Molecular and cellular responses to renal injury : a (phospho)-proteomic approach

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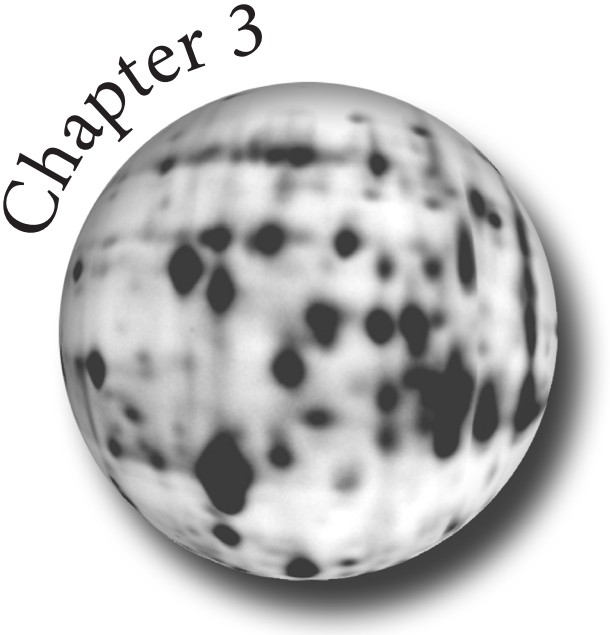
Dynamic focal adhesions in ischemia reperfusion

Chapter 3



Dynamic focal adhesion organization and differential phosphorylation of focal adhesion kinase in renal ischemia/reperfusion injury and regeneration

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ABSTRACT

Acute renal failure due to ischemia/reperfusion involves disruption of integrin-mediated adhesion of renal proximal tubular cells (PTC). We studied the dynamics of focal adhesions, F-actin organization and tyrosine phosphorylation of focal adhesion proteins in PTC during ischemia/reperfusion. Male Wistar rats were subjected to unilateral ischemia for 30 or 45 min by renal artery clamping followed by reperfusion periods from 1 h to 2 weeks. In control kidneys, focal adhesions, containing FAK, paxillin and talin, were present at the basolateral membrane of PTC and co-localized with F-actin stress fibers. These adhesion sites were rich in tyrosine phosphorylation, which was lost directly after ischemia. After 24 h of reperfusion, the size of focal adhesions was increased, compared to control. This correlated with an increase in the number and thickness of the F-actin stress fibers. Reperfusion caused increased phosphorylation of several focal adhesion proteins. The amount of PY397 and PY861-FAK as well as PY416-Src and PY118-paxillin was highest at 1 and 24 h of reperfusion; PY576-FAK was only elevated at the early time-point. We propose a concept whereby reversible loss of protein tyrosine phosphorylation during ischemia drives the dynamic dissolution/re-structuring of focal adhesions and the F-actin cytoskeleton during reperfusion and regeneration.

INTRODUCTION

Ischemia reperfusion (I/R) injury is an important, life threatening clinical problem that may occur in various vital organs like heart, brain and kidney. One of the primary events during I/R is mitochondrial dysfunction leading to ATP depletion. A general phenomenon during I/R is compromised cell adhesion [1-4]. Cell adhesion is tightly controlled by proper integrity of the actin cytoskeletal network, which requires ATP [5;6]. Likewise, I/R causes cytoskeletal disruption in various tissues [7]. It is important to better understand the basic mechanisms that drive disruption of cytoskeletal organization and cell adhesion in the course of I/R. In contrast to most other organs the kidney can completely and efficiently regenerate after I/R injury, making it a unique model to unravel the dynamics of the F-actin cytoskeleton and the cell-extracellular matrix (ECM) interactions, and molecular events involved in this process.

I/R injury is one of the most important causes of acute renal failure (ARF) and the proximal tubular cells (PTC) are the main target [8;9]. Early after the ischemic period the basolateral-apical protein polarity is disturbed and the microvilli brush border is lost in conjunction with loss of cytoskeletal integrity [7;10]. Renal I/R injury also results in perturbations of cell-cell contacts at adherens and tight junctions [11;12] and the integrin-mediated cell-ECM adhesions [4;13;14]. These changes are requirements for detachment and exfoliation of epithelial cells into the lumen, leaving a denuded proximal tubule that requires regeneration. To better understand cell adhesion *in vivo* and the role that adhesion plays during I/R it is necessary to unravel the molecular and cellular mechanisms that underlie the cellular injury and regeneration during I/R.

Cell-ECM adhesions are mediated by the integrin-family of cell adhesion receptors at focal adhesions (FAs). FAs consist of a large number of both cytoskeletal and signal transduction (adapter) proteins and are rich in tyrosine phosphorylated proteins. β1-integrin is the most prominent integrin in PTC-mediated cell-ECM interactions. β1-integrins are lost from the PTC basolateral membrane region during the ischemic period, and return to this side during reperfusion [4;15;16]. Localization of β1 integrin at the cell-ECM contacts is regulated by the integrity of the F-actin cytoskeletal network as well as signal transduction pathways. Given the fact that I/R results in ATP depletion coupled to intracellular stress responses and modulation of β1-integrins, adhesion of PTCs will largely be regulated via inside-out signalling. Protein phosphorylation is one of the principal regulatory mechanisms that controls cell adhesion, therefore determining differential kinase activities as well as protein (de)phosphorylation events is essential in understanding the mechanisms of cell detachment during I/R.

Focal adhesion kinase (FAK) is a ubiquitously expressed non-receptor protein tyrosine kinase, which is essential in cell-ECM signalling towards cell migration, survival and proliferation. Recruitment to clustered integrins at FAs allows FAK autophosphorylation on tyrosine residue Y397. This residue forms a binding site for the SH2-domain of Src kinase, which is activated and subsequently induces phosphorylation of FAK on other tyrosine residues, including Y576/Y577 in the kinase domain and Y861 in the C-terminal domain. Together the FAK/Src kinase complex phosphorylates downstream targets such

as the signalling adapter protein paxillin [17;18]. FAK is dephosphorylated after chemical anoxia in isolated rabbit proximal tubules [19] and during nephrotoxicant exposure in primary cultured rat PTCs [20]. So far, the functional role of FAK in I/R *in vivo* remains unclear. Moreover, the differential phosphorylation of FAK at different tyrosine residues in the context of restructuring of both adhesion complexes and the F-actin cytoskeleton in renal I/R injury and regeneration have not been investigated.

In this study, we report the abundant presence of FAs rich in tyrosine phosphorylated proteins at the basolateral membrane of PTC *in vivo*. These FAs contain FAK, paxillin and talin that co-localize with F-actin stress fibers. Tyrosine phosphorylation at FAs was lost directly after ischemia, which was associated with reorganization of the FAs and followed by a drastic increase in phosphorylation during reperfusion together with an increase in FA size. The ischemia-induced FAK dephosphorylation was followed by a differential phosphorylation of the different FAK tyrosine residues during reperfusion. These data indicate a dynamic restructuring of FAs in association with differential tyrosine phosphorylation of FA-associated structural and signalling proteins. The disturbances most likely drive loss of integrin-mediated cell-ECM interactions.

MATERIALS AND METHODS

Renal ischemia/reperfusion injury

For this study, a unilateral rat model of renal ischemia/reperfusion was used [4;21;22]. Male Wistar rats (170-220 grams) were anesthetized with S-Ketamine (25 mg/kg body s.c.) and Metodomine hydrochloride (0.04 mg/animal i.m.). A small incision was made over the left flank, the left renal artery was prepared and clamped with a hemostatic clamp (5-15 G/mm^2, Moria) for 30 or 45 minutes, while right kidneys were unaffected and served as internal controls. After removal of the clamp, the kidney was reperfused for indicated time intervals. After reperfusion, both left and right kidneys were harvested and prepared for further analysis as described under 'Tissue preparation'. Control kidneys were obtained from animals that underwent sham surgery without ischemia/reperfusion. Each group consisted of 5 animals.

Tissue preparation

After harvesting, both kidneys were briefly washed in ice cold PBS to remove excess blood, and sliced into two equal halves. One half was frozen in liquid nitrogen and stored at -80 ^{o}C for Western blot analysis or immunohistochemistry. One half was fixated in cold Carnoy's solution (60 % (v/v/) absolute ethanol, 30 % (v/v) chloroform and 10 % (v/v) glacial acetic acid) for 3 hours and thereafter transferred to 70% (v/v) ethanol and stored at 4 ^{o}C for histopathologic evaluation.Kidneys were embedded in paraffin and sectioned (3 μM) onto APES coated slides. Paraffin sections were deparaffinized and rehydrated before staining for hematoxylin and eosin (H&E) and examined for the characteristic morphological changes resulting from I/R injury using light microscopy (Leica DM6000B, 400x magnification).

Histopathologic evaluation

Kidneys were embedded in paraffin and sectioned (3 μm) onto APES coated slides. Paraffin sections were deparaffinized and rehydrated before staining for hematoxylin and eosin (H&E) and examined for the characteristic morphological changes resulting from I/R injury using light microscopy (Leica DM6000B, 400x magnification).

Immunohistochemistry

Frozen sections (10 μm) were cut with cryostat and thaw settled on APES coated slides and fixed in 4 % formaldehyde for 10 minutes. After washing with TBS, sections were blocked in 5 % (v/v) normal goat serum (NGS, Vector Laboratories) for 1 h and incubated overnight at 4 °C in a humidified chamber with primary antibody; total protein tyrosine phosphorylation (PY99, Santa Cruz Biotechnology), FAK (clone 77, Transduction Laboratories), PY397-FAK (BioSource), paxillin (Transduction Laboratories) and PY118-paxillin (BioSource), collagen I and III (Sigma). Thereafter, slides were washed and incubated for 1 h with secondary antibody; Alexa488-labeled goat anti-mouse or anti-rabbit (Molecular Probes), Cy3-labeled goat anti-mouse or anti-rabbit antibodies (Jackson Laboratories). Rhodamin/Phalloidin (Molecular Probes), was used for F-actin staining. After removing the secondary antibody, slides were washed and mounted on Poly Aquamount (Polysciences, Inq.). Images were made using a Bio-Rad Radiance 2100 confocal system with a 60x Plan Apo (NA 1.4:Nikon) objective lens. All images were processed with Image-Pro® Plus (Version 5.1 Media Cybernetics).

Western blot analysis

Frozen sections were lysed in 250 μl of Triton lysisbuffer (20 mM Tris pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 10 % glycerol) with inhibitors and incubated at 4 °C for 2 hours. Lysates were syringed four times though a 26 G needle, centrifuged (20 minutes at 10.000 rpm, 4°C) and immediately boiled in sample preparation buffer (125 mM Tris-HCl, pH 6.8, 20 % glycerol, 4 % SDS, bromophenol blue). Protein concentrations were determined using a Bradford assay with IgG as a standard. Equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Blots were blocked in 5% (w/v) BSA in TBS-T (0.5 M NaCl, 20 mM Tris-HCl and 0.05 % (v/v) Tween-20 pH 7.4) for 1 h. Primary antibody incubation was performed overnight at 4 °C in anti-PY99 (0.04 μg/ml, monoclonal, Santa Cruz), anti-PY397-FAK (1 μg/ml, polyclonal, Biosource), anti-FAK (monoclonal, 1 μg/ml, Transduction Laboratories), anti-PY118-paxillin (0.75 μg/ml, polyclonal, Biosource), anti-paxillin (monoclonal, 0.5 μg/ml, Transduction Laboratories). Thereafter blots were incubated with horseradish peroxidase conjugated secondary antibody (GE Healthcare) in TBS-T for 1 h at room temperature. Protein signals were detected with ECL plus method (GE Healthcare) followed by scanning of the blots with the Typhoon 9400 (GE Healthcare). Ratios of the protein band intensity were obtained using ImageQuant analysis.

RESULTS

Focal adhesions *in vivo* are tyrosine phosphorylated structures connected to F-actin stress fibers

In vitro, FAs are located at the closest contact site between the cell and the ECM and are connected to the F-actin cytoskeleton. To demonstrate the presence of FAs *in vivo* we stained frozen sections of control kidneys for the focal adhesion proteins talin, focal adhesion kinase (FAK) and paxillin. These three proteins were organized in a similar stripe-like manner at the basolateral cell surface of the proximal tubules, demonstrating the existence of FA-like structures *in vivo* (**Fig. 1A**). To visualize the localization of FAK containing FAs in more detail, a z-scan was made starting at the basolateral side of a proximal tubule and ending in the lumen (**Fig. 1B**). Using a collagen I and III staining the basolateral membrane of the proximal tubulus was defined (data not shown). FAs were solely present at this collagen-rich basolateral side where cells adhere to the ECM and not at the luminal side of the cells, thereby surrounding the proximal tubulus. Since the function of FAs proteins is often regulated by tyrosine phosphorylation, frozen sections were stained for total protein tyrosine phosphorylation (pTyr) to determine whether *in vivo* FAs are pTyr containing structures. In these sections pTyr staining was organized in similar stripe-like structures as observed for the FA proteins talin, FAK and paxillin (**Fig. 1A**). Focal adhesion proteins like FAK and paxillin can regulate the F-actin cytoskeleton and are often located at the end of F-actin stress fibers [18;23]. *In vivo*, stress fibers were found at the basolateral side of PTCs and phospho-tyrosine positive FAs co-localized at the end of these F-actin stress fibers (**Fig. 1C**).

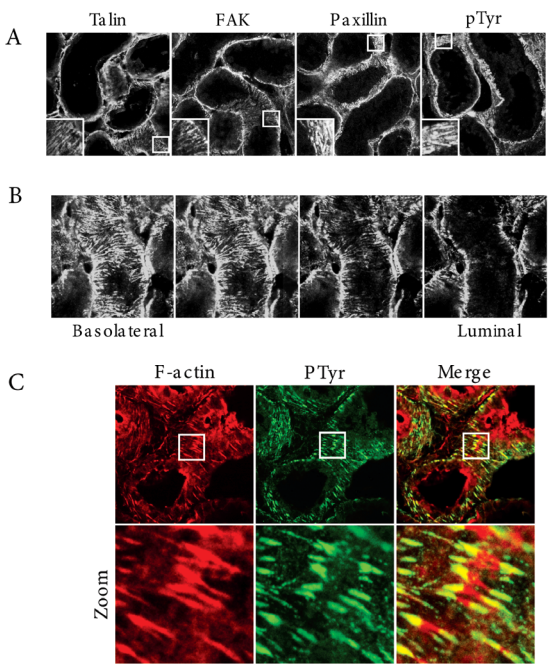

Figure 1. *In vivo co-localization of tyrosine phosphorylated focal adhesion proteins with F-actin stress fibers.* To determine localization of FAs in the proximal tubules, frozen sections (10 µm) of control kidneys were stained for the FA proteins talin, FAK, paxillin and total tyrosine phosphorylation (pTyr) (A). A z-scan was created from the basolateral site towards the lumen of the tubule to show localization of FAK (B). To indicate co-localization between pTyr containing FAs and F-actin, sections were co-stained for pTyr (green) and F-actin (red); colocalization is yellow (C). All sections were imaged using confocal laser scanning microscope. Sections are representative of proximal tubules in 3 different rats and observed in two different stainings.

Formation of F-actin stress fibers during reperfusion after mild ischemia

In PTCs of normal rat kidneys, F-actin is concentrated mainly in the brush border microvilli at the apical membrane, which is extremely dense in the cortex region, providing cell-cell interactions. The F-actin cytoskeleton is also associated with cell-extracellular matrix (cell-ECM) interaction at the basolateral membrane with stress fibers connected to FAs (**Fig. 2**).

After 30 min of ischemia, the proximal tubules were dilated in both the outer stripe of the outer medulla (OSOM) and cortex region. This mild ischemic condition did not cause severe renal injury (data not shown). However, directly after ischemia, the F-actin network was reorganized with a loss of stress fibers (zoom **Fig. 2**) and a reduction in F-actin at the microvilli. During reperfusion well-defined F-actin stress fibers appeared in some proximal tubule cells in both OSOM and cortex region at the basolateral membrane (**Fig. 2**, arrow heads). After 24 h of reperfusion the overall F-actin arrangement in these kidneys was hardly different from that seen in sham operated animals, except for stress fibers that were very pronounced in proximal tubular cells (**Fig. 2**). The arrangement of F-actin in the contralateral kidney did not differ from that seen in kidneys from control rats (data not shown).

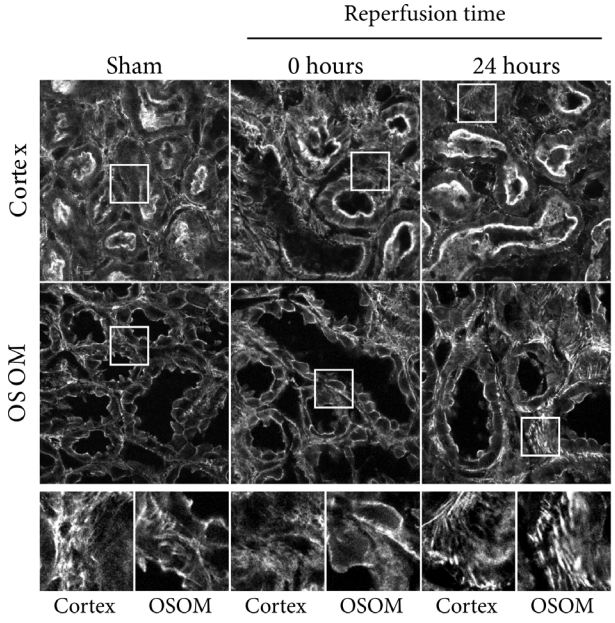

Figure 2. *Formation of F-actin stress fiber during reperfusion*. Rats were subjected to 30 min of ischemia followed by reperfusion for 0 and 24 hours. Kidneys were evaluated for F-actin reorganization in the OSOM or cortex region by staining frozen sections (10 μm) for F-actin. Images were obtained using confocal laser scanning microscopy. The bottom panel indicates a zoom of the areas containing stress fibers. Images shown are representative of proximal tubules in 3 different rats and observed in two independent stainings

Total tyrosine phosphorylation increases in a biphasic manner during reperfusion

Determining the phosphorylation state of adhesion proteins is an essential part of understanding their function, regulation and downstream signalling. To see if the prominent changes in the F-actin cytoskeleton during mild I/R have an effect on the FA organization and possibly the downstream signalling we determined the tyrosine phosphorylation status of FA proteins and their expression during ischemia and reperfusion.

In both OSOM and cortex, 30 min of ischemia resulted in dephosphorylation of proteins located at the FAs of the renal cells leaving only little pTyr at the basolateral side of the cells (**Fig. 3A-B**). Return of blood flow through the kidney resulted in an increase in pTyr after 1 and 24 h of reperfusion, whereas pTyr was decreased at intermediate time points (**Fig. 3B**). The phosphorylation pattern at 1 h reperfusion slightly differs from the pattern observed at 24 h of reperfusion, suggesting that different phospho-proteins may be activated. After 1 h of reperfusion pTyr was not solely located on the FAs, but also more cytosolic (**Fig. 3A**). During 4 and 8 h of reperfusion the overall intensity of the phospho-tyrosine staining at FAs decreased, which were now organized in dotted-like structures. In addition, we observed more cytosolic staining with some pTyr in the microvilli of the tubular cells (**Fig. 3A**). The intensity of the phospho-tyrosine staining increased dramatically after 24 h of reperfusion, with thick and striped organized focal adhesion structures (**Fig. 3A**) that co-localized with F-actin stress fibers (data not shown).

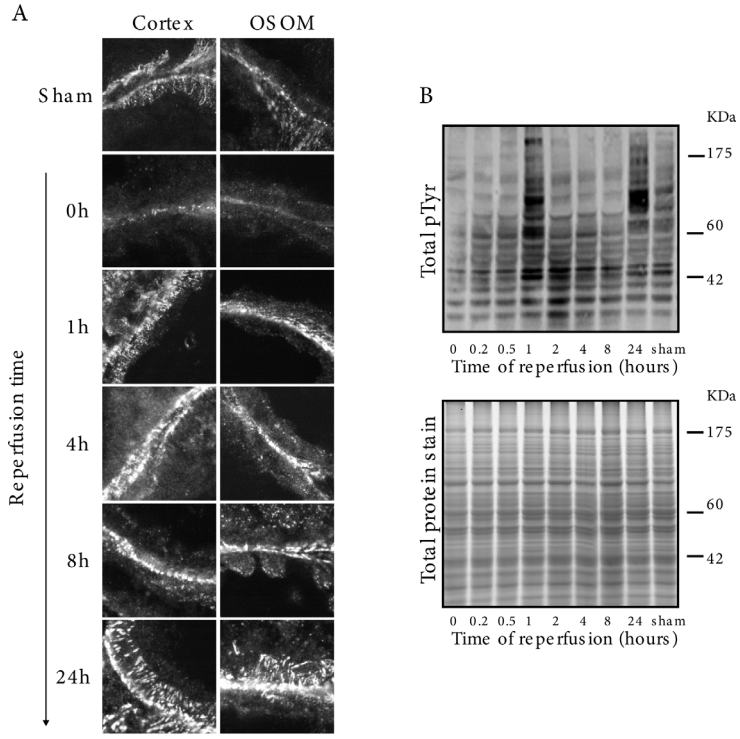

Figure 3. *A bi-phasic increase in protein tyrosine phosphorylation during mild ischemia/reperfusion.* Rats were subjected to 30 min of ischemia followed by reperfusion for the indicated time-periods. Thereafter, frozen kidney sections (10 μm) were stained for total tyrosine phosphorylation using an anti-pTyr antibody. Both OSOM and cortex region were evaluated for differential tyrosine phosphorylation using confocal laser scanning microscopy (A) and Western blot analysis by staining blots with an anti-pTyr antibody (B, upper). Total protein was measured by staining an SDS-PAGE gel with Sypro Ruby (B, lower). Data are representative for 3 different rats and observed in two different stainings.

FAK phosphorylation increases at the FA after 24 h of reperfusion concurrently with stress fiber formation

FAK activity is regulated by its tyrosine phosphorylation and is correlated with changes in F-actin stress fibers [24;25]. The changes seen in total pTyr protein expression and localization as well as the formation of stress fibers during reperfusion, raises the question what happens with FA associated proteins like FAK and paxillin. We stained rat kidneys subjected to 30 min of ischemia and reperfusion up to 24 h for FAK, PY397-FAK and F-actin. PY397-FAK co-localized with FAK and was organized in thick, small FA structures in the sham operated kidney. After 30 min of ischemia, FAK was dephosphorylated on the FAs. However, small and dotted-like FAK-containing FAs were still present, indicating that FAs were not completely disrupted during ischemia. FAK staining was also present in the cytosol and nucleus of the proximal tubular cells. After 1 h of reperfusion PY397-FAK increased (**Fig. 6A**) and located on the basolateral membrane of the proximal tubule cells organized in small, thin and stripe-like structures (**Fig. 4A**). At both 4 and 8 h after ischemia PY397-FAK was decreased (**Fig. 6A**) and FAs were rearranged to more short structures (**Fig. 4A**). PY397-FAK increased 24 h after ischemia and was organized in thick and bulky FAs, co-localizing with FAK. These data indicate that also PY397-FAK shows a biphasic pattern like observed for total pTyr staining (**Fig. 3B**). The increased tyrosine phosphorylation of FAK after 24 h correlated with an increased amount of stress fibers formed as well with an increased FA size (**Fig. 4B**). Similar observations were obtained for paxillin, which also showed a biphasic phosphorylation pattern (**Fig. 6B** and data not shown). Together these data show that the size and phosphorylation status of FA proteins changes over time during renal ischemia and reperfusion, suggesting dynamic reorganization of proteins within the FA.

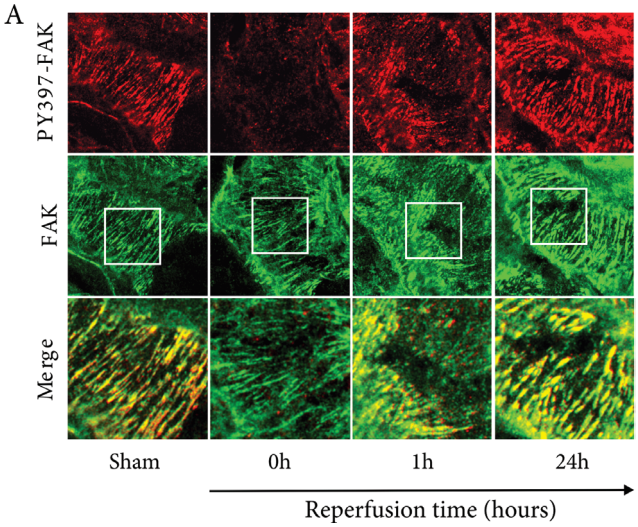

Figure 4A. *FAK phosphorylation increases and co-localizes with F-actin stress fibers after 24 h of reperfusion.* Rats were subjected to 30 min of ischemia followed by reperfusion for 0, 1 and 24 h. Frozen sections (10 µm) were stained for FAK (green) and PY397-FAK (red) to determine differential tyrosine phosphorylation and co-localization (yellow) at FAs.

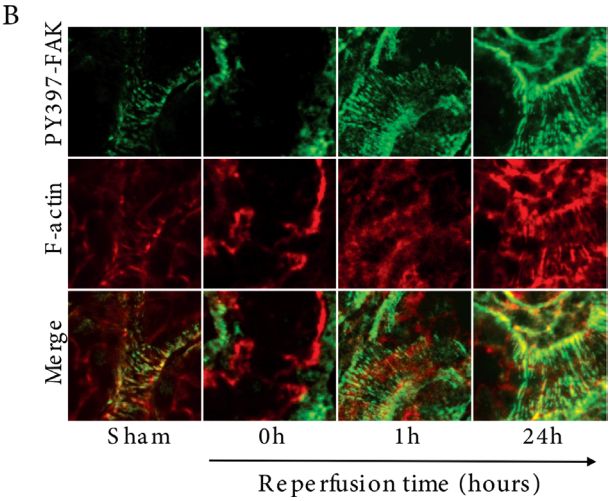

Figure 4B. To determine co-localization of FAK with F-actin stress fibers, frozen sections were stained for PY397-FAK (green) and rhodamine/phalloidin for F-actin cytoskeleton (red); colocalization is yellow. All sections were imaged using confocal laser scanning microscope. Sections are representative of proximal tubules in 3 different rats and observed in two different stainings.

Severe ischemia-reperfusion injury causes changes in focal adhesion structure and phosphorylation.

Severe I/R injury induced by 45 min of ischemia followed by 24 h of reperfusion results in clear renal injury, showing dilated, atrophic and denuded tubules, fragmented nuclei pointing to apoptosis, mainly in the OSOM region (data not shown). Two weeks after ischemia all tubules were lined with cells and, though some what more dilated, the proximal tubules resemble control proximal tubules (data not shown). Reperfusion of kidneys subjected to 45 min of ischemia resulted in marked changes in F-actin arrangement in both OSOM and cortex (**Fig. 5A**). At the apical site, F-actin containing microvilli were completely lost in the OSOM and to a lesser extent in the cortex. During reperfusion, F-actin was relocalized to microvilli and F-actin stress fibers were formed (see arrows **Fig. 5A**). In contrast to a milder form of ischemia (*i.e.* 30 min) reperfusion times of up to 2 weeks were necessary to completely regenerate the renal tubular F-actin structure (data not shown), since cells were lost from the tubules after 45 min of ischemia.

Next, we determined the phosphorylation status of FA proteins during regeneration of a more severely damaged kidney. The tyrosine phosphorylation status of FA proteins seemed to undergo a similar pattern of dephosphorylation and hyper-phosphorylation as during mild I/R. In sham-operated animals, the pTyr was present on the basolateral side of the cell in small and thin FAs. Directly after ischemia the FAs were dephosphorylated more in the OSOM than the cortex region (**Fig. 5B**). In both regions, talin was still present at the focal adhesions, indicating that the focal adhesions itself were still intact, though differently shaped (data not shown). In the cortex the pTyr at the FAs emerged after 1 h of reperfusion and formed thin and striped structures. However, in the OSOM the FAs were not as phosphorylated and the structures were very small and irregularly organized (**Fig. 5B**). 24 h of reperfusion resulted in thick, bulky and intensely stained FAs in the cortex, whereas the FAs in the OSOM were, although thick, more scarcely found. During 1 week of reperfusion the FAs became very round, large and hyper-phosphorylated. In addition,

control kidneys showed a thin and continuous line of pTyr staining at the basolateral side of the proximal tubule. During the reperfusion period of 1 week, no continuous line of pTyr proteins was present on the basolateral side of the proximal tubules (**Fig. 5B**). After 2 weeks of reperfusion, the tyrosine phosphorylated FAs were organized like FAs in the sham operated animals, but more elongated (**Fig. 5B**). Although the proximal tubules were repopulated with PTCs in the OSOM region during 2 weeks of reperfusion, the cells were still flatter and contained less F-actin in the microvilli compared to sham-operated animals. These data indicate that the proximal tubules were regenerated almost completely two weeks after a more severe ischemic insult in conjunction with similar phosphorylation-dependent dynamic reorganization of FAs and F-actin cytoskeletal network.

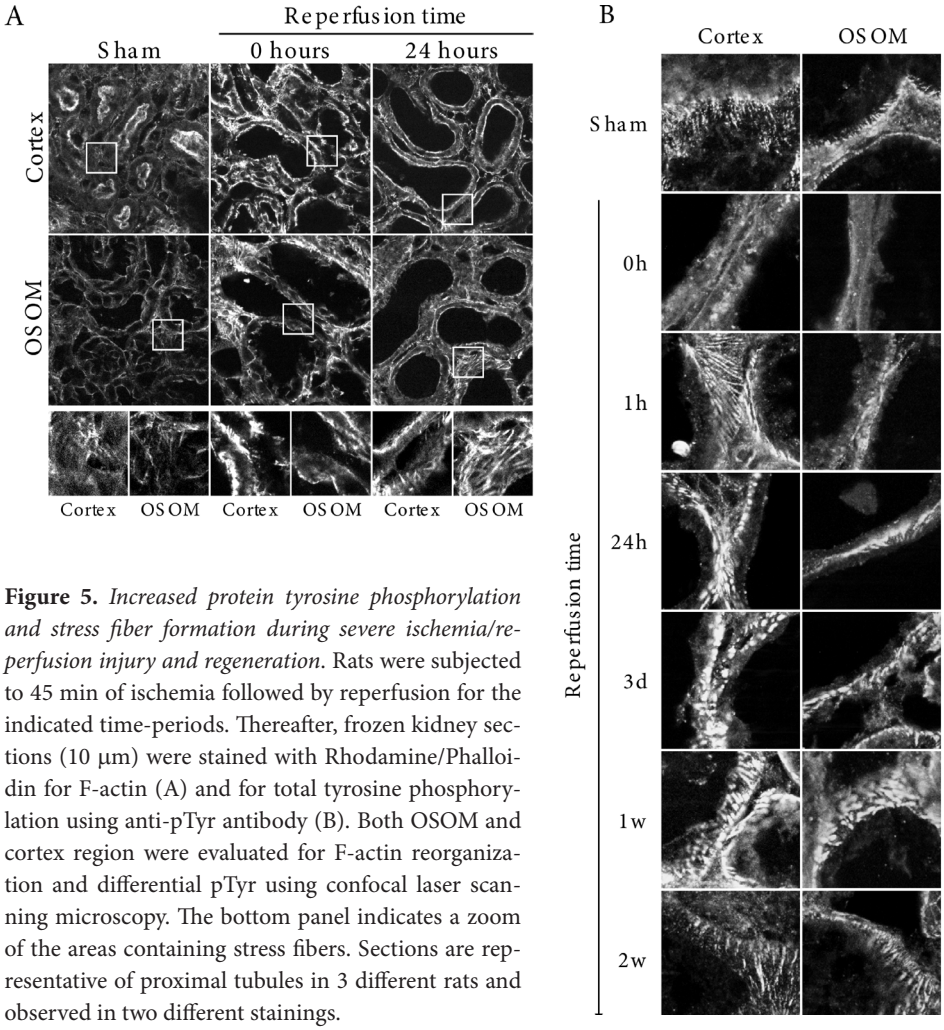

Figure 5. *Increased protein tyrosine phosphorylation and stress fiber formation during severe ischemia/reperfusion injury and regeneration*. Rats were subjected to 45 min of ischemia followed by reperfusion for the indicated time-periods. Thereafter, frozen kidney sections (10 μm) were stained with Rhodamine/Phalloidin for F-actin (A) and for total tyrosine phosphorylation using anti-pTyr antibody (B). Both OSOM and cortex region were evaluated for F-actin reorganization and differential pTyr using confocal laser scanning microscopy. The bottom panel indicates a zoom of the areas containing stress fibers. Sections are representative of proximal tubules in 3 different rats and observed in two different stainings.

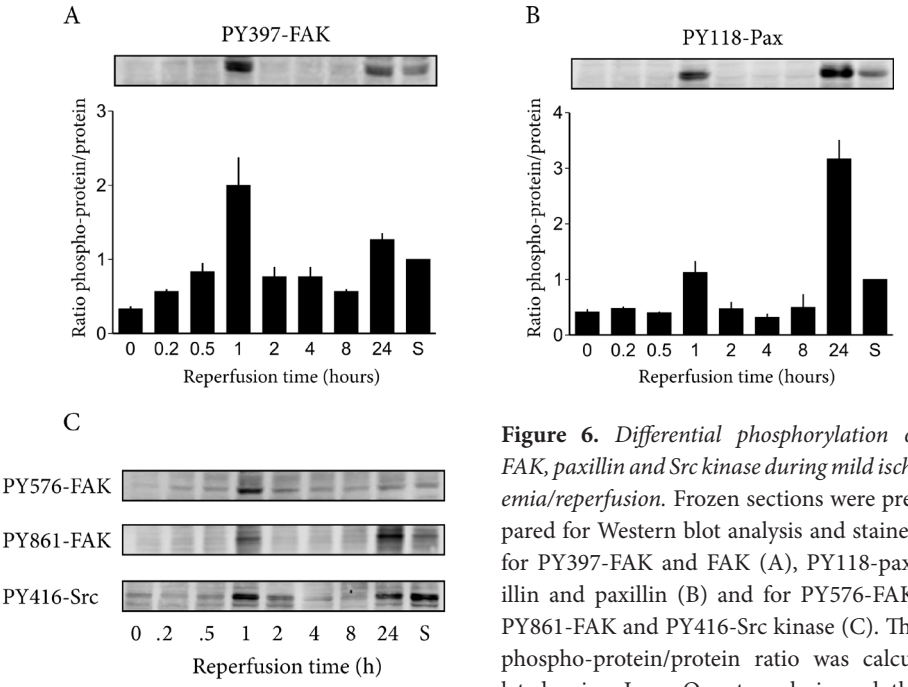

Figure 6. *Differential phosphorylation of FAK, paxillin and Src kinase during mild ischemia/reperfusion.* Frozen sections were prepared for Western blot analysis and stained for PY397-FAK and FAK (A), PY118-paxillin and paxillin (B) and for PY576-FAK, PY861-FAK and PY416-Src kinase (C). The phospho-protein/protein ratio was calculated using ImageQuant analysis and the mean value (+/- SD) of three independent experiments is plotted (A-B).

Different phosphorylation patterns of focal adhesion proteins during ischemia reperfusion

FAs are dynamic structures that vary in size and in content of FA associated proteins. FAK is auto-phosphorylated on Y397 upon integrin clustering and recruits Src kinase which is activated by phosphorylation on Y416. The FAK-Src complex can subsequently phosphorylate the adapter protein paxillin. Other tyrosine residues of FAK are phosphorylated by Src kinase and all have their own function and influence on the FA dynamics and downstream signalling. The biphasic increase in tyrosine phosphorylation and PY397-FAK and the changes in the structure of the FAs indicate activation of signalling pathways. Using the phospho-protein expression of FAK and paxillin as well as the expression of PY416-Src we systematically analyzed this FAK-Src-Paxillin signalling complex.

The auto-phosphorylation site of FAK, Y397, was highly phosphorylated after 1 h of reperfusion but just above control levels after 24 h (**Fig. 6A**), whereas paxillin, which is phosphorylated by the FAK-Src complex, was more phosphorylated after 24 h than after 1 h (**Fig. 6B**). FAK phosphorylation at Y397 promotes the Src homology domain 2 (SH2)-dependent binding of Src kinase, thereby activating the protein via phosphorylation of Y416 [26]. During ischemia, Src was dephosphorylated at Y416, but unlike FAK and paxillin this dephosphorylation was not complete (**Fig. 6C**). Src kinase was phosphorylated at 1 h and 24 h after reperfusion, the time-points that also FAK was phosphorylated

at Y397. These results indicate that the phosphorylation status of Src kinase is regulated in a similar biphasic manner. Activation of Src kinase results in phosphorylation of FAK at multiple residues, among which are Y576, thereby enhancing FAK catalytic activity and Y861, thereby creating additional interaction sites for SH2-containing proteins [27;28]. Both Y576 and Y861 were dephosphorylated during ischemia and phosphorylated after 1 h of reperfusion, with high phosphorylation content for Y576 and phosphorylation levels just above control for Y861. Like the other FA proteins Y861 was phosphorylated in a biphasic expression pattern during reperfusion, since it was highly phosphorylated after 24 h of reperfusion (**Fig. 6C**). In contrast, Y576 phosphorylation levels resembled that of control kidneys at 24 h after reperfusion, indicating that FAK catalytic activity is probably less compared to its activity after 1h of reperfusion. Together these data indicate that the phosphorylation status of FA proteins changes over time during I/R, thereby continuously changing their activation, resulting in activation of signalling cascades, which dynamically reorganize the FAs and F-actin network.

DISCUSSION

In this study we have used a unilateral renal ischemia/reperfusion model to study the temporal and spatial reorganization of FAs and F-actin stress fibers, as well as phosphorylation of FAK during I/R injury and regeneration. Firstly, our data indicate the existence of FAs under *in vivo* conditions at the basolateral membrane of the renal proximal tubular epithelial cells. The FA structures are enriched in FAK and paxillin as well as their respective tyrosine phosphorylated forms and connected to basolateral F-actin stress fibers. Secondly, protein tyrosine phosphorylation events at the FAs were lost directly after the ischemic event, which coincided with a disruption of the FA structures and the F-actin network. During the reperfusion period levels of protein tyrosine phosphorylation increased in association with an increase in FA size and F-actin stress fibers formation. Finally, our data indicate a differential phosphorylation of FAK tyrosine residues during reperfusion.

FAs are often thought of as artefacts of *in vitro* cell culture; while FA-like signalling complexes are likely to be present in a variety of cells *in vivo*, the classical FA contacts are not well studied *in vivo*. Our data clearly indicate the existence of FAs located at the basolateral side of PTCs in the S1, S2 and S3 segment. These adhesion structures have incorporated known FA proteins, like talin, paxillin and FAK (**Fig. 1A**). In primary cultured PTCs, focal adhesions are rich in tyrosine phosphorylation [20]. *In vivo*, the FAs in the proximal tubulus are also abundant in pTyr, which under steady state conditions included PY397-FAK and PY118-paxillin. *In vitro*, FAs are formed at cell-ECM and their size and stability, which depends on the molecular composition and rigidity of the ECM, is linked to the amount of cytoskeletal contractility [29;30]. This linkage suggests that the presence FAs and their attachment to F-actin stress fibers *in vivo* may allow regulation of tension in the proximal tubulus [30], thereby providing it with a possibility to regulate tubular pressure and ultrafiltration rate.

Directly after an ischemic insult we show dephosphorylation of FA-associated pro-

teins followed by reorganization of FAs together with disruption of the F-actin cytoskeleton. The dephosphorylation of basolateral associated proteins is consistent with other studies that have shown dephosphorylation *in vitro* [19]. In other organs such as brain and heart, a decrease in protein tyrosine phosphorylation is also observed [2;3;31]. In cultured MDCK cells chemical anoxia-induced ATP depletion causes a dissolution of F-actin stress fibers, which is related to decreased MLC phosphorylation [32;33]. This corresponds with loss of RhoA activity after ATP depletion in cultured LLC-PK1 cells; likewise, overexpression of active RhoA prevents the loss of the F-actin network organization and protects against loss of cell adhesion [5]. This suggests that the initial loss of F-actin stress fibers at the basement membrane of the PTC *in vivo* is related to disruption of the Rho/Rho-kinase pathway. In our hands F-actin disruption precedes the complete loss of FA organization, suggesting that the early loss of cell adhesion and exfoliation of viable cells in the urine observed during ARF is due to F-actin disruption followed by FA disorganization. Yet, alternatively, the loss of phosphorylation of FA-associated cytoskeletal and/or signalling (adaptor) proteins might also indirectly lead to disruption of stress fibers. In a second and later phase of the reperfusion period (24 h), F-actin stress fibers and FAs are reformed (**Fig. 3A** and **Fig. 4**). These F-actin fibers are more compact and the FAs are larger in size.

A biphasic protein tyrosine phosphorylation wave after ischemic injury was observed: after 1 and 24 h of reperfusion the amount of total pTyr was increased above sham operated control levels. The increase at an early time-point is most likely related to the reversal of cellular ATP levels, which can be used by protein tyrosine kinases to phosphorylate cellular proteins. Activation of the EGF receptor is already observed after 5-30 min of reperfusion [34], suggesting that activation of receptor protein tyrosine kinases indeed participates in the increased protein tyrosine phosphorylation observed at an early time-point. The increased levels of protein pTyr at later time-points are most likely directly related to increased amounts of growth factors (*i.e.* EGF, HGF and IGF) that are generated in the renal cell response to injury [35]. These factors will activate receptor tyrosine kinases that promote downstream activation of signal cascades, including activation of FAK and the c-Met receptor [36;37]. In addition, after 2-8 h protein phosphorylation levels were lower compared to 1 and 24 h of reperfusion. Although it has been shown that protein phosphatase activity can decrease after an ischemic insult [38], we did not observe changes in overall phosphatase activity (data not shown). Although we can not exclude that the activity of some phosphoatases is affected during I/R injury, this suggests that the increase in tyrosine phosphorylation at 1 and 24 h is mainly caused by activation of kinases.

The FAK phosphorylation observed during the reperfusion period was both temporal and tyrosine residue site specific. In control kidney, primarily Y397 was phosphorylated. At later time-points, an increase in Y861 phosphorylation was observed. The latter occurred in conjunction with increased activation of Src kinase (*i.e.* Y416 phosphorylation). These data suggest a potential dualistic function of FAK during the reperfusion period with possible consequences for the outcome of the regeneration process after I/R injury. Autophosphorylation of FAK at Y397 occurs upon binding of FAK to FAs through

integrin and/or talin and paxillin adaptor protein binding [17;18]. Phospho-Y397 serves as a SH2-docking site for Src kinase, resulting in activation of the latter. Subsequently, Src kinase will phosphorylate other tyrosine residues of FAK including Y576/Y577 in the kinase domain and Y861 in the c-terminal domain. Y576/Y577 phosphorylation results in increased FAK kinase activity [26-28]. The direct association with Src results in a coordinated FAK-Src kinase activity resulting in phosphorylation of downstream targets, of which, amongst others, is paxillin. Paxillin can be phosphorylated on Y31 and Y118 and is important for FA turnover and cell motility [18;23]. *In vivo* we observed both PY397-FAK and PY118-paxillin at FAs as well as by Western blotting. This indicates that under normal conditions FAK and/or the FAK-Src kinase complex mediates phosphorylation and activation of paxillin at FAs. In the reperfusion period, increased PY397-FAK, PY576/577-FAK and PY416-Src were directly associated with increased PY118-paxillin and FA remodeling [23]. Interestingly, PY861-FAK was low under control conditions, but increased considerably after I/R. FAK phosphorylation at Y861 facilitates SH3 domain-mediated binding of p130Cas to the C-terminal proline-rich domains of FAK. PY861-FAK seems important in the migratory processes and c-Jun N-terminal kinase-mediated expression of matrix metalloproteinase 9 [39]. Moreover, tyrosine phosphorylation of paxillin is required for FA turnover and cell migration [23]. We propose that the coordinated and differential phosphorylation of FAK and downstream substrates is a requirement for reorganization of FAs and the actin cytoskeletal network early after ischemia-reperfusion and it contributes to the efficient regeneration of the damaged area at later time-points.

FAs form the physical links between integrins and the cellular cytoskeleton. In the proximal tubular epithelium, β1-integrins are predominantly expressed [40]. β1-integrin localization at the basement membrane is lost during unilateral ischemia, followed by its appearance on lateral borders in epithelial cells of the S3 segment during the reperfusion period4. Moreover, increased renal injury occurred when β1 integrin binding sites were blocked by either anti-collagen IV or anti-fibronectin antibodies [4;22]. Since loss of intracellular ATP is the primary event during ischemia, it is likely that loss of integrin localization is related to inside-out signalling, most likely through loss of protein phosphorylation at FAs, including FAK and paxillin phosphorylation. Maintenance of β1-integrins in an active conformation using the HUTS-21 protected against I/R induced renal failure in mice1, indicating that outside-in integrin signalling can suppress acute renal failure. Whether under the latter conditions FA organization and phosphorylation as well as cytoskeletal architecture would be preserved needs further investigation.

FA disturbance and FAK dephosphorylation in the context of F-actin reorganization seems a common theme in acute renal cell injury responses. Previously we showed that the nephrotoxicant cysteine conjugate 1,2-dichloro-L-vinyl-cysteine (DCVC) causes actin reorganization both *in vitro* and *in vivo* preceding the onset of apoptosis [41]. The disruption of the F-actin cytoskeleton and stress fibers was associated with loss of talin from the FAs and preceded cell detachment. DCVC-induced injury to the LLC-PK1 cells also caused loss of cell-matrix interactions, which was preceded by dissociation of FAK from the FAs and dephosphorylation of FAK and paxillin on their tyrosine residues [20]. Over-

expression of a dominant negative deletion mutant of FAK in LLC-PK1 cells potentiated nephrotoxicant-induced cell detachment and apoptosis [42]. By using heterozygous FAK mice our preliminary *in vivo* data indicate that heterozygous mice are more susceptible to the DCVC-induced renal injury compared to the wild-type littermates (manuscript in preparation). These studies indicate that cell-matrix signalling may be very important in the control of injury caused by either ischemia-mediated ATP depletion of chemically-induced apoptosis.

In summary, our current data indicate a dynamic reorganization of FAs, F-actin cytoskeleton and FAK phosphorylation during renal I/R. Increased and tyrosine residue specific phosphorylation of FAK and paxillin during reperfusion may be important in the cellular stress response after ischemic injury and is linked to the actin reorganization that takes place during I/R. In addition, phosphorylation of FAK and paxillin at later time-points may assure recovery of proper actin cytoskeleton organization and cell-ECM adhesion after I/R and can be involved in the regeneration process of injured tissue by initiating cell motility and proliferation.

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