Elucidating mechanisms underlying altered renal autoregulation in diabetes

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Elucidating mechanisms underlying altered renal autoregulation in diabetes. Am J Physiol Regul Integr Comp Physiol 303: R495–R504, 2012. First published June 27, 2012; doi:10.1152/ajpregu.00217.2012—Previous studies have reported that high-salt intake paradoxically activates tubuloglomerular feedback (TGF) in type 1 diabetes. Using Zucker lean (ZL) and diabetic fatty (ZDF) rats on normal and high-salt diets, renal hemodynamics and the renin-angiotensin system (RAS) were characterized. On normal salt diet, glomerular filtration rate (GFR) was higher in ZDF than ZL rats. Autoregulation of GFR was less efficient and lithium clearance was lower in ZDF rats than ZL rats. Salt load reduced GFR in ZDF rats with restoration of lithium clearance and partial improvement in autoregulatory index (AI). The administration of 8-cyclopentyl-1,3-dipropylxanthine, a selective adenosine-1 receptor antagonist to ZDF rats on a high-salt diet abolished the improvement of AI in GFR. However, this effect was seen by neither Cx40GAP27 nor Cx37,43GAP27, which inhibits connexin (C) 40 or Cx37. Renal ANG II was higher in ZDF than ZL rats on normal salt diet, but the difference was eliminated by a salt load. The present data provide the first demonstration for a salt paradox in type 2 diabetes and implicate that in addition to Cx alterations, the enhanced proximal reabsorption attenuates TGF, underlying glomerular hyperfiltration and RAS activation.

The present findings extended our previous data that Cx alterations, the enhanced PTR in early type 2 diabetes attenuates TGF, and although P2 signal transduction resulted in almost complete abolition of renal autoregulation (32). Previous data indicated that P2 receptor inhibition resulted in partial attenuation of autoregulation, including TGF (30). Distal delivery may be reduced in ZDF rats. Pruijm et al. (22) reported that glomerular hyperfiltration was associated with high PTR in patients with type 2 diabetes (22). However, the effects of salt intake on renal hemodynamics have not been adequately assessed in type 2 diabetes.

To further examine mechanisms mediating altered renal autoregulation, experiments were performed to assess the effects of diabetes and salt on renal autoregulation and the RAS. The present findings extended our previous data that Cx abnormalities in the JGA relate to suppressing TGF signal transduction, resulting in almost complete abolition of renal autoregulation (32). Previous data indicated that P2 receptor inhibition resulted in partial attenuation of autoregulation, including TGF (30). Distal delivery may be reduced in ZDF rats. Pruijm et al. (22) reported that glomerular hyperfiltration was associated with high PTR in patients with type 2 diabetes (22). However, the effects of salt intake on renal hemodynamics have not been adequately assessed in type 2 diabetes.

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METHODS

Experiments were performed using 8–10-wk-old male Zucker lean rats on normal (ZL-L) or high-salt diet (ZL-H) and ZDF rats on normal (ZDF-L) or high-salt diet (ZDF-H) (Charles River Japan, Kanagawa, Japan) to assess changes in early diabetes (32). Experimental protocols were approved by the ethical committee of Saitama Medical University. Animals were initially allowed free access to tap water and rat chow containing 0.3% NaCl (Quick Fat, Nihon CLEA, Tokyo, Japan). For the high salt group, chow containing 6% NaCl was given for 1 wk. Thereafter, chow was pair-fed (25 g/day) for four groups of rats to make the salt load comparable between ZL and ZDF rats. Rat weights were stable for a week of the pair-fed period. Two days before acute experiments, the chow containing LiCl (5 mmol/kg) was given to obtain measurable concentrations of lithium and its clearance as an index of end-proximal tubular flow without acute diuresis (26, 37, 44).

Western blot analysis. Animals were anesthetized with pentobarbital sodium (50 mg/kg ip) and decapitated (n = 6 for each group). Both kidneys were removed. The kidney was immersed in liquid nitrogen and kept frozen at −80°C until the assay was done (11, 13, 32). On the day of the assay, renal cortex was excised and minced and then homogenized in buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.1% Triton X-100, protease inhibitor cocktail (Sigma-Aldrich Chemicals, St. Louis, MO). Homogenate was obtained in the microtube. Protein content was measured using BSA as the standard, and treated with 2-mercapto-ethanol at 95°C for 10 min. The protein (30 μg) was separated in 7.5% polyacrylamide gel, and translocated to PVDF membranes using a semi-dry system. Membranes were blocked with 5% BSA in TBS-Tween (TBS-T), and rabbit antibodies to rat Cx37 (1:2,000; Alpha-Diagnostic International, San Antonio, TX), Cx40 (1:2,000, Chemicon, Gibbstown, NJ), Cx43 (1:2,000; Invitrogen, Carlsbad, CA), or phosphorylated Cx43 (1:2,000; Santa Cruz Biotechnology, Santa Cruz, CA) were applied at 4°C overnight, and then washed with TBS-T. Labeling was visualized with chemiluminescence (ECL Plus, Amersham, Piscataway, NJ) following incubation in goat anti-rabbit-HRP (Santa Cruz) for 2 h, and washing in TBS-T. β-actin was used as a housekeeping protein.

Renal hemodynamics. On the day of the experiment, the rats were anesthetized with pentobarbital sodium and placed on a thermostatically controlled table to maintain body temperature at 37°C, as detailed previously (33, 34). The animals were infused at the rate of 1.2 ml/h with isotonic saline solution containing 6% BSA during surgery, and, thereafter, with isotonic saline solution containing 1% BSA, 7.5% Inutest (Laevosan-Gesellschaft, Linz/Donau Austria) and 1.5% para-aminohippuric acid (PAH; Merck Sharp & Dohme; West Point, PA) via the jugular vein to enable calculation of GFR and effective renal plasma flow (RPF). The left femoral artery was catheterized with polyethylene (PE-50) filled with heparinized saline (100 U/ml) to allow blood sampling and continuous arterial pressure measurements. For fatty rats, additional saline was needed to maintain hematocrit constant (normal salt: 11 ± 2 μl/min, high salt: 12 ± 2 μl/min). Mean arterial pressure (MAP) was used as renal arterial pressure. The left ureter was cannulated (PE-10) with a midline abdominal incision to collect urine in a preweighed tube.

An adjustable clamp was placed on the aorta above the left renal artery to control renal arterial pressure. The left adrenal artery was cannulated with extended PE-10 to infuse heparinized saline or GAP peptides (5 mg ia followed by 0.2 mg/min) at a rate of 0.6 ml/h (6, 30–32, 36). The solution for transjugular infusion was adjusted to contain 2% BSA and infused at the rate of 0.6 ml/h to equalize the water loads. The animals whose left adrenal artery came from the aorta were excluded from the study. Rats were allowed to breathe air enriched with 100% oxygen (aimed at FIO2 of 40%), which markedly improves the stability of arterial blood pressure. After completion of surgery, 1 h of equilibration was allowed before initiating experimental protocols.

The experiments contained three series of studies and were performed to characterize renal hemodynamic effects of three different GAP peptides (Cx37,GAP27, Cx40,GAP27, or Cx43,GAP26) and 8-cyclopentenyl-1,3-dipropylxanthenine (CPX), a selective adenosine-1 receptor antagonist (Sigma). In the first series of studies designed to test whether Cx40 participates in renal autoregulation, the effects of Cx40,GAP27 were examined. This series of studies were performed on ZL-L, ZL-H, ZDF-L, and ZDF-H rats (n = 6 for each). Two consecutive 20-min control clearances were carried out. The aortic clamp was tightened to reduce renal arterial pressure by ~20 mmHg before initiating two consecutive 20-min clearance periods. Subsequently, the aortic clamp was released. Saline was exchanged with one containing GAP peptide and infused into the adrenal artery throughout the remaining experimental periods. Because our previous studies had shown that Cx37,GAP27 and Cx40,GAP27 induced increases in blood pressure (30, 31), the aortic clamp was slightly tightened to prevent this increase and hence maintain renal arterial pressure at the control level. To obtain maximal actions of GAP peptides, at least another 60-min equilibration period was allowed before initiating two consecutive 20-min clearance periods. Subsequently, the aortic clamp was further tightened to reduce renal arterial pressure by ~20 mmHg, and two consecutive 20-min clearance studies were carried out. Then, renal arterial pressure was returned to the control level by carefully releasing the aortic clamp. CPX was intravenously administered (0.3 mg/kg) and repeated every 20 min. Two consecutive 20-min clearance experiments were performed. Finally, the aortic clamp was again tightened, and two consecutive 20-min clearance experiments were performed. The stock solution of CPX (2.5 mg/ml) was freshly prepared with 0.1 M NaOH in saline on the day of each experiment (30).

In the second series of studies, similar experiments were performed to assess the effects of Cx37,GAP27 and CPX for four groups of Zucker rats (n = 6 for each).

For the other four groups of Zucker rats (n = 6 for each), the third series of studies were performed to assess the impacts of Cx40,GAP26 and CPX on renal autoregulation.

Arterial blood samples (~0.2 ml) were taken at the midpoint of each clearance period. Cells were separated by centrifugation, and plasma was removed. Urine volume was determined gravimetrically. Immediately, the sodium, and lithium concentrations in both plasma and urine were measured by standard photometry. At the end of the experiment, the rats were euthanized with an overdose of pentobarbital, and the left kidney was removed, decapsulated, blotted dry, and weighed (31).

PTR was calculated by subtracting lithium clearance (end-proximal tubular flow) from inulin clearance (GFR). Pressure-induced natriuresis was expressed using fractional excretion of sodium (FENA), because GFR was altered in ZDF rats following changes in renal arterial pressure. Because GAP peptides altered basal RPF and GFR (26, 27) and because changes in MAPs (and thus autoregulatory stimuli) were not exactly the same among experimental periods, autoregulatory capacity was compared using the autoregulatory index (AI), calculated according to following formulas: AI for RPF = [(RPF2−RPF1)/(RPF1)](MAP2−MAP1)/MAP1 for GFR = [(GFR2−GFR1)/(GFR1)](MAP2−MAP1)/MAP1.

RPF1, GFR1, and MAP1 indicate those at control MAP. RPF2, GFR2, and MAP2 depict those at reduced MAP (30). Thus, AI of zero means perfect autoregulation, and AI of one depicts absence of autoregulation.

Renin-angiotensin. In additional experiments, plasma and renal ANG II concentrations were measured in four separate groups of rats (ZL-L, ZL-H, ZDF-L, and ZDF-H rats; n = 6 for each). Surgical procedures were the same as the above, except that neither ureter nor adrenal artery was cannulated. After an hour of equilibration, kidneys were removed, and 2 ml of blood sample was taken from the femoral artery in chilled tubes containing EDTA. Kidneys were quickly frozen with liquid nitrogen, and blood samples were centrifuged at 4°C.
Baseline characteristics and connexin expression. Regardless of diets, ZDF rats showed higher values for body weight, plasma glucose, and kidney weight than ZL rats (Table 1). Thus, the present data agreed with previous results that ZDF rats fed with Quick Fat became diabetic at the time of experiments (32) and further extended the notion that they were diabetic, regardless of salt load. However, ZDF rats did not show any sign of hydropnephrosis (17). MAP was comparable among the four groups. ZDF-L rats showed higher RPF and GFR than ZL-L and ZL-H rats. A high-salt diet lowered RPF and GFR in ZDF rats. Both RPF and GFR in ZDF-H rats were indistinguishable from those of ZL-H rats. ZDF-L rats also manifested lower lithium clearance (Cl-Li) and higher PTR than ZL-L and ZL-H rats. High-salt diet increased Cl-Li and attenuated PTR in ZDF rats, rendering them similar to those of ZL rats. ZDF rats had higher urine volume (UV) than ZL rats, and high-salt diet increased UV in ZL but not in ZDF rats. The present results extended the previous findings that urinary excretion of albumin (UAlb) in ZDF-L rats was greater than ZL-L rats (32), and further indicated that a high-salt diet reduced UAlb in ZDF rats.

Figure 1 summarizes the expression of Cxs in the kidneys of Zucker rats. We failed to find any differences in renal expressions of Cx37, Cx40, and Cx43 among the four groups of Zucker rats. However, p-Cx37 expression was considerably elevated in ZDF rats compared with ZL rats. Similar analyses for phosphorylated Cx37 and Cx40 were not performed because specific antibodies for each were not available.

Renal hemodynamics. As shown in Tables 2 and 3, renal autoregulation was well preserved in ZL-L and ZL-H rats under control conditions. During intra-renal infusion of Cx40GAP27 or Cx37,43GAP27, both RPF and GFR were slightly lowered in ZL-L and ZL-H rats (P < 0.05) following reductions of MAP. In the presence of CPX, RPF and GFR fell markedly in response to reductions of MAP in ZL-L and ZL-H rats (P < 0.01). Thus, in ZL-L and ZL-H rats, Cx40GAP27 or Cx37,43GAP27 increased AI (P < 0.05), and subsequent addition of CPX further worsened AI (P < 0.05). In contrast, RPF and GFR were markedly decreased in ZDF-L rats (P < 0.01) and slightly declined in ZDF-H rats (P < 0.05) following reductions of MAP, even under control conditions. Cx40GAP27 and Cx37,43GAP27 failed to elicit further alterations of renal autoregulation and AI in ZDF-L and ZDF-H rats. Although subsequent addition of CPX did not alter renal autoregulation in ZDF-L rats, both GFR and RPF were reduced by decreasing MAP in ZDF-H rats treated with CPX (P < 0.05). Thus, AI remained elevated throughout all experimental periods in ZDF-L rats, and CPX further worsened AI only in ZDF-H rats (P < 0.05). In addition, at basal MAP, Cx40GAP27 reduced RPF by 15 ± 5% in ZL-L (P < 0.01) and 10 ± 2% (P < 0.05) in ZL-H rats. However, Cx40GAP27 failed to reduce RPF in ZDF-L and ZDF-H rats at baseline MAP. Similarly, Cx37,43GAP27 reduced RPF in ZL-L (15 ± 3%, P < 0.01) and ZL-H rats (11 ± 2%, P < 0.05) when compared at basal MAP. But, Cx37,43GAP27 failed to reduce baseline RPF in ZDF-L and ZDF-H rats.

As depicted in Table 4, in the absence or presence of Cx43GAP26, RPF, and GFR were well autoregulated in ZL-L and ZL-H rats. The observations that Cx43GAP26 failed to alter renal autoregulation in both ZL and ZDF rats were consistent with our previous findings that Cx43 localized on endothelium in the JGA of Zucker rats (32) and further suggested that an inhibition of Cx37 by Cx37,43GAP27 should account for its ability to alter renal autoregulation. During administration of CPX, RPF and GFR were slightly decreased following the decrements of MAP in ZL-L and ZL-H rats (P < 0.05). Thus, CPX worsened AI in both groups (P < 0.05). Superimposition of CPX on Cx43GAP26 induced partial inhibition of renal autoregulation in ZL rats, uniquely serving as time controls to Cx40GAP27 and Cx37,43GAP27 studies. This also supported the notion that P2 signal through the gap junction consisting of Cx37 and/or Cx40 in JGA and A1 signal are required for full

Table 1. Baseline characters of Zucker rats

<table>
<thead>
<tr>
<th></th>
<th>ZL-L</th>
<th>ZL-H</th>
<th>ZDF-L</th>
<th>ZDF-H</th>
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<tbody>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>BW, g</td>
<td>271 ± 6</td>
<td>259 ± 7</td>
<td>363 ± 6+†</td>
<td>343 ± 7+†</td>
</tr>
<tr>
<td>KW, g</td>
<td>0.97 ± 0.03</td>
<td>0.98 ± 0.04</td>
<td>1.19 ± 0.04+†</td>
<td>1.14 ± 0.04†</td>
</tr>
<tr>
<td>KW/BW, 10⁻³</td>
<td>3.5 ± 0.1</td>
<td>3.6 ± 0.2</td>
<td>3.3 ± 0.1</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>PG, mg/dl</td>
<td>90 ± 4</td>
<td>94 ± 5</td>
<td>231 ± 0.05+</td>
<td>240 ± 0.06+</td>
</tr>
<tr>
<td>Ht, %</td>
<td>48 ± 1</td>
<td>47 ± 1</td>
<td>49 ± 1</td>
<td>48 ± 1</td>
</tr>
<tr>
<td>RPF</td>
<td>3.34 ± 0.06</td>
<td>3.33 ± 0.06</td>
<td>3.85 ± 0.07+†</td>
<td>3.52 ± 0.07+†</td>
</tr>
<tr>
<td>AI of RPF</td>
<td>0.05 ± 0.04</td>
<td>0.05 ± 0.05</td>
<td>0.89 ± 0.06+†</td>
<td>0.47 ± 0.05+†‡</td>
</tr>
<tr>
<td>GFR</td>
<td>0.95 ± 0.03</td>
<td>0.96 ± 0.03</td>
<td>1.16 ± 0.03 †</td>
<td>1.05 ± 0.03+†‡</td>
</tr>
<tr>
<td>AI of GFR</td>
<td>0.04 ± 0.04</td>
<td>0.01 ± 0.05</td>
<td>0.88 ± 0.07+</td>
<td>0.47 ± 0.05†‡</td>
</tr>
<tr>
<td>CI-Li</td>
<td>0.32 ± 0.01</td>
<td>0.33 ± 0.01</td>
<td>0.26 ± 0.01+</td>
<td>0.33 ± 0.01†‡</td>
</tr>
<tr>
<td>PTR</td>
<td>0.63 ± 0.03</td>
<td>0.63 ± 0.03</td>
<td>0.90 ± 0.03+†</td>
<td>0.73 ± 0.03+†</td>
</tr>
<tr>
<td>UV, μl/min</td>
<td>7 ± 1</td>
<td>12 ± 1*</td>
<td>19 ± 2+</td>
<td>21 ± 2+</td>
</tr>
<tr>
<td>UAlb, μg/min</td>
<td>0.41 ± 0.01</td>
<td>0.55 ± 0.02*</td>
<td>1.03 ± 0.05+†</td>
<td>1.06 ± 0.06†‡</td>
</tr>
</tbody>
</table>

ZL-L, ZL-H, ZDF-L, and ZDF-H depicted Zucker lean (ZL) and diabetic fatty (ZDF) rats with normal (L) or a high-salt diet (H). BW, body weight; KW, kidney weight; PG, plasma glucose; Ht, hematocrit; RPF, renal plasma flow; GFR, glomerular filtration rate; AI, autoregulatory index; CI-Li, lithium clearance; PTR, proximal tubular reabsorption; UV, urine volume; UAlb, urine albumin excretion. *, †, ‡Significant differences (P < 0.05) from ZL-L, ZL-H, and ZDF-L, respectively. The units for RPF, GFR, CI-Li, and PTR were milliliters per minute per gram kidney weight.

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expression of renal autoregulation (30). Furthermore, at base-
line MAP, Cx43GAP26 decreased GFR in both ZL-L (9 ± 1%;
P < 0.05) and ZL-H rats (8 ± 1%; P < 0.05). In contrast,
Cx43GAP26 altered GFR in neither ZDF-L nor ZDF-H rats at
baseline MAP. As in the studies with Cx40GAP27 or
Cx37,43GAP27, both RPF and GFR were markedly decreased in
ZDF-L rats (P < 0.01) and slightly declined in ZDF-H rats (P <
0.05) following reductions of MAP under control conditions.
Like ZL rats, Cx43GAP26 failed to elicit further alterations in
renal autoregulation in both ZDF-L and ZDF-H rats. The
superimposition of CPX on Cx43GAP26 did not provoke further
deterioration of renal autoregulation in ZDF-L rats, whereas

![Image](https://example.com/image.png)

**Table 2. Influence of Cx40GAP27 and 8-cyclopentyl-1,3-dipropylxanthine on renal hemodynamics**

<table>
<thead>
<tr>
<th>MAP</th>
<th>Control</th>
<th>GAP</th>
<th>GAP+CPX</th>
<th>Control</th>
<th>GAP</th>
<th>GAP+CPX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZL-L</td>
<td>3.33 ± 0.08</td>
<td>2.88 ± 0.07</td>
<td>2.80 ± 0.06 #</td>
<td>0.94 ± 0.05</td>
<td>0.91 ± 0.04</td>
<td>0.91 ± 0.03</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.30 ± 0.06</td>
<td>2.60 ± 0.06 *</td>
<td>2.28 ± 0.04</td>
<td>0.93 ± 0.05</td>
<td>0.83 ± 0.04 *</td>
<td>0.74 ± 0.02 *</td>
</tr>
<tr>
<td>AI</td>
<td>0.05 ± 0.06</td>
<td>0.49 ± 0.07</td>
<td>0.91 ± 0.08 #</td>
<td>0.06 ± 0.05</td>
<td>0.48 ± 0.08 #</td>
<td>0.90 ± 0.09 #</td>
</tr>
<tr>
<td>ZL-H</td>
<td>3.32 ± 0.09</td>
<td>3.02 ± 0.10</td>
<td>2.98 ± 0.11</td>
<td>0.97 ± 0.05</td>
<td>0.92 ± 0.05</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.27 ± 0.09</td>
<td>2.70 ± 0.10 *</td>
<td>2.42 ± 0.11</td>
<td>0.96 ± 0.06</td>
<td>0.83 ± 0.05 *</td>
<td>0.75 ± 0.04 *</td>
</tr>
<tr>
<td>AI</td>
<td>0.05 ± 0.06</td>
<td>0.49 ± 0.08</td>
<td>0.90 ± 0.05 #</td>
<td>0.04 ± 0.06</td>
<td>0.48 ± 0.07 #</td>
<td>0.86 ± 0.08 #</td>
</tr>
<tr>
<td>ZDF-L</td>
<td>3.85 ± 0.10</td>
<td>3.63 ± 0.09</td>
<td>3.62 ± 0.13</td>
<td>1.16 ± 0.05</td>
<td>1.14 ± 0.06</td>
<td>1.12 ± 0.04</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.22 ± 0.09 *</td>
<td>3.10 ± 0.07 *</td>
<td>3.07 ± 0.12 *</td>
<td>0.97 ± 0.05 *</td>
<td>0.97 ± 0.05 *</td>
<td>0.95 ± 0.04 *</td>
</tr>
<tr>
<td>AI</td>
<td>0.88 ± 0.07</td>
<td>0.85 ± 0.07</td>
<td>0.83 ± 0.09</td>
<td>0.87 ± 0.08</td>
<td>0.85 ± 0.06</td>
<td>0.84 ± 0.10</td>
</tr>
<tr>
<td>ZDF-H</td>
<td>3.43 ± 0.12</td>
<td>3.28 ± 0.11</td>
<td>3.27 ± 0.10</td>
<td>1.04 ± 0.08</td>
<td>1.05 ± 0.06</td>
<td>1.01 ± 0.05</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.12 ± 0.11 *</td>
<td>2.95 ± 0.10 *</td>
<td>2.72 ± 0.09 *</td>
<td>0.95 ± 0.08 *</td>
<td>0.95 ± 0.06 *</td>
<td>0.85 ± 0.04 *</td>
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<tr>
<td>AI</td>
<td>0.46 ± 0.06</td>
<td>0.51 ± 0.07</td>
<td>0.85 ± 0.09 #</td>
<td>0.47 ± 0.06</td>
<td>0.50 ± 0.06</td>
<td>0.84 ± 0.07 #</td>
</tr>
</tbody>
</table>

MAP, mean arterial pressure; CPX, 8-cyclopentyl-1,3-dipropylxanthine. *Significant difference from the respective value at basal MAP (P < 0.05).
#,$Significant difference (P < 0.05) from the control period and that with GAP, respectively. Symbols for significance among three periods at reduced MAP
were omitted for clarity.
both GFR and RPF varied substantially with decrements of MAP in ZDF-H rats ($P < 0.01$) in the presence of CPX. Thus, AI remained elevated throughout the experimental periods in ZDF-L rats, and the addition of CPX further aggravated AI only in ZDF-H rats ($P < 0.05$).

Finally, the data from the control periods in three series of experiments confirm that salt load partially improved renal autoregulation in ZDF but not in ZL rats (Tables 1–4), supporting the salt paradoxE in type 2 diabetes.

**Renin-angiotensin.** Two-way ANOVA revealed that salt decreased ($F = 23$, $P < 0.001$), and diabetes increased, renal ANG II ($F = 6$, $P < 0.05$), and that there was significant interaction between salt and diabetes ($F = 5$, $P < 0.05$). Indeed, one-way ANOVA showed that renal ANG II was higher in ZDF-L than ZL-L rats (Fig. 2). However, the difference was negated by the salt load. Behavior of plasma ANG II was similar to renal ANG II but with less significance. Two-way ANOVA revealed that salt decreased ($F = 21$, $P < 0.001$) plasma ANG II. Diabetes did not alter plasma ANG II ($F = 1$, $P = 0.3$), and there was little interaction between salt and diabetes ($F = 4$, $P = 0.5$). Although ZDF-L rats tended to show higher plasma ANG II concentrations than the ZL rats, statistical significance was not attained. A high-salt diet suppressed plasma ANG II in both groups.

**Pressure-induced natriuresis relationship among Zucker rats.** Comparisons for FENa among the four groups were performed on the baseline data from three series of experiments ($n = 18$ for each). Two-way ANOVA showed that salt ($F = 8$, $P < 0.01$) and diabetes ($F = 84$, $P < 0.0001$) increased FENa but without significant interaction. Under basal conditions, FENa was higher in ZDF rats than ZL rats at spontaneous MAP, possibly because of additional fluid load (Fig. 3). Although MAP was indistinguishable among the four groups (Table 5), a high-salt diet increased FENa in either ZL or ZDF rats at basal MAP, suggesting that reductions in tubular sodium absorption by high salt in both strains were not pressure-dependent. Of importance, while sodium excretion in ZL-L rats was smaller than ZL-H rats ($P < 0.05$), it was similar between

### Table 3. Influence of GAP and CPX on renal hemodynamics

<table>
<thead>
<tr>
<th>MAP</th>
<th>RPF</th>
<th>GAP</th>
<th>GAP+CPX</th>
<th>GFR</th>
<th>GAP</th>
<th>GAP+CPX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZL-L Basal</td>
<td>3.23 ± 0.10</td>
<td>2.76 ± 0.11#</td>
<td>2.67 ± 0.07#</td>
<td>0.92 ± 0.06</td>
<td>0.92 ± 0.05</td>
<td>0.94 ± 0.04</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.22 ± 0.08</td>
<td>2.48 ± 0.09*</td>
<td>2.20 ± 0.06*</td>
<td>0.92 ± 0.05</td>
<td>0.82 ± 0.04*</td>
<td>0.77 ± 0.04*</td>
</tr>
<tr>
<td>AI</td>
<td>0.05 ± 0.06</td>
<td>0.54 ± 0.08#</td>
<td>0.88 ± 0.09#</td>
<td>0.01 ± 0.05</td>
<td>0.52 ± 0.08#</td>
<td>0.87 ± 0.09#$</td>
</tr>
<tr>
<td>ZDF-H Basal</td>
<td>3.26 ± 0.10</td>
<td>2.92 ± 0.13#</td>
<td>2.87 ± 0.11#</td>
<td>0.95 ± 0.06</td>
<td>0.91 ± 0.05</td>
<td>0.91 ± 0.05</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.18 ± 0.08</td>
<td>2.63 ± 0.12*</td>
<td>2.37 ± 0.11*</td>
<td>0.95 ± 0.06</td>
<td>0.82 ± 0.05*</td>
<td>0.75 ± 0.04*</td>
</tr>
<tr>
<td>AI</td>
<td>0.07 ± 0.09</td>
<td>0.49 ± 0.09#</td>
<td>0.91 ± 0.08#</td>
<td>-0.03 ± 0.05</td>
<td>0.49 ± 0.05#</td>
<td>0.92 ± 0.07#$</td>
</tr>
</tbody>
</table>

*Significant difference from the respective value at basal MAP ($P < 0.05$). #Significant difference ($P < 0.05$) from the control period and that with GAP, respectively. Symbols for significance among three periods at reduced MAP were omitted for clarity.

### Table 4. Influence of “C-44GAP26 and 8-cyclopentyl-1,3-dipropylxanthine on renal hemodynamics

<table>
<thead>
<tr>
<th>MAP</th>
<th>RPF</th>
<th>GAP</th>
<th>GAP+CPX</th>
<th>GFR</th>
<th>GAP</th>
<th>GAP+CPX</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZL-L Basal</td>
<td>3.45 ± 0.13</td>
<td>3.32 ± 0.13</td>
<td>3.25 ± 0.12</td>
<td>0.98 ± 0.05</td>
<td>0.90 ± 0.04#</td>
<td>0.89 ± 0.04#</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.42 ± 0.11</td>
<td>3.28 ± 0.12</td>
<td>2.90 ± 0.11*</td>
<td>0.96 ± 0.05</td>
<td>0.89 ± 0.04</td>
<td>0.79 ± 0.04*</td>
</tr>
<tr>
<td>AI</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.06</td>
<td>0.52 ± 0.06$</td>
<td>0.04 ± 0.04</td>
<td>0.07 ± 0.06</td>
<td>0.53 ± 0.06#$</td>
</tr>
<tr>
<td>ZL-H Basal</td>
<td>3.40 ± 0.13</td>
<td>3.15 ± 0.13</td>
<td>3.20 ± 0.13</td>
<td>0.97 ± 0.04</td>
<td>0.90 ± 0.04#</td>
<td>0.90 ± 0.04#</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.33 ± 0.13</td>
<td>3.13 ± 0.12</td>
<td>2.82 ± 0.12*</td>
<td>0.96 ± 0.04</td>
<td>0.89 ± 0.03</td>
<td>0.81 ± 0.03*</td>
</tr>
<tr>
<td>AI</td>
<td>0.02 ± 0.05</td>
<td>0.07 ± 0.07</td>
<td>0.55 ± 0.07$</td>
<td>0.01 ± 0.05</td>
<td>0.03 ± 0.06</td>
<td>0.56 ± 0.06#$</td>
</tr>
<tr>
<td>ZDF-L Basal</td>
<td>3.88 ± 0.13</td>
<td>3.72 ± 0.13</td>
<td>3.65 ± 0.12</td>
<td>1.16 ± 0.05</td>
<td>1.14 ± 0.05</td>
<td>1.13 ± 0.04</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.22 ± 0.12*</td>
<td>3.12 ± 0.13*</td>
<td>3.07 ± 0.12*</td>
<td>0.97 ± 0.05*</td>
<td>0.96 ± 0.05*</td>
<td>0.95 ± 0.05*</td>
</tr>
<tr>
<td>AI</td>
<td>0.86 ± 0.07</td>
<td>0.86 ± 0.07</td>
<td>0.88 ± 0.08</td>
<td>0.84 ± 0.06</td>
<td>0.85 ± 0.06</td>
<td>0.86 ± 0.07</td>
</tr>
<tr>
<td>ZDF-H Basal</td>
<td>3.58 ± 0.13</td>
<td>3.55 ± 0.13</td>
<td>3.38 ± 0.13</td>
<td>1.05 ± 0.05</td>
<td>1.04 ± 0.05</td>
<td>0.99 ± 0.04</td>
</tr>
<tr>
<td>Reduced</td>
<td>3.25 ± 0.13*</td>
<td>3.20 ± 0.12*</td>
<td>2.85 ± 0.12*</td>
<td>0.95 ± 0.04*</td>
<td>0.94 ± 0.05*</td>
<td>0.81 ± 0.04*</td>
</tr>
<tr>
<td>AI</td>
<td>0.47 ± 0.05</td>
<td>0.51 ± 0.06</td>
<td>0.82 ± 0.07$</td>
<td>0.46 ± 0.05</td>
<td>0.50 ± 0.06</td>
<td>0.81 ± 0.08#$</td>
</tr>
</tbody>
</table>
ZDF-L and ZDF-H rats (Table 1). These results are consistent with those reported by Miller (18), providing the first demonstration for salt paradox in type 2 diabetes. In each group, reduction of MAP elicited significant decreases in FENa. Two-way ANOVA showed that although salt load failed to alter pressure natriuresis, it was reduced in ZDF rats ($F = 19, P < 0.0001$) compared with ZL rats without significant interaction between them. The slope for pressure-induced natriuresis was compared among the four groups using one-way ANOVA. Regardless of diets, pressure natriuresis was blunted in ZDF rats, compared with ZL rats (Fig. 3B).

DISCUSSION

An increase in salt reuptake at the macula densa initiates TGF signals that constrict the afferent arteriole (19, 29). Thomson et al. (38) proposed the tubular hypothesis that hyperglycemia elicits a large increase in PTR associated with...
differing results. Accordingly, the present results, which are
in SHR. Diverse strains in diabetic models would account for
ZDF rats. Brännström et al. (2) reported that TGF is enhanced
rats might shift the autoregulatory curve to a higher pressure in
intensive rats (SHR). Genetic differences between ZL and ZDF
rats was significantly reduced in ZDF-L rats, compared with ZL
hypertension by itself would reduce afferent arteriolar tone
by removing TGF, resulting in glomerular hyperfiltration. We
shown mild to moderate hyperglycemia. Although the possi-
proximal flow in the present
by removing TGF, resulting in glomerular hyperfiltration. We
showed marked osmotic diuresis, which makes Cl-Li less reli-
chloride in diabetes were lower than in the control (42). Thus,
demonstration for salt paradox in type 2 diabetes, supporting
similar additional fluid given in acute experiments. Thus, our
a reduced GFR associated with a decreased PTR despite
GFR and PTR were higher in ZDF-L rats than ZL-L rats.
and salt intake in early diabetes (36). In the present study, both
influences of hypertensive renal injury on renal hemodynam-
1 mo significantly elevates blood pressure in ZDF rats. We
increase blood pressure in ZDF rats, it did not attain statistical
Obesity is related to salt-sensitive hypertension (29). Indeed,
first, Cl-Li should not be used in the lower range of blood pressure
used Cl-Li as an index of end-proximal flow in the present
by removing TGF, resulting in glomerular hyperfiltration. We
hyperglycemia by itself would reduce afferent arteriolar tone
hyperfiltration.
MAP and suggest that distal delivery is diminished in type 2
diabetes, participating in abnormal autoregulation and glomer-
ulmonary hyperfiltration.
Glucose reuptake, thereby reducing salt delivery to the macula
densa (38). Early distal tubular concentrations of sodium and
chloride in diabetes were lower than in the control (42). Thus,
hyperglycemia by itself would reduce afferent arteriolar tone
by removing TGF, resulting in glomerular hyperfiltration. We
showed mild to moderate hyperglycemia. Although the possi-
ability remained that the influence of hyperglycemia to lower
showed mild to moderate hyperglycemia. Although the possi-
bility remained that the influence of hyperglycemia to lower
distal delivery was underestimated due to osmotic diuresis, the
present findings did constitute new demonstrations that Cl-Li
was significantly reduced in ZDF-L rats, compared with ZL
rats (Table 1). However, the present results seem different from
those of Griffin et al. (9) that renal autoregulation was pre-
served in Zucker spontaneously hypertensive rats, an offsprings
from the breeding of Zucker obese and spontaneously hyper-
tensive rats (SHR). Genetic differences between ZL and ZDF
rats might shift the autoregulatory curve to a higher pressure in
ZDF rats. Brännström et al. (2) reported that TGF is enhanced
in SHR. Diverse strains in diabetic models would account for
differing results. Accordingly, the present results, which are
consistent with Thomson et al. (38), indicate that renal auto-
regulation is markedly impaired in ZDF-L rats at spontaneous
MAP and suggest that distal delivery is diminished in type 2
diabetes, participating in abnormal autoregulation and glomer-
ular hyperfiltration.

Table 5. Mean arterial pressure in all periods at the time of hemodynamic studies

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GAP</th>
<th>GAP + CPX</th>
<th>Control</th>
<th>GAP</th>
<th>GAP + CPX</th>
</tr>
</thead>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>99 ± 2</td>
<td>101 ± 2</td>
<td>99 ± 2</td>
<td>100 ± 2</td>
<td>102 ± 1</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>Reduced</td>
<td>82 ± 2*</td>
<td>80 ± 1*</td>
<td>78 ± 1*</td>
<td>81 ± 1*</td>
<td>81 ± 1*</td>
<td>80 ± 1*</td>
</tr>
<tr>
<td>ZDF-L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal</td>
<td>102 ± 2</td>
<td>98 ± 2</td>
<td>99 ± 2</td>
<td>103 ± 2</td>
<td>98 ± 2</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>Reduced</td>
<td>81 ± 2*</td>
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<td>80 ± 1*</td>
<td>80 ± 1*</td>
<td>79 ± 1*</td>
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<td></td>
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</tr>
<tr>
<td>Basal</td>
<td>99 ± 2</td>
<td>99 ± 2</td>
<td>100 ± 2</td>
<td>101 ± 2</td>
<td>103 ± 2</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>Reduced</td>
<td>83 ± 1*</td>
<td>79 ± 1*</td>
<td>78 ± 1*</td>
<td>81 ± 1*</td>
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<tr>
<td>ZDF-L</td>
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<tr>
<td>Basal</td>
<td>100 ± 2</td>
<td>98 ± 2</td>
<td>100 ± 2</td>
<td>102 ± 2</td>
<td>98 ± 2</td>
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</tr>
<tr>
<td>Reduced</td>
<td>82 ± 2*</td>
<td>80 ± 1*</td>
<td>81 ± 2*</td>
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<td>79 ± 1*</td>
<td>81 ± 1*</td>
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<tr>
<td>Basal</td>
<td>100 ± 1</td>
<td>102 ± 1</td>
<td>100 ± 1</td>
<td>100 ± 2</td>
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<td>100 ± 1</td>
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<tr>
<td>Reduced</td>
<td>81 ± 2*</td>
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<td>79 ± 1*</td>
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<td>80 ± 1*</td>
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</tr>
</tbody>
</table>

GAP and CPX indicate GAP peptide and 8-cyclopentyl-1,3-dipropylxanthine, respectively. *Significant difference from the respective value at basal MAP (P < 0.05).

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the concept that the site of natriuretic action of salt load involves the proximal tubule in early diabetes.

Cx plays an important role in renal autoregulation. Just et al. (14) showed that Cx40 knockout animals exhibited gross impairments of the TGF-mediated component of autoregulation. Our recent findings indicated that diabetes induces posttranscriptional alterations of Cxs in the JGA and that Cx malfunction underlies the derangement of TGF in diabetes (32). Consistent with this, the present results showed that Cx phosphorylation was enhanced in ZDF rats, compared with ZL rats regardless of diets (Fig. 1). Hyperglycemia by itself or oxidative stress observed in ZDF rats activates protein kinase C and mitogen-activated protein kinase, both of which phosphorylate Cxs (4, 5, 32). Phosphorylated Cx decreases the conductance of the gap junction (15). In agreement, the present data implicated that Cx43GAP26 elicited decrements of GFR in ZL-L and ZL-H rats (Table 4). However, these effects of Cx43GAP26 on GFR were lacking in ZDF-L and ZDF-H rats, suggesting that gap junctions composed of Cx43 were already malfunctioning in ZDF kidneys. It is likely that as with Cx43, Cx37 and Cx40 are phosphorylated in ZDF rats, participating in abnormal renal autoregulation. Indeed, Cx37,43GAP27 and Cx40GAP27 reduced RPF in ZL-L and ZL-H rats (Tables 2 and 3). However, these renal hemodynamic effects were absent in ZDF-L and ZDF-H rats, suggesting that gap junctions consisting of Cx37 and/or Cx40 were malfunctioning as well.

The present studies provide new evidence that salt load restores Cl-Li in ZDF rats, improving renal autoregulation, presumably through activating TGF by raising the delivery to the macula densa (Fig. 4). Our findings also demonstrated that salt load reduced Ualb in ZDF rats (Table 1), suggesting that high salt reduces glomerular capillary pressure in early diabetes. Zatz et al. (46) reported that enarapril decreased glomerular capillary pressure and proteinuria, but not GFR in type 1 diabetes. Moreover, the present data indicated that renal autoregulation was partially restored in ZDF-H rats and that CPX, but not Cx40GAP27 or Cx37,43GAP27, abolished this amelioration of renal autoregulation. Cx plays an important role in the myogenic response (7), especially as one of the mechanisms mediating renal autoregulation (28). The possibility remains that the myogenic response is attenuated in diabetes, contributing to hyperfiltration. As discussed, however, the difference in diabetic states, but not salt intake, plays an important role as the determinant for posttranscriptional alterations of Cxs in the kidney, suggesting that salt-induced changes in renal hemodynamics are independent of Cxs. High salt intake upregulates renal adenosine (47). Previous data demonstrated that adenosine is involved in reversing diabetic hyperfiltration during high-salt feeding in mice (41). Taken together, these results support the salt paradox in ZDF rats, a model of type 2 diabetes, and suggest that salt paradox operates via Cx-independent, adenosine-dependent TGF signaling in ZDF rats, just as reported for type 1 diabetes (41).

Our previous data indicated that renal ANG II in adult Otsuka Long-Evans Tokushima Fatty rats, a model of type 2 diabetes, was higher than in Long-Evans Tokushima Otsuka rats (20). Similarly, renal ANG II was higher in ZDF-L than ZL-L rats in the present study (Fig. 2), suggesting that renal RAS was activated in early type 2 diabetes. Plasma ANG II shows trends similar to renal ANG II, but statistical significance was not obtained. Macula densa initiates TGF to constrict afferent arterioles and suppresses renin release (19). Although ANG II constricts both afferent and efferent arterioles (35), autoregulatory adjustment of vascular tone is limited to preglomerular vessels, including the afferent arteriole. ANG II has been considered as a positive modulator of TGF (29) and enhances adenosine-induced afferent arteriolar constriction (39). Adenosine and ANG II constrict afferent arterioles synergistically, and the enhancement of TGF accounts for about half of afferent arteriolar constriction by ANG II (12, 24). An elevation of renal ANG II in diabetes coupled with attenuation of autoregulatory tone would fail to considerably elevate preglomerular resistance that could exacerbate glomerular hyperfiltration. We have provided evidence that a high-salt diet markedly diminishes renal ANG II in ZDF rats, suggesting that adenosine is missing from ZDF-L due to high PTR, but not in ZDF-H, where PTR is not high. A reduction of PTR and consequent improvement of the delivery to macula densa by a high-salt diet in ZDF-H rats, at least in part, elicits a large drop in renal ANG II. Cx40 is involved in the regulation of renin in mice (43). Collectively, the present results are compatible with previous findings (16, 32) and suggest that an attenuated delivery to the macula densa or a Cx abnormality constitutes mechanisms to trigger initial RAS activation in early diabetes.

**Perspectives and Significance**

The present studies have for the first time demonstrated salt paradox in type 2 diabetes. Our results have indicated that renal ANG II is elevated in early diabetes. Furthermore, the present data suggest that in addition to malfunction of gap junctions in the JGA due to deranged Cxs, enhanced proximal reabsorption in ZDF-L rats contributes to altered renal autoregulation, presumably underlying glomerular hyperfiltration in diabetes. In addition, renal ANG II in diabetes is more sensitive to salt than the control. Our findings provide evidence that salt load...
restored decrements of end-proximal delivery in diabetes, and partially improved renal autoregulation through adenosine but was independent of Cxs. However, we should not load salt for patients with type 2 diabetes to normalize macula densa delivery, because of high prevalence of hypertension in this population. These observations support the idea that not only peptidomimetic molecules which prevent closure of gap junctions (32) but also the agents, including dipetidyl peptidase-4 or sodium-glucose transporter inhibitors, which reduce PTR, may improve renal autoregulation to exert beneficial actions on hyperfiltration and RAS activation in type 2 diabetes. Clearly, further studies are needed to elucidate these issues.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


