Changes in renal medullary transport proteins during uncontrolled diabetes mellitus in rats

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Changes in renal medullary transport proteins during uncontrolled diabetes mellitus in rats. Am J Physiol Renal Physiol 285:F303–F309, 2003. First published April 15, 2003; 10.1152/ajprenal.00438.2002.—We tested whether the abundance of transport proteins involved in the urinary concentrating mechanism was altered in rats with uncontrolled diabetes mellitus (DM). Rats were injected with streptozotocin and killed 5, 10, 14, or 20 days later. Blood glucose in DM rats was 300–450 mg/dl (control: 70–130 mg/dl). Urine volume increased in DM rats from 41 ± 7 ml/100 g body wt (BW) at 5 days to 69 ± 3 ml/100 g BW at 20 days (control: 9 ± 1). Urine osmolality of DM rats decreased at 5 days DM and remained low at 20 days. UT-A1 urea transporter protein in the inner medullary (IM) tip was 55% of control in 5-day DM rats but increased to 170, 220, and 280% at 10, 14, and 20 days DM, respectively, due to an increase in the 117-kDa glycoprotein form. UT-A1 in the IM base was increased to 325% of control at 5 days DM with no further increase at 20 days. Aquaporin-2 (AQP2) increased to 290% in the IM base at 5 days DM and 150% in the IM tip at 10 days; both showed no further increase at 20 days. NKCC2/BSC1 increased to 240% in outer medulla at 20 days DM, but not at 5 or 10 days. UT-B and ROMK were unchanged at any time point. The increases in UT-A1, AQP2, and NKCC2/BSC1 proteins during uncontrolled DM would tend to limit the loss of fluid and solute during uncontrolled diabetes.

-diabetes mellitus (DM). Rats were injected with streptozotocin and killed 5, 10, 14, or 20 days later. Blood glucose in DM rats was 300–450 mg/dl (control: 70–130 mg/dl). Urine volume increased in DM rats from 41 ± 7 ml/100 g body wt (BW) at 5 days to 69 ± 3 ml/100 g BW at 20 days (control: 9 ± 1). Urine osmolality of DM rats decreased at 5 days DM and remained low at 20 days. UT-A1 urea transporter protein in the inner medullary (IM) tip was 55% of control in 5-day DM rats but increased to 170, 220, and 280% at 10, 14, and 20 days DM, respectively, due to an increase in the 117-kDa glycoprotein form. UT-A1 in the IM base was increased to 325% of control at 5 days DM with no further increase at 20 days. Aquaporin-2 (AQP2) increased to 290% in the IM base at 5 days DM and 150% in the IM tip at 10 days; both showed no further increase at 20 days. NKCC2/BSC1 increased to 240% in outer medulla at 20 days DM, but not at 5 or 10 days. UT-B and ROMK were unchanged at any time point. The increases in UT-A1, AQP2, and NKCC2/BSC1 proteins during uncontrolled DM would tend to limit the loss of fluid and solute during uncontrolled diabetes.

PATIENTS WITH UNCONTROLLED type I diabetes mellitus (DM) are polyuric due to the osmotic diuresis caused by glycosuria. The persistent osmotic diuresis frequently results in a serious degree of volume depletion, but these patients rarely present for medical attention with shock and cardiovascular collapse. Instead, they generally present with severe hyperglycemia and diabetic ketoacidosis. This clinical presentation suggests the hypothesis that compensatory changes occur within the kidney that permit sufficient solute and water reabsorption, despite the ongoing osmotic diuresis, to prevent hypovolemic shock.

The goal of this study was to test this hypothesis using rats made diabetic by streptozotocin (STZ) injection. The STZ-treated rat is a commonly used animal model of type I diabetes. These rats rapidly develop hyperglycemia and polyuria, although they do not develop ketoacidosis. Because the renal medulla is responsible for the production of concentrated or dilute urine, we hypothesized that any compensatory mechanism to conserve water and solute may involve changes in the abundance of the medullary transport proteins involved in the urinary concentrating mechanism. Therefore, we measured the abundance of the UT-A1 and UT-B urea transporters, the aquaporin-2 (AQP2) water channel, the NKCC2/BSC1 Na⁺-K⁺-2Cl⁻ cotransporter, and the ROMK K⁺ channel from rats made diabetic for 5, 10, 14, or 20 days.

METHODS

Animal preparation. Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 125–200 g received free access to 23% protein rat chow and water for at least 3 days after delivery. Rats were injected with STZ (Sigma, St. Louis, MO; 62.5 mg/kg body wt prepared fresh in 0.1 M citrate buffer, pH 4.0) or vehicle into a tail vein at 7 AM (9). Subsequently, the STZ-injected diabetic rats or vehicle-injected control rats were fed 23% protein chow and water ad libitum until they were killed at 5, 10, 14, or 20 days after injection (6 control and 6 diabetic rats were used at each time point). At 24 h after STZ injection, diabetes was confirmed by measuring the urine glucose ( Ames-Multistix SG, Miles, Elkhart, IN). One day before death, a 24-h urine collection was obtained to measure urinary volume, osmolality, urea concentration, and creatinine concentration (10). Rats were killed by decapitation, and blood was collected and assayed for glucose (One Touch Profile Diabetes Tracking Kit, LifeScan, Milpitas, CA), blood urea nitrogen (Infinity BUN reagent, Sigma), osmolality (model 5500 vapor pressure osmometer, Wescor, Logan, UT), and creatinine concentration (Creatinine Analyzer 2, Beckman Instruments, Fullerton, CA). Vasopressin levels were determined using the Arg8-vasopressin correlate-EIA kit (Assay Designs, Ann Arbor, MI) according to the manufacturer’s instructions. Kidneys and liver were removed for tissue protein analysis (18).

Western blot analysis. The kidney medulla was dissected into three regions: outer medulla, base of the inner medulla, and tip of the inner medulla, as previously described (9, 13, 20). Tissue from liver or the pooled tissue from both kidneys of a single rat was placed into an ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 μg/ml leupeptin, and 0.1 mg/ml PMSF), homogenized, and diluted 1:1...
Control rat values were measured at each time point (n = 6 per time point). As there was no significant difference over the 20 days of study, the control values were averaged for presentation in this table (total control n = 12–24, depending on the number of time points studied). Diabetic rats: n = 6 at each time point. *P < 0.05, statistical significance relative to control values, determined by unpaired Student’s t-test. Each experimental group (6 rats) had a matching time control group (6 rats) against which the results were compared yielding the statistical significance data at different diabetes mellitus (DM) durations. %, Percentage of urea or glucose in total urinary solutes. †Statistical significance between DM duration groups as determined by ANOVA, P < 0.05, n = 6 rats per group. ND, not determined.

with 1% SDS for Western blot analysis of total cell lysate (9, 13, 20). Total protein in each sample was measured by the Bradford method (Bio-Rad, Richmond, CA). Proteins (10 μg/lane) were size separated by SDS-PAGE using 7.5, 10, or 15% polyacrylamide gels. Proteins were blotted to polyvinylidene difluoride membranes (Gelman Scientific, Ann Arbor, MI), and Western blot analysis was performed as described previously (9). Blots were quantified using an Imaging Densitometer GS670 and Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). Where multiple bands were observed resulting from multiple glycosylated forms of a single protein (UT-A1: 110–120 kDa; UT-B: 41–54 kDa; AQP2: 35–50 kDa), all bands in the group were measured together and designated the molecular mass of the major form. In all cases, parallel gels were stained with Coomasie blue to confirm uniformity of loading (data not shown). Results are expressed as arbitrary units per microgram of protein.

Deglycosylation of UT-A1 protein in inner medullary homogenates. A sample of inner medullary (IM) homogenate (45 μg) containing 0.5% SDS and 1% β-mercaptoethanol was denatured by heating to 100°C for 10 min. After addition of NP-40 detergent to 1% and addition of 2,500 U of peptide N-glycosidase F (PNGase F; catalog no. 704S, New England Biolabs, Beverly, MA), the mixture was incubated at 37°C for 60 min. The reaction was quenched by the addition of an equal volume of 2× Laemmli sample buffer. These samples were heat treated again to 100°C for 6 min before SDS-PAGE and immunoblotting.

Antibodies. Western blot analyses were probed with antibodies (diluted in TBS/Tween) to the following proteins: 1) UT-A1 and UT-A2 (13); 2) AQP2 (8, 15); 3) UT-B (22); 4) NKCC2/BSC1 (6, 7); and 5) ROMK (generous gift from Dr. M. A. Knepper, National Institutes of Health) (4). Statistics. Data are presented as means ± SE (n), where n indicates the number of rats studied. To test for statistically significant differences between two groups, a paired Student’s t-test was used. To test for statistically significant differences among three groups, an ANOVA was used followed by a multiple comparison, protected t-test (21).
RESULTS

Animal parameters. The weight gain of control rats at both 5 and 20 days was significantly greater than the weight gain of the diabetic rats (5-day control: 39 ± 1 g vs. diabetic: 19 ± 3 g; 20-day control: 170 ± 5 g vs. diabetic: 109 ± 7 g). Food intake was assessed during the final 2 days of 5- and 20-day STZ treatments. At 5 days DM, the food intake of control rats was 14.6 ± 0.4 g, whereas the DM rats consumed 19.5 ± 4.5 g/100 g body wt. The food intake of the more mature control animals of the 20-day group was 10.9 ± 0.4 g compared with 20.3 ± 1.0 g/100 g body wt for the 20-day DM rats. At both time points, the DM rats ate significantly more than control rats (P < 0.01). Blood and urinary parameters for the diabetic rats are provided in Table 1. Blood glucose was significantly increased in diabetic over control rats, but not changed by the duration of diabetes. Blood urea nitrogen of diabetic rats was not significantly different from that of controls. The urinary osmolality and urinary urea concentration of 5-day diabetic rats were significantly decreased to ~50 and 20% of the levels found in control rats, respectively, with no further decrease to 20 days. Urinary volume, total solute excretion, and urea excretion per 100 g body wt gradually increased with the duration of diabetes, but the average percentage of urea in total urinary solute was constant in both control (45%) and diabetic (18%) rats. Urinary glucose concentration and the percentage of glucose in total urinary solute were relatively constant during the 20 days of diabetes. Plasma vasopressin levels were measured in 5-day (control: 1.7 ± 0.2; DM: 2.2 ± 0.5 pg/ml) and 20-day (control: 2.2 ± 0.8; DM: 3.0 ± 0.9 pg/ml) animals. The vasopressin levels were not statistically different in diabetic animals compared with controls at either duration of DM.

UT-A1. The abundance of UT-A1 protein in IM tip of control and diabetic rats is shown in Fig. 1. UT-A1 exists as two distinct glycoproteins with molecular masses of 117 and 97 kDa (3). To determine whether the 117- and 97-kDa proteins that increase in the diabetic rats are the same glycoproteins as in control rats and represent glycosylated forms of the same base UT-A1 protein, kidney lysates from both control and 20-day diabetic rats were treated with PNGase F. Deglycosylation of UT-A1 protein from diabetic and control rats revealed the identical 88-kDa nonglycosylated base protein (Fig. 2). When reporting on the total UT-A1 protein abundance differences, we make the assumption that the antibody recognizes both glycoproteins equally. However, we are also providing information about shifts in the relative abundances of each of the two forms to better characterize the changes in UT-A1 that occur in the diabetic animal (reviewed in Ref. 19). The functional difference between these two glycoproteins, if any, is not known.

Compared with control rats, the abundance of UT-A1 in 5-day diabetic rats was significantly decreased to 55% of control rats in the IM tip. The decrease of UT-A1 in the IM tip was mainly due to a decrease in the abundance of the 97-kDa UT-A1 protein (26% of control) rather than a change in the abundance of the 117-kDa UT-A1 protein (87% of control). In contrast, the abundance of UT-A1 was significantly increased in the IM tip of rats made diabetic for 10, 14, or 20 days: UT-A1 increased to 170% of control at 10 days; 220% of control at 14 days; and 280% of control at 20 days. The amount of the 117-kDa UT-A1 protein relative to total UT-A1 protein in diabetic rats also increased with time to 56, 66, and 70% at 10, 14, and 20 days, respectively. At each time point, six control and six diabetic rats were used and the results are representative of four experiments.

Fig. 2. Deglycosylation of UT-A1 protein in CTR vs. DM rats. IM tip lysates from CTR and 20-day DM rats were treated with PNGase F (+) or vehicle (–). Western blot analysis of these samples is shown. Left: arrows indicate the glycosylated bands at 117 and 97 kDa in the untreated lysates and the deglycosylated band at 88 kDa that is common to both CTR and DM rats.

Fig. 3. UT-A1 protein in IM base of CTR vs. DM rats. The time after streptozotocin treatment is indicated as days on the left of the blots and below the bars. A: Western blot analyses of representative samples of IM base lysates probed with anti-UT-A antibody. Samples from 3 different CTR rats (left) and 3 different DM rats (right). Arrows indicate the glycosylated bands at 117 and 97 kDa. B: bar graphs showing the summary densitometry (total UT-A1 protein of CTR rats is 100%) from all rats. Bars = means, *P < 0.05, n = 6 rats/bar.
The abundance of UT-A1 protein in the IM base of control and diabetic rats is shown in Fig. 3. In contrast to the IM tip, the abundance of UT-A1 in the IM base was significantly increased to 325% of control levels by 5 days of diabetes and did not increase further at 10, 14, or 20 days (300, 390, and 340% of control, respectively). The increase in UT-A1 protein abundance in the IM base of diabetic rats was mainly due to an increase in the 117-kDa UT-A1 protein (to 2,000% of control) rather than an increase in the 97-kDa UT-A1 protein (135–190% of control). The percentage of 117-kDa UT-A1 protein to total UT-A1 protein (62, 55, 56, and 55% at 5, 10, 14, and 20 days, respectively) did not vary with the duration of diabetes in the IM base.

**UT-B.** UT-B is normally expressed in the IM tip, base, and outer medulla (22). It is found exclusively in red blood cells and descending vasa recta but not in tubules (reviewed in Ref. 19). There was no significant difference in UT-B protein abundance in any of these kidney regions at 20 days of diabetes compared with control rats (Fig. 4) nor at 5 or 10 days of diabetes (data not shown).

**AQP2 in the IM tip and base.** AQP2 protein abundance in the IM tip (Fig. 5A) was unchanged at 5 days, but significantly increased to 150% of control at 10 days of diabetes and remained elevated at 20 days. In the IM base (Fig. 5B), AQP2 protein abundance was significantly increased to 290% of control at 5 days of diabetes and remained elevated at 10 and 20 days. At each time point, six control and six diabetic rats were used and the results are representative of four experiments.

**NKCC2/BSC1 and ROMK abundance in the outer medulla.** At 5 or 10 days of diabetes, there was no significant difference in the abundance of either NKCC2/BSC1 or ROMK protein between control and diabetic rats (data not shown). At 20 days of diabetes, NKCC2/BSC1 was significantly increased to 245% of control levels (Fig. 6). In contrast, ROMK was not significantly increased at 20 days of diabetes (Fig. 7).

**UT-A in liver.** UT-A is found in several extrarenal tissues including the liver (11), the site of ureagenesis. We previously reported that the liver form of UT-A is regulated by uremia and acidosis (10, 11). The 49-kDa UT-A protein was significantly increased to 400% of control in liver from 20-day diabetic rats but not in liver from rats with diabetes for 5 or 10 days (Fig. 8). The abundance of the 36-kDa protein was not significantly different at any time point. The abundance of the 36-kDa protein was also unchanged in the liver from uremic rats (11).
However, the diabetic rats were able to maintain their ability. These current multiplication, increase urinary concentrating ability, and water reabsorption from the inner medulla, where it is needed for maximal urinary concentrating ability despite the continuing osmotic diuresis. The diabetic rats have reduced urinary osmolality, but urinary osmolality does not vary between 5 and 20 days, even though urinary volume progressively increases (Table 1). The polyuria of diabetes results from nonreabsorbable glucose in the tubule lumen. Theoretically, 300 mosmol of nonreabsorbable solute will retain about 1 liter of water in the tubular lumen and reduce urinary osmolality to ~300 mosmol/kgH₂O. However, the diabetic rats were able to maintain their urinary osmolality at 850–950 mosmol/kgH₂O. The present findings suggest that the increases in UT-A1, AQP2, and NKCC2/BSC1 proteins play a role in maintaining urinary osmolality above isotonicity. If urinary osmolality had continued to decrease as urinary volume increased with the longer duration of diabetes, it is likely that the rats would have lost more water and solute.

**UT-A1.** The findings in the present study appear to resolve some discrepancies between previous studies of the effect of diabetes on UT-A1 abundance. We previously showed that at 3 days of diabetes, UT-A1 protein abundance is downregulated in the IM tip, compared with control rats (9), and the present study shows that UT-A1 protein is downregulated in the IM tip at 5 days. In contrast, Bardoux and colleagues (1) showed that at 21 days post-STZ treatment, diabetic rats show an increase in UT-A1 mRNA and protein in the IM base. In particular, they showed that the 117-kDa band was increased without a change in the 97-kDa band (1). Consistent with Bardoux and colleagues (1), the present study shows that the 117-kDa form of UT-A1 is consistently upregulated in the IM base from 5 to 20 days of diabetes and also shows that it is increased in the IM tip from 10 to 20 days. Thus both previous studies (1, 9) were correct, but neither recognized that there are temporal and IM regional changes in UT-A1 protein during the first 3 wk after STZ.

**AQP2.** The findings in the present study also appear to resolve some discrepancies between previous studies on the effect of diabetes on AQP2 abundance. We previously showed that at 3 days of diabetes, AQP2 protein abundance is unchanged in the IM tip, compared with control rats (9), and the present study shows that AQP2 protein is unchanged in the IM tip at 5 days. In contrast, Nielsen and colleagues (14) showed that AQP2 protein is increased in the IM at 21 days post-STZ. Bardoux and colleagues (1) also show that AQP2 protein is increased in the IM at 21 days post-STZ. Consistent with these latter studies (1, 14), the present study shows that AQP2 is consistently upregulated in the IM base from 5 to 20 days of diabetes and also shows that it is increased in the IM tip from 10 to 20 days. Thus all previous studies (1, 9, 14) were correct, but again they did not recognize that there are temporal and IM regional changes in AQP2 protein during the first 3 wk after STZ. The present study does not address whether diabetic rats have an abnormality in the regulated trafficking of AQP2 nor whether there...
is a change in the abundance of AQ3 or AQ4 (located in the basolateral membrane of the collecting duct), and future studies will be needed to test these possibilities.

NKCC2/BSC1. Nielsen and colleagues (14) found no significant change in NKCC2/BSC1 protein at 15 days post-STZ. The present study shows that NKCC2/BSC1 is unchanged at 10 days but increases at 20 days. However, ROMK protein was unchanged at all time points.

Interestingly, UT-A1, AQP2, and NKCC2/BSC1 protein abundances are decreased in 6-mo-old, obese Zucker rats, a model of type II diabetes (2). Thus more prolonged periods of diabetes or type II (vs. type I) diabetes may have different effects on these transporters. Regardless, the present and previous (1, 9, 14) findings suggest a complex compensatory response in which UT-A1, AQP2, and NKCC2/BSC1 are upregulated at different times after STZ injection in different medullary regions, but all of these changes will tend to limit the loss of water and solute during uncontrolled diabetes.

Possible mechanisms. Several metabolic and hormonal abnormalities present in diabetes could contribute to the changes in medullary transport protein abundances. In normal rats, we showed that glucocorticoids downregulate UT-A1 protein abundance in the IM tip and lower basal and vasopressin-stimulated facilitated urea permeability in rat terminal IMCDs (13) by decreasing the transcription of UT-A1 promoter I (17). Rats with uncontrolled DM induced by STZ have increased corticosterone production and urea excretion at 3–6 days (12). We previously showed that glucocorticoids mediate the downregulation of UT-A1 protein abundance in rats with uncontrolled diabetes at 3 days (9). Thus the decrease in UT-A1 at 3–5 days after STZ treatment is likely to be mediated by repressive effect of glucocorticoids on UT-A1 transcription.

What about the upregulation of UT-A1, AQP2, and NKCC2/BSC1 at the later time points? Vasopressin upregulates AQP2 protein long term by a transcriptional mechanism (reviewed in Ref. 16). Vasopressin also upregulates NKCC2/BSC1 and ROMK proteins (4, 5, 7). A previous study showed that diabetic rats have polyuria despite elevated plasma vasopressin levels (23). However, we did not find a significant change in vasopressin levels in the present study. In addition, the difference in time course for AQP2 between IM base and tip, and the lack of change in ROMK, suggests that factors other than vasopressin may play a role.

Regardless of the mechanism, the increase in UT-A1 protein is likely to promote the production of a more concentrated urine. Given the sustained increase in urinary volume, and presumably urinary flow rates, the increase in UT-A1 protein may be an important compensatory mechanism to maintain urea delivery to the IM interstitium. Vasopressin does mediate the rapid increase in urea reabsorption by phosphorylating UT-A1 (24). Because vasopressin levels are not suppressed in diabetic rats, vasopressin is likely to result in UT-A1 phosphorylation and an increase in urea transport per UT-A1 molecule, potentially compensating for the reduced time for urea transport due to the increase in urinary flow rate. Consistent with this hypothesis, the percentage of urea in total urinary solute excretion remained constant from 5 to 20 days of diabetes (Table 1).

Summary. The abundance of the major medullary transport proteins involved in the urinary concentrating mechanism varies with time and kidney region after rats are made diabetic by STZ. These findings tend to support the hypothesis that increases in UT-A1, AQP2, and NKCC2/BSC1 proteins during uncontrolled diabetes are compensatory changes that prevent a progressive decline in urinary concentrating ability despite the continuing osmotic diuresis. Future studies in which knockout mice lacking one or more of these transport proteins are made diabetic may be very useful in defining the importance of each transporter to the compensatory response. However, if similar changes in these medullary transport proteins occur in patients with uncontrolled type I diabetes, they would tend to lessen the degree of volume depletion that occurs in these patients.

DISCLOSURES

Portions of this work have been published in abstract form (J Am Soc Nephrol 13: 67A, 2002). This work was supported by National Institutes of Health Grants R01-DK41707 and R01-DK63657.

REFERENCES


