Ischemia-induced cleavage of cadherins in NRK cells: evidence for a role of metalloproteinases

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The survival rate for acute renal failure (ARF) has not improved since the advent of dialysis and, in fact, mortality rates approach 30–50% (42). Although ischemia is a leading cause of ARF, the molecular targets leading to renal injury and failure are not completely understood. In ischemia-induced ARF, a loss of epithelial integrity and shedding of epithelial cells occur in the tubules. After injury, both viable and nonviable cells are shed, leaving the basement membrane as the only barrier between filtrate and interstitium (4a), which allows for backleak of the filtrate and intratubular obstruction from cellular casts (32). The loss of cell-cell attachments may occur via disruption of adherens junctions, which are composed of cadherin and catenin proteins. While evidence suggests that disruption of the cadherin/catenin complex occurs during ischemia (24), the mechanism underlying the loss of cadherin/catenin complex integrity remains unclear.

Adherens junctions are composed of cadherins and catenins and are essential for the formation and maintenance of functional tight junctions (7). Thus the loss of adherens junction integrity may lead to disruption of the normal epithelial barrier due to deficits in the functioning of multiple adhesive complexes (11). The cadherin gene superfamily encodes for transmembrane proteins that regulate Ca<sup>2+</sup>-dependent cell-cell adhesion (44), which most often requires intracellular association with catenins for adhesive activity. α-Catenin is linked to the cytoplasmic domain of cadherins via β- or γ-catenin; α-catenin does not directly bind to cadherins, but it is suggested that α-catenin links the cadherin/catenin complex to the cytoskeleton (28, 39). The renal expression of cadherin molecules is complex, with reports of at least eight cadherins present in the kidney (E-, K-, Ksp-, N-, OB-, P-, R-, VE-). OB-cadherin is a mesenchymal cadherin (35), whereas P-cadherin is expressed in glomeruli (6). The expression of K- and R-cadherin is mainly confined to the tubules in developing kidneys (6, 8, 10, 34), suggesting that E-, Ksp-, and N-cadherin are the primary cadherins expressed in the tubules of adult kidneys.

Several recent studies have examined the impact of simulated ischemia on the cadherin/catenin complex. Internalization of E-cadherin was seen in Madin-Darby canine kidney (MDCK) cells challenged with antymycin A (10 μM) and 2-deoxyglucose (10 mM) to deplete ATP (18). In addition, ATP depletion using a similar protocol was associated with a loss of full-length E-cadherin, and the generation of an 80-kDa fragment of E-cadherin in MDCK cells, which was not blocked by inhibitors of the proteasomal, lysosomal, or calpain proteolytic pathways (5). Importantly, ischemic rat kidneys demonstrated a reduction in E-cadherin protein levels (5). Taken together, the evidence indicates that ischemia disrupts adherens junctions; however, the mechanism of disruption is not known.

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes that are synthesized as zymogens, and once activated, are proteinases that regulate extracellular matrix (ECM) degradation (27, 29). Traditionally MMP substrates were thought to be limited to ECM components, but this view has changed with the discovery of non-ECM substrates, including cadherins. MMP-3 and MMP-7 can cleave E-cadherin, generating an 80-kDa extracellular fragment and a 40-kDa intracellular fragment (30). In addition, MMP-7 has been...
shown to mediate E-cadherin ectodomain shedding in injured lung epithelium (19). Induced expression of MMP-3 resulted in E-cadherin cleavage, which was blocked by GM-6001, an MMP inhibitor (17).

The objectives of this study were to adapt an in vitro model of ischemic injury to examine ischemia-induced disruption of the cadherin/catenin complex in normal rat kidney (NRK) cells and to determine a potential role for MMPs in this process.

MATERIALS AND METHODS

Cell culture. NRK-52E cells (ATCC, Gaithersburg, MD) were cultured on plastic dishes in Dulbecco’s modified Eagle’s medium containing 1.5 g/l sodium bicarbonate and 5% bovine serum in an atmosphere of 5% CO2-95% air at 37°C. At confluence (4–5 days), subcultures were prepared by treatment with 0.02% EDTA, 0.05% trypsin solution, and cells were seeded at a density of 4×10⁶ cells/cm². Cells were used between passages 3 and 20.

Simulated ischemia. Using a protocol adapted from Meldrum et al. (22), confluent cells were washed twice with PBS before the addition of PBS supplemented with 1.5 mM CaCl2 and 2 mM MgCl2. A layer (22), confluent cells were washed twice with PBS before the addition of PBS supplemented with 1.5 mM CaCl2 and 2 mM MgCl2. A layer of mineral oil (Sigma 400–5, St. Louis, MO) was added to the cell culture dish. For a 10-cm² dish, 2 ml of PBS with Ca2⁺ and Mg2⁺ and 10 ml of mineral oil were used. After 6 h of ischemia, cells were washed 5× with PBS and normal growth media was added to simulate reperfusion. To isolate conditioned PBS, the mineral oil and PBS with Ca2⁺ and Mg2⁺ were aspirated. Unless otherwise noted, control cells were incubated for 6 h in PBS with Ca2⁺ and Mg2⁺.

Western blot analysis. Cell culture plates were washed twice with PBS, scraped, and centrifuged. The supernatant was removed, and cell pellet was washed with cold PBS and then lysed in buffer (10 mM Tris-HCl, pH 7.6, 1% SDS, 1 mM PMSF, 1 mM leupeptin, 1 mM orthovanadate) and boiled for 10 min. The homogenates were spun at 18,000 g for 10 min, and the supernatant was collected. Proteins were quantified by the Bradford method and diluted to 1 μg/μl in 2× sample buffer (250 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.006% bromophenol blue). Samples were boiled for 5 min before electrophoresis, and 20 μg of protein were separated by 8, 12, or 15% SDS-PAGE. Separated proteins were transferred onto a Hybond-ECL nitrocellulose membrane (Amersham, Piscataway, NJ) in transfer buffer (25 mM Tris, 200 mM glycine, 20% methanol, and 1% SDS). Nonspecific binding was blocked by incubation with Tris-buffered saline plus Tween 20 (TBST) blocking buffer (0.1% Tween 20, 10 mM Tris, pH 7.5, 100 mM NaCl) supplemented with 5% nonfat dry milk for 1 h at room temperature. Primary antibodies (Table 1) were diluted in the same buffer and incubated at 4°C overnight. After subsequent washes with TBST, membranes were incubated with secondary antibody (1:20,000 in TBST, 5% nonfat dry milk) against the appropriate species for 1 h at room temperature. The blots were washed 3× in TBST, and proteins were detected with the Amersham ECL system and exposure to X-ray film (Kodak, Rochester, NY).

Immunofluorescence. Cells grown on Lab Tek Chamber Slides (Nunc, Rochester, NY) were washed twice with PBS, fixed in 2% paraformaldehyde for 10 min, washed two times in 0.02 M PBS for 10 min, and permeabilized in 1% Triton X-100 in 0.02 M PBS for 10 min. Slides were treated with 1:20 blocking solutions of serum related to the species in which the secondary antibody was generated at room temperature for 1 h. Primary antibodies were added at appropriate dilutions overnight. After washing (0.3% Tween in 0.02 M PBS; PBST), FITC-conjugated secondary antibodies (1:200) were added and sections were incubated in the dark at room temperature for 1 h. Slides were mounted with antifade media ( Molecular Probes, Eugene, OR) following several washes. Immunostained slides were visualized with a Zeiss Axioplan 2 microscope (Zeiss, Thornwood, NY) fitted with an Axiocam HR digital camera and Axiovision 3.0 software. Negative controls involved substituting IgG for primary antibodies and appropriate species serum for secondary antibodies. The actin cytoskeleton was detected using Alexa Fluor 488-phalloidin (Molecular Probes) staining. A Hypoxyprobe Kit-1 (Chemicon, Temecula, CA) was used to detect ischemia using the manufacturer’s protocol.

ATP levels. ATP levels were detected using an ATP assay kit based on luminescence (Calbiochem, La Jolla, CA) according to the manufacturer’s instructions.

Aggregation assay. The aggregation assay developed for cadherins, where calcium is present during trypsin treatment to preserve cadherin function, was utilized (9). Normal and ischemic NRK cells were washed with PBS containing 1.5 mM CaCl₂ and 2 mM MgCl₂, before the addition of 0.025% trypsin with Ca2⁺ and Mg2⁺ for 10–15 min at 37°C in the presence of 5% CO2. Cells were collected and resuspended at 1.0×10⁶ cells/ml in either PBS with 1 mM EDTA or PBS with Ca2⁺ and Mg2⁺. From this suspension, 1.5 ml were placed into a 12.5-mm-diameter well coated with 1.5% agarose in PBS. Cells were incubated in a rotary shaker at 80 rpm and 37°C for 20 and 60 min. Aggregated cells were counted manually in a hemacytometer.

Inhibitors. All inhibitors were added to PBS with Ca2⁺ and Mg2⁺ 30 min before ischemia. Chemical MMP inhibitors GM-6001 (Calbiochem, San Diego, CA) and TAPI-O (Peptides International, Louisville, KY) were dissolved in DMSO, and the desired concentration was added. Recombinant tissue inhibitors of metalloproteinase (TIMP)-1, TIMP-2, and TIMP-3 (Chemicon) were solubilized at 100 μg/ml in 1 mg/ml of BSA in PBS with Ca2⁺ and Mg2⁺. BSA (1 ml/ml) was added to PBS with Ca2⁺ and Mg2⁺ in control cells for recombinant TIMP experiments. NRK cells were transfected with 50–100 multiplicity of infection (MOI) of green fluorescent protein or TIMP-3 adenoviruses, constructed as previously described (4, 12).

Statistics. Data are expressed as means ± SE. Groups were compared using ANOVA followed by the Bonferroni post hoc test. P < 0.05 was defined as significant.

RESULTS

Table 1. Antibodies

<table>
<thead>
<tr>
<th>Source</th>
<th>Species</th>
<th>WB</th>
<th>IF</th>
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<tr>
<td>Primary</td>
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<tr>
<td>E-cadherin</td>
<td>Transduction</td>
<td>Mouse</td>
<td>1:2,000</td>
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<tr>
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<td>Santa Cruz</td>
<td>Rabbit</td>
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<tr>
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<td>Transduction</td>
<td>Mouse</td>
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<tr>
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<td>Transduction</td>
<td>Mouse</td>
<td>1:2,000</td>
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<tr>
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<td>Transduction</td>
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<td>p120-Catenin</td>
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<td>Mouse</td>
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<td>Rabbit</td>
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</tr>
<tr>
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<td>Rabbit</td>
<td>1:1,000</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Cell Signaling</td>
<td>Rabbit</td>
<td>1:1,000</td>
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<tr>
<td>GLUT-1</td>
<td>Chemicon</td>
<td>Rabbit</td>
<td>1:1,000</td>
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The source and specificity of the antibodies used in these studies and the dilutions used for the various assays are shown. WB, Western blot analysis; IF, immunofluorescence; GLUT-1, glucose transporter-1.

Protein expression of cadherins/catenins in ischemic NRK cells. We used a model developed by Meldrum et al. (22) that closely parallels in vivo renal ischemia, in which mineral oil is used to simulate ischemia by restricting exposure to oxygen and metabolite washout. Based on the requirement for Ca2⁺ in cadherin function, we modified the model by using PBS...
supplemented with Ca\(^{2+}\)/H\(_{11001}\) and Mg\(^{2+}\)/H\(_{11001}\) and overlayered with mineral oil to induce ischemia as opposed to a direct overlayering of the confluent cells with mineral oil. Protein expression of cadherins and catenins in ischemic NRK cells was evaluated by Western blot analysis. Using an antibody targeting the cytoplasmic domain of E-cadherin (Transduction Laboratories), cleavage of E-cadherin to a 40-kDa fragment was seen after 3 h of ischemia, and full-length (120 kDa) E-cadherin was lost following 6 h of ischemia (Fig. 1A). Although no fragmentation of N-cadherin was detected, N-cadherin expression began to decrease after 3 h and was virtually lost following 5 h of ischemia (Fig. 1A). Cadherin expression was stable in NRK cells incubated for 6 h in PBS supplemented with Ca\(^{2+}\)/H\(_{11001}\) and Mg\(^{2+}\) (Fig. 1A), suggesting that the loss of protein expression was due to the ischemic insult, i.e., the mineral oil overlay. However, the simulated mineral oil overlay model includes substrate deprivation as well as oxygen restriction, both of which may be important. As such, a similar pattern of E-cadherin fragmentation (80- and 40-kDa fragments) was seen in ischemic NRK cells in which normal growth media was substituted for PBS with Ca\(^{2+}\) and Mg\(^{2+}\); however, the extent of loss of full-length E-cadherin was not as great (data not shown). Similar results were also seen in NRK cells placed in an Anaeropack system designed for anaerobic cell culture to simulate ischemia (14), suggesting that the fragmentation of E-cadherin and loss of N-cadherin occur in multiple models and is not an artifact of the mineral oil overlay model.

While there was no decrease in \(\beta\)-catenin over the ischemia time course, a slight decrease in expression of \(\alpha\)-catenin was detected, as well as a dramatic loss of \(\gamma\)-catenin protein expression at 6 h (Fig. 1A). To examine whether the 40-kDa E-cadherin fragment was generated by extracellular cleavage, the conditioned PBS was isolated from ischemic NRK cells. Using an antibody directed against the extracellular domain of E-cadherin (Santa Cruz Biotechnology), an 80-kDa E-cadherin fragment that is released from the cell surface in ischemic NRK cells was identified (Fig. 1B). A complete recovery of full-length E- and N-cadherin was seen after 24 and 48 h of reperfusion, demonstrating the reversible nature of the ischemic insult (Fig. 2).

**Ischemic model.** To confirm that the mineral oil overlay model induced ischemia and to compare the biochemical impact of the model with other in vitro systems used to simulate ischemia, the expression of two ischemia-inducible markers was assessed. First, utilizing the Hypoxyprobe Kit-1 (Chemicon), immunofluorescence was performed on ischemic NRK cells. Because pimonidazole forms long-lived adducts with thiol groups in proteins, peptides, and amino acids in ischemic

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**Fig. 1.** Impact of ischemia on cadherin/catenin protein expression in normal rat kidney (NRK) cells. Ischemia was induced in NRK cells by layering cells with PBS supplemented with 1.5 mM CaCl\(_2\) and 2 mM MgCl\(_2\) followed by mineral oil. **A:** lysates of ischemic NRK cells were prepared at the given times and separated by SDS-PAGE (8%). Following transfer, the blots were probed with monoclonal (E-, N-cadherin, \(\alpha\)-p120, \(\beta\)-, and \(\gamma\)-catenin) or polyclonal (\(\alpha\)-catenin) antibodies. **B:** conditioned PBS was isolated from hypoxic cells and separated by SDS-PAGE. Blots were probed with a polyclonal E-cadherin antibody targeted to the extracellular domain. Signals were detected by ECL and autoradiography. Similar results were seen in 10 and 5 replicate experiments, respectively. NRK, NRK cells in normal growth media (DMEM+5% bovine serum); CON, NRK cells incubated with PBS supplemented with Ca\(^{2+}\) and Mg\(^{2+}\) for 6 h.

**Fig. 2.** Impact of ischemia-reperfusion on E-cadherin and N-cadherin protein expression in NRK cells. Ischemia was induced in NRK cells by layering cells with PBS supplemented with 1.5 mM CaCl\(_2\) and 2 mM MgCl\(_2\) followed by mineral oil. After 6 h of ischemia, cells were washed 5\(\times\) with PBS and normal growth media was added for 24–48 h. NRK cell lysates were prepared at the given times, and Western blotting was performed as previously described. Similar results were seen in 5 replicate experiments.
cells, a monoclonal antibody was used to detect the pimonidazole adducts. In control NRK cells, there was no detection of pimonidazole adducts, but after 4–6 h of ischemia, specific staining with the probe was detected (Fig. 3A). To further verify the induction of ischemia, protein expression of an ischemia-inducible protein, the glucose transporter-1 (GLUT-1) (46), was measured. A time-dependent increase in GLUT-1 protein expression was seen in ischemic NRK cells (Fig. 3B).

After 2 h of ischemia, cellular levels of ATP decreased to 46.5 ± 2% of control and rapidly declined to 9.3 ± 0.8% after 6 h of ischemia (Fig. 3C). ATP levels were restored to 51.2 ± 1% of control after 2 h of reperfusion and returned to control levels following 24 h of reperfusion. The decrease in ATP levels is similar to the 90% decrease seen in cells treated with sodium cyanide (15). In addition, caspase activation was determined by Western blot analysis using antibodies for cleaved (i.e., activated) caspase 3 and 9. There was no detection of cleaved caspase 3 or 9 at 2–6 h of ischemia, and total protein expression of caspase 3 was not affected (Fig. 3D). In addition, cleavage of caspasas was observed at 12 h of ischemia, suggesting induction of apoptosis following prolonged ischemia (Fig. 3D). As expected, cleavage of both caspasas was seen in NRK cells treated with staurosporine. Taken together, these results demonstrate that this in vitro model of ischemia is associated with hypoxia, a transient decrease in cellular ATP levels that is reversible following reperfusion, and is not associated with activation of caspasas during the 6-h ischemic insult.

**Cadherin/catenin function.** Cadherin/catenin protein localization was evaluated by immunofluorescence microscopy. While normal NRK cells expressed E-cadherin at the plasma membrane, 4 h of ischemia resulted in a decrease in E-cadherin at the cell membrane, which was further decreased after 6 h (Fig. 4). N-cadherin expression in normal NRK cells, although patchy, is located at the cell membrane, but after 4 and 6 h of ischemia N-cadherin expression was lost. After 6 h of ischemia, there was a loss of p120, γ-, and α-catenin expression, whereas no change in the expression of β-catenin was observed (Fig. 4). Changes in the actin cytoskeleton were examined using Alexa Fluor 488-phalloidin staining. In normal NRK cells, actin filaments displayed staining that outlines the cell periphery as well as actin stress fibers. Ischemia resulted in disruption of the cytoskeleton, primarily observed as a decrease in the actin stress fibers (Fig. 4). However, there was no disruption in the actin at the cell periphery, as was seen when cells were treated with 10 μM cytochalasin D (data not shown).

NRK cells viewed by phase-contrast microscopy revealed characteristically closely packed polygon-shaped cells with little light transmitted between them (Fig. 5A). Following 6 h of ischemia, cells exhibited a loss of cell-cell adhesion and increased transmission of light at cell-cell boundaries, although cells remained attached to the growing surface (Fig. 5A). After 3 h of reperfusion, cells looked similar to control, which is indicative of cell recovery from ischemia. Using the classic aggregation assay developed for cadherins (9), the function of cadherins in ischemic NRK cells was examined (Fig. 5B). Control and ischemic NRK cells were allowed to aggregate for 20 and 60 min at 37°C while being shaken at 80 rpm. A significant decrease in cell aggregation induced by 6 h of ischemia was seen at both 20 (30 ± 3% of control) and 60 min (52 ± 4% of control) of cell aggregation, and this functional deficit was still present after 3 h of reperfusion. However, after 24 h of reperfusion cell aggregation returned to control values (Fig. 5B), which corresponds to the reexpression of full-length cadherins (Fig. 2).

**Ischemia-induced cleavage of cadherins: evidence for a role of MMPs.** To determine whether MMPs were involved in ischemia-induced disruption of cadherins, the MMP inhibitors GM-6001 and TAPI-O were added to the PBS with Ca²⁺ and Mg²⁺ before ischemia. Both 10 μM GM6001 and 50 μM of...
TAPI-O blocked E-cadherin fragmentation and loss of N-cadherin (Fig. 6), suggesting that MMPs play a role in ischemia-induced cleavage and loss of cadherins. Interestingly, an increase in N-cadherin expression was seen in NRK cells treated with GM-6001. In addition, recombinant TIMPs were used to inhibit MMP activity. TIMP-1 did not prevent ischemic cleavage and/or loss of E- or N-cadherin (Fig. 7A). Although TIMP-2 protected full-length E-cadherin, there was a 40-kDa fragment of E-cadherin present and a loss of N-cadherin expression. In contrast, TIMP-3 inhibited both cleavage and/or loss of E- and N-cadherin expression (Fig. 7A). Similar inhibitory activity was also seen in NRK cells transfected with a TIMP-3 adenovirus but not the green fluorescent protein control (Fig. 7B). These data collectively indicate the involvement of a membrane-associated metalloproteinase in E- and N-cadherin cleavage following ischemic insult in NRK cells.

**DISCUSSION**

Because ischemia is a leading cause of ARF, investigating the targets of ischemic insult will provide insight into the mechanisms underlying ARF. The cadherin/catenin complex, which is critical to renal proximal epithelial cell function, is disrupted by ischemic insult. In this study, an ischemic model, which resembles ischemic renal injury in vivo, resulted in selective fragmentation/loss of E-cadherin and loss of N-cadherin expression that could be blocked by MMP inhibitors.

Most in vitro ischemic models use chemicals to induce ischemia, such as ATP depletion with deoxy-D-glucose and antimycin A or sodium cyanide (5, 15). To avoid using chemicals that may have other nonspecific molecular targets and to mimic ischemic insult in vivo (oxygen/nutrient deprivation and metabolite accumulation), an alternative model for ischemia was employed in the present study using PBS supplemented with Ca\(^{2+}\) and Mg\(^{2+}\) overlaid with mineral oil. We have...
adapted this model for a renal cell line from Meldrum et al. (22); however, the mineral oil model was first used in isolated myocytes (13, 41). In the original and subsequent studies by Meldrum et al. (20–23), mineral oil is directly overlaid onto confluent LLC-PK1 cultures. For our studies of cadherin/ catenin expression and function, we removed the confounding factor of divalent cation removal by first using a thin layer of PBS supplemented with Ca^{2+}/H11001 and Mg^{2+}/H11001. The Meldrum group uses simulated ischemia, which is much shorter than the time period used in our studies. However, when mineral oil was directly added to NRK cultures, the fragmentation and loss of E-cadherin were seen at earlier time points (data not shown), suggesting that the supplemented PBS layer may be partially attenuating the ischemic insult. Consistent with this conclusion is the fact that replacement of the supplemented PBS with complete culture media was associated with E-cadherin fragmentation, but to a lesser extent than with PBS plus Ca^{2+} and Mg^{2+} (data not shown). In agreement with previous results.

**Fig. 5. Impact of ischemia-reperfusion on cadherin function in NRK cells.** A: morphology of control, 6-h ischemic, and 3-h reperfused NRK cells assessed by phase-contrast microscopy. The width of each field is 1,600 and 800 µm. B: after ischemia and reperfusion, cells were dissociated as described in MATERIALS AND METHODS and allowed to aggregate for 20 and 60 min. The number of cell aggregates was converted to %control (n = 10). Similar results were seen in 4 replicate experiments. ‡Significant difference (P < 0.05) from positive control cell aggregation values at 20 min. †Significant difference in cell aggregation values compared with positive control at 60 min.

**Fig. 6. Evidence for involvement of matrix metalloproteinases (MMPs) in ischemia-induced loss of cadherins.** Before ischemia, cells were incubated for 30 min in 10 µM GM-6001 or 50 µM TAPI-O. Lysates of ischemic NRK cells were prepared at the indicated times and Western blot analysis was performed. Similar results were seen in 4 replicate experiments. DMSO, NRK cells incubated with DMSO as a vehicle control.
using TNF-α as a marker of ischemia (22), the induction of ischemic markers pimonidazole adducts and GLUT-1 was seen in NRK cells. In addition, the decrease in ATP levels following 6 h of ischemia in this study is similar to the 90% decrease seen in cells treated with sodium cyanide (15).

Caspase 9 is activated on cytochrome c release from the mitochondria and further activates other caspases, such as caspase 3, to initiate a cascade leading to apoptosis (36). During the 6-h time course of ischemia, no evidence of caspase activation was detected; however, prolonged ischemia (12 h) resulted in caspase activation. While caspase 3 has been shown to cleave E-cadherin to generate a 24-kDa fragment (38), the lack of temporal correlation between caspase activation and cadherin loss suggests that this protease is not responsible for cadherin/catenin disruption in this system. Cleavage of E-cadherin and loss of N-cadherin occur before caspase activation, suggesting that disruption of the cadherin/catenin complex precedes ischemia-induced apoptosis. However, the possibility remains that significant apoptosis occurs during the reperfusion phase, as reported by Meldrum et al. (20–23).

Disruption of the cadherin/catenin complex at the cell membrane is concurrent with altered cell morphology, as evidenced by the loss of cell-cell contact after 6 h of ischemia. The integrity of intracellular junctions and polarity in epithelial cells depends on cadherin-mediated cell-cell contacts (2). Therefore, cadherin disruption and loss correlate with an altered phenotype in ischemic epithelial cells that includes, but is not limited to, the loss of cell polarity and shedding of tubular epithelial cells. Given the relationship between the cadherin/catenin complex and the actin cytoskeleton, we also examined the pattern of actin staining during ischemia. In normal NRK cells, actin filaments displayed a staining pattern that outlines the cell periphery as well as actin stress fibers, similar to that seen in previous studies (1). Six hours of ischemia resulted in disruption of actin stress fibers, but the junctional staining of actin was preserved. Similar results were seen in mouse proximal tubule cells treated with cyanide to induce chemical anoxia (16).

Disruption of cadherin/catenin integrity may also be associated with activation of the transcriptional activity of β-catenin (43). However, during ischemia or reperfusion β-catenin was not seen in the nucleus. Thus far, we have not been able to provide evidence for activation of β-catenin cell signaling during ischemia in our in vitro model. In contrast, ATP depletion with sodium cyanide induces translocation of β-catenin into the nucleus in opossum kidney cells (32). The discrepancy in experiments could be due to the different methods utilized to induce ischemia or the different cell lines used.

Fragmentation of N-cadherin is seen in apoptotic hepatic cells and proliferating vascular smooth muscle cells (26, 40); however, in our model ischemia was associated with a loss of N-cadherin protein expression without detectable fragments. It is possible that the antibody used in our studies cannot recognize the cleaved fragments. However, a panel of four commercially available antibodies did not detect fragments in ischemic NRK cells. The fact that MMP inhibition blocks the loss of N-cadherin suggests that this loss may be due to proteolytic cleavage. In addition, GM-6001 was associated with a significant increase in N-cadherin levels in control NRK cells, further supporting a role for MMPs in regulating N-cadherin expression.

Although rapid internalization of E-cadherin is seen in ATP-depleted cultured renal epithelial cells (18), a longer insult leads to proteolytic clipping of E-cadherin and disruption...
of the complex (5). Previous studies have shown a loss of E-cadherin in ischemia both in vivo and in vitro (5). ATP-depleted MDCK cells expressed an 80-kDa fragment and loss of full-length E-cadherin that could not be prevented with inhibitors of the proteasomal, lysosomal, or calpain-mediated proteolytic pathways (5). The 40-kDa E-cadherin fragment is believed to correspond to the cytoplasmic and transmembrane domain of the protein. Detection of the extracellular 80-kDa fragment of E-cadherin in conditioned PBS of ischemic NRK cells suggests that the cleavage event occurs extracellularly.

Our results suggest that MMPs play a key role in the cleavage and/or loss of E- and N-cadherin by inhibition with GM-6001 and TAPI-O. Recent data indicate that MMPs play a role in ischemic ARF, as MMPs were increased in rat postischemic kidney tissue and were localized to the renal tubules (3). In addition, MMPs are thought to be involved in ischemia-induced damage to other organs, such as the brain, lung, and heart (25, 33, 37, 45). Furthermore, this study has shown that TIMP-3 completely blocks both cleavage and/or loss of E- and N-cadherin, whereas TIMP-2 protects full-length E-cadherin protein expression but not loss of N-cadherin. These differences imply that different MMP(s) may play a role in E- and N-cadherin regulation. This hypothesis is further supported by the finding that lower concentrations (10 μM) of TAPI-O blocked the loss of N-cadherin but did not attenuate E-cadherin cleavage and/or loss of E- and N-cadherin.

In summary, simulated in vitro ischemia was associated with fragmentation of E-cadherin and loss of N-cadherin before loss of α-, γ-, and p120 catenin. A loss of cell-cell contacts corresponds to the disruption of cell adhesion. Ischemia-induced cleavage and/or loss of E- and N-cadherin can be blocked by GM-6001 and TAPI-O, suggesting a potential role for MMPs. In addition, the present study shows that the MMP(s) responsible for ischemia-induced E-cadherin cleavage is inhibited by TIMP-2 and TIMP-3, and loss of N-cadherin is inhibited by TIMP-3, indicating a novel role for membrane-associated metalloproteases in these events. Future experiments will examine whether specific MMP inhibitors can decrease the pathological sequelae following ischemia-induced ARF.

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REFERENCES


