SIK1 localizes with nephrin in glomerular podocytes and its polymorphism predicts kidney injury

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Mutant α-adducin and endogenous ouabain levels exert a causal role in hypertension by affecting renal Na–K ATPase. In addition, mutant β-adducin is involved in glomerular damage through nephrin down-regulation. Recently, the salt-inducible kinase 1 (SIK1) has been shown to exert a permissive role on mutant α-adducin effects on renal Na–K ATPase activity involved in blood pressure (BP) regulation and a SIK1 rs3746951 polymorphism has been associated with changes in vascular Na–K ATPase activity and BP. Here, we addressed the role of SIK1 on nephrin and glomerular functional modifications induced by mutant β-adducin and ouabain, by using congenic substrains of the Milan rats expressing either mutant α- or β-adducin, alone or in combination, ouabain hypertensive rats (OHR) and hypertensive patients. SIK1 co-localized and co-immunoprecipitated with nephrin from glomerular podocytes and associated with caveolar nephrin signaling. In cultured podocytes, nephrin-gene silencing decreased SIK1 expression. In mutant β-adducin congenic rats and in OHR, the podocyte damage was associated with decreased nephrin and SIK1 expression. Conversely, when the effects of β-adducin on podocytes were blocked by the presence of mutant α-adducin, nephrin and SIK1 expressions were restored. Ouabain effects were also reproduced in cultured podocytes. In hypertensive patients, nephrinuria, but not albuminuria, was higher in carriers of mutant SIK1 rs3746951 than in wild-type, implying a more direct effect of SIK1 on glomerular damage. These results demonstrate that, through nephrin, SIK1 is involved in the glomerular effects of mutant adducin and ouabain and a direct effect of SIK1 is also likely to occur in humans.

INTRODUCTION

Definite protein interaction networks appear to control renal tubular sodium handling and urinary protein excretion, and their alterations may progress to hypertension and related organ complications (1). Diseases are frequently caused by genetic mutations within these networks that may be responsible for biochemical dysfunctions in proteins and signaling pathways (1,2).

Our research group has shown that two main mechanisms, among others, associate with hypertension and organ damage both in animal models and in patients: mutant α-adducin variants (3) and high concentrations of endogenous ouabain (EO) (4). Evidence has indicated that mutant adducin and EO show similar functional effects at molecular, cellular and whole-organ levels (5–7) and all are antagonized by rostafuroxin, a novel anti-hypertensive compound (6–9). In particular, we showed that mutant α-adducin and ouabain increase Src tyrosine kinase and Src-dependent Na–K ATPase phosphorylation and signaling activity, resulting in an overall enhancement of sodium reabsorption across the tubular cells and higher BP (6).

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In addition, experimental and clinical evidence in hypertensive animal models and patients support a causal role of β-adducin genetic variants and EO/ouabain in glomerular lesions and kidney injury development, associated with the down-regulation of nephrin (10,11), the glomerular podocyte marker protein (12).

Recently, salt-inducible kinase 1 (SIK1) has been identified as part of a Na+-sensing network and has been shown to associate with, and increase, renal Na–K ATPase activity in a Na+ and Ca2+-dependent manner (13). In the Milan hypertensive rats (MHS) carrying the mutant α-adducin variant, SIK1 has a permissive role on the stimulatory effect of mutant adducin on renal Na–K ATPase activity, which leads to an increase of BP (14). Besides the kidney, SIK1 has been localized in vascular smooth muscle cells in rats and humans and, in population studies, a SIK1 rs3746951 polymorphism, which results in an amino acid change (15Gly → Ser) in the protein, has been associated with changes in vascular Na–K ATPase activity, left ventricle mass and BP (15).

However, a possible role of SIK1 in glomerular podocyte function and on the previously shown effect of β-adducin and EO/ouabain on podocytes has not been explored yet. In view of the many similarities between adducin and ouabain functional effects, here we hypothesized that SIK1 expression level may also be involved in the mutant β-adducin and ouabain damaging effects on glomerular podocytes. We examined whether SIK1 may be localized in podocytes and may interact with nephrin, thus participating in the glomerular filtration processes and signaling activities. For the in vivo studies, two previously characterized rat models were used: the congenic rat substrains of the MHS hypertensive and MNS normotensive rats expressing α- and β-adducin, alone or in combination (16), and the ouabain-infused rats (6,8). Furthermore, to investigate whether SIK1 rs3746951 polymorphism identified in humans may affect podocyte function, we studied a cohort of hypertensive patients, since they have a higher probability to develop glomerular lesions than normotensives and therefore they represent an ideal cohort to investigate the influence of SIK1 polymorphism on podocyte function. This strategy allowed the identification of subjects with a high predisposition to kidney injury occurrence.

RESULTS

Localization of SIK1 in glomerular podocytes

Glomeruli and podocytes were isolated from MHS hypertensive and MNS normotensive rats or strains and the congenic rat substrain (NA), where the mutant α-adducin was introgressed from MHS into the MNS genetic background (16). Glomeruli were also isolated from human renal specimens. The presence of nephrin and SIK1 in isolated glomeruli and cultured podocytes was revealed by western blotting (Fig. 1A–C). By using an anti-nephrin antibody, it was observed that SIK1 co-immunoprecipitated with nephrin from rat glomerular podocytes (Fig. 1D). Nephrin and SIK1 co-localization in glomerular podocytes was confirmed by a double immunolabeling analysis on human renal specimens (Fig. 1F) and cultured mouse podocytes (Fig. 1G). These last images clearly indicated that the co-localization of nephrin and SIK1 occurred both in podocyte extension processes and in cell bodies (Fig. 1G). Furthermore, preliminary experiments of nephrin-gene silencing performed in a mouse podocyte cell line indicated that nephrin was down-regulated compared with control cells and a parallel reduction of SIK1 protein expression was observed (Fig. 1E).

SIK1 resides with nephrin within caveolae microdomains

Nephrin associates with signaling membrane microdomains, referred to as lipid rafts/caveolae, that localize to the podocyte slit diaphragm (17). Caveolae have been shown to functionally organize signaling events downstream of Src-homology 2 (SH2) domain-containing proteins (18).

A score matrix assisted ligand identification (SMALI) analysis showed that nephrin contains SH2-consensus sequences for several tyrosine kinases, including Fyn, Src, Yes and ABL1 (see Supplementary Material, Fig. S1). Since SIK1 contains SH2-consensus sequences for Lyn and ABL1 as well (see Supplementary Material, Fig. S1), we hypothesized that it may participate in signaling pathways within podocyte caveolae. Indeed, caveolae (low-density fractions 2–5 of a sucrose gradient) (Fig. 2A), purified from human renal specimens, resulted enriched in SIK1 together with the specific caveolar marker, caveolin 1 and nephrin, Src and α-adducin, previously shown to be here compartmentalized (Fig. 2B and C) (6,19). A similar picture was obtained from rat kidneys (not shown). By using an anti-Src antibody, SIK1 co-immunoprecipitated with nephrin from isolated human renal caveolae (Fig. 2D). Adducin was not detected in the co-immunoprecipitates, possibly because of its weak interaction.

These data suggest that at least a pool of SIK1 may be localized to the podocyte slit diaphragm and may associate with nephrin-containing caveolae.

Correlation of SIK1 and nephrin protein expression in rat and human specimens

To further substantiate the possible functional association between SIK1 and nephrin within glomerular podocytes, we investigated whether factors that induce nephrin reduction, such as adducin mutations (10) or ouabain (11), may induce parallel alterations in SIK1 protein expression leading to kidney injury.

Two different experimental rat models were studied:

i) The congenic rat substrains for α- and β-adducin from MHS and MNS rats, where the mutant α-adducin (NA) or β-adducin (NB) alone, or in association (NAB), were introgressed from MHS into the MNS genetic background (16) (the details of the congenic rat substrains are reported in Materials and Methods). As previously shown (10), and here confirmed, MNS and NB rats developed glomerular damage and high proteinuria, while MHS, NA and NAB rats, carrying the mutant α-adducin, appeared protected (Table 1). MNS rats develop an age-dependent glomerulosclerosis and proteinuria (10), sustained by an increased glomerular production of thromboxane compared with MHS (20,21). Therefore, in MNS rats, part of proteinuria may be associated with this alteration. However, the presence of β-adducin in NB rats contributes to a further...
deterioration of the glomerular damage documented with an increase of proteinuria over MNS rats. In MNS and NB rats, the glomerular podocyte injury was shown to be independent from BP levels, which were higher in MHS and NA when compared with MNS, NB and NAB (Table 1) (10).

In the present study, the expression of nephrin and SIK1 was evaluated by immunoblotting in two separate preparations of isolated glomeruli from MHS, MNS and congenic rat substrains NA, NB and NAB, at the early age of 1.5 months, when proteinuria was still normal in all strains (see Supplementary Material, Table S1). The results indicated that MNS and NB glomeruli showed an early reduction of nephrin protein expression, as previously reported (10), that was associated with low SIK1 protein levels (Fig. 3A and B). Conversely, MHS, NA and NAB glomeruli presented an elevated expression of both proteins compared with MNS and NB (Fig. 3A and B). Actin levels did not differ among rats (not shown). A positive correlation ($r = 0.904, n = 10, P < 0.001$) (Fig. 3C) was observed between nephrin and SIK1 protein expression from isolated glomeruli of MHS, MNS and congenic rat strains reported in Figure 3A and B.

(i) The OHR rats, where ouabain infusion has been shown to induce an increase of BP and urinary protein excretion, associated with the reduction of the podocyte nephrin expression (Table 2) (11). This finding was reproduced ex vivo by incubating primary cultures of podocytes with $10^{-9}$ M ouabain (Fig. 3D). In this setting, ouabain, besides lowering nephrin (Fig. 3D) (11), induced a parallel decrease of SIK1 (Fig. 3E). A positive correlation ($r = 0.801, n = 7, P < 0.01$) between nephrin and SIK1 protein expression was observed (Fig. 3F).

Figure 1. SIK1 localizes with nephrin in glomerular podocytes. (A) Isolated glomeruli (10 μg protein/lane) from 1.5-month-old MHS, NA and MNS rats and (B) cultured glomerular podocytes (10 μg protein/lane) from 7- to 10-day-old MHS, NA and MNS rats were probed with anti-SIK1 (left) and anti-nephrin (right) antibodies by western blot. Representative immunoblottings are shown. Standard molecular weights (MW) are indicated. (C) Isolated glomeruli (10 μg protein/lane) from two samples of human renal specimens were probed with anti-SIK1 (left) and anti-nephrin (right) antibodies by western blot. Representative immunoblottings are reported. Standard MW are indicated. (D) Co-immunoprecipitation experiment of SIK1 and nephrin with anti-nephrin antibody from rat glomerular podocytes. Representative immunoblottings for nephrin and SIK1 in a whole glomerular podocyte homogenate (input, 25 μg proteins), co-immunoprecipitated samples (Co-IP) and negative controls (control), obtained by incubation with a non-immune IgG, are shown. (E) Western blot analysis of mouse cultured podocytes transfected with siRNA nephrin when compared with Lipofectamine (control). Results are replicates of four samples. Nephrin resulted down-regulated by ≈50% compared with control cells and a parallel reduction of SIK1 protein expression was observed. (F) Double-stained immunofluorescence analysis for nephrin (red) and SIK1 (green) in human kidney sections, analyzed by confocal microscopy. Co-localization between nephrin and SIK1 in the glomerulus is indicated in yellow (merged signals). Magnification ×1000. Framed areas of the merged signals (yellow) are shown at a higher magnification (right panel). (G) Double immunolabeling experiments analyzed by confocal microscopy in mouse cultured podocytes for nephrin (green) and SIK1 (red). Merged signals are indicated in yellow. Magnification ×1000.
Analogously, microsomes from human kidney specimens showed a positive correlation ($r = 0.807$, $n = 47$, $P < 0.001$) between nephrin and SIK1 protein expression (Fig. 4A). Additionally, a positive correlation was detected between Src and SIK1 ($r = 0.832$, $n = 47$, $P < 0.001$) (Fig. 4B) or nephrin ($r = 0.6652$, $n = 47$, $P < 0.001$) (Fig. 4C).

A polymorphism in SIK1 rs3746951, which results in an amino acid substitution, has been identified and previously associated with BP variations by Popov et al. (15). Here, to study the influence of this specific polymorphism on protein expression, SIK1, nephrin and Src were quantified in renal microsomes from patients divided according to SIK1 rs3746951 variants. No significant difference in the protein expression levels was observed between the two groups of patients carrying SIK1 wild-type C allele ($n = 28$) and mutant T ($n = 19$) (Table 3). However, in both groups of subjects, a positive correlation between nephrin and SIK1 was found ($r = 0.865$, $n = 28$, $P < 0.001$; $r = 0.765$, $n = 19$, $P < 0.001$, respectively) (Fig. 4D).

The present findings suggest that nephrin variations parallel SIK1 modifications both in rats and in humans.

**Nephrinuria in rats and patients: influence of SIK1 polymorphism**

In the recent years, urinary excretion of nephrin (nephrinuria) has been proposed as an early prognostic indicator/biomarker...
of susceptibility of podocyte damage and kidney complications, preceding albuminuria (22, 23, V. Matafora, personal communication). Here, nephrinuria was quantified in rats and in humans by immunoblotting and the influence of SIK1 rs3746951 genetic variants on nephrinuria and albumin excretion rate was evaluated in patients, but not in rats, since they do not carry this SIK1 mutation.

In rats, nephrinuria was significantly higher in MNS and NB rats over NA already at 1.5 months of age, when proteinuria was similar among groups, and progressively increased at 6 months of age (see Supplementary Material, Table S1), while urinary creatinine excretion did not differ among groups (Table 1). This result reflects an early loss of nephrin from podocytes in MNS and NB, but not in MHS, NA and NAB that appear

Table 1. SBP and urinary parameters in male 6-month-old congenic rat strains

<table>
<thead>
<tr>
<th>Rats</th>
<th>Body weight (g)</th>
<th>SBP (mmHg)</th>
<th>Urinary volume (ml)</th>
<th>Urinary protein (mg/24 h)</th>
<th>Urinary creatinine (mg/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MNS, n = 10</td>
<td>451 ± 4</td>
<td>15 ± 1.5</td>
<td>13.1 ± 0.4</td>
<td>241 ± 13</td>
<td>15.4 ± 0.4</td>
</tr>
<tr>
<td>MHS, n = 10</td>
<td>529 ± 5</td>
<td>12 ± 1.3</td>
<td>24.1 ± 1.5*</td>
<td>26.2 ± 1.09**</td>
<td>16.2 ± 0.5</td>
</tr>
<tr>
<td>NA, n = 10</td>
<td>476 ± 6</td>
<td>13 ± 0.9</td>
<td>13.3 ± 0.5</td>
<td>144 ± 13**</td>
<td>16.1 ± 0.7</td>
</tr>
<tr>
<td>NB, n = 10</td>
<td>452 ± 4</td>
<td>14 ± 0.7</td>
<td>15.1 ± 0.7</td>
<td>359 ± 12*</td>
<td>13.8 ± 0.9</td>
</tr>
<tr>
<td>NAB, n = 10</td>
<td>462 ± 7</td>
<td>139 ± 1.6</td>
<td>14.2 ± 0.4</td>
<td>155 ± 12*</td>
<td>14.1 ± 0.4</td>
</tr>
</tbody>
</table>

Rats were housed in single metabolic cages and 24 h urine collected as described in Materials and Methods. Data are mean ± SEM of n = 10 rats per each group. The statistical significance among groups was measured by ANOVA.

*P < 0.05, **P < 0.01 versus MNS.

Figure 2. SIK1 is compartmentalized with nephrin in renal caveolar microdomains. (A) Caveolae were isolated from human kidney tissues according to a detergent free method (Materials and Methods). Thirteen fractions were automatically collected and protein content determined. (B) Western blot analysis of specific caveolar markers (caveolin1 and Src), together with SIK1, nephrin and α-adducin was determined in fractions from 2 to 9. (C) The densitometric analysis, expressed as optical density in arbitrary units, identified marker enrichment in the low-density fractions from 2 to 5. These fractions were referred to as caveolae and separated from heavier fractions containing plasma membrane and cytosol. (D) Immunoprecipitation of SIK1 and nephrin with anti-Src antibody from human renal caveolae (pool of fractions 2–5). Three independent human caveolar samples were analyzed. A sample of caveolae was incubated with a non-immune IgG and used as a negative control (not shown).
protected. Proteinuria was similar among young congenic rat strains but progressively increased with age in MNS, and even more in NB, over NA and NAB (see Supplementary Material, Table S1 and Table 1). As a result, a positive correlation between nephrinuria and proteinuria was observed in old \( (r = 0.8849, n = 24, P < 0.001) \) (Fig. 5B), but not in young rats \( (r = 0.2921, n = 24) \) (Fig.5A).

Similar studies have been conducted in humans. In particular, we studied a cohort of hypertensive patients, since it is more likely to find glomerular lesions among them compared with normotensives. Subjects were divided according to SIK1 rs3746951 polymorphism (wild-type CC variant, \( n = 22 \); mutant TC + TT, \( n = 22 \)). Their characteristics are reported in Table 4. Age, body mass index (BMI), systolic and diastolic BP (SBP, DBP) and 24 h urinary Na\(^+\) excretion (UNa) did not differ between the two groups of subjects (Table 4). Additionally, an early morning urine sample (20 ml) was collected for nephrin, albumin and creatinine evaluation. In these patients, a significant correlation between nephrinuria and albuminuria, both normalized for creatininuria \( (r = 0.6312, n = 44, P < 0.001, \text{not shown}) \), was observed. When divided according to SIK1 rs3746951 polymorphism, urinary creatinine excretion did not differ between carriers of SIK1 wild-type CC (6.79 ± 1.59 mmol/l, \( n = 22 \)) and mutant TC + TT (7.66 ± 1.25 mmol/l, \( n = 22 \)). Analogously, albuminuria, normalized for urinary

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**Table 2.** SBP and urinary parameters in OHR

<table>
<thead>
<tr>
<th>Rats</th>
<th>Body weight (g)</th>
<th>SBP (mmHg)</th>
<th>Urinary protein (mg/24 h)</th>
<th>Nephrin protein expression in renal microsomes (arbitrary units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls, ( n = 8 )</td>
<td>388 ± 4</td>
<td>151 ± 1.7</td>
<td>35 ± 4.8</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>OHR, ( n = 8 )</td>
<td>391 ± 5</td>
<td>174 ± 1.4**</td>
<td>57.5 ± 3.5*</td>
<td>2.7 ± 0.2*</td>
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Ouabain (15 μg/kg/day) was subcutaneously infused into normotensive Sprague–Dawley rats for 12 weeks (OHR rats). Normotensive controls received subcutaneously saline solution. Rats were housed in single metabolic cages and 24 h urine collected as described in Materials and Methods. At sacrifice, kidneys were excised and used to quantify nephrin protein expression in renal microsomes by immunoblotting (10 μg protein/lane). The densitometric analysis for nephrin was expressed as arbitrary units and normalized for actin content. Data are mean ± SEM of \( n = 8 \) rats per each group and refer to the values measured at the last week. The statistical significance among groups was measured by \( t\)-test. \( *P < 0.05 \), \( **P < 0.01 \) OHR versus controls.

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**Figure 3.** SIK1 and nephrin protein expression correlates in rat glomerular podocytes. \((A–C)\) Glomeruli were isolated from MHS and MNS rats and from congenic rats carrying the mutant \( \alpha\)-adducin (NA) and mutant \( \beta\)-adducin (NB) alone or in association (NAB). \((A)\) Nephrin and \((B)\) SIK1 were determined by immunoblotting in two separate glomerular preparations from each rat strain. Typical western blots are represented. Data are mean of optical density expressed as arbitrary units. \((C)\) Correlation of SIK1 and nephrin protein expression of the immunoblottings reported in \((A)\) and \((B)\); \( r = 0.904, n = 10, P < 0.001 \). \((D–F)\) Cultured glomerular podocytes from 7- to 10-day-old Sprague–Dawley rats were incubated with 10 \( \times 10^{-9} \) M ouabain for 5 days. \((D)\) Nephrin and \((E)\) SIK1 were determined by immunoblotting. Typical western blots are represented. Data are mean ± SEM of optical density expressed as arbitrary units: control (contr), \( n = 3 \); 10 \( \times 10^{-9} \) M ouabain (ouab), \( n = 4 \) separate experiments. \((F)\) Correlation of SIK1 and nephrin protein expression of the immunoblottings reported in \((D)\) and \((E)\); \( r = 0.801, n = 7, P < 0.01 \).
creatinine excretion, was similar in the two groups of patients (wild-type 0.865 ± 0.127, n = 22; mutant 0.799 ± 0.166, n = 22) (Fig. 5C). Conversely, nephrinuria, normalized for urinary creatinine excretion, resulted significantly increased in mutant (1.664 ± 0.2941, n = 22) than in wild-type carriers (0.994 ± 0.141, n = 22, P = 0.0481) (Fig. 5D).

Collectively, these findings indicate that nephrinuria is an early marker of podocyte damage and its enhanced levels in patients carrying SIK1 rs3746951 mutant T allele may predict an increased risk of kidney injury.

**DISCUSSION**

In the present study, we provide the evidence that the novel identified protein kinase SIK1 co-immunoprecipitates and co-localizes with nephrin in glomerular podocytes and both proteins are compartmentalized with Src kinase within caveolae, from where they can be co-immunoprecipitated with Src. A double immunofluorescence analysis in podocyte cultures clearly documents that SIK1-nephrin co-localization occurs both in podocytes extension processes and in cell bodies.

Furthermore, although preliminary, the selective nephrin-gene silencing in cultured podocytes induces a parallel reduction of SIK1 protein expression, suggesting that both proteins share a common regulatory pathway.

Also *in vivo*, studies in rats developing glomerular damage due to the presence of the mutant β-adducin variant (NB rats) or ouabain infusion (OHR) have indicated that SIK1 protein expression appears consensually down-regulated as nephrin, suggesting that the loss of both proteins contributes to the development of podocyte damage. Conversely, SIK1 and nephrin expression are both restored to normal levels under conditions that protect against podocyte injury, as in the presence of mutant α-adducin variant (MHS, NA, NAB rats) (10). The result is a positive correlation between nephrin and SIK1 protein expression in rats and a similar picture is observed in human renal specimens. Thus, the present findings suggest the existence of a close functional link between SIK1 and nephrin in glomerular podocytes, implying that at least a pool of SIK1 may have a slit diaphragm localization and may participate in nephrin functional signaling network within caveolar microdomains.

Caveolae are specialized membrane subdomains that functionally organize signaling cascades, including proteins containing...
SH2 domains (6,18,19). As known, the podocyte slit diaphragm proteins nephrin and CD2AP localize to podocyte filtration slits and interact with caveolin1, finding that is consistent with the lipid raft/caveole nature of the podocyte filtration slit plasma membrane (24,25).

Clustering of nephrin induces its tyrosine phosphorylation mediated by Src family kinases, also present in caveolar subdomains (24). A SMALI analysis confirmed that nephrin contains SH2-consensus sequences for Src family tyrosine kinases. Nephrin-associated kinases are implicated in the recruitment of SH2–SH3 domain-containing adapter proteins and in the phosphorylation of substrate proteins belonging to the podocyte junctions, favoring the assembly of actin filaments and the integrity of the podocyte signaling network (12). From a SMALI analysis, it can be predicted that also SIK1, which contains SH2-consensus sequences for tyrosine kinases, may be phosphorylated in tyrosine residues, participating in signaling pathways within podocyte caveolae.

However, we have not yet addressed the question of whether SIK1 may associate with nephrin directly, or through a mediated interaction via adaptor proteins or cytoskeletal proteins, such

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<tr>
<th>SIK1 rs3746951 SIK1 Nephrin Src</th>
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<tr>
<td>SIK1 CC, n = 28</td>
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<td>SIK1 TC + TT, n = 19</td>
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Table 3. Western blot analysis of human renal microsomes according to SIK1 rs3746951 polymorphism

Human renal specimens were used for microsomes preparations as described in Materials and Methods. Patients (n = 47) were genotyped and divided according to SIK1 rs3746951 polymorphism (wild-type C allele, n = 28; mutant T allele, n = 19 TC, n = 1 TT). A western blot analysis was conducted on isolated renal microsomes (10 µg protein/lane) by using specific antibodies. Data are mean ± SEM of the optical densities expressed as arbitrary units and normalized for actin content.

Figure 5. Urinary nephrin excretion in rats and patients. (A and B) Five ml urine from MNS, NA, NB, NAB rats (n = 6 per each group) at 1.5 (A) and 6 months of age (B) were used for nephrin quantification by immunoblotting. The densitometric analysis was expressed as optical density in arbitrary units and normalized for actin content. Proteinuria was determined in 5 ml rat urine and expressed as mg/5 ml. Nephriturina and proteinuria mean values are reported in Supplementary Material, Table S1. The correlations between nephrituria and proteinuria in young and old rats are shown (r = 0.2921, n = 24 and r = 0.8849, n = 24, P < 0.001, respectively). (C and D) Urinary excretion of albumin and nephrin in patients. (C) Patients were divided according to SIK1 rs3746951 polymorphism (wild-type CC, n = 22; mutant TC + TT, n = 22) and urine was used for the quantification of albuminuria, normalized for urinary creatinine excretion. (D) Urinary nephrin excretion was quantified in SIK1 rs3746951 wild-type CC and mutant TC + TT carriers by immunoblotting. The densitometric analysis was expressed as arbitrary units and normalized for urinary creatinine excretion. The difference between the two groups resulted statistically significant (P = 0.0481).
as adducin, which is known to facilitate the permissive role of SIK1 on the increase of Na–K ATPase induced by mutant adducin (14). We can propose that the activation of nephrin phosphorylation by Src family kinases may regulate protein–protein interactions and signal transduction pathways, facilitating the recruitment of downstream signaling molecules, including SIK1 and its associated adducin signaling network (10,14). This assumption is reinforced by the demonstration here reported that nephrin, SIK1 and adducin are enriched in caveolar subdomains.

In this way, SIK1 may mediate, possibly via adducin, signaling events that result in the integration of nephrin cascade with podocyte actin cytoskeleton and intercellular junctions. In line with this view, adducin mutations, or the presence of endogenous compounds, such as EO/ouabain, may interfere with actin cytoskeleton dynamics, leading to the disruption of podocyte junction formations.

Early signs of perturbed podocyte activity and damage of the filtration barrier, particularly the inter-podocyte bridge, can be manifested by nephrin release into urine. Indeed, nephrinuria has emerged as an earlier predictive indicator/biomarker of susceptibility for podocyte injury and kidney complications than albuminuria, which occurs when glomeruli already demonstrate advanced glomerulopathy and massive podocyte loss (22,23).

In the congenic rats, we provide the evidence that nephrinuria is already enhanced in young (1.5-month-old) MNS and NB rats over NA rats, despite the absence of difference in proteinuria. Nephrinuria becomes even more pronounced in older MNS and NB over NA and NAB rats, where albuminuria increases. These findings are consistent with the hypothesis that MNS, and even more NB rats, show an early deterioration of podocyte structure and function, associated with a loss of nephrin, which is most likely released from the damaged podocytes into urine. This finding reinforces the notion that nephrinuria is an early and strong predictor of podocyte damage.

SIK1 is expected to follow nephrin, since both proteins are similarly modulated in glomerular podocytes. However, no detectable SIK1 has been found by immunoblotting in urine, probably due to its extensive degradation following membrane detachment.

In humans, a polymorphism of SIK1 at rs3746951, not present in rats, resulting in an amino acid change (15Gly→Ser) in the protein, has been identified and associated with increased vascular Na–K ATPase activity, decreased left ventricle mass and lower BP levels in population studies (15). In our cohort of hypertensive patients, SIK1 rs3746951 mutant T allele carriers showed only a modest decrease (−2, −3 mmHg) of SBP and DBP compared with C allele carriers. The apparent discrepancy between the two studies should be evaluated in the context of its limitation: in our cohort, subjects are much younger (>15 years) compared with those in Popov’s study (15), therefore a follow-up evaluation will be necessary to better establish the influence of SIK1 polymorphism, and of the genetic context, on BP development (see study limitations).

Notwithstanding, here we have verified that hypertensive patients carrying the SIK1 rs3746951 mutant T allele have increased nephrinuria compared with those carrying the wild-type C allele, in the absence of changes in creatinine and albumin excretion rates. Translated from rat data, it can be inferred that patients carrying the mutant T allele of SIK1 rs3746951 are more predisposed/susceptible to an increased risk of developing kidney function impairment. We can envisage that SIK1 polymorphism may be responsible for the rearrangement of the cytoskeletal architecture and nephrin stability, participating in the disruption of the filtration barrier and in the progression towards glomerular disease.

The matter still under study concerns the possible involvement of SIK1 in the complex interplay between EO and salt intake. A direct relationship between EO and a salt excess has been already documented (4,26), although the molecular mechanisms responsible for the heterogeneous BP response to a salt intake in patients is still only partially known. We have obtained indications suggesting that plasma EO levels may be responsible for the different response to a salt intake (26) and that a SIK1 polymorphism, even though different from that described by Popov et al. (15), may interfere with the BP response of EO through the modulation of renal tubular Na–K ATPase activity (M. Ferrandi, personal communication). Thus, EO, in association with selective SIK1 genetic variants, appears to be a mediator of the dangerous effects induced by a salt excess on renal tubular cells. However, it is known that salt may also affect glomerular function, leading to podocyte derangement and proteinuria mediated via activation of specific signaling pathways (27). Thus, it is reasonable to speculate that SIK1 polymorphism may interfere with the complex interplay between EO/ouabain and salt intake also at the glomerular level, influencing podocyte damage.

This study has some limitations: (i) a follow-up evaluation of the patients is necessary to finally prove the prediction and animal models should be utilized to improve our knowledge on SIK1 polymorphism effects; (ii) since the rat models studied do not carry SIK1 rs3746951 mutation, a full gene scanning should be performed to explore whether other mutations are present that may play a functional role similar to that of humans and (iii) only the selective inhibition of SIK1 in humans may provide a more precise information of its role within the complex genetic architecture underlying the regulation of nephrin and glomerular integrity.

In conclusion, we provide the evidence that SIK1 is functionally coupled to nephrin signaling, being involved in the glomerular effects of mutant adducin and ouabain in rats, even though a
direct effect of SIK1 is also likely to occur in humans. SIK1 rs3746951 polymorphism, identified in patients, may contribute to alter podocyte architecture and activities, most likely by favoring the detachment of nephrin into urine. Nephrinuria may be considered as a strong indicator that, besides providing dynamic information on podocyte functions and hints to the pathogenesis of nephropathy, may be also useful to profile and monitor patients to optimize the pharmacologic intervention.

**MATERIALS AND METHODS**

**Rat studies**

All the experiments were carried out according to the guidelines of the Prassis sigma-tau Institute for Animal Care, approved by the Italian Ministry of Health, and complied with European Directive 86/660 and with the Italian Law (DL116, 27 January 1992). Animals were monitored by a veterinarian.

*Rat models*: (i) Congenic rat substrains from MHS, Milan hypertensive and MNS, Milan normotensive parental strains. MHS and MNS rats were derived from the internal stock colony (Prassis sigma-tau Research Institute, Settimo Milanese, Milan, Italy). Congenic rat strains were obtained from MHS and MNS rats, as described previously (16). The donor strain was the MHS and the receiving genetic background was the MNS. Three congeneric substrains were obtained: NA (carrying the mutant 316Y α-adducin from MHS), NB (carrying the mutant 529R β-adducin from MHS), and NAB (carrying the mutant α- and β-adducin from MHS).

Systolic SBP and heart rate (HR) were recorded in conscious rats from 1.5 to 6 months of age by an indirect tail-cuff plethysmographic method (BP recorder, U. Basile, Varese, Italy). These rats were used to prepare renal glomeruli for immunoblotting quantifications.

(ii) Ouabain hypertensive rats (OHR). These rats were generated by subcutaneous ouabain infusion in male Sprague–Dawley rats, 5–6 week old and weighing 20–130 g, with osmotic mini-pumps (Alzet, Charles River, Wiggens, Italy) containing an ouabain–saline solution that slow-released 15 μg/ kg/day ouabain (Sigma-Aldrich, n = 8) for 12 weeks, as described previously (8). Normotensive control rats (n = 8) received sterile saline solution through osmotic mini-pumps. SBP and HR were recorded weekly at the tail. The initial SBP of controls and OHR rats was comparable (average 130–135 mmHg). After 12 weeks of ouabain infusion, plasma ouabain levels doubled (from 0.03 ± 0.04 to 0.7 ± 0.07 nm, P < 0.001) (19). SBP increased in OHR rats but HR was not affected by ouabain. The rats were used to quantify nephrin and synaptopodin protein expression in renal microsomes by immunoblotting and immunofluorescence. This last finding was replicated ex vivo by incubating podocyte primary cell cultures from Sprague–Dawley neonatal rats with low doses of ouabain (10−5 M) for 5 days.

**Human studies**

*Patients*

Forty-four newly discovered never treated hypertensive patients were enrolled in the ‘Outpatient Clinic for Hypertension’ of San Raffaele Hospital, Milan, Italy. The Ethics Committee of the San Raffaele Hospital approved the study and informed consent was obtained from all subjects. Patients underwent clinical examination and routine biochemistry. Secondary hypertension was excluded by routine methods. Females taking contraceptive pill were not enrolled in the study protocol. All patients underwent 24 h ambulatory BP monitoring (ABPM) (Spacelab 90207; Spacelab Medical, Inc., Redmond, WA, USA). Blood samples and 24 h urine collections were obtained in the days before the 24 h ABPM recording.

*Genotyping*

Genomic DNA was extracted from peripheral blood with standard methods (28). Patients were genotyped for SIK1 rs3746951 (C wild-type allele, T mutant allele).

*Human kidney specimens*

The study of human kidney samples was approved by the San Raffaele Scientific Institute Ethical Committee. Written informed consent was obtained from all patients. Human kidney specimens, derived from human nephrectomy because of tumors, were stored at −80°C Pathology Department of San Raffaele Hospital. The kidney portions used were verified to be histologically normal before analysis.

**Biochemical assay for urinary parameter measurements in rats and patients**

Urinary parameters were measured in conscious male rats at 1.5 and 6 months of age. Rats were housed in individual metabolic cages with free access to water and food. Urine samples of 24 h were analyzed for the following parameters: volume, pH, creatinine, Na+ and K+ concentrations (IL 943 photometer), total protein excretion (total protein Kit, Sentinel Diagnostics, Milan, Italy), and creatinine (colorimetric Kit, Sentinel Diagnostics).

For the quantification of urinary nephrin excretion in rats (5 ml urine) and humans (20 ml urine, collected early in the morning), the samples were concentrated by ultra-filtration and sequentially passed through a filtration membrane of 10 kDa cutoff at 750 g, at 4°C. The concentrate was washed twice with water to remove excess salt. The final protein concentration was estimated by the Bradford method. Ten micrograms of proteins were boiled and used for western blot analysis.

In human samples, urinary concentrations of creatinine and albumin were measured by immunoturbidimetric technique on a Cobas Mira autoanalyzer (Roche, Basel, Switzerland).

**Immunostaining**

Immunofluorescence analysis was performed on unfixed rat and human renal tissue embedded in OCT compound, snap-frozen and stored at −80°C. Immunofluorescence was also performed on a mouse podocyte cell line (SV1 cell line H-2kb-tsA58, CLS Heidelberg). Kidney sections, or cultured podocytes, were incubated with anti-nephrin (Progen) and anti-SIK1 (Santa Cruz) antibodies, followed by appropriate secondary antibodies and developed.

**Glomeruli and podocyte isolation**

Glomeruli were isolated by sieving from rat and human kidney specimens, as described previously (10). For rat glomerular podocyte isolation, kidneys were taken from 7- to 10-day-old
Rats. Isolated glomeruli were seeded in culture flasks pre-coated with collagen Type IV in DMEM : F12, 1:1 mixture, supplemented with 10% heat inactivated FBS, ITS, hydrocortisone, l-glutamine and penicillin/streptomycin (Sigma-Aldrich) at 37°C in 5% CO2 atmosphere. On Days 4 to 5, podocyte growth started, and by Day 8, glomeruli were detached using trypsin–EDTA and filtered through a 36 μm mesh to eliminate glomeruli. Second passage podocytes, >90% pure as judged by light microscopy, were seeded on flasks and chamber slides, as described previously (10) and cell characterization was performed by immunofluorescence, using markers for podocytes (nephrin, podocin, synaptopodin), epithelial (cytokeratin), smooth muscle (α-SMA) and endothelial cells (CD31). Podocytes seeded on flasks were incubated for 5 days with ouabain (Sigma, 10-9 M) and then analyzed by western blotting.

**Renal microsome preparation, caveola isolation and co-immunoprecipitation experiments**

Rat and human renal tissues were homogenized in 250 mM sucrose, 30 mM histidine and 1 mM EDTA, pH 7.2 and microsomes were prepared according to published procedures (19). Caveola-enriched microdomains were purified according to a detergent-free procedure, as described previously (19). Briefly, tissues were homogenized in: 200 mM sodium carbonate, pH 11, 2 mM sodium orthovanadate, 100 mg/ml Pefabloc and centrifuged at 6000g for 10 min. The supernatant was sonicated and fractionated on a 5–45% sucrose gradient. The gradients were centrifuged at 6000g for 18 h and 13 fractions were automatically collected and protein content measured. The expression of the specific proteins in the single fractions was determined by western blotting. The distribution along the gradient of the plasmanembrane, Golgi and endoplasmic reticulum was established by measuring alkaline phosphatase, α-mannosidase II and α-glucosidase II activity, respectively. Low-density fractions (fractions 2–5), containing the specific caveolin marker, were referred to as caveolae.

For the co-immunoprecipitation experiments, washed human renal caveolae were incubated for 30 min at 30°C in: 10 mM Tris, 10 mM MgCl2, 5 mM MnCl2, 0.25 mM EDTA, 0.25 mM sodium orthovanadate, 80 mM NaCl, 2 mM ATP, 100 mg/ml Pefabloc, pH 7.4 and subsequently with 0.2% CHAPS for 15 min at 4°C. Then an anti-Src antibody (GD11 clone), conjugated to protein G-Sepharose beads (Sigma), was added for 4 h. A sample of caveolae, incubated with a non-immune IgG (Sigma), instead of the anti-Src antibody, was used as control. The immunocomplexes were precipitated, washed three times with the immunoprecipitation buffer containing CHAPS, and boiled with Laemmli sample buffer. Supernatants were subjected to SDS-polyacrylamide gel electrophoresis and western blotting analysis.

Co-immunoprecipitation experiments were also performed from isolated rat glomerular podocytes by using anti-nephrin antibody as follows. Protein extracts (500 μg) were pre-cleared with 50 μl of protein-G Sepharose beads (Sigma-Aldrich), saturated with BSA (Sigma), for 1 h at 4°C. Podocyte lysates were incubated overnight at 4°C with 2 μg/ml of anti-nephrin polyclonal antibody and 50 μl of protein-G Sepharose beads were added for 3 h at 4°C. Beads were washed and boiled in SDS sample buffer. Supernatants were used for western blotting analysis.

Small interfering RNA (siRNA) silencing of nephrin

Mouse cultured podocytes (SV1 cell line H-2kb-tsA58, CLS Heidelberg) were routinely grown in DMEM F12 medium supplemented with 1% penicillin–streptomycin, 0.12 U/ml insulin, 5 mg/ml transferrin, 5 ng/ml selenite, 0.036 mg/ml hydrocortisone and 10% fetal bovine serum (Sigma). Twenty-four hours before transfection, cells at <80% confluence were grown in a medium without antibiotics. Transfection of siRNAs was carried out by incubation with Lipofectamine™-2000 Transfection Reagent in Opti-MEM Reduced Serum Medium (Invitrogen, Milan, Italy) for 6 h, followed by replacement with fresh medium containing 10% fetal bovine serum, as described by the manufacturer. For nephrin gene silencing, a pool of three siRNAs (SASI, Mm01-00152484, sequence 1147; SASI, Mm01-00152485, sequence 1281; SASI, Mm01-00152487, sequence 1111; Sigma-Aldrich) at 100 pmol were used. After siRNA, nephrin expression was verified by western blotting analysis.

**Western blot analysis**

Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (Criterion XT, Bio-Rad), blotted onto nitrocellulose membrane (Bio-Rad) for 90 min and incubated overnight at 4°C with specific primary antibodies, followed by 1 h incubation with fluorescent secondary antibodies (Alexa Fluor, 680 nm, red, Invitrogen; IRDye, 800 nm, green, Rockland). Western blotting was analyzed and quantified by Odyssey Infrared Imaging Detection System (LI-COR Biosciences). The optical densities were expressed as arbitrary units. The following antibodies were used: anti-SIK1 (Santa Cruz); anti-caveolin (BD); anti-nephrin (GP-N2, Progen); anti-synaptopodin (Sigma-Aldrich); anti-Src (GD11 clone, Upstate); anti-α-α-adducin (raised by Dr S. Salardi in Praxis Sigma-tau, Settimo Milanese, Milan); anti-α1 Na–K ATPase (Upstate); anti-actin (Sigma-Aldrich).

**Statistical analysis**

**Patients**

The effects of SIK1 rs3746951 genotypes on BP variations and urinary nephrin excretion were analyzed by ANOVA and P < 0.05 was considered statistically significant.

**Statistical analysis**

Data were reported as mean ± SEM; the statistical significance was measured by t-test or ANOVA analysis and P < 0.05 was considered statistically significant.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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Conflict of Interest statement. At the time of the experiments, M.F., I.M., P.F. were employees of Prassis sigma-tau Research Institute, Settimo Milanese, Milan, Italy. P.F. is a consultant of CVie Therapeutics, Hong Kong, China. The other authors state no conflict of interest.

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