Protein Kinase C Is Activated in Glomeruli from Streptozotocin Diabetic Rats

Possible Mediation by Glucose

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Abstract

Glomerular inositol content and the turnover of polyphosphoinositides was reduced by 58% in 1–2 wk streptozotocin diabetic rats. Addition of inositol to the incubation medium increased polyphosphoinositide turnover in glomeruli from diabetic rats to control values. Despite the reduction in inositol content and polyphosphoinositide turnover, protein kinase C was activated in glomeruli from diabetic rats, as assessed by an increase in the percentage of enzyme activity associated with the particulate cell fraction. Total protein kinase C activity was not different between glomeruli from control and diabetic rats. Treatment of diabetic rats with insulin to achieve near euglycemia prevented the increase in particulate protein kinase C. Moreover, incubation of glomeruli from control rats with glucose (100–1,000 mg/dl) resulted in a progressive increase in labeled diacylglycerol production and in the percentage of protein kinase C activity which was associated with the particulate fraction. These results support a role for hyperglycemia per se in the enhanced state of activation of protein kinase C seen in glomeruli from diabetic rats. Glucose did not appear to increase diacylglycerol by stimulating inositol phospholipid hydrolysis in glomeruli. Other pathways for diacylglycerol production, including de novo synthesis and phospholipase C mediated hydrolysis of phosphatidylcholine or phosphatidylinositol-glycan are not excluded.

Introduction

Previous studies have suggested that many of the complications of diabetes may be related to enhanced polyol pathway activity. In diabetic nerve (1–4), lens (1–4), and renal glomeruli (4–8) sorbitol has been reported to be markedly elevated and inositol content reduced. Moreover, treatment of diabetic rats with sorbinil, an inhibitor of aldose reductase activity, prevents cataract formation (9), increases nerve conduction velocity (10), reduces hyperfiltration (8), and may prevent the development of nephropathy (6, 7). Recently it has been proposed that a reduced state of activation of protein kinase C (2, 11) may be a common feature of tissues which exhibit enhanced polyol pathway activity and reduced inositol content as a consequence of diabetes. In this proposed schema (2), reduced tissue *myo*-inositol leads to reduced inositol phospholipid content and/or turnover, with a resultant reduced generation of diacylglycerol, an endogenous activator of protein ki-

nase C. Impaired diacylglycerol availability in turn could lead to the impaired activation of protein kinase C. It has been speculated that reduced protein kinase C activity may contribute to the development of diabetic complications including nephropathy (2). Some (12) but not all (13, 14) studies support this notion. With respect to the renal glomerulus, inositol content was reported to be reduced in the streptozotocin diabetic rat (5). However, the impact of reduced inositol content on inositol phospholipid turnover and the state of activation of protein kinase C in glomeruli has not been examined. Accordingly, in the present study we examined (*a*) inositol content; (*b*) the turnover of labeled inositol phospholipids; and (*c*) the state of activation of protein kinase C in glomeruli isolated from rat kidneys 1–2 wk after induction of diabetes with streptozotocin. The results demonstrate that protein kinase C is activated in glomeruli from diabetic rats despite a reduction in glomerular inositol content and the turnover of polyphosphoinositides. Studies in vitro suggest that activation of glomerular protein kinase C in diabetes may be mediated at least in part by glucose.

Methods

Treatment of rats. Female Sprague-Dawley rats (Zivic-Miller, Pittsburgh, PA) were weighed and placed in one of four groups. The number of rats in each group is indicated separately below for each parameter measured. Controls received 1 ml/kg body wt sterile 0.01 M citric acid, 0.09% saline, pH 4, by tail vein. The diabetic rats received 60 mg/kg body wt sterile streptozotocin (60 mg/ml in 0.01 M citric acid, 0.09% saline, pH 4) by tail vein. Where indicated, insulin treated diabetic rats received 10 U/kg per d of regular Iletin II pork insulin (Eli Lilly & Co., Indianapolis, IN) by osmotic minipump (2002; Alzet Corp., Palo Alto, CA) as previously described (15). Minipumps were implanted intraperitoneally 24 h after streptozotocin injection. All rats were allowed food and water ad lib. and were studied 1–2 wk after injection of vehicle or streptozotocin. Sorbinil treated diabetic rats received 20 mg/kg per d in the diet beginning the day before streptozotocin injection as previously reported (15). At death, blood was drawn from the abdominal aorta for determination of nonfasting plasma glucose by the glucose oxidase method (16).

Preparation of glomeruli. Rats were anesthetized with pentobarbital (5 mg/100 g) and the abdominal aorta exposed through a midline incision. An intravenous catheter (20 gauge) was inserted into the abdominal aorta below the renal arteries. A 20-ml syringe was attached to the catheter and the kidneys were perfused for 20–30 s with 20–30 ml of Krebs-Ringer bicarbonate buffer containing 1 mg/ml glucose (KRBG)[1] that had been previously equilibrated with 95% O_2, 5% CO_2. Glomeruli were prepared by graded sieving as previously described (17). The final suspension was composed of > 95% glomeruli as as-

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1. *Abbreviations used in this paper:* IP, inositol-1-phosphate; IP_2, inositol-1,4-biphosphate; IP_3, inositol-1,4,5-trisphosphate; KRBG, Krebs-Ringer bicarbonate buffer with glucose; OAG, 1-oleoyl 2-acetyl glycerol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate.

sessed by light microscopy. The viability of the isolated glomeruli was 95% or greater as determined by trypan blue exclusion.

Extraction of glomerular lipids. Glomeruli were suspended in 0.55 ml of H_2O and extracted with 250 μl 0.1 M Na_2EDTA, 2 M KCl, 2.5 ml methanol and 1 ml $CHCl_3$ as described by Cohen et al. (18). If ^{32}P incorporation into phospholipids was to be assessed, ^{14}C Labeled phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine were added (50,000 cpm of each) to assess recovery. Extracts were stored at $-20°C$ in one phase. Then 1 ml $CHCl_3$ and 500 μl of 0.1 M Na_2EDTA, 2 M KCl were added to separate the layers. The lower $CHCl_3$ layer was removed and the upper layer was extracted three times with 1 ml (each time) $CHCl_3$. The $CHCl_3$ layers were combined, evaporated to dryness, and resuspended in 0.5 ml of $CHCl_3$. Neutral and phospholipids were isolated by thin-layer chromatography as described below.

Determination of labeled diacylglycerol production. Glomeruli were prepared from 20 kidneys (10–20 mg protein), resuspended in 13 ml of KRBG, 95% O_2, 5% CO_2, and incubated for 1 h with 50 μCi of [^3H]myristate in 25 ml flasks (2 ml/flask). The additions shown were present for the final 15 min of incubation. After 60 min, glomeruli were centrifuged, and lipids extracted as described above. An aliquot of the lipid extract was mixed with 100 μg of carrier diacylglycerol. The sample was applied to a silica gel thin layer plate and the plate developed in hexane/ethyl ether/acetic acid 80:20:2 (19). Spots were visualized with I_2 vapor, scraped and counted.

Determination of [^{32}P]orthophosphate incorporation into phospholipids. Glomeruli were prepared from 20 kidneys, suspended in 8 ml of KRBG, 95% O_2, 5% CO_2 and incubated for 4 h at 37°C in duplicate with or without 5 mM inositol (2 ml/25 ml flask). [^{32}P]Orthophosphate (10 μCi) was present in each incube. At the end of 4 h the glomeruli were washed three times by centrifugation in 20 ml of KRBG, resuspended in 1 ml of H_2O and lipid extracts prepared as described above. Unlabeled phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP$_2$) (100 μg each) were added to the lipid extracts as carriers. The samples were then chromatographed on oxalate impregnated silica gel G thin layer plates and developed in $CHCl_3/CH_3OH/4$ N NH_4OH (45:35:10) (20). The lipids were visualized by exposure to I_2 vapor, scraped, and counted. Results shown were corrected for recoveries based on that of added [^{14}C]phospholipids, which were routinely 40–50%.

Determination of labeled inositol phosphates. Glomeruli were prepared from 20 kidneys, suspended in KRBG equilibrated with 95% O_2, 5% CO_2, and incubated for 2 h with 75 μCi [^3H]-*myo*-inositol. The glomeruli were washed three times by centrifugation in 20 ml KRBG, resuspended in 20 ml KRBG plus 10 mM LiCl, aliquoted (2 ml/25-ml flask) and incubated in triplicate at 37°C for 10 min in a shaking incubator. Additions were present for the final 3 min of incubation. Incubations were conducted in triplicate and extracts combined for isolation of inositol phosphates. The incubation was stopped by homogenization in 0.67 ml of 10% $HClO_4$ on ice. After centrifugation, inositol-1-phosphate (IP), inositol-1,4-bisphosphate (IP$_2$), and inositol-1,4,5-trisphosphate (IP$_3$) were separated by Dowex formate chromatography as previously described (21). [^3H]IP, [^3H]IP$_2$, and [^3H]IP$_3$ were used as standards.

Preparation, partial purification, and assay of protein kinase C. Glomeruli were prepared from 10 kidneys for each experimental condition examined and incubated as described in the footnotes to the Tables. Subcellular fractions were prepared, fractionated on DEAE cellulose, and assayed as previously described by Kraft et al. (22, 23). Glomeruli were homogenized in 5 ml of 20 mM Tris, pH 7.5, 0.5 mM EDTA, 2 mM phenyl methyl-sulfonylfluoride, 0.5 mM benzamidine (buffer A) and centrifuged for 60 min at 100,000 g. The supernatant (1.5–2.5 mg protein) was applied to a DEAE cellulose column. The 100,000 g pellet was resuspended in 5 ml of buffer A plus 0.2% Triton X-100 and incubated for 1 h at 0°C with stirring. The solubilized particulate fraction was then centrifuged at 100,000 g and the supernatant (4–5 mg/protein) applied to a DEAE cellulose column (4 × 0.75 cm). The columns were washed with 10 ml each of buffer A. Activity

was eluted with 40 ml of a linear gradient of 0.1 M NaCl in buffer A. Protein kinase C activity routinely eluted as a sharp peak between 0.03 and 0.04 M NaCl. As previously reported for other systems (22, 23), protein kinase C activity in subcellular fractions of crude homogenates of glomeruli was only 20% of that found following DEAE cellulose chromatography of the fractions. Accordingly, all preparations were routinely purified by DEAE cellulose before assay.

Reaction mixtures for the determination of protein kinase C activity contained 20 mM Tris, 10 mM $MgCl_2$, 400 μg/ml histone (Type III-S), 50 μM [^{32}P]ATP (1 μCi), 1 mM $CaCl_2$ and where indicated 80 μg/ml phosphatidylserine plus 2 μM 1-oleoyl 2-acetyl glycerol (OAG) in a final volume of 75 μl. These concentrations of $CaCl_2$, phosphatidylserine, and OAG yielded optimal expression of enzyme activity. DEAE cellulose column fractions were assayed with 1 mM $CaCl_2$ and in the presence and absence of phosphatidylserine plus OAG. Protein kinase C activity is defined as the difference between activity measured with Ca^{2+} in the presence versus the absence of OAG plus phosphatidylserine. Aliquots of phosphotidylserine (10 mg/ml ethanol) and OAG (0.25 mg/ml ethanol) were evaporated under N_2 and sonicated on ice in 20 mM Tris, pH 7.6 before addition to the enzyme assay mixture. Incubations were for 5 min at 30°C. Reactions were stopped by pipetting 50 μl of the assay mixtures onto a square (1 × 1 cm) of chromatography paper (31 ET; Whatman Inc., Clifton, NJ), which had been dipped in 10% TCA, 2 mM NaH_2PO_4. Filter papers were then washed with agitation in 250 ml of ice-cold 10% TCA for 15 min followed by four changes of 10% TCA at room temperature. The papers were soaked in 95% ethanol for 5 min and allowed to air dry before counting. Enzyme activity was linear for 2–5 min under all conditions of assay employed.

Determination of inositol. Glomeruli from eight kidneys were pooled for each determination of inositol content and homogenized in 3 ml of water. [^3H]Inositol (0.375 μCi) was added to each extract to monitor recovery and the extent of derivatization. The homogenates were centrifuged at 100,000 g for 1.5 h and the supernatant incubated for 40 min at 37°C with 24 U of glucose oxidase. Supernatants were deproteinized with the Centrifree Micropartition System (Amicon Corp., Danvers, MA) and fractionated on a Sugar Pak column (6.5 mm × 30 cm; Waters Associates, Milford, MA). The inositol fraction was then derivatized with *p*-chloronitrobenzene and further fractionated on a Waters microporasil column (6.5 mm × 30 cm) as previously described (24). The *p*-chloronitrobenzoate derivative of inositol was quantitated by absorption at 254 μm.

Statistics. When more than one mean value was to be compared to a single control value, the significance of differences was first determined by analysis of variance (ANOVA). If significant differences were obtained by ANOVA the significance of differences between any two average values was then determined by Student's independent test. Experiments were conducted three or four times as indicated in the footnotes to the Tables and legends to the Figures (degrees of freedom = 4 or 6).

Materials. Sorbinil was the generous gift of Pfizer Inc., Groton, CT. The sources of all other reagents have been previously reported (25, 26).

Results

Fig. 1 illustrates the content of inositol in glomeruli isolated from control and diabetic rats. As shown, inositol content was reduced by 58% in glomeruli from diabetic rats compared to controls. Treatment of diabetic rats with insulin to achieve near euglycemia prevented the fall in glomerular inositol content. Moreover, as is also shown in Table I, treatment of diabetic rats with the aldose reductase inhibitor, sorbinil also prevented the fall in glomerular inositol content. The average weight of the rats in each group was not significantly different at the start of the study (244±5 g). The diabetic rats that were
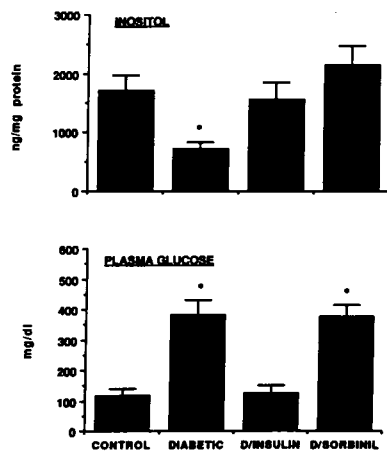
Figure 1. Glomerular inositol content and plasma glucose concentrations in control and diabetic rats. Glomeruli were isolated from eight kidneys and extracted as described in Methods for determination of free inositol. Insulin treated diabetic rats received 10 U of regular Iletin II pork insulin/kg per d by osmotic mini-pump. Sorbinil treated diabetic rats received 20 mg/kg per d in the diet. All rats were studied between 1 and 2 wk after injection of streptozotocin or its diluent (controls). Results shown are means±SE of duplicate determinations on four separate glomerular extracts ($n = 4$). *$P < 0.05$ compared to corresponding value in control rats.

not treated with insulin lost ∼ 8% of their body weight. Insulin-treated diabetic rats gained weight at the same rate as control rats. Sorbinil feedings had no effect on body weight. Animal weights at sacrifice were: control 261±14; diabetic 223±10; insulin-treated diabetic 264±16; sorbinil-treated diabetic 221±18 g±SE. Plasma glucose at the time of death was markedly elevated in the untreated and sorbinil treated diabetic rats, but near control values in the insulin treated diabetic rats (Fig. 1). Sorbinil treatment had no effect on body weight or plasma glucose in control rats, as previously reported (27).

Table I illustrates ^{32}P incorporation into phospholipids of glomeruli from diabetic versus control rats. In these studies isolated glomeruli were incubated for 4 h with [^{32}P]-orthophosphate. The results reflect the turnover of the individual labeled phospholipids. As shown, turnover of labeled PIP and PIP$_2$ were markedly reduced in glomeruli from diabetic versus control rats. By contrast, the turnover of labeled phosphatidylinositol, phosphatidylethanolamine, and phosphatidylcholine plus phosphatidylserine was not significantly different between the two groups. As also shown in Table I, addi-

tion of inositol to the incubation mixture significantly enhanced the turnover of labeled polyphosphoinositides of both control and diabetic glomeruli and abolished the differences between the two groups. These observations suggest that the reduction in turnover of labeled PIP and PIP$_2$ seen in glomeruli from diabetic rats was a consequence of the reduced inositol content of the diabetic glomeruli.

Table II illustrates the subcellular distribution of protein kinase C in homogenates of glomeruli from normal and diabetic rats. As shown, 88% of enzyme activity was recovered in the 100,000 g soluble fraction of glomeruli isolated from control rats. By contrast, only 55% of the protein kinase C activity was found in the soluble fraction of glomeruli isolated from diabetic rats. Total protein kinase C activity, calculated as the sum of activity recovered following DEAE cellulose chromatography of the soluble plus solubilized particulate fractions of glomerular homogenates, was not significantly different between diabetic and control rats. The specific activity of particulate protein kinase C was fourfold higher in glomeruli from diabetic compared to control rats, whereas the specific activity of protein kinase C in the soluble fraction was significantly lower in glomeruli from diabetic rats compared to control. These observations imply that the higher protein kinase C activity in particulate fractions from glomerular homogenates from diabetic rats was due to translocation of enzyme activity from the soluble to the particulate fraction, an index of enzyme activation. As is also shown in Table II, treatment of diabetic rats with insulin prevented the translocation of glomerular protein kinase C to the particulate fraction. By contrast, sorbinil treatment had no effect on the subcellular distribution of protein kinase C in diabetic rats.

The results presented in Tables I–II and Fig. 1 suggest that despite a reduction in glomerular inositol content and polyphosphoinositide turnover, protein kinase C is activated in glomeruli from diabetic rats compared to controls. To examine the potential mechanisms by which the diabetic state may mediate protein kinase C activation in glomeruli, we assessed the effects of glucose on the subcellular distribution of protein kinase C in glomeruli from control rats. In these studies the concentration of glucose in the incubation media was varied from 100 to 1000 mg/dl. As shown in Fig. 2, an increase in glucose concentration from 100 to 200 mg/dl resulted in a significant increase in the percent of protein kinase C activity in the particulate fraction and a concurrent decline in soluble

Table I. Effects of Inositol on [^{32}P]Orthophosphate Incorporation into Phospholipids of Glomeruli Isolated from Control and Diabetic Rats

	Phosphatidylinositol	Phosphatidylinositol-4-Phosphate	Phosphatidylinositol-4,5-bisphosphate	Phosphatidylcholine-+ Phosphatidylserine	Phosphatidylethanolamine
			cpm/mg protein		
Control	3,817±1,780	496±74	882±138	5,174±1,516	2,352±714
+Inositol	4,173±1,256	512±98	956±172	7,285±2,052	2,856±302
Diabetic	3,308±1,316	255±97*	204±98*	6,854±2,505	2,284±752
+Inositol	4,892±1,956	482±105‡	751±141‡	6,154±1,924	3,105±809

Glomeruli were prepared from 20 kidneys and incubated for 4 h in duplicate in the presence and absence of 5 mM inositol. [^{32}P]Orthophosphate (10 μCi) was present in each incubation. At the end of 4 h phospholipids were extracted and isolated by thin-layer chromatography as described in Methods. Results shown are means±SE of determination on duplicate extracts from three separate experiments. * $P < 0.05$ compared to corresponding value in glomeruli from control rats. ‡ $P < 0.05$ compared to corresponding value in the absence of inositol.

Table II. Subcellular Distribution of Protein Kinase C in Glomeruli Isolated from Control and 1–2 Wk Streptozotocin Diabetic Rats

	Specific activity		Total activity	% Soluble
	Soluble	Particulate		
	pmol/min/mg protein		*pmol/min/ mg homogenate protein*	
Control	1,651±114	93±10	474±41	88±7
Diabetic	1,262±99*	482±124*	523±46	55±3*
Insulin-treated diabetic	1,708±97	146±22‡	551±89	84±8‡
Sorbinil-treated diabetic	1,198±163*	459±94*	491±63	51±4*

Glomeruli were homogenized and a 100,000 g soluble and solubilized particulate fraction prepared as described in Methods. The soluble and solubilized particulate fractions were partially purified by DEAE cellulose chromatography before assay. Units of specific activity are pmol/min per mg of protein in the soluble or solubilized particulate fraction. Units for total activity are pmol/min per mg protein in the soluble plus solubilized particulate fraction. The percentage of activity in the soluble fraction was calculated from the total activity in the soluble plus particulate fraction that was recovered from DEAE cellulose. Where indicated rats were treated with insulin or sorbinil as described in the footnote to Table I. Results shown are mean±SE of determinations on four separate glomerular homogenates. * $P < 0.05$ compared to corresponding value in glomeruli from control rats. ‡ $P < 0.05$ compared to corresponding value in glomeruli from untreated diabetic rats.

activity. Increasing media glucose from 200 to 600 mg/100 ml further increased the percentage of protein kinase C in the particulate fraction with a concomitant decline in soluble activity. Increasing the glucose concentration to 1,000 mg/dl yielded results which were not different from those obtained at 600 mg/dl glucose. As is also shown in Fig. 2, addition of 1,000 mg/dl of the nonmetabolizable glucose analogue, 2-deoxyglucose to glomeruli incubated in KRBG, which contained 100 mg/dl glucose, had no effect on the subcellular distribution of protein kinase C in rats when compared to that observed in glomeruli incubated with 100 mg/dl glucose alone. 2-deoxyglucose had no effect on viability of glomeruli as assessed by trypan blue exclusion. Similarly, addition of mannitol (1,000 mg/dl) had no effect on the subcellular distribution of protein kinase C in glomerular homogenates (control 84±3; mannitol
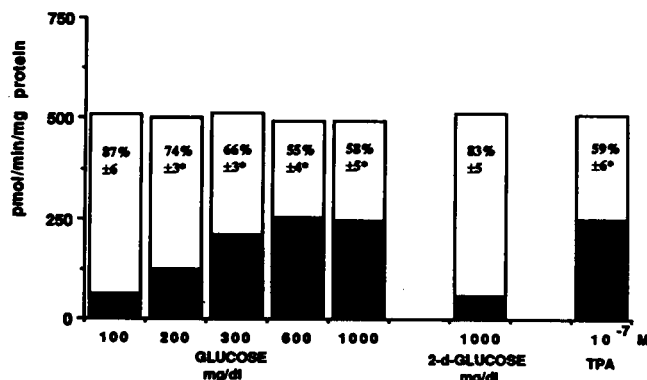

Figure 2. Effects of glucose on the subcellular distribution of protein kinase C in glomerular homogenates. Studies were conducted as described in the footnote to Table II. Where shown glucose, 2-deoxyglucose or TPA was added to give the final indicated concentration in the initial 10-min glomerular incubation. Enzyme activities were measured in the 100,000 g soluble (□) and solubilized particulate (■) fractions following partial purification on DEAE cellulose and expressed on the basis of milligrams of protein in the soluble plus solubilized particulate fraction. Two experimental parameters were studied with a single preparation of glomeruli from 10 kidneys and each experiment was repeated three times. *$P < 0.05$ compared to corresponding value obtained with 100 mg/dl glucose.

81±5% soluble activity±SE of three separate experiments). As is also shown in Fig. 2, incubation of glomeruli with 12-O-tetradecanoyl phorbol-13-acetate (TPA) a known activator of protein kinase C (22, 23), induced translocation of protein kinase C from the soluble to the particulate cell fraction.

The time course of glucose action on the subcellular distribution of protein kinase C is illustrated in Fig. 3. Glomeruli were incubated for a total of 30 min in KRBG. The concentration of glucose in the media was raised to 300 mg/dl for the final incubation times indicated in the figure. As shown in Fig. 3, percent soluble protein kinase C was significantly decreased by 2 min with maximal decline observed by 10 min after raising the glucose concentration to 300 mg/dl. The percent soluble protein kinase C remained significantly reduced 20 min after raising glucose to 300 mg/dl but by 30 min returned to values not different from those in glomeruli incubated at 100 mg/dl glucose. An analogous time course was observed when media glucose was increased from 100 to 1,000 mg/dl (not shown).

Fig. 4 illustrates the effects of glucose and 2-deoxyglucose on the turnover of labeled diacylglycerol in glomeruli from control rats. In these studies glomeruli were initially incubated for 50 min with [³H]myristic acid in KRBG which contained 100 mg/dl of glucose. Glucose or 2-deoxyglucose was then added to give the final concentration indicated in Fig. 4 and the incubation continued for 10 min. As shown, in Fig. 4, raising the concentration of glucose from 100 to 600 mg/dl progressively increased the formation of labeled diacylglycerol approximately twofold. The lowest concentration of glucose which gave a detectable increase in labeled diacylglycerol was 200 mg/dl. Raising the glucose concentration from 600 to 1,000 mg/dl had no further effect on labeled diacylglycerol turnover. As is also shown in Fig. 4, addition of 1,000 mg/dl 2-deoxyglucose to glomeruli incubated in KRBG with 100 mg/dl glucose had no effect on labeled diacylglycerol formation compared to that seen in glomeruli incubated in 100 mg/dl glucose. In contrast to the results obtained with [³H]-myristate, glucose did not stimulate labeled diacylglycerol production when glomeruli were incubated with [³H]-arachidonate. While the reasons for this are not clear, differences in the pattern of lipid labeling with these two fatty acids
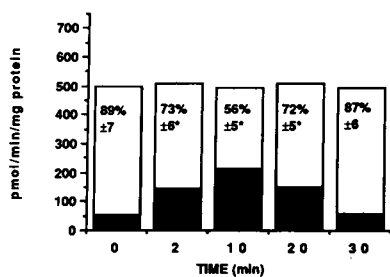
Figure 3. Time course of glucose actions on protein kinase C in glomerular homogenates. Studies were conducted as described in the footnote to Table II, glomeruli were incubated for a total of 30 min in KRBG. Where indicated the media glucose concentration was raised from 100 to 300 mg/dl for the final time of incubation shown. Enzyme activities were measured in the 100,000 g soluble (□) and solubilized particulate (■) fractions following partial purification on DEAE cellulose and expressed on the basis of milligrams protein in the soluble plus solubilized particulate fraction. Two experimental parameters were studied with a single preparation of glomeruli from 10 kidneys and each experiment was repeated three times. *$P < 0.05$ compared to corresponding value obtained when glomeruli were incubated with 100 mg/dl glucose (zero time).

Table III. Effects of Glucose and Angiotensin II on Inositol Phosphate Production in Glomeruli from Control and Diabetic Rats

	IP	IP$_2$	IP$_3$
		cpm/mg protein	
Control	3,322±498	806±134	279±32
+900 mg/dl glucose	2,953±354	765±117	312±39
+10^{-7} M angiotensin II	6,413±1,154*	1,423±173*	406±52*

Glomeruli were prepared from 20 kidneys and prelabeled with 75 μCi of [^{3}H]-*myo*-inositol. They were then washed and incubated in triplicate in KRBG which contained 100 mg/dl glucose for 10 min at 37°C. The additions shown were present for the final 3 min of incubation. IP, IP$_2$, and IP$_3$ were extracted, isolated by Dowex chromatography and counted as described in Methods. Results shown are mean±SE of determinations from three separate experiments. * $P < 0.05$ compared to corresponding value with no addition.

may be the basis for the divergent responses to glucose. Thus, although the total amount of label incorporated into lipids was similar for the two fatty acids, 70% of the label from [^{3}H]-myristate was incorporated into di- and triglycerides whereas the remainder was in phospholipids. In the case of [^{3}H]-arachidonate the reverse was true. The higher level of incorporation of [^{3}H]myristate into neutral lipids may enhance the ability to detect alterations in labeled diacylglycerol production. Secondly, [^{3}H]myristate was preferentially incorporated into phosphatidylcholine (90%) relative to other phospholipids, whereas this was not true for [^{3}H]arachidonate (49% into phosphatidylcholine). Finally, previous studies in other tissues have demonstrated the presence of myristic acid but not arachidonic acid in the phosphatidylinositol-glycan moiety (28). This lipid may also be a source of glucose stimulated labeled diacylglycerol production in glomeruli. The use of labeled myristate might thus permit detection of diacylglycerol generated from phosphatidylinositol-glycan in addition to that derived from de novo synthesis of phospholipids or phospholipase C induced breakdown of phosphatidylcholine.

The ability of glucose to increase labeled diacylglycerol production could be due to an action of glucose to enhance inositol phospholipid breakdown. This possibility was assessed directly. Table III compares the effects of glucose and angiotensin II on labeled inositol phosphate production in glomeruli
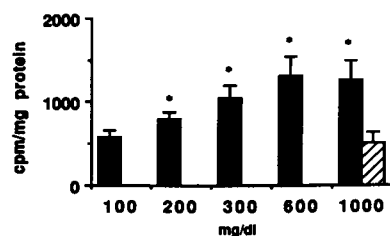

Figure 4. Effects of glucose on [^{3}H]-diacylglycerol turnover by glomeruli from control rats. Glomeruli were incubated in KRBG at 37°C for 60 min with 15 μCi of [^{3}H]-myristate as described in Methods. Where indicated, glucose or 2-deoxyglucose was added to give the indicated concentration during the final 10 min of the incubation. All six experimental parameters were studied with the same glomerular preparation, representing 20 kidneys and each experiment was repeated three times. *$P < 0.05$ compared to corresponding values obtained with 100 mg/dl glucose.

from control rats. Raising the glucose concentration of the media from 100 to 300 (not shown) or 1,000 mg/dl (Table III) had no effect on production of IP, IP$_2$, or IP$_3$. By contrast, angiotensin II stimulated labeled inositol phosphate production 1.5–2-fold.

Discussion

Previous studies in rat diabetic nerve have implicated enhanced polyol pathway activity, and reduced tissue *myo*-inositol content in the mediation of reduced nerve conduction velocity in the diabetic rat (2, 10, 11, 29). Phosphatidylinositol content of diabetic nerve is reduced and several observations have implicated the reduction in phosphatidylinositol in the mediation of reduced (Na$^+$, K$^+$)-ATPase activity and impaired nerve conduction velocity (4). It recently has been suggested that a lower phosphatidylinositol content of nerve might lead to reduced diacylglycerol production and a reduction in the state of activation of protein kinase C (2). The observation that TPA, a protein kinase C activator, enhances nerve conduction velocity in diabetic rats further suggests that a defect in protein kinase C activation may contribute to this neural defect (12). Recently, total protein kinase C has been reported to be reduced in diabetic nerve (30). However, this observation is difficult to interpret since the reduction in total protein kinase C activity was due to a loss in soluble activity alone. The percentage of enzyme activity associated with the particulate fraction, an index of enzyme activation (30), was in fact higher in diabetic compared to normal nerve. Moreover, the turnover of polyphosphoinositides (3) and the phosphorylation of specific nerve proteins which are thought to be substrates for protein kinase C in vitro (14), have both been reported to be increased, rather than decreased in diabetic nerve despite the reduction in inositol and phosphatidylinositol content. Accordingly, in diabetic nerve there does not appear to be a simple relationship between reduced *myo*-inositol content and the activity of the inositol phospholipid/protein kinase C signalling pathway.

The results of this study clearly demonstrate that the free inositol content of glomeruli isolated from streptozotocin diabetic rats is reduced by 1–2 wk after induction of diabetes. This is consistent with previous studies in diabetic nerve (2) and

lens (2) and in isolated glomeruli obtained 7–10 wk after induction of diabetes (5). The reduction in glomerular inositol content is likely related to hyperglycemia or some other consequence of the diabetic state, rather than to an action of streptozotocin, since treatment of the diabetic rats with insulin to maintain near euglycemia prevented the fall in glomerular inositol content. Indeed, glucose has been reported to inhibit the cellular uptake of inositol (31). In the present study and consistent with previous observations (2), sorbinil was found to prevent the fall in glomerular inositol content seen in the diabetic rat. This suggests that increased polyol pathway activity may contribute to the decrease in inositol content. However, the mechanism by which sorbinil prevents the fall in inositol is not known (2). Sorbinil has been shown to bind to glomeruli (32) and thus might influence membrane transport through mechanisms that do not involve aldose reductase activity. In this regard, we have been unable to demonstrate an increase in glomerular sorbitol content at 1–2 wk after induction of diabetes, at a time when inositol content was reduced (unpublished observations). Thus, sorbinil may prevent glomerular inositol depletion in diabetes through a mechanism other than inhibition of aldose reductase, although this remains uncertain.

This study also demonstrates that the turnover of the labeled polyphosphoinositides PIP and PIP_2 is reduced in glomeruli from diabetic rats. Reduced turnover of labeled PIP and PIP_2 appeared to be a consequence of reduced glomerular free inositol content, since the addition of inositol to the incubates increased labeled PIP and PIP_2 turnover to the same absolute value in glomeruli from diabetic and control rats. The reduced turnover of labeled PIP and PIP_2 was selective relative to turnover of the other major phospholipids, which did not differ in glomeruli from diabetic and control rats. The finding that phosphatidylinositol turnover was not reduced in glomeruli from diabetic rats despite the reduction in PIP and PIP_2 turnover may reflect the fact that PIP and PIP_2 are cycling more rapidly than PI, that differences in the turnover of a small pool of PI occurred, but were masked by the slower turnover of a larger pool of phosphatidylinositol, or that both of these phenomena were operative.

Despite the reduced inositol content and the reduced turnover of labeled PIP and PIP_2, protein kinase C appeared to be activated in glomeruli from diabetic rats as reflected by an increase in the percentage of enzyme activity which is associated with the particulate fraction. The increase in particulate protein kinase C observed in glomeruli from diabetics was not due to an alteration in protein content of the subcellular fractions, since the specific activity of protein kinase C in the particulate fraction of glomeruli from diabetic rats was also increased. Moreover, total enzyme activity of the soluble plus particulate fraction did not differ between glomeruli from diabetics and controls when expressed on the basis of protein. Thus, the increase in particulate protein kinase C appeared to be due to translocation of the enzyme from the soluble to the particulate fraction. Nevertheless, alternate interpretations of these findings must be considered. Several isozymic forms of protein kinase C have been described that differ in their tissue distribution and subcellular localization (33). Each isozyme is known to be encoded by a different gene and thus may be differentially expressed (33). We can not exclude the possibility that the shift in subcellular distribution of protein kinase C that we observed in glomeruli from diabetic rats reflects an altered pattern of protein kinase C isozyme expression. Furthermore, protein kinase C has been implicated in several cellular processes and some lines of evidence suggest that different isozymes may be responsible for diverse cellular responses (34–36). Thus, the functional significance of increased particulate protein kinase C in glomeruli from diabetic rats remains to be examined.

Chronic exposure to high concentrations of phorbol esters in vitro has previously been shown to cause loss of protein kinase C activity (37). If protein kinase C were chronically activated in glomeruli from diabetic rats, we might have expected to see loss of total enzyme activity. This was not observed. In this regard, it is not known whether downregulation of protein kinase C occurs in vivo with physiologic activators that increase diacylglycerol. Previous reports of increased particulate protein kinase C in mice fed a high fat diet for 4–6 wk (38), in fibroblasts from psoriatic patients (39) and in the luminal membranes of proximal tubules from 1 mo streptozotocin diabetic rats (40) suggest that downregulation may not occur with chronic stimulation in vivo. Recently, Wolfman and Macara (41) demonstrated that ras transformed fibroblasts (2 wk) possess elevated diacylglycerol and a 4–20-fold increase in 80k protein phorphorylation, a measure of endogenous protein kinase C activity. Total protein kinase C was reduced by only 25% in the transformed cells (41), suggesting that protein kinase C appeared to be both activated and modestly downregulated in these cells. Thus chronic exposure to elevated diacylglycerol, as opposed to TPA, may have minor if any effects on total protein kinase C activity.

Translocation of protein kinase C to the particulate fraction is often observed following exposure of cells to TPA or diacylglycerol analogues, known activators of this enzyme system (22, 23). In the present study, exposure of glomeruli from normal rats to TPA similarly resulted in apparent translocation of protein kinase C to the particulate fraction. Glucose had an effect on protein kinase C analogous to TPA, implying that glucose also induced enzyme activation. The ability of glucose to activate protein kinase C in normal glomeruli supports a role for hyperglycemia per se in the activation of protein kinase C observed in glomeruli isolated from diabetic rats. The observation that 2-deoxyglucose did not mimic the effects of glucose to activate protein kinase C in glomeruli suggests that a metabolic effect of glucose mediates activation of protein kinase C. Moreover, the results with 2-deoxyglucose and mannitol exclude an osmotic action of glucose in the activation of glomerular protein kinase C. Conversely, studies in diabetic rats treated with sorbinil or insulin indicate that activation of glomerular protein kinase C is dissociated from changes in glomerular inositol content. Thus, sorbinil treatment of diabetic rats restored the inositol content of glomeruli to control values without altering the magnitude of hyperglycemia, and did not prevent activation of glomerular protein kinase C. By contrast, treatment with insulin restored glomerular inositol content, corrected the hyperglycemia and prevented activation of protein kinase C.

In the present study the effects of high media glucose concentration on the subcellular distribution of protein kinase C incubated in vitro were transient. The factors responsible for this peak-decline response pattern of protein kinase C in vitro are uncertain. In vitro depletion of diacylglycerol, of critical subcellular pools of phospholipids, fatty acids, or other moieties that may participate in protein kinase C activation can

not be excluded. Notably, in a cultured cell system of bovine retinal epithelial cells, where a more complex media was employed, a high media glucose concentration produced a sustained activation of protein kinase C (42). Conversely, it is quite possible that hyperglycemia per se is not the sole mediator of the protein kinase C activation observed in glomeruli isolated from diabetic rats. Clearly, an array of hormonal and metabolic changes occur in the diabetic state that may be involved in the in vivo activation of protein kinase C in the glomeruli.

Studies in glomeruli from control rats also demonstrated that high media glucose concentrations increased the accumulation of labeled diacylglycerol, as assessed by enhanced incorporation of labeled myristate into diacylglycerol. The increase in labeled diacylglycerol occurred over the same range of glucose concentrations which was found to activate protein kinase C. This is consistent with the possibility that enhanced diacylglycerol production may mediate activation of protein kinase C seen in response to glucose.

Studies in pancreatic islets (43–47) and adipose tissue (36) have demonstrated that glucose can increase diacylglycerol production and stimulate Ca^{2+} and phospholipid dependent phosphorylation both by stimulating de novo synthesis of diacylglycerol (43, 44, 48) and by enhancing phospholipase C induced hydrolysis of inositol phospholipids (45, 47). With respect to the phospholipase C pathway, the results of the present study demonstrate that glucose does not increase diacylglycerol production in glomeruli by stimulating hydrolysis of inositol phospholipids (PI, PIP, or PIP_2). This is implied by the observation that glucose does not increase the production of inositol phosphates in glomeruli which were prelabeled with [^{3}H]inositol. By contrast, angiotensin II, a known activator of phospholipase C in glomeruli, increased inositol phosphate production twofold under the same conditions of incubation. Thus, inositol phospholipid hydrolysis was not implicated in the action of glucose. However, our studies do not exclude an effect of glucose to enhance phospholipase C-mediated hydrolysis of phosphatidylcholine (49), or of the recently described phosphatidylinositol-glycan moiety (28, 50). With respect to de novo diacylglycerol production, glucose may enhance glycerol 3-phosphate production via the glycolytic pathway (43, 44, 48). Glycerol 3-phosphate is converted to phosphatidic acid by glycerol 3-phosphate acyltransferase and phosphatidic acid is then further hydrolyzed to diacylglycerol. Accordingly, an increased flux of glucose through this pathway would be expected to increase diacylglycerol production. Additional studies are required to determine the role of de novo diacylglycerol synthesis in mediating actions of glucose on glomerular protein kinase C.

Glucose has been shown to increase cytosolic Ca^{2+} in pancreatic islets (51) and recent evidence suggests that an increase in cytosolic Ca^{2+} alone may be sufficient to trigger protein kinase C activation (52). The possibility that glucose induced increases in cytosolic Ca^{2+} may contribute to the observed activation of protein kinase C remains to be examined.

Whatever, the mechanism by which protein kinase C is activated in glomeruli of diabetic rats, this finding is of considerable interest and potential importance. Numerous previous studies have demonstrated an association between activation of protein kinase C and enhanced cell growth (53–55). Renal hypertrophy and hyperplasia are early events which occur within the first few days after induction of diabetes (56) and

may contribute to the early increase in glomerular filtration rate (57). Studies of remnant kidney after subtotal nephrectomy (58) and in cultured glomerular epithelial cells, stimulated to divide with leukotrienes (59) have also suggested a role for protein kinase C in the control of renal growth. Furthermore, diacylglycerol and protein kinase C have been implicated in both the mediation and modulation of prostaglandin synthesis in intact glomeruli and cultured mesangial cells (60–62). The synthesis of vasodilatory prostaglandins is increased by 1–2 wk in glomeruli from diabetic rats (17, 25, 63–65) and remains increased in moderately hyperglycemic rats in association with increased glomerular filtration rate (GFR). Several studies (56) conducted with inhibitors of prostaglandin synthesis have linked enhanced production of vasodilatory prostaglandin to increases in GFR (17, 65–69). The present results thus raise the possibility that an increase in the state of activation of protein kinase C in diabetic glomeruli may contribute to the increases in renal growth and GFR, which are characteristic of early diabetes.

Acknowledgments

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