Glucose Loading Induces DNA Fragmentation in Rat Proximal Tubular Cells

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A 10% glucose, 10% mannitol, or 0.9% saline solution was infused in male Wistar rats for 300 minutes via the left cervical vein. Glomerular filtration rates (GFRs) were not significantly altered in any of the three groups. DNA was extracted from isolated proximal tubular cells at the end of each infusion. Electrophoresis on agarose gels showed a distinct ladder pattern of DNA fragmentation in 10% glucose–loaded rats, but no such pattern in 10% mannitol– or 0.9% saline–loaded rats. After infusion for 300 minutes, the plasma glucose level of the 10% glucose–loaded group was higher than that of the other two groups (each $P < .005$). These results suggest that hyperglycemia led to DNA fragmentation in the DNA of proximal tubular cells, similar to the process of programmed cell death known as apoptosis. DNA fragmentation may be associated with renal proximal tubular damage in the early stages of diabetic nephropathy.

WE HAVE PREVIOUSLY reported that excretion of urinary enzymes derived from proximal tubular cells, as well as sorbitol accumulation of renal cortical tissue, increased after 10% glucose loading to the left cervical vein of male Wistar rats with normal renal function.[1-3] It has also been reported that significant changes in the urinary enzyme excretion rate occurred in diabetic patients with clinically normal renal function (serum creatinine: males, $\leq$1.3 mg/dL; females, $\leq$1.1 mg/dL) and normal urinary albumin excretion rates ($<$22 mg/d).[4] The rate of urinary enzyme excretion in diabetic patients with normal or intermediate renal function (serum creatinine levels $\leq$2.0 mg/dL) significantly changed after diet therapy, indicating a tendency to normalization.[5] These results suggest that proximal tubular cells play a role in glucose reabsorption, and that high glucose levels may cause certain alterations in renal cells.

Recently, a distinct ladder pattern of DNA fragmentation, indicative of the process of programmed cell death known as apoptosis,[6-11] was found in renal tubules after partial ureteral obstruction,[12] in the reperfusion phase after brief periods of renal ischemia,[13] and in oxidant injury–induced renal tubular epithelial cells (LLC-PK_1).[14] This suggests that excess glucose reabsorption may cause DNA fragmentation in proximal tubular cells and play a role in renal damage in diabetes.

We investigated the effect of hyperglycemia on DNA fragmentation in rat proximal tubular cells. DNA from proximal tubular tissues was extracted from 10% glucose–, 10% mannitol–, and 0.9% saline–loaded rats and analyzed by agarose gel electrophoresis. This study may be the first to report on high-glucose–induced apoptosis in a hyperglycemic animal model.

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MATERIALS AND METHODS

Animals

Experiments were performed on male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing between 250 and 350 g. All rats were allowed free access to standard laboratory chow (CE-2; Clea Japan, Tokyo, Japan) and tap water. The rats were divided into three groups as follows, according to the infusion solution they received: (1) 0.9% saline (n = 6), (2) 10% glucose (n = 6), and (3) 10% mannitol (n = 6). Each solution contained 1.0% inulin (Inutest; Lavosan-Gesellshaft, Linz, Austria) for measurement of the glomerular filtration rate (GFR). The osmolarity of the 0.9% saline solution was 0.31 osm/kg, and osmolarity of the 10% glucose and 10% mannitol solutions was 0.56 osm/kg.

Surgical Preparation

Animals were anesthetized with pentobarbital sodium (50 mg/kg body weight intraperitoneally; Abbott Laboratories, North Chicago, IL) and placed on a warm plate to keep the body temperature at 37°C. They were tracheotomized to facilitate respiration. The left carotid artery was cannulated (PE50; Becton Dickinson, Parsippany, NJ) for blood sampling. The left cervical vein was also cannulated (PE50) for infusion of each of the solutions described above. Another catheter (PE50) was inserted via a small incision into the bladder for urine sampling.

Infusion Experimental Protocol

Infusion was started immediately after surgery and continued throughout the experiment at a rate of 100 μL/min (Peristaltic pump-1; Pharmacia, Uppsala, Sweden). After an equilibration period of 120 minutes, clearance studies were performed over three consecutive 60-minute periods (120 to 180 minutes, 180 to 240 minutes, and 240 to 300 minutes). Urine samples collected from each period were used to measure urine volume and inulin concentration. At the midpoint of each clearance study (ie, at 150, 210, and 270 minutes), 400 μL blood was drawn from the carotid artery to determine plasma inulin concentrations. Concentrations of plasma and urinary inulin were determined by the anthrone method.[15] Because of the possibility that anthrone may also have reacted with glucose, a correction for each sample was made according to the glucose concentration. Glucose concentration was measured by the hexokinase/glucose-6-phosphate dehydrogenase method.[16]

Urinary Enzyme Assays

Urine samples were dialyzed against deionized water for 12 hours before analysis. *N*-acetyl-β-D-glucosaminidase ([NAG] EC 3.2.1.30) activity was determined using a Meiassay NAG-R kit (Meiji Seika, Tokyo, Japan).[17] Alanine aminopeptidase ([AAP] EC

3.4.11.2) activity was determined using L-alanine-3,5-dibromo-4-hydroxyanilide (Asahi Kasei Kogyo, Tokyo, Japan) as a chromogenic substrate.[4] γ-Glutamyltransferase ([GGT] EC 2.3.2.2) activity was determined according to the modified method recommended by the Scandinavian Society of Clinical Chemistry.[18] Dipeptidyl aminopeptidase IV ([DAPIV] EC 3.4.14.5) activity was determined using 7-glycylproline-4-methylcoumarin amide (Peptide Institute, Minoh, Japan) as a fluorogenic substrate.[19]

Dispersion of the Renal Cortex Into the Tubular Segments and Discontinuous Ficoll Gradient

Renal tubular suspension samples were obtained from all animals in each group. The protocol and solutions used in the preparation of proximal tubules were the same as those used by Tsukamoto et al.[20] Briefly, after infusion, both kidneys were perfused with ice-cold Hanks solution (137 mmol/L NaCl, 55 mmol/L KCl, 0.8 mmol/l $MgSO_4$, 0.33 mmol/L Na_2HPO_4, 0.44 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, and 10 mmol/L Tris hydrochloride, pH 7.4) containing 2.5 mmol/L $CaCl_2$ (solution A) through the inferior vena cava. The kidneys were then further perfused with an additional 20 mL Hanks solution containing 10 mmol/L $CaCl_2$, 1 mg/mL bovine serum albumin, and 1 mg/mL collagenase (Sigma, St Louis, MO) (solution B). Cortices dissected with scissors from collagenase-treated kidneys were minced with a razor blade, incubated at 37°C for 30 minutes in 3 mL solution B, and gassed with 100% O_2. The minced kidney was washed three times with ice-cold solution A with 2 mmol/L phenanthroline (Wako Pure Chemical Industries, Tokyo, Japan) to stop proteolysis. A dispersion of the collagenase-treated cortex into the suspension of tubular segments was layered on a fraction of Ficoll 400 (Pharmacia Biotech, Uppsala, Sweden). Ficoll 400 was freshly dissolved in solution A concentrations of 1%, 2%, 6%, 8%, and 12% (wt/wt, pH 7.4). After about 15 minutes, proximal tubules reached the 8%/12% interphase, the proximal tubule–rich fraction. Fifty-five percent of distal tubules were present in the 1%/2% interphase. This fraction was collected for DNA extraction.

Extraction and Purification of DNA by the Sepa Gene

DNA was extracted and purified using a Sepa Gene DNA extraction kit (Sankou Junyaku, Tokyo, Japan) by the agglutination partition method with guanidine thiocyanate.[21,22] The Sepa Gene consists of the following three steps: (1) cytolysis, (2) removal of protein components (deproteinization), and (3) purification and concentration of DNA.

DNA Fragmentation Analysis

DNA concentrations were obtained by UV spectrophotometry at 260 nm. After the DNA was incubated with 50 μg/mL RNase (Boehringer, Mannheim, Germany) at 37°C for 30 minutes, 5-μg aliquots were electrophoresed with 1.3 μmol/L ethidium bromide, stained on a 1.5% agarose gel, and visualized and photographed under UV transillumination. The molecular size marker used was DNA molecular weight marker VI (Boehringer).

Statistical Analysis

Results are expressed as the mean ± SEM. The data were analyzed using ANOVA or unpaired *t* tests, as appropriate. *P* values less than .05 were considered statistically significant.

RESULTS

Renal Function During Infusion

Table 1 shows the GFR of each group over three consecutive 60-minute periods. Infusion was started immediately after surgery and continued throughout the experiment at a rate of 100 μL/min for 300 minutes. After an equilibrium period of 120 minutes, clearance studies were performed. There was no significant difference among the groups throughout the experiment, nor was any significant difference found along the time course in each group. Therefore, judging from the glomerular function, all groups maintained similar renal function throughout the clearance period.

Table 1. Effects of the GFR of Glucose, Mannitol, and Saline Solution Infusion Over Three Consecutive 60-Minute Periods (120 to 180 minutes, 180 to 240 minutes, and 240 to 300 minutes) in Rats

Group	GFR (mL/min)		
	120-180 min	180-240 min	240-300 min
10% glucose (n = 6)	1.2 ± 0.2	1.2 ± 0.1	1.0 ± 0.1
10% mannitol (n = 6)	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
0.9% saline (n = 6)	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2

NOTE. Values are the mean ± SEM.

Accumulated Urine Volume and Plasma Glucose Concentration After Infusion

The accumulated urine volume of each group during a 180-minute period (120 to 300 minutes), as well as the plasma glucose concentration after infusion for 300 minutes, is shown in Table 2. The 10% glucose–loaded group showed a significant increase in urine volume, to a level 2.5 times that of the 0.9% saline–loaded control group ($P < .005$). The 10% mannitol–loaded group also showed a significant increase in urine volume, to a level six times that of the 0.9% saline–loaded group ($P < .005$) and 2.5 times that of the 10% glucose–loaded group ($P < .005$). Plasma glucose concentration in 10% glucose–loaded rats was higher than in the other two groups (each $P < .005$).

Urinary Four-Enzyme Excretion Studies

Figure 1 shows the results of urinary four-enzyme excretion studies conducted over a 180-minute period (120 to 300 minutes). Urinary excretion of NAG, AAP, GGT, and DAPIV was significantly increased in the 10% glucose–loaded group as compared with the 10% mannitol–loaded or 0.9% saline–loaded groups ($P < .005, P < .05, P < .005$, and $P < .01$ *v* the 10% mannitol–loaded group or 0.9% saline–loaded group, respectively). No distinct variations in urinary excretion of the four enzymes over time were observed during the three 60-minute periods (120 to 180

Table 2. Urine Volume of Infusion for 180-Minute Periods (120 to 300 minutes) and Plasma Glucose Concentration After Infusion for 300 Minutes in Glucose, Mannitol, and Saline Solution–Loaded Rats

Group	Urine Volume (mL/180 min)	Plasma Glucose Concentration (mg/dL)
10% glucose (n = 6)	6.9 ± 0.6*	751.2 ± 25.6*‡
10% mannitol (n = 6)	15.1 ± 1.0*†	183.6 ± 6.8
0.9% saline (n = 6)	2.4 ± 0.3	181.3 ± 9.1

NOTE. Values are the mean ± SEM.
*$P < .005$ *v* 0.9% saline.
†$P < .005$ *v* 10% glucose.
‡$P < .005$ *v* 10% mannitol.

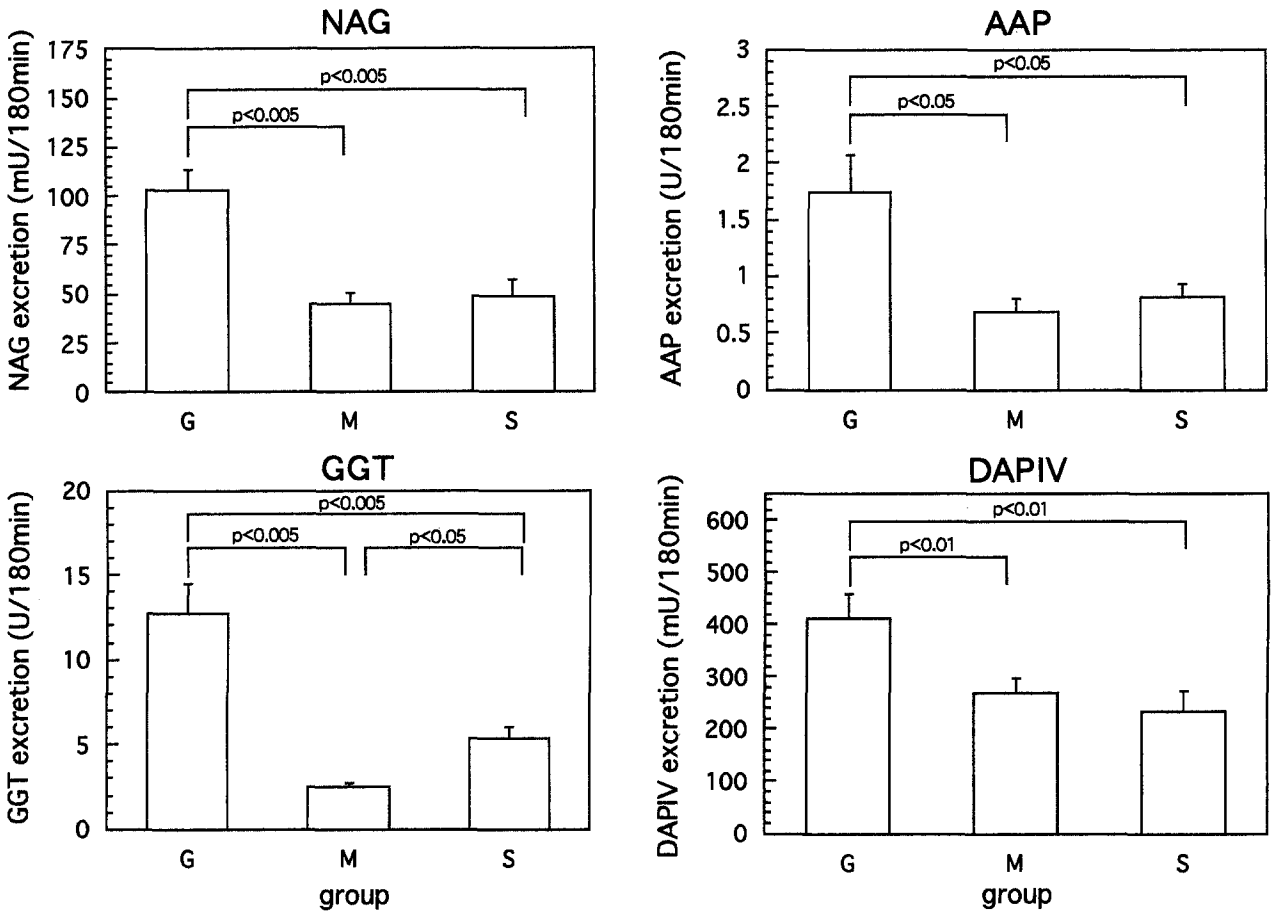

Fig 1. Rat urinary NAG, AAP, GGT, and DAPIV excretion over a 180-minute period (120 to 300 minutes). G, 10% glucose–loaded rats (n = 6); M, 10% mannitol–loaded rats (n = 6); S, 0.9% saline–loaded rats (n = 6). Each bar represents the mean ± SEM.

minutes, 180 to 240 minutes, and 240 to 300 minutes; data not shown).

DNA Fragmentation Analysis in Proximal Tubular Cells After Infusion

Figure 2 shows agarose gel electrophoresis of DNA extracted from proximal tubular cells after infusion. DNA fragments from the tubular cells of 10% glucose–loaded rats showed a typical ladder pattern (Fig 2, lane G). Ten percent glucose loading caused fragmentation of DNA into multiples of low molecular weight. The DNA ladder pattern of agarose gel electrophoresis was used as a marker of apoptosis.[6-11] However, in the 1%/2% interphase of the Ficoll gradient (the distal tubule representation fraction) of 10% glucose–loaded rats, DNA fragmentation was not observed (Fig 2, lane Gdi). DNA fragmentation was not observed in either the 8%/12% interphase (Fig 2, lanes M, S, and NL) or the 1%/2% interphase (data not shown) of the Ficoll gradient in 10% mannitol–loaded, 0.9% saline–loaded, or untreated rats. Therefore, agarose gel electrophoresis did not detect random degradation of DNA (smear pattern) that would indicate necrosis[11,13] in the Ficoll gradient of any of the groups, and no histological cytotoxicity was observed in hematoxylin-eosin–stained renal tissue from each group (data not shown).

Apoptosis was only demonstrated in DNA extracted from the proximal tubular cells of 10% glucose–loaded rats.

DISCUSSION

Based on findings that urinary excretion of enzymes that originate in the proximal tubular cells increases in non–insulin-dependent (type II) diabetic patients with normal renal function, as well as in rats with normal renal function whose left cervical vein has been loaded with glucose,[1-5] we previously suggested that hyperglycemia or excess glucose reabsorption involving proximal tubular cells induces a clinical change in proximal tubular cells before glomerular dysfunction arises, leading to advanced diabetic nephropathy.

It is possible to make a diagnosis based on the damaged area by measuring urinary enzyme activity, since the location of several of these enzymes is known.[23] The location on the brush border membrane of the proximal tubule where these enzymes are situated is a most metabolically active and damage-sensitive region.[24,25] The proximal tubular cells may be the most susceptible to glucose-loading stress, because glomerular-filtrated glucose is concentrated and reabsorbed into the renal tubular cavity.

Nonphysiological high glucose concentrations were clearly the cause of dysbolisms. However, the mechanism of proximal tubular cell change was not clear. To observe cellular level

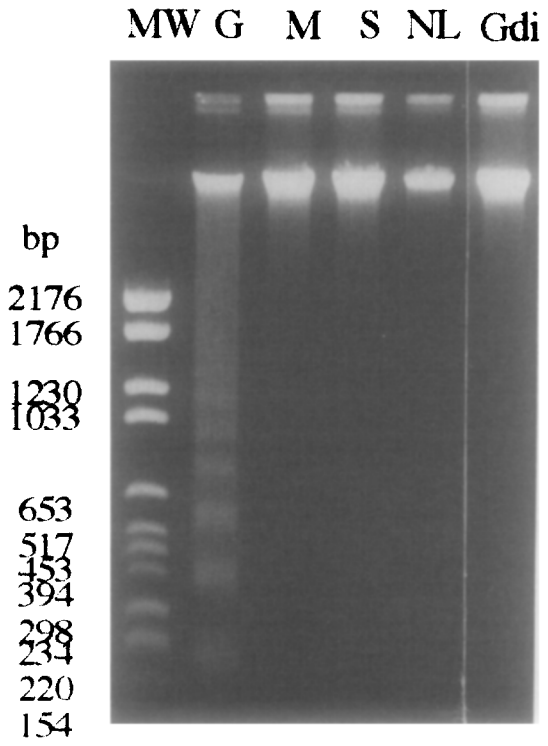

Fig 2. Agarose gel electrophoresis of rat DNA extracted from proximal tubular cells on the 8%/12% interphase of the Ficoll 400 fraction after infusion for 300 minutes. DNA fragments were made visible by UV fluorescence after staining with 1.3 μmol/L ethidium bromide. Lane G, 10% glucose– loaded rat; lane M, 10% mannitol–loaded rat; lane S, 0.9% saline–loaded rat; lane NL, untreated rat; lane Gdi, 1%/2% interphase of the Ficoll 400 fraction after infusion experiments for 300 minutes in a 10% glucose–loaded rat; lane MW, comparative molecular weight markers.

alterations in proximal tubular cells, we performed electrophoretic analysis of the proximal tubular cell DNA of hyperglycemic rats. The specific DNA ladder pattern indicative of apoptosis was detected in the proximal tubular cells of 10% glucose–infused rats.

There was no significant change in GFR during the period of infusion (Table 1). Plasma glucose levels after infusion significantly increased in the 10% glucose–loaded group as compared with the 10% mannitol– and 0.9% saline–loaded groups (Table 2). Therefore, hyperglycemic rats with normal renal function could be used to observe proximal tubular function. The accumulated urine volume of the 10% mannitol–loaded group showed a significant increase as compared with the 10% glucose–loaded group (Table 2). In the 10% glucose–loaded group, DNA fragmentation was observed and the urinary excretion of NAG, AAP, GGT, and DAPIV was significantly increased compared with the 10% mannitol– and 0.9% saline–loaded groups (Figs 1 and 2). Despite the fact that the osmolarity of the 10% mannitol solution was the same as that of the 10% glucose solution (~0.56 osm/kg), agarose gel electrophoresis did not show DNA fragmentation in 10% mannitol–loaded rats, nor was urinary excretion of NAG, AAP, GGT, and DAPIV significant, as compared with the 0.9% saline–loaded group. Therefore, DNA fragmentation induction and elevation of the urinary enzyme excretion rate may not be due to osmotic diuresis. In the 1%/2% interphase (distal tubule) of the Ficoll gradient of 10% glucose–loaded rats, neither apoptosis (ladder pattern) nor necrosis (random, smear pattern) was observed (Fig 2, lane Gdi). Excess glucose reabsorption in renal proximal tubular cells may lead to apoptosis.

DNA is cleaved at internucleosomal DNA linker regions into fragments of approximately 200 basepairs by Ca^{2+}/Mg^{2+}-dependent endonuclease activity.[9,10,26] High Ca^{2+}/Mg^{2+}-dependent endonuclease activity has also been reported in kidney cell nuclei.[27] In a preliminary study, proximal tubular nuclear Ca^{2+}/Mg^{2+}-dependent endonuclease activity was measured in 10% glucose–loaded and 0.9% saline–loaded rats by the modified method of Giannakis et al.[27] Proximal tubular nuclear Ca^{2+}/Mg^{2+}-dependent endonuclease activity in 10% glucose–loaded rats showed an increase of approximately 20% as compared with 0.9% saline–loaded rats. We did not measure intracellular Ca^{2+} or Mg^{2+} concentrations. It seems that the influx of Ca^{2+} and Mg^{2+} into proximal tubular cell nuclei was increased by the glucose relationship mechanism, and that Ca^{2+}/Mg^{2+}-dependent endonuclease was activated. Several apoptosis inducers or inhibitors have been reported.[28] However, it is not clear whether a relationship exists between glucose-induced apoptosis and Ca^{2+}/Mg^{2+}-dependent endonuclease activity.

Recent studies have indicated that the level of cyclic ADP-ribose, a Ca^{2+} mobilizer, was increased by glucose stimulation and that Ca^{2+} was released from internal stores.[29,30] Cyclic ADP-ribose was not considered in this study. We submit that glucose-dependent Ca^{2+} release mechanisms exist in rat proximal tubular cells.

Preliminary histological experiments demonstrated apoptosis in situ pathogenesis of kidney tissue tubular cell nuclei in 10% glucose–loaded rats, as determined by an in situ apoptosis detection kit using the method of terminal deoxytransferase–mediated dUTP-biotin nick end labeling[31] and the Apop Tag (Oncor, Gaithersburg, MD). The proximal and distal tubular cell nuclei of 10% mannitol–loaded, 0.9% saline–loaded, and untreated rats were unstained. In 10% glucose–loaded rats, the nuclei of both the proximal and distal tubular cells were stained by the Apop Tag. However, only the proximal tubular cells of 10% glucose–loaded rats exhibited a ladder pattern of agarose gel electrophoresis. This discrepancy seems to stem from immunospecific properties of the Apop Tag.

Baumgartner-Parzer et al[32] reported that high-glucose–induced apoptosis was found in cultured human umbilical vein endothelial cells. We performed the same experiment as a preliminary experiment using LLC-PK_1,[33-36] an oxidant injury–induced apoptosis pig renal tubular epithelial cell[14] with characteristics similar to those of mammalian proximal tubule cells.[37] However, apoptosis was not detected by agarose gel electrophoresis in attached cells under either high glucose (30 mmol/L) or low glucose (5 mmol/L) culture conditions. Furthermore, we tried cell cloning by the limiting dilution method, and derived LLC-PK_1R (round form) from the parent LLC-PK_1 cell line. Incubation of

LLC-PK$_1$R with high glucose for 72 hours increased DNA fragmentation (35.1% ± 1.6% total DNA, mean ± SD) versus cultures in low glucose (30.1% ± 2.3%, $P < .05$), as measured by [^{3}H]thymidine assays.[38]

In our study using hyperglycemic rats, we did not use diabetes-inducing drugs such as streptozotocin, because streptozotocin is a deleterious drug that induces renal tubular dysfunction.[39,40] Thus, we used a glucose solution directly infused into the left cervical vein. We reported that in 10% glucose with phlorhizin (a specific inhibitor of proximal tubular glucose transport)–loaded rats, elevations of urinary enzyme excretion and renal cortical sorbitol accumulation are prevented by a proximal tubular glucose transport blockade.[1] The intracellular sorbitol is produced by the polyol pathway, which involves two enzymes, aldose reductase and sorbitol dehydrogenase, the first of which converts glucose to sorbitol and the second of which catalyzes the oxidation of sorbitol to fructose.[41,42] Because sorbitol penetrates biological membranes only poorly, it tends to accumulate, leading to osmotic swelling.[41,42] Thus, in the kidneys of glucose-loaded rats, supernumerous glucose reabsorption may increase polyol pathway activity and sorbitol accumulation may lead to osmotically induced tissue damage. It is possible that this mechanism will trigger abnormal enzymuria in hyperglycemia, because urinary enzyme excretion is prevented by proximal tubular glucose transport blockade.[1] In our preliminary studies, epalrestat (Ono Pharmaceutical, Osaka, Japan), a specific aldose reductase inhibitor, as well as phloridzin, prevented both the accumulation of cortical sorbitol and the elevation of urinary enzyme excretion in glucose-loaded rats.[3] However, we have not yet attempted to detect apoptosis in phlorhizine- or epalrestat-treated glucose-loaded rats.

Hyperglycemia induced the following phenomena in glucose-loaded rats: (1) apoptosis of proximal tubular cells, (2) elevation of urinary enzyme excretion rates, and (3) renal cortical sorbitol accumulation. We submit that high glucose concentrations altered the proximal tubular cells of hyperglycemic rats with normal renal function. Nonfunctioning cells may be separated from normal cells to control cell death and/or apoptosis. Apoptosis of high-glucose cells may involve the biophylaxis mechanism and help other cells to survive.[11,43-45] When the biophylaxis mechanism does not function effectively, diabetic nephropathy may be caused by other mechanisms. This suggests that there is an apoptosis transition period from normal renal function to dysfunction. We could not clarify the causal relationship of these phenomena, but it may involve maintenance of homeostasis or damage of renal proximal tubules.

In the present study, cells from other organs of 10% glucose–loaded rats were not analyzed. In particular, glucose could alter retinal tissues or neurocytes, and this may play a role in diabetic complications.

These findings suggest that in the early diagnosis of diabetic nephropathy, as well as in the elucidation of its development mechanism, it may be feasible to rely on the association between apoptosis and hyperglycemia. Apoptosis-specific regulation may lead to exciting new therapies for diabetic nephropathy.

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