Protective Effect of D-α-Tocopherol on the Function of Human Mesangial Cells Exposed to High Glucose Concentrations

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Altered functions of mesangial cells (MCs) induced by high glucose levels are thought to play an important role in the pathogenesis of diabetic nephropathy. We investigate whether D-α-tocopherol (Toc), an antioxidant, can prevent malfunction of cultured human MCs induced by high-glucose media. Incubating MCs with 33 mmol/L glucose caused increased lipid peroxide (LPO) levels, disturbed cell replication, enhanced cytotoxicity, enhanced activity of the diacylglycerol (DAG)–protein kinase C (PKC) pathway, and overproduction of fibronectin and eicosanoids (6-keto prostaglandin $F_{1\alpha}$ [$PGF_{1\alpha}$] and thromboxane B_2 [TXB_2]). The amount of LPO in MCs grown in 5 mmol/L glucose was reduced by the addition of Toc in a dose-dependent manner. Since the maximum effect of Toc on decreasing LPO was achieved at a concentration of 100 μmol/L, this dose was selected for the following experiments. Addition of Toc prevented increased LPO levels and [^{51}Cr]-release from MCs induced by high-glucose media without affecting cell number. Toc decreased the total DAG level and PKC activity in membrane fractions in MCs cultured at both 5 and 33 mmol/L glucose. Furthermore, glucose-induced overproduction of fibronectin and eicosanoids from MCs was completely abolished by Toc. These results strongly suggest that Toc ameliorates glucose-induced malfunctions of MCs in vitro.

DIABETIC NEPHROPATHY is a leading cause of morbidity and mortality in diabetes. Since diabetic glomerulosclerosis is characterized by an expansion of the glomerular mesangium that eventually progresses to obliterate the capillary lumen, mesangial cells (MCs) are thought to play a central role in the pathogenesis of diabetic nephropathy.[1,2] Many reports have demonstrated glucose-induced alterations in MC functions such as abnormal proliferation,[3,4] disturbed contraction,[5] increased activity of the diacylglycerol (DAG)–protein kinase C (PKC) pathway,[6,7] and enhanced production of extracellular matrix proteins[4,8,9] and eicosanoids.[10] These changes in MC function may cause mesangial expansion or alteration of glomerular hemodynamics, leading to glomerular hyperfiltration and/or obliteration of the capillary lumen. In the meantime, glucose-induced oxidative stress has been an attractive hypothesis for the pathogenesis of diabetic complications[11] from evidence[12,13] demonstrating that various kinds of free radicals are generated in the processes of glucose autooxidation and increased nonenzymatic glycosylation in hyperglycemic conditions. In fact, several reports[14,15] have suggested that increased oxidative stress maybe involved in the development of diabetic nephropathy.

D-α-Tocopherol (Toc), the most active form of vitamin E, is known as a potent biological antioxidant in the biomembranes by scavenging oxygen radicals and terminating free radical chain reactions.[16] In addition to its role as an antioxidant, Toc has been reported to inhibit PKC activity in smooth muscle cells.[17] It has also been shown to reduce DAG levels in vascular endothelial cells.[18] These unique biological effects of Toc and evidence[19-21] described previously that Toc content in tissues is decreased in the diabetic state led us to determine whether Toc can prevent glucose-induced malfunctions in cultured human MCs. In this study, we investigated the effects of Toc on cell proliferation, lipid peroxide (LPO) formation, cytotoxicity, DAG levels, PKC activity, and fibronectin and eicosanoid synthesis in cultured human MCs exposed to low and high glucose concentrations.

MATERIALS AND METHODS

Cell Culture

Human glomerular MCs were cultured as previously reported.[22] Briefly, glomeruli were isolated from the normal cortex of kidneys removed because of renal carcinoma. The cortex was diced and pushed through a set of copper sieves (250, 150, and 75 μm) to yield decapsulated glomeruli with little tubular contamination. The isolated glomeruli were incubated in Hank's balanced salt solution (HBSS) containing 500 U/mL collagenase type IV (Sigma, St Louis, MO) at 37°C for 15 minutes. After washing twice with HBSS, collagenase-digested glomeruli were resuspended and plated in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 20% fetal calf serum (FCS) (Gibco) and 0.66 U/mL insulin (Boehringer, Mannheim, Germany) in an atmosphere of 95% humidified air with 5% CO_2. MCs were subcultured 14 to 21 days after primary culture. A single cell line between the third and eighth passages was used in the present study. MCs were characterized by morphology and biochemical characteristics as previously described.[23]

RPMI 1640 medium containing 10% FCS was supplemented with either 5 or 33 mmol/L glucose in the presence or absence of 100 μmol/L Toc (Sigma). Toc was dissolved in dimethylsulfoxide (DMSO) and added in required amounts to culture media. The final DMSO concentration did not exceed 0.1%. Control media contained DMSO at similar concentrations. These four different media were used as the experimental media. Culture media were exchanged at 24- or 48-hour intervals in the following experiments.

LPO Assay

LPO levels in MCs cultured with the RPMI 1640 media supplemented with 5 mmol/L glucose containing various (0, 1, 10, 50, 100, or 200 μmol/L) concentrations of Toc and with the experimental media for

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7 days were measured by the thiobarbituric acid method.[24] The harvested cells were mixed with 100 μL 8% sodium dodecyl sulfate, and then a reaction mixture containing 0.8% 2-thiobarbituric acid and 20% acetic acid was added. After heating this solution at 95°C for 60 minutes, thiobarbituric acid–reactive substances extracted by *n*-butanol were measured by spectrofluorometry at emission wavelength 553 nm with excitation wavelength 515 nm. The values are expressed as picomoles of malondialdehyde.

Cell Counting

MCs were removed from culture flasks by incubation with trypsin/EDTA solution (Boehringer), and equal numbers of cells were seeded into 35-mm culture dishes (Corning, Corning, NY). After 24 hours, nonadherent cells were removed and media were changed to the experimental media (day 0). After 3 and 7 days, cells were completely detached from the plates by incubation with trypsin/EDTA, and aliquots of homogenous suspension were placed in a hemocytometer for direct cell counting.

[^{51}Cr]-Release Assay

MCs were cultured in 24-well tissue culture plates (Corning) with the experimental media for 7 days. The cells were incubated with 1 μCi/mL [^{51}Cr]-sodium chromium acid (Amersham Japan, Tokyo, Japan) for 24 hours. After washing the cells five times with phosphate-buffered saline (PBS), they were incubated with RPMI 1640 medium for 24 hours, and incubation media and cells were harvested to measure radioactivity. Cell cytotoxicity was evaluated by the rate of [^{51}Cr] release derived from the following formula: [^{51}Cr] release (%) = ([^{51}Cr] released into the medium/total [^{51}Cr] incorporation in the cells) × 100.

DAG and PKC Assay

MCs were exposed to the experimental media for 7 days. For assay of DAG, the cells were extracted with chloroform:methanol:0.2 mol/L KCl/5 mmol/L EDTA (1:1:0.8). The phases were split by addition of KCl-EDTA solution, and then the chloroform phase was separated for DAG assay. DAG content was measured from the quantitative conversion of DAG to [^{32}P]-phosphatidic acid by means of a kit containing DAG kinase (Amersham International, Amersham, UK). Phosphatidic acid was separated from other phospholipids by thin-layer chromatography on silica gel G plates (Merck, Darmstadt, Germany) using chloroform:methanol:acetic acid (65:15:5 vol/vol/vol). The phosphorylated phosphatidic acid spots were counted by an Instant Imager (Packard, Meriden, CT). For PKC assay, both membrane and cytosolic fractions were obtained by the method previously described.[7] PKC activity was measured using the kit Pep Tag Non-Radioactive Assay for PKC (Promega, Madison, WI). This assay uses brightly colored fluorescent peptide substrates (amino acid sequence, D-L-S-R-T-L-S-V-A-A-K) that are highly specific for PKC. Phosphorylation by PKC of their specific substrate alters the peptide's net charge from +1 to −1. The phosphorylated version was separated by an agarose gel at pH 8.0. Negatively charged bands from the gel were removed and heated at 95°C until the gel slice was melted. Absorbance of the solubilized solution was read by a spectrophotometer at 570 nm. Active rat PKC purified by the method previously described[25] was used as a standard.

Fibronectin Assay

MCs were cultured with the experimental media for 21 days in six-well culture plates (Corning). After washing the cells twice with PBS, they were incubated with FCS-free RPMI 1640 medium for 24 hours to obtain the amount of fibronectin secreted. At the end of the incubation, the media were collected, and then the cell layer was solubilized by 0.2% Triton X-100 containing 1 mmol/L phenylmethylsulfonyl fluoride (Sigma), 25 mmol/L EDTA, and 10 mmol/L *N*-ethylmaleimide (Sigma). Fibronectin was quantified with a double-antibody enzyme-linked immunosorbent assay (ELISA) using the Human Fibronectin Enzyme Immunoassay Kit (Biomedical Technologies, Stoughton, MA). This assay kit uses human fibronectin as a standard, rabbit anti–human fibronectin antiserum as the primary antibody, and alkaline phosphatase–labeled human fibronectin as the tracer.

Eicosanoid Assay

MCs were incubated with the experimental media for 5 days. The cells were washed twice with PBS, followed by incubation with RPMI 1640 medium for 30 minutes. The incubation media were collected for the measurement of eicosanoid levels. The quantity of 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) (the stable metabolite of PGI_2 or prostacyclin) and thromboxane B_2 (TXB_2) (the stable metabolite of TXA_2) was determined using an ELISA method as described by Pradelles and Maclouf.[26]

DNA Determination

DNA content in MCs was measured by a method previously reported.[27]

Statistical Analysis

Results are expressed as the mean ± SD. Statistical analyses were performed using Bonferroni's multiple comparison procedure.[28] *P* values less than .05 were used to define statistically significant differences. For each experiment, n refers to the number of studies (each in duplicate).

RESULTS

LPO

The amount of LPO in MCs grown in 5 mmol/L glucose was 415.0 ± 89.7 pmol/μg DNA as assessed by malondialdehyde. The LPO level was reduced by addition of Toc in a dose-dependent manner. The maximum effect of Toc on decreasing LPO levels was observed at a Toc concentration of 100 μmol/L. Increasing glucose concentration in the medium from 5 mmol/L to 33 mmol/L significantly ($P < .01$) increased LPO levels (896.9 ± 93.5 pmol/μg DNA). The amount of LPO in MCs grown under elevated glucose levels was profoundly reduced by addition of 100 μmol/L Toc (199.8 ± 6.3 pmol/μg DNA), to a level similar to that seen in a low-glucose medium containing Toc (Figs 1 and 2).

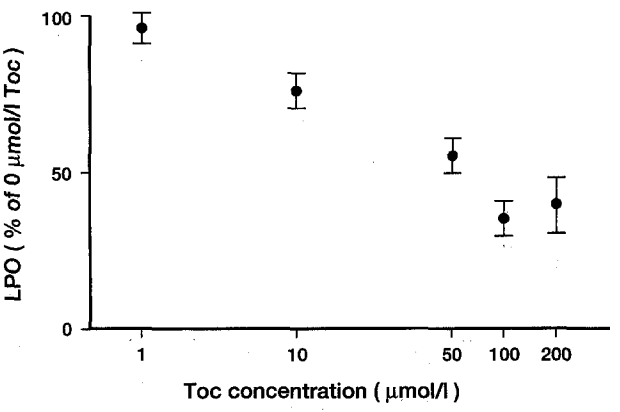

Fig 1. Effects of various concentrations of Toc on LPO levels in MCs cultured under 5 mmol/L glucose. Values are the mean ± SD from 4 experiments.

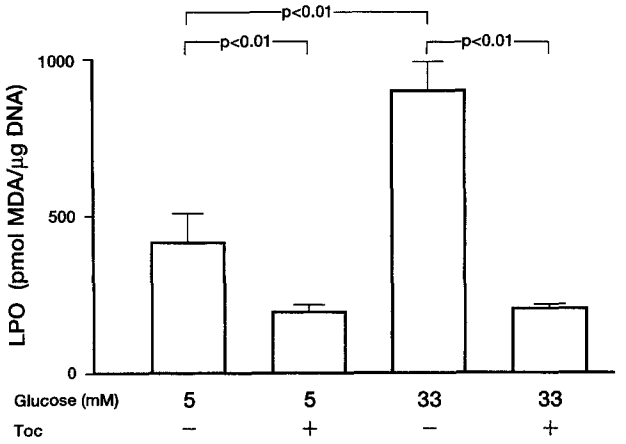

Fig 2. Effects of glucose and 100 μmol/L Toc on LPO levels in MCs (n = 5). Values are the mean ± SD.

Cell Number

At day 7, the number of MCs grown in medium supplemented with 33 mmol/L glucose (30.6×10^5/well on average) was significantly ($P < .05$) smaller than in medium supplemented with 5 mmol/L glucose (35.2×10^5/well on average). The number of MCs grown in media with 100 μmol/L Toc was not significantly different from the number grown in media without Toc under both 5 and 33 mmol/L glucose. Thus, addition of Toc to the culture media did not affect cell number in both low and high concentrations of glucose (Fig 3).

[^{51}Cr] Release

Addition of Toc had no effect on the rate of [^{51}Cr] release from MCs grown in 5 mmol/L glucose. Exposure of MCs to a high-glucose medium resulted in a significant ($P < .01$) increase in the rate of [^{51}Cr] release (26.2% ± 0.9%) compared with low-glucose medium (22.0% ± 1.3%). When Toc was added to the high-glucose medium, the rate of [^{51}Cr] release was 23.6% ± 0.8%. Thus, the glucose-induced increase in the rate of [^{51}Cr] release was totally prevented by Toc (Fig 4).

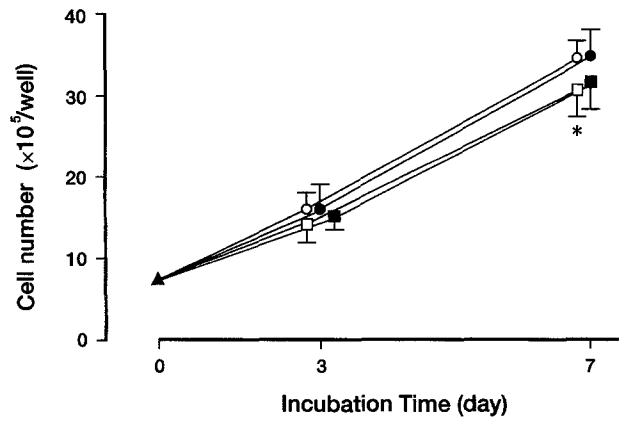

Fig 3. Effects of glucose and 100 μmol/L Toc on number of MCs. (○) 5 mmol/L glucose; (□) 33 mmol/L glucose; (●) 5 mmol/L glucose + Toc; (■) 33 mmol/L glucose + Toc. Values are the mean ± SD from 6 experiments. *$P < .05$ v 5 mmol/L glucose.

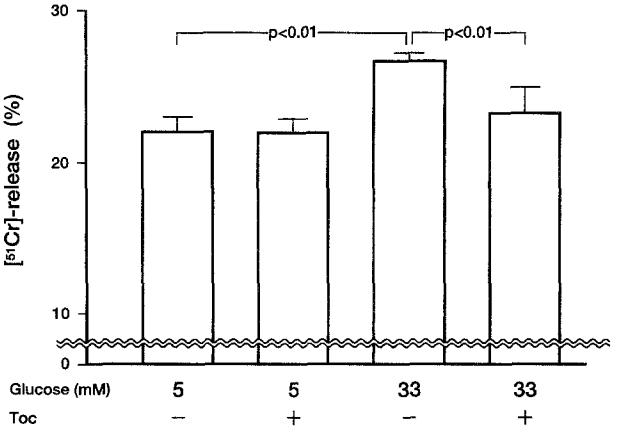

Fig 4. Effects of glucose and 100 μmol/L Toc on the rate of [^{51}Cr] release from MCs, (n = 6). Values are the mean ± SD.

DAG Levels and PKC Activities

Incubation of MCs with Toc showed a 57% reduction in DAG levels under physiological (5 mmol/L) glucose concentration. Increasing glucose levels from 5 to 33 mmol/L caused a 147% increase in DAG levels. Addition of Toc to the high-glucose medium resulted in a reduction in the level of DAG to a similar extent as observed in 5 mmol/L glucose with Toc (Table 1).

No significant differences were observed in PKC activity in cytosolic fractions among the four groups. On the other hand, PKC activity in membrane fractions showed a similar response to a high concentration of glucose and Toc as observed in DAG levels.

Fibronectin

Exposure of MCs to elevated glucose levels resulted in a 122% increase in the content of cellular fibronectin. Addition of Toc to a high-glucose medium totally prevented the glucose-induced increase in cellular fibronectin. An increase of 121% in fibronectin secretion into the medium was observed when MCs were grown in elevated glucose levels. Toc showed inhibitory effects on the release of fibronectin from MCs in both 5 and 33 mmol/L glucose (Fig 5).

Eicosanoids

Production of 6-keto $PGF_{1\alpha}$ and TXB_2 from MCs was enhanced by exposure of the cells to a high-glucose medium (156% and 134% increase v low-glucose media, respectively). Glucose-induced overproduction of 6-keto $PGF_{1\alpha}$ and TXB_2 was prevented by addition of Toc, whereas no significant changes were noted when Toc was added to low-glucose media (Table 2).

DISCUSSION

It has been reported that Toc alters cellular functions such as proliferation, collagen synthesis, and DAG-PKC pathway activity in neuroblastoma cells,[29] fibroblasts,[30] and smooth muscle cells.[17] Trachtman[31] has recently reported that vitamin E prevented glucose-induced lipid peroxidation and increased collagen production in cultured rat MCs. However, the role of Toc in the modulation of cellular signals in MCs has not been

Table 1. Effects of Glucose and Toc on DAG Levels and PKC Activities in the Cytosolic Fraction and Membrane Fraction in Human MCS

Additions	DAG (pmol/μg DNA)	PKC	
		Cytosol (pmol/μg DNA)	Membrane (pmol/μg DNA)
5 mmol/L glucose	108.4 ± 25.3	47.9 ± 4.0	112.5 ± 21.8
5 mmol/L glucose + 100 μmol/L Toc	46.8 ± 14.4 *	49.7 ± 4.1	68.4 ± 11.7 * *
33 mmol/L glucose	159.8 ± 15.7 * *	53.9 ± 1.8	163.6 ± 12.3 *
33 mmol/L glucose + 100 μmol/L Toc	46.0 ± 10.5 *	56.9 ± 7.9	58.1 ± 7.2 *

NOTE. Data are the mean ± SD (n = 5 per group).
*$P < .01$.

fully established. In this study, elevated glucose levels caused enhancement of lipid peroxidation, cytotoxicity, DAG-PKC pathway activity, and fibronectin and eicosanoid production in cultured human MCs, as previously reported.[6-8,10,32,33] This is the first demonstration that Toc can prevent or inhibit glucose-induced overproduction of fibronectin and eicosanoids, as well as suppress lipid peroxidation and DAG-PKC pathway activity, in MCs.

Since mesangial expansion is a prominent feature of diabetic nephropathy,[1,2] altered MC functions induced by a high ambient glucose have been thought to play a central role in the pathogenesis of diabetic nephropathy. Multiple studies[6,34] have established that high glucose concentrations increase PKC activity in MCs as a consequence of increased de novo synthesis of DAG from glycolytic intermediates. Enhanced DAG-PKC pathway activity may be principally involved in the regulation of several cellular functions highly relevant to the alterations seen in diabetic glomerulopathy. For example, PKC activation has been related to the modulation of matrix protein production through the action of tumor growth factor-beta.[35,36] Activation of PKC has also been linked to increased synthesis of eicosanoids in MCs, possibly via activation of phospholipase A_2 with the release of arachidonate.[10] Increased production of matrix proteins and eicosanoids from MCs could lead to the mesangial expansion and abnormal intraglomerular hemodynamics seen in patients with diabetic nephropathy. Therefore, the finding that Toc suppressed the glucose-induced overproduction of fibronectin and eicosanoids may provide insight into possible treatments for diabetic nephropathy.

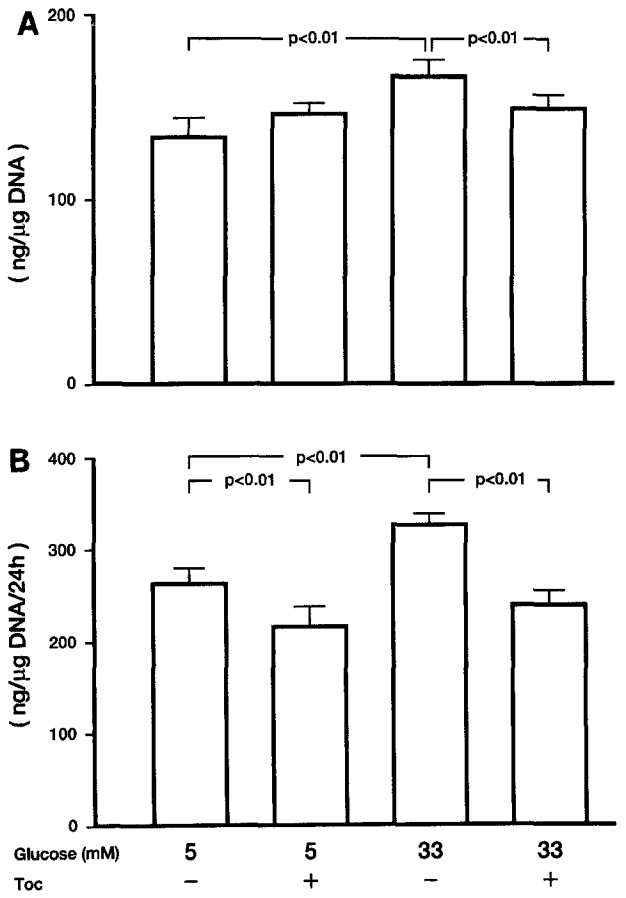

Fig 5. Effects of glucose and 100 μmol/L Toc on the amount of fibronectin in the cell layer (A) and in the medium (B) (n = 8). Values are the mean ± SD.

It is noteworthy that Toc suppressed DAG-PKC pathway activity without affecting cell number. There has been controversy as to the effect of Toc on cell proliferation. In a vascular smooth muscle cell line, Toc showed an inhibitory effect on cell growth,[17] whereas Trachman et al[37] demonstrated that Toc reversed the inhibitory effect of high glucose and advanced-glycosylation end products in rat MCs. The diverse results between the latter report and the present study may be attributed to differences in the experimental design or species used. They used the FCS-deprived medium as a experimental medium, whereas the medium was supplemented with 10% FCS in this study because a relatively large amount of FCS was required for solubilization of DMSO-treated Toc. Our results showed that Toc attenuated the glucose-induced cytotoxicity without improving the decrease in glucose-induced cell number. To clarify this discrepancy, further experiments including various doses of Toc should be performed.

The mechanisms through which Toc modulates the production of fibronectin and eicosanoids may be largely explained by the suppressive effect on the DAG-PKC pathway. However, contrary to changes seen in DAG-PKC, Toc reduced neither the amount of fibronectin accumulated in the cell layer nor the secretion of eicosanoids from MCs exposed to low-glucose medium. This discrepancy in the result for fibronectin may be partly explained by the complex regulatory mechanism for the accumulation of fibronectin in cells. The amount of fibronectin secreted into the medium directly represents production from MCs, whereas fibronectin accumulation in MCs is determined by the rate of synthesis and degradation and the rate of binding and incorporation into MCs.[38] With regard to eicosanoids, it has been proposed that a glucose-induced increase in eicosanoid

Table 2. Effects of Glucose and Toc on Secretion of 6-Keto $PGF_{1\alpha}$ and TXB_2 From Human MCS

Additions	6-Keto $PGF_{1\alpha}$ (pg/μg DNA/30 min)		TXB_2 (pg/μg DNA/30 min)	
5 mmol/L glucose	19.3 ± 6.0	*	4.1 ± 0.6	*
5 mmol/L glucose + 100 μmol/L Toc	13.4 ± 3.4		4.4 ± 0.6	
33 mmol/L glucose	30.2 ± 3.9	*	5.5 ± 1.0	*
33 mmol/L glucose + 100 μmol/L Toc	13.5 ± 4.9		3.7 ± 0.5	

NOTE. Data are the mean ± SD (n = 6 per group).
*$P < .05$.

production by MCs is mediated via glucose-induced PKC activation.[10] However, at a physiological concentration of glucose, factors other than PKC may principally modulate eicosanoid production.

Precise mechanisms for the effect of Toc on DAG are unclear. Our observation showing that Toc inhibited lipid peroxidation of MCs is consistent with the well-recognized effect of Toc as a potent biological antioxidant. Besides its effect as an antioxidant, alternative roles for Toc, such as a membrane stabilizer[39] and inhibitor of 5-lipoxygenase activity,[40] have been proposed. Toc has recently been shown to modulate DAG-PKC pathway activity in other cell lines such as vascular smooth muscle cells[17] and vascular endothelial cells.[18] Tran et al[18] have demonstrated that RRR-Toc suppressed the level of DAG in vascular endothelial cells via conversion of DAG kinase activity, resulting in increased conversion of DAG to phosphatidic acids. The exact molecular mechanism of this action remains to be determined in MCs.

Finally, these results provide evidence that Toc modulates both lipid peroxidation and the DAG-PKC pathway, which may lead to suppression of glucose-induced overproduction of mesangial matrix proteins and eicosanoids in cultured human MCs. Therefore, we suggest the possibility of Toc as a beneficial treatment for diabetic nephropathy. However, one study[41] has recently evaluated the long-term effect of vitamin E on streptozotocin (STZ)-diabetic rats. Administration of a vitamin E–enriched diet did not reduce oxidant stress and exacerbated the nephropathy as assessed by urinary protein excretion and renal histopathology. Although the investigators assumed that this effect of vitamin E may be a consequence of alterations in ascorbic acid metabolism in diabetes that convert vitamin E into a prooxidant molecule, the exact mechanism was unclear. In addition, the lack of monitoring Toc concentrations in peripheral blood or tissues may make these results unreliable. On the other hand, Kunisaki et al[42,43] have shown that intraperitoneal injection of Toc normalized the upregulated DAG-PKC activation of the aorta and retina in STZ-diabetic rats. The conflicting findings for the effect of Toc in diabetic rats may be due to differences in the mode of administration and duration of Toc treatment. Thus, our findings warrant further in vivo study on the effect of Toc in diabetic nephropathy.

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