.56 \pm 0.21 \text{ nmol/mg protein})$  were significantly increased compared with control rats  $(1.26 \pm 0.08 \text{ nmol/mg protein})$  as shown in Figure 1A. In diabetic rats treated with d- $\alpha$ -tocopherol, the total DAG contents  $(1.20 \pm 0.28 \text{ nmol/mg protein})$  did not increase and was not significantly different

Table 1. Characterization of diabetic and control rats after 2-wk treatment with or without d- $\alpha$ -tocopherol

Treatment Group	N	Body Weight (g)	Blood Glucose (mg/dL)	Kidney Weight (g/100 g body weight)
Control	24	$352.2 \pm 8.4$	$101.5 \pm 1.6$	$0.87 \pm 0.03$
Control + d- $\alpha$ -tocopherol	24	$348.9 \pm 8.4$	$104.9 \pm 7.6$	$0.77 \pm 0.04$
Diabetic	24	$263.8 \pm 37.6^{a}$	$376.1 \pm 62.4^{a}$	$1.29 \pm 0.03^{a}$
Diabetic + d- $\alpha$ -tocopherol	24	$275.6 \pm 7.8^{a}$	$374.1 \pm 32.6^{a}$	$1.15 \pm 0.04^{a,b}$

<sup>a</sup> P < 0.05 versus Control and Control + d- $\alpha$ -tocopherol.

<sup>b</sup> P < 0.05 versus Diabetic.

N = Number of rats used only in the 2-wk diabetes and treatment study.

Treatment Group	N	Body Weight (g)	Blood Glucose (mg/dL)	Kidney Weight (g/100 g body weight)
Control	14	512.5 ± 9.7	99.5 ± 8.2	$0.62 \pm 0.02$
Control + d- $\alpha$ -tocopherol	4	$505.0 \pm 6.5$	$101.5 \pm 5.0$	$0.61 \pm 0.03$
Diabetic	14	$295.0 \pm 6.5^{a}$	$407.5 \pm 12.7^{a}$	$1.02 \pm 0.02^{a}$
Diabetic + d- $\alpha$ -tocopherol	10	$287.5 \pm 15.5^{a}$	$405.0 \pm 21.0^{a}$	$0.94 \pm 0.02^{a,b}$

Table 2. Characterization of diabetic and control rats after 10-wk with or without d- $\alpha$ -tocopherol treatment

<sup>a</sup> P < 0.05 versus Control and Control + d- $\alpha$ -tocopherol.

<sup>b</sup> P < 0.05 versus Diabetic.

N = Number of rats used only in the 10-wk diabetes and treatment studies.

Table 3. d- $\alpha$ -Tocopherol concentration in plasma and kidney of control and diabetic rats after 2-wk treatment with or without d- $\alpha$ -tocopherol

Treatment Group	N	d-α- Tocopherol Levels, Plasma (µg/mL)	d-α- Tocopherol Levels, Kidney (μg/g protein)	Body Weight (g)	Blood Glucose (mg/dL)	Mean Blood Pressure (mmHg)
Control	6	$5.4 \pm 0.3$	$13.5 \pm 0.7$	$315.0 \pm 9.6$	87.0 ± 15.3	$117.7 \pm 3.0$
Control + d- $\alpha$ -tocopherol	6	$20.4 \pm 1.5^{a}$	$23.4 \pm 0.5^{a}$	$321.7 \pm 3.1$	$108.5 \pm 2.7$	$125.0 \pm 4.2$
Diabetic	5	$6.7 \pm 0.9$	$13.4 \pm 0.4$	254.0 ± 8.4 <sup>b</sup>	$346.4 \pm 31.3^{b}$	$124.0 \pm 3.8$
Diabetic + d- $\alpha$ -tocopherol	6	$20.5 \pm 3.5^{a}$	$28.9 \pm 3.5^{a}$	$260.0 \pm 8.2^{b}$	$364.2 \pm 8.8^{b}$	$116.8 \pm 3.6$

<sup>a</sup> P < 0.05 versus Control and Diabetic rats without d- $\alpha$ -tocopherol treatment.

<sup>b</sup> P < 0.05 versus Control.

N = Number of animals studied. These rats were studied as an ancillary group to the rat study as described in Table 1. This was done since additional renal tissues were needed for measurement of d- $\alpha$ -tocopherol levels. The groups in Table 1 and Table 3 have comparable physiological parameters.

from control rats (Figure 1A). The total DAG content in control rats treated with d- $\alpha$ -tocopherol (0.97  $\pm$  0.12 nmol/mg protein) was lower than that in control rats, but the results were not statistically significant.

#### Characterization of PKC Activities in Glomeruli

The PKC activity in glomeruli was quantified by *in situ* PKC assay using the phosphorylation of a highly specific peptide for PKC in digitonin-permealized glomeruli. PKC activity using this assay exhibited linearity with time for up to 30 min at 37°C (data not shown), responsiveness to phorluel 12-myristate 13-acetate (PMA), and was inhibited by PKC inhibitor, GF 109,203X (see Methods section). A significant increase in specific PKC activity was observed in the glomeruli of diabetic rats ( $28.2 \pm 1.4$  pmol/mg protein, min) compared with that in control rats ( $16.9 \pm 0.7$  pmol/mg protein/min) (Figure 1B). Treatment with d- $\alpha$ -tocopherol prevented the increases of PKC activity in diabetic rats ( $15.4 \pm 0.4$  pmol/mg protein/min) as shown in Figure 1B. There were no differences in PKC activities between control and control rats treated with d- $\alpha$ -tocopherol ( $15.1 \pm$ 0.5 pmol/mg protein min).

#### DAG Kinase Activity and Protein Levels of DAG Kinase in Glomeruli

The results described above suggest that  $d-\alpha$ -tocopherol treatment can prevent diabetes-induced increases in the PKC activities through the decreases of total DAG contents. One possible cellular mechanism by which DAG levels can be reduced is through the activation of DAG kinase, an important modulator of intracellular DAG level, by metabolizing DAG to phosphatidic acid (32). To determine whether d-a-tocopherol treatment can affect DAG kinase, DAG kinase activity was quantified in the glomeruli (Figure 2). DAG kinase-specific activity in the glomeruli was increased from 413.5  $\pm$  23.7 pmol/mg protein/min in diabetic rats without d- $\alpha$ -tocopherol treatment to  $524.0 \pm 9.4$  pmol/mg protein, min in diabetic rats with d- $\alpha$ -tocopherol treatment (Figure 3A). Similar increases in DAG kinase activity were observed in control rats treated with d- $\alpha$ -tocopherol (499.7  $\pm$  14.8 pmol/mg protein, min) compared with control rats without d- $\alpha$ -tocopherol treatment (407.7  $\pm$  17.1 pmol/mg protein, min). Because DAG kinase activities were composed of at least  $\alpha$  and  $\gamma$  isoforms (40,41), we characterized protein contents of these isoforms. The protein levels of DAG kinase  $\alpha$  and  $\gamma$  were examined for the effect of d- $\alpha$ -tocopherol by Western blot analysis using anti-DAG kinase  $\alpha$  and  $\gamma$  polyclonal antibodies. No differences in the protein levels of the DAG kinase  $\alpha$  and  $\gamma$  were observed among the control, diabetic, or d- $\alpha$ -tocopherol treatment groups (Figure 3B, left;  $\alpha$ , right;  $\gamma$ ).

### Determination of d- $\alpha$ -Tocopherol Effect on Renal Hemodynamics and Albuminuria

A recent report has suggested that the activation of DAG-PKC pathway by hyperglycemia could be responsible for the abnormal hemodynamics observed in the early stages of diabetic nephropathy, such as increases in GFR and albuminuria in diabetic rats (5). Since DAG-PKC activation was normalized in glomeruli of diabetic rats by  $d-\alpha$ -tocopherol treatment, renal clearance studies were performed to determine whether  $d-\alpha$ -tocopherol treatment has a similar normalizing effect on diabetic glomerular hyperfiltration and albuminuria. Con-



Figure 1. (A) Effect of d- $\alpha$ -tocopherol on total DAG contents in glomeruli. After extraction of total glomerular lipids from control rats, control rats treated with d- $\alpha$ -tocopherol, diabetic rats, and diabetic rats treated with d- $\alpha$ -tocopherol, total DAG contents were measured by a radio-enzymatic assay employing DAG kinase as described in Methods. Results were shown as mean  $\pm$  SE. N = 6 and number of rats measured. \*P < 0.05 versus other three groups. (B) Effect of d- $\alpha$ -tocopherol on PKC activity in glomeruli. PKC activities were measured by *in situ* PKC assay that used a specific PKC substrate, RKRTLRRL, in digitonin-permealized glomeruli from control rats, control rats treated with d- $\alpha$ -tocopherol as described in Methods. Results were shown as mean  $\pm$  SE. N = 6 and number of rats measured with d- $\alpha$ -tocopherol as described in Methods. Results were shown as mean  $\pm$  SE. N = 6 and number of rats measured is the treated with d- $\alpha$ -tocopherol as described in Methods. Results were shown as mean  $\pm$  SE. N = 6 and number of rats measured. \*P < 0.05 versus other three groups. Amount of d- $\alpha$ -tocopherol used in the treatment was described in the Methods.

cordant with the increases in DAG -PKC levels, diabetic rats exhibited elevated values for GFR (4.98  $\pm$  0.34 mL/min), compared with those found in control rats (2.90  $\pm$  0.14 mL/min), as quantified by inulin clearance (Figure 3A). The hyperfiltration was prevented in diabetic rats by treatment with d- $\alpha$ -tocopherol (2.98  $\pm$  0.10 mL/min). No differences in MBP were observed among control and diabetic rats with or without d- $\alpha$ -tocopherol treatment (Table 3). RPF estimated by



Figure 2. (A) The effect of d- $\alpha$ -tocopherol on DAG kinase activity. After extraction of total glomerular lipids from control rats, control rats treated with d-a-tocopherol, diabetic rats, and diabetic rats treated with d- $\alpha$ -tocopherol, DAG kinase activities were determined using the octyl-glucoside mixed micelle assay as described in Methods. Results were shown as mean  $\pm$  SE. N = 6 and number of rats measured. \*P <0.05 versus control and diabetic rats without d- $\alpha$ -tocopherol treatment. (B) Protein levels of DAG kinase  $\alpha$  and  $\gamma$  by Western blot analysis. Glomerular lysates (50  $\mu$ g/lane) from control rats, control rats treated with d-a-tocopherol, diabetic rats, and diabetic rats treated with d-a-tocopherol were electrophoresed in 4%-12% Tris-Glycine gradient gels in Tris-Glycine-SDS runningbuffer, transferred to PVDF membrane, and detected by using anti-DAG kinase  $\alpha$  and  $\gamma$  polyclonal antibody. Bands for DAG kinase  $\alpha$  and  $\gamma$  blot were analyzed by a densitometer, and blots shown as insets are representative of three separate experiments.

the PAH clearance did not differ among the four study groups (Figure 3B). FF calculated by GFR/RPF ratio was significantly increased in diabetic rats (0.36  $\pm$  0.02) compared with control rats (0.25  $\pm$  0.02). Again, FF in the diabetic group was normalized to 0.24  $\pm$  0.01 by d- $\alpha$ -tocopherol treatment (Figure 3C).

Finally, to confirm the beneficial effect of  $d-\alpha$ -tocopherol treatment on renal dysfunctions in diabetes, we measured the urinary albumin excretion rate in control and diabetic rats with or without 10 wk  $d-\alpha$ -tocopherol treatment. The albumin excretion rate was significantly increased, to 9.1  $\pm$  2.2 mg/day, in diabetic rats compared with 1.2  $\pm$  0.3 mg/day in control rats (Figure 4A). Similar to its effects on abnormal hemodynamics in diabetic rats,  $d-\alpha$ -tocopherol treatment



Figure 3. Effect of d- $\alpha$ -tocopherol on GFR (A), RPF (B) and FF (C) in control and diabetic rats. GFR and RPF were determined by inulin and PAH clearance as described in Methods. FF were calculated by a standard formula, GFR/RPF ratio. Results were shown as mean  $\pm$  SE. N = 6 and number of rats studied. \*P <0.05 versus other three groups.

ameliorated the increased albumin excretion rate in diabetic rats to 2.4  $\pm$  0.6 mg/day in diabetic rats (Figure 4A). Creatinine clearances (Ccr) in control and control rats treated with d- $\alpha$ -tocopherol were 0.74  $\pm$  0.10 and 0.71  $\pm$  0.06 mL/min, respectively. Diabetes increased Ccr to 1.14  $\pm$  0.25 mL/min, which was not statistically significant probably because only four rats were studied (N = 4). Treatment of diabetic rats with d- $\alpha$ -tocopherol decreased creatinine clearance to normal with 0.84  $\pm$  0.14 mL/min. Fractional excretion of albumin (FEalb) was calculated in the control and diabetic rats treated with 10 wk of d- $\alpha$ -tocopherol. The results showed that diabetes increased FEalb from 3.2  $\pm$  0.9  $\times$  10<sup>-5</sup> to 13.7  $\pm$  2.2  $\times$  10<sup>-5</sup> (Figure 4B). Treatment with d- $\alpha$ -tocopherol prevented the increase in FEalb induced by diabetes to the level of 4.8  $\pm$  0.5  $\times$  10<sup>-5</sup> (P <0.01 versus diabetic rats).

PKC activity in glomeruli of diabetic rats was increased at 10 wk after the induction of diabetes from  $18.6 \pm 0.9$  pmol/mg protein/min to  $30.4 \pm 1.9$  pmol/mg protein/min, but treatment with d- $\alpha$ -tocopherol again normalized the PKC activity to  $21.7 \pm 0.6$  pmol/mg protein/min, parallel with normalizing renal dysfunctions in diabetic rats (Figure 4C).

#### Discussion

Clinical and epidemiological studies have clearly established that hyperglycemia is responsible for alterations in renal he-



Figure 4. Effect of 10 wk of treatment with d- $\alpha$ -tocopherol on urinary albumin excretion rate (A), fractional excretion of albumin (B), and PKC activity (C) in glomeruli from control and diabetic rats. (A) Urinary albumin concentration was determined by enzyme-linked immunosorbent assay as described in Methods. 24-h urine collection from each group was performed on two consecutive days in metabolic cages. Results were shown as mean  $\pm$  SE. N = control; 14, control + d- $\alpha$ -tocopherol; 4, diabetic; 14, diabetic + d- $\alpha$ -tocopherol; 10. \*P <0.05 versus other groups. (B) FEalb was calculated by the following formula, clearance of albumin/creatinine clearance. Results were shown as mean  $\pm$  SE. N = 4. \*P <0.01 vs other groups. (C) PKC activities were measured by *in situ* PKC assay as described in Methods. Results were shown as mean  $\pm$  SE. Kidney from four rats were studied separately. \*P <0.05 versus other three groups.

modynamics, proteinuria, and glomerular pathologies in animal models of diabetes and in diabetic patients (14,44,45). The mechanism responsible for the adverse effect of hyperglycemia are probably multiple (9–13). One of these mechanisms involves the increases in DAG content resulting in the activation of PKC in renal glomeruli. The ability of hyperglycemia or elevated levels of glucose to increase total DAG content has been reported in the glomeruli of diabetic animal (5,23), renal mesangial cells (20,21,24,28), and other vascular cells (16–19) by numerous researchers, including those in our laboratory. Our findings that the glomeruli of 2-wk diabetic rats have elevated levels of total DAG and activation of PKC are consistent with previous reports. The source of the increased DAG induced by hyperglycemia is most likely from the metabolism of glucose through glycolysis, which channels glucose metabolites to the de novo synthesis pathway of DAG, as we and others have reported (22,28,46,47). It is also possible that the increase in total DAG can be derived from the metabolism of phosphatidylcholine by the actions of phospholipase D, which could be influenced by the activity changes of DAG lipase or phosphatidic acid phosphatase (30,32). There is no evidence that hyperglycemia will affect the phosphoinositide pathway in vascular tissues, which could change the DAG level by altering phosphoinositide breakdown facilitated by phospholipase C (22).

The finding of activated PKC in diabetic state glomeruli is consistent with increases in the level of DAG, which is the endogenous cellular activator of PKC (48). Other investigators have also reported that PKC activity is increased by diabetes in the renal glomeruli, as well as in cultured mesangial cells exposed to high levels of glucose (5,20,21,23,24,26-28,49). Our measurements of PKC, however, are by an in situ method that can assess PKC activity intracellularly without having to perform partial purification of PKC from the tissue first. The classical isolation procedures used to measure PKC activity into the membranous and cytosolic portion have not always reflected precisely its in vivo activity because of losses of its activity during isolation (37). Our data derived from an in situ assay have demonstrated that the DAG-PKC pathway is activated in the glomeruli of diabetic rats. We have validated this method as useful in renal tissue by its responses to known PKC activations, PMA, and inhibitors GF 109,203X. In addition, the efficiency of the in situ method was found comparable to the classical PKC assay using the isolated cytosol and membranous fractions. Studer et al. have characterized the in vivo activation of PKC by measuring the phosphorylation of the MARCKS protein, a known intracellular 80 kD protein that undergoes phosphorylation on PKC activation (49,50). This group has reported that the phosphorylation of MARCKS protein was enhanced in renal glomeruli of diabetic rats and mesangial cells exposed to high concentrations of glucose (49,50). These findings have established that the DAG-PKC pathway is actually activated in the renal glomeruli by hyperglycemia in diabetes.

The temporizing effect of d- $\alpha$ -tocopherol on PKC activation was initially reported by Tasinato *et al.*, who suggested a direct inhibitory effect of d- $\alpha$ -tocopherol on PKC activity (31). However, our results in the glomeruli showed that both DAG and activated PKC levels were decreased by d- $\alpha$ -tocopherol treatment, consistent with our previous report demonstrating that d- $\alpha$ -tocopherol's inhibitory effect on PKC was mediated by the lowering of DAG levels (18,19). This possibility is suggested by the decrease of DAG levels in the nondiabetic control rats, although the changes in DAG levels induced by vitamin E treatment were not statistically significant, possibly because of the small number of rats. One of the possible effects of d- $\alpha$ - tocopherol in preventing PKC activation could result from its DAG-lowering effect by the activation of DAG kinase, which has been suggested by Tran et al. previously (51). This possibility was supported by the results showing that d- $\alpha$ -tocopherol increased DAG kinase activities in the renal glomeruli both in control and diabetic rats. The mechanism of the increases in DAG kinase activities is not clear because the protein levels of the two main types of DAG kinase in the glomeruli,  $\alpha$  and  $\gamma$ , were not changed by diabetes and d- $\alpha$ -tocopherol treatment, suggesting that the enhancement of DAG kinase resulted from direct activation of DAG kinase. However, other DAG kinase isozymes besides  $\alpha$  and  $\beta$  have been identified (52–54). It is possible that other DAG kinase isozymes might be increased by d- $\alpha$ -tocopherol. DAG kinase activity has also been reported to be activated by phospholipids, cytokines, and its intracellular translocation (32). Supportive evidence that d- $\alpha$ -tocopherol's effect is not directly on PKC has been reported by us previously, showing that the addition of d- $\alpha$ -tocopherol to purified PKC isoforms  $\alpha$  and  $\beta$  did not have any inhibitory effect (18). The results of the study presented here suggest that an association between DAG kinase activities and PKC activities are only correlative. Definite studies are needed to establish causal relationship. It is also possible that  $d-\alpha$ -tocopherol may indirectly affect PKC activity in vivo or intracellularly because other investigators have provided evidence indicating that the activation of PKC by PMA can also be inhibited by d- $\alpha$ -tocopherol (31). Thus, it is likely that d- $\alpha$ -tocopherol is inhibiting PKC activities indirectly through activation of DAG kinase or phosphorylation of PKC itself.

The finding of many biochemical alterations in vascular tissues from diabetic animal or patients is not surprising; numerous metabolic changes are probably occurring in this chronic metabolic disorder. Therefore, it is critical to associate and correlate the biochemical changes with physiological dysfunctions or pathological changes if causal relationship are to be assigned. The activation of PKC, especially the  $\beta$  isoform, has been correlated to abnormal retinal hemodynamics and renal functions in the diabetic animal by the use of a novel PKC isoform  $\beta$  selective inhibitor LY333,531 (5). Results from the study presented here suggest that d- $\alpha$ -tocopherol treatment could prevent the onset of glomerular hyperfiltration and increases in urinary albumin excretion induced by diabetes. The close correlation between the results of the abnormal renal functions and activated the DAG-PKC pathway indicated a possible causal relationship. Our results also suggest that chronic treatment with d- $\alpha$ -tocopherol may be able to ameliorate histopathologies in the renal glomeruli of diabetic rats.

Studies of chronic treatment using vitamin E are clearly needed, especially in light of the recent publication by Trachtman *et al.*, who reported that treatment with vitamin E for more than 6 months may increase the mortality rate of diabetic rats (55). There are quite a few differences in the design of our study *versus* the study by Trachtman *et al.* In the study presented here, animals were fed with normal chow and intraperitoneal injection of d- $\alpha$ -tocopherol (vitamin E), whereas the diabetic rats in Trachtman's study were fed orally with preformulated diet containing vitamin E, but the control rats used normal chow (55). It was difficult to identify the reasons for the increase in mortality, which could be related to the preformulated chow, although Trachtman et al. stated that the normal chow was identical to vitamin E-enriched preformulated chow. However, the exact analytical comparison of the chows used was not provided. The study presented here treated diabetic rats for up to 16 weeks (data not shown); no abnormal increases were observed in blood pressure, body weight loss, food intake, or mortality in the d- $\alpha$ -tocopherol (vitamin E)treated group. Similarly, in clinical trials using high doses of vitamin E such as used in the study presented here, very few toxic side effect shave been reported (56,57). Our rats were given 40 mg/kg of d- $\alpha$ -tocopherol every other day, translated to approximately 9-10 IU/day, with the control and diabetic rats taking orally an additional 1.3 IU/day from the standard rat chow. In comparison, Trachtman et al. used 3-4 IU/day orally in the treated group versus 1.3 IU/day in the control group. Thus, our treated rats were exposed to a much greater dose of vitamin E than that used in previously reported studies. However, longer studies using orally fed vitamin E at multiple doses are needed to determine whether d- $\alpha$ -tocopherol (vitamin E) treatment can prevent the pathological changes in the renal glomeruli as well as the survival rate in a diabetic rat.

In study presented here, intraperitoneal injection of  $d \cdot \alpha$ tocopherol was used to obtain the consistent high level of vitamin E that inhibits both DAG and PKC levels (18,19). Absorption of orally administered  $d \cdot \alpha$ -tocopherol can be variable not just among control animals but between control and diabetic rats. In the study presented here, the dose of  $d \cdot \alpha$ tocopherol used was 40 mg/kg every otherda, which corresponded to human doses of approximately 1800 to 2000 IU/day in a human weighing 70 kg. This dose is clearly much more than the 200–400 IU dose taken usually in daily vitamin E supplements. However, this high dose of vitamin E was selected because of our previous finding that  $d \cdot \alpha$ -tocopherol given intraperitonealy at this dose will achieve a consistent plasma and tissue levels that prevents hyperglycemia-induced PKC activation (18,19).

The mechanism of d- $\alpha$ -tocopherol's action in unclear. The data from this study cannot determine whether vitamin E's effect results from its antioxidant actions or lipophilic properties because parameters of tissue oxidation were not assessed. It is possible that d- $\alpha$ -tocopherol could be altering cellular membrane properties through its antioxidant effect. Our previous study has showed that other antioxidants, such as probucol, which is structurally different from d- $\alpha$ -tocopherol, can also prevent the activation of the DAG-PKC pathway induced by high levels of glucose (19). These results suggest that the effect of d- $\alpha$ -tocopherol could be partly mediated by its antioxidant action. However, vitamin C, a water-soluble antioxidant, was ineffective (data not shown). Alternatively, d- $\alpha$ -tocopherol has been reported to alter plasma membrane fluidity by its direct interaction with the lipid bilayer structure of the membrane (58). Because DAG kinase and PKC are associated with plasma membrane for its actions, their activities can be affected by alterations in membrane fluidity and the oxidation state of various membranous lipids. Tasinato et al. have provided interesting evidence that the inhibitory actions of  $d-\alpha$ -tocopherol on PKC activity may be mediated through its nonantioxidant actions (31).

Our studies have found that d- $\alpha$ -tocopherol treatment can lower both the DAG and PKC levels in the renal glomeruli of diabetic rats. The amelioration of PKC activation by  $d-\alpha$ tocopherol appears to induce the normalization of renal dysfunctions as measured by renal hemodynamics and urinary albumin excretion rate. The mechanism of glomerular hyperfiltration in diabetes is not clear. Multiple factors are probably involved, with some of those factors associated with PKC activation. Vasodilatory prostanoids such as PGE<sub>2</sub> production can be increased in glomeruli of diabetic rats because we and others have reported that PGE<sub>2</sub> production is increased through the activation of cytosolic phospholipase  $A_2$ , which was due to increases in PKC activity in mesangial cells and smooth muscle cells exposed to high levels of glucose (27,59). Another possibility could be the increased production of atrial natriuretic peptide (ANP), which has been reported to be increased in the diabetic state and may cause glomerular hyperfiltration (60,61). The overproduction of ANP may also be related to PKC activation because the transcription rate of ANP in cultured arterial cells can also be increased by PKC (62).

Further studies, both in animal model of diabetes and in diabetic patients, are needed to clarify the mechanism of  $d-\alpha$ -tocopherol's action and its therapeutic significance in preventing abnormal renal functions of diabetes.

#### Acknowledgments

The authors thank Ms. Joan C. Taylor for her assistance with the preparation of this manuscript. This study was supported by the following grants: NIH EY05110, DK36433 which supported the Diabetes Endocrinology Research Center, Lions Eye Research Grant, and The Adler Foundation.

#### References

- Mogensen CE: Glomerular filtration rate and renal plasma flow in short-term and long-term juvenile diabetes mellitus. Scand J Lab Invest 28: 91-100, 1971
- Christiansen JS, Gammelgaard J, Frandsen M, Parving H-H: Increased kidney size, glomerular filtration rate and renal plasma flow in short-term insulin-dependent diabetics. *Diabetologia* 20: 451-456, 1981
- Hostetter TH, Troy JL, Brenner BM: Glomerular hemodynamics in experimental diabetes mellitus. *Kidney Int* 19: 410-415, 1981
- Jensen PK, Christiansen JS, Steven K, Parving H-H: Renal function in streptozotocin-diabetic rats. *Diabetologia* 21: 409– 414, 1981
- Ishii H, Jirousek MR, Koya D, Takagi T, Xia P, Clermont A, Bursell S-E, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL: Amelioration of vascular dysfunctions in diabetic rats by an oral PKC β inhibitor. Science 272: 728-731, 1996
- Mogensen CE, Christensen CK: Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med 311: 89-93, 1984
- Mogensen CE: Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. N Engl J Med 310: 356-360, 1984
- Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H: Microalbuminuria as a predictor of clinical nephroph-

athy in insulin-dependent diabetes mellitus. Lancet I: 1430-1432, 1982

- Anderson S, Brenner BM: Pathogenesis of diabetic glomerulopathy: Hemodynamic considerations. *Diabetes Metab Rev* 4: 163– 177, 1988
- DeRubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes: Mechanisms and potential links to the pathogenesis of diabetic nephropathy. *Diabetes* 43: 1-8, 1994
- 11. Sharma K, Ziyadeh FN: Hyperglycemia and diabetic kidney disease: The case for transforming growth factor- $\beta$  as a key mediator. *Diabetes* 44: 1139-1146, 1995
- Williamson JR, Chang K, Frangos M, Hasan KS, Ido Y, Kawamura T, Nyengaard JR, Enden MVD, Kilo C, Tilton RG: Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes* 42: 801-813, 1993
- 13. Brownlee M, Cerami A, Vlassara H: Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. N Engl J Med 318: 1315-1321, 1988
- 14. The Diabetes Control and Complications Trial Research Group: The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 329: 977–986, 1993
- Lewis EJ, Hunsicker LG, Bain RP, Rohde RD: The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. N Engl J Med 329: 1456-1462, 1993
- 16. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL: Preferential elevation of protein kinase C isoform βII and diacylglycerol levels in the aorta and heart of diabetic rats: Differential reversibility to glycemic control by islet cell transplantation. *Proc Natl Acad Sci USA* 89: 11059–11063, 1992
- Shiba T, Inoguchi T, Sportsman JR, Heath WF, Bursell S, King GL: Correlation of diacylglycerol level and protein kinase C activity in rat retina to retinal circulation. Am J Physiol 265: E783–E793, 1993
- Kunisaki M, Bursell SE, Clermont AC, Ishii H, Ballas LM, Jirousek MR, Umeda F, Nawata H, King GL : Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway. Am J Physiol 269: E239-E246, 1995
- Kunisaki M, Bursell SE, Umeda F, Nawata H, King GL: Normalization of diacylglycerol-protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. *Diabetes* 43: 1372– 1377, 1995
- 20. Kreisberg JI, Ayo SH: The glomerular mesangium in diabetes mellitus. *Kidney Int* 43: 109-113, 1993
- Kreisberg JI: Hyperglycemia and microangiopathy: Direct regulation by glucose of microvascular cells. Lab Invest 67: 416–426, 1992
- 22. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL: Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase C pathway in diabetes and hypergalactosemia. *Diabetes* 43: 1122–1129, 1994
- Craven PA, DeRubertis FR: Protein kinase C is activated in glomeruli from storeptozotocin diabetic rats: Possible mediation by glucose. J Clin Invest 83: 1667–1675, 1989
- Ayo SH, Radnik R, Garoni JA, Troyer DA, Kreisberg JI: High glucose increases diacylglycerol mass and activates protein kinase C in mesangial cell cultures. *Am J Physiol* 261: F571–F577, 1991
- 25. Williams B, Schrier RW: Characterization of glucose-induced in

situ protein kinase C activity in cultured vascular smooth muscle cells. *Diabetes* 41: 1464–1472, 1992

- 26. Kikkawa R, Haneda M, Uzu T, Koya D, Sugimoto T, Shigeta Y: Translocation of protein kinase C  $\alpha$  and  $\zeta$  in rat glomerular mesangial cells cultured under high glucose conditions. *Diabe*tologia 37: 838-841, 1994
- Williams B, Schrier RW: Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells. J Clin Invest 92: 2889–2896, 1993
- Guzman NJ, Kurian P, Andaloro VJ, Tisher CC, Grews FT: Effect of glucose on receptor-mediated phosphoinositide hydrolysis and second messenger generation in rat glomerular mesangial cells. J Am Soc Nephrol 2: 179–190, 1992
- 29. Nishizuka Y: The family of protein kinase C for signal transduction. JAMA 262: 1826-1833, 1989
- Nishizuka Y: Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607-614, 1992
- 31. Tasinato A, Boscoboinik D, Bartoli G-M, Maroni P, Azzi A: d- $\alpha$ -tocopherol inhibition of vascular smooth muscle cell proliferation occurs at physiological concentrations, correlates with protein kinase C inhibition, and is independent of its antioxidant properties. *Proc Natl Acad Sci USA* 92: 12190–12194, 1995
- Kanoh H, Yamada K, Sakane F: Diacylglycerol kinase: A key modulator of signal transduction. *Trends Biochem Sci* 15: 47–50, 1990
- 33. Ueda T, Igarashi O: New solvent system for extraction of tocopherols from biological specimens for HPLC determination and the evaluation of 2, 2, 5, 7, 8-pentamethyl-6-chromanol as an internal standard. J Micronutr Anal 3: 185–198, 1987
- 34. Bligh EG, Dyer WJ: A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* 37: 911–917, 1959
- Preiss JE, Loomis CR, Bell RM, Niedel JE: Quantitative measurement of sn-1,2-diacylglycerols. *Methods Enzymol* 141: 294– 300, 1984
- 36. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248-254, 1976
- Heasley LE, Johnson GL: Regulation of protein kinase C by nerve growth factor, epidermal growth factor, and phorbol esters in PC 12 pheochromocytoma cells. J Biol Chem 264: 8646– 8652, 1989
- Yasuda I, Kishimoto A, Tanaka S-I, Tominaga M, Sakurai A, Nishizuka Y: A synthetic peptide substrate for selectivity assay of protein kinase C. *Biochem Biophys Res Commun* 166: 1220– 1227, 1990
- Sakane F, Yamada K, Imai S-I, Kanoh H: Porcine 80-kDa diacylglycerol kinase is a calcium-binding and calcium/phospholipid-dependent enzyme and undergoes calcium-dependent translocation. J Biol Chem 266: 7096-7100, 1991
- 40. Goto K, Watanabe M, Kondo H, Yuasa H, Sakane F, Kanoh H: Gene cloning, sequence, expression and in situ localization of 80 kDa diacylglycerol kinase specific to oligodendrocyte of rat brain. Brain Res Mol Brain Res 16: 75-87, 1992
- 41. Kai M, Sakane F, Imai S-I, Wada I, Kanoh H: Molecular cloning of a diacylglycerol kinase isozyme predominantly expressed in human retina with a truncated and inactive enzyme expression in most other human cells. J Biol Chem 269: 18492–18498, 1994
- 42. Waugh WH: Photometry of inulin and polyfructosan by use of a cysteine/tryptophan reaction. *Clin Chem* 23: 639-645, 1977
- 43. Dalton RN, Wiseman MJ, Turner C, Viberti G: Measurement of

urinary para-aminohippuric acid in glycosuric diabetics. *Kidney* Int 34: 117–120, 1988

- Engerman R, Bloodworth JM Jr, Nelson S: Relationship of microvascular disease in diabetes to metabolic control. *Diabetes* 26: 760-769, 1977
- Cohen AJ, McGill PD, Rossetti RG, Guberski DL, Like AA: Glomerulopathy in spontaneously diabetic rat: Impact of glycemic control. *Diabetes* 36: 944–951, 1987
- Craven PA, Davidson CM, DeRubertis FR: Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. *Diabetes* 39: 667-674, 1990
- Inoguchi T, Xia P, Kunisaki M, Higashi S, King GL: Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. *Am J Physiol* 267: E369-E379, 1994
- 48. Bell RM: Protein kinase C activation by diacylglycerol second messengers. Cell 45: 631-632, 1986
- Craven PA, Studer RK, DeRubertis FR: Impaired nitric oxidedependent cyclic guanosine monophosphate generation in glomeruli from diabetic rats: Evidence for protein kinase C-mediated suppression of the cholinergic response. J Clin Invest 93: 311-320, 1994
- Studer RK, Craven PA, DeRubertis FR: Role for protein kinase C in the mediation of increased fibronectin accumulation by mesangial cells grown in high-glucose medium. *Diabetes* 42: 118-126, 1993
- 51. Tran K, Proulx PR, Chan AC: Vitamin E suppresses diacylglycerol (DAG) level in thrombin-stimulated endothelial cells through an increase of DAG kinase activity. *Biochem Biophys Acta* 1212: 193–202, 1994
- 52. Sakane F, Imai S-I, Kai M, Wada I, Kanoh H: Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a C-terminal tail similar to those of the EPH family of protein-tyrosine kinases. J Biol Chem 271: 8394–8401, 1996
- Bunting M, Tang W, Zimmerman GA, McIntyre TM, Prescott SM: Molecular cloning and characterization of a novel human diacylglycerol kinase ζ. J Biol Chem 271: 10230-10236, 1996

- 54. Tang W, Bunting M, Zimmerman GA, McIntyre TM, Prescott SM: Molecular cloning of a novel human diacylglycerol kinase highly selective for arachidonate-containing substrates. J Biol Chem 271: 10237-10241, 1996
- 55. Trachtman H, Futterweit S, Maesaka J, Ma C, Valderrama E, Fuchs A, Tarectecan AA, Rao PS, Sturman JA, Boles TH, Fu MX, Baynes J: Taurine ameliorates chronic streptozotocin-induced diabetic nephropathy in rats. Am J Physiol 269: F429-F438, 1995
- Meyers DG, Maloley PA, Weeks D: Safety of antioxidant vitamins. Arch Intern Med 156: 925-935, 1996
- Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ: Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 347: 781–786, 1996
- 58. Urano S, Yano K and Matsuo M: Membrane-stabilizing effect of vitamin E: Effect of  $\alpha$ -tocopherol and its model compounds on fluidity of lecithin liposomes. *Biochem Biophys Res Commun* 150: 469-475, 1988
- Xia P, Kramer RM, King GL: Identification of the mechanism for the inhibition of Na<sup>+</sup>, K<sup>+</sup>-adenosine triphosphatase by hyperglycemia involving activation of protein kinase C and cytosolic phospholipase A2. J Clin Invest 96: 733-740, 1995
- Ortola FV, Ballermann BJ, Anderson S, Mendez RE, Brenner BM: Elevated plasma atrial natriuretic peptide levels in diabetic rats: Potential mediator of hyperfiltration. J Clin Invest 80: 670-674, 1987
- 61. Sakamoto K, Kikkawa R, Haneda M, Shigeta Y: Prevention of glomerular hyperfiltration in rats with streptozotocin-induced diabetes by an atrial natriuretic peptide receptor antagonist. *Diabetologia* 38: 536-542, 1995
- 62. Suzuki E, Hirata Y, Kohmoto O, Sugimoto T, Hayakawa H, Matsuoka H, Sugimoto T, Kojima M, Kangawa K, Minamino N, Matsuo H: Cellular mechanisms for synthesis and secretion of atrial natriuretic peptide and brain natriuretic peptide in cultured rat atrial cells. *Circ Res* 71:1039-1048, 1992