ERK1/2 Mediates Cytoskeleton and Focal Adhesion Impairment in Proximal Epithelial Cells after Renal Ischemia

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Key Words

ERK1/2 • Tubular epithelial cells • Ischemia/reperfusion • Hypoxia/reoxygenation • Cell adhesion • Cytoskeleton

Abstract

ERK1/2 has been reported to be activated in the postischemic kidney but its precise role in ischemia/reperfusion (I/R) injury remains unclear. Therefore, we have studied the expression of ERK1/2 and its contribution to cytoskeleton organization and cell adhesion structures in proximal tubular cells, all affected during I/R. We observe ERK1/2 activation at 24 hours of reperfusion in an *in vivo* model of I/R, when acute tubular necrosis (ATN) is most prominent. In addition, by means of an *in vitro* model of hypoxia/reoxygenation (H/R) in rat proximal NRK-52E cells we show that p-ERK1/2 is strongly induced early during reoxygenation. Moreover, we also demonstrate that ROS generation contributed to this induction. ERK1/2 activation is contemporary with cell-cell adhesion disruption during reoxygenation but the use of U0126 did not have effect on adherens junctions (AJ) and tight junctions (TJ) disassembly, neither on epithelial monolayer permeability. On the contrary, ERK1/2

affects cytoskeleton organization and focal complexes assembly during H/R, since U0126 improved actin and tubulin cytoskeleton structure, reduced cell contraction and prevented paxillin redistribution. In summary, ERK1/2 signalling plays an essential role in I/R induced injury, mediating proximal cell adhesive alterations which lead to tubular damage and ultimately might compromise renal function.

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Introduction

Ischemia/reperfusion (I/R) injury is a major cause of acute renal failure (ARF) and is associated with a high morbidity and mortality in hospitalized patients [1, 2]. In transplanted kidneys, ATN caused by ischemia severely affects kidney graft outcome, increasing the risk of rejection and contributing to delayed graft function and chronic allograft nephropathy. In spite of the effort to find successful therapies and the advances in experimental models of renal I/R, not much progress has reached the clinical practice [3, 4].

Renal ischemia-induced damage results from a complex interplay of cellular processes including apoptosis

and necrosis, in addition to alterations in the cellular organization including loss of cell polarity, cytoskeleton disorganization or cell-matrix adhesion loss [3]. We have recently described, using an improved *in vitro* model, several events underlying proximal tubular epithelial cell impairment subsequent to H/R. This impairment has important functional implications *in vivo*, as cell shedding to the tubular lumen provokes loss of epithelium barrier function, tubular obstruction, filtrate back-leakage, edema formation, inflammation and consequently, kidney dysfunction. All these cellular alterations are the result of signalling pathways triggered by calcium mobilization, ATP depletion and ROS generation, among others, in response to changes in oxygen and nutrients availability during I/R and H/R [5].

Many reports point out protein kinase activity, in particular mitogen-activated protein kinases (MAPK) cascades (p38, JNK and ERK pathways) as important mediators in ischemic or hypoxic injury as well as during the recovery after these insults [6]. However, their precise role is not clearly determined.

ERK1/2 is activated during renal ischemia and it has been proposed as a regulator of the I/R-induced injury in kidney [7]. Recently, some reports have described the relevance of the ERK1/2 signalling pathway in the proximal tubule response to ischemia [8, 9]. Most evidences point out ERK1/2 as mediator of cell survival/cell death balance in this context. Nevertheless, ERK1/2 also controls adhesive structures by local activation in focal complexes, regulating signalling and adaptor proteins such as FAK or paxillin in response to different stimuli including growth factors [10].

In order to identify targets for an efficient therapy to kidney I/R damage, it is necessary to unravel signalling pathways involved in tubular cell response to I/R. We have addressed the role of ERK1/2 on the proximal tubule cell alterations consequent to renal ischemia. By using an *in vitro* model of H/R, we found ERK1/2 activation early during reoxygenation and the beneficial effect of its inhibition on actin and tubulin cytoskeleton and cell-matrix adhesion structures. Thus, modulating ERK1/2 signalling pathway might be relevant to ameliorate renal injury and improve renal recovery after I/R.

Materials and Methods

Cell culture and H/R protocol. Cell treatments

NRK-52E cells (ATCC), mycoplasma free, were cultured in DMEM containing 10% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), in a humidified

atmosphere with 5% CO₂ at 37°C. The H/R protocol was performed as previously described [5]. Briefly, confluent cells were made quiescent by serum deprivation for 24 hours and then cultured for 6 hours in medium without nutrients (HBSS (Invitrogen)) in a hermetic incubator in a hypoxic atmosphere (1% O₂, 94% N₂, 5% CO₂, Air Liquide). Reoxygenation was performed in complete medium and 5% CO₂ in a regular incubator. Serum-starved cells with the corresponding changes of media were used as controls for this model (Control (6h)). U0126 (Cell Signalling), a MEK1/2 inhibitor, was applied during reoxygenation (10 µM). 10 mM N-acetyl-cysteine (NAC) (Sigma) was used as an antioxidant treatment in some experiments.

I/R protocol. Tissue processing and immunohistochemistry

Pathogen free male Sprague-Dawley rats, weighting 180-200 g, were obtained from our own breeding colony, fed with standard laboratory chow *ad libitum* and given free access to water. All experimental procedures were performed according to the institutional guidelines that are in compliance with the European Community laws and the Spanish guidelines (RD 1210/2005). Animal experimental design has been approved by a Hospital Ramon y Cajal Committee for Animal Ethics.

Rats were anesthetized with isoflurane (Abbot Laboratories) and kept on a warming tap. Renal I/R injury was induced after a median abdominal incision by a 45 minutes bilateral clamping of the renal pedicle with non traumatic clamps, as previously described [11]. Sham-operated animals underwent the same surgical procedure without clamping. Animals were sacrificed at different times after reperfusion (0 and 24 hours) and blood and kidneys were harvested and processed. 5 animals per group were used. For immunohistochemistry, a piece of tissue was fixed in phosphate-buffered 10% formalin and 4 µm paraffin-embedded kidney sections were treated with peroxidase blocking solution (Dako) to inhibit endogenous peroxidase activity, blocked in 1% BSA-PBS, followed by incubation with anti-p-ERK1/2 (p42/p44Thr202/Tyr204) (1:100, Cell Signalling) and the secondary antibody conjugated with HRP (1:100, DAKO), and finally developed using DAB solution (DAKO).

Actin cytoskeleton staining and immunofluorescence

NRK-52E cells were grown onto coverslips coated with collagen IV (1 µg/ml). To visualize actin cytoskeleton, cells were fixed in 3.7% formaldehyde for 20 minutes, permeabilized with 0.5% Triton X-100-PBS, blocked in 1% BSA-PBS and stained with 165nM phalloidin-Alexa Fluor 568 (Invitrogen). For p-ERK1/2, tubulin and paxillin immunofluorescence, cells were processed as above and incubated in 1% BSA-PBS containing anti p-ERK1/2 (Cell Signalling), anti tubulin (Sigma) and anti paxillin (Upstate Biotechnologies) at 1:100 dilution, followed by the appropriate secondary antibodies: anti-rabbit or anti-mouse Alexa488 or Alexa594 (Invitrogen), at 1:1000 dilution. For E-cadherin and ZO-1 staining, cells were fixed in 1:1 methanol/acetone at -20°C for 10 minutes, blocked and incubated in PBS 1%BSA containing anti E-cadherin 1:200 (BD Transduction Laboratories) and anti ZO-1 1:100 (Zymed Laboratories) followed by the appropriate secondary antibodies

Fig. 1. ERK1/2 is activated in the post-ischemic kidney, correlating with proximal tubule damage. p-ERK1/2 immunostaining in paraffin-embedded kidney sections of Sprague Dawley male rats after 45 minutes of ischemia and 24 hours of reperfusion. Notice prominent p-ERK1/2 staining in injured proximal tubules which exhibit loss of apical microvilli, altered morphology or presence of casts. Some of these features are marked with asterisks. Representative images of each experimental group (n=5) are shown. Magnifications: x 200 and x 400.

mentioned above. After washing with 0.05% Tween20-PBS, samples were mounted with mowiol (Calbiochem) and observed using a Nikon Eclipse TE2000-U inverted microscope or Leica TCS SP5 confocal microscope. Images were obtained and processed with NIS-Elements BR Image Software or Leica Software.

Pull down assays and western blotting

Cells were lysed in 1 % Triton X-100, 1 % NP-40, 0.25% sodium deoxycholate, 1 mM DTT, the protease inhibitors aprotinin (1 µg/ml), leupeptin (1 µg/ml), AEBSF (25 mM) and the phosphatase inhibitors sodium fluoride (25 mM) and sodium orthovanadate (1 mM) (all from Sigma-Aldrich), during 20 minutes at 4°C. Pre-cleared supernatants were used for pull down assays and western blotting. Ras pull down assay was performed as previously described [12]. Briefly, GST-Raf was firstly bound to 4B Glutathion sepharose beads (Amersham), as per the manufacturer's instructions and beads were washed in lysis buffer. Then, cell lysates were incubated with 20 µg of GST-Raf during 30 minutes at 4°C. Bound Ras-GTP was resolved using 15% SDS-PAGE and detected by western blotting with anti Ras antibody (1:1000, Santa Cruz). To determine p-ERK1/2 and total ERK1/2 proteins, 12% SDS-PAGE gels (BioRad) were transferred to PVDF membranes (Millipore), blotted with the same primary antibodies used for immunofluorescence 1:1000 and developed with ECL (Amersham).

Western blots were quantified using Scion Image software (Scion Corporation) and values were corrected to actin levels.

Non selective monolayer permeability

Confluent cells grown on transwells (Costar) coated with collagen IV (1 µg/ml) were submitted to H/R and incubated for 15 minutes with 1% trypan blue (Invitrogen) at 37°C.

The amount of trypan blue contained on the medium from the lower chamber was measured by spectrometry at 595 nm.

Statistical analysis

Data are presented as means $\pm$ SEM. After the Levene test of homogeneity of variance, the Kruskal-Wallis test was used for group comparison. A $P < 0.05$ was considered significant. In case of significant differences, intergroup differences were analyzed by post-hoc Mann-Whitney U tests with the Bonferroni correction. Statistical analysis was carried out with Statistical Package for the Social Sciences (SPSS) version 11.0.

Results

ERK 1/2 is activated during I/R and H/R by ROS and Ras

ERK1/2 activation has been reported in animal models of renal I/R, mainly during reperfusion. We assessed the activation of ERK1/2 in the well established rat model of 45 minutes of ischemia followed by reperfusion. In this model, tissular and functional injury in the kidney reaches its maximum at 24 hours of reperfusion [13]. p-ERK1/2 expression was detected at 0 hours of reperfusion and more markedly at 24 hours of reperfusion, by immunohistochemistry (Figure 1). p-ERK1/2 staining showed a patched pattern typical for I/R injury in the kidney, localizing in damaged proximal tubules, which exhibited cell morphology alterations, loss of cell-cell contacts, detached cells, necrosis or even intraluminal casts.

Next, we studied ERK1/2 activation in an *in vitro*

Fig. 2. ERK1/2 is activated during reoxygenation in NRK-52E cells via Ras activation and ROS generation. (A) Western blotting for phospho and total ERK1/2 in NRK-52E cells submitted to H/R. (B) Pull down assay to detect active Ras (GTP-Ras) using Raf binding domain and western blotting for Ras. (C) Western blotting for phospho and total ERK1/2 in cells submitted to H/R in the presence of the antioxidant NAC (10 mM). Quantifications of the blots compared to actin levels are shown on top. All these assays were performed three times and representative images are shown.

model of H/R that mimics I/R injury in rat proximal tubule NRK-52E cells. This protocol includes both nutrients and oxygen withdrawal to mimic ischemia, followed by reoxygenation and nutrients repletion to reproduce reperfusion. As shown in Figure 2A, hypoxia by itself did not induce ERK1/2 activation, but it was strongly activated very early during reoxygenation, from 15 minutes to 1-3 hours, returning to basal levels thereafter. In order to evaluate whether ERK1/2 could be activated through a Ras/Raf dependent mechanism, we performed Ras pull down assays. These assays showed detectable GTP-Ras in hypoxia and early in the reoxygenation (15 minutes to

Fig. 3. p-ERK localizes in nucleus and at cell periphery during reoxygenation in NRK52-E cells. Effect of U0126 on ERK activation. (A) Localization of active ERK1/2 was determined by immunofluorescence. p-ERK immunostaining was observed in the nucleus and at the cell periphery of NRK-52E cells during reoxygenation (indicated by arrows). U0126 (10 μ M) applied during reoxygenation markedly reduces p-ERK1/2 staining both at cell periphery and in the nucleus. Magnification: x 200. (B) The specific MEK inhibitor U0126 (10 μ M) prevents ERK1/2 phosphorylation during reoxygenation, estimated by western blotting. Representative images of three independent experiments are shown. Quantifications of the blots compared to actin levels are shown on top.

1 hour), correlating with ERK1/2 activation (Figure 2B).

Both ERK1/2 and Ras activation have been reported in response to ROS. ROS are important mediators of the ischemic damage and frequently generated during reoxygenation/reperfusion as well as during hypoxia/ischemia. In our H/R protocol, ROS were generated during hypoxia and first minutes of reoxygenation, being quickly balanced [5]. Therefore, to assess the contribution of ROS to ERK1/2 activation, we used the antioxidant NAC during hypoxia and analyzed ERK1/2 activation. As shown in Figure 2C, NAC markedly reduced ERK1/2 activation in NRK-52E cells.

Fig. 4. ERK1/2 is not responsible for intercellular adhesion impairment during H/R. (A) Distribution of intercellular adhesion molecules E-cadherin and ZO-1 in NRK-52E cells during H/R, visualized by immunofluorescence. E-cadherin and ZO-1 internalized from the plasma membrane transiently during reoxygenation. 10 μ M U0126 applied during reoxygenation did not have any effect on E-cadherin and ZO-1 redistribution. Representative images of four independent experiments are shown. Magnification: $\times 200$. (B) Monolayer integrity estimated by permeability assays to trypan blue dye increases during reoxygenation but it is not affected by U0126. Data are presented as media $\pm$ SEM in comparison with monolayer permeability in the control condition, from three different experiments. Non significant differences were found between non-treated and treated cells.

These results demonstrate that ROS generation mediates ERK activation during reoxygenation. Most probably, Ras signalling also contributes to this activation.

Active ERK1/2 localizes in nuclei and at cell periphery

ERK1/2 regulates diverse biological functions by phosphorylation of numerous targets in nucleus and the cytoplasm, including proteins close to the plasma membrane, and thereby participates in the regulation of a variety of cellular processes. To identify targets for ERK1/2 in NRK-52E cells submitted to H/R, we first examined, by immunofluorescence, the localization of active ERK1/2. As presented in Figure 3A, active ERK1/2 localizes in both nuclei and at cell periphery during reoxygenation. The use of U0126 prevents ERK1/2 activation and consequently its translocation. U0126 is a specific inhibitor of the upstream kinase MEK1/2 which efficiently reduces ERK1/2 activation (Figure 3B). Thus, U0126 was used in the following experiments to determine the role of ERK1/2 in H/R-induced damage.

These findings suggest that ERK1/2 signalling activation might affect proteins localized at the nucleus and noteworthy at the cell periphery, where structures involved in cell adhesion are found.

ERK1/2 does not mediate intercellular adhesions disruption during reoxygenation

Cell-cell adhesion is compromised during I/R or H/R. In fact, we previously described transient disruption

of AJ and TJ early during reoxygenation by redistribution of Ecadherin, β -catenin and ZO-1 to the cytoplasm, without appreciable protein degradation [5]. Since ERK1/2 is activated early during reoxygenation and localizes at the cell periphery, we analyzed whether ERK1/2 might be involved in intercellular adhesions disruption, by immunofluorescence. As it is shown in Figure 4A, E-cadherin and ZO-1 internalization towards the cytoplasm was not prevented by U0126 when applied during reoxygenation. Moreover, permeability assays to verify intercellular adhesion function impairment during H/R were performed and, in correlation with the immunofluorescences, no significant changes in monolayer permeability were detected in the presence of U0126 (Figure 4B).

Hence, from these results we can conclude that ERK1/2 activation during reoxygenation does not contribute to cell-cell adhesion impairment.

ERK1/2 mediates cytoskeleton disorganization and focal complexes disruption during H/R

Since ERK1/2 activity might also affect cell-matrix adhesion [10] we also analyzed the contribution of ERK1/2 to focal adhesions stability in our H/R model. Focal adhesion establishment involves, among others, actin cytoskeleton organization and appropriate localization of adaptor/signalling proteins such as paxillin. Additionally, focal adhesion maturation is dependent on tubulin function.

Therefore, we assessed actin cytoskeleton organization by F-actin staining and confocal microscopy, in the

Fig. 5. ERK1/2 activation contributes to actin cytoskeleton remodeling during reoxygenation in NRK-52E cells. (A) F-actin staining using phalloidin-Alexa Fluor 568 revealed actin cytoskeleton reorganization: stress fibers loss during hypoxia and reassembly during reoxygenation. Longer stress fibers are observed in the presence of 10 μ M U0126. Representative confocal microscopy images of three different experiments, taken at medium cell level, are shown. Magnification: x 400, inserts x 800. (B) Cellular area during H/R was quantified in three experiments measuring three different fields per condition, indicating cell contraction. Significant differences were found, indicated by asterisks ($p < 0.05$).

presence or absence of U0126 during reoxygenation. As shown in Figure 5A, actin cytoskeleton is disorganized during H/R. Cortical actin increased during hypoxia with marked loss of stress fibers. Stress fibers were observed again during reoxygenation, although fibers were shorter than in control cells and length recovery was not complete during the first 6 hours of reoxygenation. Indeed, actin reorganization during reoxygenation could contribute to cell contraction and protrusion, as indicated by reduced cell area and as previously described [5]. Stress fibers in U0126 treated cells appeared longer and contraction was reduced.

Additionally, we examined paxillin localization by immunofluorescence and confocal microscopy during H/R in NRK-52E cells. We observed a marked redistribution of paxillin from focal complexes during hypoxia and early reoxygenation leading to loss of co-localization paxillin-actin and indicating focal adhesions disruption (Figure 6). In the presence of U0126 during reoxygenation, paxillin removal from focal contacts towards perinuclear zones is attenuated and paxillin-actin co-localization in focal complexes is partially restored

(Figure 6, inserts). Of note, paxillin staining pattern is much more organized in focal contacts in the presence of U0126 (insert at 1h). In addition, the U0126 effect on stress fibers length mentioned above can be also observed in Figure 6.

Next we addressed the ERK1/2 effect on tubulin organization. Microtubules, which regulate important functions in epithelial cells, such as focal complexes maturation, actin cytoskeleton dynamics and trafficking, can be altered during I/R. By immunofluorescence, we found that also microtubules network structure was affected by H/R and marked tubulin depolymerization was observed after 3 hours of reoxygenation (Figure 7). Tubulin network showed similar to control condition after 6 hours of reoxygenation. Moreover, we also observed that the inhibition of ERK1/2 by U0126 partially prevented microtubules depolymerization during reoxygenation.

All together these results support that ERK1/2 transient activation during reoxygenation is responsible for cytoskeleton alterations and paxillin redistribution which contribute to focal adhesion complexes disassembly or turnover in response to H/R.

Fig. 6. ERK1/2 activation provokes focal complexes disassembly. Integrity of focal adhesions was evaluated by co-localization (yellow) of F-actin staining using phalloidin-Alexa Fluor 568 (red) and paxillin immunostaining (green), using confocal microscopy and observed at basal-medium level. Co-localization actin-paxillin is lost during reoxygenation indicating focal complexes disruption. Inserts show detailed paxillin distribution in each condition. 10 μ M U0126 applied during reoxygenation reduces paxillin removal from focal contacts and increases actin-paxillin co-localization. Representative images of three different experiments are shown. Magnification: images, x 400; inserts, x 800.

Discussion

In this work, we characterize the ERK1/2 signalling pathway as an important mediator of cytoskeleton alterations and focal adhesion impairment observed in the proximal tubules in response to ischemia, pointing out this MAPK as potential target to ameliorate renal damage during I/R. ERK1/2 is activated during reoxygenation/reperfusion in response to ROS generation and Ras signalling and leads to epithelial injury.

Our results show that ERK1/2 was activated at 24 hours of reperfusion after renal ischemia in rat kidneys. We observed phosphorylation of ERK1/2 in proximal tubules which exhibited ischemic damage as evidenced by loss of epithelial cell morphology, epithelium integrity impair, cell shedding or intraluminal casts formation. In agreement with this, ERK1/2 activation has been described in the postischemic injury especially in the thick ascending limb and isolated S3 cells that had lost apical actin staining [14].

In order to identify targets for the ERK1/2 activation observed *in vivo*, p-ERK1/2 expression was studied in an *in vitro* model of H/R in rat proximal tubule NRK-52E cells, detecting a transient ERK1/2 activation early during reoxygenation. This *in vitro* model reproduces the oxygen and nutrient alterations that occur during I/R. ERK1/2 activation *in vitro* has been reported in oxidative stress models, including hydrogen peroxide treatment, often used to mimic renal I/R injury but related to proximal cell death [15, 16]. Interestingly, ERK1/2 activation is detected during reoxygenation in a model of H/R which

Fig. 7. ERK1/2 activation leads to microtubules depolymerization during reoxygenation in NRK-52E cells. Microtubules network integrity was evaluated by immunostaining for tubulin and conventional fluorescence microscopy. Notice reduction of tubulin staining during reoxygenation as well as shortening of microtubules, most evident at 3 hours. The use of 10 μ M U0126 prevents tubulin network impair. Representative images of three independent experiments are shown. Magnification: images x 200; inserts x 400.

only includes oxygen levels alteration, promoting cell proliferation [8]. However, our results link ERK1/2 activation to proximal cell organization and adhesion impairment, both contributing to I/R injury as discussed below.

ROS are generated during I/R or H/R and mediate ischemic injury by promoting mitochondrial damage, DNA alterations, lipid peroxidation, endoplasmic reticulum stress or apoptosis, among others [17]. The ERK1/2 activation reported here is a result of the ROS generated during hypoxia since the use of NAC during this period reduced significantly ERK1/2 phosphorylation. Some studies have reported ERK signalling in tubular cells after oxidant stress [18, 19] and both beneficial and deleterious effects have been attributed to it. On the other hand, ROS generation in our model does not lead to cell death [5]. This might be because, in contrast to our 6 hours of hypoxia, pro-oxidant treatments are frequently used in long exposure (over 24 hours in some cases) causing cytotoxicity.

On the other hand, Ras activation has been observed in our H/R model, contemporarily with ERK1/2 phosphorylation and consistently with the Ras/Raf/ERK1/2 activation during postischemic renal injury already reported [9]. Our results argue that hypoxia-induced ROS as well as nutrients replenishment contribute to Ras activation during reoxygenation. Supporting this hypothesis, it has been demonstrated that Ras/ERK1/2 inhibition protected against oxidative stress-induced cell death both in proximal tubule and endothelial cells [9].

In spite of the well known role of ERK1/2 controlling cell survival in response to many stimuli including ischemia, we have analyzed here the role of ERK1/2 on the maintenance of proximal cell structure, mainly guaranteed by cytoskeleton integrity and cell adhesion, since altered cellular organization is observed during sublethal ARF [3]. Indeed, the marked alterations in the cytoskeleton network as well as in the cell adhesion structures that were observed in NRK-52E cells submitted to H/R occurred while ERK1/2 activation was taking place. Unexpectedly, no significant activation of other MAPKs pathways such as JNK or p38 was observed in our model (data not shown).

As shown here, active ERK1/2 localized in the nucleus and interestingly at the cell periphery where cell-cell adhesions are established. After insults including ischemia, phosphorylation of AJ or TJ proteins such as β -catenin and ZO-1, respectively, has been reported, which would promote their cytoplasmic localization and/or their association with cytoskeleton proteins [20]. Internalization or degradation of AJ and TJ proteins causes intercellular adhesions disruption and impaired epithelial

function. We have observed here a transient but generalized internalization of AJ and TJ proteins in NRK-52E cells during H/R. This internalization was more evident early during reoxygenation when ERK1/2 is active. Nevertheless, ERK1/2 activation does not mediate the described cell-cell adhesion disruption since the use of U0126 did not prevent redistribution of E-cadherin and ZO-1 neither restored epithelial monolayer impermeability. In contrast, ERK1/2 activation is a requirement for TJ disruption in Ras-transformed MDCK cells [21] and PMA stimulated epithelial corneal cells [22]. Furthermore, ERK1/2 activation has recently been reported as a crucial regulator of TGF- β -induced EMT in proximal epithelial cells [23, 24].

Conversely, our results demonstrate a critical role of ERK1/2 activation in the transient cell-matrix interaction alterations caused by H/R. Indeed, focal adhesion disruption observed in our model by redistribution of paxillin from focal contacts as well as loss of actin stress fibers was reduced by U0126. Protein phosphorylation is one of the principal regulatory mechanisms that control cell adhesion dynamics. Thus, after localized activation, ERK1/2 can directly phosphorylate proteins such as FAK, paxillin and others or indirectly cause transphosphorylations leading to focal complex assembly or turnover, both essential for adhesion and migration [25, 26]. In fact and consistently with our *in vivo* and *in vitro* findings, Alderliesten et al., 2007 recently demonstrated that ERK1/2 activation *in vivo* enhanced dynamic paxillin, Src and FAK phosphorylation leading to dissolution and restructuring of focal adhesions during reperfusion, thus promoting renal injury.

In addition, ERK1/2 activation controls actin cytoskeleton dynamics and in particular stress fibers formation since longer fibers are observed in U0126 treated cells. Because stress fibers regulate cell contraction, ERK1/2 activation in our system can contribute to it. Indeed, U0126 showed a slight effect on cell contraction. ERK1/2 effects could be mediated through tropomyosin phosphorylation in response to oxidative stress [27] or downregulating RhoA/Rho Kinase signalling [28]. Of note, changes in RhoA activity early during reoxygenation have been previously observed in our H/R model [5] even though further experiments should be done to clarify this relationship.

On the other hand and concerning microtubule network structure, ERK1/2 inhibition by U0126 prevented the dramatic depolymerization of tubulin observed during reoxygenation. ERK and tubulin direct interaction has been described to occur on the spindle of dividing cells

[29]. Regarding the effect of ERK/tubulin interaction, it has been shown that, in the presence of active Ras, ERK associates with microtubules and promotes their destabilization [30]. Disorganization of the tubulin network might contribute to focal complexes disturbance since microtubules drive adhesive proteins to adhesion structures as well as RhoGTPases to control actin polymerization [31]. It is important to notice that we have observed tubulin network structure recovery contemporarily with focal adhesion re-assembly in NRK-52E after 6 hour of reoxygenation [5]. Additionally, microtubules are essential for trafficking [32], a crucial function of the proximal epithelial cells. It is conceivable then that the tubulin network alterations described here contribute critically to the tubule dysfunction associated to I/R injury.

In summary, our results show that ERK1/2 is responsible for cytoskeleton and focal adhesions alterations during I/R, linking ERK activation to proximal tubule injury. In spite of the controversial role of ERK1/2 in cell survival/cell death extensively reported in response to ischemic insult, our work points out ERK1/2 signalling as an important mediator of proximal cell structure impairment. Since ATN is likely caused by altered cellular organization, inhibition of ERK1/2 signalling could

be a potential intervention in the ARF caused by ischemia.

Abbreviations

ARF (Acute Renal Failure); ATN (Acute Tubular Necrosis); AJ (Adherens Junctions); EMT (Epithelial-mesenchymal transition); H/R (Hypoxia/Reoxygenation); I/R (Ischemia/Reperfusion); NA: (N-Acetylcysteine); NRK-52E (Normal Rat Kidney Epithelial Cells); ROS (Reactive Oxygen Species); TJ (Tight Junctions).

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