Reversal of redox-dependent inhibition of diacylglycerol kinase by antioxidants in mesangial cells exposed to high glucose

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Abstract. Activation of the diacylglycerol (DAG)-protein kinase C (PKC) pathway is one of the pathomechanisms of diabetic nephropathy. We previously reported that d-α-tocopherol, well known as an antioxidant, enhances diacylglycerol kinase (DGK) activity, leading to the reduction of excess DAG accumulation and PKC activation in the glomeruli of streptozotocin-induced diabetic rats. However, it remains to be determined whether the effect of d-α-tocopherol on DGK activity is exerted through its antioxidative action. DAG contents, PKC and membranous DGK activity were measured in cultured human glomerular mesangial cells under normal (5.5 mM) or high glucose (27.5 mM) conditions in the presence or absence of two antioxidants (50 μM d-α-tocopherol and probucol). Mesangial cells were exposed to hydrogen peroxide (H_2O_2) (10-1000 μM) in the presence or absence of 300 U/ml catalase, followed by measurement of DGK activity. Both antioxidants restored the high glucose-induced decrease in DGK activity, resulting in the reduction of high glucose-induced activation of the DAG-PKC pathway. In mesangial cells exposed to H_2O_2 at various concentrations, DGK activity decreased in a dose-dependent manner. The addition of antioxidative enzyme catalase to the cells reversed the H_2O_2-mediated down-regulation of DGK activity. In conclusion, DGK activity is reduced by oxidative stress in human mesangial cells cultured under high glucose conditions. Antioxidants, including d-α-tocopherol and probucol may improve hyperglycemia-induced DAG-PKC activation by enhancing DGK activity.

Introduction

Diabetic nephropathy is the leading cause of end-stage renal disease, and hyperglycemia is recognized as a major pathophysiological factor of diabetic complications (1,2). Various abnormalities, such as the activation of the polyol pathway or the diacylglycerol (DAG)-protein kinase C (PKC) pathway, increased formation and activity of advanced glycation end-products (AGEs), oxidative stress and the activation of the hexosamine pathway have been proposed as potential molecular mechanisms of diabetic vasculopathies (3). Among these, the DAG-PKC pathway has been studied extensively (4). Intracellular hyperglycemia promotes abnormal DAG accumulation from *de novo* synthesis, and high levels of DAG induce the activation of PKC. High DAG levels and PKC activation have been demonstrated in vascular tissues including the kidneys of diabetic animals (5-7), as well as in cultured renal vascular cells exposed to high glucose (7-10). DAG is converted by diacylglycerol kinase (DGK) to phosphatidic acid (PA), and enhanced DGK activity leads to a reduction in excess DAG accumulation induced by high glucose, resulting in reduced PKC activation. We previously revealed that d-α-tocopherol inhibited DAG accumulation in diabetic renal glomeruli or mesangial cells under high glucose conditions via the enhancement of DGK activity (8,11), leading to the reduction of PKC activation. Notably, Lee *et al* (9) reported that not only d-α-tocopherol but also probucol increased DGK activity in vascular smooth muscle cells, resulting in an improvement in excess DAG accumulation and increased PKC activation under high glucose conditions. D-α-tocopherol and probucol are well-known antioxidants. We have also revealed that oxidative stress is enhanced in storeptozocin (STZ)-induced diabetic glomeruli, and treatment with these two antioxidants may improve the diabetic state (12,13). The data from these studies indicate that oxidative stress contributes to the reduction of DGK activity in hyperglycemia. Thus, the present study aimed to investigate whether DGK activity is modulated by oxidative stress and d-α-tocopherol, and whether probucol is capable of increasing DGK activity through its antioxidative properties in human glomerular mesangial cells.

In the present study, we demonstrate that DGK activity is reduced in human glomerular mesangial cells exposed to high glucose conditions, and that two types of antioxidants, d-α-tocopherol and probucol, enhance DGK activity, leading to the suppression of the hyperglycemia-induced activation of the DAG-PKC pathway. Moreover, the exposure of mesangial cells to oxidative stress using H_2O_2 leads to the reduction of DGK activity in a dose-dependent manner. Our results indicate that DGK activation is modulated by oxidative stress in cultured mesangial cells under high glucose conditions.

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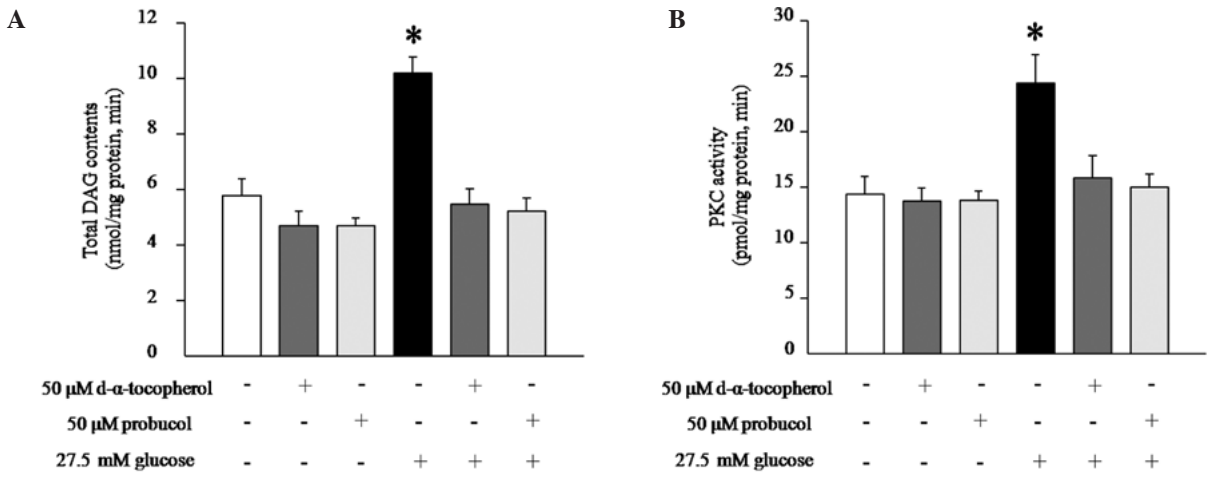

Figure 1. (A) Effect of d-α-tocopherol or probucol on total DAG contents in mesangial cells exposed to high glucose conditions. After extraction of total cellular lipids, total DAG contents were measured by a radio-enzymatic assay employing DAG kinase as described in Materials and methods. Data represent the means ± SEM of six experiments, and each experiment was performed in triplicate. *P<0.01 vs. the other groups. (B) Effects of d-α-tocopherol and probucol on PKC activity in mesangial cells exposed to high glucose conditions. PKC activity was measured by *in situ* PKC assay employing a specific PKC substrate, RKRTLRRL in digitonin-permealized mesangial cells. Data represent the means ± SEM of six experiments and each experiment was performed in triplicate. *P<0.01 vs. the other groups.

Materials and methods

Culture of human glomerular mesangial cells. Human glomerular mesangial cells purchased from Clonetics (NJ, USA) were cultured in a 10-cm culture dish in Dulbecco's modified Eagle's medium (DMEM) containing 5 μg/ml each of insulin, transferrin and selenium (Gibco BRL, Grand Island, NY, USA), 5.5 mM glucose, 100 U/ml penicillin and 100 μg/ml streptomycin with 20% fetal bovine serum (FBS). Cells from the 4th to 9th passages were plated on culture dishes and used for the following experiments.

Assay of total DAG content. Subconfluent mesangial cells in 10-cm culture dishes were exposed to 5.5 or 27.5 mM glucose for 4 days with daily changes of DMEM with 2% FBS. The cells were washed with serum-free DMEM containing and further incubated overnight in DMEM containing 2% FBS in the presence or absence of a 50 μM concentration of d-α-tocopherol or probucol (Sigma-Aldrich, Tokyo, Japan). After washing, total cellular lipids were extracted from the cells according to the method of Bligh and Dyer (14). Total DAG contents were determined with a radio-enzymatic assay kit (Habersham, Arlington Heights, IL, USA) employing DG kinase (Caliches, San Diego, CA, USA), according to the instructions provided by the manufacturer, as previously described (5,8), which quantitatively converts DAG to [γ-^{32}P]-PA in the presence of [γ-^{32}P]-ATP (NEN, Boston, MA, USA) according to the instructions provided by the manufacturer. The resulting [γ-^{32}P]-PA was separated by silica-gel thin-layer plates (EM Separations, Gibbstown, NJ, USA) in a chamber containing chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) according to the method of Priess *et al* (15). PA was visualized by autoradiography and identified by co-migration with radio-labeled PA derived from DAG standard from 31.25 to 2,000 pmol. The spots were scraped and radioactivity was counted in a liquid scintillation counter (Beckman CS6500, Fullerton, CA, USA). The values of total DAG contents were expressed relative to the amount of cellular protein as described by Bradford (16).

Assay of PKC activity. Subconfluent mesangial cells in 12-well culture dishes were exposed to 5.5 or 27.5 mM glucose for 4 days with daily changes of media with 2% FBS. The cells were further incubated overnight in DMEM containing 2% FBS in the presence or absence of 50 μM d-α-tocopherol or probucol. PKC activity was determined using the method described previously with some modification (5). Briefly, cells were rinsed twice with 2 ml of DMEM containing 20 mM HEPES (pH 7.4) and pre-incubated in DMEM containing 20 mM HEPES (pH 7.4), and in certain experiments in the presence of 100 nM phorbol-12-myristate-13-acetate (TPA) for 10 min at 37˚C. After washing, the cells were incubated for 15 min in a salt solution (137 mM sodium chloride, 5.4 mM potassium chloride, 0.3 mM sodium phosphate, 0.4 mM potassium phosphate, 5.5 mM glucose, 10 mM $MgCl_2$, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$ and 20 mM HEPES) containing 5 μg/ml digitonin 1 mM and ATP mixed with [γ-^{32}P] ATP (<1500 cpm/pmol) in the presence or absence of 100 μM PKC-specific peptide substrate, RKRTLRRL. The reaction was terminated with 5% trichloroacetic acid (TCA) buffer (final concentration). Aliquots of the reaction mixture were spotted on 3x3-cm phosphocellulose papers (Whatman P-81, Maidstone, UK) 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 the liquid scintillation counting method.

Assay of DGK activity. Subconfluent mesangial cells in 10-cm culture dishes were exposed to 5.5 or 27.5 mM glucose for 4 days with daily changes of DMEM with 2% FBS. The cells were further incubated overnight in DMEM containing 2% serum in the presence or absence of 50 μM d-α-tocopherol or probucol. In certain experiments, cells were exposed to H_2O_2

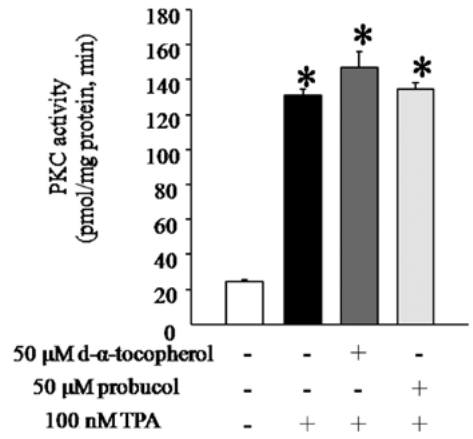

Figure 2. Effects of d-α-tocopherol and probucol on PKC activity stimulated by phorbol-12-myristate-13-acetate (TPA) in mesangial cells. PKC activity was measured by *in situ* PKC assay, employing a specific PKC substrate, RKRTLRRL, in digitonin-permealized mesangial cells. Data represent the means ± SEM (n=6), and each experiment was performed in triplicate. *P<0.01 vs. control.

(0 to 1000 μM) for 1 h in the presence or absence of 300 U/ml catalase. After washing, the cells were lysed in a buffer solution containing 25 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 μg/ml leupeptin, 20 μg/ml aprotinin and 1 mM PMSF, homogenized by a Dounce homogenizer. After centrifugation at 550 x g for 10 min, the supernatant was used for the assay of total DGK activity. In certain experiments, the supernatant was further centrifuged at 100,000 x g for 30 min to separate the cytosolic and particulate fractions. The DGK activity was measured by the octyl glucoside mixed micellar assay as previously described (8,17). Briefly, the reaction was initiated by the addition of enzyme (10-20 μg or cellular fraction) in a mixture containing 50 mM MOPS (pH 7.2), 50 mM octyl glucoside, 100 mM NaCl, 1 mM DTT, 20 mM NaF, 2.1 mM $CaCl_2$, 2 mM EGTA, 0.8 mM EDTA, 10 mM $MgCl_2$, 6.7 mM phosphatidylserine, and 1 mM [γ-^{32}P]-ATP (10,000 cpm/mmol) in the presence of 1 mM 1.2-didecanoyl-sn-glycerol (Avanti Polar Lipids, Alabaster, AL, USA) and continued for 10 min at 30˚C. Lipids were extracted from the mixture and PA was separated on thin-layer plates of silica gel, scraped and counted in a liquid scintillation counter. The values of DGK activity were expressed relative to the cellular protein content.

Statistical analysis. Results were expressed as the means ± SEM. Differences among groups were tested by one-way analysis of variance (ANOVA) followed by the Neuman-Keuls test. A value of P<0.05 was considered statistically significant.

Results

Effect of d-α-tocopherol or probucol on total DAG contents and PKC activity. Total DAG contents in mesangial cells exposed to high glucose levels were significantly increased compared to those under normal glucose (Fig. 1A). Treatment with either d-α-tocopherol or probucol normalized total DAG contents in mesangial cells under high glucose conditions. The DAG-suppressive effect of d-α-tocopherol and probucol was not observed in mesangial cells cultured under normal glucose levels. Culture under high glucose conditions resulted in the significant activation of PKC in mesangial cells compared to cells cultured under normal glucose conditions (Fig. 1B), and this PKC activation was normalized to control levels after the addition of d-α-tocopherol (Fig. 1B). Another antioxidant, probucol, also normalized high glucose-induced PKC activation (Fig. 1B). In addition, PKC activation by TPA was not inhibited by the addition of d-α-tocopherol and probucol, indicating that these antioxidants inhibit DAG-PKC pathway via the modulation of upstream effecter molecules (Fig. 2).

Effect of d-α-tocopherol and probucol on DGK activity. The cellular mechanisms that control intracellular DAG contents were then investigated. Treatment with d-α-tocopherol in mesangial cells under normal and high glucose conditions revealed a significant enhancement of membranous DGK activity (Fig. 3A). A similarly enhanced DGK activity was found using probucol (Fig. 3A). The membranous DGK activity was significantly inhibited by 40% in cells exposed to high

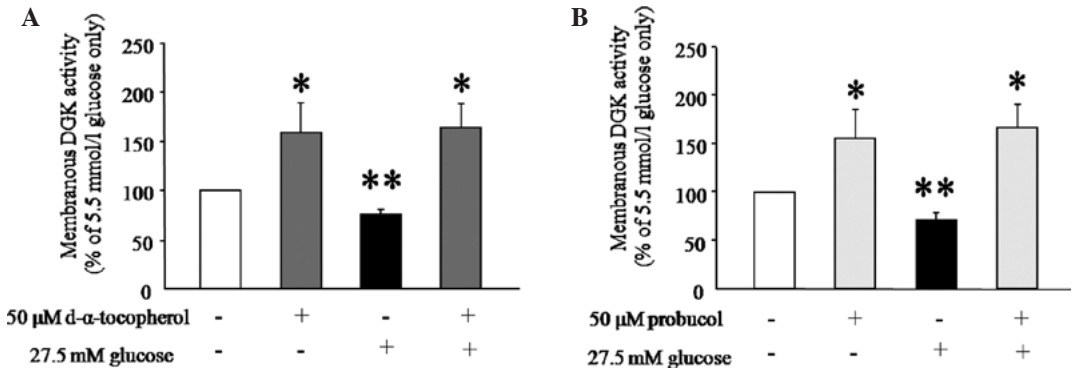

Figure 3. (A) Effects of d-α-tocopherol on the membranous DGK activity in mesangial cells exposed to high glucose conditions. After separating the cytosolic and membrane fractions, DGK activity of the membrane fraction was determined using the octyl glucoside mixed micellar assay as described in Materials and methods. Data represent the means ± SEM (n=6), and each experiment was performed in duplicate. *P<0.05 vs. normal glucose or high glucose without treatment with d-α-tocopherol and probucol. **P<0.05 vs. normal glucose without treatment with d-α-tocopherol and probucol. (B) Effects of probucol on the membranous DGK activity in mesangial cells exposed to high glucose conditions. Data represent the means ± SEM (n=6), and each experiment was performed in duplicate. *P<0.05 vs. normal glucose or high glucose without treatment with d-α-tocopherol and probucol. **P<0.05 vs. normal glucose without treatment with d-α-tocopherol and probucol.

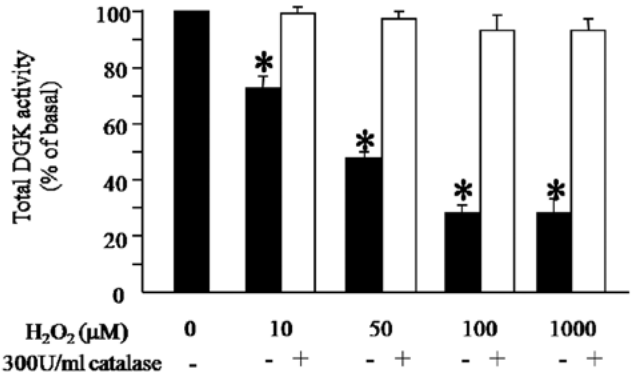

Figure 4. Effects of H_2O_2 in the presence or absence of catalase on DGK activity in mesangial cells. DGK activity was determined in mesangial cells exposed to hydrogen peroxide (H_2O_2) (10-1000 μM) in the presence or absence of 300 U/ml catalase. Data represent the means ± SEM (n=3) and each experiment was performed in duplicate. *P<0.05 vs. H_2O_2 (0 μM) and H_2O_2 (10-1000 μM) with 300 U/ml catalase.

glucose conditions compared to those under normal glucose conditions (Fig. 3A and B). The high glucose-mediated suppression of membranous DGK activity was restored by treatment with either d-α-tocopherol or probucol (Fig. 3A and B).

Oxidative stress inhibits DGK activity. Since antioxidants inhibit the DAG-PKC pathway likely via the induction of DGK activity, we hypothesized that oxidative stress itself stimulates the activation of the DAG-PKC pathway via modulation of DGK activity. To test this hypothesis, H_2O_2 and endogenous H_2O_2 scavenger catalase were used with mesangial cells. Treatment with H_2O_2 inhibited DGK activity in a dose-dependent manner and exogenous catalase completely abrogated H_2O_2-induced inhibition of DGK activity in mesangial cells (Fig. 4).

Discussion

In the present study, we provide evidence for the first time that oxidative stress by high glucose can cause the inhibition of DGK, possibly through its redox modification. Moreover, two types of antioxidants, d-α-tocopherol and probucol, can restore the reduction of DGK activity through their antioxidative properties.

Previous studies have indicated that diabetes-induced increase in DAG content and PKC activation are important cellular mechanisms involved in the development of various diabetic complications, including nephropathy (4). Moreover, we and others reported previously that d-α-tocopherol prevents the activation of the DAG-PKC pathway in diabetic vascular tissues, including renal glomeruli through the enhancement of DGK (6,7,11), acting as a regulator of PKC activity (18). Thus, the activation of DGK may have therapeutic potential for preventing diabetic vascular complications. The present study reveals that both d-α-tocopherol and probucol, prevent the high glucose-induced activation of DAG-PKC pathway by enhancing DGK activity in human glomerular mesangial cells, similar to previous reports (9,11). On the other hand, Tasinato *et al* (19) reported that d-α-tocopherol normalized TPA-induced activation of PKC. However, we show that d-α-tocopherol and probucol do not inhibit TPA-induced PKC activation. Therefore, we believe that the inhibitory effects of d-α-tocopherol and probucol on the activation of the DAG-PKC pathway are mediated through the modulation of DGK activity rather than through direct action on PKC activity.

Oxidative stress in diabetes has also been implicated in the initiation and progression of diabetic vasculopathies, including nephropathy (3). It is well known that both d-α-tocopherol and probucol have antioxidative properties, and we have also reported previously that both these antioxidants attenuate oxidative stress in the glomeruli of rats with STZ-induced diabetes (12,13). Therefore, we hypothesized that diabetes- or hyperglycemia-induced oxidative stress may contribute to the down-regulation of DGK activity in mesangial cells. In the present study, DGK activity in the membrane fraction was decreased in mesangial cells under high glucose conditions, and the addition of each of the two antioxidants restored the reduction of DGK activity, together with the attenuation of overproduction of reactive oxygen species induced by high glucose (data not shown). Thus, it seems likely that the antioxidants specifically reduce excess DAG content through the enhancement of DGK activity and oxidative stress negatively modulates its enzymatic activity in mesangial cells under high glucose conditions. In addition, our results also reveal that oxidative stress by H_2O_2 dose-dependently inhibits DGK activity and that this inhibition disappears by the addition of catalase, a H_2O_2 scavenger. The structure of DGKs commonly contains two or three characteristic cysteine-rich regions (20,21). Therefore, DGK may be oxidized and inactivated by oxidative stress probably through its redox modifications. The inhibition of DGK activity by high glucose could also be significant in oxidative stress-induced cellular dysfunction, such as the sustained PKC activation in diabetes. Further studies are required to clarify the target residue of the reversible oxidation of DGK.

In summary, the present study demonstrates that i) high glucose activates the DAG-PKC pathway in glomerular human mesangial cells, ii) the antioxidants, d-α-tocopherol and probucol, abrogate such biochemical abnormalities, iii) DGK activity is inhibited under a high glucose environment but improves in the presence of antioxidants, and iv) DGK activity can be negatively modulated by oxidative stress. Therefore, the efficacy of antioxidants in inhibiting hyperglycemia-induced DAG/PKC pathway activation via the enhancement of DGK could lead to the development of novel therapeutic strategies for diabetic nephropathy.

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References

1. 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.
2. UK Prospective Diabetes Study (UKPDS) Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 352: 837-853, 1998.

3. Kitada M, Zhang Z, Mima A and King GL: Molecular mechanisms of diabetic vascular complications. J Diabetes Invest 1: 77-89, 2010.
4. Koya D and King GL: Protein kinase C activation and the development of diabetic complications. Diabetes 47: 859-866, 1998.
5. Koya D, Jirousek MR, Lin YW, Ishii H, Kuboki K and King GL: Characterization of protein kinase C beta isoform activation on the gene expression of transforming growth factor-beta, extracellular matrix components, and prostanoids in the glomeruli of diabetic rats. J Clin Invest 100: 115-126, 1997.
6. Kunisaki M, Bursell SE, Clermont AC, *et al*: Vitamin E prevents diabetes-induced abnormal retinal blood flow via the diacylglycerol-protein kinase C pathway. Am J Physiol 269: E239-E246, 1995.
7. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W and King GL: Preferential elevation of protein kinase C isoform beta II 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.
8. Isshiki K, Haneda M, Koya D, Maeda S, Sugimoto T and Kikkawa R: Thiazolidinedione compounds ameliorate glomerular dysfunction independent of their insulin-sensitizing action in diabetic rats. Diabetes 49: 1022-1032, 2000.
9. Lee IK, Koya D, Ishi H, Kanoh H and King GL: d-Alpha-tocopherol prevents the hyperglycemia induced activation of diacylglycerol (DAG)-protein kinase C (PKC) pathway in vascular smooth muscle cell by an increase of DAG kinase activity. Diabetes Res Clin Pract 45: 183-190, 1999.
10. Geraldes P, Hiraoka-Yamamoto J, Matsumoto M, *et al*: Activation of PKC-delta and SHP-1 by hyperglycemia causes vascular cell apoptosis and diabetic retinopathy. Nat Med 15: 1298-1306, 2009.
11. Koya D, Lee IK, Ishii H, Kanoh H and King GL: Prevention of glomerular dysfunction in diabetic rats by treatment with d-alpha-tocopherol. J Am Soc Nephrol 8: 426-435, 1997.
12. Hayashi K, Haneda M, Koya D, Maeda S, Isshiki K and Kikkawa R: Enhancement of glomerular heme oxygenase-1 expression in diabetic rats. Diabetes Res Clin Pract 52: 85-96, 2001.
13. Koya D, Hayashi K, Kitada M, Kashiwagi A, Kikkawa R and Haneda M: Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats. J Am Soc Nephrol 14: S250-S253, 2003.
14. Bligh EG and Dyer WJ: A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911-917, 1959.
15. Preiss JE, Loomis CR, Bell RM and Niedel JE: Quantitative measurement of sn-1,2-diacylglycerols. Methods Enzymol 141: 294-300, 1987.
16. 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.
17. Sakane F, Yamada K, Imai S and 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.
18. Geraldes P and King GL: Activation of protein kinase C isoforms and its impact on diabetic complications. Circ Res 106: 1319-1331, 2010.
19. Tasinato A, Boscoboinik D, Bartoli GM, Maroni P and 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.
20. Merida I, Avila-Flores A and Merino E: Diacylglycerol kinases: at the hub of cell signalling. Biochem J 409: 1-18, 2008.
21. Topham MK and Prescott SM: Mammalian diacylglycerol kinases, a family of lipid kinases with signaling functions. J Biol Chem 274: 11447-11450, 1999.