Rapid Publication

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

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Hyperglycemia and diabetes have been shown to increase diacylglycerol (DAG) level and activate protein kinase C (PKC) activity in the vascular tissues, possibly altering vascular function. We have characterized the effects of D-α-tocopherol (vitamin E) on PKC activities and DAG levels in rat aortic smooth muscle cells (ASMCs) cultured with elevated glucose levels as well as in the vascular tissues obtained from control and diabetic rats. In ASMCs, the specific PKC activity from the membraneous fraction and total DAG level were increased by 31 ± 4% ($P < 0.05$) and 50 ± 7% ($P < 0.05$), respectively, when the glucose levels were changed from 5.5 to 22 mmol/l. The addition of D-α-tocopherol and another lipophilic antioxidant, probucol, prevented the glucose-stimulated increases in DAG level and PKC activity. By immunoblotting studies, D-α-tocopherol treatment was able to reduce the enhancement of PKC βII isoform in the membraneous fraction isolated from ASMCs. Comparing streptozotocin-induced diabetic rats with their nondiabetic controls, both membraneous-specific PKC activities and total cellular DAG levels were increased in aorta by 162% ($P < 0.05$) and 60% ($P < 0.05$), respectively. Intraperitoneal injection of D-α-tocopherol (40 mg/kg) every other day prevented the increases in membraneous-specific PKC activities and total DAG levels in parallel with a significant increase of D-α-tocopherol contents in the aorta and plasma. These findings have demonstrated that D-α-tocopherol can prevent the activation of PKC activities in the vascular cells and tissues induced by hyperglycemia by lowering DAG levels, possibly via its antioxidant effect.
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DAG, diacylglycerol; PKC, protein kinase C; STZ, streptozotocin; ASMC, aortic smooth muscle cell; PBS, phosphate-buffered saline; PMA, phorbol myristic acid.

Vascular complications of diabetes occur in both micro- and macrovessels, with hyperglycemia being an important risk factor (1). Multiple biochemical hypotheses have been proposed to explain the mechanism by which hyperglycemia could cause vascular dysfunctions (2–6). One mechanism is that hyperglycemia can exert its adverse effects by activating the diacylglycerol-protein kinase C (DAG-PKC) pathway (5–8). Recently, we and others have reported that these two biochemical parameters are increased in the retina (6), aorta (5), heart (5,7), and renal glomeruli (8) of diabetic rats as well as in cultured vascular cells or tissues exposed to elevated levels of glucose and galactose (5,6,9–12). Functionally, several studies have indicated that various abnormal vascular functions observed in diabetic animals can be normalized by using PKC inhibitors (4,6). For example, we have reported that abnormalities in the retinal hemodynamics of the streptozotocin (STZ)-induced diabetic rats were normalized by intravitreous injection of PKC inhibitor and mimicked in nondiabetic rats by PKC agonist (6). However, in vivo studies with PKC inhibitor have been hindered by the toxicity and nonspecificity of the available agents (13). Interestingly, recent studies have suggested that in cultured cells (14) and macrophages (15), the addition of D-α-tocopherol can prevent the activation of PKC activities via metabolic modulation rather than direct inhibition. D-α-tocopherol, the most active form of vitamin E, is an antioxidant that affects various membrane-bound enzyme systems and biochemical properties of plasma membrane via either antioxident or non-antioxidant pathway (16). Because hyperglycemia has been postulated to increase the level of oxidative stress and activate DAG-PKC levels in the vascular tissues of diabetic animals and patients (17), we determined whether D-α-tocopherol can prevent the glucose-induced increases in DAG levels and PKC activities in cultured smooth muscle cells as well as in vivo using the aorta of STZ-induced diabetic rats.

RESEARCH DESIGN AND METHODS

Cell culture. Rat aortic smooth muscle cells (ASMCs) were isolated from the thoracic and descending aorta of the Sprague-Dawley rats (Taconic Farms, Germantown, NY). These cells were then cultured

using the technique previously described for bovine ASMCs (5). Medium was changed to Dulbecco's modified Eagles's medium containing either 5.5 or 22 mmol/l glucose with or without D-α-tocopherol (50 μg/ml) and 2% serum and incubated for 3 days, when the maximal increase of DAG level was observed (11). Medium was changed daily to maintain the desired glucose and D-α-tocopherol levels.

Animals. Male Sprague-Dawley rats (Taconic Farms) weighing 230–260 g were injected intraperitoneally with STZ in citrate buffer (20 mmol/l, pH 4.5) at 65 mg/kg body weight. Twenty-four hours after injection, the development of diabetes was identified by the findings of hyperglycemia (>250 mg/dl of blood glucose). Half of the control and diabetic rats were randomly selected to receive D-α-tocopherol (40 mg/kg) or placebo vehicle (HCO, polyethylene 60-hydrogenated castor oil) provided by Eisai Company (Tokyo, Japan), which were injected intraperitoneally every other day for 2 weeks at the onset of diabetes, with the rats killed 1 day after the final injection. The levels of D-α-tocopherol in plasma and aorta in the rats were determined by the method of Ueda and Igarashi (18) using high-performance liquid chromatography determination after 2 weeks of treatment.

Extraction and assay of total DAG. After 3 days of incubation with or without D-α-tocopherol in the presence of 5.5 or 22 mmol/l glucose, the cell experiments were terminated by the addition of 2 ml of ice-cold methanol to ASMCs, and samples were transferred to chloroform-resistant tubes. Total lipid was extracted, and total DAG was determined by an enzymatic assay using DAG kinase (Lipidex, Westfield, NJ), as previously reported (5). DAG kinase used in vitro for the assay was not inhibited by D-α-tocopherol.

After the rats were treated for 2 weeks with D-α-tocopherol or vehicle, aortas were rapidly dissected, washed in ice-cold phosphate-buffered saline (PBS) (Ca^{2+} and Mg^{2+} free; pH 7.4), and frozen in liquid N_2. Aorta as crushed frozen powder was weighed and thawed in 2 ml of 100% methanol and homogenized with a Polytron for 20 s. Total DAG was determined by the same methods described previously (5).

Labeling of DAG with [^{3}H]palmitate. After 3 days of incubation with or without D-α-tocopherol in the presence of 5.5 or 22 mmol/l glucose, [^{3}H]palmitate was added at the concentration of 5 μCi/ml and incubated for 16 h. The uptake of [^{3}H]palmitate was not different among the four groups. Then the reaction was terminated, and total lipids were extracted as described above. Labeled DAG was separated on silica gel G thin layer plates developed in hexane:ether:acetic acid (60:40:1). The spot of DAG was visualized using oxaloacetate and H_2SO_4 and identified by using DAG standard as described (11,12).

Partial purification and assay of PKC. Confluent ASMCs were washed twice with PBS without Ca^{2+} and Mg^{2+} and once with buffer A (20 mmol/l Tris-HCl, pH 7.5, 2 mmol/l EDTA, 0.5 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mmol/l dithiothreitol, 0.3 mmol/l sucrose, and 25 mg/ml leupeptin). For studies using phorbol 12-myristate 13-acetate (PMA, 160 nmol/l), the cells were exposed to PMA for 15 min at 37°C. PKC proteins in the membraneous and cytosolic fractions were partially purified, and PKC activity was determined as described previously (5). D-α-tocopherol at 50–100 μg/ml did not inhibit PKC activities using partially purified PKC preparations from rat brain. Protein determination was performed according to the method of Bradford (19).

Immunoblotting of PKC isoforms. Partially purified PKC preparations from cultured ASMCs were separated on 7.5% sodium dodecyl sulfate gel under reducing conditions and transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH) as previously described (5). The nitrocellulose paper was blocked overnight with 3% bovine serum albumin in Tris-buffered saline (50 mmol/l; pH 7.5) and then incubated with monospecific anti-peptide antibodies. The specificity of each anti-peptide antibody to PKC-α and βII isoform has been described in detail previously (6). The nitrocellulose blot was incubated with ^{125}I-labeled protein A (Amersham, Arlington Heights, IL, 30 mCi/mg), and results were analyzed using videodensitometry.

Determination of PKC activity of rat aorta was performed in the same manner as described above and as previously reported (5).

Statistical analysis. Statistical analyses were conducted using the Student's *t* test. Multiple comparisons among groups were made with the Newman-Keuls test. Data are shown as means ± SE unless stated differently. $P < 0.05$ was statistically significant.

RESULTS

Effect of D-α-tocopherol on total DAG and [^{3}H]palmitate-labeled DAG levels in ASMCs. We have reported previously that total DAG level in ASMCs incubated at 22 mmol/l vs. 5.5 mmol/l glucose was increased after 2 days of incubation, with maximal effect observed at 3 days (11). In cultured ASMCs, the dose-response curve on the effect of D-α-tocopherol was determined beginning with 25 μg/ml, which inhibited glucose-induced DAG increase by 25% and normalized the increase in DAG level at 50–100 μg/ml of D-α-tocopherol. As shown in Fig. 1*A*, total DAG levels in ASMCs were increased significantly by 50 ± 7% ($P < 0.05$) when exposed to 22 vs. 5.5 mmol/l glucose. The addition of D-α-tocopherol (50 μg/ml) to media containing elevated glucose level (22 mmol/l) prevented the increases of total DAG in ASMCs, whereas no changes were noted in ASMCs incubated with 5.5 mmol/l glucose and D-α-tocopherol.

The effect of D-α-tocopherol on [^{3}H]palmitate incorporation into DAG in ASMCs was also examined to determine whether D-α-tocopherol can affect the DAG fraction that was derived from de novo pathway or phosphotidylcholine metabolism (11,12). As shown in Fig. 1*B*, exposure of ASMCs to 22 mmol/l glucose for 3 days increased [^{3}H]palmitate-labeled DAG levels by 30 ± 7% ($P < 0.05$). When D-α-tocopherol was added with 22 mmol/l of glucose, the amount of [^{3}H]palmitate-labeled DAG in the ASMCs increased only by 9%, which was not statistically significant from cells exposed to 5.5 mmol/l glucose. Similar to total DAG level, the presence of D-α-tocopherol did not affect [^{3}H]palmitate level in cells exposed to 5.5 mmol/l glucose. To characterize D-α-tocopherol's inhibitory action on glucose's effect on DAG level, we have investigated the effect of probucol (100 μmol/l), which is a potent lipophilic antioxidant but structurally different from D-α-tocopherol (20), on DAG synthesis as measured by [^{3}H]palmitate-labeled DAG fractions. Probucol also inhibited the glucose-induced DAG synthesis to a similar extent as D-α-tocopherol (103 ± 6% vs. 5.5 mmol/l glucose, $P < 0.05$ vs. 22 mmol/l glucose, $n = 3$), suggesting that D-α-tocopherol is probably mediating its effect via an antioxidative mechanism.

Effect of D-α-tocopherol on PKC activity and isoform levels in cultured rat ASMCs. Because one of the consequences of DAG increases is an increase of PKC activities in the membraneous fractions, the effect of D-α-tocopherol on PKC activities in ASMCs was characterized (Fig. 2). Specific PKC activities in the membraneous fraction were increased by 31 ± 4% ($P < 0.05$, $n = 6$), when glucose concentrations were changed from 5.5 to 22 mmol/l for 3 days, but no significant changes were observed in the cytosolic fraction. D-α-tocopherol almost completely reverses the increases of PKC activities in the membrane to control levels. The addition of PMA (160 nmol/l), a potent PKC agonist, increased PKC activity in the membraneous fraction, with an expected decrease in the cytosol. In contrast to its effect in high glucose conditions, D-α-tocopherol was not able to prevent PMA's stimulatory effect.

The effect of glucose concentration on the expression of PKC isoforms α and βII was also characterized (Fig. 3). Quantitative analyses by Western blot showed that stimulation with PMA increased the protein content in the membraneous fractions of both PKC-α and PKC-βII, consistent with properties of PKC, but a significant decrease was only observed in the cytosol fraction of βII, not in the α isoform. Exposure of ASMCs in 22 mmol/l glucose for 3 days did not affect the protein content of PKC-α in either the cytosolic or membraneous fractions, whereas the membraneous fraction of PKC-βII was increased by 110 ± 20% ($n = 3, P < 0.05$), and

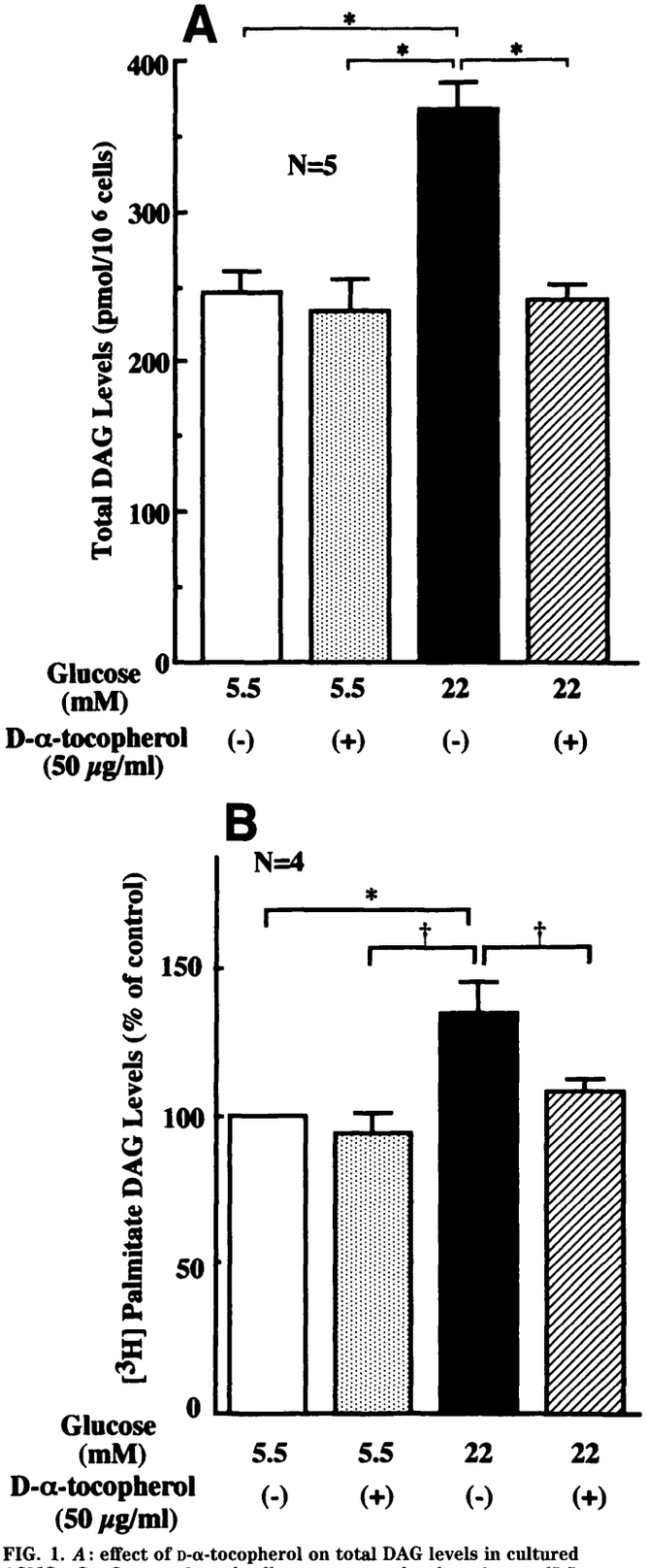

FIG. 1. *A*: effect of D-α-tocopherol on total DAG levels in cultured ASMCs. Confluent cultured cells were exposed to low glucose (5.5 mmol/l), low glucose plus D-α-tocopherol (50 μg/ml), high glucose (22 mmol/l), and high glucose plus D-α-tocopherol (50 μg/ml) for 3 days, respectively. *B*: effect of D-α-tocopherol on [^{3}H]palmitate incorporation into DAG in rat ASMCs. After 3 days incubation with low glucose (5.5 mmol/l) or high glucose (22 mmol/l) and with or without D-α-tocopherol (50 μg/ml), [^{3}H]palmitate (5 μCi/ml) was added. Radioactivity in labeled DAG was counted after 16 h. Each experiment was done in triplicate with each using three confluent 35-mm culture dishes. N represents the number of independent experiments. Results are shown as means ± SE. *, †$P < 0.05$.

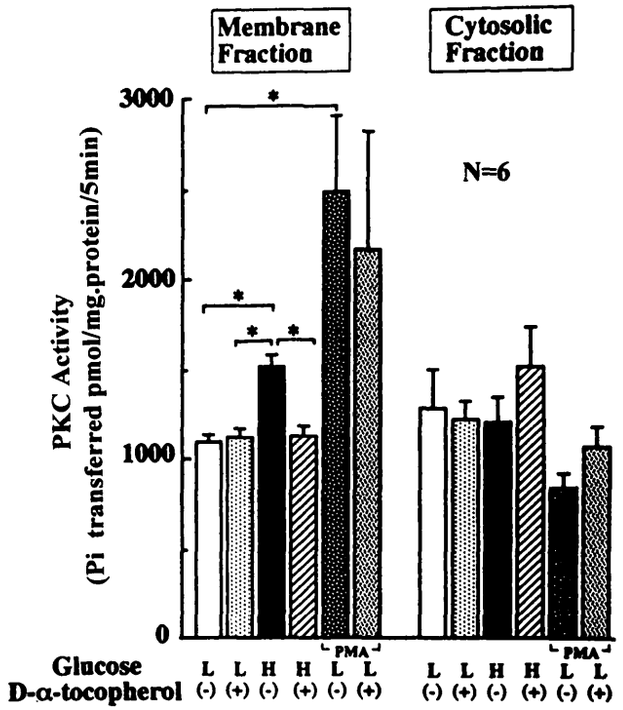

FIG. 2. Effect of glucose and D-α-tocopherol on PKC activity (picomole of Pi transferred per milligram protein per 5 min) in cultured ASMCs. Confluent cultured cells were exposed to low glucose (L, 5.5 mmol/l), low glucose plus D-α-tocopherol (50 μg/ml), high glucose (H, 22 mmol/l), and high glucose plus D-α-tocopherol (50 μg/ml) for 3 days, respectively. PKC-specific activities were measured in both membraneous and cytosolic fractions. For each study, cells from three 150-mm dishes were collected for each condition. PKC activity and protein were partially purified as described in METHODS. For studies using PMA (160 nmol/l, n = 3), the cells were exposed to the PMA for 15 min at 37°C. N represents the number of independent experiments. Results are shown as means ± SE. *$P < 0.05$.

a modest decrease was observed in the cytosolic fraction (11 ± 2%), statistically not significant. Similar to PKC activities, the addition of D-α-tocopherol prevented the glucose-induced but not PMA-stimulated increases in the membraneous fraction and the modest decrease in cytosolic fraction of PKC-βII but did not alter the cellular distribution of PKC-α in ASMCs.

Effect of D-α-tocopherol treatment on D-α-tocopherol levels in the plasma and aorta. To determine whether D-α-tocopherol can be effective in vivo as well as in cultured cells, the effect of intraperitoneally injected D-α-tocopherol on plasma and tissue level were determined (Table 1). The physiological parameters of rats used in the study showed diabetic rats had a significant elevation of glucose levels from 122 ± 7 to 397 ± 6 mg/dl that were not affected by treatment with D-α-tocopherol. Intraperitoneal injection of D-α-tocopherol at a dose of 40 mg/kg per every other day increased D-α-tocopherol levels by 633 and 410%, respectively, in the plasma and aorta of diabetic rats. For the control rats, D-α-tocopherol contents were increased by 479 and 290% in the plasma and aorta, respectively.

Effect of D-α-tocopherol treatment on total DAG levels in aorta. After 2 weeks of diabetes (Fig. 4), total DAG levels in the aorta were significantly increased by 60% (n = 6; $P <$ 0.05) compared with nondiabetic controls, similar to previous studies (5). When the diabetic rats were treated with D-α-tocopherol, the total DAG levels in the aorta of diabetic

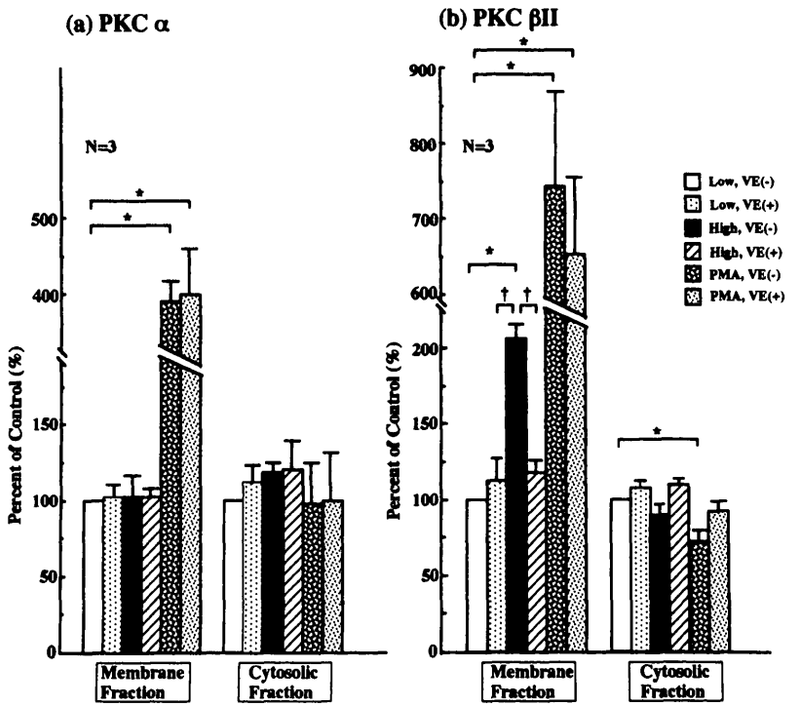

FIG. 3. Quantitative analyses of Western blot using videodensitometry. For each study, cells from three 150-mm dishes were collected for each condition. Confluent cultured cells were exposed to low glucose (5.5 mmol/l), low glucose plus D-α-tocopherol (50 μg/ml), high glucose (22 mmol/l), and high glucose plus D-α-tocopherol (50 μg/ml) for 3 days, respectively. PKC protein was partially purified as described in METHODS. For studies using PMA (160 nmol/l, $n = 4$), the cells were exposed to PMA for 15 min at 37°C. The same amount of protein was applied for cytosol and membrane. N represents the number of independent experiments. Results are shown as means ± SE. *, †$P < 0.05$.

rats did not increase significantly from those of control rats. Similar to cultured ASMCs, total DAG levels in the aorta of control rats were not affected by the D-α-tocopherol treatment (Fig. 4).

Effect of D-α-tocopherol treatment on PKC activities in the aorta. PKC-specific activities were measured in both membraneous and cytosolic fractions isolated from age-matched rats treated with either placebo or D-α-tocopherol in both control and STZ-induced diabetic rats. As shown in Fig. 5, after 2 weeks of diabetes, membraneous PKC-specific activities were significantly increased by 162% ($n = 6$, $P < 0.05$) in the aorta of diabetic rats compared with controls. D-α-tocopherol treatment normalized these diabetes-induced increases in the membraneous PKC activities. Treatment of control rats with D-α-tocopherol did not affect the PKC activities in the cytosolic or the membraneous fractions.

DISCUSSION

Multiple studies, including ours, have established that elevated levels of glucose or poorly controlled diabetes will increase total DAG levels or membraneous PKC activities in the vascular tissues as well as in cultured vascular cells (5–11,21,22). Consistent with previous studies (5,6,11,12), this study has demonstrated that both DAG levels and membraneous PKC activities are increased in rat aorta or cultured rat ASMCs when they are exposed to elevated levels of glucose or diabetic conditions. Extending these previous studies, the present studies have provided simultaneous evaluations of aorta with regard to DAG and PKC levels as well as documenting that PKC-βII, specifically, was increased by elevated levels of glucose. This result provides the first direct evidence that elevated glucose levels could specifically activate PKC isoform βII in cultured vascular cells, although previously we have reported similar findings in the aorta from diabetic rats (5). The mechanism for the preference of activating PKC βII isoform is not clear, although it is possible that PKC isoforms such as delta or zeta could be altered, since they were not evaluated. If the activation is solely because of DAG-induced translocation process, then both α and βII should have been activated because they have comparable affinities for DAG (13). However, it is possible that these PKC isoforms are located in different compartments intracellularly as some studies have postulated (13,23).

The impetus to characterize the effect of D-α-tocopherol's effect on DAG levels and PKC activities is derived from the reports of Boscoboinik et al. (14) and Mahoney and Azzi (24) that showed that the addition of D-α-tocopherol can inhibit serum-induced activation of PKC in cultured cells. They have suggested that the effect of D-α-tocopherol may be due to its lipid solubility, which could interact with the lipophilic domain of the plasma membrane and PKC, rather than due to

TABLE 1
Characterization of plasma and tissues of diabetic and control rats after 2 weeks treatment with D-α-tocopherol

		D-α-tocopherol content			
	n	Plasma (μg/ml)	Aorta (μg/g protein)	Weight (g)	Blood glucose (mg/dl)
Control placebo	4	6.8 ± 1.0	49 ± 6	340 ± 24	122 ± 7
Control D-α-tocopherol	5	33 ± 3.5*	141 ± 42*	330 ± 13	108 ± 15
Diabetes placebo	4	7.4 ± 1.1	36 ± 3	236 ± 17†	394 ± 12†
Diabetes D-α-tocopherol	8	47 ± 4.5‡	150 ± 15‡	230 ± 17†	399 ± 11†

Data are means ± SE. *$P < 0.01$ vs. control without D-α-tocopherol. †$P < 0.01$ vs. control with or without D-α-tocopherol. ‡$P < 0.01$ vs. diabetic without D-α-tocopherol.

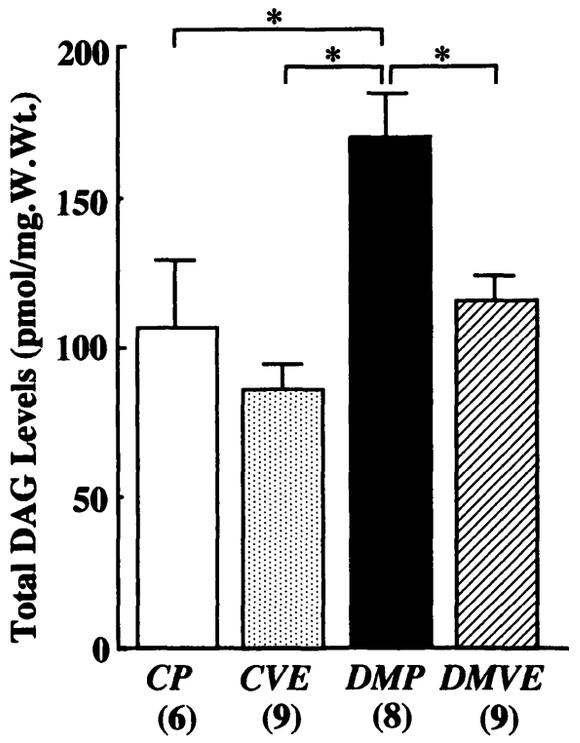

FIG. 4. Effect of D-α-tocopherol on total DAG levels were measured in aorta from age-matched controls with placebo (CP) or with D-α-tocopherol treatment (CVE), and from STZ-induced diabetic rats with placebo (DMP) or with D-α-tocopherol treatment (DMVE). DAG content was represented as picomole per weight (mg) of frozen dried tissue. Numbers in parentheses are measured numbers of preparations. Results are shown as means ± SE. *$P < 0.05$.

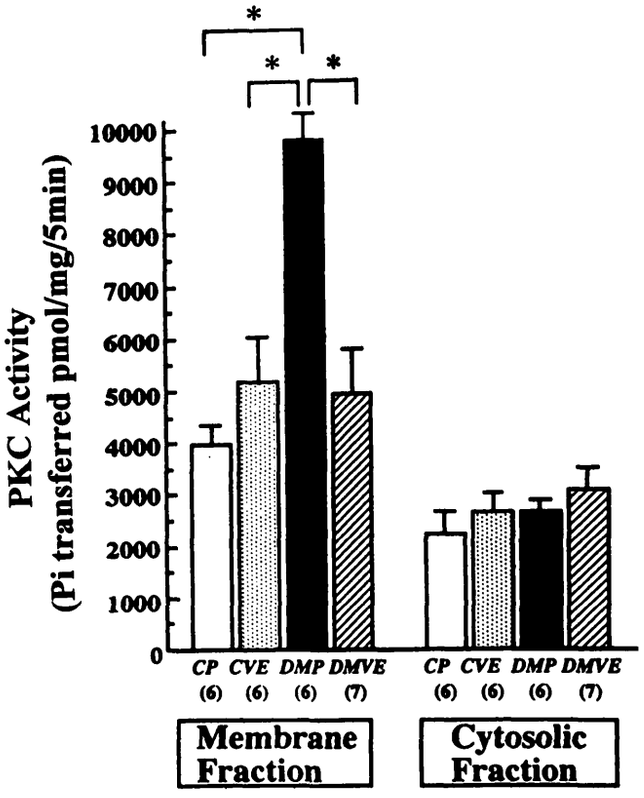

FIG. 5. Effect of D-α-tocopherol injection on PKC activity (pmol Pi · mg protein^{-1} · 5 min^{-1}) in rat aorta. PKC-specific activities were measured in both membranous and cytosolic fractions from age-matched controls with placebo (CP) or with D-α-tocopherol treatment (CVE), and from STZ-induced diabetic rats with placebo (DMP) or with D-α-tocopherol treatment (DMVE). Numbers in parentheses are measured numbers of preparations. Two rats were used for each preparation. Results are shown as means ± SE. *$P < 0.05$.

its antioxidant effects, because D-α-tocopheryl acetate, being equally potent for antioxidant as for D-α-tocopherol, was less effective in stabilizing membrane fluidity or preventing PKC activation (14). In cultured cells, this same group and others have shown that D-α-tocopherol could also inhibit the proliferation of a variety of cells, including smooth muscle cells (25), neuroblastoma cells (25), and cancer cells (26). The inhibitory effect of D-α-tocopherol was concordant with its effect on PMA-stimulated PKC activation and phosphorylation of MARCKS protein in the ASMCs, but D-α-tocopherol's effect on DAG levels was not evaluated (14). Our study was able to demonstrate that D-α-tocopherol can also prevent the activation of PKC-βII in cultured rat ASMCs when exposed to elevated levels of glucose. This effect of D-α-tocopherol is probably not due to its inhibition of PKC activities directly, because the concentration required (200 μg/ml) to inhibit PKC activities in vitro far exceeds the D-α-tocopherol concentration needed to inhibit glucose-induced changes. Most likely, the inhibitory effect of D-α-tocopherol on glucose-induced PKC activation is because of the normalization of DAG level. This effect of D-α-tocopherol has not been reported previously and suggests the effect of D-α-tocopherol in vivo may not be inhibiting PKC activity directly, but it is altering the metabolism of DAG in the cellular membrane. The results described here clearly show that D-α-tocopherol can reverse the effect of elevated glucose levels on DAG and PKC activity. One likely explanation for D-α-tocopherol's effect may be associated with its antioxidant properties (16). Our results support this possibility because probucol, a structurally different antioxidant, was able to prevent the increase in DAG levels to the same extent as D-α-tocopherol.

Multiple studies have indicated that vascular cells and tissues when subjected to elevated levels of glucose are exposed to an increased level of oxidative stress (17,27). However, it is not clear how this increased level of oxidation can affect DAG levels and PKC activities. It is also possible that this effect of D-α-tocopherol could be attributed to its actions on the membrane, such as membrane fluidity (28), inhibition of 5-lipoxygenase activity (29), and stimulation of PGI_2 synthesis (30). This is possible because both D-α-tocopherol and probucol can be concentrated in the membraneous pool because of its lipophilic properties and could reach a concentration that would alter membrane properties via non-antioxident pathways such as fluidity that are known to be affected by oxidants. The changes in the membrane properties could in turn affect the association of DAG kinase and cause its activities to decrease. Treatment with lipophylic antioxidant can prevent this series of events. Further studies should differentiate these possibilities.

Besides being effective in cultured vascular cells, we have also demonstrated that D-α-tocopherol is effective in vivo by reversing these biochemical changes in the aorta of diabetic rats. Measurements of D-α-tocopherol levels in plasma and aorta did not detect any difference between control and diabetic rats. When treated with D-α-tocopherol, the levels of D-α-tocopherol reached in diabetic animals are similar to that in nondiabetic controls, suggesting that the uptake and incorporation of D-α-tocopherol in the diabetic rats are not

altered. D-α-tocopherol treatment is able to prevent the glucose-induced increases in DAG and the activation of PKC in vivo as well as in cultured cells. It is quite interesting that treatment with D-α-tocopherol prevented the increases in DAG and PKC in spite of the fact that the diabetic rats had elevated levels of glucose. This finding has a significant clinical implication because it suggests that vascular abnormalities could be prevented even if euglycemia may not be strictly maintained.

In summary, these results have identified that high concentration of glucose can preferentially activate PKC isoform βII in vascular smooth muscle cells. The finding that D-α-tocopherol reversed hyperglycemia's adverse effects both in cultured cells and in vivo has provided insights into both the pathogenesis and possible treatments for diabetic vascular complications.

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