Ischemia Induces Surface Membrane Dysfunction

Mechanism of Altered Na^+-dependent Glucose Transport

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Abstract

Reversible ischemia reduced renal cortical brush border membrane (BBM) Na^+-dependent D-glucose uptake (336±31 vs. 138±30 pmol/mg per 2 s, $P < 0.01$) but had no effect on Na^+-independent glucose or Na^+-dependent L-alanine uptake. The effect on D-glucose uptake was present after only 15 min of ischemia and was due to a reduction in maximum velocity (1913±251 vs. 999±130 pmol/mg per 2 s; $P < 0.01$). This reduction was not due to more rapid dissipation of the Na^+ gradient, altered sidedness of the vesicles, or an alteration in membrane potential. Ischemia did, however, reduce the BBM sphingomyelin-to-phosphatidylcholine (SPH/PC) and cholesterol-to-phospholipid ratios and the number of specific high-affinity Na^+-dependent phlorizin binding sites (390±43 vs. 146±24 pmol/mg; $P < 0.01$) without altering the binding dissociation constant (K_d). 20 mM benzyl alcohol also reduced the number of Na^+-dependent phlorizin binding sites (418±65 vs. 117±46; $P < 0.01$) without altering K_d. The reduction in Na^+-dependent D-glucose transport correlated with ischemic-induced changes in the BBM SPH/PC and cholesterol-to-phospholipid ratios and membrane fluidity. Taken together these data indicate the cellular site responsible for ischemic-induced reduction in renal cortical transcellular glucose transport is the BBM. We propose the mechanism involves marked alterations in BBM lipids leading to large increases in BBM fluidity which reduces the binding capacity of Na^+-dependent glucose carriers. These data indicate that reversible ischemia has profound effects on the surface membrane function of epithelial cells.

Introduction

Reversible ischemia results in marked alterations in epithelial cellular function (1), but the mechanism(s) and cellular site(s) responsible for these alterations remain largely unknown. Morphologic studies of epithelial tissues have shown that surface membranes undergo reversible changes during ischemic injury (2–4). Correlating these morphologic changes with biochemical and physiologic alterations, however, has been difficult because of the limited ability to isolate representative membrane fractions after ischemia (4). Therefore, the extent and role surface membrane damage plays in abnormal cellular function after an ischemic insult remains largely speculative (5–7).

The surface membrane of renal cortical epithelial cells has marked lipid polarity (8, 9). The apical membrane (brush border membrane [BBM][1]) has a high content of sphingomyelin (34.5%) and phosphatidylserine (16.8%) and a high cholesterol-to-phospholipid ratio (0.9) (8). The basolateral membrane (BLM), on the other hand, has a high content of phosphatidylcholine (38.4%) and phosphatidylinositol (4.3%) (8). We have recently shown that reversible ischemia leads to partial loss of surface membrane polarity which in turn results in marked reductions in the sphingomyelin-to-phosphatidylcholine ratio (2.2 vs. 1.0) and the cholesterol-to-phospholipid ratio (0.8 vs. 0.6) (10).

Renal proximal tubule studies indicate ischemia causes a reversible reduction in transcellular glucose transport (1). As the first step of renal proximal tubular glucose transport involves the Na^+-coupled reabsorption of glucose across the apical membrane, we questioned whether ischemia reduced the ability of the apical membrane to transport glucose. Furthermore, as the Na^+-dependent glucose carrier is extremely sensitive to membrane fluidity (11), we questioned whether the marked ischemic-induced alterations in apical membrane lipids could be responsible for reduced renal glucose transport after ischemic injury. The purposes of these studies were, therefore, to first determine if ischemia reduced the ability of apical membranes to transport glucose, and second, to evaluate the mechanism(s) of this alteration.

Methods

Membrane preparation and characterization. Male Sprague-Dawley rats (220–280 g) maintained on standard chow were used in all experiments. Bilateral renal ischemia was induced while under anesthesia with sodium pentobarbital (50 mg/kg body wt) by clamping (Schwartz clip) the renal pedicle after removal of the capsule, as previously described (10).

BBM were isolated from cortical homogenates and characterized enzymatically as described in detail elsewhere (8, 10). Briefly, the procedure entailed rapid decapsulization, and removal of thin cortical slices of the entire cortex which were placed in chilled buffer (300 mM mannitol, 5 mM ethyleneglycol-bis [B-aminoethylether]-*N,N'*-tetraacetic acid, 18 mmol Tris [hydroxymethyl] aminomethane hydrochloride, 0.1 mM phenylmethylsulfonyl fluoride at pH 7.4). Both control and ischemic cortical slices were suspended using a 20-ml syringe fitted

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1. *Abbreviations used in this paper:* BBM, brush border membrane; BBMV, brush border membrane vesicle; BLM, basolateral membrane; DPH, 1,6 diphenyl-1,3,5-hexatriene; LAP, leucine aminopeptidase; SPH/PC, sphingomyelin-to-phosphatidylcholine.

with a 10-cm 16-gauge needle and centrifuged for 30 s at 121 *g* to remove contaminating erythrocytes (10). This suspension was then homogenized using a Polytron PT-10 (Brinkmann Instruments Co., Westbury, NY) 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*, and the supernatant was centrifuged at 48,000 *g* for 30 min to obtain crude apical membranes. This pellet was resuspended using a Teflon-glass Potter-Elvehjem in 30 ml of diluted buffer (1:1 with deionized water) and taken through the Mg^{2+}precipitation process again. Enrichments for various enzymes in control apical preparations were as follows: alkaline phosphatase, 12.6±3.4; leucine aminopeptidase, 15.3±5.3; Na,K-ATPase, 1.4±0.6; succinic dehydrogenase, 0.5±0.1; *N*-acetyl glucosaminidase, 0.2±0.1; and KCN-resistant NADH-dehydrogenase, 0.4±0.1. As previously reported (10), ischemia had little effect on BBM marker enzyme enrichment (alkaline phosphatase, 12.2±4.5, leucine aminopeptidase, 11.1±4.6) but did cause a significant increase in apical Na,K-ATPase enrichment even with only 15 min of ischemia (4.3±0.6; $P < 0.01$), and this enrichment remained constant throughout 50 min of ischemia (3.5±0.8). Marker enzyme enrichments for intracellular organelles in ischemic apical fractions did not differ from control preparations, as we have previously reported (10).

Enzyme and protein measurements. Protein was measured according to Lowry et al. (12) using bovine serum albumin as a standard. Enzyme determinations were carried out using standard kinetic assays as previously reported from our laboratory (8, 10). Leucine aminopeptidase (LAP) activity was determined as previously described (13) using a kinetic assay monitoring the appearance of 4-nitroanilin at 380 nm at 37°C. The reaction medium contained 100 mM mannitol, 20 mM Hepes/Tris, pH 7.4, and 0.3 mg/ml L-leucine 4-nitroanilide with a final volume of 1 ml. Immunologic inhibition was determined using a monospecific polyclonal Ab (14, 15) suspended in phosphate-buffered saline (PBS) with an activity of 0.6 U/μl. 20 μl of brush border membrane vesicles (BBMV) were preincubated for 20 min at 20°C with varying amounts (0–20 μl) of antibody solution, and the final volume was adjusted to 40 μl using PBS (13).

Lipid determinations. Lipids from ~ 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 (8, 10). Total phospholipids were determined on an extract, according to Ames and Dubin (16). Individual phospholipid species were separated by two-dimensional thin-layer chromatography on Kesilgel silica gel 60 plates, using the modified (8, 10) technique of Esko et al. (17). Individual phospholipid species were identified using brief exposure to iodine vapor and scraped off the plates. A Bligh and Dyer (18) extraction was then carried out (8, 10) on each individual spot and phosphate was determined by the method of Ames and Dubin (16) on an aliquot. Membrane free cholesterol was determined as previously described (8, 10) using coprostanol as an internal standard.

Vesicle transport studies. BBM were isolated as described (vida supra), except the final resuspension of membranes using Teflon-glass homogenization (three strokes) was done in buffer containing 200 mM mannitol, 20 mM Hepes with Tris used to adjust the final pH to 7.4. This solution was centrifuged at 48,000 *g* for 30 min at 4°C. The pellet was resuspended in 200–300 μl of the same buffer (final protein concentration, ~ 5 mg/ml) using a 20-gauge spinal needle. In studies to measure total uptake the incubation media consisted of 100 mM mannitol, 100–150 mM NaCl, 20 mM Hepes, pH 7.4, using Tris and variable concentrations of the compound under study (D-glucose or L-alanine). All transport studies were done at 25°C using Millipore filtration techniques after timed incubations of 20 μl of membrane sample in 120 μl of transport solution using 1.5 ml disposable Eppendorf tubes (Brinkmann Instruments Inc.) (19). Uptake was stopped by withdrawing 20 μl of the incubation mixture at timed intervals (15, 60, 105, 150 s) and rapidly adding this to 1 ml of ice-cold stop solution containing 100 mM mannitol, 300 mM NaCl, 20 mM Hepes, pH 7.4, with Tris. In addition, the competitive transport inhibitor phlorizin (0.25 mM) was present in the stop solution for glucose experiments. The resulting solution was then rapidly transferred to prewetted 0.45-μm HA filters (Millipore/Continental Water Systems, Bedford, MA) and washed with 3.5 ml of ice-cold stop solution. The filters were dissolved in 8 ml of PCS (Amersham Corp., Arlington Heights, IL) and counted for radioactivity. Equilibrium uptakes were determined after 120 min of incubation at 25°C. Na^+-independent glucose and alanine uptakes were determined in the presence of 0.5 mM phlorizin and 150 mM KCl (no NaCl), respectively. Na^+-dependent uptakes were calculated as the difference between total and Na^+-independent uptakes. In addition, 2-s uptake studies were conducted. In these studies 40 μl of transport buffer was placed in 5-ml disposable plastic test tubes, and 20 μl of BBM vesicle solution was added to the side of the tube. The reaction was initiated by vortexing the solution and terminated by adding 1 ml of ice cold stop solution at 2 s using a metronome to count out the seconds. Using this technique the Na^+-dependent transport of glucose and alanine was linear for up to 4 s. Na^+-independent transport of glucose and alanine was measured as above and subtracted from total uptake to determine specific Na^+-dependent uptake.

Fluorescent polarization measurements. Fluorescent anisotropy, polarization (P), lifetimes (T), and differential tangents (tanΔ) were measured by phase and modulation techniques using an SLM 4800 spectrofluorimeter and a modulation frequency of 30 MHz. Fluorescent lifetimes were measured with the excitation polarizer set at 0 and the emission polarizer set at 55. Lifetimes were quantitated relative to a reference solution of 1,6 diphenyl-1,3,5-hexatriene (DPH) in hexadecane which has a known lifetime of 9.6 ns (20). From these determinations the rotational correlation time (R) and limiting fluorescence anisotropy (r_∞) were calculated according to Lakowicz (21). The excitation wavelength was 357 nm and a 03FCG001 filter (Melles Griot, Irvine, CA) was used for the excitation beam and a KV 389 filter (Schott Glass Technologies Inc., Duryea, PA) was used for the emitted light. The cuvette temperature was maintained by a circulating water bath with continuous monitoring by a thermocouple inserted into the cuvette to a level just above the light beam. BBM samples (144 μg protein) were brought to a total volume of 2.4 ml with phosphate-Hepes buffered saline (PBS) and the fluorescent probe DPH was incorporated at 35°C for 10–20 min under argon with frequent vortexing. Probe incorporation, evaluated by total polarization values, was not altered in ischemic membranes. All values for polarization were recorded at 35°C. DPH was dissolved in tetrahydrofuran and the final probe concentration was 12.5 ng/ml. DPH was purchased from Molecular Probes, Inc., Junction City, OR.

Phlorizin binding. Specific phlorizin binding was quantitated at 25°C as the difference between total phlorizin binding and nonspecific binding determined in the presence of at least a 100-fold excess cold phlorizin. A stock 10 μM phlorizin solution containing 0.25 μCi/100 μl was made and diluted using binding buffer to the desired phlorizin concentrations (0.1, 0.5, 1, 2.5, and 5 μM). Binding was initiated by the addition of 20 μl of the binding solution which also contained 150 mM NaCl, 200 mM mannitol, 20 mM Hepes, and Tris used to bring the solution to pH 7.4. 20-μl aliquots were collected and immediately added to 1 ml of ice-cold stop solution (150 mM NaCl, 200 mM mannitol, Hepes-Tris, pH 7.4, applied to prewetted Millipore filters, HA, 0.45 μM) and washed with 3.5 ml of ice-cold stop solution. Initial experiments to evaluate association and dissociation of phlorizin revealed that both reactions were complete by 5 min as previously established by other investigators (22, 23). In addition, ischemia did not alter the time course of phlorizin association or dissociation. In all binding studies, therefore, 5 min was used for both binding and dissociation. At a phlorizin concentration of 5 μM, nonspecific phlorizin binding for control and ischemic membranes was 9.7±0.1 and 15.4±1.6% of total phlorizin binding, respectively. Scatchard analysis was conducted using a ligand Scafit program modified to an IBM-XT.

Statistics. Comparisons between control and experimental groups were made using the two-tailed unpaired Student's *t* test. Results were considered significantly different if $P < 0.05$ and data were reported as $P < 0.01$, $P < 0.05$, or NS. All results are reported as the mean±1 SD unless otherwise noted.

Materials. [^{3}H]Phlorizin (55 Ci/mmol), [6-^{3}H(N)]D-glucose (33.1 Ci/mmol), [3-^{3}H]L-alanine (70.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA). Unlabeled phlorizin, D-glucose, and L-alanine were obtained from Sigma Chemical Co., St. Louis, MO. Other chemicals were of highest purity available from commercial sources.

Results

The effect of reversible ischemic injury (50 min) on BBMV Na^+-dependent and independent uptake of glucose is shown in Fig. 1. Ischemia resulted in a large decrease in the Na^+-dependent uptake of glucose at the 15 s time point, however no difference in Na^+-independent glucose uptake was seen. Equilibrium values for control and ischemic membrane vesicles were similar and in several other similar studies this marked reduction of Na^+-dependent glucose uptake was seen again without any alteration in equilibrium or Na^+-independent glucose uptakes. To determine if this decrease in uptake was selective for glucose, the effect of ischemia on L-alanine uptake was also determined. The results in Fig. 2 show that neither Na^+-dependent alanine uptake nor equilibrium uptakes obtained at 120 min (146±16 vs. 142±22 pmol/mg) were affected significantly by ischemia.

As the earliest time point in these standard vesicle uptake studies was 15 s and uptake may not be linear for this period of time, studies were then undertaken to evaluate the initial rate (2 s) of glucose and alanine uptake in control and ischemic BBMV. The results of these studies are shown in Fig. 3. Again, 50 min of ischemia resulted in a large reduction in Na^+-dependent glucose uptake (336±31 vs. 138±30 pmol/mg per 2 s; $P < 0.01$) but had no effect on Na^+-dependent alanine uptake (143±32 vs. 139±26 pmol/mg per 2 s). In all subsequent uptake studies, 2-s determinations were used, as this value more closely approximates initial uptake rates (24). Fig. 4 shows the effect of increasing duration of ischemia on glucose uptake.

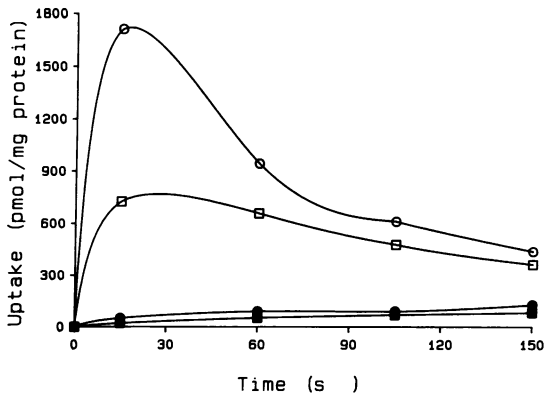

Figure 1. Effect of ischemia on renal cortical BBMV D-glucose uptake. BBMV Na^+-dependent glucose uptake (*open symbols*) was quantitated in control (○) and after 50 min of ischemia (□) in transport buffer containing 150 mM NaCl, 100 mM mannitol, 20 mM Hepes (pH 7.4) and 1–2 μCi [^{3}H]D-glucose using Millipore filtration techniques at 25°C. 20 μl of BBMV (3–5 mg protein/ml) were added to 120 μl of transport buffer, and 20-μl aliquots were sampled at the various times. All determinations were done in duplicate and this is one representative experiment. (*Solid symbols*) Na^+-independent glucose uptake. Equilibrium values obtained at 120 min were similar in control and ischemic BBMV (190 vs. 206 pmol/mg protein).

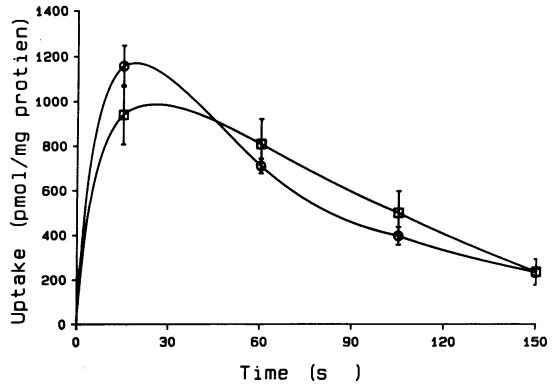

Figure 2. Effect of ischemia on renal cortical BBMV Na^+-dependent L-alanine uptake. BBMV Na^+-dependent alanine uptake in control (○) and after 50 min (□) of ischemia were quantitated as described in Fig. 1. Data represents the mean±SE of four samples done in duplicate. Equilibrium values obtained at 120 min were similar in control and ischemic BBMV (146±16 vs. 142±22 pmol/mg protein).

There was a marked reduction in Na^+-dependent uptake after only 15 min of ischemia with a more gradual decline in glucose uptake for the remaining 35 min. There was no statistical difference between 15 and 50 min of ischemia on BBMV Na^+-dependent uptake of glucose. To determine whether these effects were due to an alteration of K_t or maximum velocity (V_{max}) of glucose uptake, 2-s uptake experiments were done in the presence of varying glucose concentrations (0.03–1.0 mM). Fig. 5 shows Hofstee transformations of Na^+-dependent D-glucose transport kinetics for both control and ischemic BBM fractions. Ischemia results in a large decrease in V_{max} (1,913±251 vs. 999±130 pmol/mg per s; $P < 0.01$), whereas K_t remained constant (0.38±0.01 vs. 0.38±0.02 mM).

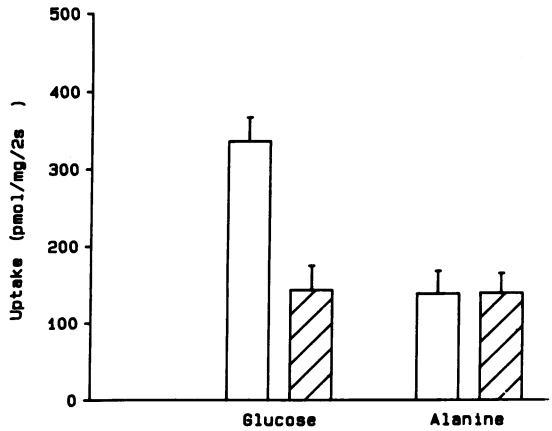

Figure 3. Effect of ischemia on initial Na^+-dependent D-glucose and L-alanine uptake rates. BBMV 2-s Na^+-dependent D-glucose and L-alanine uptake rates were quantitated for control (*open bars*) and after 50 min of ischemia (*hatched bars*) using Millipore filtration techniques. 20 μl of BBMV were added to 40 μl of transport buffer with rapid vortexing. Uptake was halted at 2 s by the addition of 1 ml ice-cold stop solution followed by rapid application of prewetted Millipore filters (HA, 0.45 μm). 2 s were quantitated using a metronome. All samples were run in duplicates and these data represent the mean±1 SD, $n = 4$. Na^+-independent transport was subtracted to correct for simple diffusion.

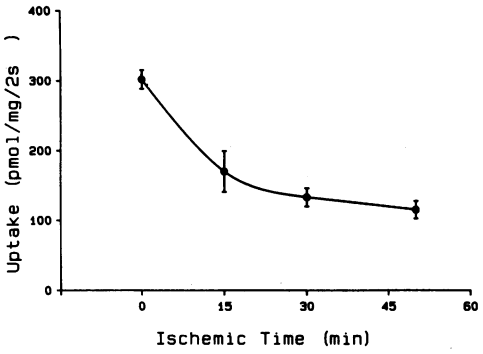

Figure 4. Effect of the duration of ischemia on Na^+-dependent D-glucose uptake by renal cortical BBMV. 2-s uptakes were carried out as described in Fig. 3. Duration of ischemia was controlled by varying the clamp time. These data show the time course of ischemia on BBMV glucose uptake and represent the mean±1 SE, $n = 3$.

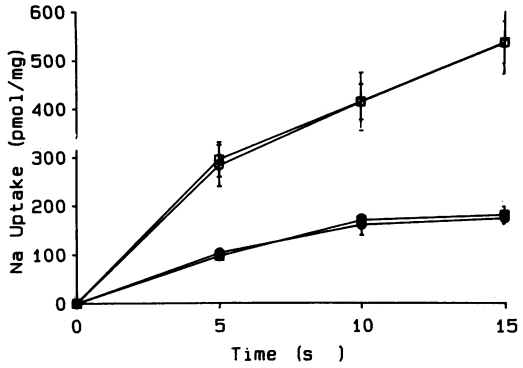

Figure 6. Effect of ischemia on renal cortical BBMV Na^+ uptake. BBMV Na^+ uptake by control (○) and after 50 min of ischemia (□) was quantitated in the presence (*solid symbols*) and absence of harmaline (10 mM), a known selective competitive inhibitor of renal BBMV Na^+-dependent transport processes (25). The buffer contained 1 mM Na^+, 200 mM mannitol, 20 mM Hepes (pH 7.4 with Tris) and 2.5 μCi ^{22}Na per transport vial. Uptake was quantitated using Millipore filtration techniques as described in Fig. 1, except 40 μl of BBMV were used. The stop solution was identical to the transport buffer except there was no Na^+ and it was ice cold. Samples were run in duplicate and these data represent the mean±1 SE, $n = 4$.

Possible explanations of the marked decrease in glucose uptake after ischemic injury include an alteration in dissipation of Na^+ gradient, an alteration in the sidedness of the BBMVs, a reduction in the number of functioning carrier units, and a reduction in the functional ability of individual carrier units. Dissipation of the sodium gradient was evaluated directly by determining $^{22}Na^+$ uptake in the presence of a 1 mM external Na^+ gradient. These results are shown in Fig. 6 and indicate that ischemia had no effect on dissipation of the Na^+ gradient and rule against this as a mechanism of ischemic-induced reduced glucose uptake. In these studies harmaline (10 mM) was used as a selective inhibitor of renal apical Na^+ transport sites (25). The lack of a difference in the presence of harmaline indicates ischemia also had no effect on nonspecific Na^+ binding to apical membranes.

As the Na^+-dependent glucose transporter may be located asymmetrically in apical membranes (26); an alteration in sidedness of BBMV could also alter Na^+-dependent glucose transport. Sidedness of the membrane vesicle population in both control and ischemic BBMV, therefore, was evaluated using a technique previously described by Haase et al. (13). Control and ischemic BBMVs were preincubated with a monospecific polyclonal antibody to leucine aminopeptidase. This enzyme is known to be localized to the external surface of the BBMVs and the antibody is known not to reach the intravesicular space (13). As is shown in Fig. 7, there was rapid neutralization of leucine aminopeptidase in both control and ischemic membranes, with control and ischemic membranes showing equivalent inactivation. In addition, 0.05% Triton X-100 had no

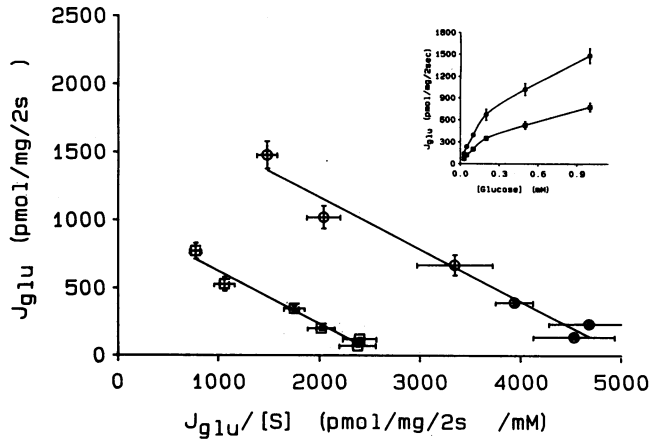

Figure 5. Kinetic plot of the initial rate of Na^+-dependent D-glucose uptake (J_{glu}) as a function of glucose concentration(s) in control (○) and ischemic (□) BBMV. Transport buffers were described in Fig. 1, glucose was varied between 0.03 and 1.0 mM, and uptake was determined after 2 s. The *y* intercepts for control and ischemic BBM were 1,930 and 1,011 pmol/mg per 2 s respectively, with a linear regression *r* value of 0.98 for each plot. Insert shows D-glucose uptake by control (○) and ischemic (□) BBM as a function of glucose concentration. The data represent the mean±SE, $n = 3$.

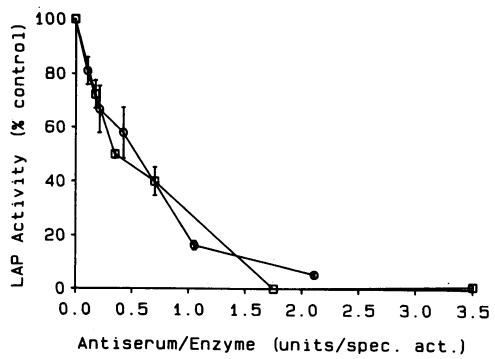

Figure 7. Immunologic inhibition of control (○) and ischemic (50 min; □) BBM leucine aminopeptidase activity. BBMV (20 μl) were preincubated for 20 min at 20°C with varying amounts (0–20 μl) of a neutralizing polyclonal antibody (6 U/μl) resuspended in PBS (13). The total preincubation volume was kept constant by adding varying amounts of PBS. Activity is expressed as a percent of the PBS control. BBM protein content was similar in control and ischemic BBM fraction (1.41±0.04 vs. 1.39±0.12). Initial LAP specific activities in control and ischemic BBMV were 56.5±3.8 U and 34.3±1.6, respectively.

effect on either control or ischemic BBMV LAP activity in the absence of the antibody, or neutralization in the presence of the antibody. These results are in close agreement with the work of Haase et al. (13), who also showed this technique gave comparable results to freeze fracture techniques and could be used to document the sidedness of renal cortical apical vesicles.

As the transport of glucose into BBMVs is electrogenic (27), it was also important to evaluate the role of an alteration in membrane potential during transport. This was done as previously described (11, 26) in the presence of 2 μM valinomycin and KCl. Under these conditions Na^+-dependent glucose uptake by ischemic BBMVs was only 22±5% of the uptake by control BBMVs and indicates the reductions in glucose transport secondary to ischemia are independent of membrane potential alterations.

To evaluate the effect of ischemia on the number and affinity of Na^+-dependent glucose carriers phlorizin binding studies were conducted. Previous studies have indicated phlorizin binding is specific, reversible, and of high affinity (28, 29). In preliminary studies, we showed that binding and dissociation equilibrium occurred in both control and ischemic membranes in < 5 min, which is in agreement with previous studies on control BBM (22, 23). Data in Fig. 8 indicate that ischemia altered specific high-affinity phlorizin binding and Scatchard transformation showed this was due to a reduction in the number of binding sites for phlorizin. Ligand Scafit analysis revealed ischemia reduced the number of binding sites from 390±43 to 146±24 pmol/mg ($P < 0.01$), but K_d was unaltered (0.47±0.08 vs. 0.58±0.16 μM). As this study could not differentiate between the capability of existing D-glucose carriers to bind phlorizin and a decrease in the total number of carriers, an additional study using benzyl alcohol was undertaken. In previous studies (30), benzyl alcohol has been shown to decrease Na^+-dependent glucose transport and induce large increases in membrane fluidity. To determine if this was due to an alteration in the binding capacity of transport carrier sites, the effect of 20 mM benzyl alcohol on phlorizin binding in control BBMVs was analyzed. This dose of benzyl alcohol has previously been shown to alter V_{max} and not K_t and in addition had no effect on dissipation of the Na^+ gradient (30). Ligand Scafit analysis revealed 20 mM benzyl alcohol reduced the number of phlorizin binding sites from 418.8±64.7 to 116.6±45.5 ($P < 0.01$), but K_d was unaltered.

As increasing membrane fluidity (benzyl alcohol) apparently reduced the phlorizin binding capacity of existing Na^+-dependent glucose carriers and ischemia results in large alterations in BBM lipids, we questioned whether these lipid changes were responsible for altered Na^+-dependent glucose transport after ischemia. To evaluate this possibility, ischemia was induced for variable lengths of time (0, 5, 10, 15, 30, and 50 min), and its effect on BBM Na^+-dependent glucose uptake, phospholipids, and DPH fluorescence polarization was quantitated. Figs. 9 and 10 show the high correlations between glucose uptake and the sphingomyelin-to-phosphatidylcholine (0.96; $P < 0.01$) and the cholesterol-to-phospholipid (0.66; $P < 0.05$) ratios in BBMVs. As a large decrease in the sphingomyelin-to-phosphatidylcholine (SPH/PC) and cholesterol-to-phospholipid ratios could result in large increases in BBM fluidity, we next correlated BBM DPH fluorescence polarization and glucose uptake. Fig. 11 shows a strong correlation (0.83; $P < 0.01$) also existed between DPH polarization and Na^+-dependent glucose uptake. Statistically significant correlations also existed between membrane fluorescence polarization and the SPH/PC ratio (0.85; $P < 0.01$) and the cholesterol-to-phospholipid ratio (0.86; $P < 0.01$).

Dynamic polarization studies, shown in Table I, were then used to determine if the effect was due to a change in the order or rate component of membrane fluidity. A large change in the order component of anisotropy was seen, but no alteration in either fluorescence lifetimes or the rate component of membrane fluidity, as measured by rotational correlation times, was observed.

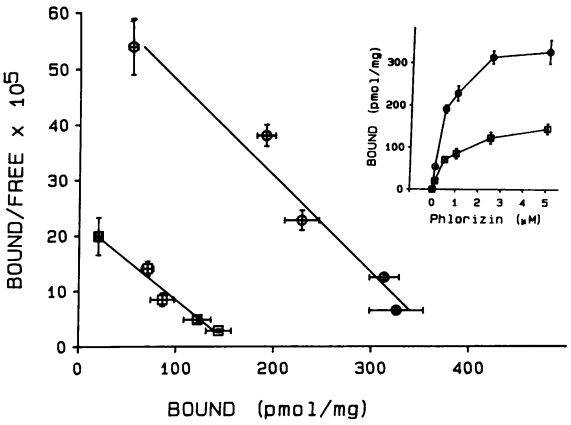

Figure 8. Effect of ischemia on specific phlorizin binding to renal cortical BBM. Equilibrium specific phlorizin binding (total minus nonspecific with > 100-fold excess of cold phlorizin) was determined in control (○) and ischemic (50 min; □) BBM at phlorizin concentrations of 0.1, 0.5, 1.0, 2.5, and 5 μm in the presence of 150 mM Na^+, 200 mM mannitol, 20 mM Hepes (pH to 7.4 with Tris). Specific phlorizin binding vs. phlorizin concentration as well as the Scatchard transformation of these data are shown. Data represent mean±1 SE, $n = 3$ of assays done in duplicate. Error bars not visualized were smaller than the symbols.

Discussion

Reversible ischemia is known to alter the transcellular transport of several compounds across epithelial cells. In particular

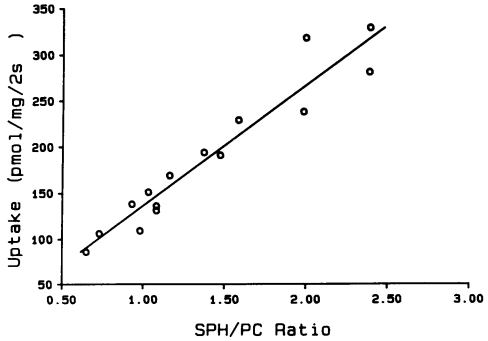

Figure 9. Relationship between BBMV Na^+-dependent glucose uptake and SPH/PC. Ischemia was induced for variable durations (0, 5, 10, 15, 30, 50 min) using the bilateral clamp technique. BBMV were isolated and 2-s glucose uptakes were performed as detailed in Fig. 3. Phospholipids were determined using 2-D thin-layer chromatography. Linear regression was performed and the correlation coefficient was 0.96; $P < 0.01$.

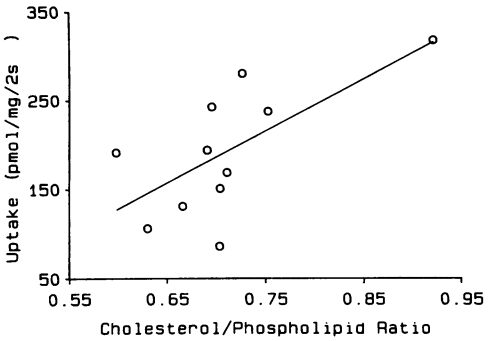

Figure 10. Relationship between BBMV Na^+-dependent glucose uptake and cholesterol-to-phospholipid ratio. See Fig. 9 for details. Linear regression was performed and the correlation coefficient was 0.66; $P < 0.05$.

there is a marked reduction in both Na^+ and glucose transport by renal proximal tubules after a reversible ischemic event (1, 31). The cellular site and mechanism responsible for these alterations, however, remained to be determined. Several lines of evidence indicated the ischemic-induced reduction in transcellular glucose transport may be due to an altered BBM. First, studies using ischemic clamp models have clearly documented morphologic alterations is the BBM (2, 3). The BBM is both lost into the lumen and internalized within the cell with the extent of membrane loss and duration of time required for regeneration dependent upon the ischemic interval. Secondly, biochemical evidence indicates the lipid composition of the BBM is markedly changed during ischemic injury. There were large reductions in the SPH/PC and cholesterol-to-phospholipid ratios resulting from ischemic-induced loss of epithelial polarity (10). These later changes would be expected to increase BBM fluidity, and because the Na^+-dependent glucose carrier is extremely sensitive to the surrounding physical environment (11), we reasoned that lipid alterations induced by ischemia in BBM might be responsible for reduced carrier-mediated glucose transport. As isolation of representative BBMVs after reversible ischemia was possible (10), we sought to determine whether this was indeed the case.

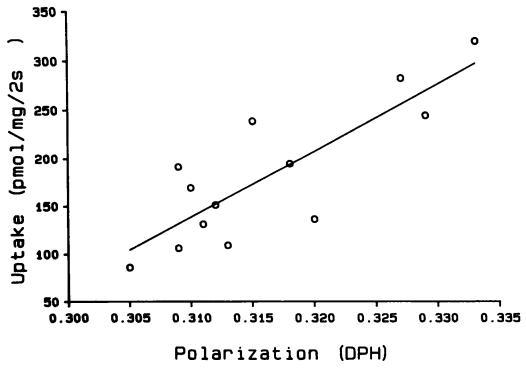

Figure 11. Relationship between BBM polarization and Na^+-dependent glucose uptake. Steady-state fluorescence polarization (DPH) using a SLM 4800 was determined on aliquots of BBMV (72 μg protein) at 35°C as described in Methods. 2-s Na^+-dependent glucose uptake was determined as indicated in Fig. 3. Linear regression revealed a correlation coefficient of 0.83; $P < 0.01$.

That ischemia did in fact reduce BBM Na^+-dependent glucose uptake is clearly shown in Figs. 1–4. Standard transport studies showed this effect to be present at 15 s, and equilibrium values (120 min) indicated intravesicular space was unaltered by ischemia. Furthermore, the effect seemed to be specific for glucose in that the Na^+-dependent transport of L-alanine was unaltered by 50 min of ischemia. To evaluate initial transport rates, we performed 2-s uptakes using Millipore filtration techniques. Again, a large reduction in Na^+-dependent glucose transport was noted, and Na^+-dependent uptake of alanine was again unaltered. This reduction in glucose transport was shown to be due to an alteration in V_{max} and not K_t. Therefore, the effect of ischemia on Na^+-dependent glucose uptake occurred rapidly due to a reduced V_{max}, and the majority of effect was present after only 15 min of ischemia. Lack of an increase in Na^+-independent glucose uptake by BBMV was also of interest. We have previously postulated that during ischemia BLM, Na,K-ATPase migrates into the BBM domain (10). Given this hypothesis, one might also expect the BLM-facilitated glucose carrier to move into the BBM domain. The present results are inconsistent with this idea. This could be due to the carrier protein being "fixed" to the BLM aspects of the cell or inactivation of the carrier during migration. Additional studies need to be conducted to determine if other Na^+-dependent and Na^+-independent processes are altered.

It was next necessary to determine the mechanism resulting in reduced Na^+-dependent glucose transport after ischemic injury. Dissipation of the Na^+ gradient and vesicle sidedness were evaluated directly and shown not to be different in control and ischemic BBMV. ^{22}Na uptake was determined in the presence of a 1 mM Na^+ gradient and showed the Na^+ gradient was not collapsed more rapidly in ischemic BBMVs. To determine the effect of ischemia on the sidedness of the BBMVs, a monospecific polyclonal antibody to leucine aminopeptidase was used. As this antibody cannot penetrate the BBMV (13), only those sites on the outside of vesicles are accessible. Therefore, right-side-out vesicles should show complete inhibition of LAP with adequate amounts of the neutralizing antibody. In both control and ischemic BBMV, LAP was readily accessible to the antibody, and nearly complete neutralization of LAP activity was seen in both control and ischemic membrane vesicles. This is important, as recent evidence indicates the Na^+-dependent glucose transporter may be asymmetric and an alteration in sidedness of the vesicle could therefore alter transport characteristics (26). This, however, did not seem to be the case after reversible ischemic injury.

The effect of ischemia on Na^+-dependent glucose carrier number was then determined using [^{3}H]phlorizin binding techniques. Phlorizin is known to bind specifically to the Na^+-dependent glucose carrier and has been used to quantitate both binding affinity (K_d) and the number of carrier units (22, 23, 28, 29). The phlorizin concentrations we used have previously been shown by other investigators to be in the range of the high-affinity Na^+-dependent glucose carrier (22, 23, 28, 29). Ischemia clearly reduced phlorizin binding, and Scatchard analysis of this data revealed that the reduction was due to an alteration in the number of binding sites and not binding affinity.

There are several mechanisms by which ischemic injury could reduce the number of phlorizin binding sites. First, loss of carrier units from BBM or destruction of binding sites within the BBM would result in reduced phlorizin binding.

Table I. Effect of Ischemia on BBM Fluorescence Lifetime, Polarization, Anisotropy, and Rotational Correlation Time

	T	P	Anisotropy		R
			Total	r_∞	
	S				
Control BBM	10.9±0.7	0.326±0.004	0.243±0.003	0.244±0.005	0.122±0.015
Ischemic BBM	10.9±0.3	0.299±0.003	0.221±0.002	0.202±0.003	0.142±0.019
P Value	NS	<0.01	<0.01	<0.01	NS

Values represent mean±1 SD; $n = 6$. T, fluorescence lifetime; P, fluorescence polarization; r_∞, limiting fluorescence anisotropy; R, rotational correlation time.

Recent evidence (32) indicates Na^+ binding to the carrier induces a conformation change in the glucose binding site necessary for glucose binding. Furthermore, both the Na^+ and glucose binding sites may be on the same polypeptide (33). The present studies do not address whether ischemic injury resulted in reduced phlorizin binding by altering Na^+ binding, the conformational change of the carrier or glucose binding of existing carrier units. This latter possibility could be due to an alteration in the equilibrium between associated and dissociated subunits. Turner and Kemper (34) and later Lin et al. (35), both using radiation inactivation studies, have suggested the Na^+-dependent glucose carrier may consist of more than one subunit and that not all of the subunits are required for glucose or phlorizin binding. Malathi and Preiser have isolated a Na^+-dependent phlorizin-inhibiting renal apical glucose carrier (36). This carrier also consisted of two subunits. Whether one subunit is necessary for binding and an additional subunit for membrane translocation is not known.

However, the data do suggest that an alteration in BBM lipids and the order component of membrane fluidity may be responsible. First, Na^+-dependent glucose uptake correlated highly with the SPH/PC and cholesterol-to-phospholipid ratios over a wide range of uptake and ratios. Secondly, glucose uptake correlated inversely with DPH fluorescence polarization also over a wide range. That the SPH/PC and cholesterol-to-phospholipid ratios are major determinants of BBM fluidity is well known (8–10). The data reported here again confirm this as both the SPH/PC and cholesterol-to-phospholipid ratios correlated highly with BBM DPH fluorescence polarization. Finally, benzyl alcohol, which increases membrane fluidity and reduces Na^+-dependent glucose uptake in both intestinal and renal BBMV (11, 30), also reduced carrier number but not carrier affinity. As the effects of benzyl alcohol are reversible (30), it is unlikely benzyl alcohol caused displacement of carriers from the membrane. Whether this is the result of the inability of the carrier to bind Na^+, have a conformational shift, or bind glucose remains to be determined. The close agreement, however, between the benzyl alcohol and ischemic experiments implies the mechanism may be similar in the two circumstances. Taken together these data indicate factors which decrease the order component of BBM anisotropy (increase BBM fluidity), decrease Na^+-dependent glucose transport by reducing the binding capability of the carriers. A similar phenomenon has been postulated for the Na^+-independent glucose carrier found in nonepithelial cells (37, 38). In those studies, however, carrier number was not quantitated. These data, however, are at odds with the observation that Na^+-dependent glucose transport increases with increasing temperature (39), which is known to increase membrane fluidity. This could be explained by a reduction in carrier number or function when BBM fluidity increases beyond its normal physiologic limits, which occurs with ischemia or benzyl alcohol but not with increasing temperature. In our studies the effect of membrane fluidity on carrier turnover can also be calculated. Ischemia seemingly increased BBM carrier turnover (V_{max}/carrier number) from 2.5 to 3.4 s. The larger reduction in carrier number, however, resulted in an overall reduction in glucose transport.

In summary, we have shown that the defect in renal transcellular glucose transport after ischemia is due to an alteration in glucose transport across the apical membrane. Furthermore, this alteration was specific to glucose, duration dependent, and the result of large alterations in BBM lipid composition. This in turn resulted in an increase in membrane fluidity and a decreased capability of the Na^+-dependent carrier to bind phlorizin. These data indicate, therefore, that reversible ischemia induces surface membrane alterations that have important physiologic significance in epithelial transport.

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