Redistribution of villin to proximal tubule basolateral membranes after ischemia and reperfusion

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Brown, Dennis, Richard Lee, and Joseph V. Bonventre. Redistribution of villin to proximal tubule basolateral membranes after ischemia and reperfusion. Am. J. Physiol. 273 (Renal Physiol. 42): F1003–F1012, 1997.—After ischemia and reperfusion, severe alterations in the cytoskeletal organization of renal tubular epithelial cells have been reported. These effects, accompanied by a modification in the polarized distribution of some membrane transport proteins, are especially evident in the proximal tubule. In normal proximal tubule cells, actin is concentrated in apical brush border microvilli, along with the actin-binding protein villin. Because villin plays an important role in actin bundling and in microvillar assembly but can also act as an actin-fragmenting protein at higher calcium concentrations, we examined the effects of ischemic injury and reperfusion on the distribution of villin and actin in proximal tubule cells of rat kidney. Using specific antibodies against villin and actin, we show that these proteins redistribute in parallel from the apical to the basolateral plasma membrane within 1 h of reperfusion after ischemia. Ischemia alone had no effect on the staining pattern. Repolarization of villin to the apical membrane begins within hours after reperfusion with enhanced apical localization over time during the period of regeneration. This apical repolarization of villin is accompanied by the migration of actin back to the apical membrane. These results show not only that villin may be involved in the initial disruption of the actin cytoskeleton during reperfusion injury but also that its migration back to the apical domain of these cells accompanies the reestablishment of a normal actin distribution in the brush border.

Immunocytochemistry; rat kidney; cytoskeleton; membrane polarity

The mechanisms underlying the remarkable ability of the kidney to regenerate after an ischemic insult are poorly understood. Although a contribution of a subpopulation of undifferentiated stem cells to the regenerative process cannot be ruled out (17), we have proposed that most sublethally damaged epithelial cells are able to undergo a cycle involving dedifferentiation and replication followed by redifferentiation (34). One rapid event that occurs after ischemia and reperfusion is a modification of the polarized distribution of some membrane proteins, particularly in proximal tubule epithelial cells, and this is accompanied by severe alterations in the cytoskeleton (23, 24, 25). Several reports have shown a rearrangement of Na⁺-K⁺-adenosinetriphosphatase (24) and integrins (27) under these conditions, and both the actin-based cytoskeleton (24) and the microtubule network (1) are disrupted within minutes of reperfusion. In addition, a degradation of ankyrin has been shown in cells of the thick ascending limb of Henle after ischemia and reperfusion (8).

In proximal tubule cells, actin is concentrated mainly in the dense brush border microvilli at the apical membrane, although some actin is also detectable in association with the basolateral plasma membrane (6, 30). The core of each microvillus contains a bundle of actin filaments that are held together by actin-binding proteins, including the cross-linking protein villin (7, 9, 13). However, although villin acts as an actin-bundling protein at submicromolar calcium concentrations, it can sever and cap actin filaments at higher calcium concentrations (13, 18). Actin-severing activity is also shared by another actin-binding protein, gelsolin, which is also activated and shortens actin filaments upon elevation of intracellular calcium (18, 35). It is possible that these actin-binding proteins are both involved in the dramatic rearrangement of actin that occurs under conditions of ischemia and reperfusion. The dual calcium-dependent activity of villin makes it a candidate for both severing and bundling of actin filaments during ischemia and reperfusion. It is known that ischemia results in disruption of apical microvilli and of the cortical actin microfilament network in these cells (24) and that reestablishment of normal microvillar structure and cellular polarity occurs slowly for several days after restoration of blood flow (25). The assembly of apical microvilli in the developing proximal tubule and intestine, as well as in cultured cells, has been shown to depend on the presence of villin at the apical pole of the cell (2, 10, 11, 14, 16, 32). Subsequent recovery of microvillar integrity in the regenerating nephron might depend critically on the apical location of villin in proximal tubule cells.

The purpose of the present study was to examine the effects of ischemia and reperfusion on the intracellular distribution and organization of villin and to determine the association of villin with the actin cytoskeleton. Using specific antibodies against villin and actin, as well as fluorescent phalloidin conjugates, we show that both actin and villin redistribute from the apical brush border to the basolateral plasma membrane of proximal tubule cells after short periods of reperfusion following ischemia. Repolarization of villin to the apical membrane occurs gradually in the hours and days following the ischemic insult, correlating with the reappearance of actin in the brush border microvilli.

MATERIALS AND METHODS
Induction of Ischemia

Ischemia followed by reperfusion was carried out as previously described (34) in male Sprague-Dawley rats (200–275 g; Charles River Breeding Laboratories, Wilmington, MA). Rats were maintained according to the NIH Guide for the Care and Use of Laboratory Animals [DHEW Publications No.
(NIH) 85–23, Revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20892, under alternating 12:12-h light-dark cycles. Briefly, rats were anesthetized by intraperitoneal administration of pentobarbital sodium (Nembutal, 6.5 mg/100 g body wt; H. Schein, Port Washington, NY), and were injected intraperitoneally with 10 ml of 0.9% saline warmed to 37°C. Animals were placed on a heated operating board. A left flank incision was made, and the left renal artery and vein were clamped with a microaneurysm clamp. The incision was temporarily closed until the clamp was removed after 40 min of ischemia. The incision was then closed, and kidneys were allowed to reperfuse for periods ranging from 1 h to 5 days prior to perfusion fixation. Postischemic and contralateral right kidneys were evaluated by immunocytochemistry. Some kidneys were fixed immediately after the period of ischemia, with no reperfusion. Other animals underwent sham surgery without ischemia and these kidneys were used as controls. Multiple tissue sections from at least three animals were examined for each condition and each time point examined.

Tissue Fixation and Sectioning

Kidneys were fixed by intravenous perfusion of paraformaldehyde-lysine-periodate fixative as previously described (1, 21). Tissue slices were then fixed overnight by immersion in the same fixative at 4°C. After rinsing several times in phosphate-buffered saline (PBS), tissue slices were immersed in 30% sucrose in PBS, prior to freezing in liquid nitrogen and sectioning at a thickness of 5 µm on a Reichert Frigocut cryostat. Sections were rinsed in PBS for 10 min and then bathed in a solution of 1% sodium dodecyl sulfate (SDS) in PBS for 5 min to improve exposure of antigenic sites (5). Sections were then rinsed two times, for 5 min each time, in PBS and incubated with PBS containing 1% bovine serum albumin for 10–20 min at room temperature to block nonspecific binding of antibodies. Sections were then incubated with primary antibody (see Primary Antibodies) for 1–2 h at room temperature or overnight at 4°C and washed for 5 min two times in high-salt PBS (PBS containing 2.7% NaCl) to reduce nonspecific binding of antibodies and finally in normal PBS.

Fig. 2. a: Higher magnification showing immunocytochemical localization of villin in S3 proximal tubule segments after ischemia and 1 h reperfusion. A marked basolateral labeling (arrows) is seen in addition to apical staining. b: Detection of actin in S3 proximal tubule segments after ischemia and 1 h reperfusion using a rhodamine-phalloidin conjugate. Although less apical staining persists than in control tubules, a strong staining of the basolateral plasma membrane is also detectable. Bars = 10 µm.

Fig. 1. Composite plate showing double immunocytochemical staining of the same sections for villin and actin in outer stripe of control (a and b) and 1 h postischemic (c and d) rat kidney. In control kidneys, villin (a) and actin (b) are concentrated in the apical brush border of proximal tubules. Actin, but not villin, is also detectable in all other cell types, in addition to proximal tubules. In kidneys reperfused for 1 h after ischemia, villin (c) and actin (d) are still detectable at the apical pole of proximal tubule cells, but, in addition, a marked basolateral localization is also apparent in proximal tubules. Bars = 20 µm.
for 5 min. Sections were then incubated for 1 h at room temperature with either 10 µg/ml donkey anti-mouse immunoglobulin G (IgG) coupled to CY3 (Jackson Immunoresearch, West Grove, PA) to detect actin, or with 15 µg/ml goat anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) (Jackson Immunoresearch) to detect villin. For double labeling, antibodies were applied in four sequential steps, with the same washing steps as above applied between each antibody application. In our hands, this procedure worked more effectively than incubations using mixed primary antibodies and mixed secondary antibodies. In some incubations, anti-actin antibodies were applied followed by goat anti-rabbit CY3, then an FITC-phycoerythrin conjugate (Sigma, 1:100 dilution) was applied for 1 h in a third incubation step.

**Photography and Digital Image Capture**

Most of the sections were evaluated and photographed by conventional epifluorescence microscopy using a Nikon FXA microscope. Photographs were taken on Kodak T-Max 400 film, push processed to 1600 ASA. Images were taken using the automatic exposure setting on the microscope, but, to ensure that different fluorescence intensity levels and film printing times did not affect the results, some images were also taken from control and ischemic kidneys using a range of overlapping, manually set exposure times ranging from 1 to 12 s. The negatives were then all printed using the same enlarger settings and print times. In such cases, although the intensity and saturation of the fluorescence staining increased with exposure time, the apical versus basolateral distribution of the proteins of interest were clearly visible under all conditions and were not affected by the photographic processing procedure.

For double staining, images were digitally captured using an Optronix color charge-coupled device camera, attached to a Macintosh 8500 Power PC running IP Lab Spectrum image analysis software. Separate images from the same field were captured using FITC or CY3 filter combinations. Images were imported into Adobe Photoshop 3.04 and merged using the “apply image” command. After construction of a composite plate, color output was generated using a Tektronix Phaser 440 dye sublimation printer.

**Western Blotting**

Kidneys were removed from control rats, and rats were subjected to 1 h unilateral ischemia for 45 min, with or without reperfusion for 1 h. Portions of the outer stripe of the outer medulla were quickly dissected and snap frozen in liquid nitrogen. Samples were solubilized in sample buffer, and proteins were separated by SDS-polyacrylamide gel electrophoresis as previously described (31). An equal amount of protein (40 µg) was loaded onto each lane. Proteins were transferred to Immobilon, and the membrane was probed with anti-villin antibody (1:1,000), followed by goat anti-rabbit IgG coupled to horseradish peroxidase. The location of labeled bands was revealed by the enhanced chemiluminescence technique.
Primary Antibodies

Rabbit polyclonal antiserum against chicken intestinal villin was a kind gift of Dr. Daniel Louvard (Institut Curie, Paris, France) and was used at a dilution of 1:200. Its specificity for villin has been previously shown (10). A mouse monoclonal antibody against chicken gizzard actin was purchased from Boehringer Mannheim. Rhodamine-phalloidin or FITC-phalloidin (Sigma) was used to detect filamentous actin, to confirm staining obtained using the anti-actin antibody. Whereas rhodamine-phalloidin detects F-actin, the anti-actin antibody can detect both F- and G-actin, although the bulk of the cellular fluorescence seen in immunostained preparations probably derives from localization of F-actin.

RESULTS

Distribution of Actin and Villin in Normal Kidneys

As previously described, normal rat kidneys showed an intense staining for actin and villin in the apical brush border microvilli of proximal tubule epithelial cells (30). Whereas villin staining was restricted to microvilli (Fig. 1a), actin staining was also found in a subapical region corresponding to the cortical cell web, as well as in distinct bundles associated with the basal (but not the lateral) plasma membrane of these cells (Fig. 1b). Villin was detectable only in proximal tubules, but actin was abundant in all cell types in the kidney. Under our tissue fixation and preparation conditions, actin staining was readily detectable using the monoclonal antibody and by rhodamine-phalloidin (or FITC-phalloidin) staining by conventional procedures. In contrast, we found that villin immunoreactivity was weak unless the tissues were pretreated with the denaturing agent SDS prior to staining.

Distribution of Actin and Villin in Postischemic Kidneys

Reperfusion for 1–3 h. After ischemia and reperfusion for 1 h, there was a marked disruption of brush border villin (Fig. 1c) and actin (Fig. 1d) staining in many epithelial cells of the proximal tubule. Some cells showed a marked reduction in the intensity of apical staining. This loss occurred mainly in the S3 segment in the outer stripe and the medullary rays, but a similar though less apparent alteration was also seen in S1 and S2 segments in the cortex. In parallel with this reduction in apical staining for villin and actin, both proteins showed a striking relocation to the basolateral pole of proximal tubule cells (Figs. 1, c and d, and 2, a and b). Again, this effect was most dramatic in the S3 segment (Fig. 2, a and b). The redistribution of
actin was detectable either with anti-actin antibodies (Fig. 2a) or with rhodamine-phalloidin (Fig. 2b). No apparent changes in actin organization were detectable in other epithelial cell types of the kidney, including the thick ascending limb of Henle (not shown). The arrangement of actin and villin in the contralateral kidney was no different from that seen in kidneys from control rats. After 3 h of reperfusion, the loss of apical staining and the basolateral redistribution of actin and villin were similar to those seen at 1 h, although, as shown in Fig. 3, some tubules had already begun to recover their apical polarization of villin and actin, with a reduction in basolateral staining. The images shown are representative of results that were consistently observed in all of the sections from at least three animals examined for each condition.

The redistribution of actin and villin was absolutely dependent on reperfusion. After ischemia alone, fol-
allowed by immediate fixation of the tissue, there was no detectable change in the cellular distribution of either actin (Fig. 4a) or villin (not shown), whereas a basolateral redistribution of actin (Fig. 4b) and villin (not shown) was apparent after 1 h reperfusion.

Codistribution of anti-actin and FITC-phalloidin staining. Because anti-actin antibodies may detect both F-actin (filamentous) and G-actin (monomeric), whereas phalloidin conjugates detect primarily F-actin, double staining was performed on control kidneys and on kidneys after ischemia and reperfusion to determine whether the actin redistribution was similar with both reagents. The color micrographs in Fig. 5, a-f, show that the bright actin staining of the proximal tubule brush border overlapped with both reagents and that the basolateral redistribution after ischemia and 1 h reperfusion was also comparable with both probes.

Postreperfusion, 1–3 days. In postschismic kidneys after 24 h reperfusion, actin and villin staining of the apical pole of proximal tubule cells was partially restored, and basolateral staining for villin was greatly diminished (Fig. 6). However, some cells retained basolateral staining for both actin and villin, and other cells showed a persisting loss of actin and villin from their apical pole. In all proximal tubule cells, actin and villin remained colocalized, regardless of the precise intracellular staining pattern. After 48 h of reperfusion, the pattern of staining was closer to that seen in contralateral kidneys, although even after 3 days of reperfusion some differences in the intensity and the homogeneity of the apical staining remained evident in postschismic kidneys. A weak basolateral staining for villin remained in some tubules (Fig. 7).

Reperfusion for 5 days. After 5 days of reperfusion, the actin and villin staining had almost returned to the normal control pattern, but some S3 segments had faint residual staining for actin associated with their basolateral plasma membranes (not shown).

Western blotting of villin. To determine whether a reduction in the amount of villin occurred during initial ischemia and reperfusion, Western blots were compared from control, ischemic, and ischemic-reperfused outer stripe of rats kidneys. The results shown in Fig. 8 demonstrate that no apparent difference in intensity of the villin band was detectable among these groups of rats. The 81-kDa band characteristic of villin was detected in all of the samples examined, indicating that no significant loss of villin occurred during ischemia and reperfusion.
DISCUSSION

Our results demonstrate that both actin and villin relocate from the apical to the basolateral pole of proximal tubule epithelial cells during reperfusion after ischemia. In previous studies from our laboratory, we reported that the model of unilateral ischemia results in a marked reduction in glomerular filtration rate 1–3 h after reperfusion (33) and a mild increase in plasma creatinine, from 0.52 to 0.68 mg/dl, and blood urea nitrogen, from 17.5 to 20.5 mg/dl (34). The increase in plasma creatinine and blood urea nitrogen are much more pronounced when bilateral occlusion is performed (22). Basolateral staining for both actin and villin was detectable as early as 1 h after reperfusion. We and others have previously demonstrated that the S3 segment of the proximal tubule is highly susceptible to ischemia-induced damage (1, 3, 34), and in the present study the redistribution of actin and villin was especially pronounced in this tubule segment. However, S1 and S2 segments also showed a qualitatively similar phenomenon, albeit less dramatic than in the S3 region. After 24 h of reperfusion, actin and villin were once again primarily located at the apical pole of the cells in most tubules, although some alterations in the normal pattern of actin staining were seen as long as 5 days after the ischemic insult. At most of the time points examined, actin and villin staining were tightly superimposed, suggesting that a close relationship is maintained between these two proteins even during the observed cytoskeletal breakdown and reorganization. The only time when a discrepancy between actin and villin localization was seen was following 5 days of reperfusion. In these kidneys, while both proteins were clearly concentrated in the apical brush border, a faint basolateral staining was still detectable for actin but not for villin in some cells. It has also been shown by confocal microscopy and Triton extraction that actin and villin remain closely associated during ATP depletion and recovery in an LLC-PK1 cell culture model of ischemic injury (28).

The actin/villin redistribution that occurs after reperfusion may represent an early event that precedes or parallels changes in membrane protein polarity and dedifferentiation of proximal tubule cells. The actin cytoskeletal network is involved in anchoring certain membrane proteins to specific domains of the plasma membrane (20, 26). It is also involved in attachment of epithelial cells to the basement membrane via its interaction with integrin molecules on the basolateral cell surface (12). Redistribution of both apical and basolateral proteins has been reported after ischemia (24), and disassembly of the cortical actin cytoskeleton may also be a prerequisite for cell shape changes that allow surviving epithelial cells to spread out over denuded areas of the peritubular basement membrane after ischemia. In our hands, little rearrangement of actin and villin localization is seen as early as 1 h after reperfusion.
In conclusion, our results demonstrate that ischemia followed by reperfusion promotes a rapid disruption of the apical cytoskeleton and a repolarization of actin and villin to the basolateral membrane of proximal tubule epithelial cells. Although our findings with actin are consistent with previous reports showing a partial loss of polarity in renal proximal tubule cells during ischemia and reperfusion (12), the present results now show that the important actin-binding protein villin is also rapidly redistributed away from the apical region of the cell under similar conditions. Recovery of cell polarity, the reestablishment of actin filament bundles and the reformation of microvilli may then depend upon the migration of villin back to its normal location at the apical pole of proximal tubule cells by an as yet undetermined mechanism.

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