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

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ABSTRACT In the present study, we have measured protein kinase C (PKC) specific activities and total diacylglycerol (DAG) level in the aorta and heart of rats, which showed that after 2 weeks of streptozotocin (STZ)-induced diabetes, membranous PKC specific activity and total DAG content were increased significantly by 88% and 40% in the aorta and by 21% and 72% in the heart, respectively. Hyperglycemia was identified as being a causal factor since elevated glucose levels increased DAG levels in cultured aortic endothelial and smooth muscle cells. Analysis by immunoblotting revealed that only α and βII PKC isoenzymes are detected in these two tissues and vascular cells among those studied. In STZ-induced diabetic rats, βII isoenzyme is preferentially increased in both aorta and heart, whereas PKC α did not change significantly. The increases in membranous PKC specific activity and DAG level are observed in both spontaneous diabetes-prone diabetic BB rats as well as in STZ-induced diabetic BB and Sprague-Dawley rats, which persisted for up to 5 weeks. After 2 weeks of diabetes without treatment, the normalization of blood glucose levels for up to 3 weeks with islet cell transplants in STZ-induced diabetic BB rats reversed the biochemical changes only in the heart, but not in the aorta. These results suggest that PKC activity and DAG level may be persistently activated in the macrovascular tissues from diabetic animals and indicate a possible role for these biochemical parameters in the development of diabetic chronic vascular complications.

Cardiovascular disease is the leading cause of mortality and morbidity in diabetic patients (1, 2). Pathological studies have shown an increased rate of atherosclerotic lesions (1, 2); cardiac muscle cell dysfunction has also been reported (3). Hyperglycemia appears to be one of the many possible risk factors in diabetic patients (4, 5). Several hypotheses have been proposed to explain the adverse effects of hyperglycemia (6–8). We have reported that protein kinase C (PKC) was activated in the retina of diabetic rats as well as in cultured vascular cells exposed to elevated levels of glucose (9). The mechanism of PKC activation may be due to increased production of diacylglycerol (DAG) in retinal microvessels due to high glucose levels (9). Other investigators have reported that hyperglycemia and glucose can activate PKC in the microvessels of renal glomeruli (10) and granulation tissues (11) due to an increase in production of DAG.

The alteration of PKC by diabetes and glucose is interesting since PKC activity has been shown to modulate smooth muscle growth (12) and contraction (13, 14), endothelial cell permeability (15), expression of signal transduction of hormones and growth factors (16, 17), and cardiomyocyte contractility (18). All of these functions have been reported to be abnormal in diabetic animals or patients. In the present study, we have determined the changes of PKC specific activities and its isoforms as well as DAG levels in the aorta and heart of diabetic rats. In addition, the normalization of glycemic control to reverse these PKC and DAG changes has been determined by islet cell transplantation. The distinctive role of hyperglycemia has been identified further by determining the effects of elevated glucose levels on DAG levels in cultured macrovascular cells.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Taconic Farms) weighing 270–290 g were injected i.p. with streptozotocin (STZ; 80 mg per kg of body weight). STZ was prepared in 20 mM citrate buffer (pH 4.5) immediately before use. STZ diabetic and control rats had plasma glucose levels of 443 ± 46 and 110 ± 6 mg/dl, respectively (mean ± SD) (P < 0.01; n = 36) after 2 weeks. Male diabetes-prone (DP) BB rats were purchased from the National Institutes of Health contract colony of the University of Massachusetts (Worcester) and received 1.5–2.2 units of subcutaneous insulin per day for maintenance without achieving euglycemia (plasma glucose = 285 ± 72 mg/dl) (19). As a control for DP BB rats, age-matched diabetes-resistant (DR) BB rats were used (plasma glucose = 114 ± 7 mg/dl; n = 14). For intensive therapy, islet cell transplantation was performed as reported (20). Untreated DR BB rats served as islet cell donors. Islet cells were isolated by a modified collagenase digestion technique (21) and 8–12 fresh islet cells per g of body weight were transplanted under the renal capsule. DR BB rats were made diabetic by STZ injection (60–70 mg per kg of body wt) and served as islet cell recipients. Two weeks after the onset of diabetes, half of the diabetic rats were randomly selected to receive syngeneic pancreatic islet cell transplants. Two or 3 weeks after transplantation, rats were sacrificed for the study. STZ diabetic and islet cell transplanted BB rats had plasma glucose levels of 473 ± 129 and 131 ± 30 mg/dl (P < 0.01).

Cell Culture. Bovine aortic endothelial and smooth muscle cells were obtained from calf aorta as described (22). Aortic endothelial cells were cultured with Dulbecco’s modified Eagle’s medium (DMEM) with 10% plasma-derived horse serum and smooth muscle cells were cultured with DMEM with 10% calf serum.

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; STZ, streptozotocin; DP, diabetes prone; DR, diabetes resistant.

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Partial Purification and Assay of PKC. Rats were sacrificed 2–5 weeks after the onset of diabetes. Aorta and heart were rapidly dissected and washed with ice-cold phosphate-buffered saline (Ca²⁺ and Mg²⁺ free; pH 7.4). Immediately, aorta and heart were frozen in liquid N₂ and then crushed into frozen powder. PKC was partially purified from aorta and heart. Briefly, the samples were homogenized at 4°C in buffer A (20 mM Tris-HCl, pH 7.5/2 mM EDTA/0.5 mM EGTA/1 mM phenylmethylsulfonyl fluoride/25 μg of leupeptin per ml/0.1 mg of aprotinin per ml/33 M sucrose) with Polytron for 20 sec and then homogenized again with 60 strokes of a Dounce homogenizer. The homogenates were centrifuged at 1000 × g for 10 min and the supernatant was ultracentrifugation at 100,000 × g for 30 min at 4°C. The resulting supernatant was retained as the cytosolic fraction. The pellets were washed and resuspended with buffer B (buffer A without sucrose) and homogenized again. The homogenates were solubilized in buffer B with 1% Triton X-100. After incubating for 45 min, soluble fractions were obtained by ultracentrifugation at 100,000 × g for 30 min and were retained as membranous fractions. Both membranous and cytosolic fractions were passed through 0.5-ml DEAE (Pharmacia) columns, washed twice in 2 ml of buffer B, and then finally eluted with 0.4 ml of buffer B containing 200 mM NaCl.

PKC activity was measured by its ability to transfer 32P from [γ-32P]ATP (NEN) into specific substrate octapeptide (RRKRTLRL) corresponding to the threonine phosphorylation sites of the epidermal growth factor receptor (residues 691–698) in the presence of Ca²⁺, phosphatidylserine (PS), and DAG (Avanti) as described (23). PKC activity was calculated by subtracting the nonspecific kinase activity obtained in the absence of Ca²⁺, PS, and DAG. Protein determination was performed according to the method of Bradford (24). In our studies, we found that the cytosol fraction contained 75% of the total PKC activities in the heart and ~85% of the activity in the aorta.

Immunoblotting of PKC Isoenzyme. Partially purified PKCs from rat aorta and heart were separated on 7.5% SDS gel under reducing conditions and transferred to nitrocellulose paper (Schleicher & Schuell). The nitrocellulose paper was blocked overnight with 3% bovine serum albumin in Tris-bismaleic acid saline (50 mM; pH 7.5) and then incubated with monospecific anti-peptide antibodies. Each anti-peptide antibody is specific for α, β, αI, and γ-PKC isoenzyme as described (23). The nitrocellulose blot was incubated with 125I-labeled protein A and results were analyzed by autoradiography and videodensitometry.

Extraction and Assay of Total DAG. Powdered frozen tissues obtained from aorta and heart as described above were weighed and thawed in 2 ml of 100% methanol and then homogenized with a Polytron for 20 sec. After addition of 2 ml of chloroform and 1 ml of H₂O to the homogenates, total lipids were extracted according to the methods of Bligh and Dyer (25). Total DAG in extracted lipids was measured as described by Priess et al. (26) with a DAG kinase kit purchased from Lipidex (Westfield, NJ). The resulting radiolabeled phosphatic acid, which was converted from DAG, was separated by thin-layer chromatography and scraped and assayed in a scintillation counter. The results for total DAG were normalized by wet weight since changes per cell are more meaningful with regard to the activation of PKC than changes per organ, which could be different in diabetic and control rats. No differences were found in the ratio of protein to wet weight of vascular tissues.

Data Analysis. Comparisons of control and diabetic groups were calculated by Student’s t test. Analysis of experiments with more than two groups was by Tukey’s method of multiple comparative analysis (27).

RESULTS

PKC Activities in Aorta and Heart from STZ-Induced Diabetic Rats. The specific activities of PKC in the membranous and cytosolic fractions of aorta and heart were measured in diabetic rats 2 weeks after the onset of diabetes. The PKC specific activities in the membrane fractions from the aorta were significantly increased by 88% (n = 11; P < 0.01; nonpaired t test) in diabetic rats as compared to those in control rats (Fig. 1). There was no significant difference in PKC activities in the cytosolic fractions between diabetic and control rats (Fig. 1). For the heart, PKC specific activities in the membrane fractions were significantly increased by 21% (n = 11; P < 0.05) in diabetic rats compared to those in control rats (Fig. 1). Again, no significant difference was found in the cytosolic fractions (Fig. 1).

Total DAG Levels in Aorta and Heart of STZ-Induced Diabetic Rats. One of the possible mechanisms for the increase in membranous PKC activities could be due to elevation of DAG levels in vascular tissue. Therefore, total DAG content was measured in aorta and heart of diabetic rats. After 2 weeks of diabetes, total DAG levels in aorta and heart were both significantly increased by 40% (n = 22; P < 0.05) and 72% (n = 23; P < 0.01), respectively, as compared to control rats.

PKC Activities and Total DAG Levels in Aorta and Heart of Diabetic BB Rats. To determine whether increased PKC activity in aorta and heart is specific for diabetes and not due to an effect of STZ, we examined PKC specific activities in aorta and heart of spontaneous autoimmune diabetic BB rats. PKC specific activities in aorta and heart of diabetic BB rats were both significantly increased in the membrane fraction

![Fig. 1](image-url) Effect of diabetes on PKC activity (pmol of P; transferred per mg per 5 min) in aorta and heart. PKC specific activities in aorta and heart were measured in both membranous and cytosolic fractions from age-matched controls (C) and STZ-induced diabetic rats (DM). N, number of preparations. For aorta, two rats were used for each preparation. For heart, one rat was used for each preparation. Results are shown as means ± SEM. N.S., not significant.
compared to age-matched controls, which were DR BB rats after 4 weeks of diabetes (3854 ± 283 vs. 2740 ± 105 pmol per mg per 5 min, \( P < 0.01; 1950 ± 154 vs. 1384 ± 48 \) pmol per mg per 5 min, \( P < 0.05 \), respectively). Similar to STZ-induced diabetic rats, no significant changes were found in the cytosolic fractions. Total DAG levels were also determined and found to be increased in both aorta and heart of diabetic BB rats (182 ± 21.6 vs. 116 ± 5.0 pmol/mg, \( P < 0.05; 134 ± 9.4 vs. 88.8 ± 5.0 \) pmol/mg, \( P < 0.01 \), respectively), after 4 weeks of diabetes.

Alteration in PKC Isoenzymes in Aorta and Heart from STZ-Induced Diabetic Rats. PKC isoenzymes in rat aorta and heart were characterized by using monospecific polyclonal antibodies against \( \alpha \), \( \beta I \), \( \beta II \), and \( \gamma \)-PKC isoenzymes. Only \( \alpha \) and \( \beta II \) PKC isoenzymes were detected in both rat aorta and heart. As shown in Fig. 2, only \( \beta II \) PKC isoenzyme in the membrane fraction was significantly increased in both aorta and heart of STZ diabetic rats by 82% and 64%, respectively. In the cytosolic fraction, there was no change in the amounts of \( \alpha \) and \( \beta II \) PKC isoenzyme in both aorta and heart. These results suggested that \( \beta II \) PKC isoenzymes were preferentially activated in macrovascular tissues in the diabetic state.

Effect of Glycemic Control by Islet Transplantation. To assess whether a glycemic control can reverse the increased PKC activities and total DAG levels in diabetic aorta and heart, islet cell transplantation was performed with STZ-treated DR BB rats (20). Two weeks after the onset of diabetes, islet cells were transplanted. Glycemic control was achieved during the next 2–3 weeks in transplanted rats. The results from transplanted rats were compared to DR nontreated diabetic rats and to similar STZ-treated diabetic BB rats without islet cell transplants. After 2–3 weeks of islet cell transplantation, PKC specific activities in the membrane fraction and total DAG levels in the heart were both completely restored to nontreated diabetic levels (Fig. 3). Surprisingly, in the aorta of islet cell-transplanted rats both membranous PKC activities and total DAG levels remained elevated to a similar degree as in nontreated diabetic rats, even after 2–3 weeks of glycemic control (Fig. 3).

Effect of Elevated Glucose Levels on Total DAG Levels in Cultured Bovine Aortic Endothelial Cells and Smooth Muscle Cells. The findings of increases in the membranous PKC specific activities and total DAG levels in diabetic aorta and heart suggest a possible causal role for hyperglycemia. Therefore, to determine whether elevated glucose levels can singularly affect DAG levels, we examined the chronic effect of elevated glucose levels on total DAG levels in cultured aortic endothelial cells and smooth muscle cells. As shown in Fig. 4, increasing glucose levels from 100 to 400 mg/dl for 4 days of incubation significantly increased total DAG levels in both

**Fig. 2.** Western blot analysis of PKC isoenzyme changes in STZ-induced diabetic rats. Autoradiography of representative experiment from heart (A) and aorta (B). (C) Videodensitometry analysis of Western blot. For one blot, preparations of PKC were pooled from three rats. n, Number of experiments performed. C, age-matched control rats; D or DM, STZ-induced diabetic rats; Memb, membrane fraction; Cyto, cytosolic fraction. Results are shown as mean percentage of control ± SEM. *, \( P < 0.05 \).

**Fig. 3.** Effect of islet cell transplantation on PKC activity (pmol of \( P_i \) transferred per mg per 5 min) (A) and total DAG levels (pmol/mg) (B) in aorta and heart. n, Number of preparations. For PKC measurements of aorta, two rats were used for each preparation. With PKC activities in heart and DAG levels in aorta and heart, preparation number is the same as rat number. C, age-matched control rats; DM, nontreated diabetic rats; Trpl, islet cell transplanted rats. Results are shown as means ± SEM. *, \( P < 0.05 \); **, \( P < 0.01 \).
The results from the present study have demonstrated that the specific activities of PKC in the membranous pool are increased in both the heart and aorta of diabetic rats. The mechanism of the increase is probably due to an increased amount of PKC in the membranous fraction since immunoblotting studies showed an increase in the amount of enzyme. The source of the membranous PKC could be due to an increase in translocation (28) or to an increase in PKC synthesis and translocation. If the latter mechanism is applicable, then a decrease should have been noted in the cytosolic PKC. The lack of changes in cytosolic PKC activities or protein cannot differentiate between these two possibilities since the assays used may not be sensitive enough to detect small changes in the amount of PKC or its activities in the cytosolic pool. Only a small change in the cytosol pool is expected since ~80% of the PKC activity is found to be located in the cytosol. Therefore, a small change in the cytosol would be magnified severalfold in the membranous fraction. Support for the activation hypothesis is also provided by the finding that total DAG levels are increased in both heart and aorta, since DAG is a physiological activator of PKC translocation (29). These increases in DAG and membranous PKC specific activities have been reported in other vascular tissues such as retina (9) and renal glomeruli (10). Okumura et al. have also reported that total DAG level was increased in the heart of diabetic rats (30) but a decrease was noted in the aorta (31). This difference in the results regarding the aorta is probably due to the fact that in Okumura's studies the aorta, but not the heart, was subjected to preincubation for 25 min at 37°C in buffer containing 100 mg of glucose per dl before undergoing a DAG extraction procedure, whereas we froze both types of tissue for DAG extraction immediately after removing them from the animals. Since DAG levels can fluctuate rapidly, the preincubation step could easily nullify the effect of diabetic milieu on aortic DAG levels (32).

The DAG increases could be due to hyperglycemia, since elevated glucose level can, by itself, increase total DAG levels in cultured aortic endothelial and smooth muscle cells. Preliminary data have also indicated that membranous PKC activity was increased similarly in cultured smooth muscle cells. Others have reported similar effects of elevated glucose levels on tissue DAG levels in renal glomeruli (33) as well as glomerular tissue (11). One possible mechanism of increased DAG level is via the de novo synthesis pathway suggested by studies with [3H]glucose to label DAG (33). This pathway of stimulating DAG synthesis has been reported in the islet cell as a possible signal for stimulation by glucose of insulin release (34–36). In vascular tissues, the source of increased level of DAG is probably from glucose as well, although the exact molecular mechanism by which the elevated glucose level causes an increase in de novo synthesis of DAG needs to be clarified.

One of the consequences of a persistent increased level of DAG as reported here is an elevation of PKC activities in the membranous pool, which persisted for up to 5 weeks without evidence of down-regulation. This effect of diabetes is not due to STZ, since the same changes are found in spontaneous autoimmune diabetic BB rats. Immunoblotting studies suggested that the increases in membranous PKC activities in both the aorta and the heart are due to an increase in PKC, chiefly the βII isoform. This is interesting since both of these vascular tissues also contain PKC isoform α, which was not significantly increased, indicating the possibility of differential regulation of these two PKC isoforms. There is some evidence that suggested various PKC isoforms may be regulated differently, such as in the rate of activation and down-regulation (37). In addition, PKC isoforms may have different subcellular distribution with the α isoforms mainly in the plasma membrane and the β isoform on the intracellular vesicular bodies (37). Therefore, it is possible, although speculative, that glucose may induce an increase in DAG levels differently at the subcellular sites, which could account for the preferential elevation of PKC βII. The findings of preferential activation of βII could also be due to differences in down-regulation of the PKC isoforms as has been reported (38). These possibilities need to be determined from additional studies.

We have also characterized the reversibility of PKC and DAG increases, with the surprising finding that after 2 weeks of STZ-induced diabetes these biochemical changes were not reversed in the aorta but were completely normalized in the heart. This again points out that the regulation of DAG and PKC levels in different vascular tissues are not the same. The institution of glycemic control by islet cell transplantation did reverse the abnormalities in the heart, but its lack of effect in the aorta does not rule out the possibility that a longer duration of glycemic control can be effective. These findings of differential reversibility of the PKC and DAG by glycemic control may have functional significance since previous studies by Pugliese et al. (39) have reported that various vascular dysfunction in the rats, such as blood flow and permeability, responded to a different extent. In some tissues such as optic nerve, sciatic nerve, and diaphragm, islet cell transplantation did not reverse the changes induced by diabetes (40). These studies have identified some biochemical changes induced by hyperglycemia in the vascular tissue and cells, which are not easily reversed by a glycemic control once they have been established, indicating that changes in PKC and DAG levels may contribute to abnormalities in vascular functions in experimental diabetic patients. A pathological mechanism responsible for the delay in reversing the biochemical and functional effects of hyperglycemia has not been deter-
mined. Since preliminary data have suggested that these biochemical findings are preventable if glycemic controls are instituted within a few hours, the lack of reversibility after several weeks of diabetes suggests more than just the involvement of protein synthesis but rather some genetic alteration or structural changes in the cells, which turn over slowly. One possible mechanism is molecular alteration induced by a nonenzymatic glycation process, although conclusive evidence needs to be presented that a significant amount of glycation with functional effect is occurring in intracellular proteins or DNA (44).

Functionally, the PKC family has been postulated to modulate vascular and cardiac contractility (13–14, 18) and to increase vascular permeability (15). Tesfamariam et al. (45) have reported that high levels of glucose will induce an impairment of endothelium-dependent relaxation of rabbit aorta, which was similar to that found in diabetic rabbits. A role for PKC was suggested by the fact that PKC inhibitor can block the effect of glucose and PKC activation can mimic the action of glucose. Clearly, further studies will be needed to document intracellular and functional effects of PKC activation.

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