

Original Article

Aldosterone-mediated apical targeting of ENaC subunits is blunted in rats with streptozotocin-induced diabetes mellitus

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Abstract

Background. Diabetes mellitus (DM) is associated with a significant polyuria and natriuresis as well as increased plasma aldosterone and anti-diuretic hormone arginine vasopressin (AVP). This study aimed to determine whether diabetic kidneys compensate for the urinary sodium and water losses by increasing apical targeting of epithelial sodium channel (ENaC) subunits and aquaporin-2 (AQP2) in the collecting duct, in addition to the previously observed changes in ENaC subunit protein expression in different kidney zones.

Methods. Female rats were investigated 2 weeks after induction of DM by streptozotocin administration. Kidneys were examined by immunohistochemistry and semiquantitative immunoblotting.

Results. We demonstrated that the protein expression of renal AQP2, Ser-256 phosphorylated AQP2, AQP3, β - and γ -ENaC (but not α -ENaC) increased consistently with an increased AVP response. In contrast, there were no significant changes in the relative apical targeting of β -, γ - and α -ENaC, and the shift in the molecular weight of γ -ENaC from 85 kDa to 70 kDa was not observed despite increased plasma aldosterone levels. These results were supported by changes in the functional data showing increased solute-free water reabsorption, increased fractional excretion of sodium and an unchanged ratio of potassium to sodium in the urine.

Conclusions. The data demonstrate that diabetic kidneys have a reduced sensitivity to the anti-natriuretic action of elevated plasma aldosterone levels with no relative increase in ENaC subunit apical targeting, whereas there is increased expression of β - and γ -ENaC, which alone may play a role in the increased sodium reabsorption in the kidney in DM.

Keywords: aldosterone; aquaporin; diabetes mellitus; ENaC; vasopressin

Introduction

Diabetes mellitus (DM) is a common endocrine disease, characterized by marked hyperglycaemia, polyuria, polydipsia and natriuresis. In response to the increased urine volume and urinary sodium excretion, DM is associated with hormonal changes for preventing extracellular fluid (ECF) volume contraction, e.g. elevated levels of the anti-natriuretic hormone aldosterone [1] and the anti-diuretic hormone arginine vasopressin (AVP) [2], which mediate their effects at the distal nephron and collecting duct via an activation of the epithelial sodium channel (ENaC) and aquaporins (AQPs).

In the kidney distal nephron and collecting duct, sodium entry from the lumen into the cells is mediated by the amiloride-sensitive sodium channel, ENaC, at the apical membrane [3] and the Na,K-ATPase at the basolateral membrane, which provides the driving force for apical sodium reabsorption [4]. ENaC is composed of three subunits, α -, β - and γ -ENaC [5], that are localized to the distal convoluted tubule (DCT2), connecting tubule, cortical, outer medullary and inner medullary collecting duct [6], with the highest expression in the cortex [7]. Importantly, gene knock-outs of the individual subunit in mice have demonstrated that any of the three subunits has significant effects on the sodium transport capacity of multimeric ENaC protein [8–10].

Sodium reabsorption via ENaC is known to be regulated by aldosterone [11]. Previous studies have demonstrated that effects of aldosterone are heterogenous and involve the changes in both the protein expression of each subunit and the subcellular localization of these subunits in the cells [12]. Dietary sodium restriction to stimulate endogenous aldosterone has been shown to increase the protein expression of α -ENaC, to cause a shift in the molecular weight

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of γ -ENaC from 85 kDa to 70 kDa and to result in increased apical targeting of all subunits to the apical plasma membrane [11]. While aldosterone is the chief regulator of sodium reabsorption by ENaC, AVP has also been demonstrated to regulate ENaC expression. (1) Water restriction of Sprague–Dawley rats, which endogenously increases plasma AVP levels, was shown to result in increased protein expression of β - and γ -ENaC, but not α -ENaC [13,14]; (2) infusion of the vasopressin V_2 -receptor-selective agonist, dDAVP (1-deamino-8D-arginine-vasopressin), for 7 days increased all ENaC subunits, but α -ENaC to a lesser extent than β - and γ -ENaC, in Brattleboro rats [13,14]; and (3) interestingly, Sauter *et al.* [15] found no increased apical targeting of β - and γ -ENaC subunits in the collecting duct principal cells in long-term dDAVP-treated rats despite increased protein expression.

The collecting duct also represents the final site for the control of water excretion into the urine. Water permeability of the collecting duct is tightly regulated under the control of the AVP, and AQP2 is the apical water channel of collecting duct principal cells and is regulated by AVP [16]. Increased circulating AVP levels result in an increase in both protein expression and protein kinase A-dependent phosphorylation, which is involved in targeting of AQP2 to the apical plasma membrane [17]. AQP3, expressed on the basolateral membrane, is also regulated by AVP [18]. Previously, we demonstrated that expressions of AQP2, phosphorylated AQP2 (p-AQP2: phosphorylated in the PKA phosphorylation consensus site, Ser 256) and AQP3, were significantly increased 2 weeks following the induction of DM [19]. Moreover, AQP2 targeting to the apical plasma membrane was enhanced, indicating a vasopressin-mediated compensatory increase in expression and targeting in response to severe polyuria. Moreover, recently, Song *et al.* [20] also extensively studied the changes in protein abundance of major renal sodium transporters and AQPs in streptozotocin (STZ)-induced DM in rats, and they found a significant upregulation of sodium transporters including all ENaC subunits and water channels AQP2 and AQP3. However, it is unclear whether the observed up-regulation of ENaC subunits is accompanied by enhanced targeting to the apical plasma membrane of the collecting duct principal cells in response to the hormonal changes for preventing ECF volume contraction. Thus, the purpose of this study was to investigate the changes of subcellular localization of ENaC subunits in the collecting duct principal cells in STZ-induced DM rats, in addition to the changes of protein expression of ENaC subunits and AQPs in different kidney zones.

Subjects and methods

Experimental animal

The animal protocols have been approved in accordance with the license for use of experimental animals issued by the Danish Ministry of Justice. Female Wistar rats (Taconic Europe, Eiby, Denmark) were divided into two groups matched for body weight. Throughout the experiments, there was a 12:12-h artificial light–dark cycle, a temperature of $21^\circ \pm 2^\circ\text{C}$ and humidity of $55 \pm 2\%$. The

rats had free access to tap water and were maintained on a standard rodent diet (1324 pellets or 1321 meal, Altromin, Lage, Germany) throughout the experiment.

Streptozotocin-induced diabetes mellitus

Female rats ($n = 20$, body weight 177–194 g) were used. After 12 h of fasting, the animals were anaesthetized with halothane and their femoral vein was exposed. Controls (Con; $n = 8$) were injected intravenously with 0.5 ml of vehicle (0.9 mg/ml saline, pH 5.4) and the diabetic rats (DM; $n = 12$) were injected with streptozotocin (STZ, Sigma; 55 mg/kg body weight) dissolved in 0.5 ml of vehicle. Throughout the experiment, rats were kept in metabolic cages and parameters (body weight, water intake, urine production, etc.) were measured every 24 h. Plasma glucose levels were checked regularly on tail-vein blood using an Accu-Chek Sensor Comfort plasma-glucose instrument (Roche, Hvidovre, Denmark). Urinary glucose and ketone bodies were measured with Combur7 Test strips (Roche, Germany). The rats were anaesthetized with halothane and a large laparotomy was made 14 days after the injection of STZ. The right kidney was removed for immunoblotting. Left kidneys were perfusion fixed as described below (immunohistochemistry). A blood sample was taken for analysis and urine samples were also analysed.

Semiquantitative immunoblotting

Semiquantitative immunolabelling was performed using methods described previously in detail [21–23]. Each kidney was immediately placed in ice-cold dissection buffer [0.3 M sucrose, 25 mM imidazol, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.2 containing $8.4 \mu\text{M}$ leupeptin, 0.4 mM pefabloc] following removal. Kidneys were dissected into cortex, inner stripe of the outer medulla (ISOM) and inner medulla (IM). The dissected tissue samples were homogenized and centrifuged at 4000 g for 15 min at 4°C . Supernatant, solubilized at 90°C for 5 min in Laemmli sample buffer, was used for immunoblotting.

Immunohistochemistry

Immunolabelling was performed on sections from paraffin-embedded preparation (2 μm thickness) using methods described previously in detail [22,23]. The kidneys were fixed by retrograde perfusion fixation with cold fixative (3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4).

Primary antibodies for semiquantitative immunoblotting and immunohistochemistry

Previously characterized polyclonal antibodies were used for semiquantitative immunoblotting and immunohistochemistry, as summarized below.

α -ENaC (3560-2g or immune serum). An affinity-purified polyclonal antibody to α -ENaC has previously been characterized [7,11].

β -ENaC (Q3755-2). An affinity-purified polyclonal antibody to β -ENaC has previously been characterized [11,21].

γ -ENaC (881g or 928). An affinity-purified polyclonal antibody to γ -ENaC has previously been characterized [11].

AQP1 (2353AP). An affinity-purified polyclonal antibody to AQP1 has previously been characterized [24].

AQP2 (H7661AP). An affinity-purified polyclonal antibody to AQP2 has previously been characterized [21,23].

p-AQP2 (AN244-pp-AP). An affinity-purified polyclonal antibody to phosphorylated AQP2 has previously been characterized [17].

AQP3 (RA1591AP or 1591AP). An affinity-purified polyclonal antibody to AQP3 has previously been characterized [24].

Presentation of data and statistical analyses

Results are expressed as mean \pm SEM. Differences between groups were determined using the unpaired Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

DM was associated with altered renal water and sodium handling

All animals in the DM group developed diabetes; blood glucose levels on Day 2 were 23.0 ± 1.5 mmol/l in the DM compared with the Con group (5.4 ± 0.1 mmol/l, $P < 0.05$). The DM group developed glucosuria but did not develop ketonuria. Water intake and urine output were significantly increased in the DM group (Figure 1A). Additionally, food consumption was significantly increased in the DM group from Day 4 and, similar to the changes of water intake and urine output, rose dramatically in the first week and began to stabilize from Day 6 (Figure 1B). Moreover, the DM group had a significantly lower body weight until Day 6, but gained weight thereafter so that the body weights of the two groups were not different on Day 14 (Figure 1C).

Plasma and urine parameters for the final day, Day 14, are outlined in Table 1. DM was associated with hyperglycaemia, polyuria and natriuresis. The DM group was characteristically associated with high plasma osmolality as well as a significantly elevated glomerular filtration rate (GFR) measured by creatinine clearance (C_{cr}) relative to the Con. Plasma potassium was not changed, whereas plasma sodium was significantly decreased relative to controls.

Urine concentration was reduced, as the urine osmolality and urine-to-plasma osmolality ratio ($(U/P)_{osm}$) were both reduced ($P < 0.05$). Osmolar clearance (C_{osm}) was increased ($P < 0.05$) in the DM group, most likely contributed mostly by highly excreted glucose and sodium. Despite this, solute-free water reabsorption (T^cH_2O) was markedly increased ($P < 0.05$) in the DM group, indicating that the diabetic kidney was reabsorbing more solute-free water than the controls despite the osmotic diuresis. DM was also associated with a significant natriuresis. The urinary sodium excretion rate in the DM group was significantly increased, to more than twice that of the Con group (Table 1). This was accompanied by a significant increase in the fractional

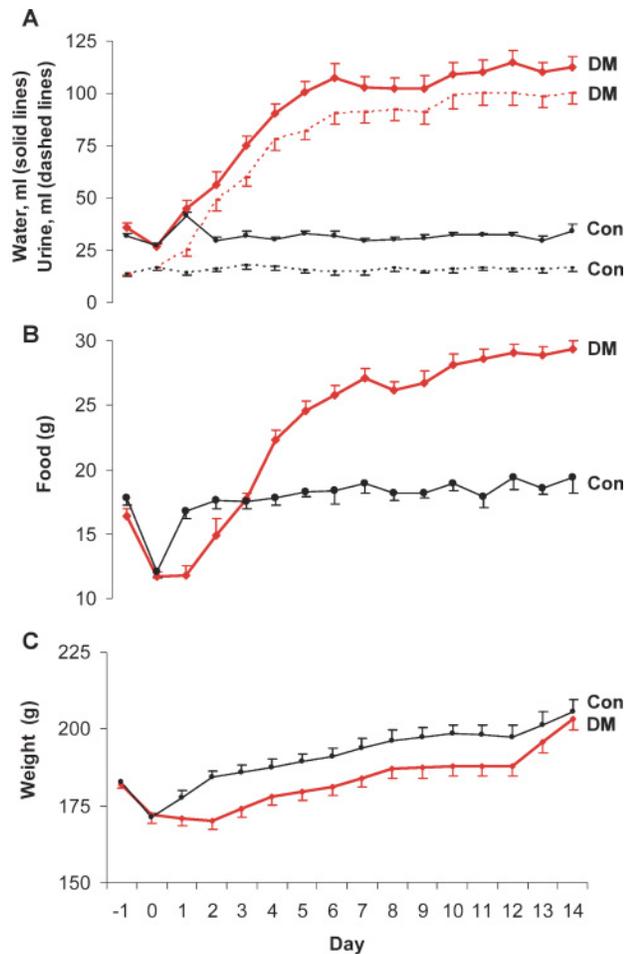


Fig. 1. Functional parameters of DM and control rats measured over the experimental period. Animals were housed in metabolic cages to allow collection and measurement of urine and measurement of water and food intake. Baseline values were obtained on Day -11. Food was removed 12 h before injection of STZ or vehicle to DM and control animals, respectively (Day 0). Animals were sacrificed on Day 14. DM group (DM, $n = 12$); control group (Con, $n = 8$). (A) Water intake, solid lines; urine production, dashed lines; (B) food consumption; (C) body weight change over the course of the study. Values are mean \pm SEM.

excretion of sodium (Table 1). In response to the significant urinary sodium loss, plasma aldosterone levels were significantly increased in the DM group, to more than four times that of the Con group (Table 1). In direct contrast to this, however, the ratio of potassium to sodium in the urine (an index of aldosterone-like activity in the kidney) was significantly reduced. Moreover, the transtubular potassium gradient and the concentration of potassium in the cortical collecting duct were also significantly reduced (Table 1). Both findings indicate that the effects of aldosterone on the renal sodium reabsorption and potassium secretion were significantly blunted in rats with DM.

DM was associated with increased protein expression of β - and γ -ENaC, but not α -ENaC

Immunoblots and the results of densitometric analyses are illustrated in Figures 2–4. DM was associated with no change in α -ENaC expression in the cortex [$137 \pm 29\%$

Table 1. Functional changes in diabetic rats

Parameter (Day 14)	DM (<i>n</i> = 6)	Control (<i>n</i> = 6)
BW (g)	203 ± 13	206 ± 15
U-volume (μl/min/kg BW)	367 ± 16*	54 ± 8
C _{Cr} (μl/min/kg BW)	7391 ± 462*	5175 ± 230
C _{urea} (mmol/l/min/kg BW)	8832 ± 452*	5151 ± 352
P-creatinine (μmol/l)	30.5 ± 1.1	33.7 ± 1.2
P-urea (mmol/l)	7.7 ± 0.3	7.5 ± 0.6
P-potassium (mmol/l)	5.1 ± 0.6	4.4 ± 0.4
P-sodium (mmol/l)	135 ± 1*	140 ± 1
P-osmolality (mOsm/kg H ₂ O)	329 ± 2*	310 ± 2
U-osmolality (mOsm/kg H ₂ O)	1065 ± 24*	1497 ± 150
(U/P) _{osm}	3.2 ± 0.1*	4.8 ± 0.5
C _{osm} (μl/min/kg BW)	1188 ± 58*	244 ± 11
T ^c H ₂ O (μl/min/kg BW)	821 ± 44*	190 ± 13
U _{Na} V (mmol/day/kg BW)	17.4 ± 1.1*	7.4 ± 0.2
FE _{Na} (%)	1.2 ± 0.1*	0.7 ± 0.0
P-aldosterone (pg/ml)	1112 ± 241*	269 ± 53
U-, K ⁺ :Na ⁺	2.3 ± 0.1*	2.7 ± 0.1
TTKG	5.0 ± 0.5*	13.5 ± 1.2
CCD _K	23.6 ± 0.7*	57.3 ± 2.5

Values are mean ± SEM.

n, number of rats; DM, diabetes mellitus; BW, body weight; P-, plasma; U-, urine; C_{Cr}, creatinine clearance; (U/P)_{osm}, urine-to-plasma osmolality ratio; Cosm, osmolal clearance; T^cH₂O, solute-free water reabsorption; C_{urea}, urea clearance; U_{Na}V, urinary sodium excretion rate; FE_{Na}, fractional excretion of sodium; K⁺:Na⁺, ratio of potassium to sodium; TTKG, transtubular K⁺ gradient; CCD_K, cortical collecting duct K⁺ concentration.

**P* < 0.05.

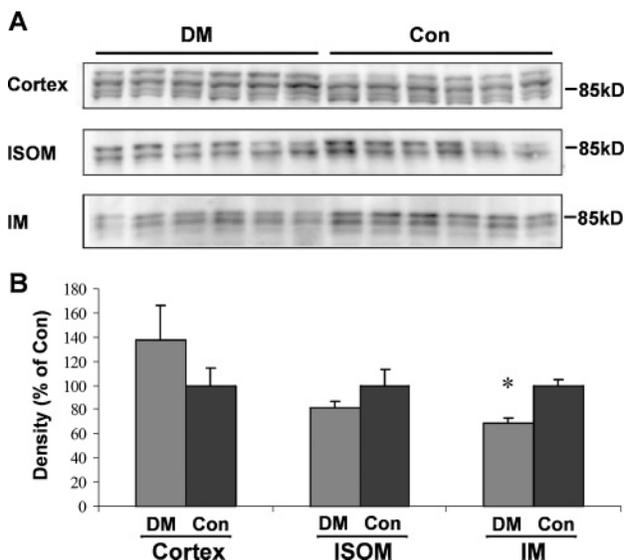


Fig. 2. Semiquantitative immunoblotting of protein samples of kidney cortex, ISOM and IM for α -ENaC. (A) Immunoblots were reacted with anti- α -ENaC and revealed an 85 kDa band. (B) Densitometric analysis revealed no change in α -ENaC expression in the cortex or ISOM, while a significant decrease was found in the IM. **P* < 0.05.

of controls (100 ± 14%, n.s.: not significant)] or ISOM [82 ± 5% of controls (100 ± 14%, n.s.)], while α -ENaC was found to be downregulated in the IM [68 ± 4% of controls (100 ± 5%, *P* < 0.05)]. DM was associated with significantly increased β -ENaC in the cortex [300 ± 74% of controls (100 ± 31%, *P* < 0.05)] and ISOM [217 ± 32% of controls (100 ± 12%, *P* < 0.05)], while no change was ob-

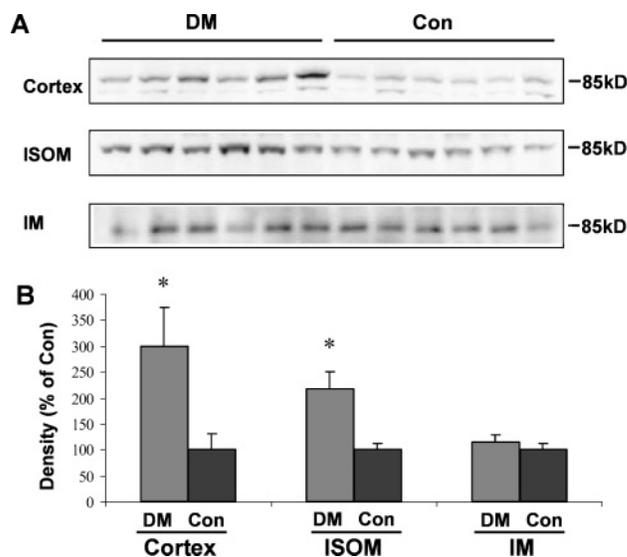


Fig. 3. Semiquantitative immunoblotting of protein samples of kidney cortex, ISOM and IM for β -ENaC. (A) Immunoblots were reacted with anti- β -ENaC and revealed an 85 kDa band. (B) Densitometric analysis revealed increased β -ENaC expression in the cortex and ISOM, while levels were unchanged in the IM. **P* < 0.05.

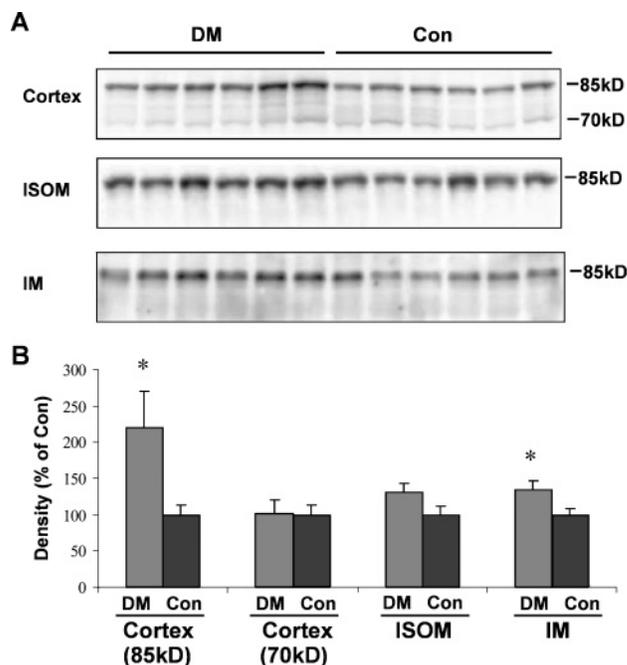


Fig. 4. Semiquantitative immunoblotting of protein samples of kidney cortex, ISOM and IM for γ -ENaC. (A) Immunoblots were reacted with anti- γ -ENaC and revealed 85 and 70 kDa bands. (B) Densitometric analysis revealed increased expression of the 85 kDa γ -ENaC band, but unchanged expression of the 70 kDa band in the cortex, and unchanged expression of the 85 kDa band in the ISOM and increased expression in the IM. **P* < 0.05.

served in the IM [115 ± 14% of controls (100 ± 11%, n.s.)]. DM was also associated with a significant upregulation of γ -ENaC (85 kDa) in the cortex [220 ± 50% of controls (100 ± 14%, *P* < 0.05)]. While a band at 70 kDa was evident, no change in expression was observed [101 ± 20%

of controls ($100 \pm 14\%$). The γ -ENaC expression in the ISOM was marginally increased [$132 \pm 12\%$ of controls ($100 \pm 12\%$, $P = 0.05$)] and it was significantly elevated in the IM [$134 \pm 12\%$ of controls ($100 \pm 9\%$, $P < 0.05$)]. While a weak band representing the 70 kDa γ -ENaC band was evident in the ISOM and IM, no change in expression levels was found when immunoblots were exposed for longer periods of time (data not shown).

DM was not associated with increased apical targeting of ENaC subunits despite increased protein expression and high plasma aldosterone levels

Despite increased protein expression, immunohistochemistry revealed that DM was not associated with increased apical targeting of α -, β - or γ -ENaC in the DCT2, CNT or CCD (Figures 5–7). No evidence for increased targeting was found in the OMCD (outer medulla collecting duct) or the IMCD (inner medulla collecting duct) either (data not shown). The α -ENaC was localized mostly to the apical plasma membrane in both DM and control rats (Figure 5). More labelling was evident in the DCT2 and CNT than in the CCD. The β - and γ -ENaC labelling was more dispersed in the cytoplasm (Figures 6 and 7), but also labelled more in the DCT2 and CNT than in the CCD. Immunohistochemistry supported the immunoblotting results insofar as no significant increase in α -ENaC labelling intensity in the different kidney zones in DM rats and more strong labelling intensity was seen for β - and γ -ENaC in the cortex (DCT2, CNT and CCD) of DM rats, relative to control rats.

DM was associated with increased levels of AQP2, p-AQP2 and AQP3, and apical targeting of AQP2 and p-AQP2

DM was associated with no change in AQP1 expression in the cortex, but with increased expression of AQP2, the phosphorylated AQP2 (p-AQP2) and AQP3 in all different kidney zones (Figure 8), consistent with previous findings [19,25]. Moreover, immunohistochemistry revealed increased targeting of AQP2 and p-AQP2 to the apical plasma membrane of the principal cells along the collecting ducts (data not shown).

Discussion

The results of this study demonstrated increased expression of AQP2, p-AQP2, AQP3, β - and γ -ENaC in 2-week diabetic rats, that was consistent with increased plasma AVP levels previously observed in rats with DM [26,27]. In contrast, no significant increase in the α -ENaC expression and apical targeting of all the ENaC subunits was observed despite increased plasma aldosterone levels. These results were consistent with the functional data showing that the diabetic rats displayed an increase in solute-free water reabsorption but did not display an increase in the ratio of potassium to sodium in the urine, an index of aldosterone-like activity in the kidney. Moreover, the fractional excretion of sodium was increased. This data could suggest that

the diabetic kidney had a reduced sensitivity to the anti-natriuretic action of elevated plasma aldosterone levels and that the AVP-mediated increase in the expression of β - and γ -ENaC alone without enhanced apical targeting was involved in mediating sodium reabsorption in DM.

DM rats demonstrated increased urinary sodium excretion despite high plasma aldosterone and AVP levels

A 2-week period of DM was chosen as it is clear from Figure 1 that DM develops during the first week following injection of STZ, with dramatic changes in water balance, food intake and to a lesser degree, body weight, that begin to stabilize during the second week and are in a steady state by the end of it. STZ-induced DM was associated with hyperglycaemia, polyuria, polydipsia, increased GFR and a marked natriuresis, consistent with the previous studies [19]. Plasma AVP levels were not measured due to the large volume of plasma required and because anaesthesia has been shown to increase plasma AVP levels [28]. Despite unchanged levels found in one study [25], several other previous studies have demonstrated increased AVP levels in association with DM [26,27]. Furthermore, because of the relative dehydration, the observed increase in solute-free water reabsorption and the upregulation of AQP2 and p-AQP2 in the present study as well as in the previous studies [19,20], plasma AVP levels are most likely elevated in these animals.

While plasma aldosterone levels were significantly increased in the DM group to more than four times that in controls, the fractional excretion of sodium was significantly increased. Importantly, the ratio of potassium to sodium in the urine, an index of aldosterone-induced ENaC-like activity in the kidney, was significantly reduced. Moreover, the transtubular potassium gradient and CCD potassium concentration were also significantly reduced, indicating the blunted effects of aldosterone on the sodium reabsorption and potassium secretion in the distal tubule and collecting duct. Taken together, these results point towards a reduced sensitivity to the anti-natriuretic action of aldosterone in the diabetic kidney.

Altered ENaC subunits protein expression in kidneys from diabetic rats

We demonstrated changes in ENaC protein expression in different kidney zones in DM. The α -ENaC expression was unchanged in the cortex and ISOM, with a decrease in the IM. In contrast, β - and γ -ENaC (85 kDa) expression was increased in the cortex; however, no shift in the molecular weight of γ -ENaC from 85 to 70 kDa was observed in any region. The β -ENaC expression was also increased in the ISOM with no change in the IM, while γ -ENaC was somewhat increased in both the ISOM and the IM. These changes could be consistent with a response to increased plasma AVP levels, since AVP has been shown *in vivo* to increase β - and γ -ENaC protein expression in water-restricted Sprague–Dawley rats without any change in α -ENaC expression [13]. In contrast, these results were not compatible with the expected response to the increased plasma aldosterone levels, since aldosterone has been shown to cause an

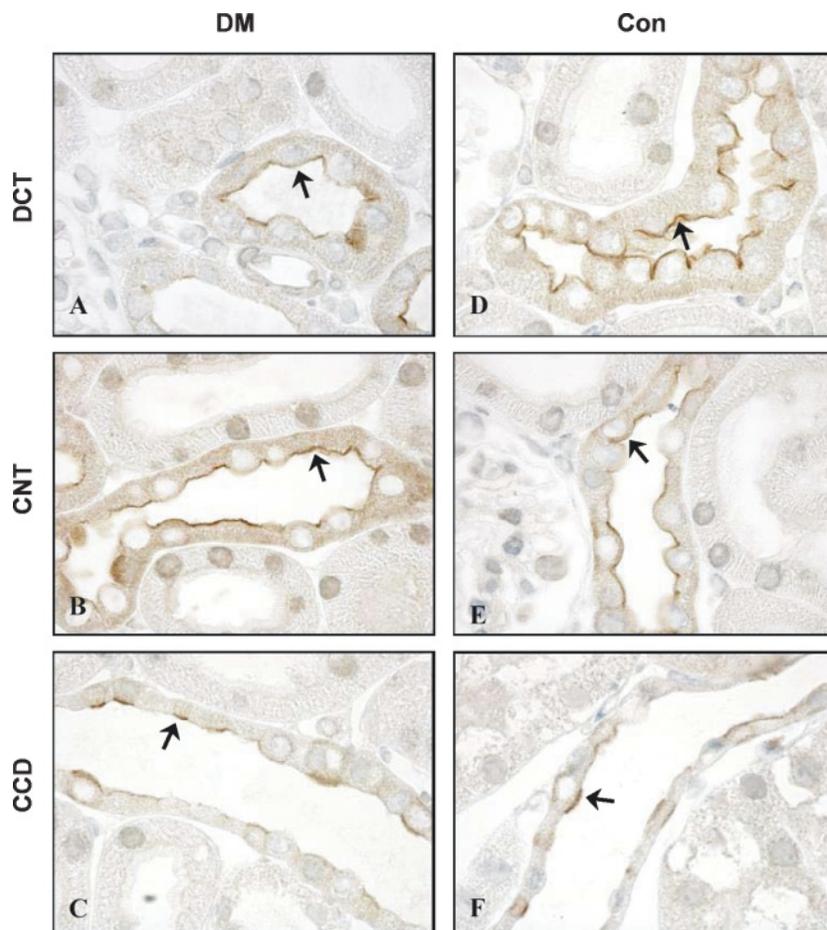


Fig. 5. Immunohistochemical analysis of α -ENaC in kidney cortex from DM (A–C; $n = 6$) and control (D–F; $n = 6$) rats. (A–F) Paraffin-embedded sections were incubated with affinity-purified anti- α -ENaC, and labelling was visualized with peroxidase-conjugated secondary antibody. α -ENaC was localized mostly to the apical plasma membrane (arrows) in both DM (A–C) and Con rats (D–F), with no change in α -ENaC labelling intensity. DCT, distal convoluted tubule (A, D); CNT, connecting tubule (B, E); CCD, cortical collecting duct (C, F).

increase in α -ENaC expression and a shift in the molecular weight of γ -ENaC from 85 to 70 kDa [11].

Apical targeting of all ENaC subunits was not increased in kidneys from diabetic rats

ENaC subunits are regulated by intracellular trafficking [29]. Increased plasma aldosterone levels have been shown to mediate a change in the subcellular localization from a dispersed cytoplasmic distribution to the apical plasma membrane domains [11,30]. Moreover, *in vitro* studies showed that vasopressin induces increased sodium reabsorption in the distal nephron cells, presumably by enhanced apical targeting of ENaC subunits [31,32]. However, importantly, we demonstrated no enhanced apical targeting of the α -, β - or γ -ENaC subunits in all renal tubular segments in DM rats, despite elevated plasma aldosterone and vasopressin levels. While α -ENaC was localized to the apical membrane in both controls and DM, increased labelling intensity was not observed. The β - and γ -ENaC were dispersed throughout the cytoplasm in controls and the cytoplasmic labelling was maintained in DM. The lack of targeting to the apical plasma membrane is not consis-

istent with the known effects of aldosterone in normal rats, indicating that kidneys in DM rats demonstrated blunted responsiveness to the effects of aldosterone, as also demonstrated by the increased fractional excretion of sodium and the reduced ratio of potassium to sodium in the urine. In contrast, the lack of targeting to the apical plasma membrane was compatible with the recent finding observed in long-term (5 days) dDAVP-treated rats [15] where β - and γ -ENaC expression was increased without enhanced apical targeting.

Thus, the results of this study may represent an aldosterone-escape phenomenon or may be due to a down-regulation or dysregulation of the mineralocorticoid receptor. In addition, other factors [e.g. atrial natriuretic peptide (ANP) or insulin deficiency] could play a role in the renal resistance to aldosterone. ANP has been shown to block aldosterone secretion and the sodium-retaining effects of aldosterone [33–35], and has been found to be increased in diabetic rats [36–38]. Furthermore, it has been shown that insulin acts synergistically with aldosterone to enhance sodium reabsorption, possibly via phosphorylation [39] or by increasing the open conformation probability [40]. Zhang *et al.* [39] demonstrated that the change in

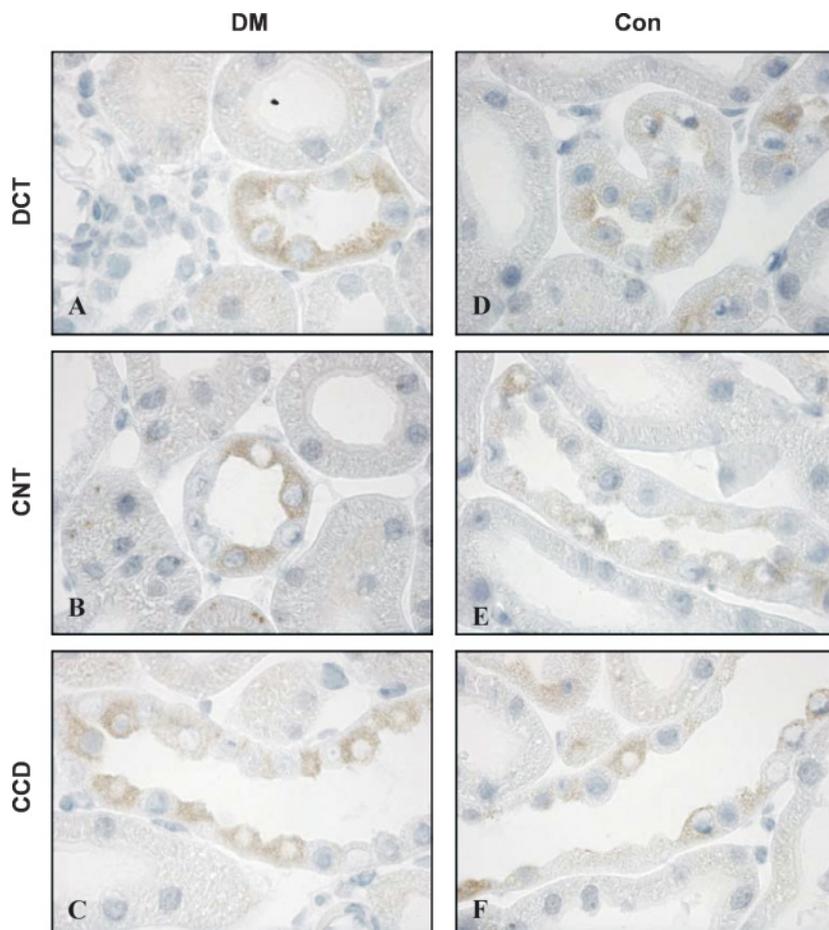


Fig. 6. Immunohistochemical analysis of β -ENaC in kidney cortex from DM (A–C; $n = 6$) and control (D–F; $n = 6$) rats. (A–F) Paraffin-embedded sections were incubated with affinity-purified anti- β -ENaC, and labelling was visualized with peroxidase-conjugated secondary antibody. β -ENaC labelling was dispersed in the cytoplasm in both DM (A–C) and Con rats (D–F). Immunohistochemistry demonstrated strong cytoplasmic β -ENaC labelling intensity in DM rats relative to Con rats, without enhanced apical targeting. DCT, distal convoluted tubule (A, D); CNT, connecting tubule (B, E); CCD, cortical collecting duct (C, F).

phosphorylation correlated with the increase in amiloride-sensitive sodium transport, indicating that insulin could play a role in the activity of ENaC. Moreover, Tiwari *et al.* [41] recently demonstrated that short-term insulin treatment *in vivo* significantly increased the expression of α -ENaC and γ -ENaC in mouse kidney homogenates. The severe insulin deficiency in STZ-induced DM rats may thus, at least in part, contribute to the severe natriuresis observed and may also be implicated in the lack of increased α -ENaC and shift of γ -ENaC to the 70 kDa band, in addition to the lack of targeting to the apical plasma membrane. Furthermore, this may explain the differences between the results of this study and the previous study, in which Song *et al.* [20] examined ENaC expression in DM rats that received insulin treatment for some of the duration of the experiment. They examined ENaC protein levels 4 days after DM [20] and it appeared that 4 days after experimentally inducing DM without insulin treatment, plasma aldosterone and all ENaC subunits were upregulated. In contrast, STZ-administered rats that were treated with insulin for 2 weeks demonstrated unchanged plasma aldosterone and vasopressin levels, and unchanged protein expression of all ENaC subunits, was

unchanged as well, despite a significant hyperglycaemia. In neither of the above studies was the apical targeting of the ENaC subunits determined. The effects of gender difference could also be considered between the two studies, since we studied female rats, while Song *et al.* [20] studied male rats. In fact, Riazi *et al.* [42] recently examined the effect of estradiol on the expression of renal sodium transporters in rats with type 1 DM. Importantly, they revealed that estradiol in ovariectomized DM rats decreased NKCC2 expression, but increased α - and β -ENaC expression compared with untreated-ovariectomized DM rats.

Upregulation of AQP2, p-AQP2 and AQP3 in kidneys of diabetic rats

AQP1 expression was unchanged, while AQP2, AQP3 and p-AQP2 protein levels were found to be upregulated in all kidney zones, with increased apical targeting of AQP2 and p-AQP2, which is consistent with elevated plasma AVP levels. AQP2 was found to have the most dramatic increase in the IM. Changes of AQP2 expression have previously been extensively investigated in the diabetic kidney. IM-tip

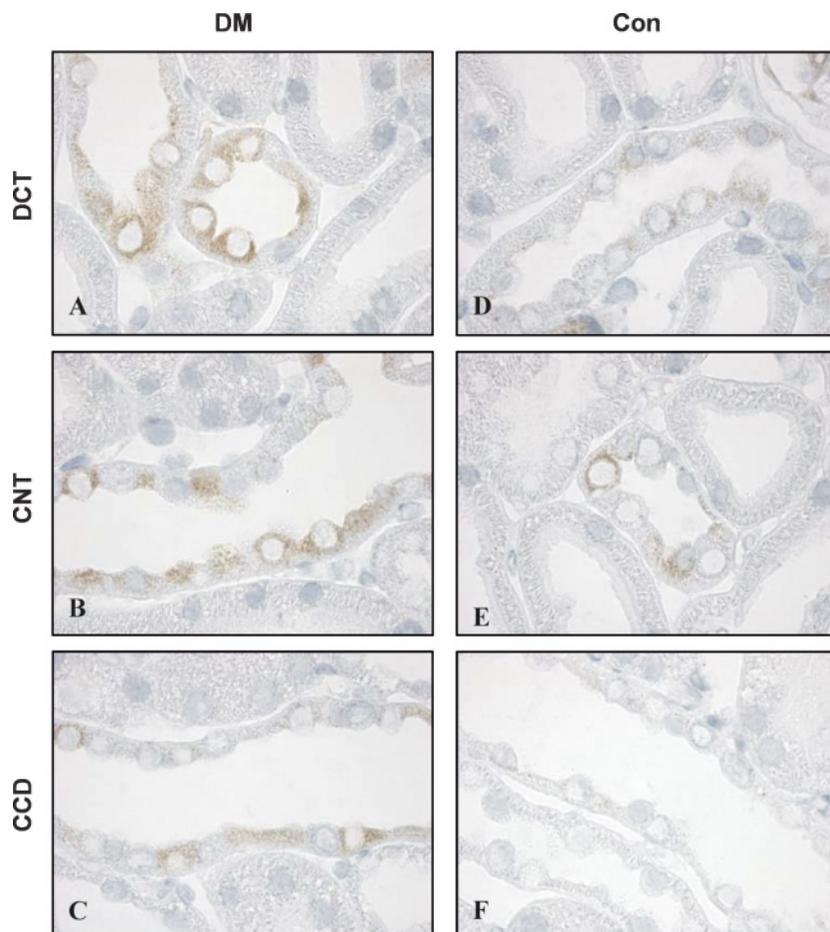


Fig. 7. Immunohistochemical analysis of γ -ENaC in kidney cortex from DM (A–C; $n = 6$) and control (D–F; $n = 6$) rats. (A–F) Paraffin-embedded sections were incubated with affinity-purified anti- γ -ENaC, and labelling was visualized with peroxidase-conjugated secondary antibody. γ -ENaC labelling was dispersed in the cytoplasm in both DM (A–C) and Con rats (D–F). Immunohistochemistry demonstrated strong cytoplasmic γ -ENaC labelling intensity in DM rats relative to Con rats, without enhanced apical targeting. DCT, distal convoluted tubule (A, D); CNT, connecting tubule (B, E); CCD, cortical collecting duct (C, F).

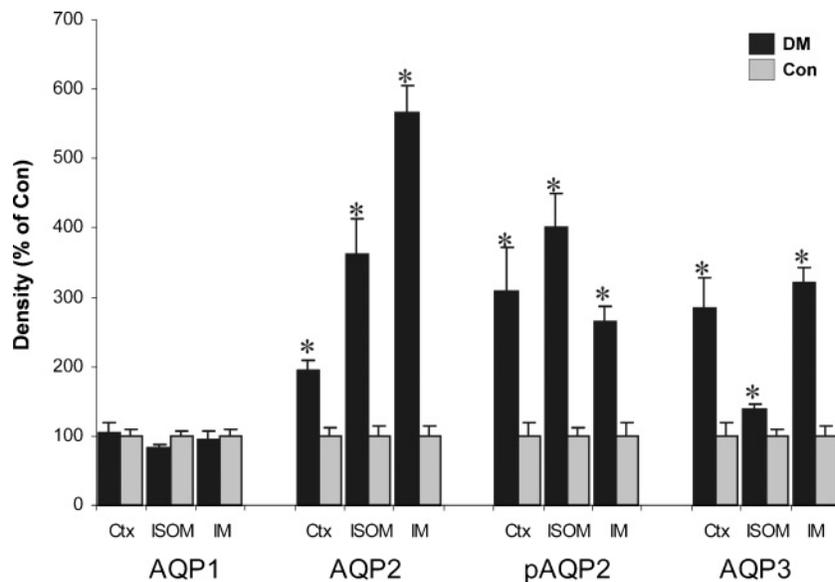


Fig. 8. Regional changes in protein expression of renal AQPs in diabetic rats. The results from semiquantitative immunoblotting of protein samples of kidney cortex, ISOM and IM are illustrated. Values are mean \pm SEM, $n = 6$ for each group. * $P < 0.05$ for DM compared to control.

expression has previously been shown to be unchanged at 3 [43] and 5 days [25] of DM, whereas upregulation has been reported after 5 days at the IM base, 10 days at the IM tip and base [25], 15 days at the whole IM [19], 20 days at the IM tip and base [25] and 21 days at the base and tip [44], consistent with our results. Bardoux *et al.* [44] also found an increase in AQP2 in the ISOM. The expression of Ser256-phosphorylated AQP2 (p-AQP2) was also increased, to ~3- to 4-fold in all kidney zones. This may reflect axial homogeneity in the renal response to the activation of the AVP receptor (V₂R) on the basolateral membrane of the collecting duct. An increase in p-AQP2 expression was also found by our previous study [19] in the IM after 15 days. AQP3 levels were also increased in all kidney zones. Like AQP2, AQP3 is thought to be upregulated in response to AVP; e.g. thirsting of rats for 48 h [18] or dDAVP treatment of Brattleboro rats for 5 days [45] have been shown to induce a marked increase in AQP3 protein expression. However, less is known of the regulation of AQP3 than AQP2. Interestingly, aldosterone has been demonstrated to play a role in the long-term upregulation of AQP3 [24]; however, it is unclear whether the increased plasma aldosterone levels played a major role in the upregulation of AQP3 in the kidneys of DM rats, where a blunted responsiveness of ENaC subunits was found in response to aldosterone in the present study.

Summary

We demonstrate that protein expression of renal AQP2, p-AQP2, AQP3, β - and γ -ENaC was increased in 2-week diabetic rats and this was consistent with the previously observed increase in plasma AVP levels in diabetic rats and consistent with the profile of AVP in regulation of ENaC subunit expression. In contrast, there was no significant increase in α -ENaC expression nor in apical targeting of all ENaC subunits despite high plasma aldosterone levels. These results were supported by the changes of the functional data showing increased solute-free water reabsorption and fractional excretion of sodium, and unchanged ratio of potassium to sodium in the urine. Taken together, the data suggest that the kidneys in untreated DM have a reduced sensitivity to the anti-natriuretic action of elevated plasma aldosterone levels with no increase in α -ENaC expression and no relative increase in ENaC subunit apical targeting, and that the AVP-mediated increase in the expression of β - and γ -ENaC alone without enhanced apical targeting may be involved in mediating increased sodium reabsorption in DM.

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Conflict of interest statement. None declared.

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