Ischemia-induced Loss of Epithelial Polarity

Role of the Tight Junction

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Abstract

In proximal tubular cells ischemia is known to result in the redistribution of apical and basolateral domain-specific lipids and proteins into the alternate surface membrane domain. Since tight junctions are required for the maintenance of surface membrane polarity, the effect of ischemia on tight junction functional integrity was investigated. In vivo microperfusion of early loops of proximal tubules with ruthenium red (0.2%) in glutaraldehyde (2%) was used to gain selective access to and outline the apical surface membrane. Under control situations ruthenium red penetrated < 10% of the tight junctions. After 5, 15, and 30 min of ischemia, however, there was a successive stepwise increase in tight junction penetration by ruthenium red to 29, 50, and 62%, respectively. This was associated with the rapid duration-dependent redistribution of basolateral membrane domain-specific lipids and NaK-ATPase into the apical membrane domain. Taken together, these data indicate that during ischemia proximal tubule tight junctions open, which in turn leads to the lateral intramembranous diffusion of membrane components into the alternate surface membrane domain.

Introduction

Epithelial cells function to provide unidirectional transport of substances across cells. This, in large part, is accomplished by having a polarized surface membrane with biochemically and physiologically distinct apical and basolateral membrane (BLM)[1] domains. Several laboratories have characterized the asymmetric distribution of proteins and lipids in these alternate membrane domains. Large differences in the phospholipid composition of apical and BLM fractions of renal proximal tubule (1, 2), small intestine (3), hepatocyte (4), and Madin-Darby canine kidney (5) cells have recently been described. The differences in renal proximal tubule (PT) cells are quite pronounced, with the apical (brush border) membrane having a sphingomyelin (SPH) to phosphatidylcholine (PC) ratio of 2.1, while the BLM ratio is only 0.4 (1). Using immunochemical, histochemical, and biochemical techniques it has been shown that during in vivo ischemia surface membrane lipid and protein polarity were lost in renal PT cells (6, 7). Specifically, NaK-ATPase redistributed from the BLM into the apical membrane, while leucine aminopeptidase (LAP) moved from the apical membrane into the BLM. This was associated with the equilibration of surface membrane cholesterol and phospholipids between the two surface membrane domains. Furthermore, this loss of lipid and protein polarity was related to specific reductions in transcellular glucose and Na+ transport (8, 9), and normalization of these cellular functions required the reestablishment of surface membrane polarity (10). Since cellular tight junctions are known to play a critical role in the maintenance of epithelial polarity (11, 12), and disruption of tight junctions results in loss of epithelial polarity (13, 14), we questioned whether the documented ischemia-induced loss of epithelial polarity was due to the disruption of cellular tight junctions. The purpose of these studies, therefore, was to determine if ischemia altered the integrity of PT cell tight junctions.

Methods

Membrane preparation and characterization. Renal ischemia was induced in male Sprague-Dawley rats (250–300 g) for variable lengths of time, and renal cortical apical membrane fractions were isolated using magnesium precipitation as previously described in detail (6–10). Enzymatic characterization of apical membrane fractions was conducted as previously described for LAP, NaK-ATPase, succinic dehydrogenase, KCN-resistant NADH dehydrogenase, cytochrome *c* reductase, and glucosaminidase (6–10). Apical membrane enrichment of LAP was 9.4±0.7 in control membrane fractions and was unaltered by ischemia (9.8±0.9) as previously reported (6, 10). In all cases specific activities of intracellular organelle marker enzymes were less than the corresponding homogenates and there were no differences between control and experimental membrane fractions. Lipids from 0.5 to 1 mg of apical membrane protein were extracted in 6 ml of chloroform/methanol (1:2), isolated by TLC, and quantitated as previously described (1, 15). Protein was measured according to the method of Lowry et al. (16) using BSA as a standard. Statistical significance was determined using a *t* test or a one-way analysis of variance, and reported as $P < 0.05$ or 0.01.

Microperfusion studies. Male Sprague-Dawley rats (250–300 g), fasted overnight, were anesthetized using pentobarbital anesthesia. Animals were placed on a thermostatically controlled heated surgical table (37°C) and a tracheostomy was performed. The left kidney was then exposed via a flank incision, dissected free of surrounding connective tissue, and, while maintaining connections to the ureter and

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1. *Abbreviations used in this paper:* BLM, basolateral membrane; LAP, leucine aminopeptidase; PC, phosphatidylcholine; PT, proximal tubule; RR, ruthenium red; SPH, sphingomyelin.

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vasculature, placed in a Lucite cup affixed to the surgical table. The surface of the kidney was bathed in physiologic saline warmed to 37°C. A PE-50 catheter was inserted into the ureter for collection of urine. After stabilization for 15–30 min individual loops of proximal tubules were identified. In all studies a micropipet (outside diameter 5 μM) was inserted, for direct infusion, into the lumen of the tubule. Individual surface PT under direct visualization were then perfused-fixed in vivo by infusing, in order, 0.2% ruthenium red (RR) and 2% glutaraldehyde in 100 mM Na+ cacodylate buffer (pH 7.4), followed by 0.2% RR in 2% O_sO_4 in 100 mM sodium cacodylate buffer each for 15 min.

Histologic techniques. After in vivo perfusion fixation individual tubules were grossly dissected, washed in distilled water, dehydrated in graded ethanol solutions, and embedded in Spurr's plastic. Ultrathin sections were obtained, stained with uranyl acetate and lead nitrate, and viewed in an electron microscope (model CM-12; Philips Electronic Instruments, Inc., Mahwah, NJ).

Tight junction penetration by RR was used to quantitate the effect of ischemia on tight junction functional–structural integrity (17, 18). Ultrathin sections were viewed. photographed, and quantitated by one investigator (Dr. Dahl) in a coded fashion. All areas photographed were viewed at low power to ensure that RR was selectively applied to the apical surface and was, therefore, not present in the vascular spaces or along the basal surface of tubular cells. Photographs (×10,000–15,000) were then taken and tight junction penetration by RR was quantitated only in tight junctions sectioned perpendicularly, in which it was possible to observe the entire junctional complex. Control and experimental tubules were processed, viewed, and photographed together.

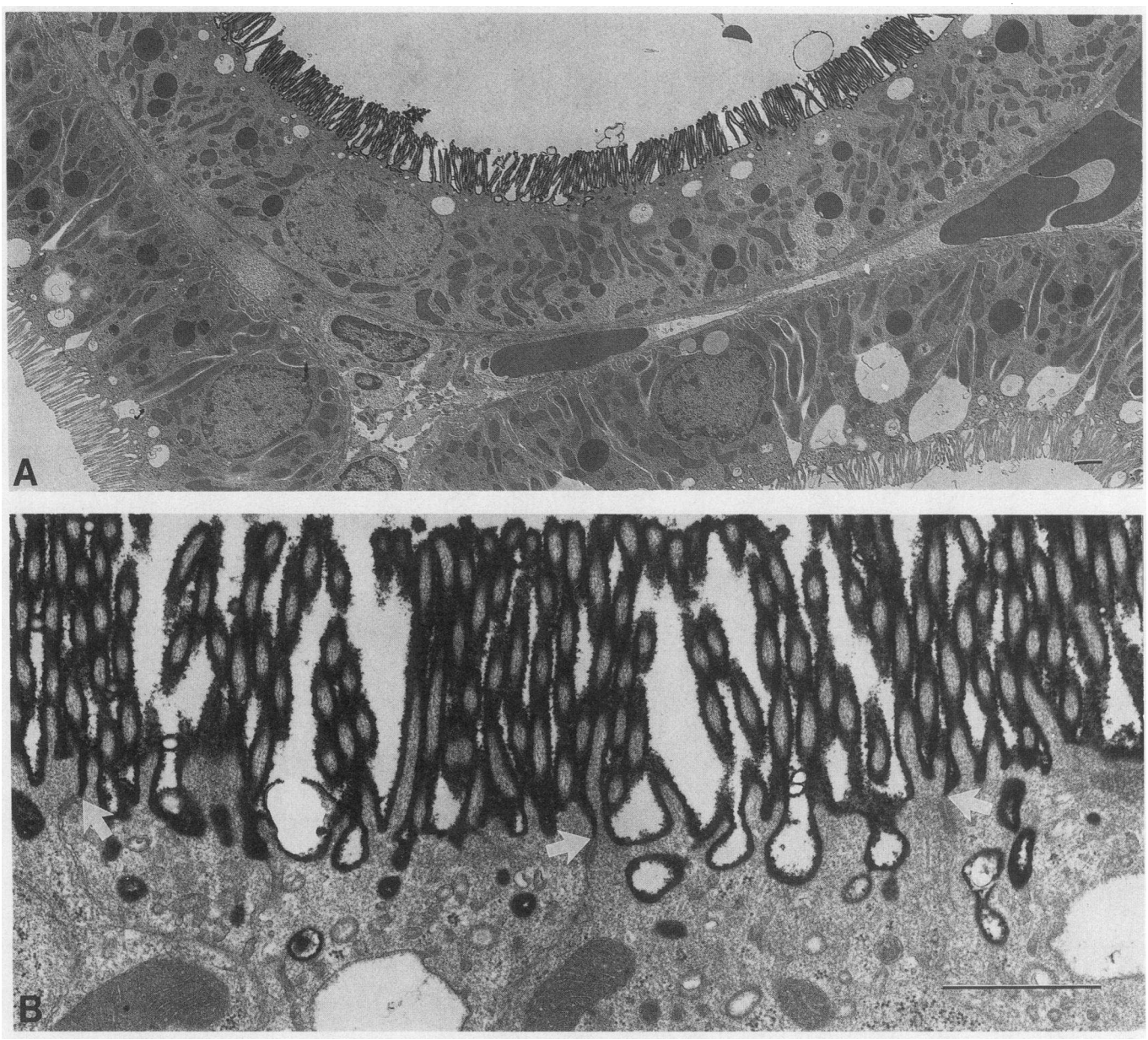

Figure 1. Selective RR staining of the apical surface of individual control PT cells using microperfusion techniques. Control PT were microperfused with a solution containing 0.2% RR and 2% glutaraldehyde in 100 mM Na+ cacodylate buffer (pH 7.4) for 15 min. This was followed by a solution containing 0.2% RR in 2% osmium. Tubules were then dissected and processed as described in Methods. *Bars,* 1 μm. *A,* Low power (×3,500) transmission electron micrograph showing an RR-perfused PT and two adjacent nonperfused tubules. *B,* Transmission electron micrograph showing complete uniform labeling of the apical surface membrane, including coated pits. RR, however, did not penetrate the cellular tight junctions (*white arrows*). ×24,000.

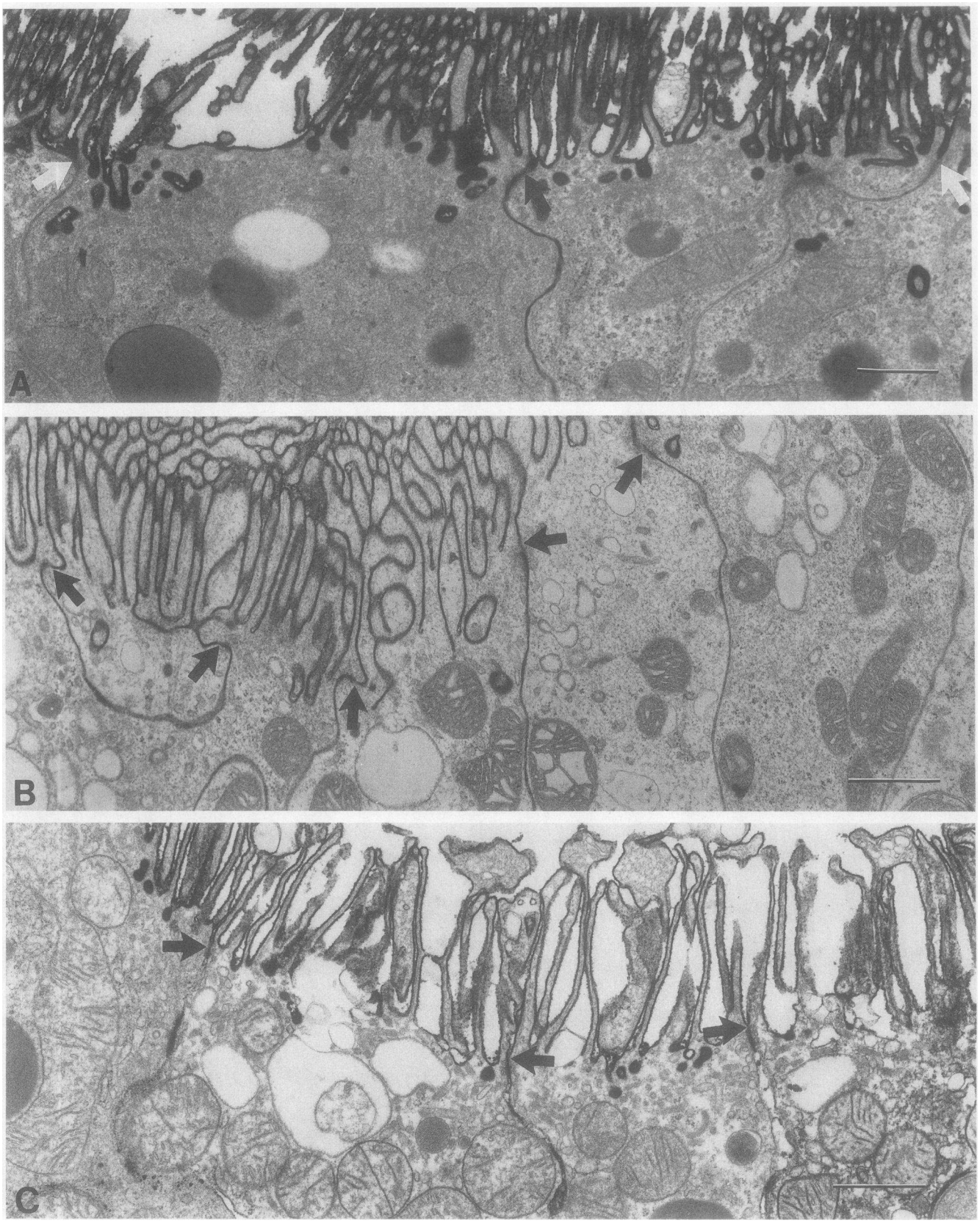

Figure 2. Effect of increasing intervals of ischemia on the integrity of PT tight junctions. After stabilization, ischemia was induced via renal pedicle clamping for 5–30 min and tubules were individually perfused and processed as described in Fig. 1. *White arrows,* closed tight junctions; *dark arrows,* open tight junctions. ×16,500. *A,* PT cells after 5 min of ischemia. *Black arrow,* a tight junction where RR is penetrating into the lateral cellular space. *B,* PT cells microperfused after 15 min of in vivo ischemia. *C,* PT cells microperfused after 30 min of in vivo ischemia. *Bars,* 1 μm.

Results

Fig. 1 *A* shows a cross-section of a control tubule in vivo microperfused with 0.2% RR in 2% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4). Selective staining of the apical membrane is apparent with no RR staining in the vascular spaces or along the lateral or basal aspects of tubule cells. Cross-sections of adjacent nonperfused and, therefore, nonstained PT are also seen. In addition, apical staining of distal nephron segments of perfused tubules was evident, indicating selective apical staining of the entire tubule (not shown). Fig. 1 *B* shows an apical portion of a control tubule perfused-fixed in vivo with the RR solution. Individual microvilli, coated pits, and the entire apical surface are darkly and evenly stained with RR. RR, however, does not penetrate cellular tight junctions (*white arrows*), and therefore lateral and basal surfaces of PT cells remain unstained.

To determine the effect of ischemia on the functional integrity of tight junctions, similar microperfusion studies were conducted in PT made ischemic for 5, 15, and 30 min. Fig. 2 *A* shows an electron micrograph of PT cells after 5 min of ischemia. Two tight junctions (*white arrows*) remain closed, while RR is seen penetrating a tight junction (*dark arrow*), forming an apical to basolateral gradient along the lateral aspect of the cell. 15 (Fig. 2 *B*) and 30 min of ischemia (Fig. 2 *C*) were associated with increased penetration of tight junctions by RR (*dark arrows*), and characteristic ischemia-induced apical membrane and mitochondrial alterations were seen.

In Fig. 3 quantitation of the effect of increasing ischemic intervals on tight junction penetration by RR is shown. In control tubules 9.7% of tight junctions were open. This increased to 29, 50, and 62% by 5, 15, and 30 min of ischemia, respectively.

To determine the effect of this rapid disruption of tight junctions on cell surface membrane lipid and protein polarity, apical membrane fractions were isolated after 5, 10, and 15 min of ischemia. Apical membrane SPH and phosphatidylserine decreased rapidly, while PC and phosphatidylinositol increased rapidly (Table I) during the ischemic interval. Both the SPH/PC and cholesterol to phospholipid ratios were significantly reduced ($P < 0.01$) after only 5 min of ischemia (Fig. 4). The disruption of tight junctions during ischemia was also associated with movement of NaK-ATPase into the apical membrane in a duration-dependent fashion (Fig. 5). The increase in apical NaK-ATPase reached significance ($P < 0.05$) after 10 min of ischemia.

Table I. Effect of Increasing Ischemic Intervals on Apical Membrane Phospholipid Composition

Time	SPH	PC	PI	PS	PE
min					
0	36.0±2.2	18.4±2.9	3.3±0.5	19.1±0.3	23.3±1.5
5	33.1±0.7*	22.7±0.7*	3.6±0.7	16.0±0.5*	24.6±0.6
10	32.0±1.9*	26.4±0.7*	4.0±0.4‡	14.6±1.4*	23.1±1.0
15	32.7±1.3‡	25.4±1.9*	4.1±0.4‡	14.0±1.2*	23.0±1.6

Phospholipid composition is expressed as the percent of total phospholipids. Data points represent the mean±SD of four individual preparations. Statistical significance compared with control (time 0) samples was determined using one-way analysis of variance (ABSTAT, version 4.23; Anderson-Bell, Parker, CO). * $P < 0.01$, ‡ $P < 0.05$.

Discussion

In epithelial cells normal cellular function is dependent on the establishment and maintenance of epithelial polarity (19). This requires not only the vectorial translocation of domain-specific lipids and proteins to the surface membrane (establishment phase), but also requires that the cell maintain these differences (maintenance phase). Recent evidence indicates that cellular tight junctions (zonula occludens) play a critical role in the maintenance of epithelial surface membrane lipid and protein polarity. Disruption of tight junction integrity in cell culture models, using microfilament inhibitors, a low Ca^{2+} bath, or MAbs to the tight junction, results in the rapid redistribution of domain-specific phospholipids and proteins into the alternate surface membrane domain (13, 14, 19–22). Also, freshly isolated dissociated epithelial cells show temperature-

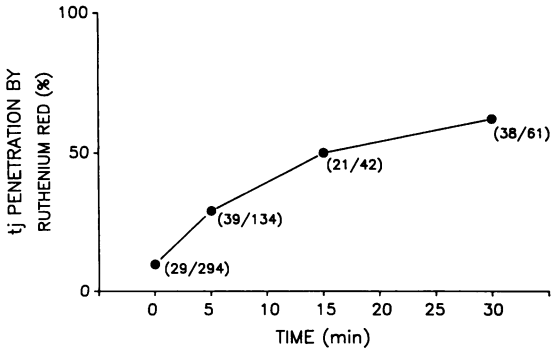

Figure 3. Quantitation of the effect of increasing intervals of ischemia on the integrity of cellular tight junctions in PT cells. RR penetration of individual tight junctions was quantitated as described in Methods. Zero time indicates control tubules. In all cases, cross-sections of at least three different stained tubules were evaluated for all time points. The numbers in parenthesis indicate the number of tight junctions open (numerator) and the total number of tight junctions evaluated (denominator).

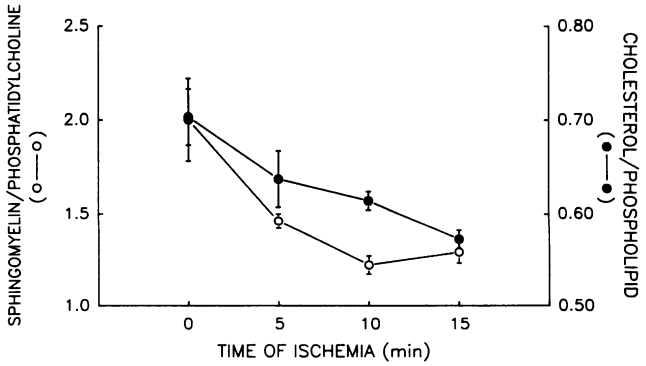

Figure 4. Effect of increasing ischemic intervals on the SPH/PC (*open circles*) and cholesterol to phospholipid (*closed circles*) ratios in apical membrane fractions isolated using Mg^{2+} precipitation techniques as described in Methods. Zero time indicates control preparations. Data points represent the mean±SD, $n = 4$.

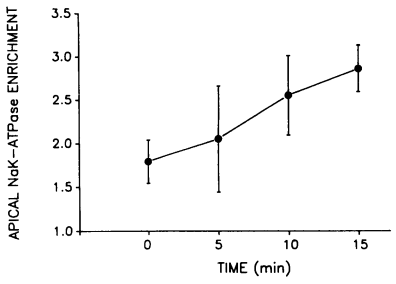

Figure 5. Effect of increasing ischemic intervals on the enrichment of the apical membrane fraction with NaK-ATPase. The data represent the mean of six individual samples for each data point and are presented as the mean±SD. The specific activity of apical NaK-ATPase increased in a stepwise fashion from 18.2 in controls to 28.3 μmol/mg protein per h in apical membranes isolated after 15 min of ischemia.

dependent redistribution of membrane components by diffusion in the plane of the membrane (23, 24). These data indicate that disruption of tight junctions results in the loss of surface membrane polarity via lateral diffusion of surface membrane components within the plane of the bilayer. They are also consistent with our previous data regarding ischemia-induced loss of epithelial polarity (6, 7) and suggest a potential role for disruption of tight junction integrity during ischemic injury.

The present studies used microperfusion techniques to evaluate the effect of ischemia on the functional integrity of PT tight junctions in vivo. Tight junctions of PT cells are composed of single or double strands that are discontinuous. This results in low transcellular electrical resistance (leaky epithelia) due to a large paracellular shunt. RR is a low molecular weight compound (859 D) that binds to mucopolysaccharides and has been used previously to evaluate tight junction integrity (17, 18). Its low molecular weight and dense uniform staining make it ideal for this purpose. Use of in vivo microperfusion was advantageous as it allowed for the selective application of RR to the apical surface in a functioning tubule. Rapid in vivo fixation of the one cell layer–thick tubule was also accomplished, minimizing potential morphologic artifacts.

Under control situations < 10% of PT tight junctions were open. This agrees with data published earlier in which control PT tight junctions were permeable to lanthanum (25). However, neither horseradish peroxidase (40,000 D) nor cationic ferritin (750,000 D) penetrated tight junctions in our studies (unreported data).

The ischemia-induced increase in tight junction penetration by RR was rapid and occurred in a duration-dependent fashion. It was associated with the rapid duration-dependent movement of phospholipids and NaK-ATPase into the alternate surface membrane domain. Rapid diffusion of both surface membrane phospholipids and NaK-ATPase in the plane of the bilayer has recently been demonstrated (21, 26). We propose, therefore, but do not have direct data to confirm, that the observed movement of lipids and NaK-ATPase into the alternate surface membrane domain occurred by diffusion through an open tight junction. Direct evidence will have to await in vitro cell culture monolayer studies. We also have not ruled out two additional possible mechanisms that could result in the loss of epithelial polarity. First, during ischemia abnormal targeting of newly synthesized lipids and proteins could occur. This, however, seems unlikely as both the synthesis and intracellular movement of surface membrane components are energy dependent. Second, apical membrane internalization as vesicles and the abnormal migration and insertion of the vesicles into the BLM could occur during ischemia. This, however, would be unlikely to explain the rapid (5 and 10 min) loss of apical lipid polarity that occurred before apical membrane internalization.

The potential mechanism(s) by which ischemia disrupts the functional integrity of PT tight junctions were not determined. Since the formation and integrity of cellular tight junctions is dependent on several cellular processes (11–14, 21–23, 27, 28), sorting this out will again require extensive in vitro studies.

In summary, ischemia resulted in the rapid duration-dependent opening of PT tight junctions. This was associated with the rapid duration-dependent redistribution of surface membrane lipids and NaK-ATPase into the alternate surface membrane domain. We propose that this occurred via lateral diffusion of membrane components through the region of the tight junction.

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References

1. Molitoris, B. A., and F. R. Simon. 1985. Renal cortical brush-border and basolateral membranes: cholesterol and phospholipids composition and relative turnover. *J. Membr. Biol.* 83:207–215.

2. Carmel, G., F. Rodriquez, S. Carriere, and C. LeGrimellec. 1985. Composition and physical properties of lipids from plasma membranes of dog kidney. *Biochim. Biophys. Acta.* 818:149–157.

3. Chapelle, S., and M. Gilles-Baillien. 1983. Phospholipids and cholesterol in brush border and basolateral membranes from rat intestinal mucosa. *Biochim. Biophys. Acta.* 753:269–271.

4. Meir, P. J., E. S. Sztul, A. Reuben, and F. L. Boyer. 1984. Structural and functional polarity of canalicular and basolateral plasma membrane vesicles isolated in high yield from rat liver. *J. Cell. Biol.* 98:991–1000.

5. Van Meer, G., and K. Simons. 1982. Virus budding from either the apical or the basolateral plasma membrane domain of MDCK cells have unique phospholipid compositions. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:847–852.

6. Molitoris, B. A., P. D. Wilson, R. W. Schrier, and F. R. Simon. 1985. Ischemia induces partial loss of surface membrane polarity and accumulation of putative calcium ionophores. *J. Clin. Invest.* 76:2097–2105.

7. Molitoris, B. A., C. A. Hoilien, R. Dahl, D. J. Ahnen, P. D. Wilson, and J. Kim. 1988. Characterization of ischemia-induced loss of epithelial polarity. *J. Membr. Biol.* 106:233–242.

8. Molitoris, B. A., and R. Kinne. 1987. Ischemia induces surface membrane dysfunction. Mechanism of altered Na+-dependent glucose transport. *J. Clin. Invest.* 80:647–654.

9. Molitoris, B. A., L. K. Chan, J. I. Shapiro, J. D. Conger, and S. A. Falk. 1989. Loss of epithelial polarity: a novel hypothesis for reduced proximal tubule Na+ transport following ischemic injury. *J. Membr. Biol.* 107:117–127.

10. Spiegel, D. M., P. D. Wilson, and B. A. Molitoris. 1989. Epithelial polarity following ischemia: a requirement for normal cell function. *Am. J. Physiol.* 256 (*Renal Fluid and Electrolyte Physiol.* 25):F430–F436.

11. Madara, J. L. 1989. Loosening tight junctions. Lessons from the intestine. *J. Clin. Invest.* 83:1089–1094.

12. Gumbiner, B. 1987. Structure, biochemistry, and assembly of epithelial tight junctions. *Am. J. Physiol.* 253 (*Cell Physiol. 22*):C749–C758.

13. Meza, I., G. Ibarra, M. Sababero, A. Martinez-Palomo, and M. Cereijido. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* 87:746–754.

14. Martinez-Palomo, A., I. Meza, G. Beaty, and M. Cereijido. 1980. Experimental modulation of occluding junctions in a cultured transporting epithelium. *J. Cell Biol.* 87:736–745.

15. Molitoris, B. A., and F. R. Simon. 1986. Maintenance of epithelial surface membrane lipid polarity: a role for differing phospholipids translocation rates. *J. Membr. Biol.* 94:47–53.

16. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:256–275.

17. Luft, J. H. 1971. Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. *Anat. Rec.* 171:347–367.

18. Wanson, J. C., P. Drochmans, R. Mosselmans, and M. F. Ronveaux. 1977. Adult rat hepatocytes in primary monolayer culture. Ultrastructural characteristics of intercellular contacts and cells membrane differentiations. *J. Cell Biol.* 74:858–877.

19. Simons, K., and S. D. Fuller. 1985. Cell surface polarity in epithelia. *Annu. Rev. Cell Biol.* 1:243–288.

20. Imhof, B. A., H. P. Vollmers, S. L. Goodman, and W. Birchmeier. 1983. Cell-cell interaction and polarity of epithelial cells: specific pertubation using a monoclonal antibody. *Cell.* 35:667–675.

21. Van Meer, G., and K. Simons. 1986. The function of the tight junction in maintaining differences in lipid composition between the apical and basolateral cell surface membrane domains of MDCK cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:1455–1464.

22. Pitelka, D. R., B. N. Taggart, and S. T. Hamamoto. 1983. Effects of extracellular calcium depletion on membrane topography and occluding junctions of mammary epithelial cells in culture. *J. Cell Biol.* 96:613–624.

23. Ziomek, C. A., S. Schluman, and M. Edidin. 1980. Redistribution of membrane proteins in isolated mouse intestinal epithelial cells. *J. Cell Biol.* 86:849–857.

24. Piman, M., and P. Ripoche. 1976. Redistribution of surface macromolecules in dissociated epithelial cells. *J. Cell Biol.* 71:907–920.

25. Tisher, C. C., and W. E. Yarger. 1973. Lanthanum permeability of the tight junction (zonnula occludens) in the renal tubule of the rat. *Kidney Int.* 3:238–250.

26. Jesaitis, A. J., and J. Yguerabide. 1986. The lateral mobility of (Na+, K+)-dependent ATPase in Madin-Darby canine kidney cells. *J. Cell Biol.* 102:1256–1263.

27. Fleming, T. P., J. McConnell, M. H. Johnson, and B. R. Stevenson. 1989. Development of tight junctions de novo in the mouse early embryo. Control of assembly of the tight junction-specific protein, 20-1. *J. Cell Biol.* 108:1407–1418.

28. Siliciano, J. D., and D. A. Goodenough. 1988. Localization of the tight junction protein, 20-1, is modulated by extracellular calcium and cell-cell contact in Madin-Darby canine kidney epithelial cells. *J. Cell Biol.* 107:2389–2399.