Ischemia activates actin depolymerizing factor: role in proximal tubule microvillar actin alterations

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Schwartz, Niles, Melanie Hosford, Ruben M. Sandoval, Mark C. Wagner, Simon J. Atkinson, James Bamburg, and Bruce A. Molitoris. Ischemia activates actin depolymerizing factor: role in proximal tubule microvillar actin alterations. *Am. J. Physiol.* 276 (*Renal Physiol.* 45): F544–F551, 1999.—Apical membrane of renal proximal tubule cells is extremely sensitive to ischemia, with structural alterations occurring within 5 min. These changes are felt secondary to actin cytoskeletal disruption, yet the mechanism responsible is unknown. Actin depolymerizing factor (ADF), a 19-kDa actin-binding protein, has recently been shown to play an important role in regulation of actin filament dynamics. Because ADF is known to mediate pH-dependent F-actin binding, depolymerization, and severing, and because ADF activation occurs by dephosphorylation, we questioned whether ADF played a role in microvilli microfilament disruption during ischemia. To test our hypothesis, we induced renal ischemia in the rat with the clamp model. Initial immunofluorescence and Western blot studies on cortical tissue documented the presence of ADF in proximal tubule cells. Under physiological conditions, ADF was distributed homogeneously throughout the cytoplasm, primarily in the Triton X-100-soluble fraction, and both phosphorylated (pADF) and nonphosphorylated forms were identified. During ischemia, marked alterations occurred. Intraluminal vesicle/bleb structures contained extremely high concentrations of ADF along with G-actin, but not F-actin. Western blot showed a rapidly occurring duration-dependent dephosphorylation of ADF. At 0–30 min of ischemia, total ADF levels were unchanged, whereas pADF decreased significantly to 72% and 19% of control levels, at 5 and 15 min, respectively. Urine collected under physiological conditions did not contain ADF or actin, whereas urine collected after 30 min of ischemia contained both ADF and actin. Reperfusion was associated with normalization of cellular pADF levels, pADF intracellular distribution, and repair of apical microvilli. These data suggest that activation of ADF during ischemia via dephosphorylation is, in part, responsible for apical actin disruption resulting in microvillar destruction and formation of intraluminal vesicles.

acute renal failure; cofilin; LIM kinase; ATP depletion; actin cytoskeleton; renal proximal tubule cell

ISCHEMIA in vivo or cellular ATP depletion in vitro results in the disruption of the actin-membrane cytoskeleton of renal proximal tubule cells (PTC) (19, 20, 26, 27). In turn, this initiates a cascade of structural and functional alterations, including loss of surface membrane polarity (28, 30, 31), apical membrane bleb formation, opening of tight junctions (3, 6, 29), detachment of cells, and redistribution of the cortical actin network throughout the cytoplasm (19, 22). These changes initiate rapidly during ischemia, are duration dependent in severity (27), and are reversible during the recovery phase (42, 43). The apical membrane of PTC is particularly sensitive to ischemia, with cytoplasmic internalization and blebbing of membrane vesicles into the lumen occurring within 5 min of ischemia. This loss of apical membrane via bleb formation results in substrate for tubular obstruction and is, in part, responsible for Na^+ wasting (10). Both tubular obstruction and proximal Na^+ wasting are known to be responsible for reduced glomerular filtration rates (GFR) in human acute renal failure (39).

The mechanism(s) underlying these actin cytoskeletal alterations during ischemia/ATP depletion has not been elucidated, but the involvement of Ca^{2+}-mediated processes, including villin or gelsolin, seems unlikely in view of recent experiments. In these studies, increased intracellular Ca^{2+} levels, induced by ionomycin, led to increases in small F-actin fragments as well as increases in cytoplasmic G-actin. However, during chemical ATP depletion or hypoxia, in the absence of ionomycin, these changes did not occur (34, 37).

Ezrin and myosin Iβ have recently been described as participating in microvillar structural alterations during ischemia (9, 44). However, the mechanism of actin microfilament bundle destruction remains unknown. A reduced intracellular pH (pH_i) also has a protective effect on cells during ATP depletion (5, 14, 15, 45), in part mediated by reduced actin alterations during injury and enhanced cellular actin recovery during ATP repletion (11). To date, the mechanism responsible for this pH_i effect has not been determined.

Recently, increasing evidence has implicated ADF/cofilin as a stimulus-response mediator of cellular actin dynamics (36, 41). ADF/cofilin proteins range in size from 15 to 19 kDa, are found in all eukaryotic cells, and bind actin in a 1:1 stoichiometry (4, 38). ADF/cofilin proteins inhibit nucleotide exchange on monomeric actin. Some members of this family preferentially bind to Mg-ADP-actin rather than to Mg-ATP-actin (8, 23). This family of proteins is not restricted to binding monomeric actin but can also bind to and depolymerize actin filaments in a Ca^{2+}-independent, pH-sensitive fashion (17, 18). The binding to F-actin changes the

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twist of the actin molecule within the filament (24) and increases the off-rate from the pointed end of the polymer (7). Binding also induces weak severing activity at the junctions between ADF-bound and unbound regions of F-actin (17, 18). Functional control over ADF is, in part, mediated by its phosphorylation state. Phosphorylation of ADF inhibits binding to actin and, consequently, any effect on actin dynamics. The amount of phosphorylated ADF in cells varies from 14% to 100% of the total ADF concentration (33, 36). Regulation of ADF phosphorylation can be modulated by bifurcating signaling pathways (25). Recently, the specific kinase responsible for rapid in vivo phosphorylation of ADF/cofilin, LIM kinase, has been identified (2, 46). Unfortunately, dephosporylation of ADF/cofilin occurs via several known phosphatases and at least one unidentified phosphatase (25). To date, it has not been possible to inhibit the dephosphorylation of ADF with known phosphatase inhibitors.

Given the actin dynamics during ATP depletion and repletion, and the characterized interactions of ADF with actin, we hypothesized that ADF played a central role in the actin cytoskeletal alterations seen during ischemia/ATP depletion. To test this hypothesis, we first conducted studies to determine the effect of ischemia and reperfusion on the amount and level of phosphorylation of ADF and on its location in normal and ischemic rat kidney tubule cells. We then examined rat urine from both normal and ischemic animals to see if ADF was associated with the luminal material responsible for tubule obstruction.

METHODS

We induced renal ischemia in the left kidney of anesthetized male Sprague-Dawley rats (200–250 g) by clamping the renal pedicle for variable lengths of time, as described previously (31). The right kidney of each animal underwent a sham operation and functioned as the control in each experiment. The kidneys were removed and the outer cortex was dissected and placed in 15 ml of PHEM buffer containing (in mM) 60 PIPES, 25 HEPES, 10 EGTA, and 2 $MgCl_2$, pH 6.9, containing 0.5% Triton X-100. The cortical tissue was homogenized using a Potter-Elvehjem tissue homogenizer, then centrifuged at 48,000 *g* for 15 min at 4°C. The supernatants were carefully separated from the pellets, and the pellets were then resuspended in a volume of PHEM extraction buffer equal to the supernatants.

SDS-PAGE and immunoblotting. We analyzed supernatant and pellets using 10–20% gradient polyacrylamide precast gels (Novex) and performed immunoblotting as described previously (44). In brief, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in a buffer containing 10% methanol, 0.1% SDS, 40 mM glycine, and 120 mM Tris, pH 8.2. The membrane was blocked in a solution of TTBS (0.01 M Tris, 0.1 M NaCl, and 0.05% Tween 20, pH 7.5), containing 10% newborn calf serum, at 4°C overnight, then washed with TTBS.

The PVDF was incubated in primary antibody to ADF (1:10,000) (4), or the phosphopeptide epitope of phosphorylated ADF (pADF) and p-cofilin (rabbit, 1:20,000) (25), for 1 h at room temperature, then washed three times in TTBS. Secondary antibody, goat anti-rabbit horseradish peroxidase (HRP), for ADF and pADF (1:2,500; Fisher Scientific, Itasca, IL) was washed five times in TTBS, then detected with enhanced chemiluminescence (ECL; Pierce, Rockford, IL). In all studies, equal quantities of total protein were applied to each gel lane.

Immunofluorescence. Rat kidneys were clamped as described above. The kidneys were then perfusion-fixed with PLP fixative (2% paraformaldehyde, 1.4% lysine, and 0.2% sodium periodate), excised, and placed in PLP overnight at 4°C. Fixed tissue was washed with PBS three times for 15 min, placed overnight in PBS containing 30% sucrose, then cut into 30- to 40-μm sections using a cryotome. The sections were incubated in blocking-extraction buffer (PBS containing 2% defatted BSA, 0.5% dry nonfat milk, and 0.1% Triton X-100, pH 7.4) for 1–2 h at 4°C. Sections were then incubated in the above blocking buffer containing primary antibody (anti-ADF; 1:400) and the monoclonal anti-G-actin antibody JLA 20 (1:100; Amersham) overnight at 4°C. This was followed by three rinses in PBS and application of secondary antibodies in the blocking buffer, with or without FITC-phalloidin (Molecular Probes, Eugene, OR), used at a 1:10 dilution of the stock methanol solution overnight at 4°C. The sections were again washed three times in PBS, mounted in Gelvatol (Monsanto, Indian Orchard, MA), and observed using the MRC-1024 confocal microscope (Bio-Rad, Hercules, CA).

RESULTS

Initial studies were undertaken to determine whether ADF was present in rat kidney cortical tissue. Figure 1 shows Triton X-100-soluble and -insoluble fractions of rat kidney cortex under physiological conditions. ADF was found in rat cortex under physiological conditions (Fig. 1*A*), primarily in the detergent-soluble portion (*lanes 1* and *3*), with only small amounts in the cytoskeletal associated fraction (*lanes 2* and *4*). Secondary bands appearing on this Western blot above and below the 19-kDa area, where the ADF standard band was found (*lane 9*), were due to background binding from the secondary antibody used, as shown in *lanes 10* and *11*. These detergent-solubility studies were also performed on cortical tissue after 25 min of ischemia to determine if any detectable redistribution of ADF occurred between detergent-soluble and -insoluble forms. After 25 min of ischemia, there was no apparent redistribution of ADF between the detergent-soluble and -insoluble pools (Fig. 1*A*, *lanes 5–8*), suggesting that the majority of ADF remained soluble. However, these studies do not rule out the possibility of rapid dissociation of ADF from F-actin during the isolation procedure. They also do not evaluate G-actin binding by ADF.

Immunofluorescence studies were then undertaken to determine tubular cell specificity and cellular distribution of ADF. Under physiological conditions, a diffuse staining throughout the cytoplasm of all tubular cells was observed, with no apparent subcellular compartmentalization noted (Fig. 1*B*). Nuclear staining was not observed.

Proximal tubule cells did appear to have enhanced total ADF staining compared with distal cells, but quantitative studies were not undertaken.

Next, a polyclonal antibody specific to pADF was used on rat cortical kidney homogenate for Western blotting and on cortical sections for indirect immunofluo-

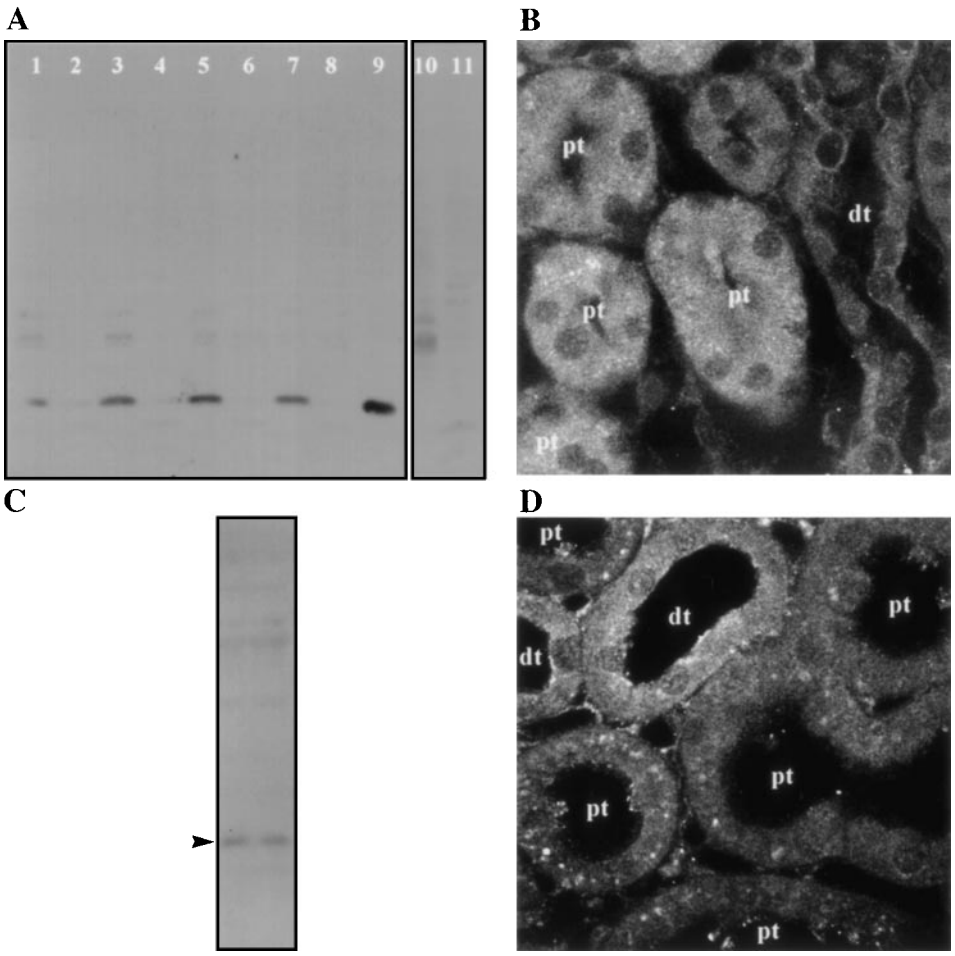

Fig. 1. Actin depolymerizing factor (ADF) in rat renal cortical tissue. *A*: Triton X-100-fractionated soluble and insoluble proteins were separated using 14% SDS-PAGE and Western blotted using polyclonal anti-ADF antibody. *Lanes 1* and *3* are Triton X-100-soluble fractions; *lanes 2* and *4* are Triton X-100-insoluble fractions, under physiological conditions. *Lanes 5* and *7* are detergent-soluble fractions and *lanes 6* and *8* are insoluble fractions after 25 min of ischemia. *Lane 9* is an ADF standard. *Lanes 10* and *11* are soluble and insoluble fractions, respectively, probed using only goat anti-rabbit horseradish peroxidase secondary antibody. *B*: cellular localization of ADF, under physiological conditions, as shown by indirect immunofluorescence. pt, Proximal tubule; dt, distal tubule. *C*: phosphorylated ADF in rat cortical kidney tissue under physiological conditions (arrowhead). *Lanes 1* and *2* show rat cortical kidney homogenate after Western blotting using antibody to phosphorylated ADF. *D*: cellular localization of phosphorylated ADF, as shown by indirect immunofluorescence and described in METHODS.

rescent staining. Figure 1*C* shows that pADF was present in this tissue under physiological conditions (*lanes 1* and *2*). Immunofluorescence staining showed that pADF appeared to be distributed throughout the cytoplasm in a homogeneous granular pattern, with some punctate accumulations, and was not associated with specific subcellular compartments (Fig. 1*D*). It was not visualized in the nucleus. The amount of pADF present in distal tubular cells seemed increased compared with PTC, but this was not quantified.

In a second series of studies, the effect of ischemia on relative amounts of pADF was determined. Time points of 0, 5, 15, and 30 min of ischemia were used. Figure 2*A* shows Western blot analysis of cortical homogenate tissue using antibodies to both ADF and pADF. We used densitometric analysis to quantify changes in ADF tissue content. The total amount of ADF in the ischemic tissue did not change significantly from control values. However, pADF levels decreased 28% after 5 min of ischemia, were maximally decreased at 15 min (81 ± 8%, $P < 0.01$), and were stable at this low level thereafter, as shown in Fig. 2*B*. Because dephosphorylation is known to activate ADF, these data imply activation of ADF under ischemic conditions.

To determine whether recovery from ischemia resulted in the rephosphorylation of rat cortical ADF, we determined tissue homogenate concentrations of ADFt and pADF (after 30 min of ischemia) after 2 and 4 h of reperfusion. As shown in Fig. 2, *B* and *C*, the amount of pADF increased progressively during 2 and 4 h of reflow. Figure 2*C* is a plot of "ADF activation" calculated as the decrease in pADF from data shown in Fig. 2*A* and graphed in Fig. 2*B*. There was a marked increase in ADF activation by 15 min of ischemia ($P < 0.01$) and no statistical difference between ADF activation at 15 and 30 min. However, there was a time-dependent decrease in active ADF at 2 h (56 ± 8%, $P < 0.05$) and 4 h (21 ± 10%, $P < 0.01$) of reflow. No statistical difference existed between cellular pADF levels after 4 h of reflow and under normal physiological conditions.

Confocal microscopy was next used to determine whether ischemia-induced dephosphorylation of ADF was associated with actin alterations (Fig. 3). Specifically, the goal was to determine whether ADF colocalized with either G- or F-actin in the cell under these conditions. After 25 min of ischemia, or 25 min of ischemia with 24 h of reflow, kidneys were fixed with 4% paraformaldehyde by perfusion fixation. G- and F-actin staining, under physiological conditions (Fig. 3*A*), showed, as previously reported, F-actin localization to the apical brush border, whereas G-actin showed a diffuse cytoplasmic staining pattern with little or no staining in the apical microvillar region (Fig. 3*A*).

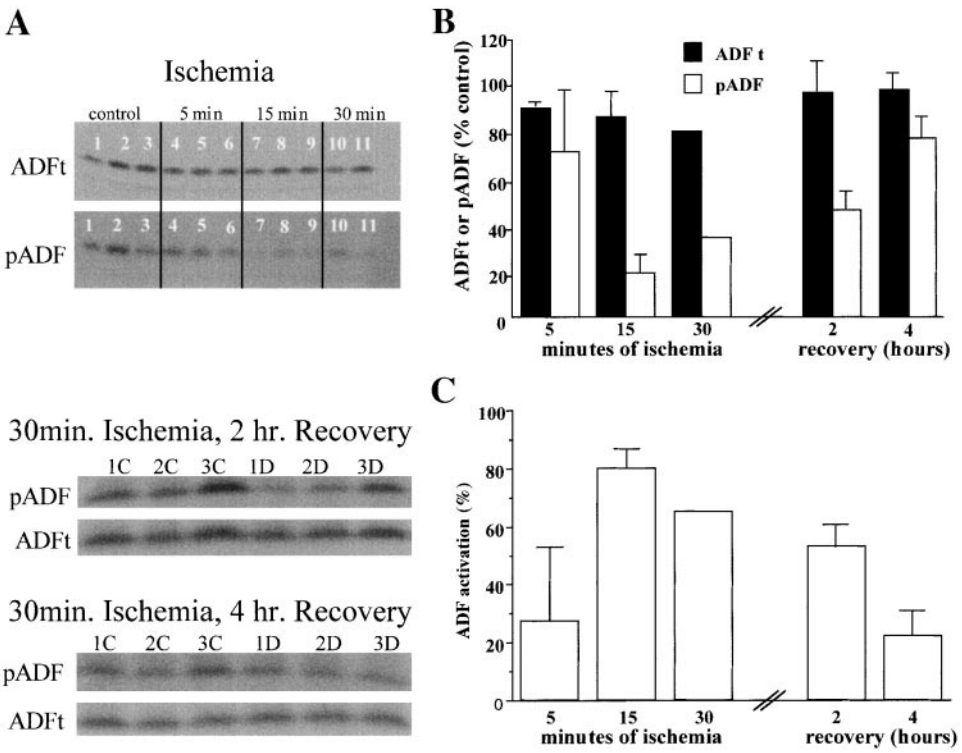

Fig. 2. Effect of 30 min of ischemia on total ADF (ADFt) and phosphorylated ADF (pADF) in rat renal outer cortical tissue. *A*, *top*: Western blot analysis of ADFt and pADF under physiological conditions (control) and after 5, 15, and 30 min of ischemia. Each lane contains 10 μg protein of homogenate from a different rat kidney. *A*, *bottom*: ADFt and pADF after 30 min of ischemia and either 2 or 4 h of reperfusion. C, control (physiological conditions); D, contralateral ischemic kidney shown in paired fashion. *B*: effect of ischemia and reperfusion on renal cortical ADFt and pADF. Western blot analysis of ADFt and pADF after 30 min of ischemia and either 2 or 4 h of reperfusion. Densitometric quantitation of outer cortical ADFt and pADF (as a percentage of control) under physiological conditions, during ischemia, and after 30 min of ischemia and either 2 or 4 h of reperfusion; $n = 3$ for 5 and 15 min of ischemia, and $n = 2$ for 30 min of ischemia. *C*: effect of ischemia and reperfusion on ADF activation. Percent activation was calculated as 100 minus the percentage of decrease in pADF from pADF levels under physiological conditions using densitometric quantitation (as shown in *B*). Error bars represent SDs.

Under ischemic conditions, disruption of apical microfilaments occurred with an apparent loss of surface membrane as vesicles into the proximal tubule lumen. These luminal vesicles stained heavily for G-actin, but not filamentous actin (Fig. 3*B*). When ischemia was followed by 24 h of reperfusion (Fig. 3*C*), the intraluminal blebs disappeared and the apical microvillar F-actin pattern region partially reformed. During reflow, the staining patterns of G- and F-actin were comparable with tissue under physiological conditions, and it appeared that the apical brush border membrane was again intact but shorter.

Immunofluorescent studies using the antibody against ADFt showed that, in proximal tubule cells, diffuse cytoplasmic staining occurred under physiological conditions (Fig. 3*D*), as seen in Fig. 1. However, in ischemic tissue, a major redistribution of ADF into intraluminal blebs occurred, as shown by the dense vesicular staining pattern and reduced cytoplasmic staining in Fig. 3*E*. There was also a loss of the homogeneous cytoplasmic staining of ADFt, with accumulations of punctate staining for both ADF and F-actin. This apparent colocalization of ADF and F-actin was also seen after 24 h of reperfusion, but to a lesser extent (Fig. 3*F*). Otherwise, 24 h of recovery was associated with an apparent recovery of cell morphology, and the ADF staining pattern resembled that observed under physiological conditions, with little or no decrease in staining intensity of the cytoplasm. Also, distal tubules again appeared to have less ADFt staining.

Immunofluorescent staining using the anti-pADF antibody showed a punctate appearance, under physiological conditions (Fig. 3*G*), with enhanced distal tubule cell staining compared with proximal tubule pADF staining. After 25 min of ischemia, staining of pADF in the luminal vesicles was observed (Fig. 3*H*). Twenty-four hours of recovery was associated with morphological improvement of the staining pattern of apical F-actin and a return to baseline pADF staining in all cell types. Taken together, these data imply higher concentrations of ADFt and a higher percentage of active ADF in proximal tubule cells.

Analysis of rat urine under these same conditions was next utilized to determine whether the G-actin/ADF-containing blebs that formed during ischemia appeared in the urine. Urine samples collected under physiological conditions contained no intact actin, pADF, or ADFt, as revealed by Western blot analysis (Fig. 4, *lanes 3*, *5*, and *7*, respectively). However, urine collected during the hour after 25 min of ischemia showed the presence of intact actin, pADF, and ADF (Fig. 4, *lanes 4*, *6*, and *8*, respectively).

DISCUSSION

Ischemia-induced apical structural alterations initiate rapidly and are duration dependent in proximal tubule cells. These changes are known to coincide with microvillar actin changes, including filament destruction and redistribution of cortical F-actin to a cytoplasmic location. Therefore, this reorganization of the actin cytoskeleton during ischemia is believed to be important in the surface membrane structural, biochemical, and functional alterations that occur in proximal tubular cell injury and acute renal failure (16). Of particular importance is the involvement of apical microvillar membrane damage, including membrane internalization into the cytoplasm and fragmentation into the

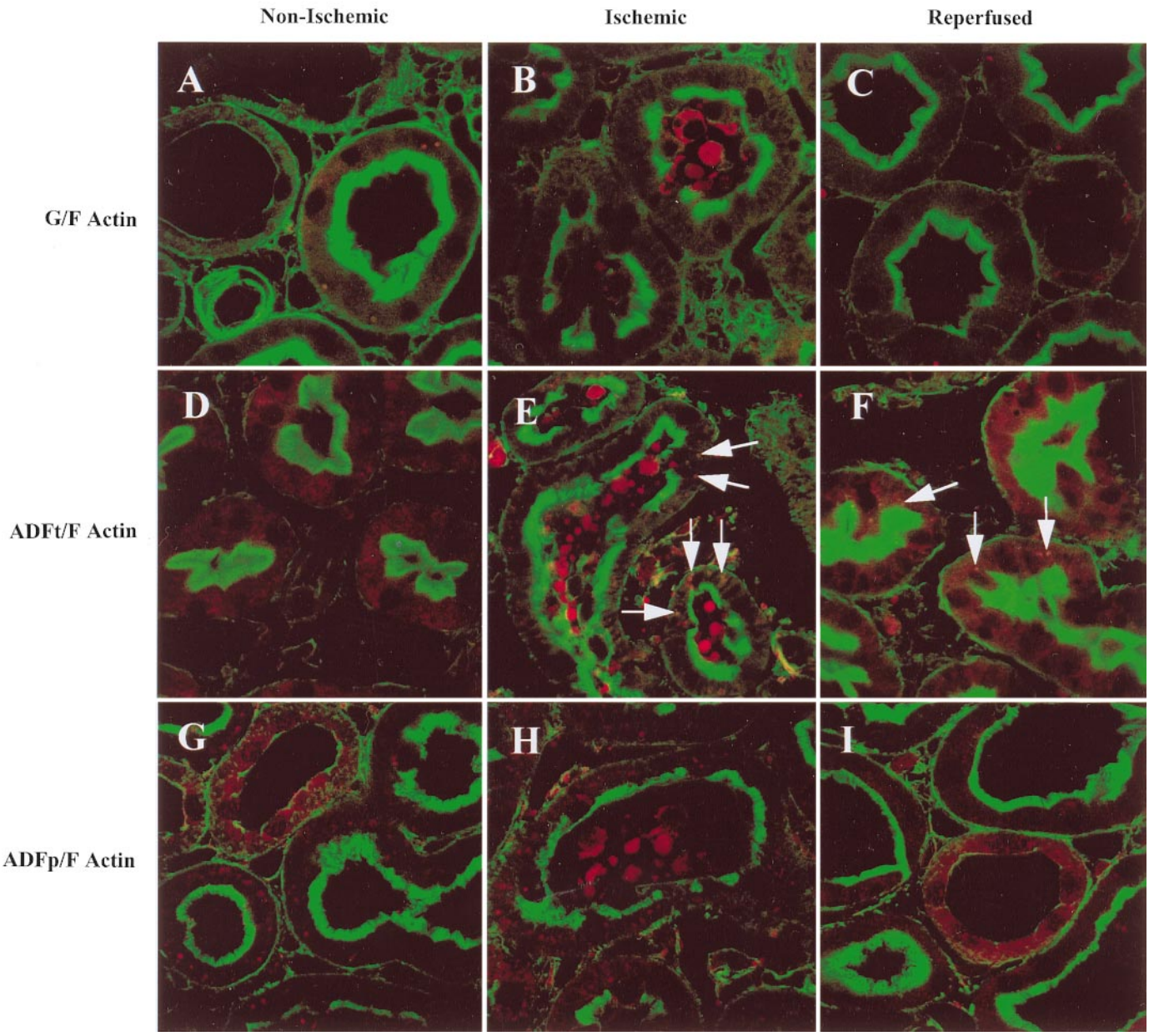

Fig. 3. Effect of 25 min of ischemia and reperfusion on cellular localization of ADFt, pADF, F-actin, and G-actin, as seen by immunofluorescence. Tissue specimens shown as follows: physiological conditions (*A*, *D*, and *G*), 25 min of ischemia (*B*, *E*, and *H*), or 24 h of recovery after 25 min of ischemia (*C*, *F*, and *I*). F-actin was stained using FITC-phalloidin (green), monomeric G-actin was stained with JLA-20 primary antibody and a Texas Red secondary antibody (red), and ADFt or pADF was stained with its respective polyclonal antibody and a Texas Red secondary antibody (red). Images were obtained and processed as described in METHODS. Arrows indicate areas of apparent colocalization of ADF and F-actin.

urinary lumen (13, 35, 43). This has important clinical implications, including tubular obstruction, abnormal tubular glomerular feedback due to reduced Na^+ reabsorption, and back-leak between cells (39). Although the mechanisms mediating these actin cytoskeletal alterations remain unknown, it is known that ischemia rapidly lowers pH_i by the accumulation of hydrogen ions. Furthermore, recent evidence has shown that a generalized protein dephosphorylation occurs during anoxia, due to the lack of ATP for kinase activity in the presence of continued phosphatase activity (9). This is known to result in microvillar ezrin dephosphorylation that mediates, in part, apical structural changes (9).

ADF is one member of a class of noncapping, Ca^{2+}-independent, actin-binding proteins that has recently been shown to markedly enhance the dynamics of actin assembly (32, 36, 41). ADF/cofilin is the major regulator of actin filament turnover in vivo (21). ADF is a 19-kDa phosphoprotein activated by dephosphorylation (1, 4, 12). The binding and severing activities of ADF are also regulated by pH_i, with decreases in pH_i resulting in enhanced F-actin binding but decreased ADF depoly-

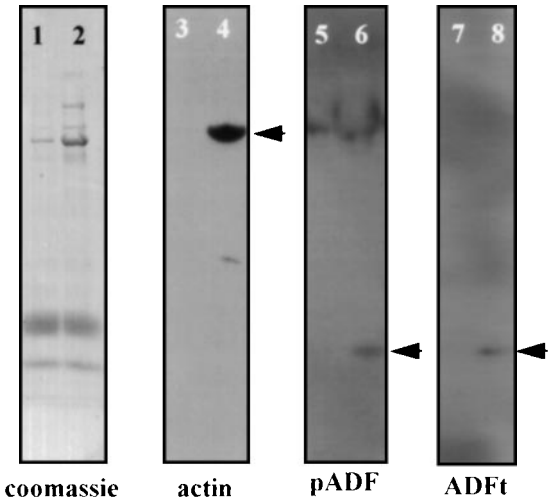

Fig. 4. Ischemia results in actin and ADF appearing in urine. Urine samples were collected before and 1 h after 25 min of pedicle clamp ischemia. Coomassie staining was used to identify all urinary proteins. Western blot analysis was used to identify actin, ADF, and pADF, as described in METHODS. *Lanes 1*, *3*, *5*, and *7* are under physiological conditions, and *lanes 2*, *4*, *6*, and *8* show urine collected for 1 h after ischemia. Arrows indicate specific protein probed for on Western blot. For all samples, 10 μg urinary protein was applied to each lane.

merizing activity (12). ADF shortens actin filaments via two different mechanisms of action. The first involves severing of the actin filament (17), whereas more recent studies point to kinetic acceleration of the on and off rates of actin monomers at the barbed and pointed ends, respectively (7, 36).

Given the above characteristics of ADF, and the fact that ischemia reduces pH_i and is associated with a generalized protein dephosphorylation, studies were undertaken to determine whether ADF could be involved in mediating the actin alterations observed during ischemia in vivo. Initial studies utilized previously well-characterized anti-ADF antibodies (4, 25) and showed that both the phosphorylated and unphosphorylated forms were present in renal cortical tissue. These data complement and extend an earlier study that showed ADF immunoreactivity with kidney tissue from other vertebrate species (4). Our indirect immunofluorescent studies showed that ADF was present in all renal tubular cells, with a homogeneous, non-nuclear cytoplasmic distribution. Proximal tubule cells had an apparently higher ADFt content compared with distal tubule cells. There was also an abundance of pADF, as shown with the antibody specific for pADF. This was especially true in distal tubule cells. Taken together, these data indicate that proximal tubule cells possess a higher content of active ADF, under physiological conditions, than distal tubule cells, although actual quantitation is necessary.

In the next series of studies, ischemia in vivo was shown to result in the rapid, duration-dependent dephosphorylation of ADF. Rephosphorylation of ADF occurred during the recovery period. This is the first demonstration that ischemia in vivo results in ADF dephosphorylation. To evaluate whether ADF activation during ischemia could be playing a role in actin cytoskeletal alterations, confocal colocalization studies were undertaken. A monoclonal antibody was used to identify and localize G-actin, and phalloidin staining was used as an indicator of F-actin. As previously described by numerous investigators, ischemia led to the rapid destruction of apical microvillar actin filamentous structures, with reperfusion resulting in the reformation of these microvilli. Twenty-five minutes of ischemia was chosen, as this is an intermediate level of injury allowing for mechanistic information to be obtained before irreversible cell injury. Notable from the present studies was the marked accumulation of G-actin within the intraluminal blebs, implying massive depolymerization of microvillar actin. This is remarkable in that the microvillar area had very little or no G-actin staining under physiological conditions. Even in studies evaluating only G-actin, in the absence of F-actin staining, no apical membrane or microvillar regional staining could be detected under physiological conditions (data not shown). Previous investigators had postulated that intraluminal blebs contained primarily G-actin and that depolymerization of microvillar F-actin was, in part, responsible for bleb formation (35). The antibody specific for G-actin now commercially available allowed us to confirm this aspect of the hypothesis.

With ischemia, there was also marked accumulation of ADF in the intraluminal vesicles, with striking colocalization with G-actin being observed. This may be secondary to the recruitment of ADF to the apical membrane, because, under physiological conditions, very little ADF was seen in the microvillar region using immunofluorescent techniques. With reflow, the alterations in G- and F-actin and ADF localization corrected. Biochemical verification of actin and ADF in the vesicles came with examination of the urine using Western blot approaches. Under physiological conditions, no actin or ADF was present, but after ischemic injury, both actin and ADF were easily detectable in the urine. We do not know if these proteins play any role in tubular obstruction, although actin polymerization would be expected to markedly enhance the viscosity of the membranous vesicles and alter vesicle shape.

Several interesting questions are posed by the present data. First, one might expect ADF activation to result in F-actin binding. However, ADF appeared primarily in the detergent-soluble fraction, even after ischemia. This could be due to dissociation of ADF from F-actin during the isolation process. However, ADF could also be bound to G-actin after ischemic injury, and this complex would appear in the detergent-soluble fraction. This is actually likely, as G-actin released during F-actin depolymerization under ischemic conditions cannot be converted from ADP to ATP-G-actin secondary to low cellular ATP levels. Because ADF has a higher affinity for ADP-G-actin, it may remain associated with G-actin. Second, pADF was identified in intraluminal vesicles after 25 min of ischemia using immunofluorescent techniques. Unfortunately, one cannot directly compare fluorescent intensities for ADF

and pADF, as the primary antibodies have different affinities. Therefore, we believe the actual quantity of pADF in the vesicles remains small. Finally, use of phosphatase inhibitors to stop ADF dephosphorylation, as was done for ezrin (9), would have been very interesting. However, to date, this has not been possible for ADF (25).

Taken together, these data indicate that ischemia in proximal tubule cells results in ADF activation, ADF recruitment to the apical domain, and microvillar actin depolymerization. In a previous study, recruitment of ADF to the surface membrane was seen after activation in neutrophil-like HL-60 cells (40). Therefore, we postulate that ischemia-induced activation of ADF mediates the severe actin alterations that occur in the apical membrane. Previous investigators were divided on the potential role of Ca^{2+}-mediated actin severing via gelsolin and villin. However, recent work by Weinberg et al. (34, 37) has again served to minimize the potential role of these Ca^{2+}-dependent, actin-severing proteins in ischemic injury.

The present studies may also have importance in understanding the protective role of reduced pH_i during ischemic injury. Several laboratories have shown the value of acidic conditions in minimizing cellular injury in ischemia or ATP depletion in both in vivo and cell culture studies (5, 14, 15, 45). We have shown that part of the protection involves minimizing actin cytoskeletal changes during ATP depletion and enhancing actin recovery during cellular recovery (11). Because a reduced pH_i inhibits the actin depolymerizing activity of ADF, it is possible that this represents a mechanism whereby acidosis is protective.

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