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Characterization of Ischemia-Induced Loss of Epithelial Polarity

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Summary. Total renal ischemia for various time intervals (0–50 min) resulted in the rapid and duration-dependent redistribution of polarized membrane lipids and proteins in renal proximal tubule cells. Following only 15 min of ischemia, apical membrane enrichment of NaK-ATPase, normally a basolateral membrane (BLM) enzyme, had increased (1.6 ± 0.6 *vs.* 2.9 ± 1.2, $P < 0.01$). In vivo histochemical localization of NaK-ATPase showed reaction product throughout the apical microvillar region. PTH-stimulatable adenylate cyclase, another BLM protein, was also found in ischemic but not control apical membrane fractions. One dimensional SDS-PAGE showed four bands, present in control BLM and ischemic apical membranes, which could not be found in control apical membrane fractions. Immunohistochemical localization of leucine aminopeptidase (LAP) showed the enzyme was limited to the apical domain in control cells. Following ischemic injury (50 min), LAP staining could be seen within the cell and along the BLM. Following 24 hr of reperfusion, the BLM distribution of LAP was further enhanced. With cellular recovery from ischemic injury (5 days), LAP was again only visualized in the apical membrane. Duration-dependent alterations in apical and BLM lipids were also observed. Apical sphingomyelin and phosphatidylserine and the cholesterol-to-phospholipid ratio decreased rapidly while apical phosphatidylcholine and phosphatidylinositol increased. Taken together, these results indicate renal ischemia causes rapid duration-dependent reversible loss of surface membrane polarity in proximal tubule cells.

Key Words ischemia · surface membrane polarity · phospholipids · NaK-ATPase · leucine aminopeptidase

Introduction

It is through the polarized distribution of ionic channels, transport proteins and lipids that vectorial transport of select compounds in epithelial cells is possible. Establishment and maintenance of epithelial polarity is therefore essential for normal cellular function. In renal proximal tubular cells, the apical and basolateral membrane segments are known to be functionally different with respect to numerous parameters including enzymes [6], hormone receptors [41], electrical resistance [12], membrane transporters [4], membrane lipids [9, 16, 33], and membrane fluidity [9, 16, 31]. We have recently reported [36] that ischemic injury, resulting in reversible acute renal failure, caused partial loss of renal proximal tubular cell surface membrane polarity. In these studies, large alterations in apical lipids and the redistribution of NaK-ATPase to the apical membrane were noted. Specifically, the sphingomyelin-to-phosphatidylcholine ratio decreased from 2.2 to 1.0, the cholesterol-to-phospholipid ratio from 0.8 to 0.6, and NaK-ATPase accumulated in the apical membrane.

The present studies were undertaken to further define ischemia-induced loss of epithelial polarity. Specifically, we wanted to determine the time course for loss of both lipid and protein polarity, whether other proteins move into the apical membrane during ischemic injury, and finally, whether apical protein markers and lipids move into the basolateral membrane during ischemic injury.

Materials and Methods

Treatment of Rats

Male Sprague-Dawley rats weighing 220–260 g were fasted overnight before induction of ischemia. Renal ischemia was induced, while under anesthesia with sodium pentobarbital (60 mg/kg body weight), by clamping (Schwartz clip) the renal pedicles after removal of the capsules [32, 36]. After placement of the clamp, a two-layer closure was carried out, and 1 ml of normal saline was injected intraperitoneally. To avoid any reperfusion, the clamp was left in place until the kidney was removed for processing. For reflow experiments, the clamps were removed and a two-layer closure was used. Rats were then maintained in warmed cages during a 4-hr recovery period.

Preparation and Characterization of Membrane Fractions

Renal cortical apical (brush border membrane, BBM) and basolateral membranes (BLM) were isolated simultaneously from the same cortical homogenate and characterized enzymatically as described in detail elsewhere [30–36]. Briefly, the procedure entailed rapid decapsulization and removal of thin cortical slices in chilled buffer (300 mM mannitol, 5 mM ethyleneglycol-bis [β-aminoethylether]-N, N'-tetraacetic acid, 18 mmol Tris [hydroxymethyl] aminomethane hydrochloride, 0.1 mM phenyl-methylsulfonyl fluoride) at pH 7.4. The slices from two kidneys were homogenized using a Polytron PT 200D (Brinkmann) in 15 ml of buffer, and Mg^{2+} precipitation (15 mmol/liter) was carried out for 20 min. The resulting solution was centrifuged for 15 min at 2,445 × g, the pellet (P_2) was saved for BLM isolation, while the supernatant was centrifuged at 48,000 × g for 30 min to obtain crude apical membranes. This pellet was resuspended using a Potter-Elvehjem homogenizer in 30 ml of diluted buffer (1 : 1 with deionized water) and taken through the Mg^{2+} precipitation process again.

To isolate the BLM fraction, P_2 was resuspended using a loose Dounce glass homogenizer followed by repeat addition of Mg^{2+} (15 mM) and then centrifugation at 2,445 × g for 15 min. The pellet (P_3) was resuspended in standard buffer, diluted with water (1 : 1), and centrifuged for 15 min at 755 × g. The supernatant was centrifuged for 30 min at 48,000 × g. The resulting pellet (P_5) was resuspended in 19 ml of 50% sucrose, overlaid with a discontinuous sucrose gradient using 41% (5 ml) and 37.5% (12 ml) in cellulose-acetate tubes, centrifuged at 88,000 × g at 4°C in a Beckman Model L8-70 ultracentrifuge for 3 hr and the top layer 37.5% was harvested.

Protein was measured according to Lowry et al. [26] using BSA as a standard. Determinations were carried out on alkaline phosphatase, leucine aminopeptidase, and NaK-ATPase as previously reported from our laboratory [30–36].

Statistical significance was determined using either a Student's t test or one-way ANOVA and reported as <0.05 or 0.01.

Assays of Adenylate Cyclase Activity

The activity of adenylate cyclase was measured according to a modification [22, 23] of the method of Morel et al. [37]. Whole homogenates, basolateral, and apical membrane fractions were washed and resuspended in cold hypo-osmotic solution (3 mM $MgCl_2$, 1 mM EDTA, 5 mM Tris-HCl, pH 7.4), and stored at −80°C until assayed.

The membranes (130–170 μg protein per assay) were incubated at 37°C for 15 min in a final volume of 50 μl containing 0.25 mM alpha-^{32}P-ATP (1 to 2 × 10^6 cpm/sample), 1 mM cAMP, 3.8 mM $MgCl_2$, 0.25 mM EDTA, 20 mM creatine phosphate (Calbiochem, San Diego, CA), 100 U/ml creatine phosphokinase (Calbiochem, San Diego, CA), and 100 mM Tris, pH 7.4, with or without addition of 10 IU/ml of parathyroid hormone (Sigma, St. Louis, MO) or 100 μM forskolin (Sigma, St. Louis, MO) in a 12 × 75 mm tube.

The reaction was stopped by addition of 150 μl cold stopping solution (3.3 mM ATP, 5 mM cAMP, 50 mM Tris HCl, pH 7.4, and ^{3}H-cAMP containing 1 × 10^4 cpm/sample to determine recovery), and 1 ml of distilled H_2O was added to each tube. Produced ^{32}P-cAMP was separated according to the method of Salomon [39] using Dowex 50-x4, 200–400 mesh (BIO RAD, Richmond, CA), and aluminum oxide (ICN, Cleveland, OH) columns, and the separated ^{32}P-cAMP and recovered ^{3}H-cAMP were counted in a liquid scintillation counter. Every assay was performed in triplicate. The enzyme activity was expressed as fmol cAMP produced/min/μg protein.

Protein Electrophoresis

One dimensional SDS-PAGE was carried out according to Laemmli [24] using 100 μg of membrane protein, a 3.5% stacking gel and 5–15% continuous gradient separating gel containing 5% SDS. Coomassie blue staining and high and low molecular weight standards (BIO-RAD Laboratories, Richmond, CA) were used for identification of membrane proteins.

Lipid Determinations

Lipids from approximately 1 mg of membrane protein were extracted in 6 ml of chloroform/methanol (1 : 2 vol/vol) isolated and quantitated as we have previously described [33, 34]. Total phospholipids were determined on an extract, according to Ames and Dubin [3]. Individual phospholipid species were separated by two-dimensional thin-layer chromatography on Kesilgel silica gel 60 plates, using the technique of Esko and Raetz [11]. Individual phospholipid species were identified by brief exposure to iodine vapor and scraped off the plates. A Bligh and Dyer [5] extraction was then carried out on each individual spot and P_i was determined by the method of Ames and Dubin [3] on an aliquot.

NaK-ATPase Cytochemistry

Tissue preparation for electron microscopic evaluation of cellular NaK-ATPase was conducted as previously described [10, 36]. Rat kidneys made ischemic by renal pedicle clamping and control kidneys were sliced (0.5–1 mm), and the cortex was fixed for 15 min at 4°C in 1% paraformaldehyde, 0.25% glutaraldehyde in 100 mM sodium cacodylate buffer containing 5% sucrose. The tissue fragments were then snap frozen on a precooled cryostat chuck. Frozen cryostat sections cut at 50 μm were incubated for 30 min at room temperature in media designed to localize NaK-ATPase [10]. This media was comprised of 100 mM Tris-HCl buffer (pH 9) containing 5% sucrose, 20 mM $MgCl_2$, 10 mM KCl, 20 mM $SrCl_2$, 5 mM sodium nitrophenyl phosphate as substrate, and 2.5 mM levamisole to inhibit alkaline phosphatase activity. Dimethylsulfoxide (25%) was added to some media as an activator [28]. Controls contained 10 mM ouabain, a specific inhibitor for NaK-ATPase. After incubation, tissue slices were washed four times for 5 min in Tris-HCl buffer, pH 9, at 4°C, post-coupled in 2% lead nitrate for two changes of 5 min, rinsed for 5 min each in Tris/HCL buffer, and post-fixed for 30 min in 1% osmium tetroxide in 100 mM sodium cacodylate buffer. After dehydration in graded ethanol and embedding in araldite, ultrathin sections were cut and viewed with a Philips 300 electron microscope either without post-staining or after staining with uranyl acetate or lead citrate.

Immunohistochemical Localization of Leucine Aminopeptidase Staining

Leucine aminopeptidase (LAP) was localized in renal cortical tissue by indirect immuno-peroxidase immuno-histochemistry as

Table 1. Effect of ischemia on apical marker enzyme enrichment

	Leucine aminopeptidase	Alkaline phosphatase	NaK-ATPase
Control	12.4 ± 3.3 (14)	10.0 ± 2.6 (11)	1.6 ± 0.6 (17)
Ischemia			
15 min	10.1 ± 2.7 (13)	9.7 ± 2.3 (11)	2.9 ± 1.2 (19)[A]
30 min	8.5 ± 3.2 (5)[a]	8.6 ± 2.0 (7)	3.4 ± 1.2 (11)[A]
50 min	7.5 ± 2.0 (5)[A]	8.6 ± 1.3 (7)	3.4 ± 0.7 (11)[A]

All values are the mean ± SD with the number of individual preparations analyzed shown in parenthesis (n). Statistical significance, as determined using one way ANOVA, is indicated by capital ($P < 0.01$) or lower case ($P < 0.05$) letters. Statistical significance was denoted between experimental and control groups by [A], between 15 and 30 and 15 and 50 min ischemic groups by [B], and between 30 and 50 min of ischemia by [C]. There was, however, no difference between any of the ischemic groups ([B], [C]). Apical membrane fractions from control and all ischemic intervals showed de-enrichment (enrichment <1) for the intracellular organelle-contaminating marker enzymes succinic dehydrogenase, cyctochrome c reductase and glucosaminadase. There was no statistical difference in contamination with intracellular organelle markers between control and ischemic membrane fractions.

described by Ahnen et al. [1] and Isobe et al. [18]. In brief, light microscopic localization of LAP was conducted using a monospecific polyclonal anti-LAP antibody (kindly provided by Dr. Gary Gary [2, 21]). Tissue was fixed in vivo by perfusion with saline followed by 25 ml of periodate-lysine-formaldehyde (PLP) [29]. Kidneys were rapidly removed, placed on ice, sliced in 1-mm cubes and further fixed for 3 hr in PLP at 4°C with constant stirring. The tissues were washed in Hales solution (30% sucrose and 1% Gum Arabic) [1, 2] at 4°C on a rocker platform for 24 hr with three exchanges of fresh solution. They were then embedded in O.C.T. compound (Miles Scientific, Naperville, IL) and frozen in a dry ice-alcohol slurry. Tissue sections (10–12 μm) were cut onto albumin coated slides, allowed to air dry and washed three times with phosphate-buffered saline (PBS). Endogenous peroxidase activity was inhibited with 0.01 M periodic acid followed by sodium borohydride (0.003 M) [30]. Normal sleep serum (1 : 250) was applied to the tissue sections (10 min, 24°C) to block nonspecific binding. After washing in PBS, rabbit anti-LAP antibodies were applied to the sections (1 hr, 24°C) (1 : 200). After washing, sheep anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1 : 75) were then applied to the sections (1 hr, 24°C). Slides were washed in PBS, developed in diaminobenzidine (DAB) (0.025%) solution containing hydrogen peroxide (0.005%) for 10 min, and then washed again in PBS. They were then dehydrated in alcohol, counterstained with methylene green and mounted with Permount. Control sections were incubated with normal rabbit serum (1 : 200) in place of the anti-LAP antiserum.

Electron microscopic localization of LAP was carried out according to Brown and Farquhar [7]. Kidneys were perfused fixed for 4–6 hr in PLP. Cortical cubes were then placed in 50 mmol NH_4Cl in PBS with three changes over 25 min, washed in PBS for 15 min, soaked in 10% DMSO in PBS for 1 hr at 4°C and snap frozen in a dry ice-alcohol bath. Tissue sections (10–20) were cut, placed in 1% filtered ovalbumin and incubated overnight in preimmune serum (1 : 200) or with the monospecific polyclonal anti-LAP antiserum (1 : 200). The sections were washed six times in PBS with 0.1% ovalbumin for 20 min, incubated for 1 hr with a sheep anti-rabbit horseradish peroxidase (HRP) conjugated IgG (1 : 50), washed six times and fixed in 1.5% glutaraldehyde and 0.1 M Na^+ cacodylate (pH 7.4) with 5% sucrose. Sections were then washed three times in 0.1 M Na^+ cacodylate (pH 7.4) with 7.5% sucrose, three times in 50 mmol Tris-HCl (pH 7.4) containing 7.5% sucrose and incubated in 0.2% DAB in Tris-HCl (pH 7.4) for 2–5 min. Hydrogen peroxide (0.01%) was added to the solution and incubated 15 min. Sections were washed three times in 50 mmol Tris-HCl (pH 7.2) with 7.5% sucrose, post fixed in 1% OsO_4 with 1% KFeCN in 0.1 M Na^+ cacodylate for 45 min at 4°C, washed in distilled H_2O, dehydrated, embedded in Epon, sectioned and viewed with a Philips CM-12 electron microscope.

Results

Apical marker enzyme enrichment in isolated membrane fractions is shown in Table 1. In control fractions, leucine aminopeptidase and alkaline phosphatase were enriched 12.4- and 10-fold with respect to the homogenate. NaK-ATPase, a basolateral membrane marker enzyme, was only enriched 1.6-fold, and these results are in agreement with our previous publications [30–34]. Ischemia caused a step-wise reduction in the enrichment of both leucine aminopeptidase and alkaline phosphatase, with only the former being statistically significant at the 30 and 50 min ischemic intervals. On the other hand, all levels of ischemic injury were shown to increase the amount of apical membrane NaK-ATPase ($P < 0.01$).

Finding NaK-ATPase in the apical membrane fraction following only 15 min of ischemia indicated that NaK-ATPase moved early during the ischemic insult. To verify the isolated membrane fraction results, in vivo cytochemical localization of NaK-ATPase was accomplished. In control specimens, the lead reaction product could be seen intracellularly but more abundantly along the basolateral membrane of intact cells (Fig. 1*A*). Following 15 min of ischemia, however, reaction product could now be seen lining the apical membrane microvilli as well as the BLM segments of the cells (Fig. 1*B*).

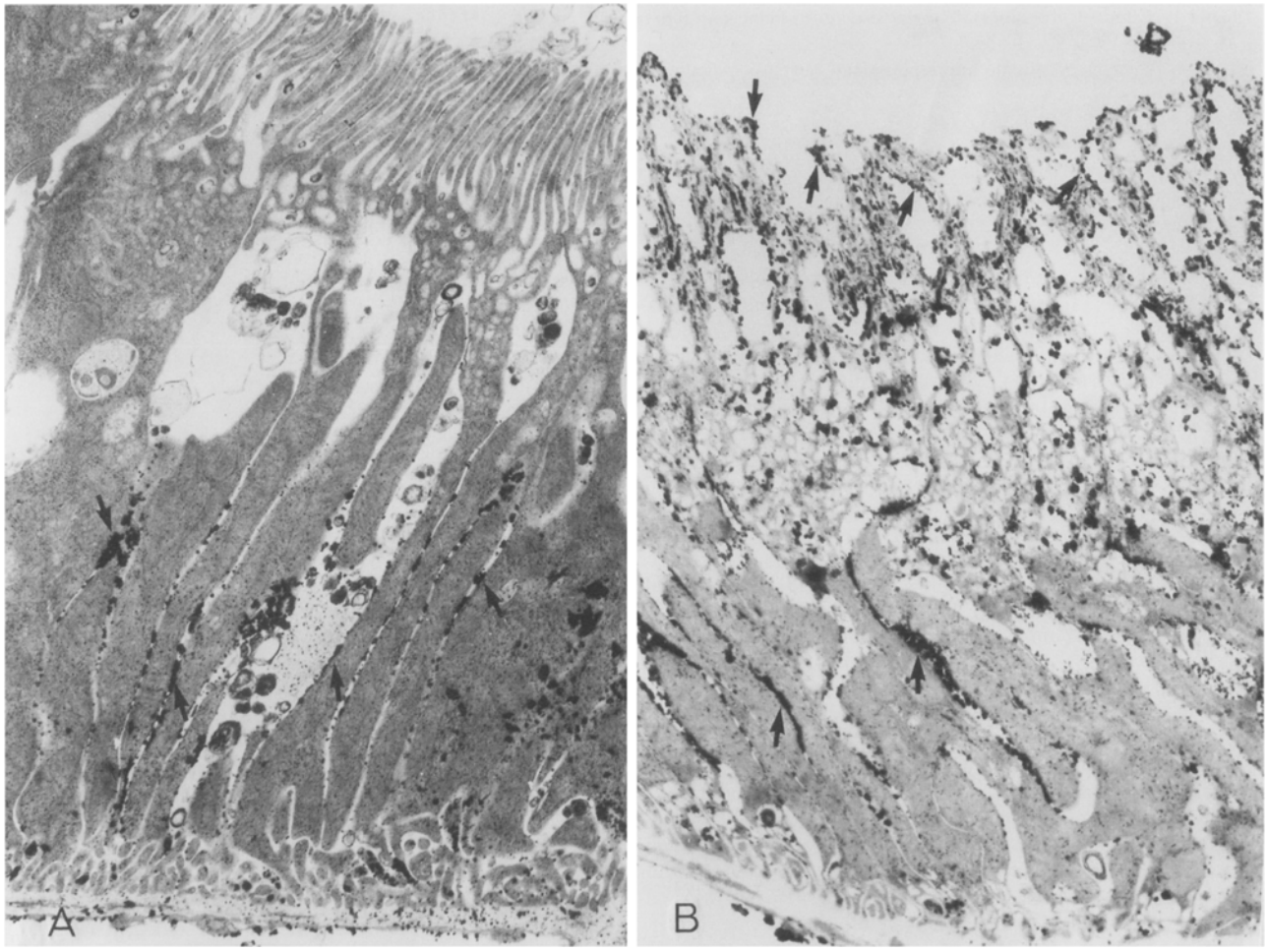

Fig. 1. Cytochemical localization of NaK-ATPase using the strontium method of Ernst [10]. Frozen sections of 1% paraformaldehyde-0.25% glutaraldehyde-fixed kidney cortex were incubated for 45 min in enzyme media. Reaction product formation was inhibited by the addition of ouabain to the medium as previously reported [6]. (*A*) NaK-ATPase in control proximal tubules. Electron-dense reaction product is seen on basolateral membrane infoldings (arrows). ×24,000. (*B*) After 15 min of ischemia, NaK-ATPase reaction product is seen at the apical surface as well as the BLM surface (arrows). ×24,000

To determine if basolateral membrane proteins, in addition to NaK-ATPase, could be found in the apical membrane following ischemia, two different approaches were undertaken. First, basal and PTH-responsive adenylate cyclase activity was assayed in control apical membrane fractions and apical membrane fractions following 50 min of ischemia. As shown in Table 2, basal adenylate cyclase activity was found in control apical fractions although a much greater specific activity was found in BLM fractions ($P < 0.01$). Stimulation of control apical adenylate cyclase was achieved with forskolin but not PTH, whereas BLM adenylate cyclase was stimulated by PTH. Following 50 min of ischemia, basal and forskolin-stimulated apical adenylate cyclase activity were unaltered. Parathyroid hormone, however, now stimulated ischemic apical membrane adenylate cyclase activity ($P < 0.01$).

One dimensional SDS-PAGE of apical membrane fractions following ischemia for variable durations and control basolateral membrane fractions showed the movement of four basolateral membrane protein bands into apical membrane fractions (Fig. 2). In addition, it should be noted that several selective basolateral membrane proteins could not be observed in apical membrane fractions following ischemic injury. These data indicate that some but not all basolateral membrane proteins move into the apical membrane fraction during ischemic injury. Finally, two bands were noted in ischemic BBM fractions but not in control BBM or BLM. Whether these bands were due to proteolysis of large molecular weight proteins was not determined.

To determine if the basolateral membrane polarity was altered during ischemia, the appearance of LAP, a specific apical marker, was evaluated using a mono-specific polyclonal anti-LAP antibody. As is shown in Fig. 3, leucine aminopeptidase was

Table 2. Effect of Ischemia on Apical adenylate cyclase activity[a]

	Adenylate cyclase activity (fmol/min/μg protein)		
	Basal	PTH	Forskolin
Control			
Apical	1.8 ± 0.4 (7)	1.9 ± 0.4 (3)	8.4 ± 2.4 (4)
BLM	8.8 ± 1.9 (3)	19.1 ± 3.9 (3)	
Ischemic			
Apical	1.6 ± 0.3 (7)	3.2 ± 0.2 (3)	6.9 ± 0.6 (4)
P value	NS	<0.01	NS

[a] All values are the mean ± SD with the number of individual preparations analyzed shown in parenthesis (*n*). Statistical significance, as determined using one way ANOVA, is given for control *vs.* ischemic apical membrane fractions.

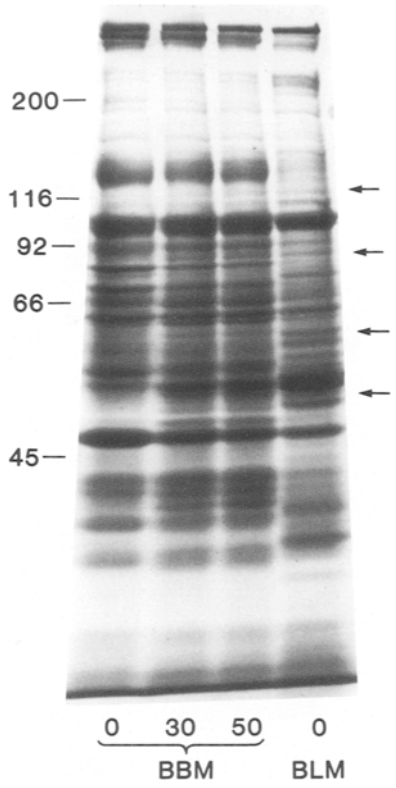

Fig. 2. Effect of ischemia on the electrophoretic profile of brush border membrane (BBM) proteins. 100 μg of membrane protein was used. For BBM, *0*, *30* and *50* represent control BBM and BBM isolated immediately following 30 and 50 min (respectively) of ischemia. The BLM is from a control animal. The arrows indicate protein bands present in the control BLM and ischemic BBM but not seen in the control BBM fraction

selectively localized to the apical region of the cell in control situations. Following 50 min of ischemic injury, however, much of the previously apical LAP could be visualized apparently within the cell and specific reaction product could also be seen along basolateral portions of the cell (Fig. 3*B*). Following 50 min of ischemia and 24 hr reperfusion, the redistribution of LAP to the basolateral membrane was further enhanced (Fig. 3*C*) and could be visualized within damaged tubules. The specific BLM localization of LAP was confirmed with EM studies (Fig. 4*C, D*) showing specific reaction deposits along apical and BLM segments of the surface membrane. The basolateral deposits were not seen in control tubular cells (Fig. 4*A, B*). Finally, following 50 min of ischemia and recovery for 120 hr, leucine aminopeptidase was now selectively localized again only to the apical membrane (Fig. 3*D*). Pre-immune serum control sections showed no staining at any point. These data indicate that during ischemic injury, apical membrane LAP moves and is incorporated into the basolateral membrane. With recovery from ischemic injury, there is a gradual remodeling such that the abnormal membrane proteins are not present and polarity is re-established at least with regard to LAP.

To determine if ischemia also caused a time-dependent alteration in apical membrane lipid composition, apical phospholipids and cholesterol were determined following ischemia for various time intervals and are shown in Table 3. Ischemia resulted in significant duration-dependent reductions in sphingomyelin, phosphatidylserine and the cholesterol-to-phospholipid ratio. There was also an increase in apical phosphatidylcholine, phosphatidylinositol, and a small but significant change was noted in phosphatidylethanolamine. The large decrease in sphingomyelin and corresponding increase in phosphatidylcholine resulted in a marked decrease in the sphingomyelin phosphatidylcholine ratio from 2.1 to 0.8 ($P < 0.01$). These lipid changes were again duration dependent; however, the greatest percentage of the alterations occurred during the first 30 min of ischemia.

Lipid changes in the basolateral membrane could also be documented following 15 min of ischemia. Sphingomyelin levels tended to increase (16.3 ± 1.7 *vs.* 18.7 ± 1.4), and phosphatidylcholine showed a significant decrease (40.9 ± 1.1 *vs.* 37.5 ± 1.5, $P < 0.01$) which resulted in a significant increase in the SPH/PC ratio (0.40 ± 0.04 *vs.* 0.50 ± 0.04, $P < 0.01$). Phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine, and the cholesterol to phospholipid ratios were not altered in the BLM following 15 min of ischemia.

Discussion

The polar distribution of specific apical and basolateral membrane components is essential for normal

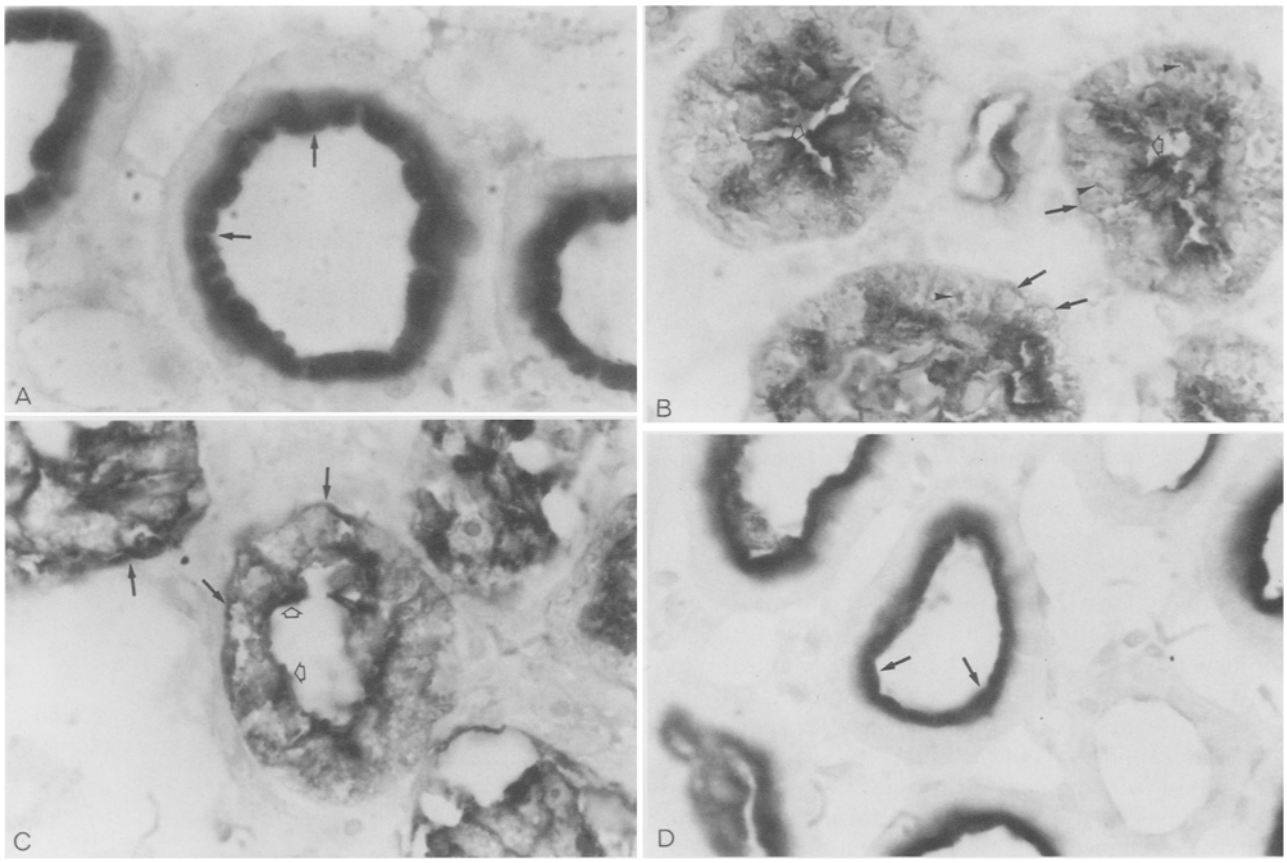

Fig. 3. Effect of ischemia on the sub-cellular localization of leucine aminopeptidase. Light microscopic localization of LAP using indirect immunohistochemical staining techniques. (*A*) Control proximal tubules, 1000×. The black LAP staining is present exclusively at the apical pole of the epithelial cells (arrows). (*B*) Following 50 min of ischemia, LAP is seen within the cell (solid arrow heads) and on the basolateral (arrows) as well as the apical membrane (open arrow heads), 800×. (*C*) After 50 min of ischemia and 24 hr of reperfusion, further redistribution of LAP from the apical membrane (open arrow heads) to the basolateral membrane (arrows) is seen, 800×. (*D*) After 50 min of ischemia and 120 hr of reperfusion the normal apical localization of LAP (arrows) is restored, 640×.

Table 3. Effect of ischemia on apical phospholipid and cholesterol composition

	n	SPH	PC	SPH/PC	PI	PS	PE	Ch/PL
Control	(14–16)	36.6 ± 3.2	17.6 ± 2.6	2.1 ± 0.4	3.0 ± 0.9	17.6 ± 2.9	25.8 ± 2.1	0.81 ± 0.06
Ischemia								
15 min	(14–16)	31.2 ± 2.1^{A}	26.8 ± 2.1^{A}	1.2 ± 0.1^{A}	4.2 ± 0.9^{A}	14.0 ± 1.4^{A}	23.7 ± 2.1^{a}	0.71 ± 0.06^{A}
30 min	(6–8)	31.7 ± 2.6^{A}	30.0 ± 2.1^{AB}	1.1 ± 0.1^{Ab}	4.7 ± 0.8^{A}	12.5 ± 1.1^{Ab}	21.6 ± 2.5^{Ab}	0.65 ± 0.02^{Ab}
50 min	(5–7)	27.2 ± 2.4^{ABC}	33.5 ± 3.9^{AB}	0.8 ± 0.1^{ABC}	5.0 ± 0.5^{A}	12.9 ± 3.5^{A}	22.6 ± 2.1^{Ab}	0.64 ± 0.04^{Ab}

[a] All values are the mean ± SD and represent the percent of total lipid phosphorus for individual phospholipid classes. SPH, sphingomyelin; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; Ch/PL, cholesterol to total phospholipid ratio. *n* refers to the number of individual preparations analyzed. Statistical evaluation and nomenclature are as denoted in Table 1.

cell function. Establishment of epithelial polarity has received great attention in recent years. Current evidence indicates that both NaK-ATPase [8] and leucine aminopeptidase [25] are selectively sorted to the BLM and apical membranes, respectively. New evidence also indicates that maintenance of this polarity requires more than the selective sorting of lipids and proteins to apical and BLM domains. Specifically, approximately half of NaK-ATPase in MDCK cells has been shown to have rapid lateral

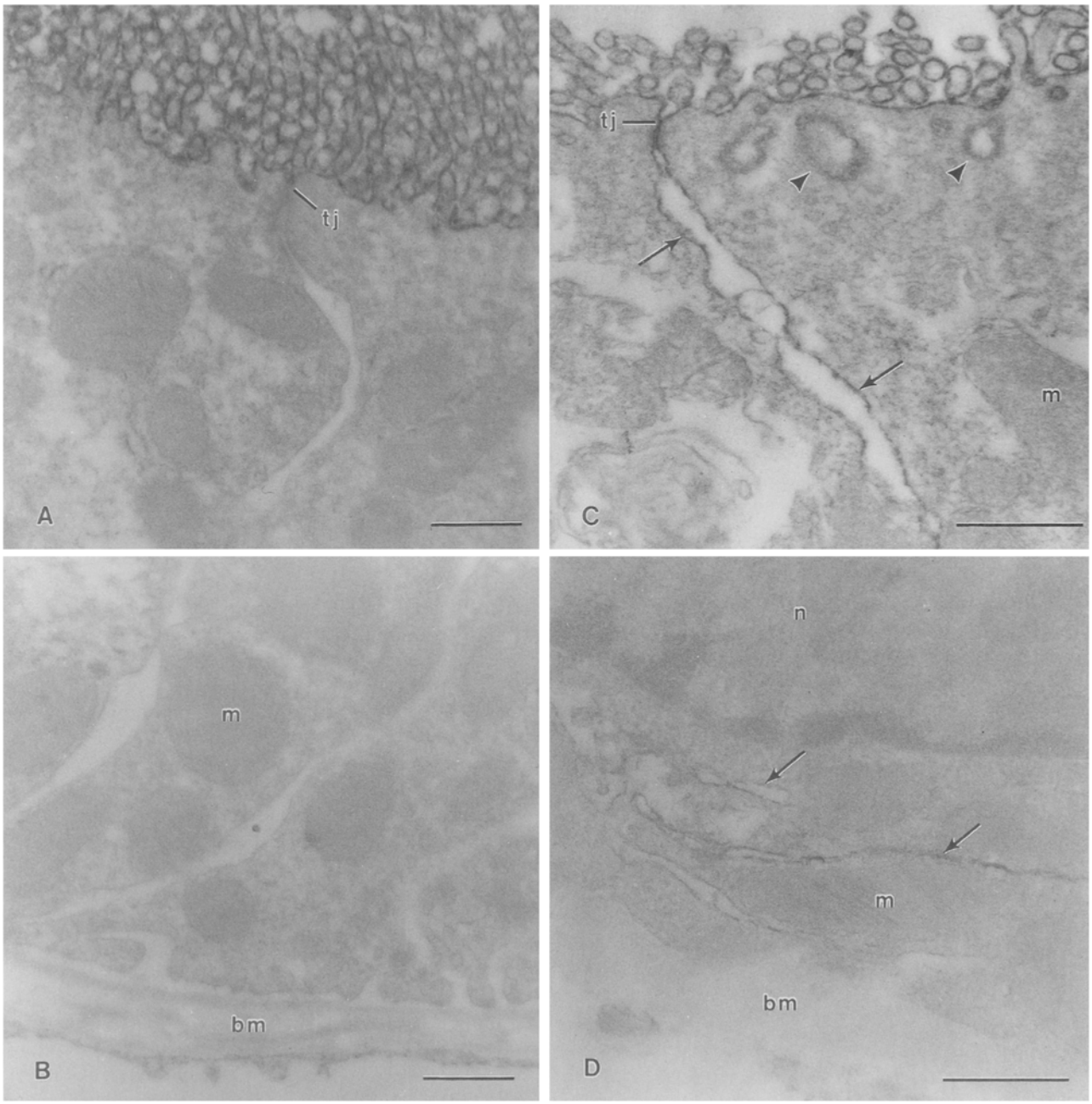

Fig. 4. Subcellular localization of LAP. (*A*) Control proximal tubule cells (32,000×) showing strong immunoperoxidase staining of apical microvilli which is not present below the tight junction (*tj*) along the lateral membrane. (*B*) Basalar aspect of control proximal tubule cells showing lack of inmmunoperoxidase staining along the BLM (32,000×). (*C*) Apical aspect of proximal tubule cell following 50 min of ischemia and 26 hr of reperfusion (43,000×). The immunoperoxidase stain is now seen around apical microvilli, along the lateral membrane of the cell below the tight junction (arrows). It is also seen in large intracellular vesicles located in the apical region of the cell (arrowheads). (*D*) Basalar aspect of proximal tubule cell following 50 min of ischemia and 24 hr of reperfusion (43,000×). Arrows indicate localization of immunoperoxidase staining along the basal BLM. *Abbreviations: m*–mitochondrion; *bm*–basement membrane; *tj*–tight junction; *n*–nucleus; magnification bars represent 0.5 μM

mobility and therefore requires additional cellular processes independent of selective sorting to maintain specific membrane domains [19]. In addition, LAP has been shown to cluster at the level of the tight junction [25]. Several investigators, primarily using cell culture techniques, have shown that disruption of tight junctions leads to loss of epithelial polarity and the redistribution of protein markers

and lipids from one surface membrane domain to the other [27, 42].

The present studies indicate that ischemia disrupts the cells' ability to maintain surface membrane polarity. Biochemical, protein electrophoretic and immunohistochemical studies clearly indicated that movement of apical and basolateral domain-specific markers into the alternate domain occurs during ischemic injury. That these selective markers remain functionally active is indicated by the biochemical assay for NaK-ATPase and the PTH sensitivity of adenylate cyclase in ischemic apical membrane fractions. Once present in the alternate domain, LAP seems to have undergone remodeling, eventually leading to complete repolarization. That this represents cellular remodeling and not new cell synthesis (regeneration) is an important point. Previous work indicates proximal tubule cells recover and do not regenerate following this level of ischemia [13, 14]. The reported changes, therefore, represent actual alterations in the surface membranes of recovering cells and are not due to cell synthesis.

The data also suggest that apical and basolateral membrane lipids undergo a rapid time-dependent movement into the alternate surface membrane domain during ischemic injury. In apical membrane fractions, this was most notable for phosphatidylcholine and spingomyelin, and resulted in a marked reduction in the SPH/PC ratio. There was also a marked decrease in the cholesterol-to-phospholipid ratio which has been shown to be a major determinant of the difference between apical and basolateral membrane fluidity [31]. The reduction in both SPH/PC and cholesterol to phospholipid ratios are probably responsible for the marked reduction in apical membrane fluidity that we have demonstrated [36]. The apparent movement of apical lipids into the BLM was also documented following 15 min of ischemia.

Ischemia is known to cause marked alterations in the function of proximal tubular cells. Specifically, net cellular transport of compounds such as sodium and glucose have been shown to be reduced [15, 20]. We believe that ischemia-induced loss of surface membrane polarity may in part explain these functional alterations. In this vain, we have shown that the ischemia-induced increases in apical membrane fluidity reduced the apical membrane's ability to transport glucose [32]. This reduction was due to a decreased availability of specific sodium-dependent glucose cotransporters. In addition, we have recently postulated, and reported in preliminary form [35], that the NaK-ATPase localized to the apical membrane may play a role in reduced sodium reabsorption following ischemic injury. Additional evidence from our laboratory [40] also indicates that normal functioning of proximal tubular cells following recovery from ischemic injury is dependent upon re-establishment of both lipid and protein polarity. Taken together, these data indicate that ischemia-induced loss of epithelial polarity may be in part responsible for altered cellular function following ischemic injury. Further studies, however, are necessary to directly prove this hypothesis.

Ischemic-induced loss of epithelial polarity could occur via at least three different mechanisms. First, during the ischemic interval, newly synthesized membrane constituents could be missorted to alternate surface membrane domains. This seems unlikely in that cellular ATP rapidly diminishes to nondetectable levels during ischemia and intracellular synthesis of both proteins and lipids is ATP dependent. The second potential mechanism involves abnormal targeting and insertion of pre-synthesized surface membrane components that are present in the cell within vesicles. Sub-apical vesicles, which have been postulated to serve as a mechanism for reshuttling endocytosed apical membrane back to the apical surface [25], could, during the ischemic interval, lose their polar distribution and unite with the basolateral membrane. This hypothesis has not been tested. Finally, movement of both proteins and lipids through disrupted tight junctional areas could explain our observations. Evidence from multiple laboratories indicate that disruption of tight junctions, using either antibodies [17] or low ionic calcium, results in the redistribution of both apical and basolateral membrane proteins and lipids [27, 38, 43]. Whether or not this is happening during ischemic injury remains to be determined.

In summary, we present evidence that ischemia induces a duration-dependent loss of the polar distribution of lipids and proteins within the plasma membrane. The redistributed proteins remain functional, and with recovery from ischemic injury, surface membrane polarity is restored. We postulate that this loss of surface membrane polarity is in part responsible for altered cellular function following ischemic injury.

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