Na^+,K^+-ATPase is modulated by angiotensin II in diabetic rat kidney – another reason for diabetic nephropathy?

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Angiotensin II (ANGII) plays a central role in the enhanced sodium reabsorption in early type 1 diabetes in man and in streptozotocin-induced (STZ) diabetic rats. This study investigates the effect of untreated STZ-diabetes leading to diabetic nephropathy in combination with ANGII treatment, on the abundance and localization of the renal Na^+,K^+-ATPase (NKA), a major contributor of renal sodium handling. After 7 weeks of STZ-diabetes (I.V. 65 mg kg^{-1}) a subgroup of control (C) and diabetic (D7) Wistar rats were treated with ANGII (S.C. minipump 33 $\mu g\,kg^{-1}\,h^{-1}$ for 24 h; CA and D7A). We measured renal function and mRNA expression, protein level, Serin23 phosphorylation, subcellular distribution, and enzyme activity of NKA α-1 subunit in the kidney cortex. Diabetes increased serum creatinine and urea nitrogen levels (C *versus* D7), as did ANGII (C *versus* CA, D7 *versus* D7A). Both diabetes (C *versus* D7) and ANGII increased NKA α-1 protein level and enzyme activity (C *versus* CA, D7 *versus* D7A). Furthermore, the combination led to an additive increase (D7 *versus* D7A, CA *versus* D7A). NKA α-1 Ser23 phosphorylation was higher both in D7 and ANGII-treated rats in the non-cytoskeletal fraction, while no signal was detected in the cytoskeletal fraction. Control kidneys showed NKA α-1 immunopositivity on the basolateral membrane of proximal tubular cells, while both D7 and ANGII broadened NKA immunopositivity towards the cytoplasm. Our study demonstrates that diabetes mellitus (DM) increases the mRNA expression, protein level, Ser23 phosphorylation and enzyme activity of renal NKA, which is further elevated by ANGII. Despite an increase in total NKA quantity in diabetic nephropathy, the redistribution to the cytosol suggests the Na^+ pump is no longer functional. ANGII also caused translocation from the basolateral membrane, thus in diabetic states where ANGII level is acutely elevated, the loss of NKA will be exacerbated. This provides another mechanism by which ANGII blockade is likely to be protective.

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Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia and long-term microvascular complications in various organs, including the kidney. Diabetic nephropathy occurs in 20–40% of patients with DM and is the leading cause of end-stage renal disease (American Diabetes Association, 2005). In type 1 DM in men and in streptozotocin (STZ)-induced diabetic rats an early sign of diabetic nephropathy is glomerular hyperfiltration, which may be due to the resetting of tubular glomerular feedback and increased Na^+ reabsorption (Vallon *et al.* 1999). There are conflicting data on the relationship between type 1 DM and the renin–angiotensin–aldosterone system (RAS) (Campbell *et al.* 1999; Vallon *et al.* 1995; Zimpelmann *et al.* 2000;

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Blüher *et al.* 2001; Zhang *et al.* 2006). However, there is no doubt that acute hyperglycaemia plays a causal role in the short term RAS activation of patients with type 1 DM (Miller, 1999). It is also obvious that both systemic and glomerular hypertension caused by increased angiotensin II (ANGII) level accelerate the progression of diabetic nephropathy. It has been well established that in the proximal tubules, the main site for renal water and salt regulation, ANGII plays a critical role in the regulation of renal Na^+ and water homeostasis by increasing Na^+ reabsorption (Burns *et al.* 1993). In the renal proximal tubules Na^+ is actively reabsorbed primarily by the apical Na^+–H^+ exchanger and the Na^+–HCO_3^- cotransporter and extruded through the basolateral sodium pump Na^+,K^+-ATPase (NKA). Previous experiments demonstrated the involvement of ANGII in the regulation of subcellular abundance of Na^+–H^+ exchanger (Beutler *et al.* 2003) and Na^+–HCO_3^- cotransporter (Leong *et al.* 2006). It has been also established that a combination of high glucose and ANGII is involved in diabetic nephropathy by regulating the Na^+–glucose cotransporter activity (Lee *et al.* 2007; Vidotti *et al.* 2008). However, there have been no literary data about the combined effect of ANGII and DM on the subcellular localization of NKA, which is the key element of Na^+ transport and the driving force of the other secondary Na^+ transporters. The transporting enzyme NKA consists of a heterotetramer of two α and two β glycoprotein subunits. The α subunits are responsible mainly for the catalytic activity (Sweadner, 1989). Under physiological circumstances NKA actively secretes Na^+ into the interstitium on the basolateral membrane of renal proximal tubular cells (Yingst *et al.* 2004). This physiological localization is essential for efficient enzyme function and Na^+ reabsorption (Woroniecki *et al.* 2003).

The catalytic α subunit of NKA is phosphorylated by protein kinase (PK) C in the N-terminal at Ser11, Ser18 and Ser23 (Logvinenko *et al.* 1996), as well as by PKA in the C-terminal at Ser943 (Feschenko & Sweadner, 1995). Reversible covalent modification by phosphorylation takes part in the regulation of NKA trafficking between subcellular compartments (Chibalin *et al.* 1999; Efendiev *et al.* 2003). Furthermore, phosphorylation is generally associated with altered enzyme activity (Bertuccio *et al.* 2007; Lal *et al.* 2000), which may be in connection with altered subcellular NKA distribution. The influence of DM and ANGII on the expression and function of NKA has been studied separately in rodents, but their common effect is still not known.

In diabetic experimental models, there is controversial data about NKA with time-, dose- and tissue-specific changes. In the kidney, STZ-induced DM resulted in increased enzyme activity (Ng *et al.* 1993), while others found a biphasic time-dependent effect (Tsimarato *et al.* 2001). DM-induced alterations of renal NKA activity are only partially restored by insulin therapy (Vér *et al.* 1995), indicating that other factors, including ANGII, may also influence the enzyme function (Féraille & Doucet, 2001). There are also conflicting data about the effect of ANGII on NKA abundance and activity. While some showed increased NKA activity with ANGII treatment (Shah & Hussain, 2006), other studies revealed no or even opposite effects (Hakam *et al.* 2006; Siddiqui & Hussain, 2007). In most studies, ANGII has been administered chronically with a subpressor dose (5–15 $\mu g\,kg^{-1}\,h^{-1}$), while short-term effects and pressor doses of ANGII in the kidney are poorly defined. To our knowledge, there are only scarce data in rat kidney that used short-term ANGII in a pressor dose of 30 $\mu g\,kg^{-1}\,h^{-1}$ (Wilcox & Welch, 1990). Based on the study of Gonzalez-Villalobos *et al.* (2008) this dose would result in an approximately 1.5-fold increase in serum ANGII level. Therefore, we postulate that 24 h infusion of 33 $\mu g\,kg^{-1}\,h^{-1}$ ANGII – chosen in our experiment – would also correspond to an approximately 1.5 times higher ANGII level reported in several studies both in DM patients and STZ-diabetic rat models (men: Hollenberg *et al.* 2004; rats: Zhang *et al.* 2006; Kobayashi *et al.* 2006; Xu *et al.* 2007). In several disease states (e.g. diabetic ketoacidosis, hyperosmolar non-ketotic hyperglycaemia, acute cardiac failure, or acute exacerbation of chronic heart failure) RAS activation, as a result of acute elevation of ANGII levels, superimposes to diabetes and diabetic nephropathy. Therefore, the goal of our study was to differentiate the effect of untreated STZ-diabetes leading to diabetic nephropathy, in combination with short-term ANGII treatment, on the abundance and localization of the renal NKA, a major contributor of renal Na^+ handling.

Methods

Experimental protocol

Experiments were performed on 4-week-old male Wistar rats (weighing 100 ± 30 g). Rats were housed at a constant temperature (20°C), humidity, 12 h light–dark cycle, and were allowed free access to standard chow and water. All experimental protocols were in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKEB 99/94).

STZ-induced diabetes, ANGII treatment and experimental groups. Rats were rendered diabetic with STZ (65 $mg\,kg^{-1}$ I.V., Sigma Chemical Co.) dissolved in 0.1 M citrate buffer (pH 4.5). Only STZ-treated animals with plasma glucose concentrations above 15 $mmol\,l^{-1}$ were

considered diabetic and included in the study. All experiments were performed 7 weeks after DM induction.

Seven weeks after the induction of DM (defined as day 0), an osmotic minipump (containing ANGII, pumping rate: 24 h, 33 $\mu g\,kg^{-1}\,h^{-1}$ was implanted subcutaneously under pentobarbital anaesthesia (40 $mg\,kg^{-1}$ pentobarbital sodium, Abbott Laboratories). After 24 h of ANGII treatment the animals were re-anaesthetized, blood and post mortem urinary samples were collected; the kidneys were removed and immediately snap-frozen for further investigations. Sham-operated, vehicle-only treated rats served as controls. The experimental protocol consisted of four groups: (1) control (C); (2) control treated with ANGII (CA); (3) STZ-induced 7 week-diabetic rats (D7); (4) STZ-induced 7 week-diabetic rats treated with ANGII (D7A) ($N = 6$, in each group) (Ruzicska *et al.* 2004).

Measurement of general and laboratory (metabolic and renal) parameters

Body and kidney weight were measured to calculate kidney/body weight ratio. Serum and urinary sodium, potassium levels, and renal parameters (blood urea nitrogen (BUN) and creatinine) were photometrically determined with commercially available kits (Boehringer-Mannheim Diagnostic Systems) on a Hitachi-917 automated spectrophotometer. Non-fasting serum glucose concentration was measured using a reagent kit from Boehringer-Mannheim. Serum fructosamine concentration was measured using a kit from Roche Diagnostics Ltd.

Fractional sodium excretion was calculated as follows:

$$(\text{Sodium}_{\text{urinary}} \times \text{Creatinine}_{\text{plasma}}) / (\text{Sodium}_{\text{plasma}} \times \text{Creatinine}_{\text{urinary}}) \times 100.$$

Measurement of mean arterial blood pressure

Mean arterial blood pressure (MAP) was monitored with TL11M2-C50-PXT radiotelemetry transmitters (Data Sciences International) implanted in the peritoneal cavity of conscious, freely moving rats. Under sterile conditions, in anaesthetized rats (pentobarbital sodium, 40 $mg\,kg^{-1}$, I.P. Abbott Laboratories) a catheter was introduced into the abdominal aorta and the body of the transmitter was sutured to the abdominal wall. After surgery the animals were treated with a single dose of antibiotic (1 $mg\,kg^{-1}$ I.M., Tardomyocel, Bayer AG).

A post-operative period of at least 7 days was allowed for the animals to recover completely. RLA1000 receivers placed under each animal's cage detected radio signals emitted by the transmitters. The data were collected, and evaluated using Dataquest IV Software (Data Sciences International). The computer was set to sample the parameters for 10 s every other minute. Both parameters were averaged for 30 min periods using the 'Sort Utility' of the Dataquest IV System. The upper and lower limits of the evaluating routine were set to exclude biologically improbable values.

RT-PCR reaction

All reagents, enzymes and isolation kits were purchased from Qiagen GmBH. Total RNA extraction, first-strand cDNA synthesis and PCR reaction for NKA α-1 and glyceraldeyde-3-phosphate dehydrogenase (GAPDH) were performed as previously described (Fekete *et al.* 2004).

Tissue homogenization and Western blot analysis

Protein determinations were performed in triplicate by Bradford analysis using bovine serum albumin as a standard. All reagents for PAGE and Western blot were purchased from Sigma Chemical Co.

Tissue homogenization and cellular protein fractionation. A sample of kidney cortex (100 mg) was homogenized in chilled extraction buffer (60 mM Hepes, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, 100 mM NaF, 0.5 mM PMSF, 0.75 $mg\,l^{-1}$ leupeptin and 0.1 mM DTT) using a Potter-Elvehjem homogenizer. The total renal cortical tissue homogenate (TOT) was centrifuged at 680 *g* for 5 min at 4°C and stored at −80°C for further analysis. Triton X-100 extraction fractionates the cellular pool of NKA into an insoluble pellet (cytoskeletal-associated fraction) and a soluble supernatant by simple differential centrifugation. This method has been used in several studies as a reproducible marker for NKA subcellular distribution (Bidmonn *et al.* 2000; Aufricht *et al.* 2002; Fekete *et al.* 2004). Therefore, cold Triton X-100 (0.1%, 4°C) was added to the extraction buffer for cellular protein fractionation: another 100 mg of renal cortical tissue was homogenized in this chilled buffer and then centrifuged at 35000 *g* for 15 min at 4°C to separate the Triton-soluble fraction (detergent soluble (DS), non-cytoskeletal) from the Triton-insoluble pellet (detergent resistant (DR), cytoskeletal). DR was resuspended in extraction buffer and both fractions (DR and DS) were saved at −80°C.

Western blot analysis. NKA Western blot analysis was performed as previously described, with monoclonal antibodies to NKA α-1 (Upstate Biotechnology) diluted to 1 : 750 (Fekete *et al.* 2004). The phosphorylation of NKA was verified by detecting the changes in the immunoreactivity to a phosphorylation state-specific Ser23 antibody (Santa Cruz Biotechnology) that had

Table 1. General and laboratory parameters in samples of control (C), ANGII-treated control (CA), 7 week streptozotocin-diabetic (D7), and ANGII-treated D7 (D7A) rats

	C	CA	D7	D7A
Body weight (g)	390 ± 37	393 ± 42	$280 \pm 32^{+}$	$273 \pm 38^{++}$
Kidney weight (g)	1.9 ± 0.4	1.8 ± 0.4	2.0 ± 0.4	2 ± 0.4
Kidney/body weight	0.4 ± 0.1	0.4 ± 0.1	$0.7 \pm 0.3^{+}$	$0.7 \pm 0.4^{++}$
Non-fasting blood glucose (mM l^{-1})	8.6 ± 1.4	8.2 ± 1.7	$30.9 \pm 2.5^{+}$	$27.9 \pm 3.2^{++}$
Fructosamine (μM l^{-1})	137 ± 4	143 ± 4	$252 \pm 8^{+}$	$265 \pm 32^{++}$
Serum sodium (mM l^{-1})	149 ± 7	142 ± 4	145 ± 10	143 ± 8
Serum potassium (mM l^{-1})	4.7 ± 0.1	4.9 ± 0.2	4.9 ± 0.7	$5.5 \pm 0.6^{++}$
Serum creatinine (μM l^{-1})	68 ± 5	65 ± 2	$81 \pm 10^{+}$	$97 \pm 5^{++}$
Blood urea nitrogen (mM l^{-1})	4.7 ± 0.3	$7.5 \pm 1.3^{*}$	$10 \pm 1.9+^{**}$	$21 \pm 3.0^{++}$
Urinary sodium (mM l^{-1})	69 ± 11	55 ± 27	$35 \pm 8+^{**}$	$13 \pm 1^{++}$
Urinary potassium (mM l^{-1})	38 ± 17	35 ± 30	16 ± 7	22 ± 3
Urinary creatinine (μM l^{-1})	1156 ± 231	$573 \pm 300^{*}$	$285 \pm 173+^{**}$	$175 \pm 96^{++}$
Urinary urea nitrogen (mM l^{-1})	83 ± 44	95 ± 63	$69 \pm 22^{**}$	$181 \pm 26^{++}$
Fractional sodium excretion (%)	2.8 ± 0.6	$4.5 \pm 0.3^{*}$	$8.1 \pm 0.3^{+}$	$6.3 \pm 0.3^{++}$
Mean arterial pressure (mmHg)	98.7 ± 1.7	$125.2 \pm 2.4^{*}$	100.7 ± 1.6	109.1 ± 5.3

$N = 6$ per group. $^{*}P < 0.05$, C *vs* CA; $^{**}P < 0.05$, D7 *vs* D7A; $^{+}P < 0.05$, C *vs* D7; $^{++}P < 0.05$, CA *vs* D7A.

been subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were developed with enhanced chemiluminescence Western blotting detection (AP-Biotech). Computerized densitometry of the specific bands was analysed with Gel-Pro Analyser 3.1 software. The values were normalized to β-actin (Sigma Chemical Co.) as an internal standard and expressed as relative optical density.

NKA enzyme activity

NKA activity was assayed in TOT renal cortical tissue homogenate by measuring the strophantidine sensitive 3-O-methylfluoresceinphosphatase activity (Vér *et al.* 1995, 1997). Briefly, the activity was determined in the presence of 19.5 mmol l^{-1} 3-O-methylfluorescein-phosphate, 4 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} EDTA, 80 mmol l^{-1} Tris-HCl (pH 7.6), 10 mmol l^{-1} KCl and 100 μg TOT homogenate, and pre-incubated with 0.1% Na-deoxycholate (pH 7.4) for 30 min at 24°C. Inhibition percentages were calculated by comparing the activities in the presence of 5 mmol l^{-1} strophanthidine. NKA activity represents the difference between the activity with or without strophantidine in units of μmol fluorescein (mg protein)$^{-1}$ h^{-1}.

Fluorescent immunohistochemistry

Kidney sections were immediately snap-frozen in 30% sucrose for immunohistochemical analysis. They were embedded in Shandon cryomatrix (ThermoElectron Co.), cut into 5–10 μm thick slices with a cryostat, and stored at −80°C. Slides were washed in phosphate-buffered saline (PBS) for 10 min and were incubated for 2 h at room temperature with the NKA α-1 antibody (diluted to 1 : 100). After repeated washing, slides were incubated with the appropriate secondary antibody (Alexa Fluor 488, Invitrogen) for 30 min at room temperature. DNA was stained with Hoechst 33342 (Sigma) for 10 min at room temperature. Slides were then rinsed in PBS and covered with Vectashield fluorescent mounting medium (Vector Laboratories). Appropriate controls were performed by omitting the primary antibodies to ensure the same specificity with Western blot methods and to avoid auto-fluorescence. Confocal images were taken on a Zeiss Axiovert LSM510.

Statistical analysis

Data were analysed on STATISTICA.6 software (StatSoft Inc.). Data are presented as means ± S.D., and were tested for normal distribution with a Kolmogorov–Smirnov test. Multiple comparisons and possible interactions were evaluated by two-way ANOVA followed by Scheffe correction as a *post hoc* test. For non-parametrical data the Kruskal–Wallis ANOVA on ranks was used. Criterion for significance was $P < 0.05$ in all experiments.

Results

Effect of STZ-diabetes and ANGII treatment on metabolic and renal parameters

General, metabolic and renal parameters are summarized in Table 1. Seven weeks after the induction of diabetes, D7 rats had lower body weight *versus* controls ($P < 0.05$ C *versus* D7; CA *versus* D7A, respectively.). Non-fasting

blood glucose and fructosamine levels were elevated in D7 animals compared to controls ($P < 0.05$ C *versus* D7; CA *versus* D7A, respectively), while ANGII treatment had no further effect on these metabolic parameters. Kidney weight to body weight ratio, serum creatinine and BUN were higher in diabetic *versus* control rats ($P < 0.05$ C *versus* D7; CA *versus* D7A, respectively) suggesting the development of diabetic nephropathy. Both STZ-diabetes and ANGII treatment increased fractional sodium excretion values compared to controls ($P < 0.05$ C *versus* D7; C *versus* CA, respectively). Elevated MAP was observed after ANGII treatment in control rats (C *versus* CA, $P < 0.05$), while D7 animals showed trends of MAP increase.

Effect of STZ-diabetes and ANGII treatment on the mRNA expression, protein level of α-1 subunit and NKA enzyme activity in renal cortical tissue homogenates

NKA α-1 subunit mRNA expression (Fig. 1*A*) and protein level (Fig. 1*B*) increased in D7 rats ($P < 0.05$, C *versus* D7, CA *versus* D7A, respectively). ANGII administration further elevated protein levels ($P < 0.05$,

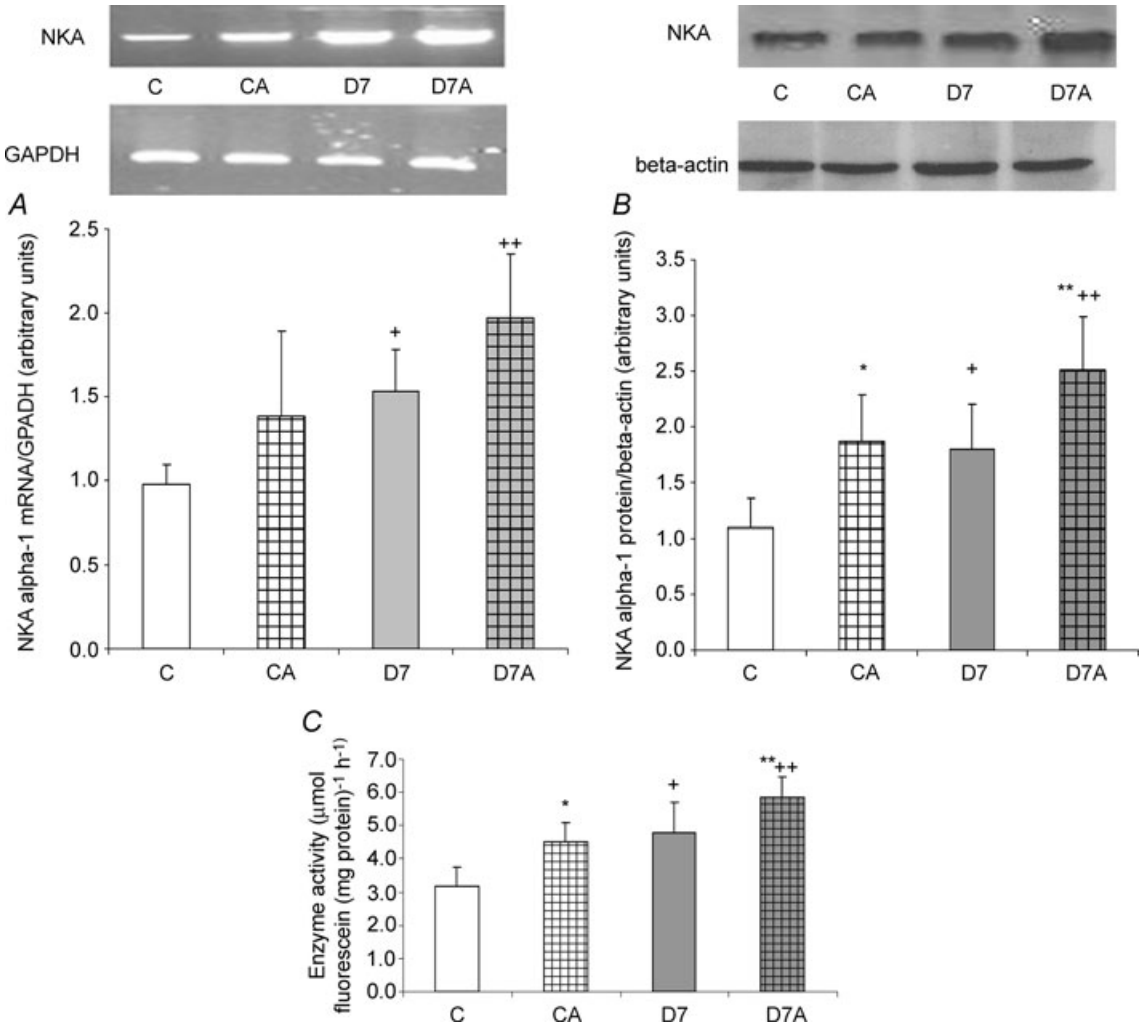

Figure 1. mRNA expression, protein level of Na+,K+-ATPase (NKA) α-1 subunit and enzyme activity in cortical tissue homogenates of angiotensin II (ANGII)-treated and streptozotocin diabetic kidneys
mRNA expression (*A*), protein level (*B*) and enzyme activity (*C*) were determined in kidney samples of control (C), ANGII-treated control (CA), 7 week diabetic (D7) and ANGII-treated D7 (D7A), rats ($N = 6$ per group). Top panel: representative examples of RT-PCR and Western blot analysis. Results are given as a ratio of intensity of NKA α-1 subunit and GAPDH mRNA, as a ratio of optical density of NKA α-1 subunit and β-actin and the difference between the activity with or without strophantidine in units of μmol fluorescein (mg protein)$^{-1}$ h^{-1}, respectively. mRNA and protein α-1 subunit: $^{*}P < 0.05$, C *vs* D7; $^{**}P < 0.05$, CA *vs* D7A. Enzyme activity: $^{*}P < 0.01$, C *vs* CA; $^{**}P < 0.05$, D7 *vs* D7A; $^{+}P < 0.0001$, C *vs* D7.

C *versus* CA, D7 *versus* D7A, respectively). In accordance with these, enzyme activity (Fig. 1*C*) was also increased in STZ-diabetic and ANGII-treated rats ($P < 0.0001$, C *versus* D7; $P < 0.001$, C *versus* CA; $P < 0.01$, D7 *versus* D7A, respectively).

Effect of STZ-diabetes and ANGII treatment on the subcellular distribution of NKA α-1 subunit

To determine whether STZ-diabetes and ANGII treatment influenced subcellular distribution of NKA, Triton X-100 extraction was used to divide TOT renal cortical tissue homogenate into Triton-soluble (DS) and Triton-insoluble (DR) fractions.

α-1 subunit in DS. Similarly to TOT homogenates, in the cytosol-representing DS fraction, protein level of NKA α-1 subunit was higher in D7 rats ($P < 0.002$, C *versus* D7; $P < 0.05$, CA *versus* D7A, respectively; Fig. 2*A*), which was further increased by ANGII treatment both in control and diabetic animals ($P < 0.01$, C *versus* CA; D7 *versus* D7A, respectively).

α-1 subunit in DR. In the DR fraction no significant changes were observed in any of the groups (Fig. 2*B*).

α-1 subunit protein level in the DS and DR fractions expressed as the percentage of TOT homogenates

Although the absolute amount of NKA α-1 protein levels were similar in the DR fraction of all groups, its relative value expressed as the percentage of TOT was lower both in diabetic and ANGII-treated animals (Fig. 2*C*). In

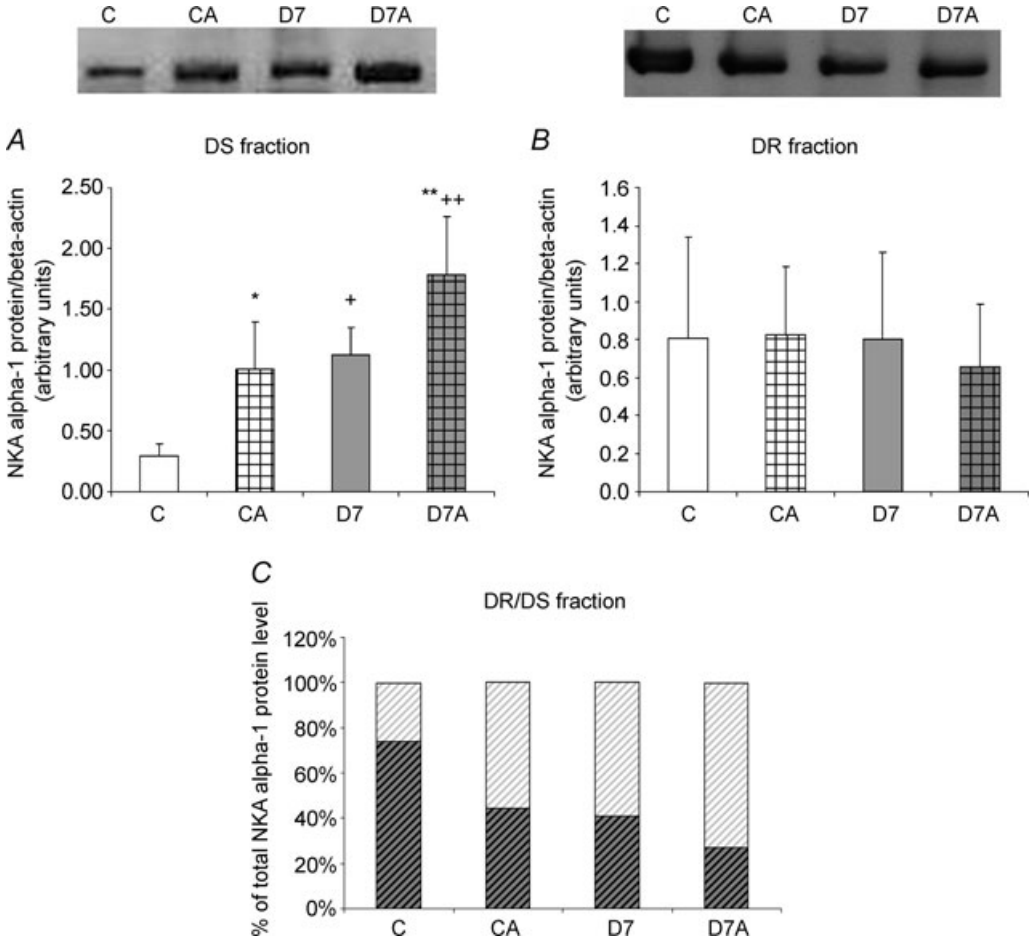

Figure 2. Protein levels of Na^+,K^+-ATPase (NKA) α-1 in (*A*) detergent-soluble (DS) and (*B*) detergent-insoluble (DR) fraction of angiotensin II (ANGII)-treated and streptozotocin-diabetic kidneys and (*C*) DR/DS ratio

Protein level of NKA α-1 subunit was measured in kidney samples of control (C), ANGII-treated control (CA), 7 week diabetic (D7) and ANGII-treated D7 (D7A) rats ($N = 6$ per group). Top panel: representative examples of Western blot analysis. Results are given as a ratio of optical density of NKA α-1 subunit and β-actin protein level. NKA α-1 protein level ratio of DR (dark hatching)/DS (light hatching) fraction is expressed in the percentage of TOT. $^*P < 0.05$, C *vs* CA; $^{**}P < 0.05$, D7 *vs* D7A; $^+P < 0.05$, C *vs* D7; $^{++}P < 0.05$, CA *vs* D7A.

parallel, with these the relative fraction of DS increased demonstrating a translocation between these subcellular compartments.

Effect of STZ-diabetes and ANGII treatment on Ser phosphorylation of Na+,K+-ATPase α-1 subunit

Ser23 phosphorylation was only detectable in the DS fraction. Ser23 phosphorylation of NKA α-1 increased in diabetes ($P < 0.05$, C *versus* D7, CA *versus* D7A, respectively, Fig. 3). ANGII treatment increased Ser23 phosphorylation both in control and D7 rats ($P < 0.05$, C *versus* CA, D7 *versus* D7A, respectively). There were no changes in the state of Ser943 phosphorylation in any of the groups or fractions (data not shown).

Fluorescent immunohistochemistry

Figure 4*A*–*C* shows representative pictures of superficial cortex marked by the presence of glomeruli (GL). Control kidney NKA α-1 stained heavily in cortical thick ascending limbs (cTAL). Weaker staining was seen mainly in the basolateral surface of intact proximal tubular epithelial cells (PT) (Fig. 4*A*). NKA staining and distribution were not affected by ANGII administration in slightly stained proximal and heavily stained distal convoluted tubules compared to controls; however, the basal membrane was slightly widened (Fig. 4*B*). In D7 kidneys, broadened staining into the cytosol and towards the apical surface was seen in the markedly sustained proximal tubular cells (Fig. 4*C*). D7A kidneys showed a patchy distribution of stain. Some of the tubular cells showed marked NKA immunopositivity along the whole width of severely damaged proximal tubular cells, while there were other cells without any staining (Fig. 4*D*).

Discussion

The pathogenesis of diabetic nephropathy and end-stage renal failure is clearly multifactorial. Both hyperglycaemia and hypertension are major contributing factors in the progression of kidney disease, which are often simultaneously present in diabetic patients. Moreover, the impact of these two factors may be different during the development and progression of diabetic nephropathy and there are several clinical situations such as diabetic ketoacidosis, hyperosmolar non-ketotic hyperglycaemia, acute cardiac failure, etc., when acute RAS activation, as a result of acute elevation of ANGII, superimposes to DM and diabetic nephropathy.

The STZ-treated rat is a widely used animal model of type 1 DM. It is associated with severe hyperglycaemia and carbohydrate disorder that we also observed in the present study. In line with previous findings (Vallon *et al.* 1999; Marwaha *et al.* 2004; Hakam *et al.* 2006), diabetic rats were shown to have higher serum creatinine and BUN levels, and increased kidney/body weight ratio, in addition to elevated urinary creatinine, urea nitrogen levels, and increased fractioned sodium excretion. All these results strongly suggest the presence of structural and functional kidney damage. ANGII treatment resulted in hypertension, reflected by higher MAP in CA animals and also deterioration of renal function.

The first important finding is that STZ-diabetes along with ANGII treatment led to a 'superimposed' nephropathy with a supposed diabetic and RAS-activated factor. These animals had severely damaged kidney function (serum creatinine and BUN levels increased in parallel with elevated serum potassium and fractional sodium excretion). MAP also tended to be higher in D7A animals, though not significantly, which can be explained also by severe diabetic exsiccosis, as reflected by a high BUN. However, it is also well known that DM leads to cardiac, vascular and renal malfunction, which in turn alters MAP, heart rate and cardiovascular homeostasis as a whole. In the present study the lack of MAP increase in D7A rats might be also explained by malfunctioning pressor responses, as it was also seen in several other experiments using animal models of type 1 DM (Yu & McNeill, 1992; Fitzgerald & Brands, 2000; Ishikawa *et al.* 2004). This phenomenon can be detected as early as 3 weeks (Nagareddy *et al.* 2005), or as late as 7 weeks after the induction of DM, as it was seen in our experiment. However, the main aim of our experiments was not to

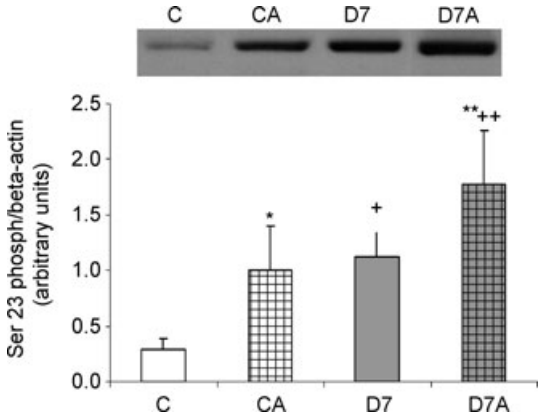

Figure 3. Serine23 phosphorylation of Na+,K+-ATPase (NKA) α-1 in angiotensin II (ANGII)-treated streptozotocin-diabetic kidneys
Serin23 phosphorylation was determined in the detergent-soluble fraction of kidney samples of control (C), ANGII-treated control (CA), 7 week diabetic (D7), ANGII-treated D7 (D7A) rats ($N = 6$ per group). Results are given as a ratio of optical density of Ser23phospho-NKA α-1 and β-actin protein level. No signal was detected in the detergent-insoluble fraction in any of the groups. $^{*}P < 0.05$, C *vs* CA; $^{**}P < 0.05$, D7 *vs* D7A; $^{+}P < 0.05$, C *vs* D7; $^{++}P < 0.05$, CA *vs* D7A.

differentiate the direct and indirect effects of ANGII on MAP. This would need further studies, considering the fact that elevated MAP has also direct effect on kidney function, maybe through up-regulated AT1 receptors, especially in diabetes (Sechi *et al.* 1994; Yoo *et al.* 2007).

Since the majority of filtered sodium is being reabsorbed by NKA in the proximal tubule, alterations of NKA may play a key role in the development of impaired renal Na^+ handling. Previous findings on intestinal (Wild *et al.* 1999) and kidney tissues (Scherzer & Popovtzer, 2002) have reported an enhanced NKA α-1 mRNA and protein expression in linear correlation with enzyme activity in STZ-diabetic animals; however, they have not investigated the effect of ANGII. Another study demonstrated that elevated ANGII level increased NKA mRNA expression in hyperinsulinaemic diabetic rat aortas. Furthermore, ANGII administration made this effect even more pronounced (Kobayashi *et al.* 2006); however, protein level and enzyme activity have not been measured.

Our study is unique in that we investigated both the effect of untreated STZ-diabetes leading to diabetic nephropathy in combination with acute RAS activation referred by ANGII administration on renal NKA. We found that STZ-diabetes and ANGII increased NKA α-1 subunit mRNA expression and protein level, as well as the enzyme activity in renal cortex. Moreover, ANGII treatment had an additive effect on NKA in STZ-diabetic

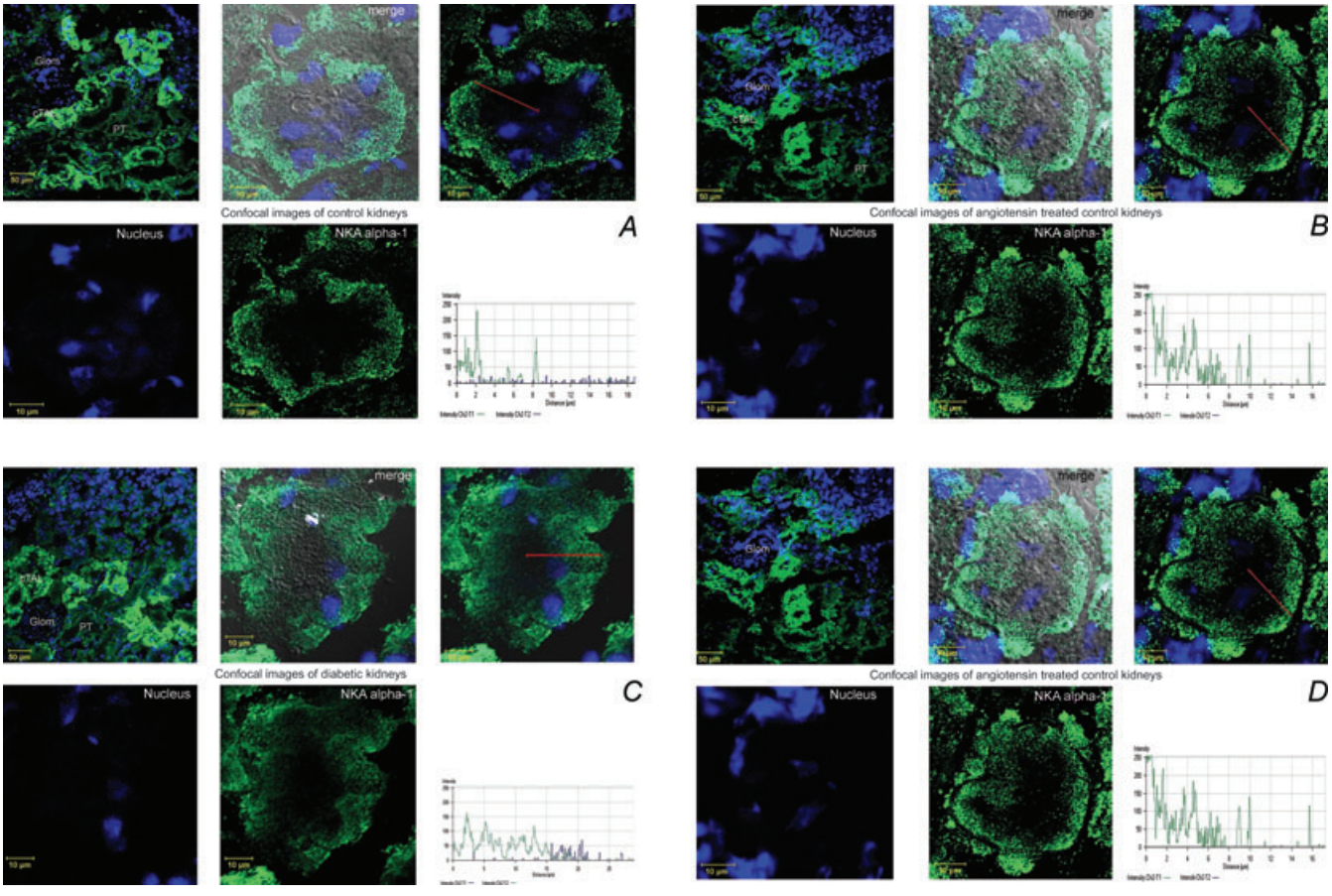

Figure 4. Confocal images of angiotensin II (ANGII)-treated streptozotocin-diabetic kidney sections labelled with Na^+,K^+-ATPase (NKA) α-1 antibody
Representative examples of NKA α-1 (green) localization in kidney sections of (*A*) control (C), (*B*) ANGII-treated control (CA), (*C*) 7 week diabetic (D7) and (*D*) ANGII-treated D7 (D7A) rats. Nuclei are stained blue. Fluorescence signal intensities of NKA α-1 (green) generated from a scanned horizontal line shown as red arrow in the merged image are shown on the bottom right of each panel. Glom, glomerulus; cTAL, cortical thick ascending limb; PT, proximal tubule. Scale bars: top left images for all panels, 50μm, all other images, 10 μm.

rats. One can hypothesize that the increased NKA expression and enzyme activity in the diabetic state and/or ANGII-related RAS activation may involve alterations in transcriptional and post-transcriptional events, and may represent an adaptive response to increased filtered and delivered load of Na^+ to the tubular epithelial cells.

Previously, a transcriptional Na^+ response mechanism has been characterized, defining a positive Na^+ response regulatory region in the NKA α-1 genes (Azuma *et al.* 1993). In vascular smooth muscles, ANGII-stimulated α-1 gene transcription is mediated through phosphatidylinositol-3 kinase and MAPK signalling (Isenovic *et al.* 2004). Therefore, it is also conceivable that similar mechanisms might be responsible for the ANGII effect in the kidney as well; however, the molecular basis should be further investigated.

In agreement with several earlier reports in the diabetic kidney (Wald & Popovtzer, 1984; Khadouri *et al.* 1987; Marwaha *et al.* 2004), we found higher NKA α-1 protein level and enzyme activity in the renal cortical tissue homogenate of STZ-diabetic rats and also after ANGII treatment. Since the absolute protein amount of cytoskeleton-representing fraction did not change significantly, one can postulate that the elevated NKA α-1 protein level observed in the total renal cortical tissue homogenate is rather a result of the higher level in the cytosol-representing soluble fraction. Our results demonstrate that both DM and ANGII promote NKA α-1 subunit translocation from the cytoskeletal fraction into the cytosol, as reflected by the DR/DS ratio. However, it is also conceivable that the newly synthesized NKA α-1 subunits have not yet reached their physiological destination on the basolateral surface of proximal tubular cells and this might be a reason for their presence in the cytosol. All these data suggest that even though the total expression and enzyme activity can be increased under experimental circumstances, the membrane-associated pump does not necessarily follow these changes.

Besides the protein amount, phosphorylation takes a major part in the regulation of NKA enzyme activity and subcellular distribution. Previous studies demonstrated that in the proximal tubule ANGII action is mediated by PKC (Rangel *et al.* 2001; Rangel *et al.* 2002). The Ser23 phosphorylation site is within a PKC motif in the NH2 terminus of α-1 and it is not phosphorylated by any other known kinases (Feschenko & Sweadner, 1995). Recent data indicate that phosphorylation of the NKA α1 subunit at Ser18 does not alter NKA activity per se, but provides the signal for the removal of NKA from the plasma membrane and endocytosis into intracellular compartments (Chibalin *et al.* 1999; Lecuona *et al.* 2006). In accordance with these data, we found a marked increment in Ser23 phosphorylation of the cytosolic α-1 subunit in both ANGII-treated and STZ-diabetic rats, indicating that the translocated NKA protein is also phosphorylated. On the contrary, no signal has been detected in the cytoskeleton-associated detergent resistant fraction.

It is conceivable that PKC in addition to directly phosphorylating NKA also phosphorylates other proteins that interact with NKA, thus altering its activity. Since the modulation of NKA by PKC is complex, the exact role of Ser23 still remains elusive (Andersson *et al.* 2004).

We confirmed the altered subcellular distribution of NKA also by confocal microscopy. In control kidneys, NKA was localized on the basolateral surface of most tubules. ANGII-treated control kidneys showed increased staining in the cytoplasm which coincided with the lack of distinct basolateral membrane in-folding. In DM, parallel with typical histological picture of diabetic nephropathy, NKA translocation was even more pronounced. ANGII treatment in DM rats led to superimposed kidney damage with markedly increased staining and altered subcellular distribution. On the basis of our results, it can be hypothesized that DM, which also changes systemic and locally produced ANGII level, provokes trafficking of NKA from the basolateral membrane into the cytoplasm of proximal tubular cells. Literary data report that ANGII stimulates rapid subcellular trafficking of other Na^+ channel in rat kidney further support our hypothesis (Sandberg *et al.* 2007); however, the question still remains as to how the translocation of NKA is controlled.

Caveolins might have a relevant role in intracellular trafficking, especially in DM: caveolin-1 and caveolae may play a dual role in the regulation of glucose homeostasis, both through a direct interaction between caveolin-1 and the insulin receptor, and as an agent for GLUT4-mediated glucose uptake (Cohen *et al.* 2003). Several other transporters and receptors, including angiotensin receptor 1, and NKA can be found associated to caveolae (Cohen *et al.* 2003; Wyse *et al.* 2003; Liu *et al.* 2002). Caveolin-dependent internalization of NKA might modulate NKA activity by regulating subcellular localization and providing a microdomain to protein–lipid or protein–protein interaction (Liu *et al.* 2002; Komers *et al.* 2006) Previous studies showed a reduced caveolin-1 protein level both in ANGII-treated and diabetic animals (Kobayashi *et al.* 2001). Based on all these observations one can also postulate that the reduced caveolin-1 level in diabetic animals might lead to altered integration of transmembrane proteins and result in impaired subcellular trafficking and activity.

In summary, our study demonstrates that: (i) DM increases the mRNA expression, protein level and enzyme activity of renal NKA, and is further elevated by ANGII treatment; (ii) Ser23 phosphorylation of the NKA α-1 subunit is higher in both DM and ANGII-treated animals, which might result in enzyme translocation and internalization from the basolateral membrane of proximal tubular cells; (iii) ANGII potentates the effect of DM

on renal NKA, which underlines the relevance of RAS blockade in diabetes therapy. This beneficial effect might be in connection with the 'conservation' of the physiological localization of NKA on the basolateral membrane of renal proximal tubules.

Owing to improving glycaemic control and stricter follow-up of diabetic patients, such high glucose levels – seen in our study – are fortunately not that common nowadays. However, it still occurs in patients of untreated or uncontrolled type 1 DM, especially in adolescents. Furthermore, due to non-compliance or dietary mistakes, also old, obese, diabetic patients with hypertension and atherosclerosis often have acute hyperglycaemia, combined with acute RAS activation caused by, for example, myocardial ischaemia (angina, infarction or arrhythmias). Our experimental model provides data supporting another mechanism by which ANGII blockade is likely to be further protective in clinical situations of acute RAS activation in diabetic patients.

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