Increased renal ENaC subunit and sodium transporter abundances in streptozotocin-induced type 1 diabetes

Song, Jian, Mark A. Knepper, Joseph G. Verbalis, and Carolyn A. Ecelbarger. Increased renal ENaC subunit and sodium transporter abundances in streptozotocin-induced type 1 diabetic rats. Am J Physiol Renal Physiol 285: F1125–F1137, 2003. First published August 5, 2003; 10.1152/ajprenal.00143.2003.—Uncontrolled diabetes mellitus (DM) is associated with copious water and sodium losses. We hypothesized that the kidney compensates for these losses by increasing the abundances of key sodium and water transporters and channels. Using targeted proteomic analysis via immunoblotting of kidney homogenates, we examined comprehensive regulation of transport proteins. In three studies, streptozotocin (STZ; 65 mg/kg) or vehicle was administered intraperitoneally to male Sprague-Dawley rats. In study 2, to control for potential renal toxicity of STZ, one group of STZ-treated rats was intensively treated with insulin to control diabetes. In another group, the reversibility of DM and related changes was assessed by treating animals with insulin for the final 4 days. In study 3, we correlated blood glucose to transporter changes by treating animals with different doses of insulin. In study 1, STZ treatment resulted in significantly increased band densities for the type 3 sodium/hydrogen exchanger (NHE3), the thiazide-sensitive Na-Cl cotransporter (NCC), and epithelial sodium channel (ENaC) subunits α, β, and γ (85- and 70-kDa bands) to 204, 125, 176, 132, 147, and 241% of vehicle mean, respectively. In study 2, aquaporin-2 (AQP2) and AQP3 were increased with DM, but not AQP1 or AQP4. Neither these changes, nor blood glucose itself, could be returned to normal by short-term intensive insulin treatment. Whole kidney abundance of AQP3, the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2), and γ-ENaC (85-kDa band) correlated most strongly with blood glucose in study 3. These comprehensive changes would be expected to decrease volume contraction accompanying large-solute and water losses associated with DM.

Kidney; epithelial sodium channel; natriuresis; diuresis; type 2 sodium-phosphate cotransporter; type 3 sodium/hydrogen exchanger; bumetanide-sensitive sodium-potassium-2chloride cotransporter; aquaporin; sodium-chloride cotransporter; hyperglycemia; proteomics

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estricted primarily to the medullary portion. AQP6–8 are also expressed in the kidney, but less is understood about their functional roles.

Streptozotocin (STZ) treatment is a widely accepted model for type 1 diabetes in which the β-cells of the pancreas are destroyed by the broad-spectrum antibiotic STZ given intravenously or intraperitoneally (29). Moreover, several groups have investigated certain aspects of the regulation of selected renal sodium, water, and urea transport proteins in the STZ-treated rat model. Several investigators (2, 17, 26) report an increase in AQP2 or phosphorylated AQP2 in kidneys harvested from STZ-treated rats. Nejsum et al. (26) also saw an increase in AQP3. Bardoux et al. (2) and Kim et al. (16) found increases in the vasopressin-regulated urea transporter of the collecting duct (UT-A1) with STZ-induced diabetes in rat kidney inner medulla. Kim et al. also reported an increase in NKCC2 abundance in the outer medulla from STZ-treated rats after 20 days, whereas Ward et al. (31) have reported an increase in the abundance of NCC in whole kidney 2 wk after STZ treatment. To our knowledge, no one has reported the effects of STZ-induced diabetes on the protein abundances of ENaC subunits.

Here, we describe our findings from three distinct studies. In the first study, rats were simply treated with STZ or vehicle and killed after 4 days. The second and third studies were designed to complement the first study and to clarify three issues regarding changes we observed: 1) whether STZ, independently of diabetes mellitus (DM), played a role (both studies); 2) whether we could reverse any of the changes with 4-day intensive insulin replacement therapy (IRT; study 2); and 3) whether these changes correlate with the degree of diabetic severity (both studies).

In all three studies, we purposely selected short time points, i.e., 4–14 days, because we wanted to assess time points that were long enough to allow changes in channel and transporter protein abundances to occur (through translation, for example) but too short to lead to large effects resulting from diabetic nephropathy, especially hypertrophy. We previously reported that diabetic hypertrophy of the kidney led to dramatic downregulation of many renal salt and water transport proteins in diabetic obese Zucker rats (4).

MATERIALS AND METHODS

Animals and Study Design

In study 1, 12 male Sprague-Dawley rats weighing 300 g were obtained from Taconic Farms (Gaithersburg, MD). After an overnight fast following a 5-day equilibration period, the rats were injected intraperitoneally with either vehicle (0.2 ml of 0.1 M citric acid) or STZ (65 mg/kg body wt, dissolved in 0.2 ml citric acid, n = 6 rats/group). The animals had free access to water and chow (LabDiet rodent chow 5001, Purina Mills, St. Louis, MO) for the course of the study. After 4 days, the rats were euthanized by decapitation, and kidneys and blood were collected.

In study 2, 24 male Sprague-Dawley rats (~310 g) were assigned to one of four treatment groups (n = 6 rats/group): 1) vehicle (citric acid); 2) STZ plus total IRT; 3) STZ plus partial IRT; and 4) STZ plus minimal IRT. In this study, insulin therapy (Humulin-R, a short-acting insulin preparation containing 100% recombinant human insulin, Eli Lilly, Indianapolis, IN) was provided subcutaneously by an osmotic minipump (model 2002, Alzet Osmotic Minipumps, Cupertino, CA) at a dose of 10 U·kg⁻¹·day⁻¹ for different time periods. A time line of the protocol is presented in Fig. 1A. To minimize the potential impact of direct renal STZ toxicity, as otherwise described (29), all STZ-treated rats were given IRT for the first 6 days to, in essence, delay the “start” of the experiment until the majority of STZ had been cleared from their kidneys. After this time, IRT was discontinued to the rats in groups 3 and 4 by removal of the minipump. However, to deduce whether the hyperglycemia and changes in transporter/channel abundances could be reversed, after 4 days use of the minipump was reinstated for group 3 to continue the same level of IRT for the final 4 days.

The protocol for study 3 is detailed in Fig. 1B. Twelve rats (rats A–L) were treated with vehicle (rats A–C) or STZ (rats D–L). Hyperglycemia was allowed to stabilize untreated for 2 days. After this time, all STZ rats were treated with IRT by subcutaneous injection once a day with Novolin 70/30 (Novo Nordisk), a mixture containing 70% human insulin suspension (slower release) and 30% regular insulin from recombinant DNA (fast acting). This initial therapy was continued for 4 additional days, again as in study 2, in the time frame needed to clear STZ from the kidneys. Thus 6 days after the initial STZ treatment, IRT therapies were individualized as follows: rats D–F were treated with insulin twice a day so that mean daily glucose levels stayed between 70 and 150 mg/dl. Rats G–I were treated so that glucose levels stayed between 250 and 350 mg/dl, and rats J–L received no IRT. This treatment was continued for 8 additional days.

For all studies, rats were anesthetized for surgery with isoflurane (IsoFlo, Abbott Laboratories, North Chicago, IL) and were fasted overnight before STZ administration. The animals were fed chow (LabDiet rodent chow 5001) and received water ad libitum. They were housed singly in Nalgene metabolic cages (Harvard Apparatus, Holliston, MA) to facilitate urine collection. In studies 2 and 3, the urine collected daily was analyzed for sodium (ion-selective electrode system, EL–ISE Electrolyte System, Beckman Instruments, Fullerton, CA) and creatinine (Jaffe rate method, Creatinine Analyzer 2, Beckman Diagnostic Systems Group). Glucose levels in blood obtained from the tails of the rats were monitored daily with a glucometer (TheraSense, Freestyle, Alameda, CA). All animals were maintained continuously under conditions and protocols approved by the Georgetown University Animal Care and Use Committee, which is sanctioned by the American Association for Accreditation of Laboratory Animal Care.

The rats were euthanized by decapitation, and trunk blood was collected into both heparinized and K₃-EDTA tubes (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). Both kidneys were rapidly removed and either frozen on dry ice for later processing or immediately homogenized.

Plasma Analyses

Whole blood was centrifuged at 3,000 rpm (Sorvall RT 6000 D, Sorvall, Newtown, CT) at 4°C for 20 min to separate plasma. Plasma aldosterone, vasopressin, and insulin levels were measured by radioimmunoassays, as previously described (4). Plasma was also analyzed for creatinine according to methods described above.

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Preparation of Samples for Immunoblotting

Whole left kidneys (all studies) and right kidney cortex, inner stripe of outer medulla, and inner medulla (study 2) were homogenized, and aliquots of whole homogenates were prepared for immunoblotting as previously described (8, 10). For immunoblotting, 5–30 μg of protein from each sample were loaded into individual lanes of minigels of 7, 10, or 12% polyacrylamide (precast, Bio-Rad, Hercules, CA). Our immunoblotting protocol and the production of polyclonal antibodies against NHE3, NaPi-2, NKCC2, NCC, α-, β-, and γ-ENaC, and AQP1–4 have been previously described (11–13, 18–21, 24, 27). The mouse monoclonal antibody to the α1-subunit of Na-K-ATPase was obtained from Upstate Biotechnology (Lake Placid, NY).

Immunoblotting

Initially, Coomassie-stained loading gels were prepared for all sample sets to assess the quality of the protein by the sharpness of the bands and to confirm the equality of loading, as previously described (8, 10). For immunoblotting, 5–30 μg of protein from each sample were loaded into individual lanes of protein gels containing 12% polyacrylamide. The electrophoresis was followed by Coomassie-stained loading gels to confirm the equality of loading. All sample sets were run under the same conditions, and the gels were run at 120 V for 2 h or until the bromophenol blue dye reached the bottom of the gel. The proteins were transferred to nitrocellulose membrane by electroblotting in Tris-glycine buffer for 2 h at 100 V. The membrane was blocked with 5% nonfat dry milk in 2× phosphate-buffered saline (PBS) containing 0.1% Tween-20 for 1 h. After blocking, the blot was incubated with primary antibodies against NHE3, NaPi-2, NKCC2, NCC, α-, β-, and γ-ENaC, and AQP1–4 for 1 h at room temperature. After washing, the blot was incubated with secondary antibodies for 1 h. The signal was visualized by chemiluminescence (ECL, Amersham BioSciences), and the membranes were exposed to x-ray film. The bands were quantified by densitometry using a computerized image analysis system (Bio-Rad, Hercules, CA). The data were analyzed by one-way ANOVA followed by Tukey’s multiple comparisons test (when significant differences were determined) was used. For other data, an unpaired t-test was used for each group. All data are expressed as means ± SEM. A probability level of P < 0.05 was considered statistically significant for all tests.

RESULTS

Study 1

Physiology. In the first study, rats were made diabetic with STZ and euthanized after 4 days, before body or kidney weights were affected. Table 1 is a summary of physiological data. Indeed, there were no significant differences between treatments in rat final body or kidney weights. Final plasma insulin levels were reduced by 62% in STZ-treated rats, whereas glucose levels were on average 205% higher.

Data were evaluated using SigmaStat (Chicago, IL). An unpaired t-test was used for study 1 data. For study 2, in which blood glucose measurements were examined over time, two-way (time × treatment) repeated-measures ANOVA followed by Tukey’s multiple comparisons test (when significant differences were determined) was used. For other data in study 2, and physiological data from study 3, one-way ANOVA followed by Tukey’s test was used. For graphic and tabular display of the results of Tukey’s test, letters and symbols, respectively, were assigned to indicate means that were significantly different from each other. That is, group means assigned letters or symbols in common would not be different from each other, but both groups would be significantly different from a group assigned another letter or symbol. For study 3, immunoblotting data were analyzed by linear regression analysis, with blood glucose as the independent variable and specific band density (% of mean vehicle level) as the dependent variable. P < 0.05 was considered statistically significant for all tests.
Table 1. Study 1: physiological data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body wt, g</td>
<td>303 ± 12</td>
<td>289 ± 6</td>
</tr>
<tr>
<td>Kidney wt, g/kg body wt</td>
<td>3.9 ± 0.1</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>2.1 ± 0.4</td>
<td>0.8 ± 0.2†</td>
</tr>
<tr>
<td>Blood glucose, mg/dl*</td>
<td>110 ± 10</td>
<td>335 ± 131†</td>
</tr>
<tr>
<td>Urinary volume, ml/day*</td>
<td>27 ± 1</td>
<td>88 ± 8†</td>
</tr>
<tr>
<td>Plasma creatinine, mM</td>
<td>43 ± 2</td>
<td>60 ± 3†</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/ml</td>
<td>41 ± 18</td>
<td>180 ± 41†</td>
</tr>
<tr>
<td>Fractional excretion of Na+, %‡</td>
<td>0.44 ± 0.07</td>
<td>1.26 ± 0.06†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats/group. *Mean of last day. †Statistically different from vehicle (P < 0.05). ‡Calculated as \( \frac{U_{\text{Na}^+} - P_{\text{renal}}}{U_{\text{crea}} - P_{\text{Na}^+}} \).

indicating efficacy of the model. Similarly, urinary volumes on the final day were increased on average by 226% with STZ treatment. Furthermore, plasma creatinine concentrations in the STZ-treated rats were significantly elevated, indicating some decrease in creatinine clearance or glomerular filtration rate in these rats. Plasma aldosterone levels were increased by 339%. Despite this, fractional excretion of sodium was significantly greater in STZ-treated rats.

Renal sodium transport proteins. In Fig. 2, we have plotted the mean densitometry values for immunoblots done on whole kidney homogenates in the STZ-treated rats as a percentage of those in vehicle-treated rats. STZ treatment after 4 days resulted in an increase in several sodium transport proteins and channel subunits, especially in distal sites along the renal tubule. However, we also saw an increase in NHE3, which is located in the proximal tubule and thick ascending limb.

Study 2

Blood glucose levels. In Fig. 3, we plot mean blood glucose levels for the rats in study 2 over the course of the experiment. In the first 6 days, all STZ-treated groups (groups 2–4) received IRT by minipump. During this time, although blood glucose levels tended to be higher on average in all STZ-treated rats, there were no significant differences among the groups. Between days 6 and 10, blood glucose levels rose in all STZ-treated groups but did so more quickly with the treatments in which the IRT pump had been removed (groups 3 and 4). Between days 10 and 14, there was no difference in glucose levels between groups 3 and 4, despite the fact that group 3 was receiving an insulin infusion. Furthermore, during days 10–14, group 2 mean glucose levels remained somewhat higher than those in vehicle-treated rats (statistically higher on days 10 and 13).

Physiology. In Table 2, we show physiological data for the rats in study 2. Final body weights were significantly lower in groups 3 and 4 rats relative to group 1 (vehicle-treated rats). Furthermore, kidney weights relative to body weights were increased in rats in groups 3 and 4 compared with vehicle-treated rats. Mean final urinary volume (ml/day) was significantly increased in group 4 rats relative to groups 1 and 2. Urinary volume in group 4 was 690% of that in group 1. Urinary osmolality, however, decreased significantly with increasingly severe diabetes, despite a 237% increase in the excretion of osmolytes in group 4 rats vs. those in group 1. The ratio of potassium to sodium in the urine was used as an index of aldosterone-like activity in the kidney, which would be independent of dietary intake. This ratio was found to be increased in groups 3 and 4 relative to groups 1 and 2, indicating relative retention of sodium to potassium or increased aldosterone-like activity in the kidney with diabetes.

Plasma creatinine levels were significantly elevated in group 4 rats, (relative to group 1), as observed in study 1. However, there were no significant differences among the groups in urinary creatinine excretion or in creatinine clearance. Fractional excretion of sodium was also not significantly different (P = 0.08) among groups, likely due to high variability in diabetic groups, although the mean was increased with diabetes. However, fractional excretion of sodium positively correlated with blood glucose levels when all groups were analyzed together (r² = 0.296, P = 0.009).

Plasma hormones. Hormones measured in the plasma of rats in study 2 are represented in Fig. 4. Final insulin levels were not significantly different in the first three groups (Fig. 4A). Group 4 rats, which were not receiving IRT at the end of the study, had lower plasma insulin levels, as expected. Aldosterone levels (Fig. 4B) were not significantly different in any of the groups. In fact, levels in group 4 rats tended to be the lowest. Aldosterone levels were relatively low, how-

![Figure 2. Study 1: effect of STZ-induced diabetes on the mean band densities for immunoblots of whole kidney homogenates probed with antibodies against major sodium transporter and channel subunits. Values are expressed as a percentage of vehicle-infused means.](http://ajprenal.physiology.org/DownloadedFrom/10.22033.6.on.June.14,2017)
followed by a multiple comparisons test (Tukey’s) to compare groups for a given day. This test was only applied when a significant difference ($P < 0.05$) was observed by ANOVA. As described in Statistics in MATERIALS AND METHODS, letters were assigned based on the outcome of the Tukey’s multiple comparisons test. Means with letters in common are not statistically different from each other. Repeated-measures ANOVA revealed a significant effect of treatment and time and the interaction between treatment and time. Glucose levels in groups 3 and 4 were not statistically different from each other throughout the course of the experiment, despite the administration of insulin to group 3 on days 10–14. Similarly, overall, glucose levels in group 2 rats were not statistically different from those in the vehicle-treated group, despite some individual time point differences, i.e., days 10 and 13.

Table 2. Study 2: physiological data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 (vehicle)</th>
<th>Group 2 [STZ+(IRT D1–14)]</th>
<th>Group 3 [STZ+(IRT D1–7, 10–14)]</th>
<th>Group 4 [STZ+(IRT D1–7)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body wt, g</td>
<td>357 ± 3*</td>
<td>324 ± 16†</td>
<td>310 ± 10†</td>
<td>309 ± 10†</td>
</tr>
<tr>
<td>Kidney wt, g/kg body wt$^{-1}$</td>
<td>3.3 ± 0.0†</td>
<td>4.1 ± 0.0†</td>
<td>4.6 ± 0.1*</td>
<td>4.5 ± 0.1*</td>
</tr>
<tr>
<td>Urinary volume, ml/day</td>
<td>20 ± 2†</td>
<td>57 ± 20†</td>
<td>115 ± 9†</td>
<td>138 ± 9*</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH$_2$O</td>
<td>1,620 ± 56*</td>
<td>1,144 ± 171†</td>
<td>883 ± 41†</td>
<td>649 ± 52†</td>
</tr>
<tr>
<td>Urinary osmolytes, mosmol–kg body wt$^{-1}$–day$^{-1}$</td>
<td>86 ± 8†</td>
<td>164 ± 60†</td>
<td>338 ± 42*</td>
<td>290 ± 13†</td>
</tr>
<tr>
<td>Urinary, KNa</td>
<td>0.80 ± 0.02†</td>
<td>0.99 ± 0.05†</td>
<td>1.62 ± 0.06*</td>
<td>1.71 ± 0.12*</td>
</tr>
<tr>
<td>Plasma creatinine, mM</td>
<td>52 ± 2†</td>
<td>55 ± 3†</td>
<td>58 ± 3†</td>
<td>100 ± 16*</td>
</tr>
<tr>
<td>Urinary creatinine, mmol–day$^{-1}$–kg body wt$^{-1}$</td>
<td>282 ± 12</td>
<td>257 ± 33</td>
<td>290 ± 19</td>
<td>317 ± 44</td>
</tr>
<tr>
<td>Creatinine clearance, l$^{-1}$–kg body wt$^{-1}$</td>
<td>5.5 ± 0.3</td>
<td>4.7 ± 0.6</td>
<td>5.0 ± 0.4</td>
<td>3.8 ± 1.0</td>
</tr>
<tr>
<td>Fractional excretion of Na$^+$, %</td>
<td>2.0 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>2.8 ± 0.2</td>
<td>3.8 ± 0.8</td>
</tr>
</tbody>
</table>

Values are means ± SE; urinary volume, osmolality, osmolytes, and KNa are averages of the last 2 days; $n = 6$ for groups 1–3 and $n = 5$ for group 4. STZ, streptozotocin; IRT, insulin replacement therapy; D, day. Symbols are assigned based on the outcome of Tukey’s multiple comparisons test, which was only applied after a statistically significant difference ($P<0.05$) was determined by 1-way ANOVA. Therefore, group means with any symbols in common are not different from each other. Thus * is different from † but not from ‡.|

Fig. 3. Study 2: changes in daily blood glucose concentrations in vehicle or STZ-treated rats coadministered insulin (IRT) for various time periods. Data were analyzed by 2-way repeated-measures ANOVA (time × treatment) followed by a multiple comparisons test (Tukey’s) to compare groups for a given day. This test was only applied when a significant difference ($P < 0.05$) was observed by ANOVA. As described in Statistics in MATERIALS AND METHODS, letters were assigned based on the outcome of the Tukey’s multiple comparisons test. Means with letters in common are not statistically different from each other. Repeated-measures ANOVA revealed a significant effect of treatment and time and the interaction between treatment and time. Glucose levels in groups 3 and 4 were not statistically different from each other throughout the course of the experiment, despite the administration of insulin to group 3 on days 10–14. Similarly, overall, glucose levels in group 2 rats were not statistically different from those in the vehicle-treated group, despite some individual time point differences, i.e., days 10 and 13.

Na-K-ATPase, NHE3, and NaPi-2. In Fig. 5, we show results for immunoblots of whole kidney homogenates probed with Na-K-ATPase $\alpha_1$-subunit, NHE3, or NaPi-2 antibodies. There were no significant differences in the abundances of any of these proteins among groups.

NCC and NKCC2. Findings with regard to NCC and NKCC2 are given in Fig. 6. For NKCC2, groups 2–4 were significantly increased relative to vehicle, and group 4 was significantly increased relative to groups 1–3. For NCC, there was significance detected by ANOVA, i.e., $P < 0.05$, indicating some increase with diabetes. However, Tukey’s multiple comparisons test revealed no significant differences between any two pairs of treatment means.

ENaC subunits. Diabetes increased the abundance of $\alpha$-ENaC (Fig. 7). $\alpha$-ENaC was significantly increased in groups 3 and 4 relative to group 1. $\beta$-ENaC was not significantly different among the groups. Both bands associated with $\gamma$-ENaC were modestly increased with diabetes. These bands are analyzed separately because they are independently regulated (8, 24). The 85-kDa band was increased in group 4 relative to group 1, and the 70-kDa band was significantly increased in groups 2 and 4 relative to groups 1 and 3, with group 3 being significantly lower than group 1.

AQP1–3. AQP1 in whole kidney homogenates was not significantly affected by treatment (Fig. 8). In con-

\[ STZ \]
contrast, AQP2 and AQP3 were significantly increased with diabetes (by ANOVA), but for AQP2 there were no significant differences between any two groups when Tukey’s multiple comparisons test was applied. AQP3 was significantly increased in group 3 relative to vehicle. AQP4 was not measured in whole kidney samples due to its being solely expressed in the inner medulla; therefore, whole kidney immunoblots do not develop well.

Regional analysis of kidney transporters. In Fig. 9, we show the summary of densitometry for immunoblots done on kidney regions, i.e., cortex, outer medulla, and inner medulla homogenates prepared from

Fig. 4. Final plasma hormone levels in study 2. Hormones were measured by radioimmunoassay. A: insulin levels were significantly increased in group 2 relative to group 4. B: aldosterone levels were not significantly different among groups. C: vasopressin levels were significantly increased in group 4 relative to all other treatments. Data were analyzed by 1-way ANOVA followed by Tukey’s multiple comparisons test when a significant difference ($P < 0.05$) was revealed by ANOVA. Letters were assigned based on the outcome of the Tukey’s multiple comparisons test. Means with letters in common are not statistically different from each other.

Fig. 5. Immunoblots of Na-K-ATPase, NHE3, and NaPi-2. A–C: sample immunoblots of whole kidney homogenates probed with anti-$\alpha_1$-Na-K-ATPase monoclonal (A), anti-NHE3 polyclonal (B), or anti-NaPi-2 polyclonal antibodies (C). Samples were loaded onto gels with a sample from 1 rat in each lane (n = 6 for vehicle-treated rats and groups 2 and 3; n = 5 for group 4). Equal amounts of total protein were loaded in all lanes. D: summary of immunoblot densitometry for these blots and repeated blots. Data are expressed as a percentage of vehicle mean and were analyzed by 1-way ANOVA. No significant differences were revealed.
the right kidneys of rats in study 2. The regional analysis was done not only to confirm what was seen in whole kidney samples but also to assess whether there might be differential regulation of any of these proteins that are expressed in different cell types or different regions of the kidney. For example, NHE3 in the cortex is primarily expressed in proximal tubule cells, but in the outer medullary samples it is primarily in thick ascending limb cells. Figure 9A illustrates the findings for cortical homogenates. Qualitatively, the findings agreed, in general, with those in the whole kidney homogenates. NKCC2, NCC, \(\alpha\)-ENaC, \(\gamma\)-ENaC (both bands), AQP2, and AQP3 increased in abundance with diabetes (as assessed by ANOVA). In contrast, in outer medullary homogenate samples (Fig. 9B), we saw a significant decrease with diabetes of the Na-K-ATPase \(\alpha_1\)-subunit, AQP1, and AQP3. NHE3 appeared to be increased in both cortex and outer medulla in all STZ-treated rats, although because of high variability, the increases were not significant. Finally, in the inner medullary homogenates (Fig. 9C), there was, similarly, a significant decrease in both the Na-K-ATPase \(\alpha_1\)-subunit and AQP1 with diabetes. For AQP2 and AQP3, there was a trend toward an increase, but it was not significant. For AQP4, there were no significant differences and a slight trend toward a decrease in all STZ-treated groups.

**Study 3**

**Physiological parameters.** Insulin therapy was individualized in study 3 to control for different responses...
Uncontrolled DM is associated with natriuresis and diuresis, to which the kidney must adapt. One way that adaptation occurs is through the regulation of protein abundances of the major sodium and water transporters and channels, which line the renal epithelium. These studies demonstrate that relatively short-term DM (4–14 days) in rats is associated with significant increases in the abundances of several major renal sodium transport proteins and channel subunits, including subunits of the ENaC.

**Study 1 Findings**

In *study 1*, we examined the renal abundances of the major sodium transport proteins in rats treated with either STZ or vehicle after 4 days. Here, we found a significant increase in the renal abundances of all three of the subunits of ENaC (including both bands of γ-ENaC), as well as for NCC and NHE3.

**STZ-Dependent Effects**

The above findings were fairly striking; however, we wanted to investigate these findings in greater depth, and thus *studies 2* and *3* were designed. In *study 2* (as well as *study 3*), to control for the potential renal toxicity of STZ we included a group of STZ-treated rats that were maintained with fairly normal blood glucose levels by insulin therapy. In both of these studies, with regard to all parameters measured, including urinary volume, osmolality, kidney size, and transporter and channel abundances, the STZ-treated rats were similar to the vehicle-treated rats. Therefore, we did not observe any clear STZ-dependent effects and thus could attribute these finding strictly to the development of insulin therapy to control blood glucose levels prevented the hypertrophy. Plasma aldosterone levels were measured in blood collected at death and were as follows: rats A–C, 313 ± 76; rats D–F, 276 ± 191; rats G–I, 562 ± 166; and rats J–L, 516 ± 207 pg/ml. There were no significant differences among the groups when analyzed by ANOVA.

**Correlation of transporters/channels abundance with blood glucose levels.** In Fig. 11, we have plotted the immunoblotting densitometric findings from *study 3*. Whole kidney homogenates were analyzed for each of 11 proteins, and the results are given in Fig. 11, A–L (*H* and *I* are shown for γ-ENaC, each representing a band shown to be independently regulated). Individual band densities for rats A–L, plotted as a percentage of the vehicle (rats A–C) means, are correlated with their average blood glucose level for the final 4 days. A significant positive correlation between blood glucose and band density was obtained for the Na-K-ATPase α1-subunit, NKCC2, NCC, β-ENaC, γ-ENaC (85-kDa band), and AQP3. The band densities for AQP2 and the 70-kDa band of γ-ENaC were weakly but not significantly correlated with glucose. In this study, α-ENaC abundance did not correlate to blood glucose.

**DISCUSSION**
DM. This was with the possible exception of NHE3, which was increased in study 1 but was not significantly different among all three STZ-treated groups in study 2. This early increase in NHE3 might have a role in the elimination of STZ from the kidney. 

Reversibility of DM and Related Findings

To our knowledge, there has been little or no study on the reversibility of DM-related changes in renal transporters and channels. We feel this is important to
rate in these rats. Nevertheless, many of the physiological parameters of DM were somewhat blunted in group 3 relative to group 4, e.g., urinary volume and plasma creatinine concentration (Table 2). However, there were no significant differences between the two groups for any of the physiological parameters measured. Similarly, there were few statistically significant differences between groups 3 and 4 for transporter protein abundances, indicating that reversal of the changes was possible. However, in the whole kidney, NKCC2 and the 70-kDa band associated with γ-ENaC were significantly decreased in group 3 relative to group 4. In the cortex, this was also true for α-ENaC and the 70-kDa band of γ-ENaC. Finally, it is unclear, given more time, whether these rats would be able to completely normalize any or all of these changes.

**Correlation of Transporter/Channel Changes With Blood Glucose Levels**

To our knowledge, this study is the first to attempt to correlate blood glucose levels (as an index of diabetic control) with the changes in abundances of renal transporter proteins in the STZ-induced model of DM. The abundances of several proteins, e.g., NKCC2, AQP3, and the 85-kDa band of γ-ENaC, were significantly positively correlated with blood glucose levels. This suggests that diabetic control (or degree of diabetic severity), at least in the short term, may be a major determinant of the degree of changes observed in transporter and channel proteins. Many of these changes are likely mediated through changes in hormones such as aldosterone and vasopressin. Surprisingly, other proteins, such as the α-subunit of ENaC and AQP2, did not correlate well with blood glucose levels. It is not clear why this was the case. Some possibilities include differences in the degree of kidney hypertrophy observed in this study relative to the first two, effects of insulin itself on renal protein abundances, and fluctuations in the levels of aldosterone and vasopressin.

**Changes Specific to ENaC Subunits**

This is perhaps the first reported study on the effects of DM on the renal abundances of ENaC subunits. In general, all three subunits of ENaC (including both bands of γ-ENaC) were increased during diabetes. This is not surprising, given that both aldosterone and vasopressin activity would be expected to be increased somewhat, at least at some time points, in DM. We have evidence of increased aldosterone activity with DM in both studies 1 and 2 and a trend toward increased plasma levels in study 3. In studies 1 and 2, we found a significant increase in the aldosterone-sensitive α-subunit of ENaC, as well as an increase in both

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Fig. 10. Blood glucose levels and kidney weight of rats from study 3. A: individual blood glucose measurements in rats A–L (measured daily) shown as the average of the final 4 days of the study. B: individual mean kidney weights (g/kg body wt) of rats in study 3. Mean glucose levels in rats treated most intensively with insulin (rats D–F) were fairly normal. Kidney weights increased with less control of diabetes.

Fig. 11. Relationship between individual band densities for the major sodium and water transport proteins in whole kidney homogenates and blood glucose levels (average of final 4 days) in rats in study 3. Band densities, presented as a percentage of the mean for vehicle-treated rats (A–C), for α1-Na-K-ATPase, NKCC2, NCC, β-ENaC, γ-ENaC (85-kDa band), and AQP3 were significantly correlated with blood glucose levels.

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bands associated with γ-ENaC on immunoblots. In study 3, the aldosterone-sensitive proteins, α-ENaC and the 70-kDa band associated with γ-ENaC, did not correlate at all that well with blood glucose levels per se. It is likely that with longer term diabetes differences might become more apparent. The top (dominant) band associated with γ-ENaC has been previously shown to be increased by vasopressin (8) and base-loading (20), the bottom band (more diffuse band) by aldosterone infusion or feeding of a low-NaCl diet (24). β-ENaC was modestly increased in abundance in all three studies. β-ENaC has also been shown to be increased by vasopressin, which was indeed significantly elevated in the group 4 rats in study 2. Increased ENaC activity (along with activation of the outer medullary potassium channel) in the collecting duct might be expected to increase the K⁺-to-Na⁺ ratio in the urine (as we observed).

Increased NKCC2 and NCC

Overall, we saw the most remarkable increase (>3-fold) in band density for NKCC2 with diabetes. In comparison, in a similar study by Nejsum et al. (26) mean band density for NKCC2 in whole kidney from STZ-treated rats was 125% that in control rats, i.e., not significantly different. On the other hand, Kim et al. (16) found a 2.4-fold increase in NKCC2 after 20 days in the outer medulla of STZ-treated rats. NKCC2 has been previously shown to be upregulated in protein abundance by vasopressin (19), dietary NaCl (12, 19), and loop diuretics (25).

Our observed increase in the abundance of NCC was in agreement with Ward et al. (31), who found a 192% increase in NCC in whole kidney homogenate from STZ-treated rats after 2 wk. Similarly, they were able to show a relative reduction in NCC abundance when insulin therapy was provided to STZ-treated rats. Nejsum et al. (26) reported band densities in STZ-treated rats. NKCC2 has been previously shown to be strongly upregulated in protein abundance by aldosterone (21). Overall, qualitatively our findings agreed with both Nejsum et al. (26) and Ward et al. (31) in that we see a one- to twofold increase in NCC abundance. Slight discrepancies may be due to differences in the degree of diabetes, length of the studies, and relative hypertrophy of the kidney with increased expression of hypertrophy-related proteins, such as matrix and collagen (32).

Na-K-ATPase

In study 3, the whole kidney abundance of the α1-subunit of Na-K-ATPase positively correlated with blood glucose levels. This was remarkable given that in studies 1 and 2 no differences were noted for the cortex or whole kidney and in study 2 a significant decrease was seen with increasingly severe DM in the medulla. Ku et al. (23) reported increased Na-K-ATPase activity in the medulla and cortex of STZ-treated diabetic rats. Nejsum et al. (26) reported no difference in Na-K-ATPase α1-subunit protein abundance in whole kidney homogenates, but they did not look separately at the medulla.

AQPs

Finally, we evaluated the effect of STZ-induced diabetes on the renal abundances of four major aquaporins (AQP1–4). Qualitatively, we found that our results in general agreed with those of others (2, 17, 26) who found increases in AQP2 (2, 17, 26) and AQP3 (26) with STZ-induced diabetes. However, we did not find a strong correlation between blood glucose levels and AQP2. This probably suggests that the increase in AQP2 may take additional time to develop. Increased renal abundance of both AQP2 and AQP3 would be expected to be adaptive in response to elevated vasopressin levels (5, 11) and ameliorate water losses due to osmotic diuresis. We also saw a significant decrease in AQP1 in the outer and inner medulla. Nejsum et al. (26) reported no change in AQP1 (90% of vehicle) in whole kidney homogenates, but they did not examine the medulla separately.

Role of Insulin?

Another potential regulatory factor that may have affected the abundance of renal sodium transport proteins in this model was the level of circulating insulin. Insulin was infused or injected into three of four groups of rats (in both studies 2 and 3) and thus was not allowed to fluctuate with insulin needs, as does endogenous insulin. Therefore, insulin levels may have been relatively high at certain times relative to vehicle-treated rats. We have previously shown that insulin infusion into rats with a minipump increases the whole kidney abundances of α-ENaC, NCC, both bands associated with γ-ENaC, and NaPi-2 (6, 7). However, this direct regulation is still under intensive study, and its role in affecting the abundances of transporters and channels in this study is not clear.

Conclusion

The kidney will adapt to sodium and water losses associated with DM via upregulation of both sodium and water transport proteins. These changes include upregulation of distal sodium transporter and channel subunits, such as the ENaC, likely due, at least partially, to increased aldosterone and vasopressin activity at these sites. These changes are critical for the maintenance of extracellular fluid volume in the face of large osmotically driven fluid losses.

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DISCLOSURES

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