Serum- and glucocorticoid-inducible kinase 1 in doxorubicin-induced nephrotic syndrome

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Artunc F, Nasir O, Amann K, Boini KM, Häring H-U, Risler T, Lang F. Serum- and glucocorticoid-inducible kinase 1 in doxorubicin-induced nephrotic syndrome. Am J Physiol Renal Physiol 295: F1624–F1634, 2008.—Doxorubicin-induced nephropathy leads to epithelial sodium channel (ENaC)-dependent volume retention and renal fibrosis. The aldosterone-sensitive serum- and glucocorticoid-inducible kinase SGK1 has been shown to account for the DOCA/high-salt-induced cardiac (43) and renal (3) fibrosis. However, no data are available on the functional significance of SGK1 in the volume retention and fibrosis of nephrotic syndrome.

Nephrotic syndrome is characterized by heavy proteinuria, edema, hypoalbuminemia, hyperlipidemia, and lipiduria (18). The mechanisms linking proteinuria to the development of edema are still under debate. So far, two opposing theories have been proposed to explain renal sodium retention and edema in this syndrome. According to the underfill theory, sodium retention is secondary to a reduced filling of the vascular bed due to decreased oncotic pressure and hypoalbuminemia (38). In contrast, according to the overfill theory renal sodium retention is the primary event leading to volume expansion and edema formation (24).

Nephrotic syndrome can be induced by treatment with adriamycin (doxorubicin) (6, 8, 14, 27, 34, 51). Due to the absence of immune-mediated injury and the lack of immune deposits, doxorubicin-induced nephrotic syndrome resembles human minimal change disease and focal segmental glomerulosclerosis (FSGS) (6).

The sensitivity to doxorubicin or the related substance daunomycin is variable in mice (10, 11, 28, 48, 54). In susceptible mice doxorubicin-induced nephrotic syndrome is followed by volume retention which is accounted for by enhanced activity of epithelial sodium channels (ENaC) and Na-K-ATPase in the collecting duct (15, 34). The volume retention and ascites formation in rats could not be prevented by blockade of the mineralocorticoid receptor but were completely abrogated by the ENaC inhibitor amiloride (15). Thus, the volume retention is mainly due to a mineralocorticoid-independent upregulation of ENaC activity. The signaling accounting for the stimulation of ENaC during doxorubicin-induced nephrotic syndrome remained elusive.

A potential candidate is the serum- and glucocorticoid-inducible kinase 1 (SGK1), which has originally been cloned as a glucocorticoid-inducible gene (20) and later as cell volume-regulated gene (46). SGK1 is expressed in the aldosterone-sensitive distal nephron (33) and stimulates the ENaC (7, 9, 12, 16, 22, 26, 32, 33, 35, 41), the renal outer medullary K+ channel ROMK (52), and the Na+K+-ATPase (40, 44, 53). As a matter of fact, nephrotic syndrome has previously been shown by some (8, 34) but not all (51) experimentators to upregulate SGK1 protein and mRNA expression. Even if SGK1 transcript levels remained constant, SGK1-dependent volume retention is mainly due to a mineralocorticoid-independent upregulation of ENaC activity. The signaling accounting for the stimulation of ENaC during doxorubicin-induced nephrotic syndrome remained elusive.
as well as plasma volume, plasma aldosterone, and serum albumin were determined.

METHODS

Animal experimentation. Experiments were done on SGK1 knockout mice (sgk1−/−) and their wild-type littermates (sgk1+/+), which were maintained on a 129S1/SvImJ background with minor genetic contamination from C57Bl/6. The mice were generated as described elsewhere (50). Mice were kept on a 12:12-h light-dark cycle and fed a standard diet (C1310, Altromin, Lage, measured Na⁺ content 123 μmol/g food) with tap water (measured Na⁺ content 0.8 mM) ad libitum. All animal experiments were conducted according to the guidelines of the American Physiological Society as well as the German law for the welfare of animals and were approved by local authorities.

To induce nephrotic syndrome, wild-type (n = 44) and sgk1−/− mice (n = 44) ≥6 mo of age were treated with a single intravenous injection of doxorubicin (15 μg/g body wt, adriblastin, Pfizer) into the left retroorbital venous plexus under light diethylether anesthesia. Daily samples of spontaneously voided urine were collected 3 days before (baseline) and up to 28 days following doxorubicin injection. The mice were put into metabolic cages with free access to fluid and standard diet after the end of the dark cycle at 8 AM and left until a urine sample was voided (maximally 6 PM). Before the urine collection, body weight was taken daily.

Blood samples were drawn weekly before and up to 4 wk following doxorubicin injection. To this end, animals were lightly anesthetized with diethylether and 75 μl of blood were withdrawn into a heparinized capillary by puncturing the right retroorbital plexus. In the same session, plasma volume was measured using a dye dilution technique with Evans Blue (Sigma, Taufkirchen, Germany).

Fig. 1. Urinary protein and albumin excretion following doxorubicin treatment. Arithmetic means ± SE of protein excretion (A), albumin excretion (B), and plasma albumin (C) concentration in nephrotic (squares) and nonnephrotic (triangles) sgk1−/− (open symbols) and sgk1−/− (closed symbols) mice before (baseline) and after injection of doxorubicin. D: SDS-PAGE of urinary proteins from sgk1−/− mice. Lane 1: control urine; lane 2: urine from a nonnephrotic mouse (1:20 dilution); lanes 3, 4, and 5: urine from nephrotic mice (1:50 dilution). A protein ladder is included for estimation of protein size. In control urine there are mainly low molecular weight proteins and very little albumin. In nephrotic mice, excretion of proteins >50 kDa in size is massively increased. In nonnephrotic mice, the band at 60 kDa is slightly enhanced corresponding to increased albuminuria. #Significant difference compared with baseline value. *Significant difference between nephrotic and nonnephrotic mice of the same genotype. §Significant difference between sgk1+/+ and sgk1−/− mice.
Beyond these 28 days, animals were followed for long-term survival. To avoid suffering, an animal was euthanized when it lost more than 20% weight within 2 days and showed signs of impaired well-being such as inappetence, reduced locomotor activity, or shaggy coat appearance. In that case, the animal was killed and perfusion-fixed to obtain kidneys, liver, heart, adrenal gland, and aorta for histology. Briefly, mice were anesthetized with tribromethanol (250 mg/kg body wt sc) and the infrarenal abdominal aorta was cannulated with a polyethylene catheter. The animals were then retrogradely perfused with 25 ml 0.9% NaCl and subsequently with 25 ml 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.4) and the organs were harvested.

**Determination of plasma volume.** Plasma volume was assessed weekly by a dye dilution technique using Evans Blue (Sigma). After taking 75 μl blood from the right retroorbital plexus, 75–100 μl of an Evans Blue stock solution (1.5 mg/ml in 0.9% NaCl) were injected intravenously into the left retroorbital plexus using a 30-gauge insulin syringe (BD micro-fine, Heidelberg, Germany). The exact applied volume was determined by weighing the syringe before and after injection. Two blood samples (less than 25 μl) were drawn from the right retroorbital plexus during superficial diethylether anesthesia 10 and 30 min, which yielded volumes of 8–15 μl plasma after centrifugation. Due to severe lipemia in the proteinuric animals, plasma samples had to be cleared before analysis. To this end, 100 centrifugation. To this end, 100

**Addition of 92 chloroform were added to the lipemic samples and a cleared plasma sample was recovered after centrifugation at 12,000 g for 10 min. Absorbance was measured at 620 nm against blank mouse serum after addition of 92 μl PBS (PBS tablets, Invitrogen, Karlsruhe, Germany).

Plasma concentrations of Evans Blue were calculated using the stock solution dissolved in mouse serum as a standard. To correct for differences in urine dilution by dividing the measured concentration by the respective urinary creatinine concentration.

**Histology.** Fixed kidneys and samples from other tissues (adrenal glands, heart, liver, aorta) were stored in 4% paraformaldehyde/0.1 M sodium phosphate buffer. Kidneys were dissected into 1-mm-thick slices perpendicular to the longitudinal axis. Using area weighted sampling 10 small pieces of the kidney cortex were selected for embedding in epon araldite. Semithin (1 μm) sections were prepared and stained with methylene blue/basic fuchsin. For electron microscopy, several ultrathin (0.08 μm) sections per animal were prepared and stained with lead citrate and uranyl acetate.

All remaining kidney slices were embedded in paraffin yielding one representative section of each slice for qualitative morphological investigations. Four-micrometer paraffin sections were cut and stained with hematoxylin/eosin (HE), periodic acid-Schiff stain (PAS), and a fibrous tissue stain (Sirius red). Sections were evaluated for signs of glomerular damage, i.e., mesangial cell and matrix expansion, focal segmental sclerosis, and podocyte damage, as well as for tubulointer-

**Measurements.** Plasma and urinary concentrations of Na⁺ and K⁺ were measured by flame photometry (AFM 5051, Eppendorf, Germany). Urinary creatinine concentrations were measured manually using a kinetic Jaffé method, and plasma urea was measured by an enzymatic method (both Lehmann, Berlin, Germany). Plasma aldosterone was measured using a RIA kit (Demeditec, Kiel, Germany). Hematocrit was measured after centrifugation.

Urine dipstick testing for proteinuria was done with the Combur dipstick (Roche, Mannheim, Germany). Urinary and plasma albumin were measured fluorometrically using the albumin-sensitive dye albumin blue 580 at 595-nm excitation and 642-nm emission on a multilabel counter (Victor 1420, PerkinElmer) according to the manufacturer’s instructions (microfluor, Progen, Heidelberg, Germany). Standard curves were generated with mouse albumin (Sigma) and measurements were performed within the linear range (0–156 mg/l).

Urineary total protein was measured quantitatively using Coomassie Brilliant Blue G-250 dye (BioRad protein assay, Hercules, CA). A standard curve was generated with bovine albumin (Sigma).

Urinary excretion of Na⁺, K⁺, albumin, and protein was corrected for differences in urine dilution by dividing the measured concentration by the respective urinary creatinine concentration.

**Fig. 2.** Body weight following doxorubicin treatment. A: arithmetic means ± SE of body weight in nephrotic (squares) and nonnephrotic (triangles) sgk1+/− (open symbols) and sgk1−/− (closed symbols) mice before (baseline) and after injection of doxorubicin. B: body weight gain calculated as the difference between peak body weight and minimal body weight at day 4 or 5 after injection. #Significant difference compared with baseline value. *Significant difference between nephrotic and nonnephrotic mice of the same genotype. §Significant difference between sgk1+/− and sgk1−/− mice.
stitial damage, i.e., tubular atrophy, tubular dilatation, interstitial inflammation, and interstitial fibrosis.

Tissues from the adrenal glands, heart, liver, and aorta were embedded in paraffin, sectioned, and stained with HE, PAS, and Sirius red for qualitative inspection. The histological analysis was done blinded, i.e., the investigator did not know genotype and in vivo data of the animals analyzed.

**Immunofluorescence.** For the analysis of SGK1 protein expression in the kidney, kidneys of nephrotic and nonnephrotic wild-type mice were studied 10, 20, and 30 days after doxorubicin injection (n = 2 each). Mice were killed by CO2 and cervical dislocation and the kidneys were rapidly frozen in liquid nitrogen. Frozen sections (3 μm) were fixed in acetone (10 min at −20°C), air dried, and stored in Tris buffer for 5 min. Then, blocking was performed with normal goat serum (blotto, 1:5, 45 min). Afterwards, the primary antibody (rabbit anti-SGK1, 1:50) was applied (1 h; 37°C) and the sections were washed in Tris buffer (3 × 5 min). Polyclonal monospecific antibodies against the SGK1 protein were raised by a commercial antibody service (Dr. Pineda, Berlin, Germany) as previously described (23). Afterwards, the secondary antibody (goat anti-rabbit, Alexa 488, 1:200) was applied for 30 min. 11β Steroid dehydrogenase type 2 was probed with a commercially available sheep anti-11β HSD2 antibody (AB 1296, Chemicon) and a secondary biotinylated anti-sheep antibody with subsequent detection by Streptavidin-Alexa 568. DAPI was used to stain nuclei (1:1,000 in distilled water for 5 min) followed by rinsing in Tris buffer (3 × 5 min). Finally, sections were covered with mowiol and analyzed.

**Statistics.** Data are provided as arithmetic means ± SE, with n representing the number of independent experiments. All data were tested for significance with parametric or nonparametric repeated-measures ANOVA, paired or unpaired Student’s t-test, or Mann-Whitney U-test where applicable using GraphPad InStat and Prism 4, GraphPad Software (San Diego, CA, www.graphpad.com). A P value <0.05 was considered statistically significant.

**RESULTS**

**Induction of nephrotic syndrome.** To elucidate whether lack of SGK1 influences the susceptibility to doxorubicin-induced

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**Fig. 3.** Urinary Na⁺ and K⁺ excretion following doxorubicin treatment. A: arithmetic means ± SE of urinary Na⁺ excretion in nonnephrotic sgk1⁺⁻ (open triangles) and sgk1⁻⁻ (closed triangles) mice before (baseline) and after injection of doxorubicin. Note significant difference in the y-intercept. B: arithmetic means ± SE of urinary Na⁺ excretion in nephrotic sgk1⁺⁻ (open squares) and sgk1⁻⁻ (closed squares) mice before (baseline) and after injection of doxorubicin. C: arithmetic means ± SE of urinary K⁺ excretion in nonnephrotic sgk1⁺⁻ (open triangles) and sgk1⁻⁻ (closed triangles) mice before (baseline) and after injection of doxorubicin. Note significant difference in the y-intercept. D: arithmetic means ± SE of urinary K⁺ excretion in nephrotic sgk1⁺⁻ (open squares) and sgk1⁻⁻ (closed squares) mice before (baseline) and after injection of doxorubicin. #Significant difference compared with baseline value. *Significant difference between nephrotic and nonnephrotic mice of the same genotype. §Significant difference between sgk1⁺⁻ and sgk1⁻⁻ mice.
nephrotic syndrome, the effect of doxorubicin (15 μg/g body wt) was studied in gene-targeted mice lacking SGK1 (sgk1−/−) compared with their wild-type littermates (sgk1+/+). After intravenous injection of a single dose of 15 μg/g body wt doxorubicin, heavy proteinuria developed in 15 of 44 treated sgk1+/+ mice as evidenced by a threefold positive urine dipstick test on day 4 after injection. Due to acute toxicity within 7 days, seven mice had to be euthanized. The remaining mice (n = 22) showed no or mildly positive proteinuria. In sgk1−/− mice, doxorubicin treatment (15 μg/g body wt) was followed by heavy proteinuria in 15 of 44 treated mice. Ten mice had to be euthanized due to acute toxicity leaving n = 19 nonnephrotic sgk1−/− mice. Based on these responses to doxorubicin treatment, the animals were divided a posteriori into groups with heavy proteinuria (n = 15 sgk1+/+ and 15 sgk1−/−), which developed severe nephrotic syndrome and the remaining mice (n = 22 sgk1+/+ and 19 sgk1−/−) with nonnephrotic proteinuria.

Before doxorubicin treatment, urinary protein excretion was similar in sgk1+/+ mice (7.0 ± 0.7 mg protein/mg creatinine) and sgk1−/− mice (7.1 ± 0.7 mg protein/mg creatinine). After doxorubicin injection, urinary protein excretion increased to a similar extent in nephrotic sgk1+/+ and sgk1−/− mice and, on day 11 after injection, reached 116 ± 19 mg protein/mg creatinine in sgk1+/+ mice and 125 ± 17 mg protein/mg creatinine sgk1−/− mice. As shown in Fig. 1A, this increase was sustained throughout the study. In the remaining nonnephrotic mice, urinary protein excretion tended to increase (17 ± 7 mg protein/mg creatinine in sgk1+/+ mice and 11 ± 6 mg protein/mg creatinine sgk1−/− mice), an effect escaping statistical significance.

In addition to urinary protein excretion, daily urinary albumin excretion was measured as a marker of glomerular proteinuria. Urinary albumin excretion was low in untreated sgk1+/+ mice (0.31 ± 0.02 mg albumin/mg creatinine) and sgk1−/− mice (0.34 ± 0.04 mg albumin/mg creatinine). On day 11 after injection, urinary albumin excretion increased significantly to 87 ± 9 mg albumin/mg creatinine in sgk1+/+ mice and to 87 ± 10 mg albumin/mg creatinine in sgk1−/− mice corresponding to >300-fold increases. In the remaining nonnephrotic mice, urinary albumin excretion increased significantly to 12 ± 5 mg albumin/mg creatinine in sgk1+/+ mice and to 5 ± 4 mg albumin/mg creatinine in sgk1−/− mice on day 11 after doxorubicin injection (Fig. 1B). Albuminuria was not significantly different between sgk1+/+ and sgk1−/− mice (Fig. 1B).

Figure 1C depicts the course of plasma albumin concentration. At baseline conditions, plasma albumin concentration was significantly higher in untreated sgk1+/+ mice (3.0 ± 0.2 g/dl) than in sgk1−/− mice (2.4 ± 0.1 g/dl). After doxorubicin injection, plasma albumin concentration declined slightly in nonnephrotic mice of both genotypes, whereas a sharp drop was seen in the nephrotic groups of both genotypes after the first week.

Electrophoresis of urinary proteins using SDS-PAGE revealed that proteinuria in nephrotic mice consisted of mainly albumin but also proteins sized ~80 kDa (Fig. 1D). In nonnephrotic mice, a slight increase in the albumin band was detectable without any additional band.

Course of body weight, urinary Na+, and urinary K+ excretion. The course of the body weight is shown in Fig. 2A. Baseline body weight was similar in both genotypes (32.6 ± 0.5 g in sgk1+/+ and 33.5 ± 0.9 g in sgk1−/− mice) and dropped during the first 4 days after doxorubicin injection by −2.2 ± 0.3 g in sgk1+/+ mice and by −2.5 ± 0.3 g in sgk1−/− mice. In nonnephrotic sgk1+/+ and sgk1−/− mice, body weight stabilized and remained constant throughout the whole study, yet stayed below the baseline body weight. In nephrotic sgk1+/+ and sgk1−/− mice, marked body weight gains were observed that sharply peaked between days 9 and 10 after injection and were accompanied by severe ascites formation. Interestingly, between days 11 and 14, the body weight gain was completely reversed. The maximal body weight gain calculated as the difference between peak and minimal body weights was significantly higher in sgk1+/+ (+6.6 ± 0.7 g) compared with sgk1−/− mice (+4.1 ± 0.8 g; Fig. 2B). During the course of nephrotic syndrome, sgk1−/− mice consistently showed lower body weight than sgk1+/+ mice.

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**Fig. 4.** Plasma volume, hematocrit, and plasma aldosterone concentrations following doxorubicin treatment. Arithmetic means ± SE of plasma volume (A), hematocrit (B), and plasma aldosterone concentration (C) in nephrotic (squares) and nonnephrotic (triangles) sgk1+/+ (open symbols) and sgk1−/− (closed symbols) mice before (baseline) and after injection of doxorubicin. *Significant difference compared with baseline value. §Significant difference between nephrotic and nonnephrotic mice of the same genotype.
Sodium handling during nephrotic syndrome was studied by daily collection of spot urine. Under baseline conditions urinary Na⁺ excretion was significantly higher in sgk1⁻/⁻ mice (494 ± 28 µmol/mg creatinine) than in sgk1⁺/⁺ mice (394 ± 21 µmol/mg creatinine) consistent with a salt-loosening phenotype. After doxorubicin injection, urinary Na⁺ excretion in nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice showed day-to-day variations but remained in the range of the baseline value (Fig. 3A). Linear regression did not yield a slope significantly different from zero. In contrast, urinary Na⁺ excretion in the nephrotic mice dramatically dropped during the first 10 days of doxorubicin injection indicating sodium retention (Fig. 3B). Between days 9 and 11, a minimum was reached that was significantly lower in sgk1⁺/⁺ mice (15 ± 5 µmol/mg creat) than in sgk1⁻/⁻ mice (35 ± 5 µmol/mg creat). Between days 10 and 15 after injection, urinary Na⁺ excretion was strongly enhanced in both genotypes and, after day 16, it reached the range of baseline values again. Plasma Na⁺ concentrations were similar in both genotypes at baseline conditions (149 ± 1 vs. 146 ± 3 mM, respectively) and remained unchanged in nonnephrotic mice following doxorubicin treatment. In nephrotic sgk1⁺/⁺ mice, there was significant increase of plasma Na⁺ concentration at week 4 (155 ± 2 mM), whereas in nephrotic sgk1⁻/⁻ mice plasma Na⁺ concentration increased only slightly (149 ± 3 mM).

Since K⁺ excretion is coupled to Na⁺ excretion in the distal nephron, daily urinary K⁺ excretion was also determined. Under baseline conditions, daily urinary K⁺ excretion was not different between genotypes (634 ± 29 µmol/mg creatinine in sgk1⁺/⁺ and 629 ± 22 µmol/mg creatinine in sgk1⁻/⁻ mice). After doxorubicin injection, daily urinary K⁺ excretion showed large variations in nonnephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice (Fig. 3C) without a significant change over time. In contrast, linear regression of the urinary K⁺ excretion in the nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice revealed a significant increase with time that was significantly more pronounced in sgk1⁻/⁻ mice (Fig. 3D). Plasma K⁺ concentrations were not different between both genotypes at baseline conditions (4.8 ± 0.2 vs. 4.5 ± 0.2 mM), and they significantly increased in nephrotic mice of both genotypes (within 4 wk to 5.6 ± 0.4 mM in sgk1⁻/⁻ and 5.0 ± 0.2 mM in sgk1⁻/⁻ mice).

Assessment of volume status during nephrotic syndrome. Volume retention and edema formation in nephrotic syndrome have been partially explained by an underfilling of the vascular bed leading to secondary renal Na⁺ retention. To assess the filling of the vascular bed during the course of volume retention, plasma volume was measured with a dye dilution technique using Evans Blue (Fig. 4A). During baseline conditions, plasma volume was similar in sgk1⁺/⁺ mice (51 ± 2 µl/g body wt) and sgk1⁻/⁻ mice (52 ± 2 µl/g). After doxorubicin injection, plasma volume remained stable in the nonnephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice and tended to increase in the nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice. Additionally, hematocrit was measured, which was during baseline conditions not different between sgk1⁺/⁺ mice (51 ± 1%) and sgk1⁻/⁻ mice (52 ± 2%). After doxorubicin injection, hematocrit significantly decreased in the nonnephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice after 1 wk and recovered at the end of 4 wk after doxorubicin treatment (Fig. 4B). In nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice, hematocrit similarly decreased after 1 wk, but progressively decreased during the further course of the study. Under baseline conditions, plasma aldosterone concentrations were significantly higher in sgk1⁻/⁻ mice (1.026 ± 0.25 pg/ml) than in sgk1⁺/⁺ mice (288 ± 79 pg/ml) which is consistent with type 2 pseudohypoaldosteronism. As shown in Fig. 4C, plasma aldosterone levels further increased in both nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice. The plasma aldosterone levels remained, however, significantly higher in nephrotic sgk1⁻/⁻ mice than in nephrotic sgk1⁺/⁺ mice.

Renal function and survival. Serum urea concentrations were utilized to estimate renal excretory function. As shown in Fig. 5A, serum urea concentrations were similar in sgk1⁺/⁺ and sgk1⁻/⁻ mice under baseline conditions (53 ± 2 vs. 51 ± 3 mg/dl, respectively). After doxorubicin injection, serum urea concentrations remained stable in the nonnephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice. In the nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice, a progressive increase during the study was observed that was significantly more pronounced in sgk1⁻/⁻ mice (Fig. 5A).

After 28 days blood and urine collections were stopped and the animals were followed for long-term survival. Between days 25 and 61, all nephrotic sgk1⁺/⁺ animals deteriorated and were finally euthanized. In contrast, all animals of the nonne-
phrotic group remained alive and were killed for organ collection on day 60. The corresponding survival curve is given in Fig. 5B, and the median survival of the nephrotic mice was 40 days. In nephrotic sgk1−/− mice, median survival was significantly lower at 29 days (Fig. 5B).

Tissue analysis. Immunofluorescence was performed to explore whether doxorubicin-induced nephrotic syndrome affects the expression of SGK1 protein in kidneys of sgk1+/+ mice. As shown in Fig. 6, SGK1 expression was higher in nephrotic sgk1+/+ mice 10 to 30 days after doxorubicin treatment. Staining was confined to the distal nephron as confirmed by colocalization with 11β-hydroxysteroid dehydrogenase type 2. These experiments suggested a role of SGK1 in volume retention.

Histologic analysis of the kidneys from nephrotic sgk1+/+ mice revealed severe changes of glomeruli and the interstitium featuring focal segmental and global glomerulosclerosis with hyalinosis as well as massive tubulointerstitial inflammation and tubular atrophy (Fig. 7). Protein droplets were visible in the proximal tubules indicative of avid reabsorption. In non-nephrotic mice, no histologic changes were observed. Histologic analysis of the adrenal glands, heart, liver, and aorta did not show significant differences between both groups (not shown). Electron microscopy of nephrotic kidneys on day 5 of treatment revealed podocyte foot process fusion and loss (Fig. 8). Histological changes of nephrotic kidneys tended to be more severe in sgk1−/− mice than in sgk1+/+ mice. Accordingly, Sirius Red Stain showed increased fibrosis in sgk1−/− mice (Fig. 9).

DISCUSSION

In this study, we describe the induction of a full-blown nephrotic syndrome in strain 129 mice by doxorubicin featuring massive proteinuria, hypoalbuminemia, volume retention, and lipidemia. Histologically, early proteinuria coincided with normal light microscopy and podocyte changes seen in electron microscopy at day 5 after treatment, reminiscent of human minimal change disease. In agreement with the morphological injury, proteinuria in the nephrotic mice was almost completely due to albuminuria consistent with selective glomerular proteinuria. SDS-PAGE, however, detected additional proteins (Fig. 1D) with similar size as albumin (<80 kDa). Retrospectively, only animals with protein excretion >100 mg/mg crea or albuminuria >100 mg/mg crea developed nephrotic syndrome, while protein or albumin excretion in the remaining nonnephrotic mice ranged from 1 to maximally 40 mg/mg crea. In view of these data, a threshold value of >50–100 mg protein/mg crea for the development of nephrotic syndrome in mice was assumed. Doxorubicin treatment of BALB/c mice led to maximal proteinuria of 10–20 mg/24 h and no edema formation (10, 11, 45, 48). Given a daily creatinine excretion of −500 μg/day in this mouse strain (36), substantially higher proteinuria and albuminuria were achieved in our model (>50 mg/day) explaining the different course of doxorubicin-induced nephropathy in our study.

The full syndrome did not develop in all animals at the applied dose of 15 μg/g body wt doxorubicin and the remaining animals exhibited slight to moderate increases in proteinuria that might be related to genetic contamination from the
C57Bl/6 background, which is known to be resistant against doxorubicin (54). At the applied dose, 17 of 89 mice (19%) showed signs of acute systemic toxicity requiring euthanasia, and higher doses >15 μg/g body wt were associated with >50% mortality, keeping with published LD₅₀ doses for doxorubicin in mice (17 μg/g body wt) (25).

To gain insight into the mechanisms of edema formation and to distinguish between over- and underfill theories, the chromatin organization and nuclear morphology were analyzed. The results showed distinct differences between the conditions, providing evidence for the involvement of chromatin remodeling in nephrotic syndrome.

Fig. 7. Light microscopy of kidneys following doxorubicin treatment. Light microscopy of HE-stained renal tissue from nephrotic mice (right) and nonnephrotic mice (left) killed 8 wk after treatment with doxorubicin. Marked segmental and global glomerulosclerosis and tubulointerstitial inflammation with interstitial fibrosis are seen in nephrotic mice.

Fig. 8. Electron microscopy of kidneys following doxorubicin treatment. Electron microscopy of glomeruli 5 days after treatment with doxorubicin in a nephrotic (right) and a nonnephrotic (left) mouse at 3,000- and 12,000-fold magnification. Podocyte enlargement, vacuolization of cytoplasm, and foot process fusion are visible.
nological relationship between renal sodium excretion and plasma volume as well as plasma aldosterone and serum albumin was studied. After induction of nephrotic syndrome, plasma aldosterone levels were significantly enhanced in the nephritic mice compared with the nonnephritic group and serum albumin levels were markedly reduced after the first week due to renal loss. Plasma volume was not decreased at any time and tended to increase in weeks 3 and 4 which was paralleled by a drop in hematocrit. In particular, plasma volume was not lower during the period of avid sodium retention observed in the first 10 days which argues against the underfill theory. It should be kept in mind that the measurement of plasma volume does not reflect arterial filling, as the majority of the plasma volume resides in the venous system. The development of hyperaldosteronism during nephrotic syndrome is suggestive for arterial underfilling.

Surprisingly and unexpectedly, renal sodium excretion was strongly enhanced in the nephritic mice after day 10 and the ascites spontaneously disappeared despite constant proteinuria and albuminuria. This paradoxical behavior was also observed in studies with nephrotic rats (5, 14) and seems to represent a general feature of experimental nephrotic syndrome in rodents. The reason, however, remains elusive. Up to now, the majority of studies in rats focused only on the first 6 days after induction of nephrotic syndrome and could not contribute to the elucidation of the underlying mechanisms (8, 13, 27, 34, 51). The increased potassium excretion observed in nephritic mice over time might be a consequence of increased plasma K⁺ levels and hyperaldosteronism.

The present study also addressed the role of SGK1 in the development of volume retention. In rats, volume retention has been attributed to increased ENaC and Na-K-ATPase activity in the collecting duct (15, 34). Both ENaC (7, 9, 12, 16, 22, 26, 32, 33, 35, 41) and Na⁺-K⁺-ATPase (40, 44, 53) are stimulated by SGK1. SGK1 is upregulated by mineralocorticoids and is assumed to participate in the mineralocorticoid regulation of renal sodium excretion (7, 9, 12, 16, 22, 26, 32, 33, 35, 41). It is involved in the volume retention during mineralocorticoid excess (3) or following treatment with the PPARγ agonist pioglitazone (4). Our data show that SGK1 protein expression was strongly enhanced in nephritic wild-type mice suggesting an involvement of the kinase in the observed volume retention. Urinary sodium retention was blunted in sgk1⁻/⁻ mice during the first 10 days, despite the significantly higher plasma aldosterone levels in sgk1⁻/⁻ mice than in sgk1⁺/⁺ mice. This was accompanied by a significantly lower body weight gain in sgk1⁻/⁻ mice. Thus, SGK1 does participate in the stimulation of renal Na⁺ reabsorption during nephrotic syndrome. However, significant Na⁺ retention was observed even in sgk1⁻/⁻ mice, pointing to the contribution of SGK1-independent mechanisms to renal salt retention. Accordingly, body weight increased significantly in both sgk1⁺/⁺ and sgk1⁻/⁻ mice, indicating that activation of SGK1 contributes to but does not account for the development of edema in nephrotic syndrome.

In experimental nephrotic syndrome, hyperaldosteronism is not a prerequisite for edema formation, as it was shown in adrenalectomized rats (13, 34). Furthermore, blockade of the mineralocorticoid receptor was not effective in preventing ascites formation (15) as was the case with the ENaC inhibitor amiloride pointing to a mineralocorticoid receptor-independent activation of ENaC during nephrotic syndrome. However, rats with nephrotic syndrome display an increase in apical targeting of ENaC (27) that is absent in adrenalectomized rats with nephritic syndrome (13), suggesting an involvement of aldosterone in the volume retention. In those studies, the fractional sodium excretion remained higher in nephritic adrenalectomized rats (1.1%) compared with nephritic rats without adrenalectomy (0.3%). This allows the conclusion that hyperaldosteronism and

Fig. 9. Fibrous tissue stain of kidneys from nephrotic wild-type and sgk1⁻/⁻ mice following doxorubicin treatment. Sirius Red stain of kidneys from nephrotic sgk1⁺/⁺ and sgk1⁻/⁻ mice (top ×10, bottom ×20 magnification). Note the increased fibrosis (red) in kidneys from sgk1⁻/⁻ mice.
subsequent activation of SGK1 during nephrotic syndrome are able to worsen edema formation. The observed absence of ENaC targeting in adrenalectomized rats is likely to reflect a lack of activation of SGK1 and explains the reduced sodium retention and body weight gain in nephrotic sgk1−/− mice.

Although glomerular filtration rate was not measured directly, increasing plasma urea levels indicated severely reduced renal excretory function after 4 wk. Histologically, focal segmental and also global glomerular sclerosis were present in nephrotic mice along with severe tubulointerstitial inflammation and fibrosis. This was also reported in doxorubicin-induced nephropathy in BALB/c mice (48). In contrast, the histological appearance of the kidneys from the nonnephrotic mice was unremarkable. The podocyte-toxic effects of doxorubicin leading to podocyte loss and effacement and at later stages to focal segmental or global glomerulosclerosis are well established (6, 39). A direct tubulotoxic effect, however, is not typical, at least in rats (42). Hence, the development of tubulointerstitial inflammation and fibrosis seen in this model more likely reflects the tubulotoxic effects of proteinuria and albuminuria. Unlike in rats (37) and in BALB/c mice (48), all nephritic wild-type mice had reduced survival and died within 61 days after induction of nephrotic syndrome, most likely due to renal failure and azotemia. Compared with wild-type mice, nephritic sgk1−/− mice experienced a faster progression of renal failure as evidenced by a more rapid increase in plasma urea concentrations and increased wasting. Their median survival was significantly reduced. These findings might point to hitherto unknown SGK1-dependent protective effects during doxorubicin-induced nephrotic syndrome. Our data clearly show that SGK1 expression is upregulated in nephrotic wild-type mice. Similarly, SGK1 mRNA expression was found to be upregulated in human kidney biopsies from patients with glomerulonephritis (21) or diabetic nephropathy (31). In vitro, SGK1 is a stress-induced survival factor and exerts antiapoptotic effects, which have been shown in vitro in cardiomyocytes (2) or more recently in kidney cancer cells (1). The lack of these antiapoptotic effects in sgk1−/− mice could, at least in theory, cause a more severe apoptosis following doxorubicin or faster loss of renal cells thereafter. In a study examining children with FSGS (17), apoptosis was evident in both proximal and distal tubule cells and was related to the degree of proteinuria. Also, apoptosis was found to independently predict the development of end-stage renal disease. It should, however, be pointed out that the rather subtle differences between between sgk1+/+ and sgk1−/− mice do not allow postulation of a significant protective effect of SGK1 in nephrotic syndrome.

In any case, however, lack of SGK1 does not protect against doxorubicin-induced renal injury. The increase of plasma urea concentration is even significantly more pronounced in sgk1−/− mice. Similarly, glomerular sclerosis was, if anything, enhanced in sgk1−/− mice. This observation may be of pathophysiological importance, as SGK1 is expressed in a wide variety of fibrosing diseases (30). SGK1 is downstream of TGF-β (31, 47) and upstream of CTGF (43) and DOCA/high salt-induced cardiac (43) and renal fibrosis (3) are virtually absent in sgk1−/− mice. The present observation shows for the first time that fibrosis could be triggered without the permissive or active role of SGK1. Future studies will have to define the SGK1-dependent and SGK1-independent mechanisms of renal fibrosis.

In summary, we describe a murine model for experimental nephrotic syndrome featuring heavy proteinuria, volume expansion, and FSGS using strain 129 mice. Elements of both underfilling and overfilling were present. We demonstrate that gene-targeted mice lacking SGK1 have a decreased antinatriuresis but were not fully protected from the volume retention in experimental nephrotic syndrome. More importantly, we demonstrate that genetic knockout of SGK1 does not prevent proteinuria and subsequent tubulointerstitial inflammation and fibrosis in this experimental model.

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REFERENCES

DOXORUBICIN-INDUCED NEPHROTIC SYNDROME AND SGK1


