Effects of Glucose on Receptor-Mediated Phosphoinositide Hydrolysis and Second Messenger Generation in Rat Glomerular Mesangial Cells[1,2]

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ABSTRACT

The phosphoinositide system plays a critical role in mesangial cell contraction. *myo*-inositol depletion occurs in glomeruli from diabetic animals and may result in mesangial cell dysfunction. The hypothesis that mesangial cell exposure to high concentrations of glucose could lead to abnormalities in phosphoinositide metabolism and receptor-mediated inositol phosphate release was tested. When compared with controls (5 mM glucose), inositol phosphate release in mesangial cells exposed to 28 mM glucose was decreased by 27% after maximal stimulation with angiotensin II, by 41% after arginine vasopressin, and by 63% after the thromboxane A_2 analog, U46619. Increasing the concentration of glucose to 50 mM caused a further reduction (from 27 to 54%) in maximal angiotensin II stimulation of inositol phosphate release. High glucose decreased incorporation of *myo*-inositol into phospholipids but did not change phosphoinositide mass. High glucose also resulted in increased *de novo* synthesis of diacylglycerol which was associated with membrane translocation of protein kinase C. *myo*-inositol supplementation prevented the reduction in phosphoinositide hydrolysis whereas sorbinil did not. It was concluded that high concentrations of glucose cause abnormalities in *myo*-inositol metabolism in mesangial cells which lead to reduced receptor-mediated phosphoinositide hydrolysis. These abnormalities appear to be related to desensitization of receptor-mediated phosphoinositide responses due to negative feedback by protein kinase C which becomes activated as a result of enhanced *de novo* diacylglycerol formation from glucose. These changes are unrelated to the polyol pathway and can be prevented by *myo*-inositol supplementation.

Key Words: *Hyperglycemia, mesangium, diacylglycerol, protein kinase C*

The kidney disease of diabetes mellitus is characterized in its early stages by abnormal glomerular hemodynamics and hyperfiltration (1). Although the mechanisms responsible for these abnormalities have not yet been identified, some investigators have suggested that alterations in glomerular contractility could play an important role in their pathogenesis. In fact, glomeruli isolated from streptozotocin-induced diabetic rats show decreased contractility in response to angiotensin II (AII) when compared with glomeruli from normal rats (2). Studies in diabetic animals have shown that the content of *myo*-inositol in glomeruli as well as in a number of other tissues is reduced and that this reduction occurs as a result of prolonged hyperglycemia (3). More recent studies have also demonstrated depletion of intracellular *myo*-inositol in mesangial cells exposed to high concentrations of glucose (4). Phosphatidylinositol (PtdIns) is an essential component of cell membranes and signal transduction processes (5). Activation of mesangial cell membrane receptors by vasoconstrictor agonists leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] by phospholipase C and generation of the second messengers inositol trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG) (6). Ins(1,4,5)P_3 causes calcium release from the endoplasmic reticulum which leads to a rapid increase in cytoplasmic calcium concentrations and mesangial cell contraction (7–10). DAG causes activation of protein kinase C, and this appears to play a role in the maintenance of a more prolonged tonic phase of contraction (11,12). *myo*-inositol depletion in glomeruli could lead to abnormal phosphoinositide metabolism and functional alterations

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in the various cellular elements. Recent studies have also shown that hyperglycemia causes an increase in *de novo* glomerular DAG synthesis via glycerophospholipids and that this is associated with protein kinase C activation (13). Protein kinase C exerts downregulatory influences in a number of cellular processes including the uncoupling of receptors from their signalling elements (14–16).

Because the mesangial cell is the contractile element in the glomerulus and the polyphosphoinositide signaling system plays a critical role in mediating mesangial cell contraction (6–8), it has been hypothetized that the decrease in contractility observed in diabetic glomeruli is due to mesangial cell dysfuntion. Moreover, the presence of aldose reductase activity and the polyol pathway in mesangial cells grown in medium with high concentrations of glucose (17) raises the possibility that intracellular accumulation of sorbitol as a result of prolonged hyperglycemia could play a role in the pathogenesis of glomerular diabetic injury. It was the purpose of this study to investigate the effects of hyperglycemia on receptor-mediated phosphoinositide hydrolysis and inositol phosphate (IP) production, and on *myo*-inositol metabolism in glomerular mesangial cells in culture. We report here that agonist-induced stimulation of phosphoinositide hydrolysis is reduced in mesangial cells cultured in high glucose. The glucose-induced decrease in phosphoinositide hydrolysis appears to be due to activation of protein kinase C through enhanced *de novo* DAG formation and not secondary to the accumulation of polyols or the depletion of phosphoinositides.

MATERIAL AND METHODS

Sprague-Dawley rats were obtained from the University of Florida animal breeding facilities. Hank's balanced salt solution (HBSS), insulin-transferrin-selenium, AII, arginine vasopressin (AVP), D-glucose, D-fructose, and D-*myo*-inositol were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI 1640, minimum essential medium, fetal bovine serum, penicillin, and streptomycin were purchased from Fisher Scientific (Orlando, FL). [^{3}H]*myo*-inositol, [^{3}H]phorbol 12,13-dibutyrate ([^{3}H]PDBu), and [^{14}C(U)]glucose were purchased from Dupont, NEN Research Products (Boston, MA). Lipid standards were from Avanti Polar Lipids (Pelham, AL). Dowex AG 1-X8 anion-exchange resin (formate form) was obtained from Bio-Rad Laboratories (Richmond, CA).

Preparation of Mesangial Cell Cultures

All experiments were performed in primary cultures of rat mesangial cells between passages 2 and 5. Mesangial cells were harvested from male Sprague-Dawley rats weighing between 150 and 200 g by previously published techniques (18,19). Twelve kidneys from six animals were used to set up each primary culture. The cultures were prepared by standard aseptic techniques. Halothane-anesthetized animals were sacrificed by decapitation, and their kidneys were removed and placed in ice-cold HBSS containing 10 mM HEPES, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The kidneys were then transferred to a laminar flow hood where they were decapsulated and transected. The renal cortices were separated and minced into a fine paste. The tissue was then passed through a series of stainless steel sieves arranged by decreasing pore size as follows: top to bottom, 250, 106, and 75 μm. By this method, the glomeruli become trapped in the 75-μm-pore-size sieve. After the sieves were washed with HBSS, the glomeruli were recovered, transferred to centrifuge tubes, and washed by centrifugation at 1,000 rpm for 5 min. These were then digested for 30 min with type IV collagenase (750 U/mL) at 37°C. After digestion with collagenase, the glomerular "cores," which contain mostly mesangial and endothelial cells, were washed by centrifugation with HBSS, plated in 75-cm^2 plastic tissue culture flasks, and incubated at 37°C in a 5% CO_2–95% air incubator. The culture medium, which was supplemented every 3 to 4 days, consisted of RPMI 1640 containing 16% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 5 μg/mL of insulin, 5 μg/mL of transferrin, and 5 ng/mL of selenium. After 3 to 4 wk, the mesangial cells overgrow the other cells, become confluent, and constitute greater than 90% of the cells in the culture. After two passages, no endothelial or epithelial cells were seen and the cultures consisted exclusively of mesangial cells. These were then subcultured every 7 to 10 days by mild trypsinization and were transferred to six-well cluster dishes for the specific experiments.

Mesangial cells were identified by using the following characteristics: (*1*) spindle or stellate shape under phase-contrast microscopy, (*2*) presence of microfilament bundles under transmission electron microscopy, (*3*) ability to proliferate in medium containing D-valine—a condition which inhibits fibroblast growth, (*4*) resistance to the effects of the glomerular epithelial cytotoxin, aminonucleoside of puromycin (100 μg/mL), (*5*) cell detachment and lysis after an overnight exposure to the mesangial cytotoxin, mitomycin (10 μg/mL), (*6*) presence of immunofluorescence staining for anti-smooth muscle-specific myosin antibodies, (*7*) absence of immunofluorescence staining for anti-factor VIII antibodies, which excludes the presence of endothelial cells, and (*8*) contractile response to AII (18–20).

Experimental Conditions

Seven to nine days before an experiment, cells matched for cell line, number of passages, age, plat-

ing density, and culture conditions were divided into three groups: one control group was incubated in minimum essential medium containing 4% fetal bovine serum, 30 μM *myo*-inositol (total concentration taking into account *myo*-inositol present in serum) (21), 5 μg/mL of insulin, antibiotics, and 5 mM glucose; one experimental group was incubated in the same medium but with 28 mM glucose (high glucose), and a second experimental group was incubated in the same medium but with 28 mM fructose (high fructose). The osmolarities were 282 to 300 mosmol/kg of H_2O in the control, 300 to 320 mosmol/kg of H_2O in the high glucose, and 300 to 316 mosmol/kg of H_2O in the high-fructose media. We used this last group as a control for osmolarity. Seventy-two hours before an experiment, cell growth was arrested by the reduction of the concentration of serum to 0.5%, *myo*-inositol was adjusted to maintain a concentration of 30 μM, glucose concentrations were maintained (e.g., control, 5 mM; or high, 28 mM glucose), and insulin was withdrawn from the media. Cell viability, as assessed by morphologic characteristics of the culture (absence of cell detachment, vacuolization, or abnormal granularity) and trypan blue exclusion, was judged to be greater than 95% under all conditions studied.

Phosphoinositide Hydrolysis

Phosphoinositide hydrolysis was determined by measuring release of IP from membrane phospholipids by a modification of the method of Gonzalez, Crews, and colleagues (22–24). Mesangial cells were incubated in 2 mL of either control or experimental medium containing 2 μCi/mL of [^{3}H]*myo*-inositol. Preliminary experiments with various concentrations of *myo*-inositol had shown that radiolabeling of membrane phospholipids reached steady state at 48 h, and, therefore, we chose this time for maximal labeling. After 48 h, the unincorporated label was washed off with Krebs-Ringer-bicarbonate buffer having the following composition (in mM): NaCl, 118; KCl, 4.7; Ca_2Cl, 0.75; KH_2PO_4, 1.18; $MgSO_4$, 1.18; $NaHCO_3$, 24.8; and D-glucose, 10. The cells were then treated with AII, AVP, the stable thromboxane A_2 analog, U46619, or their respective vehicles at the indicated concentrations (see below). The stimulation phase was carried out in 1 mL of Krebs-Ringer-bicarbonate buffer containing 10 mM LiCl plus 10 μL of either vehicle or agonist. The reaction was stopped after 10 min by the addition of 1 mL of methanol. The cells were then scraped off the plates with a rubber policeman and transferred to test tubes. After the plates were rinsed with an additional 0.5 mL of methanol, 1 mL of chloroform was added and the aqueous and lipid phases were separated by agitation and centrifugation. The aqueous phase was transferred into a Dowex AG 1-X8 (100 to 200 mesh, formate form) anion-exchange column. The column was washed with 20 mL of water, and the IP were eluted with 5 mL of formate buffer (1 M NH_4 formate–0.1 M formic acid). The eluate was collected in scintillation vials and counted for radioactivity. The organic phase was evaporated under nitrogen and counted for lipid radioactivity. Results are expressed as fractional release of IP. IP fractional release (FR) is calculated as the ratio of total IP released to total *myo*-inositol (Ins) incorporated into membrane phospholipids by using the following formula (22,23):

$$FR = \frac{\text{dpm } [^3\text{H}]\text{IP in aqueous phase}}{\text{dpm } [^3\text{H}]\text{IP in aqueous phase} + \text{dpm } [^3\text{H}]\text{Ins in organic phase}}$$

myo-Inositol Uptake into Phospholipids

Mesangial cells were incubated with [^{3}H]*myo*-inositol (2 μCi/mL) in 2 mL of culture medium (control or high glucose) for 6, 24, 48, or 72 h. At the end of the incubation period, the cells were washed three times with ice-cold phosphate buffer saline (PBS) to remove the unincorporated radiolabel, gently scraped off the wells with a rubber policeman, and transferred into a total volume of 2 mL of ice-cold PBS. The cell suspension was then homogenized in a Polytron while kept on ice and was divided into two samples which were then pelleted by centrifugation at 10,000 rpm for 10 min at 4°C. One pellet was resuspended in 1 mL of 1 N NaOH for measurement of protein content by the Lowry method (25), and the other was resuspended in 1 mL of chloroform, 2 mL of methanol, and 1 mL of H_2O for lipid extraction. Lipid extraction was performed as described above, and the aqueous phase was discarded. The lipids in the organic phase were then pooled and concentrated by being dried under a nitrogen stream, and radioactivity was determined by scintillation counting. Results are expressed as disintegrations per minute of [^{3}H]*myo*-inositol per milligram of protein.

Determination of Phosphoinositide and Phospholipid Mass

Lipids were extracted with chloroform, methanol, and 0.1 N HCl as described previously. Phosphoinositides were separated by thin-layer chromatography (TLC) in silica gel plates impregnated with potassium oxalate as described by van Dongen *et al.* (26). Fifty microliters of the concentrated lipid suspension were spotted, and phosphoinositides were resolved in the following solvent system: chloroform:acetone:methanol:acetic acid:water (80:30:26:24:14). Phosphatidylinositol 4-monophosphate [PtdIns(4)P] and PtdIns(4,5)P_2 spots were identified by using authentic

standards (Sigma), scrapped, and assayed by phosphorus analysis as described by Rouser *et al.* (27). Phospholipids including PtdIns were separated and assayed by two-dimensional TLC (27).

Measurement of [^{3}H]Phorbol Dibutyrate Binding to Intact Cells

Protein kinase C activation was determined by the measurement of specific high-affinity binding of [^{3}H]PDBu to intact cells (28,29). This technique measures the degree of protein kinase C translocation from the cytosol to its site of activation in the membrane and has been shown to correlate very well with phosphorylation assays of protein kinase C activity (29). High-affinity [^{3}H]PDBu binding is considered to be a very precise index of protein kinase C activation (29). Mesangial cells under control or experimental conditions were washed thrice with binding HEPES-salt buffer (in mM: HEPES, 20; NaCl, 140; KCL, 5; $CaCl_2$, 1; $MgSO_4$, 2.5; glucose, 5 mM; and fatty acid-free BSA, 0.1%; pH 7.40) and incubated in the same buffer containing 5 nM [^{3}H]PDBu for 15 min at 22°C. Preliminary experiments confirmed published findings that binding equilibrium occurs within 10 min and remains constant for 60 min. The cells were then washed three times with ice-cold PBS and dissolved in 0.5 N NaOH. Samples were used for scintillation counting and protein measurement. Nonspecific binding was determined in the presence of 1 μM phorbol myristate acetate.

De Novo DAG Synthesis from [^{14}C]Glucose

Mesangial cells were incubated in Krebs buffer containing 5 or 30 mM glucose and [^{14}C(U)]glucose for 0, 6, and 12 min. The specific activity of the isotopic glucose (2.0 mCi/mmol) was maintained constant under both conditions. The reaction was terminated by the addition of ice-cold methanol, and lipids were extracted as described above in chloroform:methanol:0.05% aqueous $CaCl_2$ (3:48:47). After addition of the appropriate standards, the samples were evaporated under nitrogen and resuspended in chloroform and lipids were separated by TLC by using the following solvent system:benzene:diethylether:ethyl acetate:acetic acid (80:10:10:0.2). Bands corresponding to DAG and triglycerides were scrapped and counted for radioactivity.

Statistical Analysis

Unless otherwise specified, the results are presented as mean ± SE of 6 to 12 experiments performed in triplicate in six different cell lines. EC_{50} was determined by the method of logits. Statistical analysis was performed by using the Student's *t* test for paired and unpaired data.

RESULTS

Effects of Glucose on Receptor-Mediated Phosphoinositide Hydrolysis

To determine if high concentrations of glucose altered receptor-stimulated phosphoinositide hydrolysis, mesangial cells were incubated over 7 to 9 days in medium containing either 5 or 28 mM glucose, and AII-stimulated IP release was measured over a 10-min period. This 10-min time course was found in preliminary experiments to correspond to maximal IP release (data not shown). Basal fractional IP release over the 10-min experimental period was always less than 0.01, and no differences were found between the control and experimental groups. When compared with controls, mesangial cells incubated in medium containing 28 mM glucose showed a 27% reduction in phosphoinositide hydrolysis after stimulation with the vasoconstrictive agonist, AII (100 nM) (0.055 ± 0.004 versus 0.040 ± 0.006 fractional release; $P < 0.005$). To study the effects of high glucose on AII stimulation of phosphoinositide hydrolysis at different AII concentrations, several concentration-response curves were determined. The inhibitory effect of high glucose on AII stimulation of IP release was evident along the entire range of concentrations studied (1 to 300 nM) (Figure 1). Maximal agonist response, or agonist efficacy, relative to the control group was decreased to 66 ± 5% ($P < 0.005$) in the high-glucose group. Analysis of the concentration-response curves showed similar agonist poten-

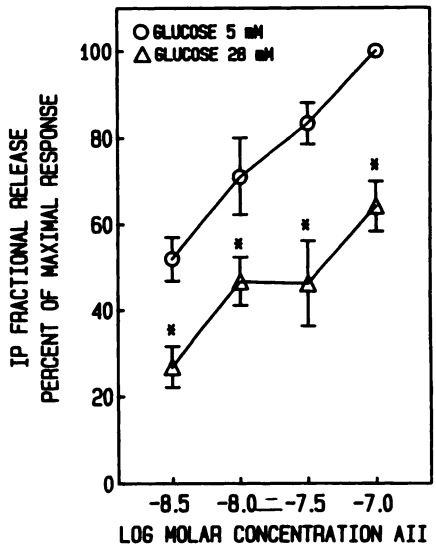

Figure 1. Concentration-response curves for AII stimulation of IP release under physiologic (5 mM) and high (28 mM) glucose concentrations. Results are shown as percent release relative to maximal IP fractional release in 5 mM glucose. *$P < 0.05$. Statistical significance is reported for differences in response between 5 and 28 mM glucose at each concentration of AII. $N = 6$.

cies in both groups, with an approximate EC_{50} of 11 ± 5 and 9 ± 6 nM in the control and high-glucose conditions, respectively. A similar effect was seen in experiments in which total IP release was studied as a function of the amount of total cellular protein or as release per well (data not shown) instead of fractional release. In all of these experiments, AII agonist efficacy in high-glucose conditions relative to the control group was reduced to 67 ± 9% ($P < 0.001$) whereas the EC_{50} were 8 ± 3 and 11 ± 3 nM for the control and high-glucose groups, respectively. Thus, incubation with high glucose reduced AII-stimulated phosphoinositide hydrolysis at all concentrations studied without shifting the EC_{50}.

To determine if the effects of glucose were unique to AII stimulation of phosphoinositide hydrolysis or generalized to phosphoinositide hydrolysis by other vasoconstrictive agonists, a series of experiments were performed with arginine vasopressin (10 nM) and the thromboxane A_2 analog, U46619 (10 μM). As was the case with AII, when compared with controls, fractional release of IP in cells grown in high-glucose medium was reduced by 41% ($P < 0.005$) after stimulation with AVP and by 63% ($P < 0.05$) after stimulation with U46619 (Figure 2). Thus, incubation with high glucose reduces phosphoinositide hydrolysis by a variety of different agonists.

To evaluate whether glucose could have a concentration-response relationship in its effect on receptor-mediated phosphoinositide turnover, a group of experiments were designed to study the effects of increasing concentrations of glucose on AII-stimulated phosphoinositide hydrolysis. Increasing the concentration from 28 to 50 mM doubled the inhibitory effect of glucose, causing a further reduction (from 27 to 54%) in maximal AII stimulation of IP release (Figure 3). Experiments were also performed in cells that had been incubated in 28 mM fructose to determine if the inhibitory effect on receptor-mediated phosphoinositide turnover was specific for glucose. Agonist-stimulated IP release in mesangial cells incubated in medium containing 28 mM fructose was not statistically different from that in cells grown in medium containing 5 mM glucose (Figure 4). Thus, the degree of inhibition of phosphoinositide hydrolysis is dependent on the concentration of glucose and the inhibitory effect is specific for this sugar.

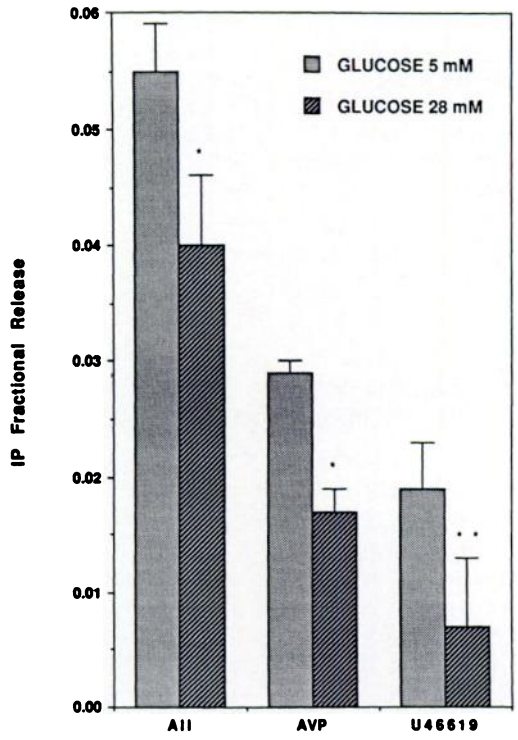

Figure 2. Effects of high glucose concentration (28 mM) on maximal AII, AVP, and U46619 stimulation of IP release in mesangial cells. Basal IP release was less than 0.01 and was not different between control and experimental conditions. *$P < 0.005$; **$P < 0.05$. $N = 12$.

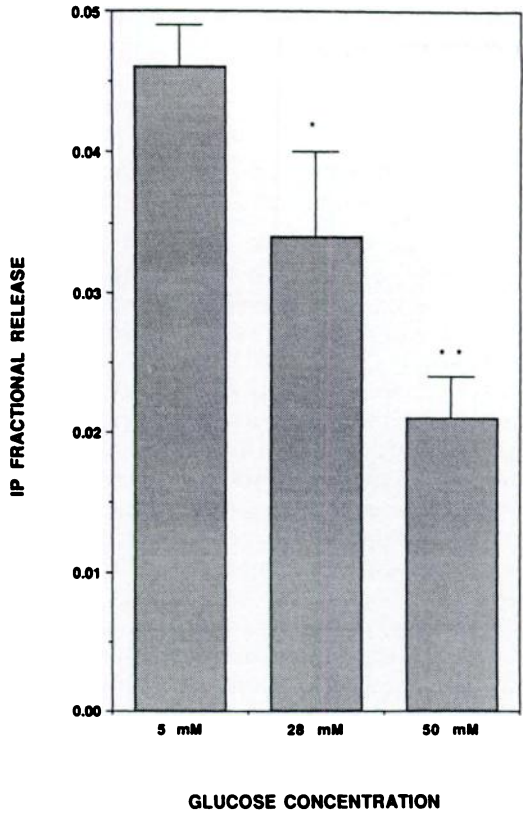

Figure 3. Effects of increasing concentrations of glucose on maximal AII stimulation of IP release. *$P < 0.005$ versus 5 mM glucose; **$P < 0.001$ versus 28 mM glucose. $N = 9$.

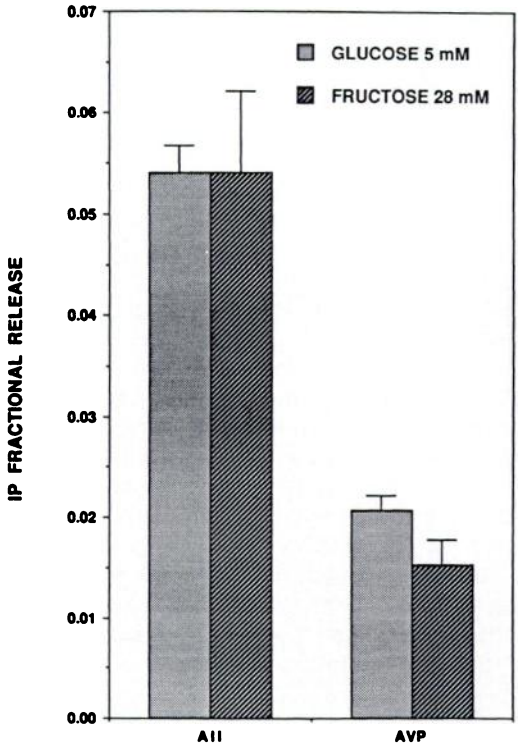

Figure 4. Effects of high fructose concentration (28 mM) on maximal AII and AVP stimulation of IP release. Differences were not statistically significant. $N = 6$.

Effects of Glucose on *myo*-Inositol Incorporation into Phospholipids and Phospholipid Mass

Because intracellular *myo*-inositol depletion has been postulated as one of the mechanisms responsible for diabetic injury and has been shown to occur in mesangial cells during hyperglycemia (4), we studied the possibility that the reduction in receptor-mediated phosphoinositide hydrolysis caused by glucose could be related to decreased synthesis of PtdIns. Experiments were designed to follow incorporation of *myo*-inositol into membrane phospholipids and to measure total phospholipid mass. Incubation of mesangial cells in high-glucose medium significantly reduced the amount of [^{3}H]*myo*-inositol incorporated into membrane phospholipids (Figure 5). This effect of glucose was seen at all time points studied with *myo*-inositol incorporation under both control and high-glucose conditions, reaching a plateau after 48 h. Phosphoinositide and phospholipid mass, however, were unchanged as compared with controls after 9 days of incubation in high glucose (Table 1). We can thus conclude that, although the rate of *myo*-inositol incorporation into cellular phospholipids is decreased, the absolute mass of phosphoinositides is not reduced.

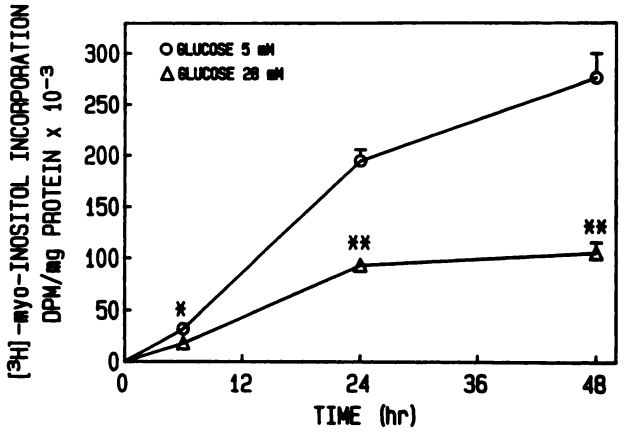

Figure 5. Effects of glucose on (^{3}H)*myo*-inositol incorporation into membrane phospholipids. $^*P < 0.05$; $^{**}P < 0.0001$. $N = 12$.

TABLE 1. Effect of 9-day incubation in high glucose on phospholipid mass[a]

	Glucose	
	5 mM	28 mM
PtdIns(4,5)P_2	3.2 ± 0.5	2.9 ± 0.1
PtdIns(4)P	4.5 ± 0.6	5.3 ± 0.4
PtdIns	153.0 ± 8.1	140.7 ± 19.5
Phosphatidic acid	2.9 ± 1.5	2.5 ± 0.7
Phosphatidylserine	172.7 ± 5.0	149.0 ± 12.5
Phosphatidylcholine	311.0 ± 33.6	300.7 ± 60.6
Phosphatidylethanolamine	160.0 ± 24.1	125.7 ± 13.8
Sphingomyelin	53.3 ± 5.7	48.0 ± 7.6

[a] Values represent mean ± SE (nanomoles/milligram of phospholipid) of three experiments.

Effects of Sorbinil on the Abnormalities in Phosphoinositide Hydrolysis Induced by Glucose

To assess the potential role of the polyol pathway on the abnormalities in phosphoinositide turnover induced by glucose, we incubated cells simultaneously in high glucose and 0.4 mM sorbinil, an aldose reductase inhibitor, and generated concentration-response curves of AII stimulation of IP release. Sorbinil at concentrations known to effectively inhibit aldose reductase in mesangial cells (4) failed to reverse the abnormalities in AII-stimulated phosphoinositide hydrolysis induced by high glucose (Figure 6). AII agonist efficacy relative to controls was decreased (67 ± 9%; $P < 0.001$) in the sorbinil plus high-glucose-treated group, which was not different from the high-glucose-only group (67 ± 9%). The EC_{50} in the sorbinil plus high-glucose-treated group was

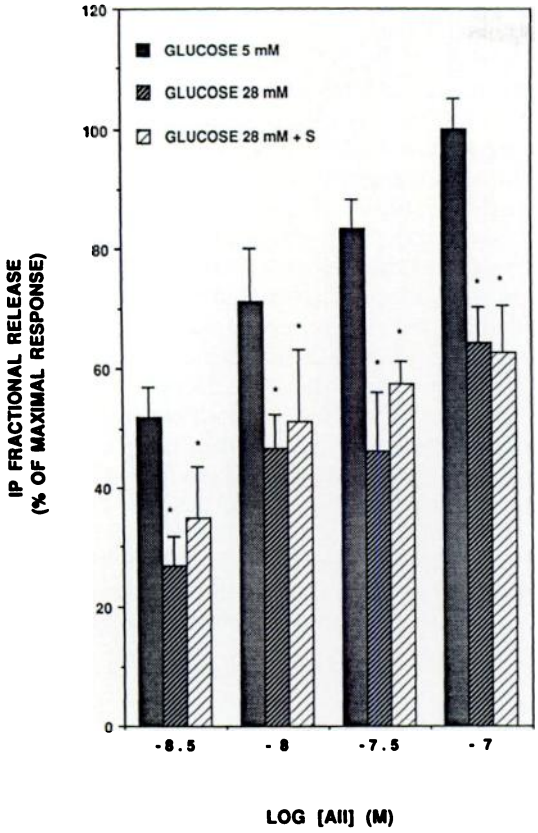

Figure 6. Effects of sorbinil (S; 0.4 mM) on the concentration-response curve of AII stimulation of IP release under high-glucose conditions. Results are shown as percent release relative to maximal IP fractional release in 5 mM glucose. *$P < 0.05$ versus 5 mM glucose at each concentration of AII. No difference in IP release was seen between high glucose in the absence or presence of sorbinil. $N = 6$.

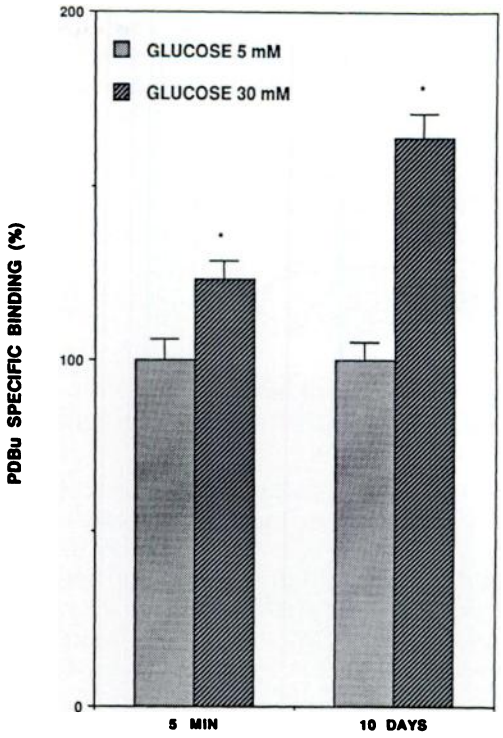

Figure 7. Effects of short-term (5 min) and long-term (10 days) high-glucose exposure on specific high-affinity (^{3}H) PDBu binding to active membrane-bound protein kinase C. Results were calculated as disintegrations per minute per milligram of protein and expressed as percent (^{3}H)PDBu binding in comparison to controls (5 mM glucose). Actual results were (in dpm/mg of protein): 1,976 ± 118 versus 2431 ± 107 (at 5 min) and 637 ± 34 versus 1,042 ± 47 (at 10 days) for 5 versus 30 mM glucose, respectively. *$P <$ 0.05. $N = 6$ (5 min) and 9 (10 days).

not significantly different from that in the two other groups (4 ± 1 versus 8 ± 3 nM in the controls and 11 ± 3 nM in the high-glucose-alone group). Sorbinil alone did not have an effect on agonist-stimulated phosphoinositide hydrolysis (data not shown). Thus, inhibition of aldose reductase activity does not prevent the inhibitory effects of high glucose on phosphoinositide hydrolysis.

Effects of High Glucose on Protein Kinase C Translocation and Activation

To determine if high glucose could cause protein kinase C translocation to its site of activation in the membrane, we measured specific high affinity [^{3}H] PDBu binding in intact mesangial cells. [^{3}H]PDBu binding increased as early as 5 min after exposure to high glucose and remained elevated for as long as hyperglycemia was maintained (experiments terminated at 10 days; Figure 7). This effect was not seen during incubation with fructose or mannitol at similar concentrations (data not shown), indicating that high glucose induced translocation of protein kinase C to its site of activation in a specific manner. The degree of protein kinase C translocation induced by high glucose was greater after long-term incubation (65 versus 23% increase in protein kinase C translocation at 10 days versus 5 min, respectively; $P <$ 0.05). Thus, elevated glucose increases membrane-associated protein kinase C which is likely to reflect increased activation of the enzyme.

Effects of High Glucose on *De Novo* Synthesis of DAG

Because other studies have demonstrated that glucose in high concentrations can be metabolized to DAG via glycerophospholipids and that this can result in activation of protein kinase C (13,30), we

measured the formation of glycerolipids by following their *de novo* synthesis from [^{14}C]glucose. Incubation of mesangial cells in high glucose resulted in a rapid increase in *de novo* synthesis of 1,2-DAG and triglycerides (Figure 8). Incorporation of [^{14}C]glucose into total cellular phospholipids was also increased; however, we did not detect changes in the levels of phosphatidic acid or monoglycerides. We can thus conclude that incubation with high glucose enhances *de novo* synthesis of DAG and triglycerides in mesangial cells.

Effects of *myo*-Inositol Supplementation on the Abnormalities in Phosphoinositide Hydrolysis Induced by Glucose

Previous studies in isolated rat pancreatic islets have demonstrated that high glucose causes an increase in ^{32}P labeling of CDP-diglyceride and PtdIns and that *myo*-inositol supplementation suppresses the enhanced labeling of CDP-diglyceride and further increases PtdIns labeling (31). By enhancing DAG incorporation into PtdIns, *myo*-inositol appears to be acting as a scavenger of DAG. This would decrease intracellular DAG levels inside cells and could constitute an important modulatory mechanism of protein kinase C activity. Given our previous results regarding the effects of high concentrations of glucose on the incorporation of *myo*-inositol into membrane phospholipids, we decided to study the effects of supplementing the culture medium with *myo*-inositol on the abnormalities in phosphoinositide turnover seen with high glucose. Mesangial cells were incubated simultaneously with high glucose and 490 μM *myo*-inositol over a period of 7 to 9 days. Forty-eight hours before an experiment, the concentration of *myo*-inositol was reduced to 30 μM (physiologic concentration) and the cells were labeled with [^{3}H] *myo*-inositol (2 μCi/mL). *myo*-Inositol supplementation completely reversed the inhibitory effects of high glucose on maximal AII and AVP-stimulated IP release (Figure 9). Supplementing the medium with *myo*-inositol actually caused an increase in the agonist-induced response. Thus, *myo*-inositol supplementation prevents the inhibitory effect of high glucose on agonist-stimulated phosphoinositide hydrolysis.

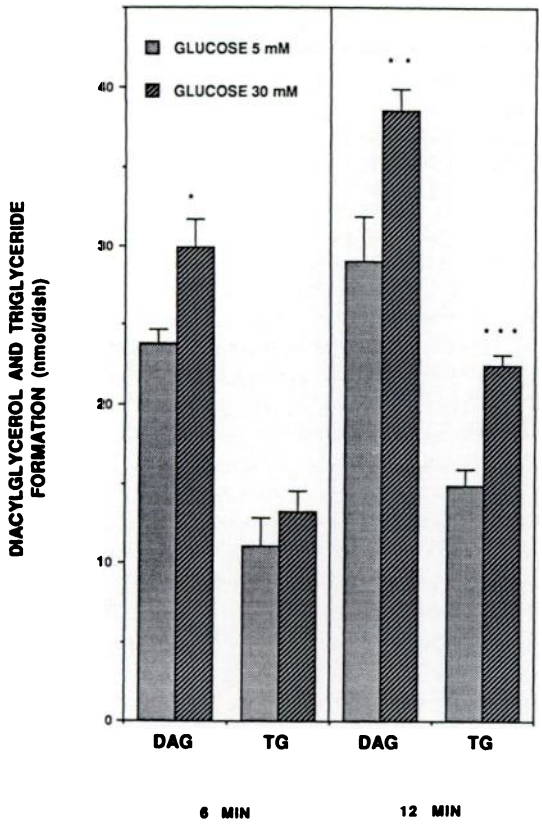

Figure 8. Effects of high glucose on *de novo* synthesis of DAG and triglycerides (TG) from ([^{14}C]glucose. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$. $N = 6$.

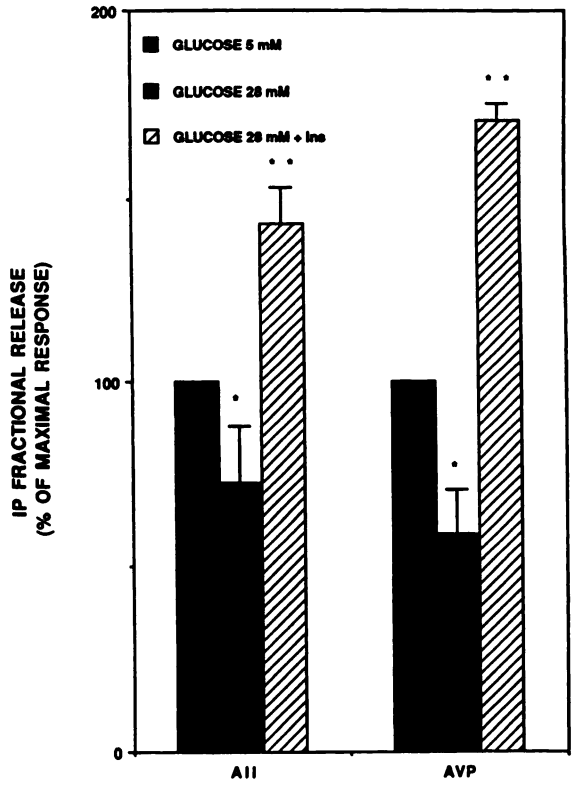

Figure 9. Effects of *myo*-inositol supplementation (Ins; 490 μM) on maximal AII and AVP stimulation of IP release under high-glucose conditions. Results are shown as percent release relative to maximal IP fractional release in 5 mM glucose. $^{*}P < 0.005$; $^{**}P < 0.001$. $N = 3$.

DISCUSSION

A variety of studies have suggested that a disruption of *myo*-inositol metabolism may be responsible for the development of some complications of diabetes mellitus (3). Our study demonstrates that exposure of mesangial cells in culture to high concentrations of glucose at levels not uncommonly seen in patients with diabetes mellitus results in a marked decrease in the generation of second messenger inositol phosphates by three different and potent vasoconstrictive agonists: AII, AVP, and the thromboxane A_2 analog, U46619. This effect was specific for glucose and not due to changes in osmolarity, because fructose, a hexose that has the same molecular weight as glucose but does not interfere with *myo*-inositol uptake or metabolism (32,33), did not induce any significant changes in agonist-stimulated IP release. In addition, our results suggest that the effects of glucose follow a concentration-response relationship, with the higher concentrations of glucose causing greater reductions in AII-stimulated phosphoinositide hydrolysis. We have been careful to quantitate our inositol phosphate release data relative to changes in incorporation, i.e., fractional release; however, similar changes were observed when IP release was expressed as a function of protein content or per well.

The inhibitory effect of glucose on receptor-stimulated phosphoinositide hydrolysis could underlie changes in mesangial cell function in diabetes. One study has shown that, when compared with normal controls, glomeruli isolated from streptozotocin-induced diabetic rats exhibit decreased ability to contract after stimulation with AII (2). This abnormality could not be attributed to changes in AII receptor density because the number of receptors was actually increased in the diabetic glomeruli. This upregulation of receptors could have been due to a decrease in circulating levels of AII, which were not reported in this study, to compensatory changes in response to a postreceptor defect, or to both. The authors concluded that the observed changes were most likely due to mesangial cell dysfunction (2).

There are several possible explanations for the inhibitory effect of glucose on receptor-stimulated IP formation observed in this study. Previous studies in streptozotocin-induced diabetic rats have shown that the content of *myo*-inositol in the sciatic nerve, the autonomic superior cervical ganglion, and the renal glomerulus is reduced and that this reduction appears to be related to the level of hyperglycemia (3,34,35). *myo*-Inositol depletion could lead to abnormal phosphoinositide metabolism and mesangial cell dysfunction and could explain our findings.

Two mechanisms have been proposed to explain the observed reduction in *myo*-inositol content in these tissues: activation of the polyol pathway with intracellular accumulation of sorbitol and competitive inhibition of cellular *myo*-inositol uptake by elevated glucose.

In the first instance, glucose at high extracellular concentrations enters the cell and is reduced to sorbitol by aldose reductase. Sorbitol is then itself reduced to fructose by sorbitol dehydrogenase. The activity of this enzyme, however, is more than sevenfold lower than that of aldose reductase, and, therefore, rapid intracellular accumulation of sorbitol occurs. Activation of the polyol pathway resulting in intracellular accumulation of sorbitol has been associated with decreased total cell and cell membrane levels of *myo*-inositol (32) by an as yet unknown mechanism. Aldose reductase inhibitors prevent the formation of sorbitol and have been shown in some studies to prevent the reduction in tissue *myo*-inositol content induced by hyperglycemia (3,32,34,35). In addition, the presence of aldose reductase activity and accumulation of sorbitol have been demonstrated in mesangial cells exposed to high concentrations of glucose (17). Even though the concentrations of glucose used were very high (55 mM), the accumulation of sorbitol was completely prevented by the use of an aldose reductase inhibitor. Those authors postulated that activation of the polyol pathway with intracellular accumulation of sorbitol in mesangial cells could play a role in the pathogenesis of diabetic nephropathy. More recent studies in diabetic patients, however, have cast some doubt on the meaning and universality of the association between the polyol pathway and the complications of diabetes (36,37). In our *in vitro* studies, the aldose reductase inhibitor sorbinil failed to prevent the inhibitory effects of glucose on phosphoinositide hydrolysis, suggesting that the polyol pathway does not play a significant role in their genesis.

Another possible explanation for the abnormalities in phosphoinositide hydrolysis observed in our study is that glucose could have an inhibitory effect on *myo*-inositol uptake, its incorporation into membrane phospholipids, or both. A number of studies have shown that high glucose levels cause both competitive and noncompetitive types of inhibition of *myo*-inositol uptake and incorporation into membrane phospholipids in a variety of tissues (4,32,33). Incorporation of *myo*-inositol into membrane phospholipids in mesangial cells, however, has not been investigated. Our findings indicate that mesangial cells exposed to high glucose concentrations exhibit a decreased rate of *myo*-inositol label incorporation into membrane phospholipids. This finding suggested that decreased incorporation of *myo*-inositol and decreased *myo*-inositol uptake might reduce cellular Ptdins(4,5)P_2 and thereby reduce agonist-stimulated hydrolysis. However, because the synthesis of phosphoinositides is a very complex process and label incorporation can be complicated because of

changes in specific activity, we decided to measure phosphoinositide mass. Surprisingly, total cellular phosphoinositide mass was unchanged after several days of incubation with high glucose. One possible explanation for this discrepancy could be that glucose interferes with *myo*-inositol incorporation into small and discrete agonist-sensitive PtdIns(4,5)P_2 pools that are below the sensitivity levels of our phosphoinositide mass determination assay. Some studies have suggested the existence of a unique fraction of inositol phospholipid pools which may be depleted during diabetes mellitus (38); however, recent studies favor the hypothesis that inositol polyphosphates are not formed from a rapid turnover pool separate from the bulk of cellular phosphoinositides (39). In any case, we did not see a decrease in PtdIns(4,5)P_2 mass in the presence of high glucose suggesting that our finding of decreased PtdIns(4,5)P_2 hydrolysis is not secondary to a large depletion in PtdIns(4,5)P_2 mass although depletion of a small receptor-sensitive pool cannot be excluded. Whether the reduction in *myo*-inositol incorporation into phospholipids caused by glucose is the result of a decrease in total cellular *myo*-inositol or is a direct metabolic alteration in *myo*-inositol shuttling within the cell or both is currently unknown and deserves further investigation.

Our data could be explained by reductions in AII, AVP, and thromboxane A_2 receptor density. However, the observation that the inhibitory effect of glucose on receptor-mediated phosphoinositide hydrolysis can be prevented by *myo*-inositol supplementation, taken together with the reported finding of increased AII receptor density in diabetic glomeruli, makes this possibility unlikely.

Recent studies in a number of systems including mesangial cells have demonstrated that activation of protein kinase C by phorbol esters and physiologic agonists can inhibit receptor-mediated phosphoinositide hydrolysis (14–16). Thus, an alternative and more likely explanation for the decrease in receptor-mediated phosphoinositide hydrolysis induced by high glucose is that activation of protein kinase C through *de novo* DAG synthesis could lead to receptor uncoupling and desensitization of the response (Figure 10). Our results showing an increase in *de novo* DAG synthesis from glucose coupled with increased protein kinase C activation are consistent with previous findings in endothelial cells and glomeruli (13,30) and could explain the desensitization of phosphoinositide responses observed during hyperglycemic conditions.

The potential DAG scavenging effect of *myo*-inositol via DAG shunting into PtdIns raises the interesting possibility that *myo*-inositol may play a modulatory role on protein kinase C by reducing intracellular levels of DAG (Figure 10). Our finding that supraphysiologic concentrations of *myo*-inositol prevent the effects of glucose is interesting and may represent repletion of small agonist-sensitive phosphoinositide pools depleted by glucose, scavenging of excess DAG synthesized via glycerophospholipids from glucose, or both. Although there is evidence in the literature that these mechanisms can be operative under certain circumstances (31,38), the design of this study does not allow us to elucidate the specific mechanisms by which this effect is occurring.

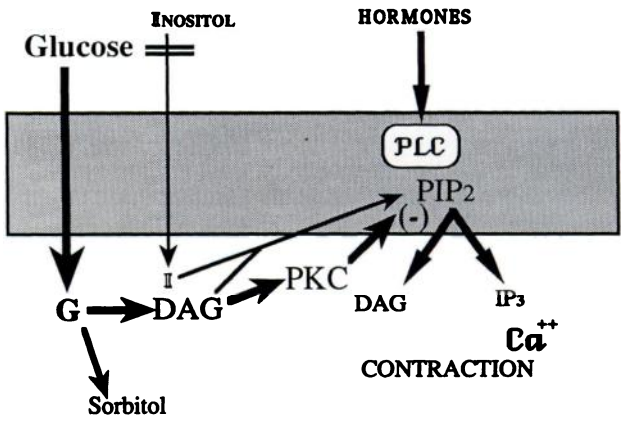

Figure 10. Schematic on glucose and inositol metabolism in mesangial cells. Hormone stimulation of phospholipase C (PLC) causes hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) forming DAG and inositol 1,4,5-trisphosphate (IP_3), releasing intracellular calcium, and causing contraction. Glucose can block inositol transport into cells reducing its incorporation into PIP_2. Glucose can be metabolized to sorbitol and/or DAG. DAG could activate protein kinase C (PKC) leading to inhibition of PIP_2 hydrolysis. Inositol and DAG combine to form phosphoinositides. Inositol supplementation may increase cellular inositol levels tending to decrease levels of DAG formed *de novo* from glucose. Inositol by reducing DAG levels and PKC activation could antagonize the inhibition of PIP_2 hydrolysis by PKC.

The results of this study are clinically relevant because of the potential implications regarding the effects of glucose on different tissues that use the phosphoinositide cascade as the major signalling system for the generation of physiologic responses to extracellular stimuli. One important circumstance, for example, could occur during the early stages of diabetes mellitus where hyperglycemia could lead to altered second messenger production in mesangial cells and thus make them unable to respond appropriately to critical stimuli by different vasoactive hormones that are continuously and very precisely modulating glomerular capillary blood flow. This mesangial cell dysfunction could lead to impaired glomerular contractility, resulting in increased capillary blood flow and hyperfiltration, and could perhaps trigger some of the other pathologic changes that occur in diabetic nephropathy (e.g., mesangial expansion and glomerulosclerosis). Similar effects of glucose on vascular smooth muscle could, at least in

part, explain the abnormal vascular reactivity and other abnormalities in vascular smooth muscle function seen in diabetes mellitus. It will be important to determine if the described changes in second messenger generation have an effect on their respective physiologic responses such as calcium mobilization and cell contraction. It will also be important to determine the mechanisms by which supplemental *myo*-inositol prevents the changes induced by glucose. Studies are currently underway to elucidate these important questions.

In conclusion, high concentrations of glucose cause alterations in mesangial cell *myo*-inositol metabolism that lead to a reduction in phosphoinositide turnover and second messenger generation in response to vasoconstrictive agonists. These alterations appear to be unrelated to the polyol pathway, are associated with increased protein kinase C activation through *de novo* DAG synthesis from glucose, and can be completely prevented by *myo*-inositol supplementation. The abnormalities in mesangial cell signal transduction induced by high concentrations of glucose may play an important role in the pathogenesis of the alterations in glomerular hemodynamics seen early in diabetes mellitus, as well as in the progression of diabetic nephropathy. These same mechanisms may also play a role in the development of other complications of diabetes mellitus.

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