

Renal ischemia induces tropomyosin dissociation-destabilizing microvilli microfilaments

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Ashworth, Sharon L., Sarah E. Wean, Silvia B. Campos, Constance J. Temm-Grove, Erica L. Southgate, Bernadette Vrhovski, Peter Gunning, Ron P. Weinberger, and Bruce A. Molitoris. Renal ischemia induces tropomyosin dissociation-destabilizing microvilli microfilaments. *Am J Physiol Renal Physiol* 286: F988–F996, 2004. First published December 23, 2003; 10.1152/ajprenal.00168.2003.—Ischemic-induced cell injury results in rapid duration-dependent actin-depolymerizing factor (ADF)/cofilin-mediated disruption of the apical microvilli microfilament cores. Because intestinal microvillar microfilaments are bound and stabilized in the terminal web by the actin-binding protein tropomyosin, we questioned whether a protective effect of tropomyosin localization to the terminal web of the proximal tubule microfilament cores is disrupted during ischemic injury. With tropomyosin-specific antibodies, we examined rat cortical sections under physiological conditions and following ischemic injury by confocal microscopy. In addition, Western blot analysis of cortical extracts and urine was undertaken. Our studies demonstrated the presence of tropomyosin isoforms in the proximal tubule microvillar terminal web under physiological conditions and their dissociation in response to 25 min of ischemic injury. This correlated with the excretion of tropomyosin-containing plasma membrane vesicles in urine from ischemic rats. In addition, we noted increased tropomyosin Triton X-100 solubility following ischemia in cortical extracts. These studies suggest tropomyosin binds to and stabilizes the microvillar microfilament core in the terminal web under physiological conditions. With the onset of ischemic injury, we propose that tropomyosin dissociates from the microfilament core providing access to microfilaments in the terminal web for F-actin binding, severing and depolymerizing actions of ADF/cofilin proteins.

actin-depolymerizing factor; cofilin; brush border

RENAL ISCHEMIA INDUCES severe biochemical, physiological, and morphological alterations to polarized kidney proximal tubule cells (PTC) (16, 21, 22, 35). The extent of ischemic injury depends on its length and the severity of the insult (17). Even very short ischemic insults provoke cellular changes in surface membrane polarity, junctional complexes, and the actin architecture. The apical microvillar region of PTC is particularly sensitive to ischemic insult with rapid and severe, but repairable, damage occurring (17). Within 5 min of ischemia, the microvillar F-actin core, as well as its overlying plasma membrane, shows signs of degeneration (16, 21). The plasma membrane no longer maintains its finger-like microvillar struc-

ture, but it becomes more amorphous, whereas the internal F-actin core begins to deteriorate. With longer ischemic insults, the plasma membrane and microvillar F-actin cores degenerate further.

Previous data from our laboratory demonstrated that the actin-binding protein family of actin-depolymerizing factor (ADF) and cofilin proteins plays a critical role in the apical microvilli breakdown in response to ischemia (3, 4, 31). ADF, an important regulator of F-actin dynamics, binds F-actin in a pH-dependent manner and mediates F-actin severing and depolymerization (5, 11). The phosphorylated form of ADF is inactive and cannot bind actin (1, 24, 25). Renal ischemia induces rapid dephosphorylation/activation and relocation of ADF in PTC (3, 31). Under physiological conditions, ADF has a diffuse cytoplasmic localization pattern and is absent in apical microvilli (3). With the onset of ischemia, ADF staining also localizes to the apical microvilli and to plasma membrane-bound vesicles that have been shed into the PTC lumen (3).

With the demonstration that ischemic injury induced dephosphorylation/activation and penetration of ADF into the apical microvillar region, we next questioned why ADF does not localize to the apical microvilli under physiological conditions. In polarized intestinal epithelial cells, tropomyosin, the F-actin binding and stabilizing protein, localized to the microvilli terminal web, a region where the ADP-actin-rich microfilament cores are located (10, 13). Several *in vitro* biochemical and structural studies demonstrated that ADF competes with tropomyosin for F-actin binding (6, 26, 27). Based on these *in vitro* studies, localization of tropomyosin at the terminal web may not only stabilize the microfilaments but may protect the filaments from binding by active ADF/cofilin proteins, preventing filament severing and depolymerization.

Therefore, we hypothesized the F-actin stabilization role of tropomyosin in PTC is disrupted through ischemia-induced tropomyosin dissociation from the terminal web microfilaments. This then may allow for the rapid disruption of microvilli microfilament cores and subsequent destabilization of the apical membrane domain by ADF/cofilin proteins.

METHODS

Tissue preparation for immunofluorescence. Renal ischemia was induced in the left kidney of anesthetized male Sprague-Dawley rats

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(200–250 g) by clamping the left renal pedicle (23). The right kidney was untreated and used as the contralateral control for each experiment. Kidneys were perfused and fixed with 4% paraformaldehyde in PBS (pH 7.4) and then excised. The excised kidneys were further fixed overnight at 4°C in 4% paraformaldehyde. The fixed tissue was washed with PBS and vibratome-sectioned into 60- to 75- μ m sections. Tissue sections were blocked for 2 h at 25°C in a PBS-blocking extraction buffer (PBS containing 2% defatted BSA and 0.1% Triton X-100, pH 7.4). The blocked tissue was incubated overnight at room temperature (RT) with primary antibody at a dilution of 1:200. The polyclonal rabbit antibodies used were α f/9d [raised against the peptide encoded by the α f/9d exon of the fast α -tropomyosin gene (30)] and anti-megalin, made against the synthetic peptide derived from the cytoplasmic tail of megalin (32). The mouse monoclonal antibodies, TM311 (Sigma, St. Louis, MO) and MP10 (made against the TM4-recombinant protein product) (34), were used. The tissue was washed in blocking buffer for 6 h at RT followed by incubation overnight at RT in blocking buffer containing Texas red-, FITC-, or Cy5-labeled goat or donkey anti-rabbit or anti-mouse secondary antibodies (1:200; Jackson ImmunoResearch Laboratories, West Grove, PA) and Texas red-labeled phalloidin (1:200; Molecular Probes, Eugene, OR). Confocal images were acquired with a MRC-1024 laser-scanning confocal microscope (Bio-Rad, Hercules, CA), using a Nikon Diaphot 200 inverted microscope with a \times 60, 1.2 numerical aperture water immersion objective or \times 100, 1.4 numerical aperture oil-immersion objective. Metamorph software (Universal Imaging, West Chester, PA) was used to process the images.

Rat urine analysis. Male rats were anesthetized with Metafane followed by a peritoneal injection of Nembutal sodium solution. The abdominal cavity was surgically opened, and the urinary bladder was gently massaged to release urine for untreated samples. The renal pedicle of both kidneys was clamped for 25 min to induce ischemia. The bladder was then gently massaged, and urine was collected for 2 h and kept on ice. Control or ischemic urine was either combined with Laemmli buffer for Western blotting or centrifuged for 10 min at 14,000 rpm in an Eppendorf centrifuge at 4°C. Supernatants were removed, and the resultant pellets were fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight at 4°C. Fixed pellets were washed, permeabilized with 0.1% Triton X-100, and blocked with 2% BSA in PBS. The pellets were probed with rabbit anti-tropomyosin α f/9d, and anti-megalin antibodies, or mouse anti-tropomyosin antibodies, MP10 or TM311 (1:200), followed by FITC-labeled goat anti-rabbit or anti-mouse secondary antibody (1:200) and Texas red-labeled phalloidin (1:200). The labeled pellets were resuspended in PBS and mounted in a glycerol-PBS medium containing DABCO.

Tropomyosin isoform identification. With the use of isoform-specific antibodies, multiple isoforms of tropomyosin were identified in rat renal cortical homogenates. Kidneys from untreated rats were perfuse-flushed with PBS, removed, and decapsulated. The outer renal cortex was dissected and minced in cold PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgSO₄) containing 0.5 mM PMSF and 0.1 mM DTT. The tissue was homogenized for 5 min in PHEM buffer containing 1.0% Triton X-100, 0.5 mM PMSF, and 0.1 mM DTT. The resulting cortical homogenate was mixed with 2 \times Laemmli buffer, electrophoresed, and transferred to PVDF membranes. The membranes were probed with the following primary antibodies to identify tropomyosin isoforms in renal cortical tissue: sheep α 2a (made against the α -gene exon 2a peptide) recognizes TM6 (BV, PG, and RW, unpublished data); rabbit WD4/9d (made against the TM4 gene exon 9d peptide) recognizes TM4 (15); rabbit α -fast/9d (made against the α -gene exon 9d peptide) recognizes TM1, TM2, TM3, TM5a, TM5b, and TM6 (34); mouse TM311 (Sigma; reacts with an epitope located in the 1a exon of both the α - and β -TM genes) recognizes TM1, TM2, TM3, and TM6; MP10 (made against the TM4 recombinant protein product) recognizes primarily TM4 and TM5; and mouse LC24 recognizes TM4 (19, 36) and mouse CG3 [reacts with an epitope found in exon 1b of the TM5NM gene (18, 28)]

recognizes the multiple TM5NM products (12). Horseradish peroxidase-labeled goat anti-rabbit, goat anti-mouse, or donkey anti-sheep secondary antibodies (Bio-Rad) were used and signal was detected with Pierce SuperSignal (Pierce, Rockford, IL) chemiluminescent reagent.

Triton X-100 solubility studies. Renal pedicle clamping of Sprague-Dawley rats for 5, 15, or 30 min was used to induce renal ischemia of increasing severity, and tissue was processed as previously described by our laboratory (23). Kidneys were perfuse-flushed with PBS, removed, and decapsulated. The outer renal cortex was dissected and minced in cold PHEM buffer containing 0.5 mM PMSF and 0.1 mM DTT. The tissue was homogenized for 5 min in PHEM buffer containing 1.0% Triton X-100, 0.5 mM PMSF, and 0.1 mM DTT and then centrifuged at 47,800 g for 15 min at 4°C. The pellets were separated from the supernatants and mixed with 2 \times Laemmli buffer. Protein concentration was determined using the Pierce Coomassie microtiter plate method. Equal total protein concentrations of Triton X-100-soluble and -insoluble fractions were loaded on a 8–16% SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 10% new born calf serum in Tris-buffered saline with Tween, probed with rabbit anti-tropomyosin polyclonal antibody, α f/9d and mouse anti-tropomyosin monoclonal antibodies, TM311 (Sigma) and MP10, followed by horseradish peroxidase-labeled goat anti-rabbit or anti-mouse secondary antibodies (Bio-Rad). Signal was detected with Pierce SuperSignal chemiluminescent reagent.

RESULTS

Tropomyosin localized to the proximal tubule terminal web. Localization of tropomyosin in untreated rat PTC was pursued in perfused-fixed rat cortical kidney sections. Three tropomyosin-specific antibodies were used, α f/9d, TM311, and MP10. With the use of immunocytochemistry followed by fluorescent microscopy, tropomyosin fluorescent signal was observed in the region of the PTC terminal web beneath the apical microvillar F-actin brush border with all three antibodies (Fig. 1, C, E, G, arrows), whereas only a minor diffuse signal was found in the cytoplasm. In addition to the tropomyosin signal found in PTC, strong tropomyosin staining was observed in the interstitium as well as microvascular tissue located between PTC in cortical sections. Megalin, a proximal tubule membrane marker, localized to the apical membrane of PTC (Fig. 2, C and G, green). Twenty-five minutes of ischemia altered the cellular architecture of F-actin (Fig. 1B) and tropomyosin (Fig. 1, D, F, H). The F-actin present in the apical brush border under physiological conditions (Fig. 1A) decreased and became disorganized (Fig. 1B), and the megalin-stained apical membrane was discontinuous, often extending into the luminal space surrounding blebs (Fig. 2, D and H, green) that contained tropomyosin (Fig. 2, F and H, blue). The polyclonal antibody, α f/9d, and the monoclonal antibodies, TM311 and MP10, no longer detected tropomyosin primarily in the proximal tubule terminal web of ischemic PTC. The tropomyosin signal was diffusely distributed in the apical membrane region of the degenerating microvilli by all three tropomyosin antibodies (Fig. 1, D, F, H, arrows). After ischemic injury, tropomyosin staining was still observed in the interstitium and vascular tissue (Fig. 1, D, F, H).

Tropomyosin-containing vesicles were found in urine of ischemic rats. Under physiological conditions rat urine appeared clear and contained few, if any, microscopically observable elements (data not shown). Urine collected from rats whose renal artery and vein were clamped on both kidneys for

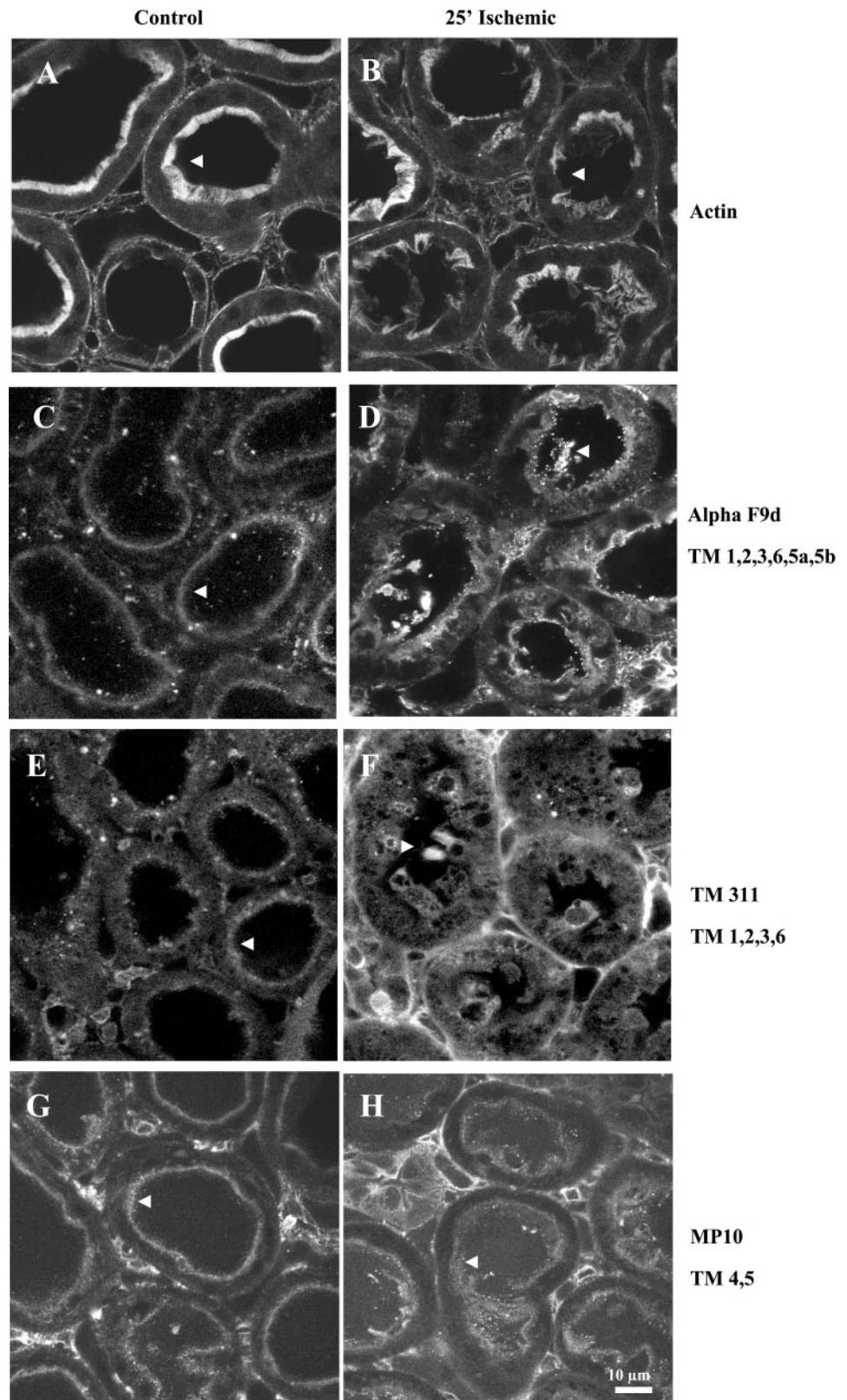


Fig. 1. Tropomyosin localization changes in response to ischemic injury in rat proximal tubule cells. Rat cortical sections collected from specimens under physiological conditions (A, C, E, and G) or following 25 min of clamp-induced ischemia (B, D, F, and H) were probed with Texas red-labeled phalloidin (A, B) to detect the F-actin cytoskeleton and with Cy5-labeled tropomyosin antibodies α f/9d (C, D), TM311 (E, F), and MP10 (G, H) to detect tropomyosins. Under physiological conditions, Texas red-phalloidin stained the apical microvillar F-actin core (A, arrow) and the basal region of the proximal tubule cells, in addition to staining of vascular tissue in the interstitium (A). The tropomyosin-specific antibodies labeled the terminal web region (C, E, G, arrows) of the proximal tubule cells just beneath the apical microvilli with minor staining of the cytoplasm (C, E, G). Strong staining of the vascular tissue in the interstitium was also observed with the tropomyosin antibodies. With induction of 25 min of ischemia, tropomyosin staining was localized in the degenerating apical region (arrows) of the proximal tubule cells and in blebs (D, F, H). The vasculature and interstitium were still stained for tropomyosin in ischemic tissue. Bar = 10 μ m.

25 min was cloudy and contained urinary vesicles of varying shapes and sizes in addition to the presence of white and red blood cells, a few amorphous crystals, epithelial cells, and bacteria. Urinary sediments from ischemic rats contained many

vesicles stained for tropomyosin, in addition to Texas red-phalloidin-stained F-actin aggregates and bundles and FITC-stained, megalin-labeled membranes (Fig. 3, A and B). Colocalization of signals from tropomyosin, megalin, and F-actin

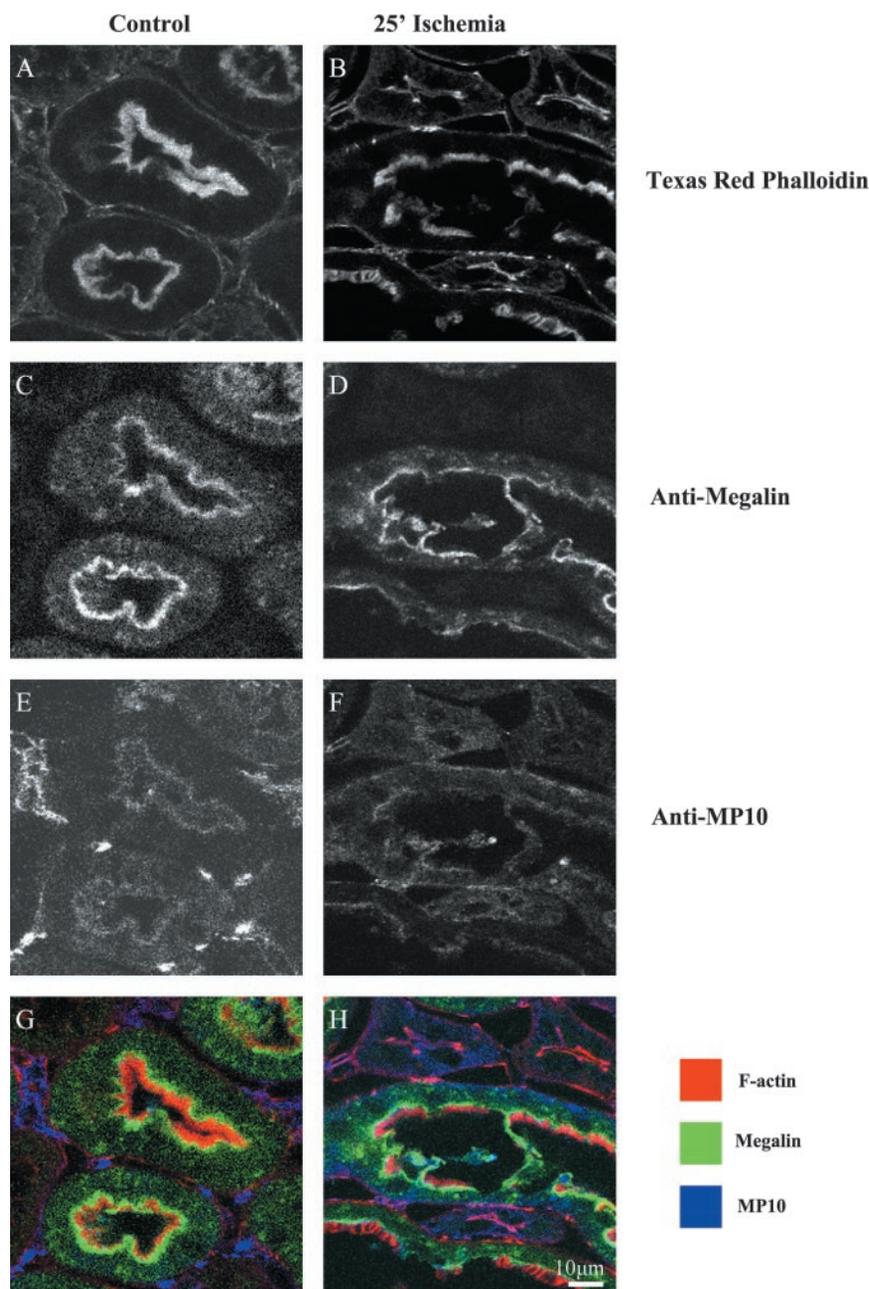


Fig. 2. Tropomyosin and F-actin locate to proximal tubule apical blebs with ischemia. Under physiological conditions, megalin stained apical membranes lining the proximal tubule lumen (C, G, green). But, in response to 25 min of renal clamp-induced ischemia, the megalin-stained apical membrane degenerates and participates in bleb formations that protrude into the tubular lumen (D, H, green). These blebs contain both F-actin (B, H, red) and tropomyosin (F, H, blue). Bar = 10 μ m.

was observed in many, but not all, vesicles. Western blot analysis (Fig. 3C) of urine from rats following ischemic injury demonstrated the presence of tropomyosin, as detected with the mouse monoclonal TM311 antibody. Urine from untreated rats had no detectable tropomyosin.

Multiple tropomyosin isoforms expressed in cortical homogenates. Analysis of rat kidney cortical homogenates by SDS-PAGE followed by Western blot analysis using tropomyosin isoform-specific antibodies demonstrated expression of multiple tropomyosin isoforms (Fig. 4). Using α 2a (TM6), WD4/9d (TM4), α f/9d (TM6, TM1, TM5a, and/or TM5b), TM311 (TM6, TM1), MP10 (TM4, TM5NM), LC24 (TM4), and CG3 (TM5) antibodies, we demonstrated expression of high molecular mass tropomyosins TM6, TM1, and low molecular mass tropomyosins TM4, TM5NM, and TM5a and/or TM5b in renal cortical homogenates (Fig. 4, see accompanying key).

Tropomyosin dissociation from F-actin increases with longer intervals of renal ischemia. To further confirm tropomyosin released from F-actin in response to renal ischemia, outer renal cortical tissue sections from untreated and ischemic-treated rat kidneys were processed for detection of F-actin-associated and -soluble tropomyosin as previously described (23). Proteins from pellets and supernatants for each timed treatment were separated by electrophoresis and transferred to blots for probing with the tropomyosin-specific antibodies, α f/9d, TM311, and MP10. Under physiological conditions, and even up to 5 min of induced ischemia, minimal quantities of the high molecular mass tropomyosin isoforms were detected in the soluble fraction with tropomyosin principally localizing to the pellet or insoluble fraction (Fig. 5, Aa, b). But, 15 min of clamp-induced ischemia resulted in increased concentrations of the high-molecular-mass tropomyosin isoforms in the soluble

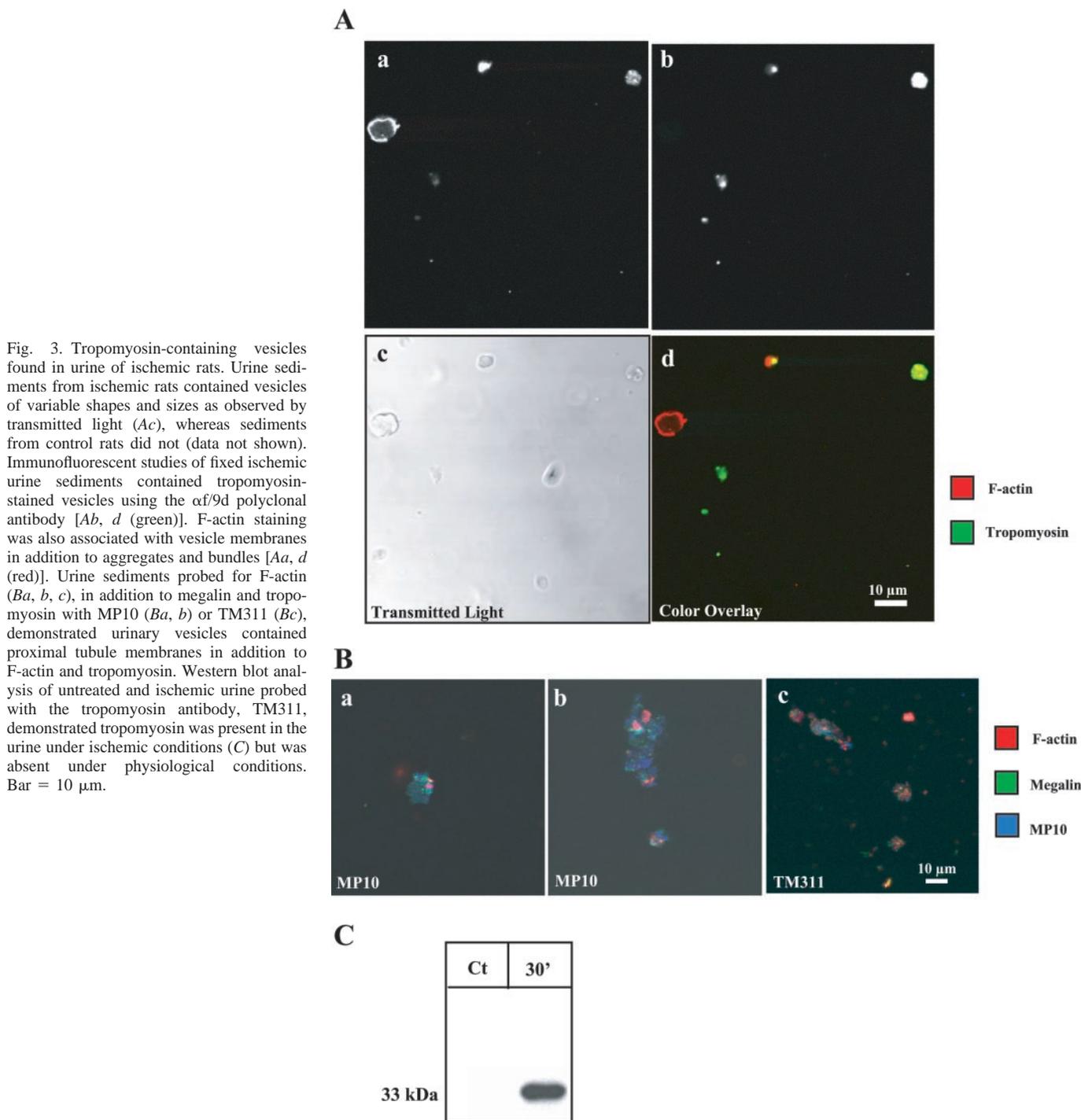


Fig. 3. Tropomyosin-containing vesicles found in urine of ischemic rats. Urine sediments from ischemic rats contained vesicles of variable shapes and sizes as observed by transmitted light (*Ac*), whereas sediments from control rats did not (data not shown). Immunofluorescent studies of fixed ischemic urine sediments contained tropomyosin-stained vesicles using the α f/9d polyclonal antibody [*Ab, d* (green)]. F-actin staining was also associated with vesicle membranes in addition to aggregates and bundles [*Aa, d* (red)]. Urine sediments probed for F-actin (*Ba, b, c*), in addition to megalin and tropomyosin with MP10 (*Ba, b*) or TM311 (*Bc*), demonstrated urinary vesicles contained proximal tubule membranes in addition to F-actin and tropomyosin. Western blot analysis of untreated and ischemic urine probed with the tropomyosin antibody, TM311, demonstrated tropomyosin was present in the urine under ischemic conditions (*C*) but was absent under physiological conditions. Bar = 10 μ m.

fraction. The low-molecular-mass tropomyosins detected with MP10 antibody demonstrated an increase over control levels in either soluble TM4 or 5 or both at 15 min of ischemia. By 25 min tropomyosin in both the soluble and insoluble fractions decreased, suggesting loss of these proteins from the degenerating apical membrane into the urine.

Zipper protein, a tropomyosin-like protein, dissociates from F-actin in response to renal ischemia. Zipper protein, a 49.3-kDa transmembrane protein with homology to tropomyosin and B-G proteins, has been localized to the intestinal brush border in close proximity to brush border myosin I (8, 9).

Zipper's 28-kDa cytoplasmic domain resembles tropomyosin and competes with tropomyosin for F-actin binding (8). We probed rat physiological and ischemic urine, in addition to Triton X-100-soluble and -insoluble rat cortical tissue fractions, with the antibody to zipper, 1092, a kind gift of D. Bikle (Fig. 5B). Under physiological conditions, little to none of the cytoplasmic domain of zipper protein was detected in the urine, but the protein was detected in both the Triton X-100-soluble and -insoluble rat kidney cortical fractions. In response to ischemic injury, the cytoplasmic domain of zipper protein was substantially increased in the ischemic urine and was absent

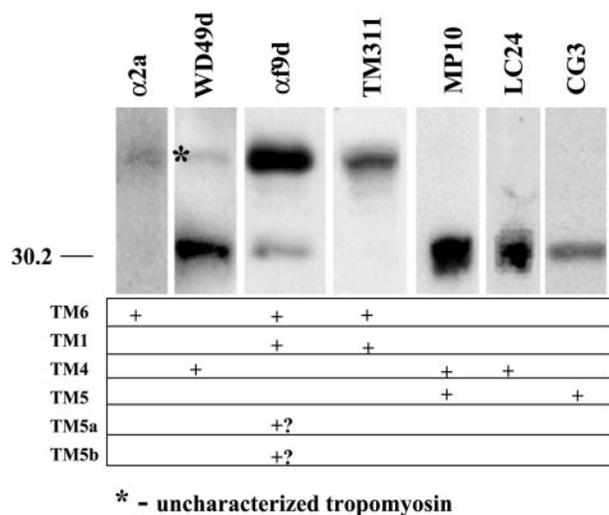


Fig. 4. Kidney cortical homogenates contain multiple tropomyosin isoforms. With the use of isoform-specific antibody probes, tropomyosin isoforms in kidney cortical homogenates were identified. High molecular mass tropomyosin isoforms, TM6 and TM1, were identified with $\alpha 2a$, $\alpha f/9d$, and TM311. Expression of low molecular mass tropomyosins, TM4, TM5NM, TM5a, and/or TM5b, was detected with WD4/9d, $\alpha f/9d$, MP10, LC24, and CG3 antibodies. The key illustrates which tropomyosin isoforms were detected with each antibody as described in METHODS.

from the Triton X-100-insoluble fraction. These data support ischemia-induced dissociation of zipper protein from F-actin (Fig. 5B).

DISCUSSION

Actin cytoskeletal integrity plays a critical role in the normal absorptive and secretory functions of kidney PTC, providing the structural basis for cellular polarization (33). Under physiological conditions, the PTC has a well-defined actin cortical network at the surface membrane. This complex network interacts with the actin-rich apical microvilli, terminal web, junctional complexes, cell-substratum attachment, and indirect anchoring of basolateral membrane proteins. Each of these interactions plays a critical role in maintaining cellular equilibrium. When the cellular equilibrium is challenged through an ischemic event, these actin cytoskeletal interactions are altered, resulting in disruption of cellular morphology, altered biochemical activities, and changes in the cell's physiology leading to compromised PTC function and viability (33). These actin cytoskeletal alterations appear rapidly and result in loss of apical membrane surface to luminal membrane blebs, loss of junctional complexes, and loss of cellular polarity due to membrane protein and lipid interfusion of apical and basolateral membrane domains. Therefore, understanding the structural and dynamic nature of the actin cytoskeleton, and its associated proteins, under physiological and ischemic conditions may help elucidate what initial mechanisms lead to cellular injury.

To better understand the actin cytoskeleton's role in response to ischemia, we focused our attention on the proximal tubule F-actin-rich apical region because it is easily visualized and ischemia has a quick and dramatic impact on its integrity. Although microvilli have been well studied in intestinal epithelial cells, less is known about their structural proteins and their function in kidney PTC. It is documented that the mem-

brane surrounding these apical fingerlike projections has distinct ion channels, transport proteins, and specific enzymes and is supported structurally by bundled F-actin (2). Actin bundling proteins, villin and fimbrin, link microfilaments to form a F-actin core that is tethered to the apical membrane by ezrin and myosin I/calmodulin complexes. The polarized filament assembly extends the length of the microvilli with the barbed (or plus end) filament end at the microvillar tip and the pointed (or minus end) filament end in the region of the terminal web. In addition to tropomyosin, the actin-binding proteins fodrin,

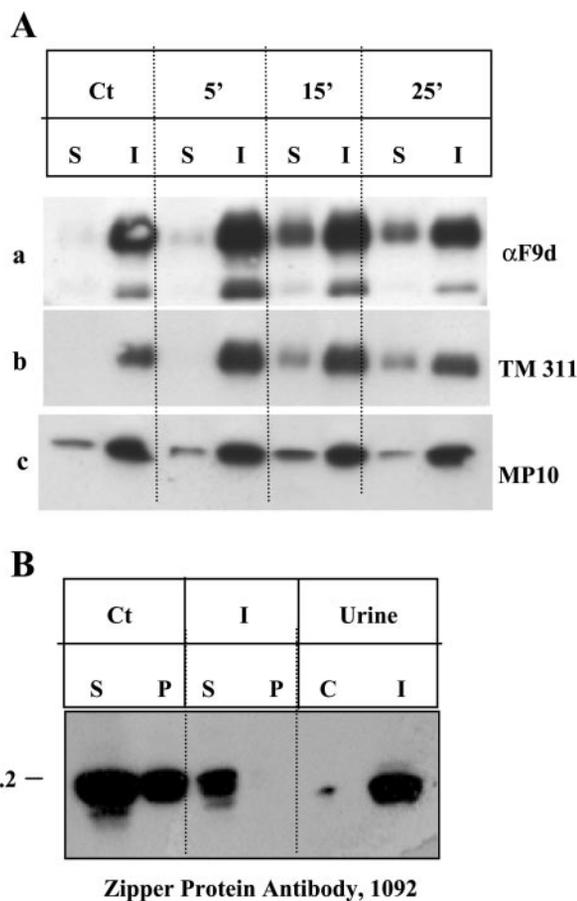


Fig. 5. Soluble tropomyosin and zipper protein detected in ischemic cortical extracts. *A*: clamp-induced renal ischemia of Sprague-Dawley rats resulted in increased concentrations of tropomyosin in the Triton X-100-soluble fractions of ischemic renal cortical tissue as identified in Western blots of the soluble and insoluble fractions probed with $\alpha f/9d$ (*Aa*), TM311 (*Ab*), and MP10 (*Ac*) antibodies. Under physiological conditions, and even up to 5 min of induced ischemia, minimal concentrations of high-molecular-mass tropomyosins were detected in the soluble fractions with the majority of detectable tropomyosin found in the insoluble fraction. The low-molecular-mass tropomyosins detected by MP10 were more soluble under physiological conditions. With as little as 15 min of clamp-induced ischemia, increased concentrations of tropomyosin were detected in the soluble fractions by all 3 antibodies. By 25 min of clamp-induced ischemia, the concentration of tropomyosin in the soluble and insoluble fractions was reduced. *B*: zipper protein, a tropomyosin-like protein, dissociated from F-actin in response to ischemia. Western blot analysis (*B*) of control and ischemic urine and Triton X-100-soluble and -insoluble fractions demonstrated the zipper protein cytoplasmic domain was present in both the soluble and insoluble Triton X-100 cortical tissue fractions and little to no protein was present in the urine under physiological conditions. Twenty-five minutes of ischemic insult resulted in loss of zipper protein from the Triton X-100-insoluble fraction, with a substantial increase in zipper protein observed in the urine.

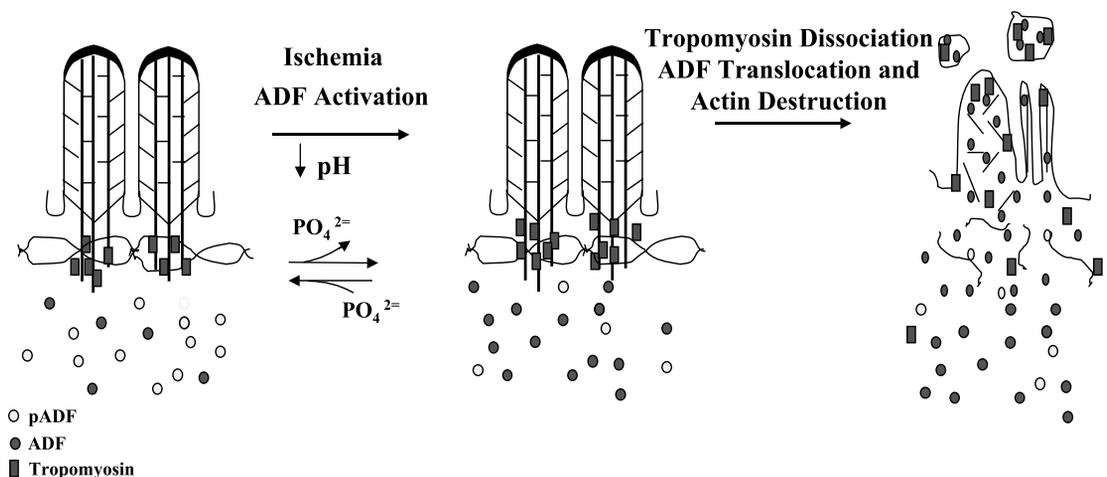


Fig. 6. Proposed model for ischemia-induced microvilli microfilament breakdown. Under physiological conditions, we propose tropomyosin binds the pointed end of the microvilli microfilaments located in the terminal web of the proximal tubule cells. We believe with the onset of renal ischemia, actin-depolymerizing factor (ADF)/cofilin becomes dephosphorylated/activated and enters the apical region of the proximal tubule in the same time frame that tropomyosin dissociates from the terminal web. Therefore, we hypothesize active ADF/cofilin is positioned to compete with tropomyosin for binding to the ADP-rich actin subunits in the region of the terminal web. ADF/cofilin has been shown to have higher affinity for ADP-actin subunits especially at the decreased pH levels associated with the onset of renal ischemia. Therefore, as the concentration of active ADF/cofilin increases in the terminal web with ischemia, ADF/cofilin would be expected to compete with tropomyosin for F-actin binding at the pointed end of the microfilament cores. Such ADF/cofilin binding would result in F-actin severing and depolymerization that could disrupt the microvilli microfilaments, leading to microvilli membrane coalescing and formation of membrane vesicles shed into the lumen or endocytosed into the cytoplasm.

α -actinin, myosin II, and tropomodulin also localize to the intestinal epithelial terminal web. Tropomyosins comprise a family of actin filament binding proteins with over 40 known isoforms, generated from alternative promoters and alternative splicing of RNA transcripts, with molecular mass ranging from 32 to 40 kDa (19, 29). Tropomyosin, a rod-shaped dimeric α -helical coiled-coiled structure (homo- and heterodimers), binds head to tail cooperatively along the groove of the actin filament, binding 6 to 7 actin monomers depending on the tropomyosin isoform bound.

To gain an understanding of actin dynamics in physiological and ischemic PTC, our previous studies focused on the localization and interactions of the actin-associated protein, ADF/cofilin, an actin-severing and -depolymerizing protein (3, 5, 31). We established in PTC that ADF had a diffuse cytoplasmic distribution with no detectable ADF signal in the actin-rich apical brush border. Interestingly, with as little as 5 min of ischemia, ADF signal was observed in the apical region of the microvilli and penetrated to the apical tip with longer insults coinciding with microvilli destruction. Because *in vitro* studies documented that ADF competes in a concentration-dependent manner for F-actin binding with tropomyosin (6, 26, 27), and because tropomyosin has been localized to the terminal web at the base of microvilli in intestinal epithelial cells (10), we questioned whether tropomyosin bound F-actin in the rat proximal tubule terminal web, and if ischemia-induced tropomyosin dissociation from F-actin or more specifically, if penetration of increasing concentrations of ADF into the apical regions competed with tropomyosin for F-actin binding resulting in release of tropomyosin from the terminal web.

In the present study, we used immunofluorescence microscopy to demonstrate tropomyosin isoforms localized to the proximal tubule terminal web basal to the microvillar

microfilament core (Figs. 1 and 2). Tropomyosin pelleted with the insoluble F-actin fraction in Triton X-100 extraction studies (Fig. 5), suggesting tropomyosin binds F-actin in the terminal web. With the onset of 25 min of renal ischemia, destructive changes occur in PTC with breakdown of the actin cytoskeleton being one of the earliest detected events (Figs. 1 and 2). Ischemic injury also induced tropomyosin dissociation from the terminal web of the microfilament cores, resulting in the tropomyosin signal found diffusely distributed in the region of the degenerating apical membrane region (Figs. 1 and 2) and to membrane-bound vesicles shed in the urine (Fig. 3). Triton X-100 solubility studies support tropomyosin dissociation from F-actin in response to ischemia (Fig. 5). Tropomyosin solubility increased in Triton X-100 cortical extracts with induction of ischemia. This change was small compared with the dramatic alterations in actin and tropomyosin observed by immunofluorescence studies. A potential explanation for this disparity could be that while a change occurs in the distribution of actin and tropomyosin in PTC, a similar change may not occur in the surrounding vascular tissue. Tropomyosin dissociation from the microfilament cores in the terminal web region is important to note because its absence in this region provides access to the ADP-actin-rich pointed ends of the microvillar microfilaments for ADF binding, with subsequent ADF-mediated F-actin severing and depolymerization.

Our studies do not address the reason(s) tropomyosin dissociates from microfilaments in response to ischemia. Tropomyosin dissociation from F-actin may result from changes in local cellular environment of the terminal web altering tropomyosin's affinity for F-actin. One potential consequence of tropomyosin dissociation from the microfilament core in the terminal web region of the PTC is that the ADP-rich actin pointed

ends become more accessible. ADF has a higher affinity for ADP-actin than ATP-actin (20). We do know an initial decrease in cellular pH occurs with ischemia onset (14). In vitro biochemical binding studies indicate the affinity of ADF for F-actin increases with decreased pH (5), and in vivo studies demonstrated intracellular pH changes also affect ADF/cofilin's affinity for F-actin (7). To date, there is no evidence Ca^{2+} fluctuations directly regulate the interaction of ADF or tropomyosin with actin. Once ADF is bound to the microvillar microfilaments, ADF can sever and depolymerize the microfilaments.

We also questioned the role of zipper protein in ischemia. Very little is known about the functional characteristics of zipper protein, but it has structural homology to tropomyosin, competes with tropomyosin for binding to F-actin, locates to the apical microvilli in intestinal epithelial cells, and affects myosin ATPase activity (8, 9). Although the antibody to zipper protein worked well with Western blot analysis, we were unable to localize zipper protein by immunocytochemistry in rat kidney cortical sections. Analyses of physiological and ischemic tissue extracts and urine suggested ischemia-induced dissociation of zipper protein from the microvilli actin filaments and excretion in the urine (Fig. 5B). Although no biochemical evidence exists, it is possible zipper protein may compete with ADF for binding to F-actin under physiological conditions, protecting the microvilli microfilaments from ADF binding, severing, and depolymerizing.

In summary, tropomyosin localizes to the terminal web of physiological PTC. It is attractive to speculate that tropomyosin may stabilize and protect the pointed ends of the tethered microvillar microfilaments from destruction by the actin-severing and -depolymerizing protein ADF. With the onset of renal ischemia, tropomyosin dissociation from the terminal web microfilaments may allow for ADF binding, which would result in F-actin severing, depolymerization, and breakdown of microvillar microfilaments. Alternatively, the increased concentration of active ADF in the microvillar region may effectively compete with tropomyosin for binding to F-actin. Both of these alternatives are consistent with our in vivo observations with the induction of renal ischemia and with previous published in vitro studies. These new data suggest that tropomyosin may play a critical role in maintaining the integrity of the bundled actin filaments present in the terminal web of the apical microvilli of rat PTC and that ischemia rapidly alters this interaction.

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