Glomerular proteins related to slit diaphragm and matrix adhesion in the foot processes are highly tyrosine phosphorylated in the normal rat kidney

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Abstract

Background. Tyrosine phosphorylation of proteins has been a focus of extensive studies since it plays crucial roles in regulation of diverse biological reactions. To understand the involvement of tyrosine phosphorylation in kidney functions, a comprehensive proteomic study for tyrosine-phosphorylated proteins was performed in the normal rat kidney.

Methods. Two-dimensional gel electrophoresis and immunoprecipitation using anti-phosphotyrosine antibodies were employed to detect tyrosine-phosphorylated proteins. The proteins were analysed by mass spectrometry and validated by immunological analyses using specific antibodies.

Results. Most of tyrosine-phosphorylated proteins were confined to the glomerulus and predominantly localized along the glomerular capillary wall, especially in the foot processes of podocytes. Our systematic proteomic analysis identified nephrin, SHPS-1 (tyrosine-protein phosphatase non-receptor-type substrate 1), FAK1 and paxillin as major tyrosine-phosphorylated proteins and Neph1, talin and vinculin as minor tyrosine-phosphorylated proteins. In the present study, SHPS-1 was identified as a novel tyrosine-phosphorylated protein in the glomerulus and was also predominantly localized at the foot processes. Mass spectrometric analysis identified in vivo phosphorylation sites of SHPS-1 on Y460, Y477 and Y501.

Conclusion. This study identified tyrosine-phosphorylated proteins in normal rat kidney, which were prominently rich in the glomerulus and localized at the podocyte foot processes. These proteins were categorized as cell-to-cell or cell-to-matrix adhesion complex-related molecules, suggesting their pivotal roles in the glomerular ultrafiltration.

Keywords: glomerulus; proteomics; tyrosine-phosphorylated proteins

Introduction

Tyrosine phosphorylation participates in diverse cellular processes, and tyrosine kinase signaling pathways are now a major focus of biochemical research [1]. In recent studies on kidney function, tyrosine phosphorylation of some transmembrane proteins at the slit diaphragm, such as nephrin [2–6] and Neph1 [7,8], has emerged as a critical process for the glomerular filtration function. A line of evidence demonstrated that these proteins recruit adaptor proteins to their cytoplasmic domains and then connect to the podocyte actin cytoskeleton on a tyrosine phosphorylation-dependent manner [9,10]. Therefore, the actin cytoskeleton of foot processes becomes the common denominator in kidney function and dysfunction through the association with the signaling proteins [11,12].

A systematic study for identification and characterization of proteins phosphorylated in vivo on tyrosine residues in the normal kidney will provide further insights into the understanding of the regulation of kidney functions in health and disease. However, it has not been well done. In this study, we aimed to identify tyrosine-phosphorylated proteins in the normal rat kidney by a high-confidence proteomic approach. Interestingly, tyrosine-phosphorylated proteins were highly concentrated in the glomerulus rather than other parts of the kidney. Proteomics in combination with immunological analyses unambiguously identified a group of tyrosine-phosphorylated proteins in the glomerulus. Those were nephrin, Neph-1, FAK1, paxillin, talin, vinculin and SHPS-1 (tyrosine-protein phosphatase non-receptor-type substrate 1). These results provide a panel of in vivo tyrosine-phosphorylated proteins in the glomerulus and support the crucial role of tyrosine phosphorylation of signaling pro-
teins at the slit diaphragm and focal adhesion of the foot process in the regulation of glomerular filtration and other functions in health and disease.

Materials and methods

Animals

Male 8-week-old Wistar rats (Charles River) were used in this study, which was approved by the Animal Committee at Niigata University School of Medicine.

Antibodies

Primary antibodies used in this study were monoclonal anti-phosphotyrosine antibodies P-Tyr-100 (Cell Signaling, Danvers, MA, USA) and 4G10 (Upstate, Charlottesville, VA, USA), polyclonal anti-SHPS-1 (Upstate, Charlottesville, VA, USA), polyclonal anti-FAK (Cell Signaling, Danvers, MA, USA), monoclonal anti-paxillin (Zymed, Carlsbad, CA, USA), monoclonal anti-synaptopodin (Progen, Heidelberg, Germany) and monoclonal anti-GAPDH (Ambion, Tokyo, Japan). Polyclonal anti-nephrin and anti-NepH1 antibodies were raised against synthetic peptides as described [8,13]. Secondary goat antibodies used herein were gold-labeled anti-mouse and anti-rabbit IgG (GE Healthcare, Chalfont, St. Giles, UK), FITC-conjugated anti-rabbit IgG (pre-absorbed with rat IgG, Immuno-Biological Laboratories, Gunma, Japan), Texas-Red-conjugated anti-mouse IgG (Rockland Immunchemicals, Gilbertsville, PA, USA), HRP-conjugated anti-mouse and anti-rabbit IgG (DakoCytomation, Hamburg, Germany). HRP-conjugated anti-mouse antibody was pre-absorbed with rat serum prior to use. Mouse IgG and rabbit IgG (Jackson Immune-Research Laboratories, Suffolk, CB8 7SY England), which were known not to react with antigens of rat kidneys in immunochemical analyses, were used as negative controls.

Preparation of protein samples

Rat kidneys were removed after perfusion through abdominal aorta with PBS in the presence or absence of 1mM Na2VO4. Glomeruli were isolated from cortices by the standard sieving method. The isolated glomeruli, cortex and medulla were homogenized in lysis solution (1% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, 1mM Na3VO4, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na2VO4, 25 mM NaF, 50 mM Tris–HCl, pH 7.5, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A) and centrifuged at 14 000 g for 20 min to obtain supernatants as protein samples. All the tyrosine-phosphorylated proteins were recovered in the supernatants as no specific bands for phosphotyrosine were detected in the pellets by Western blot (data not shown).

Immunoblotting

Protein samples were separated on 10% SDS–PAGE gels and transferred onto PVDF membranes and incubated with primary antibody overnight at 4 °C, followed by the appropriate secondary antibody (dilution 1:1000). Immunoreactive proteins were visualized by an enhanced chemiluminescence detection system (ECL Plus, GE Healthcare, Chalfont, St. Giles, UK).

Immunohistochemistry and immunoelectron microscopy

Normal rat kidneys were perfused with paraformaldehyde-lysine-periodate (PLP) and immersed in the same fixative at 4 °C overnight. The tissues were dehydrated and embedded in hydrophilic methacrylate resin. Sections of 1 μm thickness were prepared for immunohistochemistry and incubated with P-Tyr-100 or irrelevant mouse IgG at room temperature for 1 h followed by incubation with gold-labeled anti-mouse IgG (dilution 1:20) for 1h. The immunogold staining was enhanced by treatment with IntenSEM (GE Healthcare, Chalfont, St. Giles, UK) for 10 min. After washing with distilled water, the sections were stained with toluidine blue. For immunoelectron microscopy, ultrathin sections were prepared from kidneys after perfusion with PLP as described [14,15]. They were incubated with P-Tyr-100 or anti-SHPS-1 followed by gold-labeled anti-mouse IgG (dilution 1:20) or anti-rabbit IgG (dilution 1:20) and observed under an electron microscope (H-600A, Hitachi).

Results

Western blot analysis

We firstly analysed expression of tyrosine-phosphorylated proteins in glomerulus, cortex and medulla of normal rat kidneys by Western blot using authentic anti-phosphotyrosine antibodies, P-Tyr-100 and 4G10. Rat kidneys were perfused with PBS with or without 1mM Na2VO4, a protein tyrosine phosphatase inhibitor. As shown in Figure 1A, P-Tyr-100-immunoreactive proteins were intensely detected in the glomerulus, compared with the cortex and medulla. The perfusion with PBS containing
The intensity of the immunoblotted protein bands. Four predominant bands with molecular masses of 220, 145, 120 and 70 kDa were detected in the glomerulus. Specific detection of phosphotyrosine was confirmed by a pre-absorption experiment, where the immunoreactive bands to P-Tyr were abolished by the pre-absorption of the antibody with phosphotyrosine but not with either phosphoserine or phosphothreonine (Figure 1B). Similar results were obtained with 4G10 (Supplementary material 1).

Immunolocalization of tyrosine-phosphorylated proteins in the kidney

The localization of tyrosine-phosphorylated proteins was examined by immunohistochemistry with P-Tyr-100. In the cortex, immunostaining was demonstrated prominently along the glomerular capillary walls and also sparsely along the basolateral side of tubular epithelial cells (Figure 2A). Their localization was further explored by immunoelectron microscopy (Figure 2C). The immunogold particles were largely confined to the basal membranes of foot processes and sparsely at slit diaphragms and fenestrated glomerular endothelial cells.

Identification of tyrosine-phosphorylated proteins by mass spectrometry

In our first approach for identification of tyrosine-phosphorylated proteins, glomerular lysate was separated by two-dimensional gel electrophoresis (2-DE). Tyrosine-phosphorylated proteins were well separated on the 2-DE gels and detected by Western blot. However, only talin (220 kDa) and vinculin (120 kDa), two proteins of focal adhesion complex [17,18], were identified by mass spectrometric analysis (Supplementary material 2). Other glomerular tyrosine-phosphorylated proteins were elusive.

We, therefore, immunoprecipitated tyrosine-phosphorylated proteins with P-Tyr-100 and analysed them by mass spectrometry. P-Tyr-100 specifically reacted with phosphotyrosine but with neither phosphothreonine nor phosphoserine in Western blot (Figure 1B) and in immunoprecipitation (Figure 3A). The glomerular lysate was pre-cleared twice with protein A-Sepharose beads before immunoprecipitation, but nonspecific proteins could not be removed completely. The irrelevant mouse IgG was used instead of P-Tyr-100 for immunoprecipitation as a negative control. As shown in Figure 3B, all four protein bands were efficiently immunoprecipitated with P-Tyr-100, whereas no specific bands were detected in the proteins nonspecifically bound to the beads and in the immunoprecipitate with irrelevant mouse IgG. As the fractions 2, 4, 5 and 9 corresponded to the tyrosine-phosphorylated protein bands of 220, 145, 120 and 70 kDa, respectively (Figure 3C), these four fractions were subjected to mass spectrometry for protein identification. Proteins were excluded from the candidate list if they were also identified in the corresponding fractions in the lane of the bead-bound protein.

Through three independent analyses, 12 proteins were selected by the following identification criteria: identified with two or more peptide matches and commonly identified in all the three analyses (Table 1). Of these proteins,
Fig. 2. Tyrosine-phosphorylated proteins are mainly localized to glomerular capillary walls, especially at basal membranes of foot processes and slit diaphragms, as revealed by immunohistochemistry and immunoelectron microscopy. (A) 1 μm-thick sections were incubated with the primary antibody to phosphotyrosine, P-Tyr-100, followed by incubation with gold-labeled goat anti-mouse IgG and treated with IntenSEM for enhancing the immunogold staining. The lower panel is the magnified image of the area indicated by the rectangle in the upper photograph. Arrows show some immunogold particles along the capillary walls, and arrowheads indicate some particles along the basal membranes of tubular epithelial cells. The asterisk indicates a podocyte. (B) The section was incubated with irrelevant mouse IgG followed by incubation with the same second antibody and enhancement reagent used in A. (C) Ultrathin sections were probed with P-Tyr-100, followed by gold-labeled anti-mouse IgG. Besides the immunoreaction to basal membranes of foot processes (arrows) and slit diaphragms (asterisks), Glomerular endothelial cells were also sparsely and positively stained (arrowheads). Symbols: P, podocyte; GBM, glomerular basement membrane; E, endothelial cell; pY, P-Tyr-100.

Fig. 3. Glomerular tyrosine-phosphorylated proteins are specifically immunoprecipitated by the anti-phosphotyrosine antibody, P-Tyr-100. (A) Glomerular lysate was immunoprecipitated by P-Tyr-100 (lane 5) or the antibody pre-absorbed with phosphotyrosine (lane 2), phosphothreonine (lane 3) or phosphoserine (lane 4). Results of Western blot analysis of these immunoprecipitates are shown. Lane 1 represents glomerular lysate not subjected to immunoprecipitation. See ‘Materials and methods’ section for more details. (B) Glomerular lysate was first pre-cleaned with protein A-Sepharose and subjected to immunoprecipitation with P-Tyr-100 or irrelevant mouse IgG. Protein eluates from protein A-Sepharose beads used for precleaning (NS), immunoprecipitation with P-Tyr-100 (imppt) or irrelevant mouse IgG (NS*) were analysed by Western blot with P-Tyr-100. (C) Eluates from protein A-Sepharose beads used for precleaning (NS) and immunoprecipitation with P-Tyr-100 (imppt) were separated on SDS–PAGE gel and silver stained. The parts of both lanes corresponding to 60–270 kDa were each sliced into nine fractions and subjected to mass spectrometric analysis for protein identification. Among the nine fractions, fractions 2, 4, 5 and 9 correspond to the tyrosine-phosphorylated protein bands of 220, 145, 120 and 70 kDa, respectively.
Table 1. Identification of tyrosine-phosphorylated proteins in glomerulus of normal rat kidney

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<th>Fraction no.</th>
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<th>Protein score</th>
<th>Sequence coverage (%)</th>
<th>No. of matched peptides</th>
<th>Phosphorylation site (database: Swiss-Prot)</th>
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<td>43</td>
<td>45</td>
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<td>13</td>
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Proteins were analysed by a nanoflow LC-linear iontrap-time-of-flight mass spectrometer (Hitachi NanoFrontier LD). Proteins were identified by Mascot search engine (version 2.2) against Swiss-Prot (release 54.30). Fraction number corresponds to gel slice of SDS–PAGE gel as depicted in Figure 3. Protein score was provided by Mascot search engine using MudPIT scoring algorithm.

Fig. 4. Nephrin is the major component for the band of 220 kDa. (A) Protein extracts (10 μg/lane) from the glomerulus, cortex and medulla of normal rat kidneys were separated on 10% gel, transferred to PVDF membranes and immunoblotted with either anti-nephrin or anti-GAPDH. Arrows indicate the double bands for nephrin in glomerulus. Symbols: G, glomerulus; C, cortex; M, medulla. (B) Glomerular lysate was immunoprecipitated either with P-Tyr-100 (a and b) or with anti-nephrin (c and d). Each of immunoprecipitate was divided in halves. One-half was immunoblotted with P-Tyr-100 (a and d) and the other with anti-nephrin (b and c). The lysate used for immunoprecipitation and the resultant supernatant were also analysed by Western blot with the indicated antibodies. Irrelevant mouse IgG and rabbit IgG were used for immunoprecipitation, and the immunoprecipitates were analysed by Western blot as negative controls. Arrowheads and long arrows indicate nephrin, while short arrows indicate IgG heavy chain. Symbols: IP, immunoprecipitation; WB, Western blot; pY, P-Tyr-100; lys, lysate; sup, supernatant; imppt, immunoprecipitate.
nephrin in fraction 2, SHPS-1 and Neph1 in fraction 4, FAK1 in fraction 5 and paxillin in fraction 9 were considered as major tyrosine-phosphorylated proteins as estimated by the number of matched peptides, sequence coverage and Mascot protein score and subjected to validation by immunochemical analyses.

LC-MS/MS analyses unambiguously identified several tyrosine phosphorylation sites for nephrin, Neph1 and SHPS-1: nephrin, Y1186 [Q9R044 in Swiss-Prot, corresponds to Y1204 (AAF14884 in NCBInr)]; Neph1, Y654 (Q6X936 in Swiss-Prot); SHPS-1, Y460, Y477 and Y501 (P06685 in Swiss-Prot) (Table 1 and Supplementary material 3).

**Confirmation of glomerular tyrosine-phosphorylated proteins**

**Nephrin.** Nephrin was almost exclusively detected in the glomerulus as a doublet around 220 kDa in 10% SDS–PAGE (Figure 4A). Since nephrin was previously described as a doublet around 180 kDa in 5 or 7.5% SDS–PAGE [19,20], we examined the size of nephrin using 7% gels and found that the molecular mass of nephrin was around 180 kDa (data not shown). So we presumed that the migration of nephrin was retarded much more in 10% gels than in gels with lower concentration, possibly due to physical nature attributable to its glycosyl or phosphate groups. Tyrosine phosphorylation of nephrin was confirmed by immunoprecipitation with P-Tyr-100 followed by Western blot with anti-nephrin and vice versa (Figure 4B, panels a and b). Furthermore, only the upper band of nephrin doublet was phosphorylated (Figure 4B, panel b). Nephrin was almost undetectable in the supernatant after immunoprecipitation with anti-nephrin, and the 220-kDa band of tyrosine-phosphorylated protein was also almost eliminated from the supernatant and intensely detected in the immunoprecipitate (Figure 4B, panels c and d), indicating that nephrin is the major tyrosine-phosphorylated protein for the 220-kDa band. While P-Tyr-100 precipitated almost all tyrosine-phosphorylated proteins from the lysate, intensive nephrin band was still detected in the supernatant (Figure 4B, panels a and b), suggesting that only a small portion of nephrin molecules were tyrosine-phosphorylated in vivo.

**SHPS-1 and Neph1.** To confirm SHPS-1 and Neph1 identified by the mass spectrometric analysis as glomerular tyrosine-phosphorylated proteins for the 145-kDa band, the same experimental procedures as those for nephrin were employed. By Western blot, SHPS-1 and Neph1 were present in the glomerulus predominantly, compared with the cortex and medulla (Figures 5A and 6A). Tyrosine phosphorylation of SHPS-1 was demonstrated by immunoprecipitation using anti-SHPS-1 followed by Western blot using P-Tyr-100 or vice versa (Figure 5B, panels b and d). Furthermore, SHPS-1 was confirmed as the major tyrosine-phosphorylated protein for the 145-kDa band.
(Figure 5B, panels c and d). Also, it was revealed that only a small portion of SHPS-1 molecules in the glomerulus were tyrosine-phosphorylated (Figure 5B, panels a and b). Neph1 was also confirmed as a tyrosine-phosphorylated protein for the 145-kDa band (Figure 6B, panel d). However, reciprocal immunoprecipitation with P-Tyr-100 followed by Western blot with anti-Neph1 demonstrated no distinct band for Neph1 (Figure 6B, panel b). The result may indicate that Neph1 is a low-abundance tyrosine-phosphorylated protein in the glomerulus. The intensity of the 145-kDa band for phosphotyrosine in the supernatant after Neph1 immunoprecipitation remained almost the same as in the lysate (Figure 6B, panel d), suggesting that Neph1 is a minor tyrosine-phosphorylated protein component for the 145-kDa band.

FAK1. FAK1 was demonstrated predominantly in the glomerulus at 120 and 90 kDa by immunoblotting analysis (Figure 7A). The intense 120-kDa band and the weak 90 kDa band were detected with anti-FAK1 in the P-Tyr-100 immunoprecipitate and also in the reciprocal experiment (Figure 7B, panels b and d), proving that FAK1 is tyrosine-phosphorylated in the glomerulus. Furthermore, FAK1 was demonstrated as the major tyrosine-phosphorylated 120-kDa protein (Figure 7B, panel d). The 90-kDa band detected in the immunoprecipitate was less intense than the 120-kDa band, suggesting that the 90-kDa band was the minor tyrosine-phosphorylated form of FAK1. It should be noted that, in contrast to other phosphorylated proteins, most of FAK1 seemed to be tyrosine-phosphorylated as P-Tyr-100 precipitated most of FAK1 (Figure 7B, panel b).

Paxillin. Western blot showed an intense 70-kDa band and a weak 55-kDa band for paxillin in the glomerulus (Figure 8A). As the 70-kDa band corresponded to the size of paxillin, the 55-kDa band, which was consistently observed, may be a short isoform or a degraded form of paxillin. Paxillin was also tyrosine-phosphorylated in the glomerulus (Figure 8B, panels b and d) and demonstrated as the major protein for the 70-kDa band (Figure 8B, panel d). In addition, the 70-kDa band for paxillin detected in the anti-P-Tyr-100 immunoprecipitate showed a slightly lower mobility than those two in the lysate and supernatant (Figure 8B, panel d), possibly reflecting the tyrosine phosphorylation of paxillin. Since the size of the lower band for paxillin is 55 kDa, which is close to that of IgG heavy chain, it was difficult to distinguish these two bands in the immunoprecipitate. The lower band detected in the anti-P-Tyr-100 immunoprecipitate (Figure 8B, panel b) was likely to be IgG heavy chain as paxillin could not be identified by the additional mass spectrometric analysis (data not shown). We presume that the 55 kDa band in the anti-paxillin immunoprecipitate (Figure 8B, panel d) also represented IgG heavy chain since this band could not be observed in the glomerular lysate.
Immunolocalization of SHPS-1 in the normal rat glomerulus

As SHPS-1 was identified as a novel tyrosine-phosphorylated protein in the glomerulus, we examined its localization by double-labeled immunofluorescence with anti-SHPS-1 and anti-synaptopodin (Figure 9A). SHPS-1 was co-localized with synaptopodin, an actin-associated protein expressed exclusively in podocyte foot processes. Immunoelectron microscopy revealed subcellular localization of SHPS-1 at plasma membrane of foot processes: the basal and apical membranes and also the base of slit diaphragm (Figure 9B). Since anti-phosphotyrosine antibody predominantly labeled the basal membranes of foot processes, we presume that the staining of SHPS-1 in this area represents its tyrosine-phosphorylated form.

Discussion

Podocytes are structurally adapted to facilitate the plasma ultrafiltration and endure the high glomerular hydraulic pressure probably through their cell–cell interaction (slit diaphragm) and cell–matrix adhesion (focal adhesion complex). Slit diaphragm and focal adhesion complex in the function of the foot processes, not only as the structural framework but also as the signaling platform for inside-out and outside-in signals [9]. Further, these cell–cell and cell–matrix contacts are physically and functionally linked to the actin cytoskeleton of podocyte foot processes, which is of crucial importance for the glomerular filtration function [21]. Interestingly, most of the tyrosine-phosphorylated proteins identified in this study are either transmembrane proteins of slit diaphragm (nephrin and Neph1) or cytoskeleton-associated proteins in focal adhesion complex (FAK1, paxillin, vinculin and talin).

Recent studies on nephrin [2–6] and Neph1 [7,8] demonstrated that they recruit adaptor molecules in tyrosine phosphorylation-dependent manner. Upon tyrosine phosphorylation by Fyn, a Src family kinase, nephrin recruits Nck adaptor proteins, which modulates the organization of cytoskeletal architecture of the podocyte foot process [3,6,22]. Tyrosine phosphorylation of nephrin was also shown to bind phosphoinositide-3-kinase (PI3K), which induces actin reorganization through activation of Akt and Rac as mediators [9]. Furthermore, phosphorylation on Y1204 of nephrin has been recently demonstrated to induce recruitment of phospholipase C-γ1, which is known to regulate Ca²⁺ signaling through production of inositol 1,4,5-trisphosphate [23]. Phosphorylation of Y1204 and Y1228 residues in rat nephrin was shown to be required to bind Nck proteins [3], while phosphorylation of Y1152 was shown to be necessary for binding to PI3K [4]. The present study identified Y1204 as a phosphorylation site of nephrin. Neph1 was demonstrated to be phosphorylated by Fyn at Y604, Y637, Y638 and Y654 in vitro [8]. We identified Y654 of Neph1 as an in vivo phosphorylation site.
SHPS-1 is a transmembrane glycoprotein belonging to the immunoglobulin superfamily [24]. While SHPS-1 was shown abundantly expressed in the neuronal and hematopoietic cells, it has not been well characterized in podocytes. It has been reported that tyrosine phosphorylation of SHPS-1 is regulated by integrins [25] and involved in cytoskeletal reorganization and cell motility [26]. Tsuda et al. reported that tyrosine phosphorylation of SHPS-1 requires both FAK and Src family kinase [27]. In our study, we found that SHPS-1 was tyrosine-phosphorylated in the glomerulus under physiological conditions and predominantly localized in podocytes. Phosphorylation of three tyrosine residues in the SHPS-1 cytoplasmic domain (Y460, Y477 and Y501) was demonstrated by mass spectrometric analysis. We speculate that tyrosine phosphorylation of SHPS-1 also plays a critical role in maintaining the unique actin cytoskeleton organization and focal adhesion complex of the foot process.

Fig. 8. Paxillin is the major component for the band of 70 kDa. (A) Protein extracts (10 μg/lane) from the glomerulus, cortex and medulla of normal rat kidney were analysed with anti-paxillin and anti-GAPDH. Symbols: G, glomerulus; C, cortex; M, medulla. (B) Glomerular lysate was immunoprecipitated either with the antibody P-Tyr-100 (a and b) or with anti-paxillin (c and d). The immunoprecipitates and supernatants together with the glomerular lysates were used for Western blot with the indicated antibodies. Two bands for paxillin were detected at 70 and 55 kDa in glomerular lysate (b and c), and the 70-kDa band was the tyrosine-phosphorylated form of paxillin (b and d, indicated by long arrows). Short arrows indicated IgG heavy chain except the lane imppt in panel c, where both IgG heavy chain and the lower band for paxillin were closely arrayed around 55 kDa. Irrelevant mouse IgG was used for immunoprecipitation as negative controls. Symbols: IP, immunoprecipitation; WB, Western blot; pY, antibody to phosphotyrosine, P-Tyr-100; lys, lysate; sup, supernatant; imppt, immunoprecipitate.

Conclusion

The proteomic analysis identified nephrin, Neph1, SHPS-1, FAK1, paxillin, vinculin and talin as tyrosine-phosphorylated proteins in normal rat glomerulus. These molecules are components of either slit diaphragm or focal adhesion complex. We speculate that these proteins may play a crucial role in the regulation of glomerular ultrafiltration and the maintenance of physical structure resistant to the high glomerular hydraulic pressure through phosphorylation-mediated signaling pathways.

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Conflict of interest statement. None declared.
Supplementary data

Supplementary data is available online at http://ndt.oxfordjournals.org.

References


Fig. 9. SHPS-1 is predominantly localized in the podocyte of glomerulus. (A) Double-labeled immunofluorescence microscopy using anti-SHPS-1 and anti-synaptopodin antibodies. Frozen sections of normal rat kidney were fixed by PLP before incubated with the mixture of anti-SHPS-1 and anti-synaptopodin antibodies, then sequentially with FITC-conjugated anti-rabbit IgG and Texas-Red-conjugated anti-mouse IgG. Arrows indicate glomerular capillary walls. (B) Immunoelectron microscopy using anti-SHPS-1 antibody. Ultrathin sections from PLP-perfused kidney were probed with anti-SHPS-1, followed by gold-labeled anti-rabbit IgG. Symbols: P, podocyte; GBM, glomerular basement membrane; E, endothelial cell. Arrows indicate the basal membrane of foot processes, asterisks indicate slit diaphragms and arrowheads indicate the apical membranes of foot processes.
Role of p21 and oxidative stress on renal resistance

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Abstract

Background. Subsequent ischaemic episodes may induce renal resistance. P21 is a cell cycle inhibitor that may be induced by oxygen-free radicals and may have a protective effect in ischaemic acute kidney injury (AKI). This study aimed at evaluating the role of oxidative stress and p21 on tubular resistance in a model of acquired resistance after renal ischaemia and in isolated renal tubules.

Methods. Wistar rats were divided into: Group 1—sham; Group 2—sham operated and after 2 days submitted to 45-min ischaemia; and Group 3—45-min ischaemia followed after 2 days by a second 45-min ischaemia. Plasma urea was evaluated on Days 0, 2 and 4. Serum creatinine, creatinine clearance and oxidants (thiobarbituric acid-reactive substances) were determined 48 h after the second procedure (Day 4). Histology, immunohistochemistry for lymphocytes (CD3), macrophages (ED1), proliferation (PCNA) and apoptosis (TUNEL) were also evaluated. Rat proximal tubules (PTs) were isolated by collagenase digestion and Percoll gradient from control rats and rats previously subjected to 35 min of ischaemia. PTs were submitted to 15-min hypoxia followed by 45-min reoxygenation. Cell injury was assessed by lactate dehydrogenase release and hydroperoxide production (xylenol orange).

Results. Ischaemia induced AKI in Group 2 and 3 rats. Subsequent ischaemia did not aggravate renal injury, demonstrating renal resistance (Group 3). Renal function recovery was similar in Group 2 and 3. Plasma and urine oxidants were similar among in Group 2 and 3. Histology disclosed acute tubular necrosis in Group 2 and 3. Lymphocyte infiltrates were similar among all groups whereas macrophages infiltrate was greater in Group 3. Cell proliferation was greater in Group 2 compared with Group 1. Treatment with adrenaline increased cell proliferation in Group 2 and 3. The oxidative stress marker 3-Phosphono-1,2-propanediol increased cell proliferation in Group 2 but not in Group 3. Ischaemia increased cell proliferation in Group 3 but not in Group 2. In addition, ischaemia increased cell proliferation in PTs isolated from control rats but not in PTs isolated from rats subjected to ischaemia.

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