Rho-kinase regulates myosin II activation in MDCK cells during recovery after ATP depletion

TIMOTHY A. SUTTON, HENRY E. MANG, AND SIMON J. ATKINSON
Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202
Received 31 January 2001; accepted in final form 22 June 2001

Sutton, Timothy A., Henry E. Mang, and Simon J. Atkinson. Rho-kinase regulates myosin II activation in MDCK cells during recovery after ATP depletion. Am J Physiol Renal Physiol 281: F810–F818, 2001.—Alterations in the actin cytoskeleton of renal tubular epithelial cells during periods of ischemic injury and recovery have important consequences for normal cell and kidney function. Myosin II has been demonstrated to be an important effector in organizing basal actin structures in some cell types. ATP depletion in vitro has been demonstrated to recapitulate alterations of the actin cytoskeleton in renal tubular epithelial cells observed during renal ischemia in vivo. We utilized this reversible cell culture model of ischemia to examine the correlation of the activation state and cellular distribution of myosin II with disruption of actin stress fibers in Madin-Darby canine kidney (MDCK) cells during ATP depletion and recovery from ATP depletion. We found that myosin II inactivation occurs rapidly and precedes dissociation of myosin II from actin stress fibers during ATP depletion. Myosin II activation temporally correlates with colocalization of myosin II to reorganizing stress fibers during recovery from ATP depletion. Furthermore, myosin activation and actin stress fiber formation were found to be Rho-associated Ser/Thr protein kinase dependent during recovery from ATP depletion.

myosin light chain kinase; Rho-associated serine/threonine protein kinase; adenosine 5'-triphosphate depletion; stress fibers; ischemic acute renal failure; Madin-Darby canine kidney cells

DISRUPTION OF THE ACTIN CYTOSKELETON that accompanies renal ischemia and cellular ATP depletion has important consequences for renal tubular epithelial cell structure and function. Cellular alterations that correlate with disruption of the actin cytoskeleton include loss of cell polarity, loss of apical microvilli, loss of tight junction function, and loss of cell-cell and cell-matrix adhesion (1, 3, 20, 21, 26, 27, 29, 39). Disruption of the actin cytoskeleton occurs rapidly after ischemic injury and is dependent on the severity and duration of the insult (18). Importantly, these alterations are reversible during the recovery phase. Although these alterations may fall short of being lethal to the cell, they can disrupt the ability of the renal epithelia to maintain normal renal function. Proximal tubular Na+ wasting due to loss of polarity and loss of apical microvilli, tubular obstruction due to detached epithelial cells and shed apical microvilli, and backleak of glomerular filtrate due to loss of tubular epithelial monolayer integrity or loss of tight junction function have all been implicated in the decrement of glomerular filtration rate (GFR) that is the hallmark of acute renal failure (11, 35, 37, 42).

The basal actin network plays an integral part in cell-matrix adhesion, and actin stress fibers are an important component of the basal actin network in renal tubular epithelial cells (28). Disruption of the basal actin network, including stress fibers, during ischemia and cellular ATP depletion alters cell-matrix interaction and leads to renal tubular epithelial cell detachment (20). The regulatory mechanisms involved in alterations of the actin cytoskeleton after ischemia/reperfusion injury and the concomitant depletion and repletion of ATP have not been fully elucidated.

Normal structural and functional properties of distinct aspects of the actin cytoskeleton depend extensively on the spatial and temporal association with various actin-binding proteins. The myosin superfamily of proteins is a somewhat diverse group of actin-binding motor proteins with at least 15 distinct classes that demonstrate a variety of intracellular functions. The myosin II class is the prototypical two-headed myosin best characterized in studies of muscle but found in all vertebrate cells (34). Smooth muscle myosin II and vertebrate nonmuscle myosin II form a subclass of myosin II that has similar structural, enzymatic, and regulatory properties (5). Phosphorylation of the 20,000-Da myosin regulatory light chain (MLC) at serine-19 by myosin light chain kinase (MLCK) plays an important role in regulating the actin-activated Mg2+-ATPase activity in this subclass of myosin II (30). A number of studies have demonstrated a dependence on MLC phosphorylation by MLCK for myosin activation (36). Myosin II activity is downregulated by a heterotrimeric myosin phosphatase composed in part of a type I protein phosphatase and a myosin-binding subunit (MBS) (14).

A variety of studies have demonstrated myosin II to play a vital role in cellular processes that may have important implications for altered epithelial function...
during ischemic injury of the kidney. Myosin II-based contractility plays an important role in actin stress fiber formation (7). Loss of myosin II contractility disrupts actin stress formation that could lead to renal tubular epithelial cell detachment and subsequent tubular obstruction by sloughed epithelial cells, as well as backleak of glomerular filtrate through a denuded epithelial monolayer. The state of myosin II activation regulates tight junction and paracellular permeability in epithelial cells (16, 40). Loss of normal tight junction function could promote backleak of glomerular filtrate. Myosin II may also play an important role in closing defects in the epithelial monolayer either through epithelial cell migration or through a purse string mechanism (4, 25). Loss of these two reparative mechanisms could also promote backleak of glomerular filtrate.

Interestingly, there is evidence for a signaling pathway linking the Rho family GTPases, which are known to play an important role in regulating distinct aspects of actin rearrangement involved in normal cellular function (13), to myosin II activation and actin stress fiber formation (7). In this proposed pathway, Rho-GTP binds to and activates the Ser/Thr kinase Rho-kinase (ROCK) (23). ROCK can lead to activation of myosin II by two potential mechanisms that may act separately or together. The first mechanism is based on the evidence that ROCK can phosphorylate the MBS of myosin phosphatase (19). Phosphorylation of the MBS of myosin phosphatase by ROCK inhibits myosin phosphatase activity and indirectly activates myosin II (17, 19). ROCK can also directly activate myosin II via phosphorylation of MLC at the identical site phosphorylated by MLCK (2). Recent evidence suggests that in 3T3 fibroblasts, ROCK and MLCK may play distinct roles in the spatial regulation of MLC phosphorylation and subsequent myosin II activation (38). However, the relative contribution of MLCK and ROCK to MLC phosphorylation and subsequent myosin II activation in epithelial cells is unknown.

Recently, RhoA inactivation has been implicated as a mechanism for disruption of the actin cytoskeleton during ATP depletion in renal tubular epithelial cells (31). In this same study, RhoA activation was implicated as a requisite condition for recovery of normal cell architecture after anoxic injury (31). It is our hypothesis that myosin II is an important distal effector in the Rho-regulated alterations of the actin cytoskeleton that occur during ischemia and recovery from ischemia in renal tubular epithelial cells. Therefore, the goal of this study is to examine the activation and spatial distribution of myosin II as well as the relative contribution of MLCK and ROCK in the regulation of myosin II during ATP depletion and recovery in Madin-Darby canine kidney (MDCK) cells. We show that in MDCK cells 1) myosin II inactivation precedes disruption of actin stress fibers during ATP depletion, 2) myosin II dissociates from actin stress fibers during ATP depletion, 3) reactivation of myosin II spatiotemporally correlates with reorganization of actin stress fibers during recovery from ATP depletion, and 4) myosin II reactivation and actin stress fiber reorganization during recovery from ATP depletion are prevented by inhibitors of ROCK but not by inhibitors of MLCK.

METHODS

Reagents and antibodies. The MLCK inhibitors ML-9 and ML-7 were from Sigma (St. Louis, MO). The Rho-kinase inhibitor Y-27632 was from Upstate Biotechnology (Lake Placid, NY). Other reagents were purchased from Sigma or Midwest Scientific (St. Louis, MO) unless otherwise stated. Monoclonal IgM antibodies to MLC (clone MY-21) were from Sigma, and polyclonal antibodies to nonmuscle myosin IIA were from Covance (Richardson, CA). Affinity-purified fluorescein-labeled goat anti-rabbit antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA), affinity-purified horseradish peroxidase (HRP)-conjugated goat antimouse IgM antibodies were from Zymed Laboratories (South San Francisco, CA), and HRP-conjugated donkey anti-rabbit IgG antibodies were from Amersham Pharmacia Biotech (Piscataway, NJ).

Cell culture. MDCK type II cells were selected for this study on the basis of the potential for development of specific molecular tools in our lab to further investigate the role of myosin II regulation in recovery from ATP depletion. MDCK type II cells were maintained in DMEM (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum and penicillin-streptomycin. Cells were grown in humidified atmosphere (95% air-5% CO2) at 37°C. Cells were fed 24 h before experiments and were 1 day past confluence at the time of the experiment. Chemical anoxia was induced by incubation of the cells in substrate-free media (amino acids and glucose omitted) containing 0.1 μM antimycin A as previously described (6). Three washes with warm PBS were performed before experimental conditions were started.

ATP and GTP measurements. ATP and GTP measurements were performed as previously described by Dagher (8). Briefly, MDCK monolayers grown in 100-mm plastic culture dishes were extracted at the end of an experimental protocol by scraping the cells into 300 μl of ice-cold acetonitrile followed by 700 μl of cold water. The soluble and precipitated fractions were centrifuged at 16,000 × g for 10 min at −20°C. The supernatant fraction, kept on ice, was then gassed with N2 for 30 min to evaporate acetonitrile. The pellet was solubilized with 1 N NaOH, and the protein content was analyzed with Coomassie blue assay (Pierce Chemical, Rockford, IL) using BSA as a standard. Nucleotides were separated by high-performance liquid chromatography (HPLC) by utilizing a 4.6×250 mm Nova-Pack C18 cartridge equipped with a radial compression chamber (Waters, Millford, MA). The buffer consisted of 20% acetonitrile, 10 mM ammonium phosphate, and 2 mM PIC-A ion pairing reagent (Waters) and was run isocratically at 2 ml/min (12). Samples were diluted by one-half, and the injection volume was 100 μl. An HP Chemstation model no. 1100 was used (Hewlett-Packard, Wilmington, DE), and the ultraviolet detector was set at 254 nm. HPLC-grade nucleotide standards were used to calibrate the signals.

Cell extraction and myosin II measurements. Triton X-100 extractions were performed by a method similar to that described by Molitoris et al. (27). Briefly, MDCK monolayers grown in 100-mm plastic culture dishes under control, ATP-depleted, and ATP-repleted conditions were quickly placed on ice and washed with ice-cold PBS followed by the addition of 350 μl of PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgSO4; pH 6.9) containing 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml antipain, 10 μg/ml pepstatin A, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 10 mM 1,4-dithiothreitol (DTT).
The samples were rocked on ice for 10 min and then gently scraped from the culture dish. The samples were then centrifuged for 15 min at 15,000 rpm and at 4°C. The supernatants were carefully removed, and the pellet was resuspended in a volume of extraction buffer equal to the starting volume. Aliquots of equal total volume from each sample were processed by SDS-PAGE by use of 5% gels and the Laemmli buffer system (22). The proteins were transferred to nitrocellulose. After transfer to nitrocellulose, Western immunoblotting was performed utilizing polyclonal anti-nonmuscle myosin IIA antibodies and then HRP-conjugated donkey anti-rabbit IgG antibodies. Immunoreactive bands were identified by a chemiluminescence method utilizing Super Signal-West Dura (Pierce Chemical). The blot was scanned on a Bio-Rad Fluor-S Multimager (Hercules, CA). Protein content was analyzed with Coomassie blue assay (Pierce Chemical) with the use of BSA as a standard.

**Measurement of LC20 phosphorylation.** MDCK monolayers grown on collagen-coated coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Coverslips were blocked in PBS containing 10% goat serum and 0.2% BSA for 30 min (block solution). Coverslips were incubated with a 1:100 dilution of polyclonal anti-nonmuscle myosin IIA antibodies in block solution for 1 h at room temperature, followed by a brief wash and additional incubation with a 1:200 dilution of monoclonal IgM anti-MLC antibodies in block solution for 1 h at room temperature followed by a brief wash. The proteins were transferred to nitrocellulose. After the proteins were transferred, the nitrocellulose was incubated in 0.1% SDS in a buffer containing 25 mM Tris (pH 8.6) and 20 mM glycine at 30°C for 15 min, followed by Western immunoblotting with monoclonal IgM anti-MLC antibodies and then HRP-conjugated goat anti-mouse IgM antibodies. Immunoreactive bands were identified by a chemiluminescence method utilizing Super Signal-West Dura (Pierce Chemical). The blot was scanned on a Bio-Rad Fluor-S Multimager (Hercules, CA). Protein content was analyzed with Coomassie blue assay (Pierce Chemical) with the use of BSA as a standard.

**Fluorescence microscopy.** MDCK monolayers grown on collagen-coated coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Coverslips were blocked in PBS containing 10% goat serum and 0.2% BSA for 30 min (block solution). Coverslips were incubated with a 1:100 dilution of polyclonal anti-nonmuscle myosin IIA antibodies in block solution for 1 h at room temperature, followed by a brief wash and additional incubation with a 1:200 dilution of monoclonal IgM anti-MLC antibodies in block solution for 1 h at room temperature followed by a brief wash. The proteins were transferred to nitrocellulose. After the proteins were transferred, the nitrocellulose was incubated in 0.1% SDS in a buffer containing 25 mM Tris (pH 8.6) and 20 mM glycine at 30°C for 15 min, followed by Western immunoblotting with monoclonal IgM anti-MLC antibodies and then HRP-conjugated goat anti-mouse IgM antibodies. Immunoreactive bands were identified by a chemiluminescence method utilizing Super Signal-West Dura (Pierce Chemical). The blot was scanned on a Bio-Rad Fluor-S Multimager (Hercules, CA). Protein content was analyzed with Coomassie blue assay (Pierce Chemical) with the use of BSA as a standard.

**Stoichiometry of MLC phosphorylation**

\[
\text{Stoichiometry of MLC phosphorylation} = \frac{\text{MLC-P} + 2(\text{MLC-PP})}{\text{MLC} + \text{MLC-P} + \text{MLC-PP}}
\]

where MLC is the unphosphorylated MLC band, MLC-P is the monophosphorylated MLC band, and MLC-PP is the diphosphorylated MLC band.

**Fluorescence microscopy.** MDCK monolayers grown on collagen-coated coverslips were fixed with 3.7% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.05% Triton X-100 in PBS for 5 min. Coverslips were blocked in PBS containing 10% goat serum and 0.2% BSA for 30 min (block solution). Coverslips were incubated with a 1:100 dilution of polyclonal anti-nonmuscle myosin IIA antibodies in block solution for 1 h at room temperature, followed by a brief wash and additional incubation with a 1:200 dilution of monoclonal IgM anti-MLC antibodies in block solution for 1 h at room temperature followed by a brief wash.
dilution of fluorescein-labeled goat anti-rabbit IgG antibodies in block solution for 1 h at room temperature. F-actin was labeled by including 0.1 μg/ml rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR) in the incubation with the secondary antibody. Immunostained coverslips were mounted in 50% glycerol-PBS containing 100 mg/ml diaminobicyclo-[2.2.2] octane to minimize photobleaching. Images were collected with an LSM-510 Zeiss confocal microscope (Heidelberg, Germany) equipped with argon and helium/neon lasers. Z-series stacks of images were collected that included the entire cell volume, with a separation of 0.5 μm between each confocal section. Images were analyzed by use of Metamorph software (Universal Imaging, West Chester, PA).

**Statistics.** Results are expressed as means ± SD and were analyzed for significance by paired and unpaired Student’s t-test and ANOVA.

**RESULTS**

*Myosin II inactivation during ATP depletion.* Chemical anoxia of MDCK cells by metabolic inhibition with

![Fig. 3](http://ajprenal.physiology.org/) Effect of ATP depletion on stress fibers and myosin II distribution in MDCK cells. MDCK cells were incubated in depleted media containing 0.1 μM antimycin A for 0 (A and B), 30 (C and D), and 60 min (E and F). Cells were fixed, permeabilized, and labeled for F-actin with rhodamine-phalloidin (A, C, and E) and myosin II with rabbit polyclonal antibodies to nonmuscle myosin IIA and fluorescein-labeled goat anti-rabbit antibodies (B, D, and F). Myosin II colocalizes to stress fibers under control conditions. Myosin II staining begins to become more diffuse by 30 min of ATP depletion. By 60 min of ATP depletion, stress fibers are disrupted and myosin II staining is diffuse throughout the cytoplasm. Arrows indicate larger actin aggregates. Bar = 10 μm.
substrate depletion and antimycin A resulted in a fall of ATP levels to ~10% of the physiological level by 15 min and to <3% of the physiological level by 30 min (Fig. 1) as has been reported previously (9). After 60 min of chemical anoxia followed by 120 min of recovery in normal media, ATP levels recovered to ~50% of the physiological level. During chemical anoxia by this method, GTP levels decreased to ~10% of the physiological level by 30 min and remained at this level at 60 min as has been recently reported for LLC-PK cells (8). During recovery in normal media after 60 min of chemical anoxia, GTP levels recovered more rapidly than ATP levels. Although chemical anoxia by substrate depletion and antimycin A resulted in ATP and GTP depletion, we will subsequently refer to this process as ATP depletion.

Because phosphorylation of MLC plays an important role in regulating the actin-activated Mg\(^{2+}\)-ATPase activity of myosin II, we examined the effects of ATP depletion on phosphorylation of MLC. The stoichiometry of MLC phosphorylation was 0.65 mol PO\(_4\)/mol MLC under physiological conditions (Fig. 2). We found that the MLC was rapidly dephosphorylated during ATP depletion. The stoichiometry of MLC phosphorylation decreased to 10% of the physiological level (0.06 mol PO\(_4\)/mol MLC) after 15 min of ATP depletion and was essentially zero at 30 min of ATP depletion.

Myosin II organization is disrupted during ATP depletion. Given that myosin II was rapidly dephosphorylated and thus inactivated during ATP depletion of MDCK cells, we next examined the effects of ATP depletion on the localization of myosin II to actin stress fibers. Myosin II localized to actin stress fibers during physiological conditions (Fig. 3). The majority of actin stress fibers remained intact after 30 min of ATP depletion, and myosin II remained localized to the actin stress fibers. Disruption of actin stress fibers was extensive by 60 min of ATP depletion. Amorphous phalloidin-bound cytoplasmic aggregates were noted in the perinuclear region of MDCK cells after 60 min of ATP depletion and were consistent with F-actin aggregates observed previously (27). Myosin II immunostaining was very diffuse throughout the cytoplasm by 60 min of ATP depletion and in general did not correlate with phalloidin-bound actin (Fig. 3).

To substantiate our observation by confocal microscopy that the spatial association between actin and myosin II decreased with ATP depletion, we next examined the association of actin and myosin II during ATP depletion by use of a biochemical fractionation technique. Under physiological conditions, we found that 68% of the myosin II was soluble after extraction with 1% Triton X-100 (Fig. 4). This value is similar to what has been observed with myosin I\(\beta\) in LLC-PK\(_1\) cells (41). Extraction of MDCK cells with 0.5% did not significantly alter the distribution of myosin II in the soluble fraction. We found that myosin II is significantly increased in the Triton X-soluble fraction during ATP depletion (Fig. 4). After 60 min of ATP depletion, 82% of the total myosin II was soluble after extraction with 1% Triton X-100. Total cellular myosin content did not change significantly with ATP depletion. Both the imaging data and the biochemical fractionation data suggest that there is a decreased association of myosin II with the actin cytoskeleton during ATP depletion.

Myosin II activation and reorganization during recovery after ATP depletion. Because we observed that myosin II is inactivated during ATP depletion, we next examined the importance of the state of myosin II activation in reorganization of actin and myosin II in MDCK cells during recovery from ATP depletion. We postulated that myosin II activation, as represented by MLC phosphorylation, is an important regulatory step in reorganization of the basal actin cytoskeleton during recovery from ATP depletion. Consequently, we examined the temporal activation and localization of myosin II and the reorganization of actin stress fibers during recovery from ATP depletion. After 60 min of ATP depletion followed by recovery in normal media for 60 min, the stoichiometry of MLC phosphorylation was >40% of the physiological level (0.28 mol PO\(_4\)/mol MLC) and returned to over 80% (0.53 mol PO\(_4\)/mol MLC) of the physiological level after 120 min of recovery (Fig. 5) despite the fact that ATP levels remained at 50% of the physiological level. The temporal reorganization of actin stress fibers closely correlated with myosin II activation after recovery from ATP depletion (Fig. 6). Sixty minutes into recovery, there was a component of myosin II colocalization to the nascent stress fibers. After 120 min of recovery, F-actin staining, myosin II staining, and the respective colocalization to stress fibers were similar to that observed in MDCK cells that were not ATP depleted. This temporal relationship between myosin II activation and stress fiber reorganization during recovery from ATP depletion supports myosin II activation as an important regulator in stress fiber formation.

Role of ROCK (Rho-kinase) and MLCK in reorganization of stress fibers during recovery from ATP depletion. To investigate the mechanisms involved in regulation of myosin II activation and reorganization of
actin stress fibers during recovery from ATP depletion, we utilized the ROCK inhibitor Y-27632 and the MLCK inhibitors ML-9 and ML-7. In the presence of the ROCK inhibitor Y-27632, the stoichiometry of LC20 phosphorylation remained ~12% of the control value (0.079 mol PO4/mol MLC) after 120 min of recovery from 60 min of ATP depletion (Fig. 7). This stoichiometry of MLC phosphorylation was significantly less than the stoichiometry of MLC phosphorylation observed in cells recovering from ATP depletion in the absence of Y-27632. In contrast, in the presence of the MLCK inhibitors ML-9 and ML-7, the stoichiometry of LC20 phosphorylation during recovery increased to ~100% of control value (0.68 mol PO4/mol MLC) and 68% of control value (0.42 mol PO4/mol MLC), respectively. These values are not significantly different from control cells recovering in regular media alone. The effect of the inhibitors on actin stress fiber and myosin II reorganization during recovery from ATP depletion is depicted in Fig. 8. The ROCK inhibitor Y-27632 clearly inhibited reorganization of actin stress fibers and localization of myosin II to actin during recovery from ATP depletion. Interestingly, Y-27632 did not result in the disruption of actin stress fibers in MDCK cells under physiological conditions. The MLCK inhibitors ML-7 (Fig. 8) and ML-9 (data not shown) had

Fig. 5. MLC phosphorylation in MDCK cells during recovery from ATP depletion. MDCK cells were incubated in depleted media containing 0.1 μM antimycin A for 60 min and then allowed to recover in regular media and in the absence of antimycin A for 60 (60/60) and 120 (60/120) min. Stoichiometries of MLC phosphorylation were determined by analysis of urea-glycerol gels as described in Fig. 3. Results ± SD are given. The stoichiometry of MLC phosphorylation after 120 min of recovery was not significantly different from control cells at time 0 (n = 4).

Fig. 6. Stress fiber and myosin II reorganization in MDCK cells during recovery from ATP depletion. MDCK cells were incubated in depleted media containing 0.1 μM antimycin A for 60 min and then allowed to recover in regular media and in the absence of antimycin A for 60 (A and B) and 120 min (C and D). Cells were fixed, permeabilized, and labeled for F-actin with rhodamine-phalloidin (A and C) and myosin II with rabbit polyclonal antibodies to nonmuscle myosin IIA and fluorescein-labeled goat anti-rabbit antibodies (B and D). Myosin II begins to colocalize to nascent stress fibers by 60 min of recovery and is similar to 0 time point control by 120 min of recovery. Bar = 10 μm.
little effect on actin stress fiber reorganization and myosin II localization to actin stress fibers during recovery from ATP depletion compared with control. These data suggest that ROCK may play an important role in myosin activation and actin stress fiber formation in MDCK cells recovering from ATP depletion and possibly less of a role in actin stress fiber maintenance in MDCK cells under physiological conditions.

**DISCUSSION**

To further the understanding of cellular alterations that occur during renal ischemia, we have utilized an in vitro model of ischemia to test our hypothesis that myosin II is an important distal effector in the Rho-regulated alterations of the actin cytoskeleton that occur during ischemia and recovery from ischemia in renal tubular epithelial cells. Although these findings done under conditions of severe ATP depletion do not directly apply to the full range of clinical renal ischemic injury, investigations of cell culture models such as the one utilized in this study have previously provided clues into the cellular mechanisms involved in ischemic renal injury (24). We found that myosin II dephosphorylation and therefore myosin II inactivation occurs rapidly during severe ATP depletion. The time course and extent of MLC dephosphorylation and therefore...
myosin II inactivation generally parallel the time course and extent of ATP depletion. Although this result may initially seem predictable, a recent study indicates that phosphorylation states of proteins do not necessarily parallel cellular ATP content during severe ATP depletion (32).

Although myosin II inactivation is complete by 30 min of ATP depletion, disruption of actin stress fibers is minimal at this time point and does not become extensive until 60 min of ATP depletion. Furthermore, both our imaging and our biochemical extraction data suggest there is decreased association of myosin II with actin after 60 min of ATP depletion. These findings suggest that during severe ATP depletion, myosin II first becomes inactivated and then dissociates from the actin cytoskeleton. This observation is consistent with the mechanism for regulation of the actomyosin ATPase cycle by MLC phosphorylation (33). Phosphorylation of MLC primarily stimulates P1 release and the shift from a low-affinity state for actin to a high-affinity state for actin. Consequently, dephosphorylation of MLC would favor myosin remaining in the low-affinity state for actin and subsequently dissociating from actin.

Although our findings suggest that myosin II does not form extensive rigor complexes with actin during severe ATP depletion as has been suggested for myosin I(polymerized) in LLC-PK1 cells (41), there was still evidence of myosin II colocalization to the larger perinuclear F-actin aggregates. Moreover, addition of ATP to the Triton X-100 extraction buffer did increase the extent of myosin II in the soluble fraction (unpublished data) after 60 min of ATP depletion. This would suggest that although there is not extensive formation of rigor complexes, at least a portion of the cellular myosin II is caught in a high-affinity state for actin during severe ATP depletion and is trapped in a rigor complex with actin composing the larger phalloidin-stained aggregates.

Our data demonstrate that myosin II dephosphorylation and therefore myosin II inactivation occurs rapidly during severe ATP depletion, that myosin II inactivation precedes stress fiber disruption, and that myosin II dissociates from actin during severe ATP depletion. Taken together, these data demonstrate a spatiotemporal relationship between myosin II inactivation and disruption of actin stress fibers and suggest that myosin II inactivation may contribute to destabilization of the actin cytoskeleton during severe ATP depletion. However, the temporal difference between myosin II inactivation and full disruption of actin stress fibers would suggest that other processes, such as actin severing, are also involved in the full disruption of actin stress fibers.

In contrast to ATP depletion, reorganization of actin stress fibers temporally correlates with myosin II activation and myosin II redistribution to stress fibers during recovery from severe ATP depletion. This spatiotemporal relationship between myosin II activation and stress fiber reorganization during recovery from ATP depletion supports myosin II activation as an important regulator in stress fiber formation and is consistent with a proposed model of stress fiber formation in fibroblasts (7).

Our investigation into the mechanisms regulating myosin II activation and reorganization of stress fibers during recovery from severe ATP depletion suggests myosin activation and actin stress fiber formation to be ROCK dependent. Our data do not discern whether ROCK is important in activating myosin II by directly phosphorylating MLC, by phosphorylating and inhibiting myosin phosphatase, or both. Interestingly, the MLCK inhibitors ML-7 and ML-9 had no significant effect on myosin II activation or reorganization of stress fibers during recovery from ATP depletion. These findings are consistent with a recent study in serum-starved 3T3 cells that provided evidence that ROCK is the kinase responsible for directly phosphorylating MLC and activating myosin II during organization of actin stress fibers (38).

In summary, our study demonstrates that myosin II inactivation occurs rapidly and precedes disruption of actin stress fibers in a cellular model of ischemia and that activation of myosin II during recovery in a cellular model of ischemia is spatiotemporally related to actin stress fiber reorganization. Initial characterization of the mechanisms regulating myosin II activation and reorganization of actin stress fibers during recovery in this model of ischemia suggests these processes to be ROCK dependent. More extensive characterization of these mechanisms may provide further insight into the regulation of the actin cytoskeleton during ischemic injury and recovery from ischemic injury.

We thank Bruce Molitoris for critical reading of the manuscript, Pierre Dagher for assistance in measuring cellular nucleotide levels and insightful discussions, Mark Hallett and Patricia Gallagher for helpful discussions, and Ruben Sandoval for assistance with microscopy. We are very grateful to Bruce Molitoris for advice and support throughout this project.

This work was supported in part by grants from the National Kidney Foundation-Indiana (to T. A. Sutton), the National Institute of Diabetes and Digestive and Kidney Diseases (no. DK-53194; to S. J. Atkinson), and the Grace M. and Ralph W. Showalter Research Trust (to S. J. Atkinson).

REFERENCES