Membrane repolarization is delayed in proximal tubules after ischemia-reperfusion: possible role of microtubule-organizing centers

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SEVERAL PROCESSES INVOLVED in cellular injury during ischemia and after ischemia-reperfusion (I/R) have been reported in recent years in an effort to understand the pathophysiology of ischemic acute renal failure (ARF) (7, 17). It is generally accepted that disorganization of the cytoskeleton and loss of membrane polarity are significant pathophysiological mechanisms of ischemic injury in proximal tubule cells (44). Most attention has been focused on the disarrangement of the actin cytoskeleton (20, 26), although it is known that cortical fodrin, villin, and tight junctions are also affected (9, 16, 27). Interestingly, however, it has been recently reported that disorganization of the actin cytoskeleton alone is not sufficient to explain the anoxic disruption of the plasma membrane (13). In addition, Brown and co-workers (1) also showed depolymerization of microtubules (MTs) in proximal tubules in vivo during the first 24 h after I/R.

Our laboratory and others have reported that microtubule-organizing centers (MTOCs) are apical in simple epithelia (3, 4, 10, 25, 34, 37), thus organizing the MTs in a polarized fashion with the minus ends toward the apical membrane (5). Although this apicobasal polarization of MTs is not absolutely essential for polarization (19, 38), it is thought to participate in the vesicular traffic bound to the apical membrane (24), especially for vesicles involved in transcytosis from basolateral to apical membrane (8). In this regard, the nucleating activity of MTOCs must be necessary to repolymerize MTs after I/R. Because MTOCs cap the minus ends of MTs (45), it is expected that MTOCs positioned in their normal (apical) localization will reorganize MTs with the correct orientation (minus end apical). Conversely, if MTOCs become delocalized during ischemia, the MTs formed during the recovery period will have abnormal orientations. In that scenario, aberrant MTs may transport the carrier vesicles that originate in the trans-Golgi network and contain apical cargo (28) to incorrect regions of the cytoplasm. Therefore, newly formed MTs with an incorrect orientation may contribute to the mispolarization of the plasma membrane more seriously than a simple depolymerization of MT.

Because our previous work suggests that the apical localization of MTOCs in simple epithelia depends on binding to apical intermediate filament (IF) (37), we decided to analyze whether the IFs are also disrupted by anoxia and whether the MTOCs are still bound to IF and apically localized in proximal tubule kidney cells after ischemia. We performed the analysis in two epithelial cell lines, and in vivo, using a mild I/R protocol of unilateral clamp of the renal vessels, which is known to disrupt the MTs (1) but causes little or no necrosis and only modest amounts of apoptosis only within the first 24 h after ischemia (31). The results indicate that MTOCs become delocalized after chemical anoxia in
tissue culture or as a result of I/R in vivo. Furthermore, the anomalous localization of MTOCs results in a disorganized array of MTs and correlates with poor membrane polarization of proximal tubule cells, delayed by days respect to the formation of the F-actin brush border.

**MATERIALS AND METHODS**

**Cell culture and ATP depletion.** LLC-PK1 (porcine proximal tubule) and CACO-2 (human colon carcinoma epithelial cells) were obtained from ATCC and maintained by weekly passages in tissue culture plastic flasks in DMEM-F-12 nutrient mixture (DMEM/F-12, Gibco) supplemented with 10% fetal bovine serum (Cellgro). For chemical anoxia experiments, the method described by Molitoris and co-workers (11) was used, with the following modifications. Monolayers confluent for 4 (LLC-PK1) or 9 days (CACO-2) were incubated in Earle’s balanced salt solution (GIBCO formulation) without glucose or other nutrients for 30 min and then changed to the same solution supplemented with 0.5 μM antimycin A (Sigma), 1 mM adenosine, and 0.2 mM allporphin (15) for 1 h (ATP depletion). Recovery was initiated by four washes and incubation in the standard DMEM/F-12 culture medium for various times. ATP determinations were performed using a luciferase-based kit (Calbiochem).

**Kidney ischemia in vivo.** Animal handling was in compliance with Public Health Service Policy on Humane Care and Use of Laboratory Animals. Male Sprague-Dawley rats (Charles River Laboratories) weighing 300–400 g were anesthetized with 1.5% isoflurane and kept on a warm plate to maintain temperature. The peritoneal cavity was opened, and the left renal vessels were clamped for 30 min with two microvascular (no. 1) clamps. The success of the clamp, and, later, the reperfusion, was visualized by rapid changes in the color of the kidney. The animals were killed by an overdose of pentobarbital and were separated in 10-ml 20% gradients in a SW41 swinging bucket rotor at 15,000 rpm for 10-s periods of sonication and 15-s lapses to dissipate heat. The sonication fragments of the Triton-insoluble cytoskeleton were separated in 1% Triton X-100, in PBS supplemented with 2 mM EDTA, and a complete cocktail of antiproteases (Sigma), extracted in 1% Triton X-100, in PBS supplemented with 2 mM EDTA, and a complete cocktail of antiproteases (Sigma), and the pellet was extensively sonicated (3 min total, with 10-s periods of sonication and 15-s lapses to dissipate heat). The sonication fragments of the Triton-insoluble cytoskeleton were separated in 10-ml 20–60% sucrose continuous gradients in a SW41 swinging bucket rotor at 15,000 rpm for 50 min at 4°C. Usually, the top five 1-ml fractions of the gradient were used. Immunoprecipitation was done following standard procedures. The protein A-agarose beads were centrifuged through a 0.5-ml 30% sucrose cushion. All these centrifugations were done at 14,000 rpm for 2 s to minimize nonspecific copelleting of unbound cytoskeletal fragments. Immunoblotting was performed as described before (37).

**RESULTS**

**Relocalization of centrosomes during chemically induced ATP depletion and ischemia.** To study the effects of chemical anoxia in tissue culture cells, we used a well-established protocol that is known to cause a rapid and reproducible depletion of ATP (11). The efficiency of chemically induced ATP depletion in our cells and the kinetics of recovery after 1-h depletion were assessed in LLC-PK1 (porcine proximal tubule) and CACO-2 (human colon carcinoma) cells using a standard luciferase assay. In both cell lines, ATP levels fell >90% within the first 10 min after treatment with antimycin A, and significant recoveries of ATP levels were started between 2 and 4 h after the cells were replaced in DMEM (Fig. 1). Thus the kinetics of ATP depletion/recovery for LLC-PK1 were similar to those in previous reports.

To analyze the localization of centrosomes and to extend the observations to a different cell line, LLC-PK1 and CACO-2 monolayers were subjected to ATP depletion for 1 h and 1-h recovery. Double-immunofluorescence experiments were performed colocalizing the γ-tubulin signal (Fig. 2, green) with the cortical IF cytoskeleton (Fig. 2, red). To show the apical domain
alone, K19 was used as a marker of IF in the CACO-2 cells. As in CACO-2 cells (37), K18, the other type I keratin, distributed under the apical and lateral domains in LLC-PK1 cells, but the IF cortical signal was thicker in the kidney cells than in the intestinal cells (Fig. 2, a, c, e, g, and i). More importantly, ATP depletion did not affect the IF cytoskeleton in either LLC-PK1 or CACO-2 cells (Fig. 2). A similar result was observed, reciprocally, using anti-K18 or anti-K19 antibodies in CACO-2 or LLC-PK1 cells, respectively (not shown).

In Fig. 2, examples of fields with centrosomes within the same confocal optical section in nearby cells are shown for control monolayers (a–d) and ATP-depleted cells (e–j). As expected from previous publications, the centrosomes in control cells were observed always embedded in the apical IF submembrane cytoskeleton (Fig. 2, a–d). In ATP-depleted cells, a proportion of the centrosomes was found separated from the apical IF cytoskeleton (Fig. 2, arrows). The proportion was smaller in LLC-PK1 cells (28%) than in CACO-2 cells (49%) but in both cases significantly larger than the proportion of centrosomes normally found separated from the IF in control interphasic cells (0% in all our samples). In control cells, a modest proportion of centrosomes (usually <10%) does appear attached to the lateral domain IF (37), which was not the case in the ATP-depleted cells either. In some cases, the γ-tubulin

![Fig. 1. ATP levels in CACO-2 (A) and LLC-PK1 (B) epithelial cells in tissue culture during and after chemically induced ATP depletion. The ATP from quadruplicate confluent monolayers (~3 x 10⁶ cells each) was extracted at various times of incubation in nutrient-free medium supplemented with antimycin A, adenosine, and allopurinol (depletion) and after a 1-h depletion at various times of recovery in normal culture medium. ATP was measured by luciferase luminescence by integrating light emission for 1 min.](image)

![Fig. 2. Centrosomes detach from their normal apical location at the apical intermediate filament (IF) network after 1-h ATP depletion in LLC-PK1 and CACO-2 cells. Monolayers of LLC-PK1 (a, c, e, g, i) and CACO-2 cells (b, d, f, h, j) were grown on Transwell filters. Some cells were subjected to ATP depletion for 1 h (e–j). The cells were fixed after 1-h recovery and processed for double immunofluorescence with monoclonal Abs against K18 (LLC-PK1 cells) or K19 (CACO-2 cells) (red channel) and a polyclonal antibody against a conserved NH₂-terminal polypeptide of γ-tubulin (green channel). Two examples of each control and 3 examples of ATP-depleted cells were analyzed by confocal microscopy, deconvolution, and 3D reconstruction and are shown in the XZ plane (perpendicular to the monolayer), with the apical side up. Arrows point at γ-tubulin signal separated from the apical domain IFs. Scale bars: 5 μm (a–i) and 2 μm (j).](image)
signal usually interpreted as centrosomes (−0.4-μm spheres, often observed in pairs) appeared fragmented in ATP-depleted cells (Fig. 2, h and i). The noncentrosomal γ-tubulin signal (smaller dots or diffuse signal), normally at or within 1 μm of the terminal web (25, 37), also appeared separated from the apical submembrane cytoskeleton, especially in LLC-PK1 cells (Fig. 2c).

Because co-localization does not necessarily imply attachment, the physical connection of insoluble γ-tubulin-containing structures and IF was analyzed as before (37) by fragmenting the Triton-insoluble cytoskeleton of LLC-PK1 cells with extensive sonication and separating the smallest fragments by size in sucrose gradients by rate centrifugation. This procedure was originally devised to perform immunoprecipitation in highly insoluble multiprotein complexes (34). We collected the top five fractions of the gradient that contain fragments small enough to be immunoprecipitated (larger fragments tend to nonspecifically copellet with agarose beads). Each aliquot was divided into two equal parts, one of them immunoprecipitated with an irrelevant rabbit IgG as a negative control (Fig. 3, − lanes), and the other with anti-γ-tubulin Ab (Fig. 3, + lanes). The immunoprecipitates were analyzed by immuno blot using anti-K18 or anti-K19 monoclonal antibody. In general, the previous findings in CACO-2 (37) were extended to LLC-PK1 cells. In control cells, there was coimmunoprecipitation of K18 and γ-tubulin in fractions 1, 3, and 4 and of K19 and γ-tubulin in fractions 1–5 (Fig. 3). This coimmunoprecipitation was mostly abolished in LLC-PK1 cells subjected to 1-h ATP depletion. K18 blots showed only background levels, and K19 showed only some ATP depletion-insensitive coimmunoprecipitation with γ-tubulin in fraction 4 (Fig. 3). Controls showing that this coimmunoprecipitation cannot be explained by simple physical trapping or artificial protein binding as a result of detergent extraction have been performed elsewhere (37, see Fig. 8). These results indicate that the attachment between MTOC and IFs is largely broken after 1-h ATP depletion in LLC-PK1 and CACO-2 cells in tissue culture.

Ischemic injury in vivo causes detachment of MTOCs in proximal tubules. To test whether MTOCs also detach from the apical domain in vivo after ischemia, rat kidneys were subjected to a 30-min clamp of the renal vessels. The contralateral kidneys were used as a sham operation control. We analyzed the kidneys at 24 h after I/R, a time that has been widely studied but that yields results difficult to analyze because of the complexity of the effects. We also analyzed the kidneys at 3, 5, 7, and at 8 days after I/R. The latter was chosen because our preliminary experiments showed that the changes described in this section last up to 7 days after I/R, and kidney functional parameters with a 30-min bilateral clamp protocol have been reported to be nearly normal as early as 5 days after I/R (23). In addition, Hropot and co-workers (21) found plasma creatinine and sodium still abnormal after 7 days for a 40-min bilateral clamp model. Even in a different system, the human renal allograft, patients recovering well achieve acceptable glomerular filtration rates on day 7 (14).

Frozen sections from reperfused or sham-operated kidneys were analyzed by immunofluorescence with anti-γ-tubulin antibody and FITC-phalloidin to stain F-actin. In the control proximal tubules, very few 0.3- to 0.4-μm spots of γ-tubulin signal (centrosomes) were easily recognizable and were mostly localized in the apical pole of the cells. However, a continuous subapical layer of γ-tubulin signal was observed in most tubules both in kidneys subjected to a sham operation (Fig. 4a) and in kidneys from animals not subjected to surgery (not shown). When analyzed in sections perfectly perpendicular to the axis of the tubule, the γ-tubulin signal (Fig. 4a, inset, green) could be localized immediately below the F-actin signal in the brush border (Fig. 4a, inset, red; arrows point at basal actin signal). This image is consistent with findings in our laboratory and by others as well that a layer of noncentrosomal MTOCs lies under the apical domain (3, 25). During the first 24 h after I/R, the apical γ-tubulin layer became very discontinuous or disappeared altogether (not shown). However, because the entire apical domain is degraded (44), it is difficult to draw any conclusions about the fate of MTOCs at that time. More interesting were the results from kidneys at 3 days following I/R. At this stage, the F-actin component of the brush border was mostly repaired in many (but not all) cells, as judged by a phalloidin label (Fig. 4d, arrows). However, no subapical layer of γ-tubulin was observed, even in tubules (or cells) with a complete brush border (Fig. 4, c vs. d, arrows). Instead, γ-tubulin signal was either diffuse in the cytoplasm or concentrated under the lateral domain in some cells (Fig. 4c,
Fig. 4. Polarization of microtubule-organizing centers (MTOCs) is delayed respect to the formation of the brush border in proximal tubules. Frozen sections from the kidneys of an animal that was subjected to a 30-min clamp of the left renal vessels followed by a 3-day recovery (c and d) and sham-operated kidney (sections from the right kidney; a and b) were processed with fluorescent phalloidin (F-actin; b and d) or for indirect immunofluorescence with anti-γ-tubulin antibody (a and c). Inset: higher magnification view of a segment of a proximal tubule overlapping pseudocolor images of phalloidin (red) and anti-γ-tubulin (green) signals. L, lumen; arrowheads, basal actin signal. Arrows point out depolarized γ-tubulin signal (c) and fully reassembled F-actin brush border (d). Scale bars: 20 μm (a–d) and 5 μm (inset).

Fig. 5. MTOCs are detached from the cytoskeleton in the renal cortex 3 days after an ischemic injury. The left kidneys of rats were subjected to a 30-min clamp followed by 1, 3, or 8 days of reperfusion (I). The corresponding right kidneys were used as sham controls (S). Each S and I pair corresponds to a different animal. Pieces of cortex were homogenized in nonionic detergent, and the cytoskeletal (detergent insoluble) fraction was pelleted at low-speed centrifugation, which was devised to maintain γ-tubulin (tub.) complexes (γ-TurC; 42) in the supernatant. The pellets were rehomogenized by sonication. Then, 30 μg total protein of each sample were separated by SDS-PAGE and analyzed by immunoblot with an anti-γ-tubulin antibody (pellets) or an anti-α-tubulin antibody ( supernatants) to independently assess protein load in the same lanes.
**Table 1. γ-Tubulin signal in the cortex of kidneys subjected to I/R as a percentage of the band from the contralateral (sham surgery) kidney**

<table>
<thead>
<tr>
<th>Days After I/R</th>
<th>Cytoskeletal Pellets, %</th>
<th>Supernatants, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>37 ± 30*</td>
<td>117 ± 18</td>
</tr>
<tr>
<td>3</td>
<td>6 ± 7*</td>
<td>89 ± 19</td>
</tr>
<tr>
<td>8</td>
<td>113 ± 27</td>
<td>106 ± 28</td>
</tr>
</tbody>
</table>

Values are shown as means ± SD (n = 8). Extracts from kidney cortex were processed as described in Fig. 5, normalized by protein, and analyzed by Western blotting using an anti-γ-tubulin antibody. I/R, ischemia-reperfusion. The bands were measured by densitometry and for each pair of animals the value of the band from the kidney subjected to I/R was normalized with respect to the value of the band from the contralateral kidney (sham surgery), which was taken as 100%. *Significantly different from the 8-day pairs (P < 0.01).

**Fig. 6.** Microtubules remain disorganized 3 days after ischemia-reperfusion (I/R) in tubules with normal F-actin distribution. Sections of the right (sham; a) and left (I/R; b and c) kidneys fixed 3 days after renal artery clamp operation were stained with an anti-α-tubulin monoclonal Ab (red channel) and subsaturation concentrations of FITC-phalloidin to minimally counterstain the brush-border F-actin (green channel). The sections were analyzed by confocal microscopy at 0.7 Airy units. Arrows, abnormal microtubule distribution. Scale bars: 10 μm.

Lack of polarity of apical plasma membrane markers at 3 days after I/R in proximal tubules showing an apparently normal F-actin brush border. Because MTOCs cap the minus end of MTs (45) and because MTOCs seem to be scattered in the cytoplasm at 3 days after I/R (Fig. 4) free from their normal cytoskeletal attachment (Fig. 5), we reasoned that the disorganized MTs at 3 days after I/R (Fig. 6) must have their minus ends randomly distributed in the cells. Therefore, MTs would not only not contribute to the normal polarization of the plasma membrane but would actually tend to randomize apical membrane proteins. To test this hypothesis, we analyzed the polarization of two apical membrane markers normally expressed in S2 and S3 segments of the proximal tubule, CAIV (S2 and S3 marker) (40) and iAP (S3 marker) (30). At 3 days after I/R, F-actin in the brush borders was similar to control tubules, as judged by phalloidin distribution (Fig. 7,d vs. b). CAIV and iAP were observed in the brush border in all tubules where they are expressed in sham-operated kidneys (Fig. 7a, Table 2). At 1 day after I/R, the iAP signal could not be observed and the CAIV signal was weak and depolarized in at least one cell in 72% of the S2–3 sections (Table 2). This result is consistent with previous publications describing the loss of apical membrane and depolarization of the cells immediately after ischemia (2, 44). Strikingly, however, on day 3 significant levels of basolateral CAIV (Fig. 7c, arrows) were observed in one or more cells in 55% of the S3 sections (Table 2). Similarly, 64% of the sections positive for iAP showed at least one cell depolarized (Table 2). The number of depolarized cells decreased significantly on day 5, although isolated depolarized cells were observed up to 7 days after I/R for CAIV (Table 2).

To obtain a more precise assessment of the magnitude of the depolarization of CAIV and iAP, we measured the total signal in the apical and basolateral domain in high z-axis resolution confocal optical sections (Fig. 7, e and g). For each cell, the signal was weighed as total pixels × average pixel intensity in each area. With care taken to avoid images in which high-intensity pixels are saturated, this method can provide a fair estimation of the relative distribution of a membrane marker in a way that accounts for the differences in membrane folding (36). In proximal tubules subjected to sham surgery, only 3.6 ± 4.2% of...
total CAIV signal was localized to the basolateral domain (Fig. 7, a and e; only detectable with digital microscopy). In kidneys at 3 days after I/R, on the other hand, proximal tubule cells showed 48.2 ± 13.3% of total cellular CAIV signal localized to the basolateral domain (Fig. 7, c, arrows, and g). In other words, these cells “look” partially polarized because the apical signal is localized in a smaller area of the section with a high level of membrane folding (brush-border microvilli), whereas the basolateral signal is in a much more stretched membrane array. However, nearly equal amounts of the apical membrane marker are localized in each domain, and, thus these cells are almost totally depolarized.

**Intracellular localization of Na⁺-K⁺-ATPase after I/R.** To analyze the polarity of basolateral membrane proteins during the same period after I/R, we conducted experiments similar to those depicted in Fig. 7 but using an anti-Na⁺-K⁺-ATPase antibody instead. The antibody showed a basolateral image in sham kidneys. In 3-day I/R kidneys, a large proportion (72% of the proximal tubules, an average of 8 cells/section, Table 2) of the proximal tubule cells showed intracellular images, even in cells with an already fully reassembled actin brush border (Fig. 8, arrows). At this stage, however, little or no apical distribution of Na⁺-K⁺-ATPase was observed. At 5 days after I/R, only scattered cells (21% of the sections, an average of 1.2 cells/section) were still showing the intracellular distribution of Na⁺-K⁺-ATPase, and with few exceptions (one shown in Fig. 8C), no Na⁺-K⁺-ATPase signal was found in the apical domain either (Fig. 8). These results suggest that Na⁺-K⁺-ATPase also redistributes at the 3-day stage after I/R but not in the apicobasal axis but rather into an intracellular compartment and fully confirm the observations of Alejandro and coworkers (2) in human allografts. In addition, the results also indicate that different mechanisms govern

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**Fig. 7.** Substantial amounts of basolateral carbonic anhydrase IV (CAIV) signal remain 3 days after I/R in tubules with a reestablished F-actin brush border. Sections of the right (sham) and left (I/R) kidneys fixed 3 days after renal artery clamp operation were processed for indirect immunofluorescence with an anti-CAIV antibody (a, c, e, g) followed by a specific secondary antibody coupled to CY3 and FITC-phalloidin to show F-actin (b, d, f, h). e–h: Confocal images of the same preparations at higher magnification. Scale bars: 20 μm (a–d) and 10 μm (e–h).
the polarization of apical and basolateral markers, as described before in Madin-Darby canine kidney cells (43) and that reassembly of the brush-border actin is not an indicator of full polarization.

**DISCUSSION**

The results in this work point at three general conclusions. First, IFs are stable after ATP depletion in

![Image](https://example.com/image.png)

**Fig. 8. Intracellular localization of “depolarized” Na⁺-K⁺-ATPase after I/R in proximal tubules. Sections of the right (sham; 3 days after operation) and left (I/R) kidneys fixed 3 and 5 days after renal artery clamp operation were processed for indirect immunofluorescence with an anti-Na⁺-K⁺-ATPase antibody (red channel) and FITC-phalloidin (green). The arrows point to cells with intracellular signal such as those shown in Fig. 8, arrows.**

### Table 2. Delayed recovery of epithelial polarization in S3

<table>
<thead>
<tr>
<th>Days of Recovery</th>
<th>% Tubules With at Least 1 Depolarized Cell/Section*</th>
<th>Depolarized Cells/Section†</th>
<th>t-Test</th>
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<tr>
<td>CAIV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Ischemia 8.3 ± 10.7</td>
<td>1.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Ischemia 72 ± 18</td>
<td>6.9</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ischemia 55 ± 20</td>
<td>6.7</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ischemia 18 ± 12</td>
<td>1.3</td>
<td>P &lt; 0.005</td>
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<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
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<tr>
<td>7</td>
<td>Ischemia 12 ± 1.4</td>
<td>1.2</td>
<td>P &lt; 0.005</td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
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<tr>
<td>8</td>
<td>Ischemia 7 ± 5.4</td>
<td>1.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
<td></td>
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<tr>
<td>iAP</td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>Ischemia SND</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham 0 ± 0</td>
<td></td>
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<tr>
<td>3</td>
<td>Ischemia 64 ± 20</td>
<td>7.2</td>
<td>P &lt; 0.005</td>
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<tr>
<td></td>
<td>Sham 0 ± 0</td>
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<tr>
<td>8</td>
<td>Ischemia 0 ± 0</td>
<td>NS</td>
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<td>Sham 0 ± 0</td>
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Values are means ± SD (n = 12 animals). 0 Days of recovery refers to 1-h recovery after I/R. *Total no. of tubules taken as sections positive to carbonic anhydrase IV (CAIV), intestinal alkaline phosphatase (iAP), or Na⁺-K⁺-ATPase. SND, signal not detectable; NS, not significant. †Average depolarized cells per transverse section: iAP and CAIV refer to cells with basolateral signal of the corresponding protein; Na⁺-K⁺-ATPase refers to cells with intracellular signal such as those shown in Fig. 8, arrows.
culture or I/R in vivo (Figs. 2 and 5). Second, I/R leads to a separation of MTOCs from the cytoskeleton, in culture (Fig. 2) and in vivo (Figs. 4 and 5). This detachment results in a nonpolarized distribution of MTOCs and a disorganized architecture of MTs that last up to 1 wk after a mild ischemic injury, well beyond the time when the microfilaments are fully reorganized (2–3 days after I/R) (Fig. 6). Third, at least some plasma membrane proteins remain depolarized after the reorganization of the brush border, as defined by F-actin, and repolarize roughly with the same kinetics as MTOCs and MTs reacquire their normal subcellular organization (Fig. 7).

The initial experiments in this work were performed in two epithelial cell lines in tissue culture: LLC-PK1, derived from the proximal tubule, and Caco-2, derived from the intestine. The results from both cell lines indicate that ATP depletion has a significant impact on the apical localization of MTOCs. In proximal tubules, centrosomes were difficult to identify, and perhaps they are absent altogether in most cells. Therefore, we rather focused on the layer of noncentrosomal MTOCs that has been visualized in vivo in other epithelia as well (3). The dramatic fate of the apical pole in vivo as a result of the I/R injury, extensively described by Molitoris and co-workers (11), made any comparison of the redistribution of MTOCs in culture vs. in vivo nearly impossible. In addition, the analysis of the initial 24 h after I/R is complicated by the presence of apoptotic cells (31). Biochemical and morphological data in this work (Figs. 4 and 5) indicate that between 24 and 72 h after I/R, nearly all MTOCs become detached from the cytoskeleton and are scattered throughout the cytoplasm. Importantly, the data in tissue culture cells (Fig. 2) and in vivo (Fig. 5) consistently indicate that the detachment of MTOCs is not a consequence of depolymerization of IFs, the normal anchor of MTOCs (18, 37), that in all cases remained unaffected by ATP depletion or I/R (Figs. 2 and 5, respectively). The immediate consequence of the detachment of MTOCs was that MTs, although fully repolymerized during the same period of time (1–3 days after I/R), became a disorganized network, instead of the normal array of bundles highly oriented in the apicobasal axis (Fig. 6). Although we cannot assert a cause-and-effect relationship, it is safe to conclude that the disorganization of MTs correlates with poor levels of plasma membrane polarity, which only revert over a period of days to nearly normal at 5 days after I/R and, in some isolated depolarized cells, up to 7 days after I/R (Fig. 7, Table 2).

There are a number of possible explanations for the delayed recovery of distribution of MTOCs at 3 days after I/R. The first is that apoptosis, could, in principle, account for the separation of MTOCs from the cytoskeleton by a simple action of caspases. However, proximal tubule cells at 3 days after I/R displayed a nearly normal F-actin cytoskeleton and full-length MTs (although in abnormal orientations) (Figs. 6b and 7d). Furthermore, and in agreement with previous publications using the same I/R protocol (31), we found only modest proportions of apoptotic cells (5–8%) during the first 24 h after I/R, and none at 3 days (not shown). This was the reason the protocol was chosen in the first place, as opposed to more drastic ischemic injuries (e.g., 1 h) that result in higher percentages of apoptotic cells, and, eventually, a late secondary peak of apoptosis (41). In addition, we have demonstrated that, at least in intestinal epithelia, apoptosis does not disrupt epithelial polarity as long as the cell maintains the integrity of its plasma membrane (3). On the other hand, we cannot rule out that the early detachment of MTOCs from IF found in tissue culture cells may be due to caspase activation. Second, dedifferentiation, in a general sense, may occur because tens of genes are either upregulated or downregulated using the same I/R protocol at 1 or 3 days after I/R (48). More specifically, the expression of vimentin has been implicated as a reporter of dedifferentiation in proximal tubule cells after ischemia, and, along with the expression of proliferating cell nuclear antigen (PCNA), it was found in a substantial number of S3 cells at 2–5 days postischemia using a harsher ischemic injury (46). In our system, vimentin was expressed in <2% of the cells at 3 days after I/R. Interestingly, some of the vimentin-expressing cells actually had a polarized distribution of CAIV (not shown). Therefore, while it is quite likely that changes in the pattern of gene expression may be responsible for the detachment of MTOCs from the cytoskeleton, the phenomenon cannot be assigned to a simple epithelial-to-mesenchymal transition. Third, changes in the intracellular signaling/regulatory pathways, on the other hand, are more likely to be responsible for a phenomenon that occurs >24 h after I/R. Although the molecular identity of the “glue” attaching MTOCs to IFs has not yet been established, we have recently identified an ~190-kDa protein that, on phosphorylation with p34cdc2, seems to mediate the detachment of MTOCs from IF (18). Although we have no evidence that the same protein is responsible for the detachment of MTOCs from IF at day 3 after I/R, our working hypothesis is that a posttranslational modification of one of the components of the glue may be implicated in this phenomenon. For this reason, our laboratory is presently engaged in an attempt to identify the molecular components that mediate the binding of MTOCs to IFs. After I/R, specific signaling pathways become activated (39). The activation of “stress” kinases (p39 MAP, e-Jun, and ATF3), however, seems to be an early event after I/R (3 h) (47), and the exact nature of all the signaling/regulatory pathways active at 3 days after I/R is still unknown. Fourth, mitotic responses have been observed in S3 with harsher ischemic injuries, i.e., 40- (46) and 50-min ischemia (29). PDGF receptors have been implicated in this response (29) as well as stress kinases (39). In mitotically active tissue culture cells, MTOCs cycle from an IF-attached state in interphase to a free and mobile state during mitosis (18, 33). While there is no doubt that MTOCs are apical in quiescent cells, it is still conceivable that they may not reattach to the apical cytoskeleton in
mitotically active S3 cells. We cannot rule out this scenario, either.

One lesson that we can draw from various studies of the acquisition of membrane polarity in tissue culture cells is that the time course of polarization of various components may be different and sometimes counterintuitive. For example, we have demonstrated that apical polarity can be established before basolateral polarity and in the absence of tight-junctions (43). Grinsdstaff et al. (19) demonstrated that membrane polarity can precede the polarization of MTs. The hierarchy of events during the establishment of polarity is, therefore, still poorly understood. Our data here suggest a sequence of two events: 1) an early reestablishment of the F-actin-based brush border, along with an incipient, partial, establishment of membrane polarity completed at 3 days after I/R, entirely consistent with the findings of Brown and co-workers (1); and 2) despite the repolymerization of MTs completed at the same time as the apical F-actin reorganization, a much delayed reestablishment of the polarized arrangement of MTs, along with the full polarization of the plasma membrane, both accomplished ~1 wk after I/R. In other words, the reestablishment of the submembrane F-actin does not seem to be sufficient for a full polarization of the cells.

An obvious possibility is that depolarization of apical membrane proteins may be the consequence of open tight junctions and be unrelated to the disorganization of MTs. In this regard, Kwon and co-workers (22) have reported up to 57% backleak of the glomerular filtrate in transplanted patients with sustained ARF, as demonstrated by fractional clearance of dextrans. However, it is of note that the same group in the above-mentioned and previous publications (2) found redistribution of Na\(^+\)-K\(^+\)-ATPase to a cytoplasmic pool but not to the apical membrane. The same result was confirmed here for the 30-min unilateral ischemia paradigm in the rat (Fig. 8). Those results would suggest a dissociation between the “fence” role of tight junctions, apparently conserved, and the “gate” role, clearly disrupted. In fact, in tissue culture cells, the gate function, measured by transepithelial resistance, can be disrupted with much shorter times of ATP depletion than the fence function (6).

Although we have not analyzed the polarity of apical ion transporters, the basolateral mislocalization of such will only potentiate the effects of mislocalized Na\(^+\)-K\(^+\)-ATPase by shortcircuiting the remaining pumps at the basolateral membrane and contributing to the failure in proximal sodium reabsorption. Recently, it has been speculated that it may even contribute to elevated tubule pressure (32), the predominant cause of hypofiltration in ARF. While it is clear that the modest proportion of depolarized cells in the 5- to 7-day period after I/R may not be significant for overall kidney function in the 30-min clamp paradigm, they highlight the possibility that slow apical polarization may play a role in delaying recovery after ARF under other ischemic conditions, such as allografts, as well. Other consequences of a sustained depolarization of apical proteins may go beyond ionic transport. Meprin, a brush-border protease, for example, has been found relocalized to the basolateral domain, where it caused fragmentation of the extracellular matrix after I/R in rats (12). These potential connections with the pathogenesis of ARF warrant the need for future investigations into the molecular mechanisms involved in the attachment-detachment of MTOCs in response to I/R injury in kidney.

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DISCLOSURES

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