Effect of Inhibition of Aldose Reductase on Glucose Flux, Diacylglycerol Formation, Protein Kinase C, and Phospholipase A_2 Activation

Rosemary J. Keogh, Marjorie E. Dunlop, and Richard G. Larkins

Activation of the polyol pathway under hyperglycemic conditions is proposed to contribute to the development of diabetic nephropathy. The mechanisms by which this activation may lead to functional and structural changes within the kidney are yet to be definitively established. We have examined in vitro the steps linking increased polyol pathway activity resulting from hyperglycemia to prostaglandin production. Following the demonstration of increased prostaglandin E (PGE) levels in glomeruli from diabetic rats (14.9 ± 2.5 *v* 59.1 ± 19.4 ng PGE/mg protein), a specific inhibitor of aldose reductase, HOE-843, was used in vitro to analyze the response to hyperglycemia of the steps preceding prostaglandin production. In explants of glomeruli from control animals, increasing the glucose concentration in vitro from 5.6 mmol/L to 25 mmol/L resulted in a significant increase in the flux of glucose through the pentose phosphate pathway ([PPP] 1.29 ± 0.08 *v* 2.00 ± 0.11 nmol/h), de novo diacylglycerol synthesis (2.2 ± 0.1 *v* 3.1 ± 0.2 μmol/mg protein), membrane protein kinase C (PKC) activity (18.7 ± 0.5 *v* 24.3 ± 0.75 pmol/μg protein), and in vitro phospholipase A_2 (PLA_2) activity (2.18 ± 0.46 *v* 3.83 ± 1.07 nmol arachidonic acid hydrolyzed/min/mg cytosolic protein). For all parameters measured, the increase resulting from the increased glucose concentration could be prevented by in vitro addition of HOE-843 for 24 hours before measurement. These findings provide evidence to suggest a mechanism linking increased polyol pathway activity and an increase in PLA_2 activity to increased prostaglandin production, which is observed in diabetes of recent onset and may ultimately lead to changes associated with the development of diabetic nephropathy.

SEVERAL BIOCHEMICAL mechanisms have been implicated as contributing to the development of diabetic nephropathy with its characteristic structural changes of enlargement of the mesangial matrix[1,2] and thickened glomerular capillary basement membrane.[3] These mechanisms include increased nonenzymatic glycation of proteins,[4] decreased mesangium degradation,[5] increased oxidative stress resulting from excess free-radical production[6] and impairment of free-radical scavenging,[7-9] increased aldose reductase pathway activity,[10] and increased diacylglycerol synthesis with protein kinase C (PKC) activation and increased prostaglandin production.[11] Although there is abundant evidence from experimental diabetes that the aldose reductase pathway is important in the development of diabetic nephropathy, the mechanisms by which it might cause the functional and structural changes have not been elucidated.

Initial changes in kidney function in diabetes that precede excess matrix synthesis include glomerular hyperfiltration due to an increase in the renal blood flow and an associated increase in glomerular filtration rate. Glomerular hyperfiltration has been demonstrated both in experimental animal models of diabetes[12,13] and in humans.[14,15] The vascular reactivity of the renal glomerular efferent arterioles has been shown to be controlled at least in part by the release of endogenously synthesized prostaglandins, allowing autoregulation of the glomerular capillary pressure and thus the ultrafiltration rate within the glomerulus.[16] Due to the role prostaglandins play in controlling renal function, it is proposed that changes in prostaglandin production may contribute to the hemodynamic changes seen in diabetes. Several studies have shown that renal prostaglandin production is increased at the onset of diabetes.[17-20] However, the cellular mechanisms underlying this change are yet to be definitively established.

It has been demonstrated that glomerular hyperfiltration of diabetes can be reduced in both rats[21] and humans[22] by inhibition of the polyol pathway enzyme aldose reductase. Furthermore, glomerular production of the vasodilatory prostaglandin E_2 (PGE_2) and prostacyclin can be suppressed in diabetic rats treated with an aldose reductase inhibitor in vivo.[23-25] This suggests a potential connection between the polyol pathway and increased prostaglandin production in diabetes.

A hypothesis that involves an enhancement of polyol pathway activity may be proposed to link the increased glucose levels seen in diabetes with the observed alterations in renal prostaglandin production. At elevated glucose concentrations, there is a concomitant increase in the activity of the polyol pathway. This has been shown as increased sorbitol production not only in diabetic kidney[24,26] but also in peripheral nerve and retina[27] and lens.[28] As a consequence of the acceleration of the polyol pathway, there are changes in the ratios NADPH/$NADP^+$ (following glucose conversion to sorbitol via aldose reductase) and NAD^+/NADH (following sorbitol conversion to fructose via sorbitol dehydrogenase) that have been shown to be associated with increased pentose phosphate pathway (PPP) activity.[29]

The PPP is one source of glucose-derived triose phosphate precursors used in the de novo formation of diacylglycerol, a physiologic activator of PKC, and increasing glucose levels have been shown to increase de novo diacylglycerol formation.[30] PKC has been demonstrated to be activated in glomeruli from diabetic rats[31] and in glomerular mesangial cells exposed to high glucose concentrations in vitro.[32] PKC is able to phosphorylate and activate a cytosolic form of phospholipase

From the Department of Medicine, University of Melbourne, Royal Melbourne Hospital, Parkville, Victoria, Australia.

Submitted February 12, 1996; accepted August 23, 1996.

Supported by a National Health and Medical Research Council of Australia Program Grant and grants from the Diabetes Australia Research Trust and Juvenile Diabetes Foundation International.

Address reprint requests to Marjorie E. Dunlop, PhD, Department of Medicine, University of Melbourne, PO Royal Melbourne Hospital, Parkville, Victoria 3050, Australia.

0026-0495/97/4601-0008$03.00/0

A_2 (PLA_2),[33] the enzyme that releases arachidonic acid from phospholipids for the production of prostaglandins, and PLA_2 activity is increased in glomeruli from diabetic rats.[34] In this way, increased prostaglandin production in diabetes can be seen to result from a chain of cellular events initiated by hyperglycemia, and a metabolic pathway can be potentially mapped from glucose to prostaglandin synthesis.

In the present investigation, following the demonstration of increased prostaglandin levels in glomeruli from diabetic rats in vivo, we wished to examine in vitro the steps in the proposed link between increased aldose reductase activity and the pathway leading from hyperglycemia to prostaglandin production. Specifically, an inhibitor of aldose reductase, HOE-843, was used to block the first step of the polyol pathway, the conversion of glucose to sorbitol. The inhibitor has been used previously in a study of vasoreactivity in streptozotocin (STZ)-diabetic rats. In vivo administration of HOE-843 resulted in marked prevention of sorbitol and fructose accumulation in sciatic nerve of treated animals.[35] In the current study, the effect of inhibiting aldose reductase with HOE-843 on the early cellular events of glucose flux via the PPP, diacylglycerol production, PKC activity, and PLA_2 activity in explant cultures of glomeruli from control animals was determined.

MATERIALS AND METHODS

Materials

[5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2, 1-stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine, and a PKC assay kit were purchased from Amersham International (Buckinghamshire, England). D-[1-^{14}C]glucose, D-[6-^{14}C]glucose, [U-^{14}C]glucose, and 2-[U-^{14}C]ketoglutarate were from DuPont/NEN (Boston, MA). PGE_2 antiserum was from PerSeptive Diagnostics (Cambridge, MA). HOE-843 (imirestat: M_r 286.24, M_f $C_{15}H_8F_2N_2O_2$, spiro-(2,7-difluoro-fluorene-9,4′-imidazolidine)-2′5′-dione), a novel and potent aldose reductase inhibitor in vitro acting at the active site of the enzyme with a 50% inhibitory capacity of 8.5×10^{-9} mol/L in rat lens (technical information provided), was a gift from Dr P. Teichmann of Hoechst (Frankfurt am Main, Germany). All solvents were from BDH (Poole, England) and were analytical grade. All other reagents were from Sigma Chemical (St Louis, MO) and were of analytical grade.

Induction of Diabetes

Male Sprague-Dawley rats weighing approximately 180 g were made diabetic with a single intraperitoneal injection of STZ at a dose of 70 mg/kg body weight in sodium citrate buffer (100 mmol/L, pH 4.0). Control rats received an injection of vehicle alone. Diabetes was confirmed by a positive result on a dipstick urinalysis test for glucose. All rats were maintained for a 3-week period before killing, and STZ-diabetic animals did not receive any insulin treatment.

Preparation of Glomeruli

Mesangial cell–enriched glomerular cores were isolated by differential sieving of kidney cortices from control and 3-week STZ-diabetic rats as previously described.[24] Following limited collagenase digestion (0.5 mg/mL for 30 minutes), the cores were divided among culture dishes at approximately 5,000 to 7,000 cores per dish in RPMI supplemented with 2% (vol/vol) fetal calf serum (FCS) and penicillin and streptomycin (10 U/mL), with either 5.6 mmol/L (control) or 25 mmol/L (STZ-diabetic) glucose. These freshly isolated glomerular cores were maintained for 24 hours in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Preparation of Explant Cultures of Glomerular Cores

Mesangial cell–enriched glomerular cores were isolated from control male Sprague-Dawley rats weighing approximately 180 to 200 g using the technique already described. Following the limited collagenase digestion, the cores were divided among culture dishes at approximately 5,000 to 7,000 cores per dish and maintained in Dulbecco's modified Eagle's medium at 5.6 mmol/L glucose supplemented with 20% (vol/vol) FCS and penicillin and streptomycin (10 U/mL) for 48 hours. The medium was then changed to Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) FCS and penicillin and streptomycin (10 U/mL) with either 5.6 or 25 mmol/L glucose for another 5 days. After this time, explants were maintained under serum-free conditions for a further 24 hours in RPMI at 5.6 or 25 mmol/L glucose supplemented with penicillin and streptomycin (10 U/mL). The aldose reductase inhibitor HOE-843 at a concentration of 10^{-8} mol/L was added over this latter time. All cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

PGE Production

Total immunoreactive PGE activity was measured in the 24-hour incubation medium either from freshly isolated control and STZ-diabetic glomerular cores or from explant cultures of glomerular cores by a specific radioimmunoassay, as described previously.[24] An anti-PGE_2 antibody with 50% cross-reactivity for PGE_1 was used at a final dilution of 1:45,000 with [5,6,8,11,12,14,15 (*n*)-^{3}H]PGE_2 (specific activity, 180 to 190 Ci/mmol). Antibody-bound PGE was separated from free ligand with dextran-coated charcoal.

Measurement of Sorbitol

Sorbitol content of explanted glomerular cores was measured using a previously described radioisotopic method.[36] Briefly, explant cultures were scraped into 2.5% (vol/vol) perchloric acid and centrifuged at $10,000 \times g$ for 1 minute, and the supernatants were collected and neutralized to pH 7.0. These samples were incubated for 60 minutes at 20°C in glycine buffer (50 mmol/L), pH 9.7, containing NAD^+ (2 mmol/L) with or without sorbitol dehydrogenase (0.1 mg/mL), and the reaction was stopped by heating the samples for 10 minutes at 75°C. Subsequently, NADH formed by the action of sorbitol dehydrogenase was determined by a 30-minute incubation at 20°C in HEPES buffer (100 mmol/L), pH 7.0, containing 100 μmol/L 2-[U-^{14}C]ketoglutarate (final specific activity, 5 mCi/mmol), adenosine diphosphate (1 mmol/L), and beef liver glutamate dehydrogenase (6 U/mL). ^{14}C-glutamate formed was separated by ion-exchange chromatography on Dowex 50 (H^+ form) and eluted with ammonium acetate (1 mol/L), and radioactivity was determined by liquid scintillation counting in a β-counter (Beckman Instruments, Fullerton, CA). Sorbitol content was calculated from the difference in radioactivity (glutamate formed) in incubations in the presence and absence of sorbitol dehydrogenase compared with sorbitol standards, and was expressed as picomoles of sorbitol per milligram of protein.

Glucose Flux

Glucose utilization in explanted glomerular cores was determined by the production of $^{14}CO_2$ from cultures in the presence of 0.5 μCi D-[1-^{14}C]glucose (C_1; specific activity, 53.4 mCi/mmol) or D-[6-^{14}C]glucose (C_6; specific activity, 51.8 mCi/mmol) sealed under 95% O_2/5% CO_2 at 37°C. The flux of glucose through the PPP was calculated as the difference of C_1 glucose hydrolysis (TCA cycle + PPP) and C_6 glucose hydrolysis (TCA cycle).[26]

Measurement of Diacylglycerol

Diacylglycerol formation was measured in explanted glomerular cores following the incorporation of [U-^{14}C]glucose with subsequent

thin-layer chromatography to identify the lipid products, as described previously.[37]

Measurement of PKC Membrane Activity

Membrane PKC activity was measured on membrane/cytoskeletal fractions prepared from explanted glomerular cores as previously described.[38] Briefly, explant cultures were permeabilized in a calcium-free medium with 60 μg/mL digitonin. Following centrifugation to separate cytosol from membrane, the membranes were solubilized in 0.1% (vol/vol) Triton X-100 for inclusion in the PKC assay, which measured the ability to phosphorylate a specific PKC substrate octapeptide in a mixed-micelle kit assay.

PLA_2 Activity

Total PLA_2 activity was measured in vitro using the method of Lin et al.[39] PLA_2 activity is measured as total activity and represents activity from all forms of PLA_2 present in the cytosolic preparations. The activity is thus the relative contribution from cytosolic and secretory PLA_2. A reported calcium-independent form[40] does not contribute to total activity, since the assay does not contain the necessary cofactors for the activity and no activity is detectable in the absence of calcium (data not shown). Briefly, explant cultures were washed and then homogenized with 25 strokes in a Dounce homogenizer in a sucrose (250 mmol/L)/HEPES (50 mmol/L) buffer, pH 7.4, containing EDTA (1 mmol/L), EGTA (1 mmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (20 μmol/L), and trasylol (100 KIU/mL). A cytosol fraction was prepared for assay by centrifugation of the homogenates at 100,000 × *g* for 1 hour at 4°C in a Beckman TL-100 ultracentrifuge. 1-Stearoyl-2-[1-^{14}C]arachidonyl phosphatidylcholine (initial specific activity, 55.6 mCi/mmol) was used as a substrate at a final concentration in the assay of 15 μmol/L. Samples were incubated with substrate for 30 minutes at 37°C in the presence of 5 mmol/L $CaCl_2$. The reaction was stopped by addition of 2% (vol/vol) acetic acid in ethanol containing 100 μg/mL unlabeled arachidonic acid. Samples were spotted onto heat-activated (110°C for 30 minutes) Whatman (Clifton, NJ) LK5D TLC plates, and the plates were developed with the organic phase of ethyl acetate:iso-octane:acetic acid:water (55:75:8:100 vol/vol/vol/vol). Following identification by autoradiography, the bands were scraped and counted in a Beckman β-counter. PLA_2 activity was calculated as nanomoles of arachidonic acid hydrolyzed from substrate per minute per milligram of cytosolic protein.

Protein Measurement

The protein content of cytosol fractions or of cells solubilized in 1 mol/L NaOH was measured using Coomassie Brilliant blue dye[41] with bovine serum albumin as a standard.

Statistical Analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS-PC; SPSS, Chicago, IL). Results were compared by one-way ANOVA. Where significant differences were indicated (F ratio with $P < .05$), Levene's test for equality of variance using an F distribution was used to determine the appropriate analysis. Since the data were found to be normally distributed but differing in variance, the Wilcoxon signed-rank test for nonparametric data was applied. Significance was taken as P less than .05 unless otherwise indicated. Data are presented as the mean ± SEM.

RESULTS

Prostaglandin Production

Prostaglandin production was measured in the 24-hour incubation medium from glomerular cores following isolation from control and STZ-diabetic rats. There was a significant increase in the amount of PGE produced by the glomerular cores from STZ-diabetic animals (control *v* diabetic, 14.9 ± 2.5 *v* 59.1 ± 19.4 ng PGE/mg protein, $P < .05$ for n = 5 separate experiments with triplicate determinations). When PGE production was measured in the 24-hour incubation medium from explanted glomerular cores, a significant increase was observed in response to culture in 25 mmol/L glucose (Table 1). This increase could be inhibited by incubation of the cultures with HOE-843 (10^{-8} mol/L) for 24 hours (Table 1).

HOE-843 at a concentration of 10^{-8} mol/L was without effect on prostaglandin synthase activity in glomerular mesangial cells. This was determined by measuring PGE production at 5.6 mmol/L glucose following stimulation with a supramaximal dose of arachidonic acid (30 μmol/L) for 30 minutes. In arachidonic acid–stimulated cells, PGE production was found to be 490.6 ± 85.9 ng PGE/mg protein in the presence of HOE-843 and 452.1 ± 93.7 ng PGE/mg protein in the absence of HOE-843 (nonsignificant).

Sorbitol Production

The ability of HOE-843 to act as an aldose reductase inhibitor was confirmed by measuring sorbitol content in explanted glomerular cores following a 24-hour incubation with 25 mmol/L glucose in the presence or absence of the inhibitor. Sorbitol content of explanted glomerular cores was significantly increased from 137.1 ± 13.4 pmol sorbitol/mg protein at 5.6 mmol/L glucose to 199.1 ± 11.2 pmol sorbitol/mg protein at 25 mmol/L glucose ($P < .01$, n ≥ 5 from two independent determinations). This increase in sorbitol content could be prevented by incubation of the explanted glomerular cores with HOE-843 (10^{-8} mol/L) for 24 hours before measurement. Sorbitol content in the presence of HOE-843 at 5.6 mmol/L glucose was 121.5 ± 4.5 pmol/mg protein, versus 123.5 ± 16.2 pmol sorbitol/mg protein at 25 mmol/L glucose (nonsignificant). This ability of HOE-843 to block an increase in sorbitol accumulation demonstrates its action as an inhibitor of aldose reductase.

Glucose Fluxes

In explanted glomerular cores cultured with elevated glucose, there was a significant increase in the flux of glucose through the PPP when measured at a single glucose concentration (5.6 mmol/L; Table 2). Addition of the aldose reductase inhibitor HOE-843 (10^{-8} mol/L) to the culture for 24 hours before

Table 1. Effect of Aldose Reductase Inhibition With HOE-843 on PGE Production by Glomerular Explants From Control Kidneys Cultured at 5.6 or 25 mmol/L Glucose

Culture Condition	PGE Production (% control)
Glucose	
5.6 mmol/L	100.0 ± 5.8
5.6 mmol/L + HOE-843	114.2 ± 8.7
25 mmol/L	204.2 ± 27.4*
25 mmol/L + HOE-843	144.4 ± 17.2†

NOTE. Results are the mean ± SEM for 5 independent experiments with ≥3 determinations each, and are expressed for each experiment as a percentage of the 5.6-mmol/L glucose value for that experiment.

*$P < .01$ *v* 5.6 mmol/L glucose.

†$P < .04$ *v* 25 mmol/L glucose in the absence of HOE-843.

Table 2. Effect of Aldose Reductase Inhibition With HOE-843 on Glucose Utilization by the PPP in Glomerular Explants From Control Kidneys Cultured at 5.6 or 25 mmol/L Glucose

Culture Condition	Glucose Flux (nmol/h)	PPP/TCA ($[C_1\text{-}C_6]/C_6$)
Glucose		
5.6 mmol/L	1.29 ± 0.08	4.8 ± 0.3
5.6 mmol/L + HOE-843	1.20 ± 0.11	4.5 ± 0.3
25 mmol/L	2.00 ± 0.11*	7.6 ± 0.4*
25 mmol/L + HOE-843	1.25 ± 0.05†	5.4 ± 0.4†

NOTE. Results are the mean ± SEM from 2 independent experiments each with duplicate determinations.

*$P < .05$ *v* 5.6 mmol/L glucose.

†$P < .05$ *v* 25 mmol/L glucose in the absence of HOE-843.

measurement of the fluxes was without effect on basal flux through the PPP in explants cultured at 5.6 mmol/L glucose. However, in explants cultured at 25 mmol/L glucose, HOE-843 was able to completely inhibit an increase in flux through the PPP observed in its absence, so that the flux was not significantly different from the level seen at 5.6 mmol/L glucose (Table 2). Thus, in explants previously cultured at 25 mmol/L glucose, an increase in flux through the PPP could be demonstrated at 5.6 mmol/L glucose.

De Novo Diacylglycerol Production

The synthesis of diacylglycerol from glucose in explant cultures is shown in Fig 1a. Increasing the glucose concentration to 25 mmol/L resulted in a significant increase in de novo synthesis of diacylglycerol. Preincubation of the cultures with HOE-843 (10^{-8} mol/L) for 24 hours before measuring diacylglycerol production had no effect on the basal synthesis at 5.6 mmol/L glucose. In cultures previously maintained at 25 mmol/L glucose, the aldose reductase inhibitor reduced the de novo synthesis of diacylglycerol to a level not significantly different from the basal 5.6-mmol/L level (Fig 1a).

Membrane PKC Activity

The activity of PKC was determined in membrane fractions prepared from explant cultures of glomerular cores and is shown in Fig 1b. Increasing the glucose concentration of the explant cultures to 25 mmol/L caused a significant increase in membrane-associated PKC activity. This increase was prevented by preincubation of the explant cultures for 24 hours with the aldose reductase inhibitor HOE-843 (10^{-8} mol/L).

PLA_2 Activity

PLA_2 activity measured in cytosolic extracts from explant cultures of glomerular cores in the presence or absence of the aldose reductase inhibitor HOE-843 (10^{-8} mol/L) is shown in Fig 1c. Culture of explants at 25 mmol/L glucose resulted in a significant increase in the basal level of PLA_2 activity measured in vitro. Addition of the aldose reductase inhibitor HOE-843 was without effect on basal PLA_2 activity at 5.6 mmol/L glucose. In explant cultures that had been maintained in 25 mmol/L glucose, addition of HOE-843 for 24 hours totally prevented the increase in basal activity observed with elevated glucose.

DISCUSSION

The results of this study provide evidence to suggest that there is a biochemical link between increased polyol pathway activity and an increase in PLA_2 activity that may be associated with the increased renal prostaglandin production seen in diabetes of recent onset. The demonstration of increased PGE production by glomerular cores from STZ-diabetic rats in the present study is in accordance with our previous findings and those of other groups. Craven[19] showed that glomerular production of PGE_2, 6-keto-$PGF_{1\alpha}$, and thromboxane B_2 significantly increased in Sprague-Dawley rats 9 to 15 days after induction of STZ-diabetes, and Schambelan[18] observed a similar increase for PGE_2 and $PGF_{2\alpha}$ but not for thromboxane B_2 production in glomeruli isolated from rats with STZ-diabetes of 9 to 23 days in duration. A previous study from our laboratory demonstrated

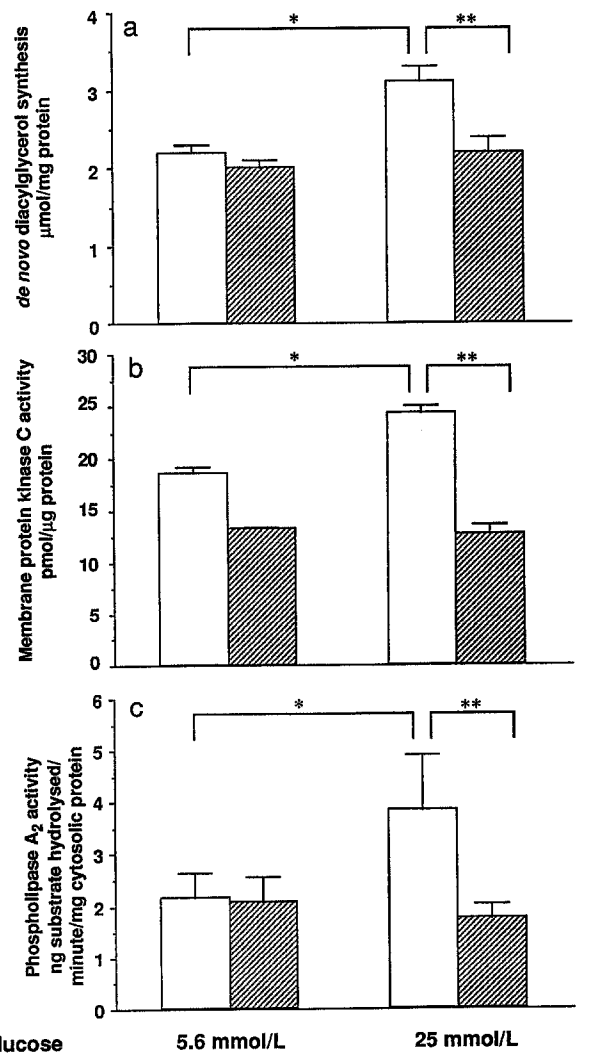

Fig 1. Effect of aldose reductase inhibition with HOE-843 on (a) de novo diacylglycerol synthesis, (b) membrane PKC activity, and (c) in vitro PLA_2 activity in glomerular explants from control kidneys cultured at 5.6 or 25 mmol/L glucose. (▨) With HOE-843 (10^{-8} mol/L); (□) without HOE-843. Results are the mean ± SEM for ≥3 separate experiments with ≥3 determinations each. *$P < .05$ *v* 5.6 mmol/L glucose and **$P < .03$ *v* 25 mmol/L glucose in the absence of HOE-843.

that the increase in PGE production resulting from the induction of STZ-diabetes can be reduced by in vivo administration of an inhibitor of the polyol pathway enzyme aldose reductase.[24] To understand the cellular events underlying this, we have used a culture system of explanted control glomerular cores maintained in low (5.6 mmol/L) or high (25 mmol/L) glucose concentrations to demonstrate increased PGE production, and have examined in vitro the effect of increasing glucose on some of the biochemical steps proposed to precede prostaglandin production. Inhibition of aldose reductase in vitro was able to normalize to a basal (low glucose) level the glucose flux through the PPP, de novo diacylglycerol production, PKC activity, and PLA_2 activity in explant cultures, all of which were increased in response to increased glucose concentrations.

The contribution of enhanced polyol pathway activity to the development of diabetic nephropathy, particularly the role of the enzyme aldose reductase, has been the subject of much investigation. Craven and DeRubertis[23] were able to demonstrate an effect of an aldose reductase inhibitor, sorbinil, to reduce glomerular filtration rate and glomerular prostaglandin production in 1- to 2-week STZ-diabetic rats when the inhibitor was given in vivo. This occurred without any change in sorbitol levels, and hence they concluded that the action of sorbinil was distinct from an effect on aldose reductase. However, results from our laboratory showed that two structurally dissimilar aldose reductase inhibitors (sorbinil and ponalrestat) both reduced the increased vasodilatory prostaglandin production in experimental diabetes, and this effect was correlated with a reduction in renal cortical sorbitol production, suggesting that the effect was indeed due to aldose reductase inhibition.[24] Our current results confirm that inhibition of aldose reductase prevents the increase in flux through the PPP, de novo diacylglycerol synthesis, PKC, and PLA_2 activation, demonstrating a plausible mechanism for the effects previously observed in vivo. The postulated pathway is shown in Fig 2.

Other studies also support a role for increased polyol pathway activity as an initiating factor leading to increased prostaglandin production. Sochor et al[26] were able to demonstrate an increase in the flux of glucose through the PPP in rats with STZ-diabetes of 9 days' duration that could be prevented by in vivo administration of the aldose reductase inhibitor ponalrestat. Our results in vitro also suggest that an increased flux through the PPP resulting from exposure to a high glucose concentration can be returned to the basal, low glucose level by treatment with the aldose reductase inhibitor HOE-843. Sochor et al[26] also found that after 3 weeks of STZ-diabetes, a further 9 days of in vivo administration of ponalrestat resulted only in a minor reduction in glucose flux through the PPP in STZ-diabetic animals. They proposed that after longer-term diabetes, the metabolic changes in glucose metabolism in the kidney are sufficiently well established and refractory to pharmacologic reversal.

It is possible that alterations in the level of aldose reductase mRNA or its activity could contribute to the observed increase in glucose flux through the polyol pathway. Although not measured in the current study, there is now some preliminary evidence that aldose reductase gene expression is increased in diabetes. Recently, a retinal pigmented epithelial cell line was reported to show an increased basal expression of aldose

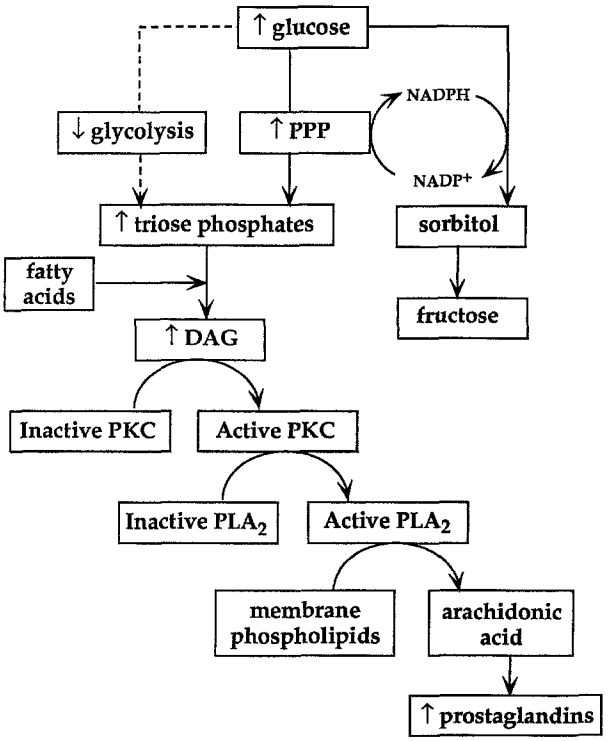

Fig 2. Mechanism proposed to link increased glucose concentrations and increased prostaglandin production via increased PPP flux, de novo diacylglycerol synthesis, PKC, and PLA_2 activation.

reductase mRNA in response to 20 mmol/L glucose.[42] This was thought to be the first report of a response by the aldose reductase gene to the pathophysiologic levels of glucose seen in diabetes. Osmotic induction of aldose reductase mRNA and activity by hyperglycemia has previously been reported, but only at exceedingly high glucose levels. Kaneko et al[43] showed an induction of aldose reductase mRNA in rat kidney mesangial cells; however, this occurred only after glucose levels reached 600 mOsm/kg.

Oxidative stress has been postulated as a common factor to link all the diverse mechanisms that appear to lead to diabetic complications.[44] An indication of the altered redox state of diabetes is apparent from the decrease in the reduced-glutathione to oxidized-glutathione ratio.[45] Free-radical scavengers such as superoxide dismutase and probucol can prevent changes in blood flow and vascular albumin permeation that result from acute hyperglycemia,[46,47] and D-α-tocopherol is able to prevent glucose-stimulated increases in the diacylglycerol level and PKC activity.[48] It has been suggested that the effects of aldose reductase inhibitors are due to an ability to act as general antioxidants rather than to specific inhibition of the enzyme. However, even if aldose reductase inhibitors exert an effect via their antioxidant properties, this still impacts on the activity of the polyol pathway. Diabetes[45] and high glucose[49] both cause a reduction in NADPH levels, resulting in a decreased NADPH/$NADP^+$ ratio. This is proposed to occur due to overutilization of NADPH by activation of the polyol pathway.[49] Hence, direct inhibition of aldose reductase or an antioxidant action to restore the normal redox state will both have the same effect with

respect to returning an activated polyol pathway and the downstream events to a normal state.

Recent studies suggest that the oxidation of sorbitol to fructose may be more closely linked to the development of vascular dysfunction in diabetic ocular, aortic, and peripheral nerve tissue.[27] The results of the present study do not delineate whether aldose reductase or sorbitol dehydrogenase is the critical step, since inhibition of aldose reductase may automatically cause a reduction in sorbitol dehydrogenase activity via reduced sorbitol substrate. However, studies on galactose feeding that leads to the development of early changes in neural and vascular function mimicking those of diabetes have shown that these changes can be prevented by administration of aldose reducatase inhibitors. Although other explanations for the galactose effect are possible, these findings support the possibility that the major effects of the aldose reductase pathway are due to the reduction of glucose to sorbitol with the associated conversion of NADPH to $NADP^+$.

Many mechanisms can be put forward to explain glomerular hyperfiltration at the onset of diabetes, but it is apparent from our results that high glucose causes activation of the polyol pathway, leading to de novo diacylglycerol synthesis, activation of PKC and PLA_2, and increased prostaglandin production. Prostaglandins may contribute to hyperfiltration, both by effects on glomerular blood flow and hydrostatic pressure[16] and by inhibition of mesangial cell contractility.[50] Moreover, subsequent effects of PGE_2 on hyaluronan synthesis[51] may contribute more directly to the structural changes of diabetic nephropathy.

REFERENCES

1. Ayo SH, Radnik RA, Glass WR II, et al: Increased extracellular matrix synthesis and mRNA in mesangial cells grown in high-glucose medium. Am J Physiol 260:F185-F191, 1991
2. Pugliese G, Pricci F, Pugliese F, et al: Mechanisms of glucose-enhanced extracellular matrix accumulation in rat glomerular mesangial cells. Diabetes 43:478-490, 1994
3. Cohen MP, Khalifa A: Renal glomerular collagen synthesis in streptozotocin diabetes. Reversal of increased basement membrane synthesis with insulin therapy. Biochim Biophys Acta 500:395-404, 1977
4. Brownlee M, Vlassara H, Cerami A: Nonenzymatic glycosylation and the pathogenesis of diabetic complications. Ann Intern Med 101:527-537, 1984
5. McLennan SV, Fisher EJ, Yue DK, et al: High glucose concentration causes a decrease in mesangium degradation. A factor in the pathogenesis of diabetic nephropathy. Diabetes 43:1041-1045, 1995
6. Sato Y, Hotta N, Sakamoto N, et al: Lipid peroxide level in plasma of diabetic patients. Biochem Med 21:104-107, 1976
7. Crouch R, Kimsey G, Priest DG, et al: Effect of streptozotocin on erythrocyte and retinal superoxide dismutase. Diabetologia 15:53-57, 1978
8. Loven DP, Schedl HP, Oberley LW, et al: Superoxide dismutase activity in the intestine of the streptozotocin-diabetic rat. Endocrinology 111:737-742, 1982
9. Matkovics B, Varga SI, Szabo L, et al: The effect of diabetes on the activities of the peroxide metabolism enzymes. Horm Metab Res 14:77-79, 1982
10. Larkins RG, Dunlop ME: The link between hyperglycaemia and diabetic nephropathy. Diabetologia 35:499-504, 1992
11. DeRubertis FR, Craven PA: Activation of protein kinase C in glomerular cells in diabetes. Diabetes 43:1-8, 1994
12. Hostetter TH, Troy JL, Brenner BM: Glomerular hemodynamics in experimental diabetes mellitus. Kidney Int 19:410-415, 1981
13. Carney SL, Wong NLM, Dirks JH: Acute effects of streptozotocin diabetes on rat renal function. J Lab Clin Med 93:950-961, 1979
14. Mogensen CE: Glomerular filtration rate and renal plasma flow in short-term and long-term juvenile diabetes mellitus. Scand J Clin Lab Invest 28:91-100, 1971
15. Mogensen CE, Andersen MJF: Increased kidney size and glomerular filtration rate in untreated juvenile diabetes: Normalization by insulin-treatment. Diabetologia 11:221-224, 1975
16. Arima S, Ren Y, Juncos LA, et al: Glomerular prostaglandins modulate vascular reactivity of the downstream efferent arterioles. Kidney Int 45:650-658, 1994
17. Kreisberg JI, Patel PY: The effects of insulin, glucose and diabetes on prostaglandin production by rat kidney glomeruli and cultured glomerular mesangial cells. Prostaglandins Leukot Med 11:431-442, 1983
18. Schambelan M, Blake S, Sraer J, et al: Increased prostaglandin production by glomeruli isolated from rats with streptozotocin-induced diabetes mellitus. J Clin Invest 75:404-412, 1985
19. Craven PA, Caines MA, DeRubertis FR: Sequential alterations in glomerular prostaglandin and thromboxane synthesis in diabetic rats: Relationship to the hyperfiltration of early diabetes. Metabolism 36:95-103, 1987
20. DeRubertis FR, Craven PA: Eicosanoids in the pathogenesis of the functional and structural alterations of the kidney in diabetes. Am J Kidney Dis 22:727-735, 1993
21. Goldfarb S, Ziyadeh FN, Kern EFO, et al: Effects of polyol-pathway inhibition and dietary *myo*-inositol on glomerular hemodynamic function in experimental diabetes mellitus in rats. Diabetes 40:465-471, 1991
22. Pedersen MM, Christiansen JS, Mogensen CE: Reduction of glomerular hyperfiltration in normoalbuminuric IDDM patients by 6 mo of aldose reductase inhibition. Diabetes 40:527-531, 1991
23. Craven PA, DeRubertis FR: Sorbinil suppresses glomerular prostaglandin production in the streptozotocin diabetic rat. Metabolism 38:649-654, 1989
24. Chang WP, Dimitriadis E, Allen T, et al: The effect of aldose reductase inhibitors on glomerular prostaglandin production and urinary albumin excretion in experimental diabetes mellitus. Diabetologia 34:225-231, 1991
25. Frey J, Zager P, Jackson J, et al: Aldose-reductase activity mediates renal prostaglandin production in streptozotocin diabetic rats. Kidney Int 35:292A, 1989 (abstr)
26. Sochor M, Kunjara S, McLean P: The effect of aldose reductase inhibitor statil (ICI 128436) on the glucose over-utilization in kidney of diabetic rats. Biochem Pharmacol 37:3349-3356, 1988
27. Tilton RG, Chang K, Nyengaard JR, et al: Inhibition of sorbitol dehydrogenase: Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. Diabetes 44:234-242, 1995
28. Lou MF, Dickerson JE Jr, Garadi R, et al: Glutathione depletion in the lens of galactosemic and diabetic rats. Exp Eye Res 46:517-530, 1988
29. Sochor M, Baquer NZ, McLean P: Regulation of pathways of glucose metabolism in kidney. The effect of experimental diabetes on the activity of the pentose phosphate pathway and the glucuronate-xylulose pathway. Arch Biochem Biophys 198:632-646, 1979
30. Craven PA, Davidson CM, DeRubertis FR: Increase in diacylglycerol mass in isolated glomeruli by glucose from de novo synthesis of glycerolipids. Diabetes 39:667-674, 1990
31. Craven PA, DeRubertis FR: Protein kinase C is activated in

glomeruli from streptozotocin diabetic rats. J Clin Invest 83:1667-1675, 1989

32. Williams B, Schrier RW: Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells. J Clin Invest 92:2889-2896, 1993

33. Nemenoff RA, Wintz S, Qian N-X, et al: Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. J Biol Chem 268:1960-1964, 1993

34. Craven PA, Patterson MC, DeRubertis FR: Role of enhanced arachidonate availability through phospholipase A_2 pathway in mediation of increased prostaglandin synthesis by glomeruli from diabetic rats. Diabetes 37:429-435, 1988

35. Stevens EJ, Willars GB, Lidbury P, et al: Vasoreactivity and prostacyclin release in streptozotocin-diabetic rats: Effects of insulin or aldose reductase inhibition. Br J Pharmacol 109:980-986, 1993

36. Sener A, Malaisse WJ: A sensitive radioisotopic method for the measurement of NAD(P)H: Its application to the assay of metabolites and enzymatic activities. Anal Biochem 186:236-242, 1990

37. Dunlop ME, Larkins RG: Activity of endogenous phospholipase C and phospholipase A_2 in glucose stimulated pancreatic islets. Biochem Biophys Res Commun 120:820-827, 1984

38. Dunlop M, Keogh R, Larkins RG: Fibronectin-induced increase in mesangial cell prostaglandin release. Diabetes 42:183-190, 1993

39. Lin L-L, Lin AY, Knopf JL: Cytosolic phospholipase A_2 is coupled to hormonally regulated release of arachidonic acid. Proc Natl Acad Sci USA 89:6147-6151, 1992

40. Hazen SL, Gross RW: ATP-dependent regulation of rabbit myocardial cytosolic calcium-independent phospholipase A_2. J Biol Chem 266:14526-14534, 1991

41. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254, 1976

42. Henry DN, Rood S, Spielman W: Heterogeneity of aldose reductase (AR) gene expression: Increased steady state abundance of AR mRNA is not due to differences in basal transcription. J Am Soc Nephrol 6:362A, 1995 (abstr)

43. Kaneko M, Carper D, Nishimura C, et al: Induction of aldose reductase expression in rat kidney mesangial cells and Chinese hamster ovary cells under hypertonic conditions. Exp Cell Res 188:135-140, 1990

44. Baynes JW: Role of oxidative stress in development of complications in diabetes. Diabetes 40:405-412, 1991

45. Grunewald RW, Weber II, Kinne-Saffran E, et al: Control of sorbitol metabolism in renal inner medulla of diabetic rats: Regulation by substrate, cosubstrate and products of the aldose reductase reaction. Biochim Biophys Acta 1225:39-47, 1993

46. Tilton RG, Chang K, Allison W, et al: Glucose-induced increases in blood flow and ^{125}I-albumin permeation: Roles of nitric oxide and superoxide. FASEB J 7:A905, 1993 (abstr)

47. Tilton RG, Chang K, Faller AF: Probucol prevents vascular protein leakage induced by diabetes, glucose, and glycated proteins. Diabetes 42:89A, 1993 (suppl 1, abstr)

48. Kunisaki M, Bursell S-E, Umeda F, et al: Normalization of diacylglycerol–protein kinase C activation by vitamin E in aorta of diabetic rats and cultured rat smooth muscle cells exposed to elevated glucose levels. Diabetes 43:1372-1377, 1994

49. Kashiwagi A, Asahina T, Ikebuchi M, et al: Abnormal glutathione metabolism and increased cytotoxicity caused by H_2O_2 in human umbilical vein endothelial cells cultured in high glucose medium. Diabetologia 37:264-269, 1994

50. Dunlop ME, Larkins RG: Insulin-dependent contractility of glomerular mesangial cells in response to angiotensin II, platelet-activating factor and endothelin is attenuated by prostaglandin E_2. Biochem J 272:561-568, 1990

51. Mahadevan P, Larkins RG, Fraser JRE, et al: Increased hyaluronan production in the glomeruli from diabetic rats: A link between glucose-induced prostaglandin production and reduced sulphated proteoglycan. Diabetologia 38:298-305, 1995