Acute and chronic effects of SGLT2 blockade on glomerular and tubular function in the early diabetic rat

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AN ONGOING DISTURBANCE in salt transport anywhere along the nephron will eventually be compensated by a combination of changes in glomerular filtration of salt, salt transport elsewhere along the nephron, and salt intake to fulfill the requirement for long-term balance. The kidney cannot obviate this requirement, but exerts some control over how it is achieved. Tubuloglomerular feedback (TGF) is one mechanism that the kidney can employ to influence how the response to an outside disturbance is compensated. TGF senses the amount of fluid and salt reaching the macula densa and evokes countercalibrating changes in single nephron glomerular filtration rate (SNGFR), thereby reducing the impact that an outside disturbance in proximal reabsorption would otherwise have on distal delivery. The usual way to measure the effectiveness of TGF is by in vivo microcirculation technique, which is most adapted to studying the TGF responses to events that occur on a time scale of several minutes (17). TGF responses over periods of 1–2 h have been partially characterized using carbonic anhydrase inhibitors to perturb proximal reabsorption (3). But little is known about the influence of TGF over the compensatory response to an outside disturbance lasting several days, which is the critical time frame over which the kidney regulates salt balance and blood pressure (5).

The present studies were performed to determine the extent to which TGF participates in the chronic response to a decrease in proximal reabsorption. The model chosen for these studies is selective blockade of the high-capacity sodium glucose cotransporter SGLT2 in rats with streptozotocin (STZ) diabetes. In moderate hyperglycemia, SGLT2 accounts for a large share of overall proximal reabsorption, such that blocking SGLT2 is expected to cause a major decline in proximal reabsorption (25). Experiments comparing the acute and chronic effects of SGLT2 blockade on various aspects of nephron function revealed that the long-term adaptation to a proximal diuretic incorporates, in roughly equal parts, a compensatory increase in reabsorption by Henle’s loop and a chronic TGF response.

MATERIALS AND METHODS

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with an institutional animal care and use committee-registered protocol.

Overview

We began by studying the effects of chronic SGLT2 blockade on proximal reabsorption, glomerular filtration rate (GFR), and the state of TGF activation in Wistar rats with early STZ diabetes. SGLT2 blockade proved to be an effective long-term suppressor of proximal reabsorption and activator of TGF, but its long-term effect on GFR was ambiguous. So, we performed a second series of experiments, this time to examine the acute TGF response to SGLT2 blockade so that acute and chronic responses could be compared. The two sets of experiments in diabetic Wistar rats were performed 9 mo apart, and each had its own group of controls. The combined result of these chronic and acute studies implied that the initial impact of SGLT2 blockade on GFR becomes less over time. To understand how the impact of SGLT2 blockade on GFR might dissipate in transition from acute to chronic blockade, we performed further experiments to compare the effects of acute versus chronic SGLT2 blockade on chloride concentration at the macula densa and to correlate distal chloride delivery with the tonic influence of TGF over SNGFR. These latter experiments were done in Wistar Froemter (MWF) rats, which are more amenable than Wistar rats to early distal microcirculation.

Diabetes

Adult male Wistar rats (Harlan, Indianapolis, IN) or MWF rats (Veterans Affairs San Diego Healthcare System breeding colony) were made diabetic with STZ (65 mg/kg ip × 1 dose; Sigma). Thereafter, blood glucose was measured daily in late morning by glucometer and injection of long-acting insulin (PZI; Blue Ridge
Pharmaceuticals, Memphis TN) was administered subcutaneously on a sliding scale. Rats were housed in pairs, given free access to tap water, and free fed standard rat chow (Teklad 7001) containing 0.4% Na, 1% K, 0.6% Cl, 25% protein, and 3 kcal/g energy density. Food and water consumption were recorded daily per cage and half of the total consumption ascribed to each animal for the days that two animals were housed together. Animals housed together received the same treatment.

**Surgical Preparation and Micropuncture**

Micropuncture experiments were performed after 10–14 days of diabetes. Rats that were housed together were studied on consecutive days. Animals were surgically prepared for micropuncture according to previously established protocols (19). Briefly, animals were anesthetized with Inactin (100 mg/kg ip; Research Biochemicals, Natick MA), and body temperature was maintained on a servocontrolled heating table. The airway was maintained with tracheostomy. Catheters were placed in the jugular vein, femoral artery, and urinary bladder. The left kidney was exposed through a flank incision, immobilized in a Lucite cup, and bathed with warm Ringer saline. The left ureter was cannulated for separate urine collection. Ringer saline containing $[^3]H$]inulin (60 μCi/ml) was infused at 3.5 ml/h for maintenance fluid and as a marker of GFR. Blood pressure was monitored throughout by intra-arterial catheter. At the beginning and end of each micropuncture period, blood samples were obtained to measure glucose concentration, hematocrit, and radioactivity. One hour was allowed for equilibration between the end of surgery and the start of micropuncture. During micropuncture, tubular fluid was collected from late proximal or early distal tubules and assayed for volume, $[^3]H$]inulin content, and/or chloride concentration. After micropuncture, kidneys were harvested and weighed. Urine collected during micropuncture was used to measure urine flow rate, whole kidney GFR, and glucose and electrolyte excretion.

**Analytical Methods**

Tubular fluid chloride concentration was determined by the electrometric titration method of Ramsey et al. (10) as modified by Windhammer and Giebisch (28) and as previously applied by us (9). Urinary Na and K were assayed by flame photometry (Cole Parmer). Volumes of tubular fluid collections were determined by transfer to a microbore capillary of known diameter. Urinary and tubular fluid inulin clearances were determined by scintillation counting of plasma, urine, and tubular fluid samples (Packard).

**Chronic Effects of SGLT2 Blockade on Nephron Function in Wistar Rats**

Male Wistar rats ($n = 15$) were given SGLT2 blocker or placebo beginning from day 0 of STZ. Chronic SGLT2 blockade was achieved by administering dapagliflozin (Bristol Myers Squibb) (7) twice daily by oral gavage (1 mg per kg at 7 AM and 2 mg per kg at 4 PM). The final dose was given ~16 h before micropuncture. Insulin was targeted to blood glucose 20 mM by daily adjustment. SNGFR was determined from collections of distal tubular fluid to establish the value of SNGFR at the TGF operating point. SNGFR and proximal reabsorption (Jprox) were measured at both extremes of TGF activation by collecting from the late proximal tubule while manipulating the TGF signal by orthograde perfusion of Henle’s loop. These microperfuosions were done with a Hample nanoliter pump (Univ. of Tuebingen) filled with artificial tubular fluid and positioned just downstream of a wax block in the last proximal segment. Collections were done upstream of the wax block. Paired collections were done in each nephron at 0 and 50 nl/min perfusion rates to establish values for SNGFR and Jprox at the shoulder and elbow if the TGF curve. The order of collection was alternated between nephrons.

**Acute Effects of SGLT2 Blockade on Nephron Function in Wistar Rats**

Male Wistar rats ($n = 9$) were subjected to micropuncture on days 10–12 of insulin-treated STZ diabetes. Daily insulin (0.5 units) was given throughout the course of diabetes if blood glucose exceeded 25 mM in midmorning. Each micropuncture study was divided into two experimental periods. Control data were obtained during the first micropuncture period after which dapagliflozin (1 ml/kg iv) was given, followed by a second micropuncture period. Thirty minutes was allowed to elapse between administration of the SGLT2 blocker and the start of the second period and the glucose content of the maintenance Ringer saline was increased from 5% to 20% (wt/vol) to prevent a rapid decline in blood glucose that otherwise follows acute SGLT2 blockade. Distal and proximal micropuncture collections and loop of Henle perfusions were done as described above for experiments with chronic SGLT2 blockade.

**Acute and Chronic Effects of SGLT2 Blockade on Distal Chloride Delivery in MWF Rats**

The point of these experiments was to compare the effects of acute and chronic SGLT2 blockade on the TGF signal. MWF rats were used because they have surface glomeruli with early distal tubules that are identifiable near to the macula densa. Closeness to the macula densa does not affect the flow rate or inulin clearance, but it does affect the tubular fluid chloride concentration, which declines along the distal convoluted tubule. The acute effect of SGLT2 blockade was determined in two-period micropuncture studies on days 10–12 of STZ diabetes. Insulin (0.5 units) was administered each day from the onset of diabetes if midmorning blood glucose exceeded 25 mM. Data from the first micropuncture period served as control for both acute and chronic SGLT2 blockade in MWF rats. Acute and chronic SGLT2 blockade were achieved as described above in the preceding paragraphs ($n = 5$ animals in each group). Fluid was obtained from early distal tubules of nephrons with superficial glomeruli and from the late proximal tubules of those same nephrons. SNGFR was determined from inulin clearance. Tubular flow rates were determined volumetrically. The state of TGF activation was determined from the difference between proximal and early distal SNGFR. Glycemic effects of acute and chronic SGLT2 blockade were confirmed to be similar.

**Statistical Analysis**

Results are expressed as means ± SE. Statistical testing was by ANOVA or ANCOVA with design for repeated measures and by nesting of individual tubular fluid collections within experiments using proprietary software (Systat, Evanston, IL). To discriminate direct effects of treatments on Jprox from changes in Jprox due to glomerulotubular balance (GTB), we applied ANCOVA with the state of SGLT2 blockade or experimental series as independent categorical variables and SNGFR as covariate. ANCOVA estimates the impact of SNGFR on Jprox by linear regression and computes a variance for Jprox as average distance from the regression line. Then it assigns means ± SE to the variate (Jprox*), which represents Jprox at the mean value for SNGFR from the pooled data. Doing ANOVA on Jprox* is the equivalent of doing ANCOVA on Jprox. This approach was previously described (13). Correction for multiple group comparisons was by Tukey’s honestly significant difference test.

**RESULTS**

**General Data**

Data describing somatic growth, glycemic control, and food intake during the 7 days prior to micropuncture are shown in Table 1. Diabetic rats generally lose weight for 2–3 days after
receiving STZ before they stabilize and resume growing. Daily food intake increased throughout the week prior to micropuncture ($P < 0.001$) regardless of rat strain or SGLT2 blockade. Effects of rat strain and SGLT2 blockade on food intake were, therefore, examined by two-way ANCOVA with time as a covariate. Wistar rats ate 28% more food than MWF rats ($P < 0.001$) and grew faster than MWF rats ($P < 0.001$). This is consistent with our past experience with these strains of rat (S. C. Thomson, personal observation). Rats receiving the SGLT2 blocker ate 12% more food than those receiving placebo ($P = 0.001$). The impact of SGLT2 blockade on food intake was not different between rat strains ($P = 0.7$). We suppose, without proof, that the increase in food intake is driven by energy concerns tied to glucosuria rather than to salt craving.

By design, blood glucose over the 7 days prior to micropuncture was not different between Wistar rats subjected to chronic SGLT2 blockade and their respective placebo controls. Those rats were targeted for 20 mM blood glucose and wound up receiving more insulin than later groups in which insulin was only given for blood glucose $> 25$ mM ($P < 0.01$). Loss of calories into the urine of SGLT2-blocked rats was compensated by increased food intake, which eliminated potential confounding effects of blood glucose or insulin dose within each experimental series.

**Urinary Excretion and Clearance Data During Terminal Micropuncture**

Data describing whole kidney GFR, urine flow rates, glucose transport, and electrolyte excretion are shown in Tables 2 and 3 and Fig. 1. Average body weight was not different between groups at the time of micropuncture. The study was not powered to study effects of SGLT2 blockade on blood pressure, and minor differences in blood pressure were not statistically significant. By design, blood glucose was unaffected by chronic SGLT2 blockade during the days prior to micropuncture. Blood glucose remained matched between chronically SGLT2 blocked and respective controls during micropuncture. Given the fluxes and volumes of distribution, it was anticipated that blood glucose would decline rapidly in response to acute SGLT2 blockade. We attempted to mitigate this by adding 20% dextrose to the maintenance infusion in the second period of the two-period studies. Nonetheless, blood glucose declined by $\sim 25\%$ during the second period of micropuncture in studies where dapagliflozin was given acutely ($P < 0.001$).

Within each level of SGLT2 blockade (acute, chronic, or none) urine flow rate was strongly influenced by blood glucose ($P < 0.0005$). Urine flow rate was not independently affected by the strain of rat, nor was it different between the two sets of control Wistar rats. By ANCOVA with Tukey test for post hoc comparisons, urine flow rate for each level of SGLT2 blockade was different from the other two levels (acute $> \text{chronic} > \text{none}; P < 0.001$) See Fig. 1.

All animals were glucosuric, making net glucose reabsorption a fair estimate of the transport maximum (Tm). Among the groups not receiving dapagliflozin, Tm was greatest for the second series of Wistar controls, which were exposed to slightly higher blood glucose in the days leading up to micropuncture. Glucose reabsorption was potently inhibited by acute ($P < 0.0005$) or chronic ($P < 0.0005$) SGLT2 blockade. Tm tended to be lower with acute vs. chronic blockade and in MWF versus Wistar rats, but these effects were not statistically significant (see Table 2).

Urinary electrolyte data were obtained from Wistar rats before and during acute SGLT2 blockade and from MWF rats at all three levels of SGLT2 blockade (acute, chronic, none). Acute SGLT2 blockade increased sodium and chloride excretion threefold independent of rat strain ($P < 0.001$), whereas,

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### Table 1. Data obtained during 7 days leading up to micropuncture

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Water Intake, ml/day</th>
<th>Food Intake, g/day</th>
<th>Blood Glucose, mM</th>
<th>Weight Gain, g/day</th>
<th>Insulin, U/day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar (8)</td>
<td>Placebo chronic</td>
<td>169 ± 8</td>
<td>40 ± 1</td>
<td>19.3 ± 1</td>
<td>3.5 ± 1.7</td>
<td>0.52 ± 0.11</td>
</tr>
<tr>
<td>Wistar (7)</td>
<td>Dapa chronic</td>
<td>205 ± 7*</td>
<td>46 ± 1*</td>
<td>19.8 ± 1</td>
<td>3.9 ± 1.0</td>
<td>0.48 ± 0.12</td>
</tr>
<tr>
<td>Wistar (9)</td>
<td>Control for acute Dapa</td>
<td>157 ± 6</td>
<td>43 ± 1</td>
<td>26.8 ± 0.84*</td>
<td>1.8 ± 0.8</td>
<td>0.18 ± 0.035*</td>
</tr>
<tr>
<td>MWF (5)</td>
<td>Placebo</td>
<td>122 ± 9</td>
<td>32 ± 2*</td>
<td>24.0 ± 1.5*</td>
<td>2.0 ± 0.8</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>MWF (5)</td>
<td>Dapa chronic</td>
<td>173 ± 8†</td>
<td>36 ± 1</td>
<td>23.4 ± 1.7</td>
<td>1.8 ± 0.9</td>
<td>0.13 ± 0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are number per group. Time was a covariate for food intake, but not for other variables. $*P < 0.01$ vs. Placebo of same strain. †$P < 0.01$ vs. other Wistar groups. Average daily weight gain for individual animals obtained by linear regression of body weight vs. time. Chronic Wistar series targeted for tighter glucose control gained more weight ($P < 0.04$) and received more insulin ($P < 0.005$). *Some missing data in final 3 days from this group, which wound up with higher glucose than corresponding MWF-Dapa group on the day of micropuncture. MWF, Wistar Froment rats; Dapa, dapagliflozin.

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### Table 2. Whole kidney data obtained during micropuncture

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>Body Weight, g</th>
<th>Blood Pressure, mmHg</th>
<th>Blood Glucose, mM</th>
<th>GFR, ml/min</th>
<th>Urine Flow Rate, μl/min</th>
<th>Filtered Glucose, μmol/min</th>
<th>Reabsorbed Glucose, μmol/min</th>
<th>Urine Glucose, μmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar, (8)</td>
<td>Placebo chronic</td>
<td>268 ± 13</td>
<td>115 ± 4</td>
<td>18 ± 1</td>
<td>2.8 ± 0.2</td>
<td>19 ± 3</td>
<td>50 ± 3</td>
<td>38.8 ± 6.3</td>
<td>8.9 ± 3.8</td>
</tr>
<tr>
<td>Wistar, (7)</td>
<td>Dapa chronic</td>
<td>269 ± 10</td>
<td>110 ± 2</td>
<td>16 ± 1</td>
<td>3.9 ± 0.1</td>
<td>37 ± 5</td>
<td>47 ± 4</td>
<td>14.5 ± 5.4†</td>
<td>33.0 ± 5.6†</td>
</tr>
<tr>
<td>Wistar, (9)</td>
<td>Control for acute Dapa</td>
<td>298 ± 11</td>
<td>116 ± 6</td>
<td>27 ± 2</td>
<td>3.5 ± 0.2</td>
<td>57 ± 7</td>
<td>94 ± 7</td>
<td>58.8 ± 4.9</td>
<td>34.8 ± 4.5</td>
</tr>
<tr>
<td>Wistar, (9)</td>
<td>Dapa acute</td>
<td>298 ± 11</td>
<td>112 ± 5</td>
<td>20 ± 2†</td>
<td>2.9 ± 0.2†</td>
<td>106 ± 11</td>
<td>59 ± 7</td>
<td>6.3 ± 5.3†</td>
<td>52.6 ± 5.5†</td>
</tr>
<tr>
<td>MWF, (5)</td>
<td>Control</td>
<td>304 ± 7</td>
<td>120 ± 4</td>
<td>23 ± 3</td>
<td>2.7 ± 0.2</td>
<td>29 ± 8</td>
<td>62 ± 9</td>
<td>49.2 ± 4.7†</td>
<td>23.1 ± 5.9</td>
</tr>
<tr>
<td>MWF, (5)</td>
<td>Dapa Acute</td>
<td>304 ± 7</td>
<td>121 ± 3</td>
<td>18 ± 2†</td>
<td>2.0 ± 0.1†</td>
<td>105 ± 11</td>
<td>38 ± 5</td>
<td>-1.6 ± 1.1†</td>
<td>53.8 ± 4.5†</td>
</tr>
<tr>
<td>MWF, (5)</td>
<td>Dapa chronic</td>
<td>261 ± 8</td>
<td>119 ± 8</td>
<td>24 ± 3</td>
<td>2.0 ± 0.2†</td>
<td>73 ± 2</td>
<td>46 ± 5</td>
<td>2.1 ± 5.9†</td>
<td>38.5 ± 3.6†</td>
</tr>
</tbody>
</table>

Values are means ± SE. Numbers in parentheses are number per group. GFR, glomerular filtration rate. $*P < 0.05$ vs. respective control.
Table 3. Additional whole kidney data obtained during micropuncture

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>FR Glucose, %</th>
<th>Urine Na, μmol/min</th>
<th>Urine K, μmol/min</th>
<th>Urine Cl, μmol/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wistar</td>
<td>Placebo chronic</td>
<td>81 ± 7</td>
<td>1.0 ± 0.1</td>
<td>3.0 ± 0.3</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Wistar</td>
<td>Dapa chronic</td>
<td>30 ± 10</td>
<td>2.7 ± 0.5†</td>
<td>3.0 ± 0.4</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Wistar</td>
<td>Control for acute Dapa</td>
<td>64 ± 4</td>
<td>1.0 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>Wistar</td>
<td>Dapa acute</td>
<td>8 ± 7</td>
<td>3.8 ± 0.6†</td>
<td>3.9 ± 0.6†</td>
<td>7.8 ± 0.1†</td>
</tr>
<tr>
<td>MWF (5)</td>
<td>Control</td>
<td>69 ± 4</td>
<td>1.2 ± 0.4</td>
<td>2.4 ± 0.4</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>MWF (5)</td>
<td>Dapa acute</td>
<td>−3 ± 2</td>
<td>1.4 ± 0.4</td>
<td>1.6 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>MWF (5)</td>
<td>Dapa chronic</td>
<td>1 ± 14</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE. †P < 0.05 vs. respective control. FR, fractional reabsorption.

chronic SGLT2 blockade did not significantly affect sodium or chloride excretion. Unlike urine flow rate, neither sodium or chloride excretion was independently affected by blood glucose. Acute SGLT2 blockade increased K excretion by 50% (P < 0.0005), while reducing the urinary molar ratio of K/Na (P < 0.02). Chronic SGLT2 blockade did not measurably affect K excretion or the urinary ratio of K/Na (Table 3).

GFR was lower in MWF than Wistar rats, overall (P < 0.0005). Acute SGLT2 blockade reduced GFR by ~20% in both rat strains (P = 0.004 by repeated-measures ANOVA). The effect on GFR of chronic SGLT2 blockade was less obvious. Chronic SGLT2 blockade did reduce GFR by ~15% (P < 0.04 by two-way ANOVA on the combined data where chronic SGLT2 blockade and rat strain were treated as categorical variables). In MWF rats, chronic blockade appeared to reduce GFR to the same extent as acute blockade, but the comparison of chronic blockade to placebo lacked the statistical power of paired comparisons and did not achieve statistical significance (P = 0.09). In the first series of Wistar rats, the placebo group did not appear to hyperfilter, and there was no apparent effect of chronic SGLT2 blockade on GFR. The second series of Wistar controls, which were exposed to slightly higher blood glucose, had higher GFR than the first series (P = 0.005). Blood glucose was not a significant covariate for GFR.

**Micropuncture Results**

**Glomerular filtration.** SNGFR at the TGF operating point (SNGFRd) was measured in 137 early distal micropuncture collections. These included experiments during acute and chronic SGLT2 blockade in Wistar and MWF rats. By two-way ANOVA on the pooled data from all experiments, the level of SGLT2 blockade (chronic, n = 49; acute, n = 30; or none, n = 58) was an independent predictor of SNGFRd (P < 0.0005), whereas rat strain (Wistar n = 74, MWF n = 63) was not (P = 0.95). Post hoc comparisons between the three levels of SGLT2 blockade were done by Tukey test on the pooled data from all groups. Compared with controls, acute SGLT2 blockade reduced SNGFRd by 33% (P < 0.0005), and chronic blockade SGLT2 blockade reduced SNGFRd by 16% (P < 0.03). The difference between acute and chronic blockade was also significant (P < 0.05). See Fig. 2. The effect of chronic blockade remained significant when the analysis was limited to contemporaneous controls (P < 0.01), in which most of the chronic effect was owed to the contribution from MWF rats.

**Proximal reabsorption.** Data on proximal reabsorption were collected from Wistar rats at both extremes of TGF activation and from MWF rats without TGF activation. SNGFR is always the strongest determinant of proximal reabsorption (Jprox). To account for this, primary effects of treatments on Jprox were analyzed by ANCOVA with SNGFR as the covariate. Proximal reabsorption cannot be measured without interrupting TGF, which makes the operating point indeterminant. TGF was manipulated for late proximal collections in Wistar rats such that the operating point was guaranteed to lie within the domain of SNGFR used in the ANCOVA. Late proximal collections in MWF were all done at the TGF shoulder, which is a less rigorous approach, but more common in the micropuncture literature. Since the approaches were different for the two strains of rats, the data were analyzed separately.
We first describe proximal reabsorption in the two series of Wistar rats (See Fig. 3): The strong influence of SNGFR over Jprox was confirmed for all groups of Wistar rats ($P < 0.0005$). The effects of chronic and acute SGLT2 blockade and primary differences in proximal reabsorption between the two series of experiments were tested by including SGLT2 blockade and experimental series (chronic vs. acute) as categorical variables and performing a $2 \times 2$ analysis on 221 late proximal tubular fluid collections obtained with and without TGF activation. Henceforth, we refer to the values of Jprox adjusted for SNGFR by the least-squares ANCOVA as Jprox*. Direct effects of treatments on proximal reabsorption appear as differences in Jprox*. Jprox* was 20% higher in the second series of experiments than in the first ($P = 0.002$). Exposure to SGLT2 blockade reduced Jprox* by 24% ($P < 0.0005$). The effects of acute and chronic SGLT2 blockade on Jprox* were indistinguishable ($P > 0.6$ for cross-term in the $2 \times 2$ ANCOVA (blockade $\times$ experimental series)). The outcome was the same when analysis was restricted to samples with SNGFR overlap between the four groups to comply with assumptions of ANCOVA that the covariate be equally distributed. Henceforth, we refer to the values of Jprox adjusted for SNGFR by least-squares ANCOVA as Jprox*.

SGLT2 blockade but not in controls. Variations in CED and SNGFRd arising from the TGF system and other random sources. Variations in SNGFRd that arise from changes in Jprox* will introduce a negative correlation between SNGFRd and CED due to TGF. Conversely, variations in CED that arise from changes in SNGFRd will introduce a positive correlation between SNGFRd and CED due to TGF. In control nephrons, SNGFRd and CED were uncorrelated ($r = 0.02$). In acute blockade, there emerged a strong negative correlation between SNGFRd and CED ($r = -0.50$), indicating variations in the effect of SGLT2 blockade on Jprox accounted for much of the variability in SNGFR. In chronic blockade, CED remained variable, and there was a weak negative correlation ($r = -0.22$). So, TGF responses arising from variations in CED assume a major role in determining SNGFRd during acute SGLT2 blockade and a minor role during chronic blockade.

**TGF responsiveness.** Range of the TGF response was determined in Wistar rats by measuring SNGFR at both extremes of TGF activation. Analysis included 192 tubular fluid collections from 96 nephrons to examine effects of chronic and acute SGLT2 blockade but not in controls. Variations in CED and SNGFRd arising from the TGF system and other random sources. Variations in SNGFRd that arise from changes in Jprox* will introduce a negative correlation between SNGFRd and CED due to TGF. Conversely, variations in CED that arise from changes in SNGFRd will introduce a positive correlation between SNGFRd and CED due to TGF. In control nephrons, SNGFRd and CED were uncorrelated ($r = 0.02$). In acute blockade, there emerged a strong negative correlation between SNGFRd and CED ($r = -0.50$), indicating variations in the effect of SGLT2 blockade on Jprox accounted for much of the variability in SNGFR. In chronic blockade, CED remained variable, and there was a weak negative correlation ($r = -0.22$). So, TGF responses arising from variations in CED assume a major role in determining SNGFRd during acute SGLT2 blockade and a minor role during chronic blockade.

**Distal chloride delivery.** Early distal chloride concentration (CED) was measured in 63 tubular fluid samples from MWF rats. SGLT2 blockade increased CED ($P < 0.04$). This effect was more pronounced with acute (70%) than chronic (35%) blockade (see Fig. 5). Individual values of CED are plotted against SNGFRd in Fig. 6, where it is shown that variations in CED correlated inversely with variations in SNGFRd during SGLT2 blockade but not in controls. Variations in CED and SNGFRd arising from the TGF system and other random sources. Variations in SNGFRd that arise from changes in Jprox* will introduce a negative correlation between SNGFRd and CED due to TGF. Conversely, variations in CED that arise from changes in SNGFRd will introduce a positive correlation between SNGFRd and CED due to TGF. In control nephrons, SNGFRd and CED were uncorrelated ($r = 0.02$). In acute blockade, there emerged a strong negative correlation between SNGFRd and CED ($r = -0.50$), indicating variations in the effect of SGLT2 blockade on Jprox accounted for much of the variability in SNGFR. In chronic blockade, CED remained variable, and there was a weak negative correlation ($r = -0.22$). So, TGF responses arising from variations in CED assume a major role in determining SNGFRd during acute SGLT2 blockade and a minor role during chronic blockade.

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SGLT2 blockade. Each treatment had its own control group, as described above. Results for individual nephron at each extreme of TGF activation are depicted in Fig. 7. TGF responsiveness was unaffected by chronic or acute SGLT2 blockade ($P > 0.6$), although TGF was more reactive in the initial series of experiments, which were dedicated to the effects of chronic SGLT2 blockade ($P < 0.003$). TGF responses in both series were within the range of prior observations from this lab.

**TGF activation state.** The TGF operating point normally resides near the midpoint between the shoulder and elbow of the TGF curve (17). All else remaining equal, a primary increase in Jpro will deliver less NaCl to the macula densa and reduce the level of TGF activation. Conversely, a primary decrease in Jpro will deliver more NaCl to the macula densa and increase the level of TGF activation. As the state of TGF activation increases, the operating point moves farther from the shoulder and closer to the elbow of the TGF curve. For the experiments done in Wistar rats, SNGFR was measured at the shoulder, elbow, and operating point of the TGF curve, and the tonic state of TGF activation was indexed as the difference between SNGFRd and the TGF midpoint. Both chronic ($P = 0.03$) and acute ($P = 0.03$) SGLT2 blockade increased the ambient degree of TGF activation. Acute blockade fully saturated the TGF response whereas chronic blockade did not (see Fig. 8). The experiments in MWF rats did not include determinations of the TGF elbow and in these we used the difference between proximal and distal SNGFR (P-D difference) to assess the state of TGF activation. P-D difference was small in control nephrons, large after acute SGLT2 blockade, and intermediate in chronic blockade (see Fig. 9).

**DISCUSSION**

There is evidence beyond doubt that TGF engages in short-term control of nephron function. This evidence comes in several forms, including over 100 published micropuncture studies (30 from our group alone) showing SNGFR and/or tubular stop flow pressure responses within 0.5 to 2.0 min of imposing a change in flow through Henle’s loop. The main novelty of the present study is in showing that TGF can exert control over GFR for extended periods of time to wit, these data point to the existence of chronic TGF.

Thurau and Boylan (22) proposed TGF as a mechanism to prevent excessive salt wasting should there arise a prolonged impairment of proximal reabsorption and termed this acute renal success. The idea of sustained TGF action over GFR continues to receive mention from time to time, usually as an hypothesis (1, 4, 11). However, the relevance of TGF to long-term kidney function was brought into question by the absence of an obvious GFR phenotype in the TGF-less adenosine A$_1$-receptor knockout mouse (2, 16, 26). Furthermore, TGF appears adapted for short-term autoregulation by maintaining its operating point within the narrow range of inputs where the TGF curve is steep (17). A sustained decline in proximal reabsorption that saturates the TGF response will render TGF impotent to perform dynamic autoregulation unless the TGF somehow adapts to reposition the operating point to the steep portion of the TGF curve. In prior studies, we observed such adaptation within 30–60 min of a saturating stimulus, which restored the ability of TGF to stabilize late proximal flow (18, 20). The methodology of those experiments did not allow for measurement of SNGFR and could provide no information about adaptations that may occur beyond 1 h. We subsequently showed that the TGF adaptation during 1 h of carbonic anhydrase inhibition need not include a normalization of GFR (3). This opened the door to the possibility of chronic TGF, although the role of TGF in the renal hemodynamics of carbonic anhydrase inhibition has since been called into question (6) and chronic carbonic anhydrase inhibition need not include a normalization of GFR (3). This opened the door to the possibility of chronic TGF, although the role of TGF in the renal hemodynamics of carbonic anhydrase inhibition has since been called into question (6) and chronic carbonic anhydrase inhibition has not been employed to test for chronic TGF. We have now addressed the possibility of long-lasting TGF and come to discover that TGF is, indeed, capable of exerting a chronic influence over nephron function.

When proximal reabsorption is chronically suppressed by SGLT2 blockade, the nephron essentially splits the difference...
between baseline and acute SGLT2 blockade with respect to the TGF signal and the tonic influence of TGF over the TGF operating point (SNGFRd). This point is made in Fig. 10 in which three separate GTB relations and a single TGF curve are superimposed on operating points for the three levels of SGLT2 blockade in Wistar rats. The single TGF curve in Fig. 10 happens to pass through all three operating points, even though its slope was independently derived from the open-loop gain in an acute perturbation analysis of TGF in diabetic Wistar rats (23). Thus a single TGF curve is sufficient to account for the acute and chronic responses to SGLT2 blockade. Even if TGF curves are drawn with arbitrary slopes, it is not possible to draw separate curves through the acute and chronic operating points where the chronic curve is right shifted relative to the acute curve. This implies that the transition from acute to chronic SGLT2 blockade occurred with no desensitization of the TGF response; to wit, the TGF behavior is stationary. Meanwhile, SNGFRd is higher and CED is lower in chronic than acute SGLT2 blockade, which can only be explained by increased reabsorption somewhere between Bowman’s space and the macula densa during the transition from acute to chronic blockade. This is shown graphically as shifting GTB relations in Fig. 10. Since the transition from acute to chronic blockade included no increase in Jprox*, the increased reabsorption must have occurred in Henle’s loop. The method for parameterizing the curves in Fig. 10 is described in the APPENDIX.

The present test for the existence of chronic TGF was made possible by the advent of a safe and convenient tool to impose a long-term decrease in proximal reabsorption, namely administration of dapagliflozin to diabetic rats. Fortunately, the acute and chronic effects of dapagliflozin on proximal reabsorption proved to be indistinguishable, which allowed for a simple comparison between responses to perturbations of the same magnitude but different duration (Figs. 3–4). The apparent lack of compensation for SGLT2 blockade by other glucose transporters in the early proximal tubule is consistent with our recent observation that glucose transport is absent altogether in the S1 segment of the SGLT2 knockout mouse (24).

The acute response to dapagliflozin was analogous to our prior experience with the nonspecific SGLT inhibitor, phlorizin, which, when delivered directly into Bowman’s space of a diabetic nephron, abruptly increased distal delivery of fluid and electrolytes, activated TGF, and lowered SNGFR by the same amount as presently observed with acute SGLT2 blockade (25). Phlorizin blocks both renal and intestinal isoforms of SGLT and is unsuitable for chronic or oral administration due to unpleasant consequences of blocking the latter (27). Dapagliflozin is specific for SGLT2, which is uniquely active in proximal tubule (7). Of historical note, phlorizin was shown, by Homer Smith in 1935, to completely inhibit renal glucose reabsorption in man. Shannon and Smith (12) also noted that acute phlorizinization could lower inulin clearance and cited this as a nuisance, since it complicated the interpretation of glucose transport experiments. In our hands, the acute effect of phlorizin on transport and TGF activity in nondiabetic rats was proportionately smaller and more difficult to detect than in diabetes, given the lesser amount of glucose available for reabsorption (25). Hence, there was no point in attempting the current experiments with dapagliflozin outside of diabetes.

Fig. 8. Left: SNGFR at shoulder, operating point and elbow of the TGF curve in Wistar rats during chronic or acute SGLT2 blockade. Each series had its own control group. Data include 3 collections in each nephron, n = 35 nephrons for chronic series, n = 21 for acute series. SNGFRd superimposed on TGF curves for illustrative purposes, with no scaling of the abscissa. Right: difference between proximal and distal SNGFR (P-D difference) normalized to the range of the TGF response. Both chronic and acute SGLT2 blockade increased the relative degree of TGF activation. Acute blockade saturated the TGF response, whereas chronic blockade did not.*P < 0.05 vs. respective control.
to other nephron segments. At the same time, chronic SGLT2 block will chronically reduce proximal reabsorption and hence, one might focus on reversing glomerular hyperfiltration by reversing the process that leads to hyperfiltration.

**APPENDIX**

We conclude that tubuloglomerular feedback (TGF) resetting is not required to explain the observed transition from acute to chronic SGLT2 blockade. This conclusion is based on the finding that the chronic operating point fits nicely along the acute TGF curve. This alignment depends on the slope of the acute TGF curve, which was determined as follows.

The fractional compensation (C) of an intact negative feedback loop for an outside disturbance is given by

\[ C = \frac{\text{OLG}}{1 + \text{OLG}} \]

where OLG is the negative open loop gain. For the TGF system, OLG = \( \alpha \beta \gamma \), where \( \gamma \) is the slope of the TGF curve, \( \alpha \) is the slope of the dependence of late proximal flow on single nephron glomerular filtration rate (SNGFR), and \( \beta \) is the slope of the dependence of early distal chloride concentration (CED) on late proximal flow. The product \( \alpha \beta \) reflects to the impact of glomerulotubular balance (GTB) on CED. The saturable TGF response is often represented with a hyperbolic tangent

\[ \text{PDD} = \frac{R}{2} \left\{ 1 + \tanh \left( \frac{2\gamma}{R} (\text{CED} - \text{CED}') \right) \right\} \]

where PDD is the proximal-distal difference in SNGFR, R is the range of the TGF response and (') applies to \( \gamma \) or CED at the TGF inflection point. A published closed-loop perturbation analysis in diabetic Wistar Froemter (MWF) rats, previously yielded peak C = 0.40, corresponding to OLG = 0.67 at the TGF inflection point (23). In perfusions of Henle’s loop, we found \( \beta = 1.1 \pm 0.1 \text{ meq}^{-1} \text{nl}^{-1} \text{min}^{-1} \) (23). Linear regression applied to current data yields \( \alpha = 0.63 \).
Solving for the TGF slope gives γ′ = 0.95 nl·min⁻¹·meq·l⁻¹. Once γ′ is assigned, \( R = 11.6 \text{ nl·min} \) and \( \text{CED}^* = 32 \text{ meq/l} \) are obtained by fit to the acute response to SGLT2 blockade. Slopes for the three GTB relations shown in Fig. 10 are \( (\alpha \beta)^{-1} \).

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AUTHOR CONTRIBUTIONS

S.C.T., J.W., and V.V. conception and design of research; S.C.T., T.R., C.M., H.M., and P.S. performed experiments; S.C.T. analyzed data; S.C.T. and P.S. edited and revised manuscript; S.C.T. and P.S. approved final version of manuscript.

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