Synergistic Interaction of Hypertension and Diabetes in Promoting Kidney Injury and the Role of Endoplasmic Reticulum Stress

Zhen Wang, Jussara M. do Carmo, Nicola Aberdein, Xinchun Zhou, Jan M. Williams, Alexandre A. da Silva, John E. Hall

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Abstract—Diabetes mellitus and hypertension are major risk factors for chronic kidney injury, together accounting for >70% of end-stage renal disease. In this study, we assessed interactions of hypertension and diabetes mellitus in causing kidney dysfunction and injury and the role of endoplasmic reticulum (ER) stress. Hypertension was induced by aorta constriction (AC) between the renal arteries in 6-month-old male Goto-Kakizaki (GK) type 2 diabetic and control Wistar rats. Fasting plasma glucose averaged 162±11 and 87±2 mg/dL in GK and Wistar rats, respectively. AC produced hypertension in the right kidney (above AC) and near normal blood pressure in the left kidney (below AC), with both kidneys exposed to the same levels of glucose, circulating hormones, and neural influences. After 8 weeks of AC, blood pressure above the AC (and in the right kidney) increased from 109±1 to 152±5 mm Hg in GK rats and from 106±4 to 141±5 mm Hg in Wistar rats. The diabetic-hypertensive right kidneys in GK–AC rats had much greater increases in albumin excretion and histological injury compared with left kidneys (diabetes mellitus only) of GK rats or right kidneys (hypertension only) of Wistar–AC rats. Marked increases in ER stress and oxidative stress indicators were observed in diabetic-hypertensive kidneys of GK–AC rats. Inhibition of ER stress with tauroursodeoxycholic acid for 6 weeks reduced blood pressure (135±4 versus 151±4 mm Hg), albumin excretion, ER and oxidative stress, and glomerular injury, while increasing glomerular filtration rate in hypertension-diabetic kidneys. These results suggest that diabetes mellitus and hypertension interact synergistically to promote kidney dysfunction and injury via ER stress.

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Key Words: blood pressure ■ glomerular filtration rate ■ kidney disease ■ oxidative stress ■ tauroursodeoxycholic acid ■ type 2 diabetes mellitus

The coexistence of diabetes mellitus and hypertension, especially when they are not adequately controlled, substantially increases the risk for onset and progression of chronic kidney disease and cardiovascular morbidity and mortality. Although current therapeutic options may slow progression of diabetic-hypertensive nephropathy, many of these patients ultimately progress to end-stage renal disease. Several pathological mechanisms, such as activation of renin–angiotensin–aldosterone system, mechanical stretch, oxidative stress, endoplasmic reticulum (ER) stress, mitochondrial dysfunction and apoptosis, have been postulated to contribute to diabetic-hypertensive nephropathy but the importance of these factors and their interactions are still unclear.

Chronic mechanical stresses associated with increases in BP may interact synergistically with hyperglycemia to cause kidney injury and some studies suggest that hypertension may be required for rapid progression of diabetic nephropathy. In 2 separate case reports, patients with long-standing diabetes mellitus and coexisting unilateral renal artery stenosis had no evidence of nephropathy in the kidney distal to the arterial stenosis, which was protected from hypertension, whereas the contralateral kidney exposed to increased BP had severe

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nephropathy. However, follow-up studies to replicate these results and to investigate potential mechanisms by which BP interacts with hyperglycemia to cause chronic kidney injury have not, to our knowledge, been reported.

Researchers have tried to replicate the hemodynamic effects of diabetes mellitus and hypertension in rodent models by administering high protein diets or by uninephrectomy, which both elevate glomerular hydrostatic pressure, or by superimposing diabetes mellitus on genetic models of hypertension. For example, in transgenic rodents with excessively activated renin–angiotensin–aldosterone system to induce hypertension, there is accelerated development of diabetic nephropathy. However, it has been challenging in these studies to separate potential contributions of neural, hormonal, metabolic, and other factors from BP effects on the kidneys. Furthermore, the cellular and molecular mechanisms by which hemodynamic effects may amplify hyperglycemia effects in causing kidney injury are still unknown.

Abnormal function of the ER, the specialized cytosolic organelle responsible for synthesis, packaging, and assembly of secretory and membrane proteins, has attracted attention for its potential role in development of cellular injury. Stimuli that disrupt normal ER function may cause accumulation of unfolded or misfolded proteins, overwhelming the chaperones and causing ER stress. Although ER stress may serve as a defense mechanism against external stresses, excessive ER stress eventually triggers pathological responses and has been implicated in obesity, diabetes mellitus, and other cardiovascular diseases including hypertension. However, the role of ER stress and its relationship to renal dysfunction during development of diabetic-hypertensive nephropathy is poorly understood.

In this study, we used a rodent model of mild type 2 diabetes mellitus, the Goto-Kakizaki (GK) rat, combined with hypertension to test our hypothesis that diabetes mellitus and hypertension may interact synergistically to amplify oxidative stress and ER stress, and to promote progressive renal injury. The GK rat is a polygenic, nonobese model of hypertension. For example, in transgenic rodents with excessively activated renin–angiotensin–aldosterone system to induce hypertension, there is accelerated development of diabetic nephropathy. However, it has been challenging in these studies to separate potential contributions of neural, hormonal, metabolic, and other factors from BP effects on the kidneys. Furthermore, the cellular and molecular mechanisms by which hemodynamic effects may amplify hyperglycemia effects in causing kidney injury are still unknown.

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In this study, we used a rodent model of mild type 2 diabetes mellitus, the Goto-Kakizaki (GK) rat, combined with hypertension to test our hypothesis that diabetes mellitus and hypertension may interact synergistically to amplify oxidative stress and ER stress, and to promote progressive renal injury. The GK rat is a polygenic, nonobese model of type 2 diabetes mellitus with insulin resistance, deficient insulin production, and mild diabetes mellitus. Renal injury in the GK rat is mild and slow to develop but can be amplified when hypertension is induced by administration of deoxycorticosterone acetate and a high-salt diet. However, the importance of hemodynamic effects compared with other effects of mineralocorticoid receptor activation and high salt intake in causing kidney injury in previous studies is unclear.

To investigate the direct impact of increased BP in causing kidney injury when combined with diabetes mellitus, we developed a model that induces hypertension in one kidney of diabetic GK rats by aorta constriction (AC) between the renal arteries. The unique aspect of this model is that both kidneys are exposed to the same levels of hyperglycemia, circulating hormones, and neural influences but the left kidney below the AC has normal to slightly reduced BP, whereas the right kidney above the AC is exposed to elevated BP.

To further understand the molecular mechanisms of diabetic-hypertensive nephropathy, we also used the ER stress inhibitor tauroursodeoxycholic acid (TUDCA) to examine the role of ER stress during development of kidney injury caused by diabetes mellitus and hypertension. Our results suggest a synergistic interaction between hyperglycemia and hypertension that enhances ER stress and amplifies renal injury. Inhibition of ER stress markedly attenuates kidney injury in diabetic-hypertensive nephropathy. These findings suggest that ER stress may be a therapeutic target to prevent development of diabetic-hypertensive nephropathy.

Materials and Methods

Animals

The experimental procedures described in this study followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. Six-month old male GK and Wistar rats (Charles River Laboratories International, Inc, MA) were used in this study. The GK rat was developed as a model of type 2 diabetes mellitus by selective breeding of Wistar rats with the highest blood glucose levels during an oral glucose tolerance test over 35 generations.

Rats had free access to standard rat chow (Harlan Laboratories, Inc, IN) and were housed in individual cages maintained at 21±2°C and a 12:12-hour light-dark cycle. Rats were randomly divided into 6 groups in this study: (1) GK rats with sham surgery (GK-sham), (2) GK rats with AC surgery (GK–AC), (3) Wistar rats with AC surgery (Wistar–AC), (4) GK–AC rats with TUDCA treatment (GK–AC+TUDCA), (5) GK–sham rats with TUDCA treatment (GK–sham+TUDCA), and (6) GK–AC rats that received phosphate buffered saline vehicle treatment (GK–AC+vehicle). The dose of TUDCA (200 mg/kg/d, S.C., EMD Millipore, MA) was based on previous studies and our preliminary experiments showing effective inhibition of kidney tissue protein levels of the ER stress markers CHOP (C/EBP homologous protein) and GRP78, as assessed by Western blot.

Surgical Procedures

A telemetric pressure transmitter device (model PA-C400, Data Sciences Int., MN) was implanted in the left common carotid artery and advanced into the aorta for 24-h/day measurements of BP and heart rate (HR) in conscious rats as previously described. A 7- to 10-day recovery period after telemetry implantation was permitted before measuring baseline BP and HR for at least 5 additional days.

After stable baseline BP and HR measurements, the AC surgery was performed (see online-only Data Supplement for additional details). To constrict the aorta, a 22-gauge needle was placed next to the aorta between left and right renal arteries, and a suture was snugly tied around the needle and the aorta. After ligation, the needle was removed and the muscles and skin were closed. The suture band between 2 renal arteries was carefully gauged to cause ≈36% reduction of the outer diameter of aorta, a mild reduction in BP in the left kidney, and ≈30 mm Hg increase in BP of the right kidney after several days (Figure 1A). Rats were allowed 7 days to recover from AC surgery before the telemetric pressure transmitters were turned on again and BPs were recorded.

To determine BP gradient above and below the constriction, a femoral artery catheterization was also performed under isoflurane anesthesia on the last day of the experiment. After cannulation, the catheter was connected to Power Laboratory data acquisition system (ADInstruments, CO) to record BP. Simultaneously, BP was measured from the telemetry catheter implanted in the common carotid artery and advanced into the aortic arch, above the AC, and the pressure gradient across the AC was calculated. More detailed description of the procedures is provided in the online-only Data Supplement.

Separate Kidney GFR and Urine Collection

Rats were anesthetized with 2% isoflurane and a midline abdominal incision was performed. A catheter was inserted into the femoral vein...
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to infuse saline or 0.1% fluorescein isothiocyanate–inulin (FITC–inulin; Sigma-Aldrich, MO) at the rate of 2.4 mL/h. To collect the urine from the right kidney, the right ureter was exposed and a catheter (RenaPulse RT 040, Braintree Scientific, Inc, MA) was inserted into the middle of the ureter. A flanged PE-40 size catheter was inserted into the bladder to collect urine from the left kidney. Glomerular filtration rate (GFR) of each kidney was measured over 2 hours using FITC–inulin clearance under isoflurane anesthesia as previously described.30

Figure 1. A, Aortic constriction between the renal arteries induced higher BP in right kidney and normal or slightly reduced blood pressure (BP) in the left kidney. After 8 weeks of aorta constriction (AC), mean arterial pressure above and below the constriction in Goto–Kakizaki (GK)–AC rat were significantly different (n=9, *P<0.05, compared with left kidneys). B, Mean arterial pressure measured by telemetry above the AC, (C), heart rate, (D), daily food intake, (E), body weight, and (F), blood glucose measured at baseline, 1, 2, 3, 4, and 8 weeks after AC or sham surgery in GK and Wistar rats (n=6 in Wistar–AC and GK–sham groups, n=9 in GK–AC group; *P<0.05 compared with GK–sham rats at eighth week after AC or sham surgery and †P<0.05 compared with Wistar–AC rats at eighth week after AC or sham surgery by 2-way analysis of variance followed by Tukey’s post hoc test).
Blood and Urine Biochemistry Measurements

Fasting blood glucose levels were measured using a glucose meter and strips (ReliOn Prime Blood Glucose Test Strips). Fasting plasma insulin and leptin concentrations were measured with enzyme linked immunosorbent assay (R&D Systems, MN and Crystal Chem Inc, IL, respectively). Twenty-four–hour total urine albumin levels were determined by enzyme linked immunosorbent assay (Crystal Chem Inc, IL) from urine collections for 72 hours in rat metabolic cages.

Western Blot for ER Stress Marker Protein

Proteins of renal cortex of left and right kidneys were isolated after being homogenized in radioimmunoprecipitation assay lysis buffer. Mouse polyclonal anti-CHOP (CCAAT-enhancer-binding protein homologous protein, 1:1000, Cell Signaling, MA) antibody was used to examine CHOP expression level.

4-Hydroxyxynonenal Immunohistochemistry Staining

Both left and right kidneys from GK and Wistar rats were harvested and fixed in 10% formalin for 24 hours and then embedded in paraffin and cut (5 μm) for 4-hydroxynonenal (HNE) staining. Sections were rehydrated, and antigens were unmasked in 10 mmol/L sodium citrate, pH 6.0 heated at 95°C for 30 minutes, serum-free protein blocker (Vector Laboratories, CA) was added, and then the sections were incubated with polyclonal anti-4-HNE antibody (Abcam, MA) diluted 1:500 overnight in a humid chamber. After rinses with phosphate buffered saline, sections were incubated with secondary antibody provided in Vector ABC-HRP kit.

Renal Histology

Paraffin-embedded sections (5 μm) were prepared from kidneys fixed in 10% phosphate-buffered formalin. Periodic acid–Schiff stain was used for analysis of renal morphological changes and Masson’s trichrome stain was performed to observe fibrosis and collagen deposition in the kidney. Sections were scored in a blinded, semiquantitative manner using an established scoring scale. For each animal, at least 10 high power (×400) fields were examined. The percentage of glomeruli that displayed basement membrane thickening, mesangial expansion, nodular sclerosis, and global glomerulosclerosis were scored as follows: 0=none, 1≤25%, 2=25% to 50%, 3=50% to 75%, 4≥75%.

Transmission Electron Microscopy

Renal cortical tissues were cut into small pieces and rapidly immersed in tissue fixative buffer. After thin sectioned (70 nm in thickness) and applied on copper grids, the stained grid was loaded in a JOEL JEM1400 transmission electron microscopy with an ANT camera system. At least 5 sections from each sample were examined under transmission electron microscopy. The entire sections were thoroughly viewed at low magnification (×300) for integrity and quality of stained tissues. Details of ultrastructural alterations were further investigated at high magnifications (×20000).

Statistical Analysis

Data are expressed as mean±standard error of the mean. A P value of <0.05 indicates significant difference. Significant differences between 2 groups were determined by Student’s t test. Significant differences between multiple groups at different time points or between left and right kidneys were determined by 2-way analysis of variance followed by Tukey’s or Bonferroni’s (for comparing left and right kidneys from the same animals) multiple comparison tests. Histological scoring in kidneys was assessed by a pathologist who was blinded to the experimental protocols to avoid bias and the results were assessed using nonparametric Kruskal–Wallis test followed by Dunn’s multiple comparison test. Individual statistical analyses are described in the figure legends.

Table 1. Baseline Parameters of 6-Month-Old GK and Wistar Rats

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Wistar (n=10)</th>
<th>GK (n=10–13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>649.4±10.3</td>
<td>432.1±10.1*</td>
</tr>
<tr>
<td>Food intake, g/24 h</td>
<td>24.4±1.1</td>
<td>19.1±0.6*</td>
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<td>Normalized food intake, mg/24 h per gram of BW</td>
<td>36.0±2.2</td>
<td>44.0±1.6*</td>
</tr>
<tr>
<td>Urine output, mL/24 h</td>
<td>47.5±4.0</td>
<td>37.3±8.0</td>
</tr>
<tr>
<td>Normalized urine output, mL/24 h per gram of BW</td>
<td>0.07±0.01</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>Urinary albumin excretion, mg/24 h</td>
<td>1.5±0.6</td>
<td>2.6±0.6</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>87±2</td>
<td>162±11*</td>
</tr>
<tr>
<td>Fasting insulin, ng/mL</td>
<td>0.9±0.2</td>
<td>1.8±0.2*</td>
</tr>
<tr>
<td>Fasting leptin, ng/mL</td>
<td>2.0±0.3</td>
<td>3.7±0.3*</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>105±1</td>
<td>110±1*</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>341±4</td>
<td>304±5*</td>
</tr>
</tbody>
</table>

BW indicates body weight; and GK, Goto-Kakizaki.
*P<0.05 between the groups.

Results

Anthropometric, Metabolic, and Cardiovascular Characteristics of GK and Wistar Rats

Table 1 shows baseline characteristics of control Wistar and GK rats at 6 months old. Data are presented as average of all groups of GK or Wistar rats before AC or sham surgery. GK rats were lighter and ate less food than Wistar control rats. However, fasting plasma glucose in GK was significantly increased compared with Wistar rats (162±11 versus 87±2 mg/dL) and accompanied with slightly higher fasting leptin and insulin levels. GK rats exhibited higher mean arterial pressure, similar 24-hour urine output, and 24-hour urinary albumin excretion (UAE), but had reduced HR compared with Wistar control rats.

Impact of AC on BP, Heart Rate, Body Weight, Food Intake, and Blood Glucose in GK and Wistar Rats

AC rapidly increased mean arterial pressure in GK and Wistar rats 1 week after surgery, from 109±1 to 134±6 mm Hg in GK–AC and from 106±4 to 126±3 mm Hg in Wistar–AC rats. After 8 weeks of AC, mean arterial pressure averaged 152±5 and 141±5 mm Hg in GK–AC and Wistar–AC rats, respectively (Figure 1B). In GK–sham rats, mean arterial pressure averaged 112±5 mm Hg at baseline and did not change significantly during the 8-week study period. AC did not significantly alter heart rate, body weight, food intake, or fasting blood glucose at 1, 2, 3, 4, 6, or 8 weeks in GK and Wistar rats (Figure 1C, 1D, 1E, 1F). There was no significant difference in BP gradient above and below AC in GK–AC and Wistar–AC rats (Figure S1 in the online-only Data Supplement).

Urinary Albumin Excretion

Total 24-hour urines, including excretion from the left and right kidneys, were collected at baseline and 8 weeks

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after AC or sham surgery to assess renal function in GK and Wistar rats. Total UAE was not significantly different at baseline in GK and Wistar rats, averaging only 1.5 to 2.6 mg/24 h (Table 1). After 8 weeks of AC, total UAE was significantly increased in GK–AC rats compared with baseline values. At 8 weeks AC, UAE in GK–AC rats increased to 46.5±13.6 mg/24 h compared with only 11.0±6.0 mg/24 h in Wistar–AC rats and 20.3±5.4 mg/24 h in GK–sham rats (Figure 2A).

Figure 2. A, Twenty-four–hour total urinary albumin secretion at baseline and 8 weeks after aorta constriction (AC) or sham surgery (n=6 in all groups; *P<0.05 compared with baseline of Goto-Kakizaki (GK)–AC rats; †P<0.05 compared with Wistar–AC rats at 8 weeks after AC surgery; #P<0.05 compared with GK–sham rats at 8 weeks after AC surgery by 2-way analysis of variance (ANOVA) followed by Bonferroni’s test). B, Urinary albumin excretion from left and right kidneys in Wistar–AC, GK–AC, and GK–sham rats after 8 weeks after AC or sham surgery (n=6 in all groups; *P<0.05 compared with left kidneys in GK–AC rats; †P<0.05 compared with right kidneys of Wistar–AC rats; #P<0.05 compared with right kidneys of GK–sham rats by 2-way ANOVA followed by Bonferroni’s test). C, Kidney weight, (D) urine output, and (E) GFR from left and right kidneys in Wistar–AC, GK–AC, and GK–sham rats at 4 and 8 weeks after AC or sham surgery (n=6 in all groups; *P<0.05, compared with left kidneys within the same group of rats; †P<0.05 compared with right kidneys of Wistar–AC rats at 8 weeks after AC surgery; #P<0.05 compared with right kidneys of GK–AC rats at 4 weeks after AC surgery by 2-way ANOVA followed by Bonferroni’s test).

Measurement of total 24-hour urinary albumin from both kidneys, however, does not differentiate which kidney is responsible for the large increase in albumin in GK–AC rats. Therefore, we also measured UAE from each kidney in Wistar–AC, GK–sham, and GK–AC rats at 8 weeks after AC or sham surgery. Figure 2B shows that UAE was markedly increased in the right kidneys of GK–AC rats (28.2±8.9 µg/min, exposed to hyperglycemia and high BP) compared with the left kidneys of the same animals (2.9±0.8 µg/min, exposed only to hyperglycemia), the right kidneys from Wistar–AC rats (7.0±3.3 µg/min, exposed only to high BP), and the right kidneys in GK–sham rats (7.6±2.2 µg/min, exposed only to hyperglycemia). These results indicate that increased urinary albumin in diabetic-hypertensive GK–AC rats mainly occurs in the hypertensive right kidneys exposed to increases in BP and hyperglycemia and suggest that coexistence of hypertension and diabetes mellitus may have synergistic effects to increase UAE.

Kidney Function

To evaluate interactions of high BP and diabetes mellitus on kidney function, kidney weight, urine output, and GFR as well as were measured in left and right kidneys of GK and Wistar rats at 4 and 8 weeks after AC. In both GK–AC and Wistar–AC rats, the right kidney weights were significantly greater than left kidney weights. Weight of right kidneys (exposed to hypertension plus diabetes mellitus) of GK–AC rats slightly increased from 1.7±0.1 g at 4 weeks of AC to 2.0±0.1 g after 8 weeks of AC. However, there were no significant differences in left kidney weight at 4 and 8 weeks of AC in GK–AC rats (Figure 2C). Urine output in the hypertensive-diabetic
right kidneys was significantly increased compared with the left kidneys in GK rats at 4 and 8 weeks after AC (Figure 2D). After 4 weeks of AC, GFR in the right kidneys of GK–AC rats was higher than in the left kidneys. However, after 8 weeks of AC, GFR in the right kidney declined substantially compared with the fourth week (from 1.1±0.1 to 0.5±0.1 mL/min/g, Figure 2E). GFR in the left kidney of GK–AC rats did not change significantly from 4 to 8 weeks after AC. These results indicate that combined hypertension and diabetes mellitus in the right kidney causes an initial increase in GFR followed by a decline to normal within 4 weeks, associated with increased UAE.

Renal Glomerular Structural Changes

Renal morphological changes were assessed by periodic acid–Schiff and trichrome staining of kidney sections. Glomerular injury scores were assigned based on the severity of renal damage. As shown in Figure 3A, marked renal morphological changes were detected in diabetic-hypertensive right kidneys compared with left kidneys in GK–AC rats. PAS staining showed glomerulopathy (arrow) in diabetic-hypertensive right kidneys in GK–AC rats (a, mesangial expansion; b, increased thickness of Bowman's capsule; c, glomerulosclerosis; d, tubular metaplasia formation in Bowman's capsule) compared with (e) right kidney in GK–sham rats, (f) left kidney in GK–AC rats, and (g) right kidney in Wistar–AC rats. Masson's trichrome staining showed collagen staining in (h) right kidney of GK–sham rats, (i) left kidney of GK–AC rats, and (j) right kidney of GK–AC rats. Increased blue staining can be observed in glomeruli of the right kidneys of GK–AC rats (empty arrow). Scale bars (40 µm in e, f, g; 20 µm in a–d and h–j).

Figure 3. A, Periodic acid–Schiff (PAS) and trichrome staining of kidney slices from Wistar–aorta constriction (AC), Goto-Kakizaki (GK)–AC, and GK–sham rats after 8 weeks of AC or sham surgery. PAS staining showed glomerulopathy (arrow) in diabetic-hypertensive right kidneys in GK–AC rats (a, mesangial expansion; b, increased thickness of Bowman's capsule; c, glomerulosclerosis; d, tubular metaplasia formation in Bowman's capsule) compared with (e) right kidney in GK–sham rats, (f) left kidney in GK–AC rats, and (g) right kidney in Wistar–AC rats. Masson's trichrome staining showed collagen staining in (h) right kidney of GK–sham rats, (i) left kidney of GK–AC rats, and (j) right kidney of GK–AC rats. Increased blue staining can be observed in glomeruli of the right kidneys of GK–AC rats (empty arrow). Scale bars (40 µm in e, f, g; 20 µm in a–d and h–j). B, Renal injury was scored based on the renal morphological changes at 8 weeks of AC or sham surgery in GK and Wistar rats. (n=6 in GK–sham, n=7 in Wistar–AC, and n=10 in GK–AC rats. *P<0.05 compared with other groups by Kruskal nonparametric test followed by Dunn's multiple comparisons test). C, Representative images of transmission electron microscopy scans from (a) the right kidneys of GK–sham rats, (b) left kidneys of GK–AC rats, and (c) right kidneys of GK–AC rat. Arrow indicates endothelial cells, asterisk indicates glomerular basement membrane, and empty arrow head indicates the foot process of podocytes.
morphological changes, such as thickening of Bowman’s capsule, expansion of glomerular mesangial matrix, increased cellularity, and tubular metaplasia formation in Bowman’s capsule were observed in the right kidneys of GK–AC rats (Figure 3Aa–d) compared with the right kidneys of diabetic GK–sham rats, the left kidneys of GK–AC rats, and the right kidneys of Wistar–AC rats and (Figure 3Aa–g) in periodic acid–Schiff staining. Trichrome stain showed more collagen in Bowman’s capsule and between the capillary loops of glomeruli in the right kidneys of GK–AC rats (Figure 3Aj) compared with the right kidney of GK–sham rats (Figure 3Ah) and left kidney of GK–AC rats (Figure 3Ai). A significantly higher renal injury score (Figure 3B) was found in the right kidneys of GK–AC rats compared with the left kidneys of GK–AC rats. However, kidneys from GK–sham rats (exposed only to diabetes mellitus) or from Wistar–AC rats (exposed only to hypertension) did not show significant increases of glomerular injury scores.

Glomerular ultrastructural changes can also be observed by electron microscopy in the right kidneys of GK–AC rats compared with the left kidneys in the same animals or the right kidneys from GK–sham rats. Disrupted glomerular ultrastructure, including detached endothelial layer in the glomerular capillary, thickening of the glomerular basement membrane, effacement of podocytes, and fusion of podocytes foot processes (Figure 3C) were observed in the right kidneys of GK–AC rats.

ER Stress and Oxidative Stress
To investigate the molecular pathways that mediate the synergistic effects of hypertension and diabetes mellitus on kidney structure and function, the ER stress marker, CHOP was measured by Western blot of kidney cortex homogenates of GK–sham, GK–AC, and Wistar–AC rats. CHOP expression in the right kidneys of GK–AC rats exposed to high BP and high glucose was significantly greater than in the left kidneys exposed only to diabetes mellitus or the right kidneys of Wistar–AC rats exposed only to hypertension (Figure 4A and 4B).

We also performed immunohistochemistry for 4-HNE, an indicator of lipid peroxidation and oxidative stress, and found a much stronger 4-HNE staining in the right hypertensive kidneys of GK–AC rats compared with the left normotensive kidneys of the same diabetic rats or the right hypertensive kidneys of Wistar–AC rats after 8 weeks of AC (Figure 4C and 4D). Thus, increased 4-HNE staining and increased CHOP were observed only in kidneys exposed to the combination of hypertension and diabetes mellitus.

Treatment With ER Stress Inhibitor TUDCA Attenuates Hypertension
After 6 weeks of TUDCA treatment, there was a 52% reduction of CHOP in both left and right kidneys of GK–AC rats compared with untreated GK–AC rats (Figure 5A). TUDCA treatment also significantly attenuated the rise in BP in GK–AC rats (Figure 5B). At the end of TUDCA treatment, BP above the AC in GK–AC rats was 135±4 mm Hg compared with 151±4 mm Hg in vehicle-treated GK–AC rats. TUDCA did not cause significant changes of BP in GK–sham rats. We also found no significant changes in heart rate, food intake, body weight, and blood glucose between vehicle and TUDCA-treated GK–AC rats (data shown in Figure 5A, 5B, 5C, 5D) indicating that the BP effects of TUDCA treatment were not because of reductions in food intake, body weight, and blood glucose.

Treatment With TUDCA Improves Renal Function
TUDCA treatment significantly reduced 24-hour total UAE to 15.1±4.2 mg/24 h in GK–AC rats compared with 59.2±11.4 mg/24 h in saline-treated GK–AC rats (Figure 5C). Urinary albumin secretion was markedly reduced in the right kidneys of GK–AC rats treated with TUDCA compared with the right kidneys of GK–AC without treatment (5.6±1.3 versus 30.9±10.5 µg/min, Figure 5D). GFR in the right kidneys of GK–AC rats treated with TUDCA was significantly higher than GFR in the right kidneys of GK–AC rats without treatment (0.9±0.1 versus 0.6±0.1 mL/min/g of kidney weight; Figure 5E). We found no significant changes in GFR in the left kidneys of GK–AC treated with TUDCA or vehicle.

Treatment With TUDCA Improves Oxidative Stress and Preserves Normal Glomerular Structure
Chronic TUDCA treatment reduced oxidative stress in kidneys of GK–AC rats (Figure 5F, upper right panel). Positive 4-HNE staining area (Figure S3) was significantly reduced by 76% in the right kidneys of GK–AC+TUDCA rats when compared with the right kidneys of GK–AC+vehicle rats. TUDCA treatment also attenuated renal glomerular injury, as indicated by reduced thickness of glomerular basement membranes and attenuated expansion of mesangial matrix in the right kidney (Figure 5F, lower right panel). The overall kidney glomerular injury score in the right kidney of GK–AC+TUDCA rats was significantly reduced from 2.5±0.3 to 1.2±0.3 when compared with the right kidneys of vechicle-treated GK–AC rats (Figure S4).

Discussion
An important finding of our study is that diabetes mellitus and hypertension have synergistic effects to promote renal dysfunction, albuminuria, ER stress, oxidative stress, and glomerular injury in GK rats. This synergy was apparent even with mild hyperglycemia and moderate increases in BP, and significant renal injury developed rapidly over 8 weeks. Our results also demonstrated that inhibition of ER stress markedly attenuated renal dysfunction, albuminuria, oxidative stress, and glomerular injury in kidneys exposed to hypertension and diabetes mellitus while producing mild reductions in BP in AC-induced hypertension.

We used GK rats for our studies since they develop mild spontaneous type 2 diabetes mellitus early in life, usually between 3 to 4 weeks of age, as a result of impaired ontogenic development of islet cells, impaired insulin release following a glucose load, insulin resistance, hyperinsulinemia, and abnormal glucose metabolism similar to changes observed in humans with type 2 diabetes mellitus.21–23 GK rats at the age used in our studies do not have hypertension, glomerulosclerosis, tubulointerstitial fibrosis, or significant
albuminuria and kidney dysfunction which are observed only at older ages (>18 months) when hypertension may also develop. These findings are similar to those observed in the preclinical phase of human diabetic nephropathy. Another attractive feature of GK rats is that they are not obese or hyperlipidemic, which may cause lipotoxic renal injury, making GK rats an excellent model to test the interaction of mild hyperglycemia and secondary injurious factors such as hypertension in contributing to chronic kidney disease.

Previous studies have shown that renal injury in GK rats can be markedly amplified when hypertension is induced.
Figure 5. A. Optical density analysis of renal cortex CHOP expression in Goto-Kakizaki (GK)–aorta constriction (AC) and GK–AC+tauroursodeoxycholic acid (TUDCA) rats at 8 weeks of AC (n=5 in all groups *P<0.05, compared with left kidneys from GK–AC rats and †P<0.05, compared with right kidneys from GK–AC rats by t test). Inset, Immunoblot of CHOP expression. B. Mean arterial pressure change in GK–AC rats with TUDCA (n=7) or vehicle (n=6) treatment and GK–sham rats with TUDCA treatment (n=5) at baseline, 2, 3, 4, 6 and 8 weeks after AC or sham surgery. (*P<0.05 compared with GK–sham+TUDCA; †P<0.05 compared with GK–AC+vehicle at 8 weeks after AC or sham surgery by 2-way analysis of variance (ANOVA) followed by Tukey’s post hoc test). C, 24-hour urinary albumin excretion (n=6 in all groups; #P<0.05 compared with GK–AC+vehicle rats by 1-way ANOVA followed by Tukey’s test); (D) Individual left and right kidney urinary albumin excretion and (E) individual kidney GFR measurements in GK–AC rats with or without TUDCA (Continued)
by administration of deoxycorticosterone acetate and a high-salt diet.\textsuperscript{25} However, the importance of increased BP compared with other effects of mineralocorticoid receptor activation and high salt intake in causing kidney injury in these studies is unclear, especially because there is evidence that mineralocorticoids may have BP-independent effects to cause renal injury and fibrosis.\textsuperscript{35,36} Similar difficulties are encountered when interpreting results from studies in which diabetes mellitus-induced renal injury is accelerated by infusion of pressor agents such as AngII, transgenic overexpression of the renin–angiotensin–aldosterone system, inhibition of nicotinic oxide synthesis, or other experimental approaches to cause hypertension in diabetic animals.\textsuperscript{37,38} In these types of studies, it has also been challenging to separate potential contributions of multiple hormonal, metabolic, and neural changes induced by diabetes mellitus and the experimental method used to create hypertension from direct effects of increased BP and hyperglycemia on the kidneys.

In the present study, we induced hypertension in GK diabetic rats by AC between the 2 renal arteries. An important aspect of this model is that both kidneys were exposed to the same levels of hyperglycemia, circulating hormones, and neural influences but different perfusion pressures. This model therefore permitted us to compare the impact of differences in BP in the left and right kidneys of the same rats with or without high blood glucose. We also were able to investigate the effects on kidney function of diabetes mellitus alone in GK rats or hypertension alone in Wistar rats with AC and normal blood glucose.

Our results demonstrated that coexistence of hypertension and diabetes mellitus exerted synergistic effects to cause renal dysfunction and injury as reflected by increases in 24-hour UAE, ER stress, oxidative stress, histological injury of glomeruli, and slowly declining GFR. Although our study was not designed to provide detailed, quantitative histological assessment of kidney injury, the renal injury score based on periodic acid–Schiff staining was consistently elevated only in the right kidneys of GK rats exposed to hypertension and diabetes mellitus. We also observed, using electron microscopy, glomerular ultrastructural changes including endothelial cell damage, increased basement membrane thickness thickening, podocyte effacement, and fusion of podocyte foot processes in the right kidney exposed to hypertension and diabetes mellitus in GK–AC rats. In the absence of hypertension, moderate hyperglycemia and hyperinsulinemia in GK rats were not associated with major kidney injury, severe albuminuria, glomerulosclerosis, ER stress, or kidney dysfunction. Eight weeks of moderate hypertension induced by AC between the renal arteries of Wistar rats also caused only modest albuminuria, ER stress and oxidative stress, and did not increase the renal injury score in the absence of diabetes mellitus.

Our results should not be interpreted as evidence that diabetes mellitus or hypertension alone cannot cause kidney dysfunction and injury. In fact, experimental studies have shown that chronic severe hyperglycemia can cause kidney injury, although renal lesions are often slow to develop. Also, in many experimental models and in humans with diabetes mellitus, chronic hyperglycemia may be associated with obesity and other metabolic or genetic abnormalities. For example in type 2 diabetes mellitus models such as Zucker fatty rats and db/db mice,\textsuperscript{39–41} the leptin receptor mutations cause severe obesity and hyperlipidemia, as well as hyperglycemia, that may contribute to kidney injury. Although chemical methods of inducing type 1 diabetes mellitus (eg, streptozotocin and alloxan) are not complicated by obesity, the amount of kidney injury is often mild despite severe chronic hyperglycemia and the agents used may themselves have toxic effects on the kidneys.\textsuperscript{42}

Previous studies have shown that superimposition of hypertension on type 1 or type 2 diabetes mellitus produces much more severe kidney injury. Our results and clinical studies showing that tight BP control is at least as important as glycemic control in slowing progression of kidney disease support a major role for hemodynamic factors in the pathogenesis of diabetic kidney injury.\textsuperscript{43,44} In fact, it has been suggested that increased BP may be a prerequisite for progression of diabetic nephropathy.\textsuperscript{45}

There is compelling evidence that hyperglycemia impairs normal autoregulation of GFR and renal blood flow.\textsuperscript{46,47} To the extent that renal autoregulation is impaired, increases in systemic arterial pressure would be transmitted to the glomerular capillaries in diabetic kidneys to a greater degree than in normal kidneys. In our studies, there was initially a substantial rise in GFR that accompanied the moderate increase in BP of the right kidneys of diabetic GK–AC rats, suggesting impared renal autoregulation. This initial glomerular hyperfiltration was followed by a decline in GFR to normal after only 8 weeks of diabetes mellitus and hypertension in the right kidneys of GK–AC rats. The rapid decrease of GFR in the hypertensive kidneys GK–AC rats is similar to the decline observed in diabetic patients who first undergo glomerular hyperfiltration followed by reductions of GFR to normal that are associated with nephron injury preceding a further decline of GFR to subnormal levels as diabetic nephropathy progresses.\textsuperscript{48,49} Therefore, one potential mechanism for the synergy of hypertension and diabetes mellitus is greater mechanical stress on the glomerular capillaries because of impaired renal autoregulation, renal vaso dilatation, glomerular hyperfiltration, and greater transmission of increases in systemic arterial pressure to the glomerulus.
Potential Role of ER Stress in Synergistic Effects of Diabetes Mellitus–Hypertension–Induced Kidney Injury

Although the underlying molecular mechanisms responsible for the synergy of hypertension and hyperglycemia to promote kidney injury are poorly understood, our results, suggest that ER stress may play an important role. The ER stress marker CHOP was markedly increased in the right kidneys of GK–AC rats exposed to increased BP and hyperglycemia, whereas kidneys subjected to chronic hypertension or diabetes mellitus alone had minimal increases in CHOP. Also, chronic administration of the ER stress inhibitor TUDCA not only reduced CHOP but also markedly decreased albumin excretion and the glomerular injury score while attenuating the decline in GFR in kidneys of GK–AC rats exposed to hypertension and diabetes mellitus. In fact, TUDCA administration essentially normalized albumin excretion and the renal injury score of diabetic-hypertensive kidneys in GK–AC rats. To our knowledge, this is the first report showing that inhibition of ER stress attenuates kidney dysfunction and injury induced by diabetes mellitus and hypertension together.

TUDCA administration also attenuated the rise in systemic arterial pressure in GK–AC rats. The mechanisms responsible for the BP lowering effects of TUDCA are unclear but could be related, in part, to attenuation of renal injury. However, TUDCA was administered systemically in our studies and we cannot ascertain the contribution of the direct effects on the kidneys compared with extrarenal actions that may have contributed to reductions in BP in GK–AC rats.

Inhibition of ER stress with TUDCA has been reported to reduce blood glucose in type 2 diabetic mice. In our study, however, TUDCA treatment for 6 weeks did not significantly alter blood glucose in GK–AC rats. Therefore, reductions in blood glucose cannot explain the beneficial effects of TUDCA on kidney structure and function in GK–AC rats.

The cellular mechanisms that lead to ER stress in kidneys subjected to diabetes mellitus and hypertension are unclear but may be related, in part, to increased mitochondrial ROS production. Previous studies have suggested that mitochondrial metabolic overload results in increased cellular oxidative and ER stress, which leads to the activation of the unfolded protein response. During its early phase, the unfolded protein response either refolds the accumulated unfolded proteins or degrades them via the ubiquitin–proteasome pathway. When the unfolded protein and cellular damage exceed a threshold and the chronic cell stress is not relieved, proapoptotic responses are initiated and cell death ultimately ensues. Although our studies were not designed to assess the role of ROS in promoting kidney injury, we found that 4-HNE, an indicator of lipid peroxidation and oxidative stress, was markedly upregulated in hypertensive kidneys of GK–AC rats compared with normotensive kidneys of the same diabetic rats or hypertensive kidneys of Wistar–AC rats after 8 weeks of AC. However, further studies will be needed to determine whether crosstalk between ER and the mitochondria may account for excess ROS and kidney dysfunction and injury when diabetes mellitus and hypertension coexist.

Perspectives

Our results indicate that hypertension and diabetes mellitus interact synergistically to promote renal dysfunction, albuminuria, ER stress, oxidative stress, and glomerular injury in a rodent model of type 2 diabetes mellitus. This synergy occurs even with mild hyperglycemia and hypertension. We also found that inhibition of ER stress markedly attenuated dysfunction and injury in kidneys exposed to hypertension and diabetes mellitus. The molecular mechanisms by which mechanical forces interact with high glucose levels to induce ER stress and renal injury warrant further investigation, especially because type 2 diabetes mellitus is often associated with hypertension and metabolic abnormalities that are difficult to control. An important implication of these studies is that simultaneous tight control of hypertension and hyperglycemia may be required to slow, or prevent, progression of diabetic nephropathy. Inhibition of ER stress may be a new therapeutic strategy for diabetic nephropathy.

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Disclosures

None.

References


What Is New?
• Moderate increases in blood pressure and mild hyperglycemia interact synergistically to promote renal dysfunction, albuminuria, endoplasmic reticulum stress, oxidative stress, and glomerular injury in a rodent model of type 2 diabetes mellitus.
• Pharmacological inhibition of endoplasmic reticulum stress attenuates increases in blood pressure and kidney injury in hypertensive-diabetic nephropathy.

What Is Relevant?
• Hypertension and metabolic abnormalities are often difficult to control in patients with type 2 diabetes mellitus and current therapeutic options for diabetic-hypertensive nephropathy only slow rather than halt the progression of chronic renal disease.
• Our study illustrates the synergistic interaction of mild diabetes mellitus and hypertension in promoting kidney injury and highlights the importance of tight control of blood pressure and glycemia in arresting kidney injury.

Summary
We created a new experimental model to investigate the mechanisms of kidney injury in type 2 diabetes mellitus. Our findings indicate that mild hyperglycemia and hypertension interact synergistically to promote renal dysfunction, albuminuria, endoplasmic reticulum stress, oxidative stress, and glomerular injury. We also found that inhibition of endoplasmic reticulum stress markedly attenuated dysfunction and injury in kidneys exposed to hypertension and diabetes mellitus, suggesting a new therapeutic strategy for hypertensive-diabetic nephropathy.

Novelty and Significance
• Our study provides a better understanding of the pathogenesis of diabetic-hypertensive renal injury and may provide novel therapeutic strategies that, when combined with current therapeutic interventions, help prevent progression to end-stage renal disease in diabetic patients.
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Synergistic Interaction of Hypertension and Diabetes in Promoting Kidney Injury and the Role of Endoplasmic Reticulum Stress

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MATERIALS AND METHODS

Surgical Procedures

For the AC surgery, rats were placed in the right lateral position and body temperature was maintained at 37°C with a heating pad. A ~2-3 cm skin incision was made in the left lateral flank about 2 cm below the rib cage. After exposing the left kidney and following the left renal artery to the aorta, a 3-0 silk suture was wrapped around the aorta between the left and right renal arteries. A cut sterile 22-gauge needle (1 cm long) was placed next to the aorta and a surgeon’s knot was snugly tied around both the needle and aorta to produce a consistent and precise degree of aorta constriction (about 36% reduction of outer diameter of the aorta). Finally, the needle was removed from the suture loop and the muscles and skin were closed.

To determine the BP gradient above and below the constriction, rats were anesthetized with 2% isoflurane, prepared for surgery and positioned under a dissecting scope in dorsal recumbency. A 2-cm ventral skin incision was made along the crease formed by the abdomen and left thigh. Blunt dissection of the adductor muscles was used to visualize the left femoral artery. A sterile catheter was inserted into the femoral artery, advanced into the aorta and secured with suture. Simultaneously, BP was measured from the telemetry catheter implanted in the common carotid artery above the AC, and the pressure gradient across the AC was calculated (Figure S1).

Separate Kidney GFR and Urine Collection

For measuring single kidney GFR and collecting urine from each kidney, rats were anesthetized with 2% isoflurane. A femoral vein catheterization was performed to allow i.v. infusion and a femoral artery catheterization was performed for blood collection. After a 30 min stabilization period of isotonic saline infusion at 2.4 ml/h, an i.v. injection of 1 ml 0.1% FITC-inulin (3 µg/g body weight) was given. Then, 0.1% FITC-inulin was infused at 2.4 ml/h for 2 hours and urine samples from each kidney were collected for 2 hours. Blood samples (200 µl) were collected at the middle and end of the 2-h infusion period and GFR from each kidney was calculated from the average rate of inulin clearance.

Western Blot for ER Stress Marker Protein

The cortex of left and right kidneys was homogenized in RIPA lysis buffer, sonicated, and cleared by centrifugation (12,000 rpm, 20 min at 4°C). 30 µg of total protein was separated in 4–15% SDS-polyacrylamide gels (Bio-rad, CA). After being transferred to nitrocellulose membranes, blots were rinsed in PBS and blocked in Odyssey blocking buffer (LI-COR, NE) for 1 h at room temperature, and with mouse polyclonal anti-CHOP (CCAAT-enhancer-binding protein homologous protein, 1:1000, Cell Signaling, MA), at 4 °C overnight. Membranes were probed with LI-COR fluorescent dye-labeled antibodies (1:3,000) for 1 h at room temperature. Antibody labeling was visualized using the Odyssey Infrared Scanner (LI-COR, NE). Fluorescence intensity analyses were performed using Odyssey software and protein levels were normalized to β-actin.

Transmission electron microscopy

Renal cortical tissues were cut into small pieces and rapidly immersed in tissue fixative buffer (10% formaldehyde, buffered to pH 7.4, Carson-Millonig formulation, RICCA Chemical Company, TX) at 4°C for at least 8 h. The fixed tissues were trimmed to 1 mm³ and stained with OsO4 for 1 h. Tissue slices were washed with acetone, 15 min twice, 1:1 acetone:Epon 1h, and then embedded in 100% Epon for 30 min. After 60°C overnight incubation, the Epon block was sectioned semi-thin (1µm in thickness, 10-12 sections) using MT-6000-XL and a glass knife. The semi-thin sections were stained with 1% Toluidine, and observed under light microscope to locate areas of interest for ultrathin sections. The trimmed Epon block was thin sectioned (70 nm in thickness), applied on copper grids, and then stained with 2% uranyl acetate for 3 min and calcinated lead citrate for 30 seconds. The stained grid was loaded in a JOEL JEM1400 transmission electron microscopy (TEM) with an ANT camera system. At least 5 sections from each sample were examined under TEM. The entire sections were thoroughly viewed at low
magnification (300×) for integrity and quality of stained tissues. Details of ultrastructural alterations were further investigated at high magnifications (20,000 x).
Figure S1. BP above and below the AC in GK and Wistar rats. MAP above the AC was measured from the telemetry catheter implanted in the common carotid artery and MAP below the AC was measured by the catheter inserted to aorta through a femoral artery. (n=9 in GK-AC rats and n=7 in Wistar-AC rats; *, P<0.05, comparing MAP below and above AC by t-test).
Figure S2. A, Blood glucose; B, Body weight and C, Food intake measurements in GK-AC rats with TUDCA (n=7) or Vehicle (n=6) treatment and GK-Sham rats with TUDCA treatment (n=5) at baseline, 2, 3, 4, 6 and 8 weeks after AC or sham surgery.
Figure S3. Pixels area of positive staining of 4-HNE in kidney samples from GK-AC and GK-AC+TUDCA rats. (n=5 in all groups *, P<0.05, compared to right kidneys of GK-AC rats by two-way ANOVA followed by Bonferroni’s multiple comparisons test)
Figure S4. Renal injury was scored based on PAS staining and morphological changes at 8 weeks after AC or sham surgery of GK-Sham+TUDCA (n=5), GK-AC+Vehicle (n=6) and GK-AC+TUDCA (n=6) rats. (*, $P<0.05$, compared to right kidney of GK-AC+Vehicle rats by Kruskal nonparametric test followed by Dunn’s multiple comparisons test)