Ischemia induces alterations in actin filaments in renal vascular smooth muscle cells

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Kwon, Osun, Carrie L. Phillips, and Bruce A. Molitoris. Ischemia induces alterations in actin filaments in renal vascular smooth muscle cells. Am J Physiol Renal Physiol 282: F1012–F1019, 2002. First published January 2, 2002; 10.1152/ajprenal.00294.2001.—Although altered renal vascular reactivity is known to occur after ischemia, the structural basis explaining the phenomenon has not been clarified. To evaluate for structural damage to the renal vasculature in ischemic acute renal failure (ARF), F-actin in the renal vasculature of rat kidneys and cultured vascular smooth muscle cells was examined using confocal fluorescence microscopy. The left renal artery was clamped for 15 or 45 min in Sprague-Dawley rats. In other experimental groups, 45 min of renal arterial clamping was followed by 1 or 3 h of reperfusion. Controls were procured without any preceding intervention. F-actin was labeled with either fluorescein or Texas red-conjugated phalloidin. Serial optical sections were collected by confocal microscopy, and image volumes were rendered three dimensionally. The degree of cytoskeletal damage in the vasculature was assessed by semiquantitative scoring of the staining for F-actin. Disorganization/disarray of F-actin, reflected by disruption and clumping of the actin filaments, was observed in arteries, arterioles, and the vascular space of the kidney after ischemia or ischemia-reperfusion. Smooth muscle cells from arteries and arterioles showed significant damage to F-actin after either 15 or 45 min of ischemia in a duration-dependent manner. The actin cytoskeleton tended to recover from damage 45 min of ischemia 1 and 3 h after reperfusion. The vascular space did not demonstrate significant damage to F-actin after 15- or 45-min ischemia. However, significant damage to the vascular space was manifest 3 h after the reperfusion following 45 min of ischemia. In summary, disorganization/disarray of F-actin in vascular smooth muscle cells of the kidney was observed after ischemia or ischemia-reperfusion. A similar finding was observed in cultured vascular smooth muscle cells. We suggest that this disorganization of the actin cytoskeleton may play a contributory role in the loss of autoregulation of renal blood flow and the aberrant vascular reactivity in postischemic ARF.

METHODS

Animal model and surgery. Anesthetized male Sprague-Dawley rats (200–250 g) were subjected to renal ischemia by clamping of the renal pedicle for either 15 or 45 min as
previously reported (24). In separate groups of rats, 45 min of renal arterial clamping was followed by 1 or 3 h of reperfusion. Control kidneys were procured without any preceding operative procedures.

**Tissue preparation for confocal fluorescence microscopy.** Kidneys from a control and four experimental groups (I-15, I-45, I-45R-60, I-45R-180) were fixed by perfusion with 4% paraformaldehyde in PBS. The kidneys were divided into four pieces and kept in the above fixative at 4°C for at least 24 h and then transferred to 0.25% paraformaldehyde in PBS. Kidneys were stored in this buffer at 4°C until they were sectioned.

**Immunofluorescence staining.** Fixed kidney tissue was cut into 60-μm-thick sections in 1 × PBS using a vibratome. Sections were incubated in blocking buffer (PBS containing 2% defatted bovine serum albumin and 0.1% Triton X-100) for 2 h. The sections were then incubated overnight with the blocking buffer containing fluorescein or Texas red-conjugated phalloidin (Molecular Probes, Eugene, OR) at a dilution of 1:300. The sections were washed in the blocking buffer for 4–6 h. Each step of the incubation and washing was done on a rotating stand at room temperature. The tissue sections were then mounted onto glass slides with glass coverslips in PBS containing 50% glycerol and viewed using an MRC-1024 confocal microscope (Bio-Rad, Hercules, CA) with a ×60 water objective lens.

**Assessment of renal vasculature smooth muscle cell cytoskeletal disruption.** To evaluate for structural damage to the renal vasculature, serial images were taken at 0.4-μm intervals throughout the 60-μm depth of tissue sections using single-photon confocal microscopy, and 3D reconstructions of these images were generated with Confocal Assistant (written by Todd Clark Brelje). The degree of cytoskeletal damage in vascular smooth muscle cells was assessed by semiquantitative scoring of staining for F-actin. Five blinded observers scored each 3D rotating video image using the following criteria: 0, “intact” filamentous structures in most cells (rare or no “damaged” fibrils); 1 (mild), more intact filamentous structure than damaged ones; 2 (moderate), more damaged structure than intact ones; and 3 (severe), damaged structures in most cells (few intact fibrils). Intact was defined as discernable, distinctly filamentous, or fibrillar structures, and damaged referred to disorganized, disarrayed, aggregated, clumped, or indiscernible structures. Staining patterns for F-actin showing respective degrees of damage are illustrated in Figs. 1, 2, and 3. Vasa rectae are often difficult to be distinguished from thin limbs of Henle in the inner stripe of the outer medulla and inner medulla. This is even true when double staining is done with a marker for endothelial cells, such as von Willebrand factor antibodies or *Ulex europaeus* agglutinin, because these structures are in fine layers adjacent to each other. To avoid this potential obstacle, in >90% of cases vasa recta staining was assessed only in areas of dense outer medullary capillary networks of vascular bundles in the outer stripe of the outer medulla.

**ATP depletion and repletion in cultured vascular smooth muscle cells.** Vascular smooth muscle cells were obtained by primary culture of bovine carotid artery. The cells were cultured in minimum essential Eagle’s medium (M 4655, Sigma) at 37°C under 5% CO₂. After reaching confluency,
cells were divided and placed on coverslips in a 12-well plate (22.2 mm in diameter for each well) and incubated in the same media for 5 days. Then, cells were washed twice with PBS. ATP was depleted by incubating cells in media depleted of glucose, serum, or amino acid and containing 0.1 μM antimycin A for 15 and 45 min. One set of cells depleted of ATP for 45 min was washed in PBS and incubated in minimum essential Eagle's medium for 3 h to replete ATP levels. Control cells were not treated with antimycin A-containing media. After each treatment, cells were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Cells were extracted by 0.1% Triton X-100 in PBS for 10 min. Then cells were blocked by 2% BSA in PBS for 30 min. Cells on coverslips were incubated overnight with 2% BSA containing Texas red-conjugated phalloidin (Molecular Probes). After each step, cells were washed three times in PBS. Nuclei were stained with 4,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes). Cells were then mounted in PBS containing 50% glycerol and 0.5% 1,4-diazabicyclo(2,2,2)octane to retard photobleaching (Sigma, St. Louis, MO) and viewed using a MRC-1024 confocal microscope (Bio-Rad) with a ×60, 1.2 numerical aperture water objective lens. Images were taken using zoom factor 1, 1.5, or 2.

SDS-PAGE and immunoblotting of smooth muscle cell actin. Supernatants and pellets from cultured vascular smooth muscle cells of bovine carotid artery were obtained after treatment by 0.1% Triton X-100 and electrophoresed using precast Tris-glycine gels containing 2.6% bis-acrylamide (Novex). Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) in a buffer containing 20% methanol, 0.1% SDS, 190 mM glycine, and 25 mM Tris. The membrane was blocked for 30 min in a wash buffer (0.1 M NaCl, 0.01 M Tris·HCl, and 0.05% Tween 20, pH 7.5) containing 10% newborn calf serum. The membrane was incubated overnight in mouse anti-actin monoclonal antibody (clone C4, Chemicon) at a dilution of 1:3,000 in the blocking buffer at 4°C. The membrane was washed three times for 5 min each in wash buffer and incubated in goat anti-mouse horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL) at 1:100,000. It was washed five times in wash buffer and then detected on Kodak BioMax ML film with enhanced chemiluminescence (Pierce, Rockford, IL). The film was scanned, and bands were quantified in a densitometer to determine the actin content of each sample. The amount of actin in the pellet and supernatant per microgram of protein was calculated to represent F-actin and G-actin, respectively. Percentages of F- and G-actin in each sample were analyzed among experimental conditions. Each ATP depletion experiment was done in triplicate.

Statistical analysis. Statistical significance was evaluated by one-way ANOVA with Dunnett’s post hoc multiple comparisons test. Statistical analysis was carried out using the SPSS 10.0 software. Results are expressed as means ± SE.

RESULTS

F-actin staining in the arterial vascular tree in kidney tissue sections from control rats demonstrated distinct, circumferential filamentous or fibrillar struc-
tures in vascular smooth muscle cells of arteries and arterioles (score of 0 in Figs. 1 and 2) and mural cells or pericytes of the vasa rectae (score of 0 in Fig. 3). Disorganization or disarray of F-actin, reflected by disruption and/or clumping of phalloidin-labeled filaments, was prominent in arteries, arterioles, and the vasa rectae of kidneys after ischemia or ischemia-reperfusion (scores of 1, 2, and 3 in Figs. 1, 2, and 3.).

**Effect of ischemia and ischemia-reperfusion on renal arterial smooth muscle cell actin cytoskeleton.** As shown in Table 1 and Fig. 4, arteries in the kidney sustained significant damage to vascular smooth muscle cell F-actin after 15 and 45 min of ischemia compared with control (1.73 ± 0.66 and 2.31 ± 0.48 vs. 0.78 ± 0.81, respectively; *P < 0.005*). The severity of damage tended to be duration dependent. After 1 and 3 h of reperfusion, after 45 min of ischemia, the damaged arteries showed a tendency to recover. The scorings of the reperfusion groups (*I*-45R-60 and *I*-45R-180) were not significantly different from the control values. Figure 4 illustrates the average scoring by five blinded observers of each 3D image in each group. The median scores were 0.5, 1.5, 2.4, 1.2, and 1.6 in control and experimental groups *I*-15, *I*-45, *I*-45R-60, and *I*-45R-180, respectively. These data indicate that ischemia induces significant damage to the actin cytoskeleton of the arterial vascular smooth muscle cells in the kidney and that the damaged arteries tend to recover during reperfusion.

**Effect of ischemia and ischemia-reperfusion on renal arteriolar smooth muscle cell actin cytoskeleton.** In untreated rats, the scoring of F-actin integrity in arteriolar smooth muscle cells was higher than those of arteries (1.21 ± 0.87 vs. 0.78 ± 0.81) but was without

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**Table 1. Semiquantitative scoring of renal vasculature**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 2)</th>
<th>I-15 (n = 3)</th>
<th>I-45 (n = 3)</th>
<th>I-45R-60 (n = 3)</th>
<th>I-45R-180 (n = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arteries</td>
<td>0.78 ± 0.81 (27)</td>
<td>1.73 ± 0.66* (15)</td>
<td>2.31 ± 0.48* (12)</td>
<td>1.58 ± 1.02 (7)</td>
<td>1.55 ± 0.64 (8)</td>
</tr>
<tr>
<td>Arterioles</td>
<td>1.21 ± 0.87 (74)</td>
<td>1.87 ± 0.74* (38)</td>
<td>2.39 ± 0.52* (28)</td>
<td>1.36 ± 0.64† (24)</td>
<td>1.78 ± 0.68 (25)</td>
</tr>
<tr>
<td>Vasa rectae</td>
<td>1.07 ± 0.71 (56)</td>
<td>1.87 ± 0.67 (12)</td>
<td>1.35 ± 0.70 (57)</td>
<td>1.08 ± 0.54‡ (12)</td>
<td>2.28 ± 0.55‡ (8)</td>
</tr>
</tbody>
</table>

Values are means ± SE with nos. of images in parentheses. n, No. of rats; *I*-15, *I*-45, *I*-45R-60, and *I*-45R-180: groups subjected to 15-min ischemia, 45-min ischemia, and 60- and 180-min reperfusion after 45-min ischemia, respectively. *P < 0.005 vs. control. †P < 0.005 vs. *I*-45. ‡P < 0.005 vs. *I*-45R-180.
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Fig. 4. Box plot of semiquantitative scoring of damage to actin cytoskeleton in renal arteries. I-15, I-45, I-45R-60, and I-45R-180: experimental groups of rats subjected to 15-min ischemia, 45-min ischemia, 60-min reperfusion after 45-min ischemia, and 180-min reperfusion after 45-min ischemia, respectively. *P < 0.005 vs. control.

statistical significance (Table 1). However, the trend of the damage to arterioles after ischemia and ischemia-reperfusion was similar to that in arteries. As shown in Table 1 and Fig. 5, arteriolar smooth muscle cell F-actin sustained significant damage after 15 and 45 min of ischemia compared with control (1.87 ± 0.74 and 2.39 ± 0.52 vs. 1.21 ± 0.87, respectively; P < 0.005). The severity of damage tended to be duration dependent. After 1 and 3 h of reperfusion, after 45 min of ischemia, the level of actin damage was reduced. The severity scoring of renal arterioles in group I-45R-60 was significantly lower than that in group I-45 (1.36 ± 0.64 vs. 2.39 ± 0.52; P < 0.005) and did not differ from that of control (1.36 ± 0.64 vs. 1.21 ± 0.87). The scoring in group I-45R-180 was not statistically significant from control (1.78 ± 0.68 vs. 1.21 ± 0.87). Figure 5 shows a box plot of the average scoring by five blinded observers of each 3D image in each group. Median values are 1, 1.9, 2.5, 1.2, and 2 in control and experimental groups I-15, I-45, I-45R-60, and I-45R-180, respectively. These data indicate that ischemia induces significant damage to the actin cytoskeleton of the arteriolar smooth muscle cells in the kidney and that the damaged arterioles recovered during reperfusion.

Effect of ischemia and ischemia-reperfusion on vasa rectae actin cytoskeleton. The vasa rectae were difficult to image and score consistently. In untreated kidneys, scoring of vasa rectae F-actin was 1.07 ± 0.71 compared with 0.78 ± 0.81 and 1.21 ± 0.87 for arteries and arterioles, respectively. Scoring values for arteries, arterioles, and the vasa rectae under physiological conditions were not significantly different. As shown in Table 1 and Fig. 6, there was no statistically significant difference noted in the scoring of the damage in groups I-15, I-45, and I-45R-60 compared with the untreated kidneys (1.87 ± 0.67, 1.35 ± 0.70, and 1.08 ± 0.54 vs. 1.07 ± 0.71, respectively). However, significant damage to F-actin in mural cells or pericytes of the vasa rectae was noted in group I-45R-180 compared with either the control group or group I-45R-60 (2.28 ± 0.55 vs. 1.07 ± 0.71, P < 0.005; 2.28 ± 0.55 vs. 1.08 ± 0.54, P < 0.005).

Effect of ATP depletion and repletion in cultured vascular smooth muscle cells. Under physiological conditions, F-actin was primarily present in the form of stress fibers. In contrast to the organized filamentous structure of smooth muscle cell actin under physiological conditions, actin filaments were disorganized and clumped and the cells were retracted after ATP depletion. The severity of disorganization was duration dependent, with marked changes being noted after only 15 min of ATP depletion (Fig. 7B). After 3 h of ATP repletion, after 45 min of ATP depletion, actin filaments demonstrated a fairly organized pattern similar to that seen under physiological conditions. Therefore, this was a reversible alteration.

SDS-PAGE and immunoblotting of smooth muscle cell actin. As shown in Table 2, with ATP depletion the relative proportion of the vascular smooth muscle cell F-actin content increased compared with that in the

Fig. 5. Box plot of semiquantitative scoring of damage to actin cytoskeleton in arterioles in the kidney. *P < 0.005 vs. control. **P < 0.005 group I-45 vs. group I-45R-60.

Fig. 6. Box plot of semiquantitative scoring of damage to actin cytoskeleton in vasa rectae of the kidney. *P < 0.005 vs. control. **P < 0.005 group I-45R-60 vs. group I-45R-180.
untreated cells. Correspondingly, G-actin decreased in ATP-depleted cells. This is consistent with previous findings in epithelial and endothelial cells (15, 16, 22, 26, 29).

DISCUSSION

In the present study, we examined alterations in F-actin in vascular smooth muscle cells and pericytes of the renal arterial vasculature after ischemia and after ischemia-reperfusion. With light microscopy, gross abnormalities are not usually detected in blood vessels of the kidney after an ischemic insult, even when physiological abnormalities of renal vasculature, such as increased vascular tone and impaired autoregulatory ability, are prominent. At the ultrastructural level, myonecrosis of renal arteries and arterioles has been observed in rats with ischemic ARF (8).

Conger et al. (5–7, 9) have reported a series of studies of vascular physiology after an ischemic insult, suggesting intrinsically abnormal smooth muscle cells in this condition.

The actin cytoskeleton is essential for maintaining the structural and functional integrity of cells. The involvement of the actin cytoskeleton in numerous cellular processes has been studied extensively, especially in epithelial cells (22, 23, 26). Actin is known to be critical in maintaining polarity, which is essential for proper localization and function of various vital proteins such as Na+/K+-ATPase, tight junction proteins, and cell-cell adhesion molecules (22, 23). Disorganization and abnormal distribution of F-actin have been shown in renal epithelial cells after ischemia or ATP depletion with recovery after ATP repletion (26, 29). Similar changes in the actin cytoskeleton have also been reported in cultured endothelial cells after ATP depletion and repletion (15, 16). The question remained as to whether similar actin cytoskeletal changes occurred in vascular smooth muscle cells and, if so, whether they mediated altered reactivity.

Table 2. Proportion of F-actin and G-actin in cultured smooth muscle cells after ATP depletion

<table>
<thead>
<tr>
<th>ATP depletion</th>
<th>F-Actin</th>
<th>G-Actin</th>
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<tbody>
<tr>
<td>Control</td>
<td>77.6</td>
<td>22.4</td>
</tr>
<tr>
<td>5 min</td>
<td>89.6</td>
<td>10.4</td>
</tr>
<tr>
<td>15 min</td>
<td>87.7</td>
<td>12.3</td>
</tr>
<tr>
<td>30 min</td>
<td>91.2</td>
<td>8.8</td>
</tr>
<tr>
<td>60 min</td>
<td>88.8</td>
<td>11.2</td>
</tr>
</tbody>
</table>

Values are expressed as % of total actin.
In this study, F-actin in arteries, arterioles, and vasa rectae in renal tissue sections from rats was analyzed by confocal fluorescence microscopy using fluorophore-conjugated phalloidin. The degree of damage to each vascular structure was assessed on 3D reconstruction of serial optical slices of the tissue sections captured by confocal microscopy. This was required as changes in the actin cytoskeleton were difficult to detect in single cross-sectional images. Disorganization of F-actin, reflected by disruption and/or clumping of the filamentous structures, was observed in arteries and arterioles from renal tissues with sustained ischemia. The severity of the alteration in F-actin was duration dependent. The observed alterations tended to reverse with 1 and 3 h of reperfusion. However, in the vasa rectae, the disorganization of F-actin was only observed 3 h after reperfusion after ischemia.

These findings indicate that smooth muscle cells in arteries and arterioles of the kidney are susceptible to ischemic injury. In contrast, mural cells or pericytes in vasa rectae seemed more vulnerable to ischemia-reperfusion injury than an ischemic insult. Pericytes containing actins, not smooth muscle cells, are known to be present in vasa rectae of rabbits (12, 14). It is not known whether vascular smooth muscle cells are present in vasa rectae of rats. However, assuming the same cellular composition of renal vasculature in rats as in rabbits, we speculate that pericytes may differ in their response to an ischemic insult. These data also suggest that damage to vasa rectae may result from increased vascular tone initiated with the damage to the resistance vasculature, i.e., arteries and arterioles, further jeopardizing the blood supply to the vasa rectae region.

Decreased renal blood flow associated with increased renovascular resistance and impaired autoregulation has been reported in rodents up to 1 wk after an initial ischemic insult and in human cadaveric renal allografts with sustained ARF 1 h after reperfusion (1, 2, 8, 10, 11, 20, 27). In the present study, the actin cytoskeleton in smooth muscle cells of arteries and arterioles tended to recover from the morphological damage from ischemia 1 and 3 h after reperfusion. The timing of the physiological abnormalities seems to extend further beyond the time of the most drastic cytoskeletal damage to resistance vessels.

Activation of tubuloglomerular feedback by increased delivery of sodium to the macula densa due to damage to proximal tubule cells has been suggested as a mechanism resulting in afferent arteriolar vasoconstriction in cadaveric renal allografts with sustained ARF from ischemic injury (17). However, the vascular pathophysiology precipitating increased renovascular resistance and the ensuing decreases in renal blood flow in ischemic ARF is not clear.

Factors affecting vascular tone can be intrinsic and extrinsic. Extrinsic factors such as angiotensin II, endothelin-1, and other vasoconstrictor agonists, including thromboxan A2, prostaglandin H2, adenosine, platelet-activating factor, and leukotrienes, have been suggested to play a role in reduced renal blood flow in ischemic kidneys (3, 4, 13, 18, 19, 25). However, failure of the antagonists blocking or decreasing the production of the vasoactive agents, except for the endothelin A receptor antagonist, to improve the glomerular filtration rate may suggest that intrinsic factors contribute more to the vasoconstriction induced in ischemic ARF (4–6, 13, 28). In human cadaveric renal allografts with sustained postischemic injury, adequate density of glomerular atrial natriuretic peptide receptors and enhanced cGMP generation have been reported, suggesting that constricted afferent arterioles are unresponsive to the vasorelaxant action of endogenous atrial natriuretic peptide in this form of postischemic ARF (27). With an attenuated response to endothelium-derived relaxing factor-dependent dilators such as acetylcholine and bradykinin and increased vasoconstrictor sensitivity to the nitric oxide synthase inhibitor N\(^{\text{G}}\)-nitro-L-arginine methyl ester reported, it has been speculated that constitutive nitric oxide synthase activity and nitric oxide production may be maximal rather than decreased and cannot be further stimulated by endothelium-dependent vasodilators (5).

Intrinsic factors resulting in low blood flow may be narrowing of the lumen due to swelling of the vascular wall and extraluminal compression associated with perivascular edema, vasospasm associated with smooth muscle \(\text{Ca}^{2+}\) entry, and a series of cellular physiological events and structural alterations in smooth muscle cells (6, 21). The structural alterations in the actin cytoskeleton in smooth muscle cells after ischemia had not been previously elucidated. The actin cytoskeleton is known to play an essential role in maintaining the functional and structural integrity of cells. In the present study, disorganization of F-actin was demonstrated after ischemia in smooth muscle cells of resistance blood vessels, i.e., arteries and arterioles, in the kidney. This study also showed a similar finding in cultured vascular smooth muscle cells. This finding may indicate that ischemia induces disruption and subsequent aggregation of F-actin in vascular smooth muscle cells, incapacitating their ability to maintain normal vascular function in response to hormonal and neural regulation. This phenomenon could contribute to increased vascular tone resulting from the loss of normal distensibility of the vasculature in ischemic ARF.

We suggest that this disorganization of the actin cytoskeleton in smooth muscle cells in the renal vasculature may play a contributory role in the increased vascular tone, impaired autoregulation of renal blood flow, and aberrant vascular reactivity in postischemic ARF.

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REFERENCES


