Proteinuria in mice expressing PKB/SGK-resistant GSK3

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1Department of Physiology, University of Tübingen, Tübingen; 2Department of Pathology, University of Erlangen, Erlangen, Germany; and 3MRC Protein Phosphorylation Unit, University of Dundee, Nethergate, Dundee, Scotland, United Kingdom

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Boini KM, Amann K, Kempe D, Alessi DR, Lang F. Proteinuria in mice expressing PKB/SGK-resistant GSK3. Am J Physiol Renal Physiol 296: F153–F159, 2009. First published November 5, 2008; doi:10.1152/ajprenal.90398.2008.—SGK1 is critically important for mineralocorticoid/salt-induced glomerular injury. SGK1 inactivates GSK3, which downregulates Snail, a DNA-binding molecule repressing the transcription of nephrin, a protein critically important for the integrity of the glomerular slit membrane. PKB/SGK-dependent GSK regulation is disrupted in mice carrying a mutation, in which the serine in the SGK/PKB-phosphorylation consensus sequence is replaced by alanine. The present study explored whether PKB/SGK-dependent GSK3 regulation influences glomerular proteinuria. Gene-targeted knockin mice with mutated and thus PKB/SGK-resistant GSK3α,β (gsk3KI) were compared with their wild-type littermates (gsk3WT). gsk3KI and gsk3WT mice were implanted with DOCA release pellets and offered 1% saline as drinking water for 21 days. Under standard diet, tap water intake and absence of DOCA, urinary flow rate, glomerular filtration rate, and urinary albumin excretion were significantly larger and blood pressure was significantly higher in gsk3WT mice. Within 18 days, DOCA/salt treatment significantly increased fluid intake and urinary flow rate, urinary protein and albumin excretion, and blood pressure in both genotypes but the respective values were significantly higher in gsk3KI than in gsk3WT mice. Plasma albumin concentration was significantly lower in gsk3KI than in gsk3WT mice. Proteinuria was abrogated by lowering of blood pressure with α1-blocker prazosin (1 μg/g body wt) in 8-mo-old mice. According to immunofluorescence, nephrin at 3 and 8 mo and podocin expression at 3 mo were significantly lower in gsk3WT mice. After 18 days, DOCA/salt treatment renal glomerular sclerosis and tubulointerstitial damage were significantly more pronounced in gsk3WT than in gsk3KI mice. The observations reveal that disruption of PKB/SGK-dependent regulation of GSK3 leads to glomerular injury with proteinuria, which may at least partially be secondary to enhanced blood pressure.

glomerular filtration rate; water; DOCA; albumin; nephrin

THE SERUM and glucocorticoid-inducible kinase SGK1 is critically important for the development of proteinuria and glomerular fibrosis during mineralocorticoid and salt excess (1). Accordingly, gene-targeted mice lacking functional SGK1 are protected against the glomerular injury following treatment with DOCA/high salt (1). Similar to protein kinase B Akt/PKB (8, 33), SGK1 phosphorylates and thus inactivates glycogen synthase kinase GSK3β (32). GSK3β, in turn, phosphorylates and thus inactivates the DNA-binding molecule Snail (3, 9) and inhibition of GSK3β is followed by upregulation of snail (9, 13). Snail represses the expression of nephrin (17, 18), which is critically important for the integrity of the glomerular slit membrane (2, 10, 14, 16, 23, 27, 29, 31, 35, 43). Defective nephrin leads to proteinuria with subsequent development of renal failure (15, 28). Moreover, deranged regulation of nephrin is considered to participate in the proteinuria of diabetic nepropathy (21, 34, 43), focal segmental glomerulosclerosis (26), experimental nephrotic syndrome (25), and preeclampsia (7).

In theory, SGK1 could have at least partially been effective through phosphorylation and thus inhibition of GSK3β with subsequent snail upregulation, suppression of nephrin expression, and development of glomerular injury.

The present study explored whether PKB/SGK-dependent inhibition of GSK participates in the pathophysiology of proteinuria and glomerular injury during salt and mineralocorticoid excess. The signaling of PKB/SGK to GSK3 could be disrupted by replacement of serine within the PKB phosphorylation site by alanine (GSK3α21A/21A, GSK3β9A/9A) thus yielding GSK3, which is resistant to inactivation by PKB/SGK (19). In knockin mice carrying these mutations (gsk3KI), the effect of insulin on muscle glycogen synthase is abrogated (19). To elucidate the role of SGK1-dependent regulation of GSK3 in glomerular function following salt/mineralocorticoid excess, kidneys from gsk3KI mice were compared with their wild-type littermates (gsk3WT) after treatment with DOCA/high salt. As shown previously (5), gsk3KI mice have significantly higher blood pressure, hematuria and proteinuria, which is at least partially due to enhanced blood pressure.

METHODS

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.

Mice were generated, in which the codon encoding Ser9 of GSK3β gene was changed to encode nonphosphorylatable alanine (GSK3βA9A9), and simultaneously the codon encoding Ser21 of GSK3α was changed to encode the nonphosphorylatable GSK3α21A/21A thus yielding the GSK3αβ21A/21A9A9 double knockin mouse (gsk3KI) as described previously (19). The mice were compared with their wild-type littermates (gsk3WT). The mice (6–7 females, 6 males, age 8 mo) were fed a control diet (1314, Altromin, Heidenau, Germany). Where indicated, the mice

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were offered saline. All other mice had free access to tap drinking water. For evaluation of renal excretion, both gsk3\textsuperscript{KI} and gsk3\textsuperscript{WT} mice were placed individually in metabolic cages (Techniplast, Hohenpeissenberg, Germany) for 24-h urine collection as described previously (36). They were allowed a 3-day habituation period when food and water intake, urinary flow rate, urinary excretion of salt, fecal excretion, and body weight were recorded every day to ascertain that the mice were adapted to the new environment. Subsequently, 24-h collection of urine was performed for 3 consecutive days to obtain the urinary parameters. To ensure quantitative urine collection, metabolic cages were siliconized and urine was collected under water-saturated oil.

To induce mineralocorticoid excess, gsk3\textsuperscript{KI} and gsk3\textsuperscript{WT} mice (8 mo old, \(n = 12\) each group) were implanted with 21-day-release 50-mg DOCA (2.4 mg/day) pellets (Innovative Research of America, Sarasota, FL) in the neck area (12, 38) during superficial general anesthesia (5 mg/kg ip midazolam + 50 mg/kg ip ketamin), which was partially antagonized by flumazenil (0.5 mg/kg ip) afterwards (1). Before the pellet implantation (control period), the mice had free access to plain tap water. After the implantation, the tap water was replaced by 1% NaCl (high salt). Throughout the entire study, mice had free access to a standard mouse diet (C1314, Altromin). Renal excretion was determined before and after 18 days of DOCA/salt treatment.

To obtain blood specimen, animals were lightly anesthetized with diethylether (Roth, Karlsruhe, Germany) and 200 \(\mu\)l of blood were withdrawn into heparinized capillaries by puncturing the retro-orbital plexus. Creatinine concentration in urine was determined using the Jaffe reaction (Sigma, St. Louis, MO), and creatinine concentration in serum was measured using an enzymatic kit (creatinine PAP, Lehmann, Berlin, Germany) according to the manufacturer’s instructions. Plasma proteins were separated by capillary electrophoresis in a Paragon CZE 2000 (Beckman Coulter). Electrophoresis was performed using the manufacturer’s instructions and reagents. Fraction limits were manually adjusted. Total plasma protein was measured using the Biuret method, and urinary total protein was measured quantitatively using Coomassie Brilliant Blue G-250 dye (Bio-Rad protein assay, Hercules, CA). A standard curve was generated with bovine albumin (Sigma). Urinary and plasma albumin was measured fluorometrically using the albumin-sensitive dye albumin blue 580 at 595-nm excitation and 642-nm emission on a multi-label counter (Victor 1420, PerkinElmer) according to the manufacturer’s instructions (microfluoral, Progen, Heidelberg, Germany). Standard curves were generated with mouse albumin (Sigma, Taufkirchen, Germany) and measurements were performed within the linear range (0–156 mg/l).

**Fig. 1.** Fluid intake, urinary flow rate, and creatinine clearance in genetargeted knockin mice with mutated and thus PKB/SGK-resistant GSK3\(\alpha,\beta\) (gsk3\textsuperscript{KI}) and wild-type littermates (gsk3\textsuperscript{WT}) before and following DOCA/salt treatment. Arithmetic means ± SE (\(n = 9–13\) each group) of fluid intake (top), urinary flow rate (middle), and creatinine clearance (bottom) in gsk3\textsuperscript{KI} (filled bars) and gsk3\textsuperscript{WT} (open bars) mice before and following an 18-day DOCA/salt treatment. \#\(P < 0.05\) vs. respective value before DOCA/salt treatment. *\(P < 0.05\) vs. respective value of gsk3\textsuperscript{WT} mice.

**Fig. 2.** Urinary protein and albumin excretion in gsk3\textsuperscript{KI} and gsk3\textsuperscript{WT} mice before and following DOCA/salt treatment. Arithmetic means ± SE (\(n = 11–12\) each group) of urinary protein (top) and albumin (bottom) excretion in gsk3\textsuperscript{KI} (filled bars) and gsk3\textsuperscript{WT} (open bars) mice before and following an 18-day DOCA/salt treatment. \#\(P < 0.05\) vs. respective value before DOCA/salt treatment. *\(P < 0.05\) vs. respective value of gsk3\textsuperscript{WT} mice.
Systolic arterial blood pressure was determined by the tail-cuff method before and 21 days following the initiation of DOCA/salt treatment. As reviewed recently (20), the tail-cuff approach to determine arterial blood pressure requires certain precautions to reduce the stress of the animals, including appropriate training of the mice over multiple days, prewarming to an ambient temperature of 29°C, measurement in a quiet and semidarkened and clean environment, and performance of the measurements by one person (KMB) and during a defined day time, when blood pressure is stable (between 1 and 3 PM). All these precautions were taken in the present study. The readings from 2 days were then averaged to obtain a mean blood pressure. All recordings and data analysis were done using a computerized data-acquisition system and software (Power Lab 400 and Chart 4, ADInstruments).

In a further series of experiments, gsk3KI and gsk3WT mice (male 8 mo old, n = 6 each) were treated after a control period with daily injections of prazosin (1 mg/g body wt ip) for 5 days. Systolic blood pressure was measured before and after 3 days of prazosin treatment. Renal protein excretion was determined before and after 5 days of prazosin treatment.

Fig. 3. Plasma protein concentration in gsk3KI and gsk3WT mice before and following DOCA/salt treatment. Arithmetic means ± SE (n = 6 each group) of plasma protein concentration in gsk3KI (filled bars) and gsk3WT (open bars) mice before (left) and following (right) a 21-day DOCA/salt treatment. *P < 0.05 vs. respective value before DOCA/salt treatment. #P < 0.05 vs. respective value of gsk3WT mice.

Fig. 4. Systolic blood pressure in gsk3KI and gsk3WT mice before and following DOCA/salt treatment. Arithmetic means ± SE (n = 10–12 each group) of systolic blood pressure in gsk3KI (filled bars) and gsk3WT (open bars) mice before (left) and following (right) a 21-day DOCA/salt treatment. *P < 0.05 vs. respective value before DOCA/salt treatment. #P < 0.05 vs. respective value of gsk3WT mice.

Fig. 5. Urinary flow rate and urinary protein excretion in gsk3KI and gsk3WT mice before and following a prazosin treatment. A: arithmetic means ± SE (n = 6 each group) of urinary flow rate (top) and urinary protein excretion (bottom) in gsk3KI (filled bars) and gsk3WT (open bars) mice before and following a prazosin treatment. *P < 0.05 vs. respective value before prazosin treatment. #P < 0.05 vs. respective value of gsk3WT mice. B: correlation between blood pressure and proteinuria before and following a prazosin treatment.

For histology, fixed kidneys and samples were stored in 4% paraformaldehyde.01 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 later embedding in epon araldite. 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 using well-established semiquantitative scoring indexes (score: 0–4) for glomerular, tubulointerstitial, and vascular damage (1). Criteria of glomerular damage were mesangial cell and matrix expansion, focal segmental sclerosis, and podocyte damage. Tubulointerstitial damage...
consisted of tubular atrophy, tubular dilatation, interstitial inflammation, and interstitial fibrosis and vascular damage was assessed as wall thickening, vascular inflammation, or fibrinoid necrosis, respectively. All semiquantitative, morphometric, and stereological measurements were performed in a blinded manner by an observer who was unaware of the study protocol.

Nephrin and podocin expression in the kidney were analyzed by immunofluorescence in 3-mo-old and nephrin expression in 8-mo-old mice. To this end, the mice were killed by CO₂ and cervical dislocation and the kidneys were rapidly frozen on liquid nitrogen. Frozen sections (3 μm) were fixed in aceton (10 min at −20°C), air-dried, and stored in Tris-HCl CSA buffer for 20 min. Then, blocking was performed with 20% goat serum (blotto, 1:5, 30 min). Afterwards, the primary antibodies against nephrin (polyclonal anti-nephrin, guinea pig, Acris, 1:100 in Tris-CSA) and podocin (polyclonal anti-podocin, rabbit, Sigma, 1:100 in Tris-CSA) were applied (1 h; room temperature) and the sections were washed in Tris-CSA buffer (3 × 5 min). Afterwards, the secondary antibody (goat anti-guinea pig, Alexa, 1:250, Molecular Probes) was applied for 30 min at room temperature followed by three Tris-CSA washings. DAPI was used to counterstain nuclei (1:1,000 in distilled water for 5 min) followed by rinsing in Tris-CSA buffer (3 × 5 min). Finally, sections were covered with mowiol and stored in darkness at 4°C until analysis.

Data are provided as means ± SE; n represents the number of independent experiments. All data were tested for significance using...
excretion of albumin was larger in gsk3KI mice, but not reaching statistical significance (Fig. 1). The 18-day treatment with DOCA/high-salt was followed by a significant increase of both fluid intake and urinary flow rate in both phenotypes. Both values remained significantly larger in gsk3KI than in gsk3WT mice. The treatment tended to decrease creatinine clearance, an effect, however, not reaching statistical significance (Fig. 1).

Urinary protein excretion was significantly enhanced in gsk3KI compared with gsk3WT mice (Fig. 2). Similarly, urinary excretion of albumin was larger in gsk3KI than in gsk3WT mice (Fig. 2). Urine electrophoresis again confirmed that urinary proteins are of glomerular origin (Fig. 2).

Under control conditions, plasma albumin concentration was not significantly different between the two genotypes. The 18-day treatment with DOCA/high salt resulted in a significant increase of plasma albumin concentration in both genotypes. Following DOCA/high-salt treatment, the plasma albumin concentration was significantly lower in gsk3WT than in gsk3KI mice (Fig. 3).

As demonstrated earlier (5), blood pressure was significantly higher in gsk3KI than in gsk3WT mice before DOCA treatment. DOCA/high-salt treatment significantly increased the blood pressure in both genotypes. Following DOCA/high-salt treatment, the blood pressure was again significantly higher in gsk3KI than in gsk3WT mice (Fig. 4).

As proteinuria could at least partially have been due to increased blood pressure, a further series of experiments aimed to define the influence of systolic blood pressure on proteinuria. To this end, blood pressure and proteinuria were determined following administration of the α1-blocker prazosin. Before the prazosin treatment, the systolic blood pressure was in this series again significantly higher in gsk3KI mice (108.2 ± 0.8 mmHg, n = 6) than in gsk3WT mice (90.0 ± 1.7 mmHg, n = 6). Prazosin treatment was followed by a decrease of blood pressure, which was significantly more pronounced in gsk3KI mice (90.7 ± 0.7 mmHg, n = 5) than in gsk3WT mice (86.4 ± 1.5 mmHg, n = 4). Before the prazosin treatment, urinary flow rate and urinary protein excretion were in this series again significantly higher in gsk3KI than in gsk3WT mice (Fig. 5). Prazosin treatment significantly decreased the urinary flow rate and urinary protein excretion in gsk3KI mice and abrogated the significant difference between gsk3KI and gsk3WT mice (Fig. 5).

Immunohistochemistry revealed that both glomerular nephrin and podocin expression were less pronounced in 3-mo-old gsk3KI mice than in 3-mo-old gsk3WT mice (Fig. 6). Nephrin expression has further been determined in 8-mo-old animals without or with DOCA/high-salt treatment. As illustrated in Fig. 7, the DOCA/high-salt treatment decreased the nephrin abundance in both genotypes. Following DOCA/high-salt treatment, nephrin abundance was again lower in gsk3KI than in gsk3WT mice (Fig. 7).

Histomorphological analysis was performed following DOCA/high-salt treatment to define the renal injury due to mineralocorticoid/salt excess. The results are illustrated in Fig. 8 and listed in Table 1. Volume densities of the medulla and cortex and mean and total glomerular volume were similar in gsk3KI and gsk3WT mice. Mesangiolysis score (MSI) tended to be higher, and kidney weight, kidney volume, medulla and cortex volume tended to be lower in gsk3KI than in gsk3WT mice. None of these parameters were significantly different between the two genotypes. Glomerular number was moderately but significantly smaller and volume density of the glomerula was significantly higher in gsk3WT than in gsk3KI mice. Most importantly, the glomerular sclerosis index and tubulointerstitial damage index were significantly higher in gsk3KI mice than in gsk3WT mice (Fig. 8).

DISCUSSION

The present study reveals that mice lacking PKB/SGK-dependent regulation of GSK3 activity are not protected against glomerular injury following mineralocorticoid and salt excess. Instead, glomerular integrity is compromised in mice lacking PKB/SGK-dependent regulation of GSK3 activity.

Table 1. Histomorphological data after 18 days of DOCA/high-salt treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Gsk3WT</th>
<th>Gsk3KI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSI</td>
<td>0.86±0.04</td>
<td>1.31±0.08</td>
<td>0.001*</td>
</tr>
<tr>
<td>MSI</td>
<td>1.46±0.01</td>
<td>1.56±0.08</td>
<td>0.234</td>
</tr>
<tr>
<td>TSI</td>
<td>0.30±0.05</td>
<td>0.58±0.03</td>
<td>0.001*</td>
</tr>
<tr>
<td>Volume density of the medulla, %</td>
<td>35.2±0.9</td>
<td>35.8±1.3</td>
<td>0.712</td>
</tr>
<tr>
<td>Volume density of the cortex, %</td>
<td>64.8±0.9</td>
<td>64.2±1.3</td>
<td>0.712</td>
</tr>
<tr>
<td>Volume density of the glomerula, %</td>
<td>12.7±0.5</td>
<td>14.8±0.6</td>
<td>0.033*</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>0.26±0.02</td>
<td>0.20±0.02</td>
<td>0.991</td>
</tr>
<tr>
<td>Kidney volume, mm³</td>
<td>250±15</td>
<td>197±22</td>
<td>0.091</td>
</tr>
<tr>
<td>Volume cortex, mm³</td>
<td>163±12</td>
<td>127±15</td>
<td>0.109</td>
</tr>
<tr>
<td>Volume medulla, mm³</td>
<td>87.5±3.2</td>
<td>70.1±7.4</td>
<td>0.075</td>
</tr>
<tr>
<td>Volume glomerula, mm³</td>
<td>20.5±1.7</td>
<td>18.7±2.1</td>
<td>0.519</td>
</tr>
<tr>
<td>Glomerular number per kidney</td>
<td>20.04±700</td>
<td>16.33±1.036</td>
<td>0.014*</td>
</tr>
<tr>
<td>Total glomerular volume, mm³</td>
<td>20.5±1.7</td>
<td>18.7±2.1</td>
<td>0.519</td>
</tr>
<tr>
<td>Mean glomerular volume, 10³ μm³</td>
<td>1.028±84</td>
<td>1.137±101</td>
<td>0.438</td>
</tr>
</tbody>
</table>

Arithmetic means ± SE (paraffin sections), n = 5–6 each group. *The P value indicates significant difference between GSK3 knockin mice (gsk3KI) and their wild-type littermates (gsk3WT). GSI, glomerular sclerosis index; MSI, mesangiolysis score; TSI, tubulointerstitial damage index.
carrying a mutation of GSK3β, in which the serine of the PKB phosphorylation site was replaced by an alanine (GSK3βS9A/9A) and at the same time carrying a mutation of GSK3α, in which the serine of the PKB phosphorylation site was replaced by an alanine (GSK3βAla21/A). The mice carrying the PKB/GSK-resistant GSK mutants (gsk3KI) have significantly enhanced glomerular injury compared with their wild-type littermates (gsk3WT).

Glycogen synthase 3 (GSK3) is phosphorylated and thus inhibited by SGK1 (32) and protein kinase B (8, 33). Inhibition of GSK3 mediates the effect of insulin on glycogen synthase (6, 19). Accordingly, the effect of insulin on muscle glycogen synthase is abrogated in gsk3KI mice (19). Glycogen synthase kinase GSK3β further phosphorylates and thus inactivates Snail, a repressor of nephrin transcription (18). Loss of inhibition by PKB/SGK would be expected to rather enhance GSK activity and thus to repress Snail leading to increased expression of nephrin. The present data show the opposite, i.e., a decreased expression of nephrin in gsk3KI mice. Accordingly, the animals are proteinuric even in the absence of DOCA/high-salt treatment. The DOCA/high-salt treatment enhances proteinuria in both genotypes, but again, proteinuria is more pronounced in gsk3KI than in gsk3WT mice. The enhanced proteinuria under DOCA/high-salt treatment again correlates with decreased glomerular nephrin expression in gsk3KI mice. The proteinuria, in turn, correlates with renal glomerular and tubular injury apparent from histomorphological analysis.

Even though the decreased nephrin expression in glomerular tissue is suggestive for an intrarenal cause of proteinuria (42), enhanced salt intake and increased blood pressure may, at least under DOCA and high-salt treatment, have contributed to the development of proteinuria. As reported earlier (5), gsk3KI mice have significantly enhanced blood pressure as early as at an age of 3 mo. Lowering of blood pressure by the α1-antagonist prazosin was followed by a significant decrease of proteinuria under DOCA/high-salt treatment again correlates with decreased glomerular nephrin expression in gsk3KI mice. The proteinuria, in turn, correlates with renal glomerular and tubular injury apparent from histomorphological analysis.

The present study did not aim to elucidate the cause for the enhanced blood pressure in gsk3KI mice. Inhibition of GSK3 by lithium has been reported to upregulate endothelial nitric oxide synthase (eNOS) (24). Accordingly, resistance of GSK to the inhibitory effect of PKB/SGK could, at least in theory, decrease eNOS activity and thus favor an increase of blood pressure. Moreover, NOS participates in the regulation of thirst (30) and reduced NOS activity could, in theory, contribute to the increased fluid intake of the gsk3KI mice. On the other hand, the exquisite sensitivity of blood pressure in gsk3KI mice to α1-blockade may point to enhanced peripheral sympathetic nerve tone in those mice.

In any case, PKB/SGK resistance of GSK does not confer protection of glomerula against injury caused by mineralocorticoid/salt excess. In contrast, lack of PKB/SGK-dependent regulation of GSK3 leads to glomerular and tubular injury. Gene-targeted mice lacking SGK1, on the other hand, are protected against DOCA/high salt-induced renal (1) and cardiac (40) injury. According to the present observations, GSK resistance does not protect against SGK1-mediated renal injury. Along those lines, GSK3β phosphorylation did not correlate with cardiac injury following DOCA/high-salt treatment (41).

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GRANTS

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