Effect of cell shrinkage on permeability of cultured bovine aortic endothelia and frog mesenteric capillaries

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1. We have tested the hypothesis that a reduction in endothelial cell volume increases microvessel permeability and that the degree of endothelial cell attachment to their basement membranes determines the magnitude of permeability changes caused by a reduction in endothelial cell volume.

2. A decrease in endothelial cell volume was imposed on both intact microvessels and cultured endothelial monolayers by raising osmolarity by 100 mosmol l^{-1}.

3. We found that hypertonic solutions did not increase the hydraulic permeability (L_p) of individually perfused venular microvessels in frog mesentery when the perfusate contained albumin. Hypertonic solutions did increase L_p, however, after we perfused the microvessels with the peptide Gly-Arg-Gly-Asp-Thr-Pro (GRGDTP; 0·3 mmol l^{-1}), to disrupt integrin-dependent endothelial cell (EC) attachment to the extracellular matrix (ECM).

4. After albumin was removed from the perfusate, hypertonic solutions increased L_p of microvessels and the permeability of endothelial monolayers to α-lactalbumin.

5. Our findings indicate that endothelial cell integrin–ECM binding plays a role in transducing changes in cell volume and/or shape into changes in permeability. We hypothesize that removal of albumin from the vascular perfusate may compromise EC–ECM interactions via an integrin-dependent mechanism.

The aim of these experiments was to investigate the mechanisms whereby a change in endothelial cell volume is transduced into a change in the microvessel permeability. We have used changes of endothelial cell volume to investigate further the role of endothelial cell size and shape in the control of permeability properties of endothelial barriers. Previous studies using cultured endothelial monolayers have established that exposure of endothelial cells to hypertonic solutions causes an immediate reduction of intracellular volume with a subsequent restoration of cell volume mediated by increased uptake of Na^+, K^+ and Cl^- via the Na^+–K^+–$2Cl^-$ cotransport system (O'Neill & Klein, 1992; O'Donnell, 1993). A number of studies have provided evidence that hypertonic solutions, which decrease endothelial cell volume, cause an increase in permeability of the endothelial barrier, both in cultured endothelial monolayers and *in situ*. Rapoport and his colleagues showed that perfusion with hypertonic solutions increased blood–brain barrier solute transport and postulated that this occurred by cell shrinkage-induced opening of the tight junctions (Rapoport, Fredericks, Ohno & Pettigrew, 1980). Granger and his colleagues reported increased permeability of cat intestinal capillaries to plasma proteins following hypertonic perfusion (Granger, Granger, Brace, Parker & Taylor, 1979). Other investigators have also shown that hypertonic solutions cause a transient increase in macromolecule permeability of capillaries and cultured endothelial monolayers (Rasio, Bendayan & Goresky, 1981; Shepard, Goderie, Brzyski, Del Vecchio, Malik & Kimelberg, 1987). These observations have led to the hypothesis that reduction of the endothelial cell volume increases permeability by increasing the area for exchange and by decreasing the path length for exchange between adjacent endothelial cells (Shepard *et al.* 1987; O'Neill & Klein, 1992; O'Donnell, 1993). In contrast, some experiments in which water and small solute permeability were measured did not produce a permeability increase upon exposure to hypertonic solutions (Pappenheimer, Renkin & Borrero, 1951; Curry, Mason & Michel, 1976).

The mechanisms which underlie these differences are not understood. Most experiments described above were not specifically designed to investigate the effect of cell shrinkage by hypertonic solutions on endothelial barrier permeability modulation. Thus one focus of this study was to test the hypothesis that cell shrinkage by hypertonic

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solutions increased endothelial barrier permeability, using well-defined experimental conditions *in vitro* and *in vivo*. In the system *in vitro*, we evaluated permeability of cultured endothelial monolayers to α-lactalbumin. In the system *in vivo*, we measured hydraulic permeability (L_p) of perfused frog mesenteric microvessels. In both systems, hypertonic solutions that were 100 mosmol l^{-1} above the normal osmolarity were used. Assuming that endothelial cells respond to these hypertonic solutions as perfect osmometers, 26 and 30% reductions in cell volume are expected for the bovine and frog endothelia, respectively (normal extracellular fluid osmolarities for the bovine and frog endothelia are 290 and 230 mosmol l^{-1}, respectively). The validity of this assumption is supported by many studies of cell volume responses to hypertonic solutions, including endothelial cells (Eveloff & Warnock, 1987; O'Donnell, 1993).

A second focus of the present study was to test the hypothesis that the response of the endothelial barrier to cell shrinkage may be influenced by the extent of endothelial cell attachment to the basement membrane. We reasoned that if endothelial cell (EC) attachment to extracellular matrix (ECM) proteins contributes to maintenance of endothelial barrier permeability, then disruption of the attachment sites is likely to increase the permeability response to hypertonic solutions. EC–ECM attachment is mediated primarily by integrins. Integrins are present in frogs and have a role in cell attachment (see Discussion). These heterodimeric integral-membrane glycoproteins interact with ECM proteins via a ligand-binding domain that recognizes arginine–glycine–aspartate (RGD, where R is arginine, G is glycine and D is aspartate) sequences. Previous studies *in vitro* have suggested that disruption of integrin–ECM binding increases endothelial barrier permeability. Qiao and his colleagues have shown that the treatment of bovine pulmonary microvascular endothelial cell monolayers with a peptide containing the RGD sequence, which competes for the integrin-binding site, results in a 3-fold increase in L_p (Qiao, Yan, Lum & Malik, 1995). In the present study, we used a system of *in vivo*, single perfused frog microvessels, to investigate the response of the endothelial barrier to cell shrinkage under conditions which disrupt endothelial cell attachment to the basement membrane. Preliminary results have been described in abstract form (Kajimura, O'Donnell & Curry, 1994*a*; Kajimura, O'Donnell & Curry, 1994*b*).

METHODS

In vitro

Endothelial cell monolayer preparations

Bovine aortic endothelial cells (BAECs) were harvested from calf thoracic aorta and were evaluated for the presence of factor VIII-related antigen. BAECs were grown in Eagle's minimal essential medium (MEM; JRH Biosciences, Lenexa, KS, USA) containing 7% fetal bovine serum (FBS; Hyclone Labs, Logan, UT, USA) and maintained in collagen-coated 75 cm^2 tissue culture flasks. BAECs (passages 8–15) were then seeded onto filter inserts (Transwell-Col, 12 mm diameter, 0·4 μm pore size; Costar Corp., Cambridge, MA, USA) designed to be placed into the wells of a 24-well tissue culture plate. Prior to plating cells, inserts were additionally coated with purified rat-tail collagen, Type IV (Collaborative Research, Bedford, MA, USA) and fibronectin (Boehringer Mannheim, Indianapolis, IN, USA). Cells were plated at a density of $2·5 \times 10^5$ cells cm^{-2}, grown in MEM supplemented with 7% FBS with a medium change every 3 days and incubated at 37 °C with 5% CO_2–95% air. It is well established that BAECs cultured under these conditions do, in fact, produce a basement membrane (Cheresh, 1987; Cheng & Kramer, 1989). All experiments were performed on the monolayers 2 or 3 days after they reached confluence.

Confluence was assessed by light microscopy after silver staining. Membranes were washed once with phosphate-buffered saline (PBS) right after the assay and then fixed with 3% glutaraldehyde in 0·1 mol l^{-1} cacodylate buffer for 5 min. Immediately after the fixative, membranes were placed in five solutions in succession; 0·9% NaCl for 2 min, 5% aqueous glucose for 30 s, 0·1% $AgNO_3$ for 1 min, 5% glucose for 30 s, then back into the fixative again. The silver halide was developed by a 15 min exposure to light of a fibre optic illuminator (FiberLite® Model 1780 D; Dolan-Jenner, Industries, Inc., MA, USA). The filter inserts were cut out, mounted and photographed on a Zeiss Photomicroscope II (Planapochromat × 25 NA, 0·65; or × 40 NA, 1·0). Each process was carried out at room temperature (18–20° C). Monolayers which were not confluent or showed damage were excluded from the study.

Measurement of α-lactalbumin flux across endothelial monolayers

Experiments were performed in Transwells, 12-well tissue culture trays containing twelve Transwell-Col inserts set in the wells. The insert was designated the luminal compartment and the outer well the abluminal compartment. Aliquots of 0·5 ml and 1·5 ml assay media were added to the luminal and abluminal compartments, respectively. These volumes produced equal liquid levels in both compartments to avoid hydrostatic pressure gradients. Permeability was assessed as the flux of α-lactalbumin labelled with tetramethylrhodamine isothiocyanate (TRITC) across the BAEC monolayers. Both luminal and abluminal compartments were equilibrated for 30 min in isotonic Hepes–MEM with 0 or 10 mg ml^{-1} bovine serum albumin (BSA: Fraction V) at 37 °C in an air atmosphere, then the media were replaced with fresh Hepes–MEM, which were either isotonic (290 mosmol l^{-1}) or hypertonic (390 mosmol l^{-1}, by addition of NaCl) containing 0 or 10 mg ml^{-1} BSA. The combination of the initial wash and the test replacements with albumin-free Hepes–MEM would have diluted FBS in the culture media by $(1–3) \times 10^4$-fold. Thus we expected to reduce albumin concentration to below the threshold (0·1 mg ml^{-1}) for maintaining permeability in intact microvessels (Mason, Curry & Michel, 1977; Huxley & Curry, 1985). At the beginning of the assay, aliquots (0·5 ml) of 40 μM TRITC-labelled α-lactalbumin were added to the luminal compartment. Aliquots of 200 μl from the abluminal compartment medium were sampled every 6 min for 36 min and sample volumes were replaced with the appropriate α-lactalbumin-free medium. Throughout the assay, monolayers were kept at 37 °C in a gyrotary water bath shaking at 60 cycles min^{-1}, to ensure adequate mixing. α-Lactalbumin content of the sample was determined by fluorescence quantification. The fluorescence intensity of each sample was measured by a cytofluor 2300 Dynatech MR 5000

(Chantilly, VA, USA). The solute permeability coefficient of the endothelial monolayers to α-lactalbumin (P_s) was calculated as:

$$P_s = \frac{\Delta C_a V_a}{\Delta C \Delta t S},$$

where ΔC_a is the change in TRITC-labelled α-lactalbumin concentration in the abluminal compartment over the sampling interval (Δt), V_a is volume of media in the abluminal compartment, S is the surface area of endothelial cell monolayer (cm^2) and ΔC is solute concentration difference between the two compartment ($\Delta C = C_l - C_a$), where C_l is solute concentration in the luminal compartment and C_a is solute concentration in the abluminal compartment).

In vivo

General methods

The preparations of both the frog mesentery and the micropipettes used to perfuse individual microvessels have been described in detail elsewhere (Curry, Huxley & Sarelius, 1983). Briefly the mesentery of a brain-pithed, male leopard frog (*Rana pipiens*, 6–7·5 cm in length supplied by J. M. Hazen, Alburg, VT, USA) was gently arranged on the surface of a Lucite pillar. This allows transillumination of the mesenteric microvasculature. The upper surface of the mesentery was superfused continuously with frog Ringer solution at 16–18 °C. The microvessels chosen for study were mostly postcapillary venules and some were true capillaries, 10–35 μm in diameter. The tissue was observed with an inverted microscope (Leitz Diavert) using a ×6 objective lens (NA, 0·18).

Measurement of hydraulic permeability (L_p)

The modified Landis micro-occlusion technique was used for the measurement of L_p (Michel, Mason, Curry, Tooke & Hunter, 1974). The fluid flux across a segment of a single perfused microvessel was measured from the motion of a flow marker immediately after occlusion of the vessel. Transcapillary fluid flux per unit area (J_v/S) was calculated as:

$$J_v/S = (dl/dt)(r/2l),$$

where dl/dt is the initial velocity of the flow marker after the microvessel is occluded, r is the vessel radius, and l the length between the flow marker and the point of occlusion. J_v/S was plotted against hydrostatic pressures. The regression of J_v/S with pressure yielded L_p as the slope. In most experiments, however, J_v/S was measured at a single pressure (30 cmH_2O) to detect the time course of the L_p change. L_p was calculated as:

$$L_p = (J_v/S)/(P_c - \pi_c),$$

where P_c is the capillary pressure, and π_c is the effective oncotic pressure of the perfusate (3·6 cmH_2O was used as π_c for the perfusate containing 10 mg l^{-1} BSA). The interstitial pressure was negligible and the interstitial oncotic pressure was assumed to be the same as that of the superfusate.

Solutions

Frog Ringer solution was used as the bathing solution for the dissection of the mesentery, the perfusates and the initial superfusate. All chemicals were purchased from Sigma unless specified. The composition of isotonic frog Ringer solution was (mmol l^{-1}): 111 NaCl, 2·4 KCl, 1·0 $MgCl_2$, 1·1 $CaCl_2$, 0·195 $NaHCO_3$, 5·5 glucose, and 5 Hepes and Na-Hepes. The pH was adjusted to 7·4 by the ratio of Hepes acid to base. The osmolarity of the isotonic Ringer solution was 230 mosmol l^{-1} as determined by freezing point depression (Advanced Wide-Range Osmometer 3W2; Advanced Instruments, Inc., Norwood, MA, USA). The hypertonic Ringer solution was prepared by increasing NaCl concentration from 111 to 166 mmol l^{-1} to raise the osmolarity to 330 mosmol l^{-1}. The perfusate containing albumin was prepared by adding 10 or 1 mg ml^{-1} BSA to Ringer solution. To eliminate osmotic gradients across the capillary wall, microvessels were perfused with a solution prepared by dialysing perfusate in 8000 MW cut-off dialysis tubing (Spectro/Por®; Spectrum, CA, USA) against three 2-litre changes of Ringer solution of equal osmolarity over 24 h at 5 °C.

Synthetic peptides

The peptides used in this study were GRGDTP (Peninsula Laboratories, San Diego, CA, USA) and Gly-Arg-Gly-Asp-Thr-Pro (GRGETP; Protein and Nucleic Acid Facility, Beckman Center, Stanford University, Medical Center, Palo Alto, CA, USA). Peptides were made up as 3 mmol l^{-1} stock solutions in albumin-free Ringer solution and stored at −70 °C for up to 1 week before use.

Statistical analysis

Values are reported as means ± S.E.M. throughout, unless specified. The significance of differences between means was calculated using both Student's paired t test and the non-parametric Wilcoxon signed-rank test. Significance was set at 5% level ($P < 0·05$).

RESULTS

Effect of hypertonic solutions on the permeability of cultured endothelial monolayers

To test the effect of hypertonic solutions on the permeability of monolayers formed by endothelial cells in culture, we measured the permeability of monolayers to α-lactalbumin (P_s) before and after exposure to hypertonic solutions. The cultured cells used have previously been shown to undergo cell volume regulation (O'Donnell, 1993). The solute α-lactalbumin was chosen because in continuous endothelial barriers *in vivo*, it shares a pathway common with the principal water pathway responsible for 90% of the L_p in the experiments described above (Curry *et al.* 1976).

Figure 1*A* shows no significant difference between the values of P_s measured in the isotonic ($(8·7 \pm 1·2) \times 10^{-6}$ cm s^{-1}) and hypertonic ($(9·5 \pm 0·9) \times 10^{-6}$ cm s^{-1}) groups when the media contained 10 mg ml^{-1} BSA ($n = 12$, $P > 0·05$, paired t test). Figure 1*B* shows the results when assay media did not contain BSA. The mean control P_s values were $(11·5 \pm 1·0) \times 10^{-6}$ cm s^{-1} and this significantly increased to $(14·5 \pm 1·2) \times 10^{-6}$ cm s^{-1} in the hypertonic group ($n = 16$, $P < 0·05$, paired t test). We noted that P_s in the absence of albumin was the mean of the first three sampling intervals (6, 12 and 18 min) excluding the later sampling points (24, 30 and 36 min), because in some experiments (but not all) with hypertonic media in the absence of albumin there was a tendency for the P_s to fall with time. This tendency for P_s to fall back towards values measured in the presence of albumin with time might be expected if cell volume began to be restored over periods longer than 20–30 min.

In eight independent experiments, the effect of BSA on the α-lactalbumin permeability in isotonic solutions was investigated. P_s of the isotonic group was significantly lower ($P < 0{\cdot}05$, paired t test) in the presence of BSA than in the absence of BSA in six out of eight experiments (BSA-free $(12{\cdot}4 \pm 1{\cdot}2) \times 10^{-6}$ cm s^{-1}; compared with BSA present at $(9{\cdot}9 \pm 1{\cdot}2) \times 10^{-6}$ cm s^{-1}). Thus, these monolayers show a 'protein effect', i.e. permeability is increased by removal of albumin from the perfusate. This will be further considered below and in the Discussion.

Effect of hypertonic solutions and albumin on microvessel permeability

Perfusion with albumin-containing solutions

A 100 mosmol l^{-1} increase in the osmolarity of both perfusate and superfusate (230 to 330 mosmol l^{-1}) did not increase L_p of single perfused microvessels in frog mesentery perfused with frog Ringer solution containing albumin. Figure 2*A* shows L_p measured as a function of time, after microvessels were exposed to the hypertonic solutions when the perfusate contained 10 mg ml^{-1} BSA. In the nine microvessels studied, the mean control L_p was $(4{\cdot}0 \pm 0{\cdot}8) \times 10^{-7}$ cm s^{-1} (cmH_2O)$^{-1}$ (range from $0{\cdot}6 \times 10^{-7}$ to $7{\cdot}4 \times 10^{-7}$ cm s^{-1} (cmH_2O)$^{-1}$). This range falls within the range of normal L_p values in microvessels perfused with frog Ringer solution containing BSA (Michel, 1984). Hypertonic solutions did not increase L_p in the presence of BSA. In fact, there was a slight tendency for L_p to fall with time after exposure to hypertonic solutions, becoming significantly less than control after 4·5 min ($n = 9$, $P < 0{\cdot}05$, Wilcoxon signed-rank test). This time is shorter than the time period expected for the restoration of cell volume (greater than 15–20 min if similar to mammalian endothelial cells) suggesting a different mechanism is responsible for the reduced L_p. In a further seven microvessels perfused with frog Ringer solution containing 1 mg ml^{-1} BSA, hypertonic solutions similarly failed to increase L_p but here there was no tendency for L_p to fall with time (Fig. 2*B*). In these experiments the initial L_p varied from $1{\cdot}9 \times 10^{-7}$ to $9{\cdot}2 \times 10^{-7}$ cm s^{-1} (cmH_2O)$^{-1}$ with a mean value of $(4{\cdot}5 \pm 1{\cdot}2) \times 10^{-7}$ cm s^{-1} (cmH_2O)$^{-1}$.

Perfusion with albumin-free solutions

In contrast to the results obtained when albumin was present in the perfusate, hypertonic solutions increased L_p in the vessels perfused with albumin-free Ringer solution. Figure 3*A* shows a sustained increase in L_p immediately after exposure to hypertonic solutions in the absence of BSA. Figure 3*B* summarizes the results of the complete experimental protocol in which at least four measurements were made on each of the nine microvessels. A mean control L_p measured during the perfusion with isotonic BSA–Ringer solution was $(5{\cdot}8 \pm 1{\cdot}1) \times 10^{-7}$ cm s^{-1} (cmH_2O)$^{-1}$. This was increased 1·7-fold by the removal of BSA from the perfusate. Exposure of microvessels to hypertonic solutions caused an additional increase in L_p of $3{\cdot}5 \pm 0{\cdot}6$-fold. L_p returned towards control values after 1 min of reperfusion with the perfusate containing 10 mg ml^{-1} BSA in isotonic Ringer solution. Values for each of the individual microvessels are given in Table 1.

To test whether the increase in L_p was not simply the result of perfusing the vessel a second time with frog Ringer solution, we evaluated the effect of two consecutive isotonic Ringer solution perfusions. Figure 4 shows an experiment in which we found that a second isotonic Ringer solution perfusion did not increase L_p but that a subsequent perfusion with a hypertonic Ringer solution did increase permeability as described above. In an additional five vessels a second perfusion with isotonic frog Ringer solution caused no significant increase in permeability. Thus the increased L_p was caused by exposure to hypertonic solutions, not by the second frog Ringer solution perfusion.

Effect of RGD peptides on L_p response to hypertonic solutions

Perfusion with Ringer solution without albumin has been suggested to result in alteration of the glycocalyx, the structure on the luminal side of the microvessels (Mason, Curry, White & Michel, 1979; Clough, 1991). It is possible, however, that it may alter structures associated with the cell surface on the abluminal side. We, therefore, tested the hypothesis that modification of the attachment of endothelial

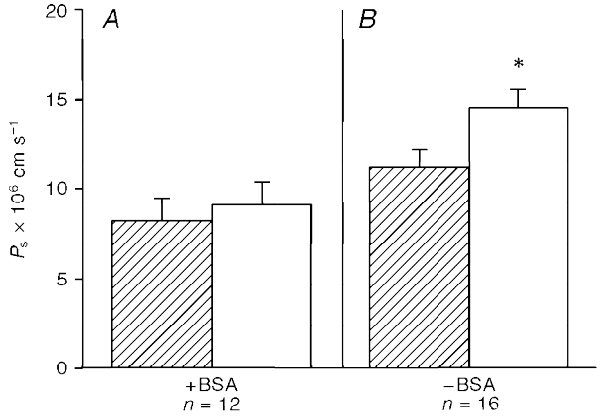

Figure 1. Effect of hypertonic solutions on permeability of cultured BAECs to α-lactalbumin

Permeability coefficient (P_s) of α-lactalbumin across monolayers with either isotonic (290 mosmol l^{-1}) or hypertonic (390 mosmol l^{-1}, by addition of NaCl) solutions is shown. *A*, the assay media of both compartments contained BSA (10 mg ml^{-1}). The mean P_s values of the isotonic (▨) and hypertonic (□) groups are not different ($n = 12$). *B*, the assay media contained no BSA. The hypertonic group (□) showed significantly higher P_s values ($n = 16$). Data are represented as means ± S.E.M. * $P < 0{\cdot}05$ compared with its respective isotonic group (paired t test).

Table 1. Effect of hypertonic solutions on L_p in the absence of BSA

Vessel number	Control (isotonic BSA)	Isotonic Ringer	Hypertonic Ringer	Isotonic BSA
1	5·5 ± 1·1	8·8 ± 2·2	44·0 ± 7·1	12·7 ± 2·5
2	6·3 ± 2·8	14·1 ± 1·2	30·7 ± 11·9	8·2 ± 0·8
3	12·6 ± 2·4	20·4 ± 6·9	41·1 ± 5·1	8·5 ± 2·3
4	6·4 ± 1·7	19·0 ± 2·7	49·6 ± 10·1	9·9 ± 0·4
5	5·7 ± 0·6	8·0 ± 2·8	60·6 ± 11·9	13·3 ± 2·2
6	4·4 ± 0·7	2·8 ± 1·0	11·6 ± 3·8	3·5 ± 0·8
7	7·4 ± 0·9	18·2 ± 6·1	27·6 ± 10·1	6·7 ± 1·7
8	2·1 ± 0·1	1·6 ± 0·3	4·3 ± 1·0	1·7 ± 0·1
9	1·6 ± 0·4	2·0 ± 0·4	7·4 ± 2·9	2·4 ± 0·1
Means ± S.E.M.	5·8 ± 1·1	10·5 ± 2·5	30·8 ± 6·6	7·4 ± 1·4

Values are mean (L_p ± S.D.) × 10^{-7} cm s^{-1} $(cmH_2O)^{-1}$.

cells to the basement membrane may determine the magnitude of the change in permeability of the endothelial barrier caused by exposure to hypertonic solutions. To do this we attempted to disrupt the attachment sites between the endothelial cells and the ECM component. We carried out these experiments on individually perfused microvessels by exposing microvessels to small peptides containing the RGD sequence which is the integrin-recognition motif found in many ECM components.

In each of twelve microvessels, L_p was first measured with the perfusate containing 1 mg ml^{-1} BSA. The microvessels were then recannulated with a pipette in which GRGDTP (0·3 mmol l^{-1}) was added to the perfusate in addition to

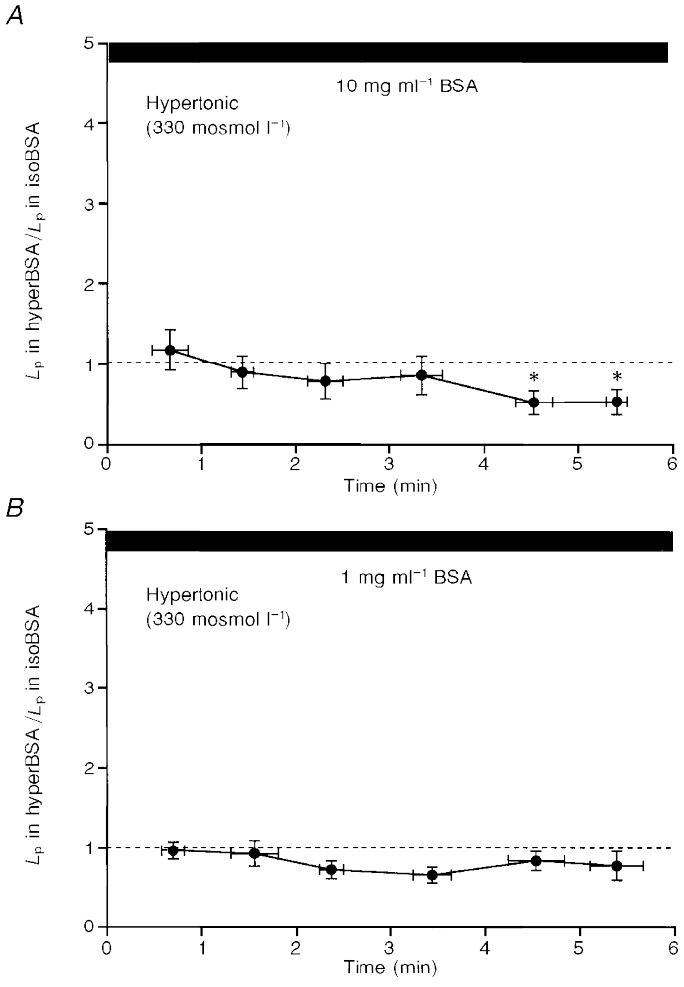

Figure 2. Effect of hypertonic solutions containing albumin on L_p of intact microvessels
Individual microvessels were first cannulated with control perfusate (not shown) then recannulated with hypertonic perfusate. L_p is expressed as a ratio relative to control values (means ± S.E.M.). The time intervals are means ± S.D. *A*, the perfusate contained 10 mg ml^{-1} BSA. After switching both superfusate and perfusate to hypertonic solutions, L_p tended to fall with time, becoming significantly reduced relative to control after 4·5 min ($n = 9$). * $P < 0·05$ (Wilcoxon signed-rank test). *B*, the perfusate contained 1 mg ml^{-1} BSA. After switching both superfusate and perfusate to hypertonic solutions, L_p did not significantly change over the 5·4 min time course ($n = 7$).

BSA. There was an increase in L_p in all the microvessels perfused with GRGDTP. In ten out of twelve microvessels the increase was small with a mean value for $L_{p,GRGDTP}/L_{p,control}$ over the first 4 min after perfusion of $1{\cdot}27 \pm 0{\cdot}06$ (S.E.M.) which was significantly greater than 1 (50 determinations of L_p, $P < 0{\cdot}05$, Wilcoxon signed-rank test). After 5 min of perfusion L_p tended to fall back towards control and L_p was not significantly increased relative to control after this time. Mean values are shown in Fig. 5*A* (isotonic). In one microvessel perfused with GRGDTP, L_p was 3·5 times higher than control after perfusion and remained elevated at this level for the 8 min of perfusion. In a second microvessel, L_p was initially increased 1·9 times higher than control, further increased to 6·5 times higher than control after 3 min of perfusion and was unstable thereafter, varying between 2 and 6 times higher than control.

Each of the twelve microvessels was then exposed to hypertonic solution (330 mosmol l^{-1}) in the presence of both GRGDTP (0·3 mmol l^{-1}) and BSA. In all vessels L_p increased more than 2-fold relative to control. The largest increases (8·7- and 12·3-fold during the first minute of perfusion) occurred in the two microvessels which had the largest increase in L_p in the presence of GRGDTP alone. The mean increase in L_p relative to control values during the first minute of perfusion in the presence of GRGDTP and hypertonic solutions was $4{\cdot}62 \pm 0{\cdot}98$-fold. L_p remained significantly elevated (and more than 2 times higher than control) for 6·3 min after perfusion began ($n = 10$, $P < 0{\cdot}05$, Wilcoxon signed-rank test; Fig. 5*A*). In seven other

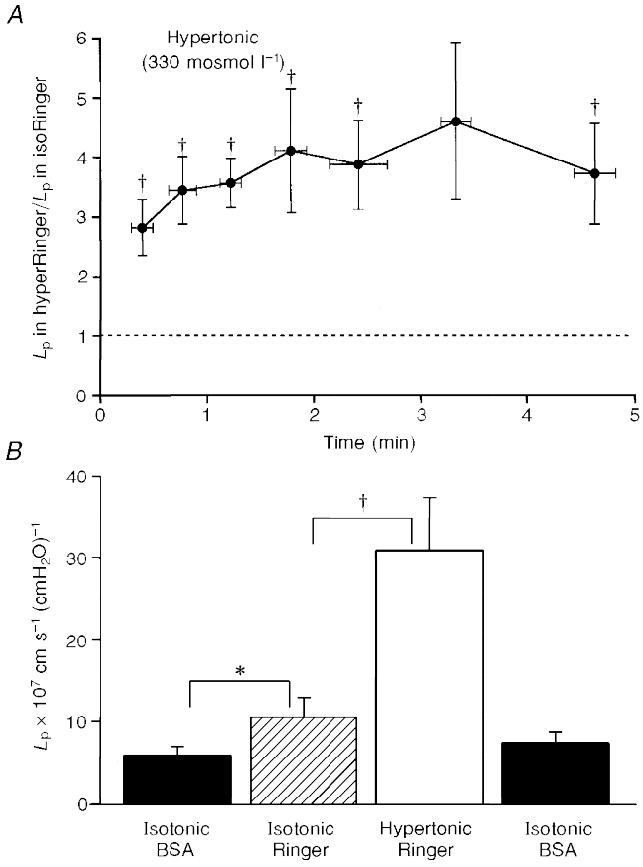

Figure 3. Effect of hypertonic albumin-free solutions on L_p of intact microvessels

A, the time course of the L_p increase after exposure to hypertonic Ringer solution is shown. L_p is expressed as the ratio relative to the L_p with isotonic frog Ringer (albumin-free) perfusion (means ± S.E.M.). The time intervals are means ± S.D. L_p increased immediately after exposure to hypertonic Ringer solution and remained elevated after 5 min. † $P < 0{\cdot}05$ compared with L_p measured with isotonic Ringer perfusion ($n = 9$, Wilcoxon signed-rank test). *B*, summary of the results from 9 microvessels which had a mean control L_p of $(5{\cdot}8 \pm 1{\cdot}1) \times 10^{-7}$ cm s^{-1} $(cmH_2O)^{-1}$ during perfusion with isotonic Ringer solution containing 10 mg ml^{-1} BSA. Mean L_p after BSA was removed from the perfusate was $(10{\cdot}5 \pm 2{\cdot}5) \times 10^{-7}$ cm s^{-1} $(cmH_2O)^{-1}$ (* $P < 0{\cdot}05$ compared with baseline, Wilcoxon signed-rank test). Mean L_p within the first 2 min of hypertonic Ringer perfusion was $(30{\cdot}8 \pm 6{\cdot}6) \times 10^{-7}$ cm s^{-1} $(cmH_2O)^{-1}$ († $P < 0{\cdot}05$ compared with L_p measured with isotonic Ringer perfusion, Wilcoxon signed-rank test). Mean L_p after 1 min reperfusion with the perfusate containing 10 mg ml^{-1} BSA was $(7{\cdot}4 \pm 1{\cdot}4) \times 10^{-7}$ cm s^{-1} $(cmH_2O)^{-1}$. Data are represented as means ± S.E.M.

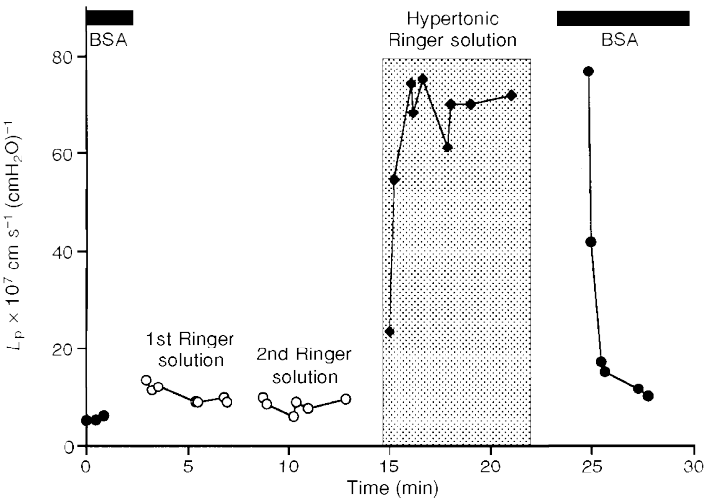

Figure 4. Effect of a second isotonic Ringer perfusion on L_p
Measurements of L_p are shown during multiple cannulations of a single vessel with different perfusates. The vessel was perfused twice with isotonic Ringer perfusate prior to exposure to hypertonic Ringer solution. There was no difference in L_p between the first and the second Ringer perfusion. Hypertonic Ringer solution caused L_p to increase to the peak value of $74{\cdot}3 \times 10^{-7}$ cm s^{-1} (cmH$_2$O)$^{-1}$. L_p returned towards the control level after reperfusion with isotonic perfusate containing BSA (10 mg ml^{-1}).

microvessels the same protocol was followed using another synthetic peptide GRGETP (containing the RGE sequence instead of RGD) which does not compete for the ligand binding site. GRGETP did not cause an increase in permeability after the microvessels were exposed to hypertonic solutions (Fig. 5*B*). In fact, as observed in BSA (10 mg ml^{-1}) and a hypertonic perfusate, L_p tended to fall with time in these vessels, becoming significantly reduced relative to control after 3·4 min ($n = 7$, $P < 0{\cdot}05$, Wilcoxon signed-rank test). The mechanisms causing the decrease in permeability are not known.

In three additional microvessels, the concentration of GRGDTP peptide was reduced to 0·1 mmol l^{-1} and the protocol shown in Fig. 5*A* was repeated. At this concentration, the peptide had no effect on L_p during

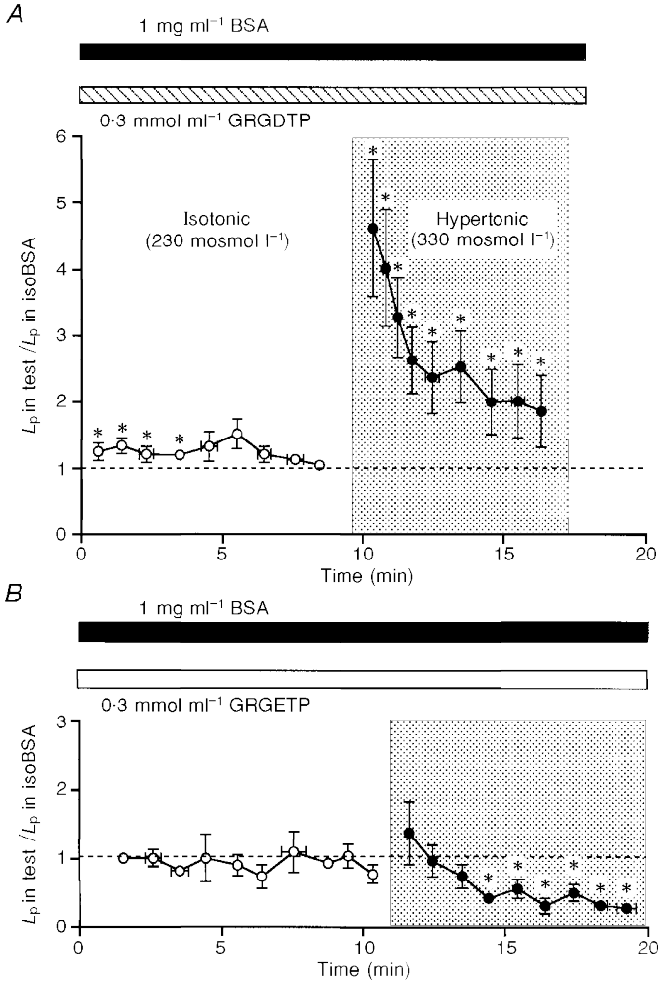

Figure 5. Effects of exposing microvessels to hypertonic solutions after RGD peptide perfusion
A, microvessels were pretreated with GRGDTP (0·3 mmol l^{-1}) in the presence of BSA (1 mg ml^{-1}). There was a small but significant increase in L_p over the first 4 min after perfusion with the RGD peptide. After 5 min of perfusion, L_p tended to fall back towards control. After exposure to hypertonic solutions (330 mosmol l^{-1}) L_p transiently increased 4·6-fold within 1 min and then fell but remained elevated by more than 2-fold after 6·3 min ($n = 10$). *B*, L_p did not increase in the vessels perfused with GRGETP (0·3 mmol l^{-1}) in the presence of BSA (1 mg ml^{-1}). After exposure to hypertonic solutions L_p tended to fall with time and values were significantly reduced relative to the control values after 3·4 min ($n = 7$). (* $P < 0{\cdot}05$ compared with baseline, Wilcoxon signed-rank test.) The time intervals are means ± S.D. and values of L_p expressed in the ratio are means ± S.E.M.

perfusion with the hypertonic solution. Thus, 0·3 mmol l^{-1} is close to the threshold concentration for observing an effect of the peptide on the response to a hypertonic frog Ringer solution. This observation was consistent with preliminary experiments with the peptide (concentration range, 0·1–1 mmol l^{-1}), which showed that a GRGDTP peptide concentration of 0·3 mmol l^{-1} was the threshold to increase baseline L_p in microvessels perfused with no albumin in perfusate. The combined effect of both frog Ringer perfusion and exposure to the RGD peptide was not investigated further, but the observation that there was a similar threshold concentration in both Ringer solution-perfused and albumin-perfused vessels to increase L_p indicates that the action of the peptide to modify EC–ECM interactions was not significantly modified by diffusion barriers at the capillary wall. If barriers were significant, the threshold concentration would have been higher in vessels perfused with albumin in Ringer solution, which have lower permeability to solutes than vessels perfused with Ringer solution above.

The reversibility of the effect of the RGD peptide was checked in preliminary experiments. The effect of the RGD peptide on increasing L_p during albumin-free Ringer solution perfusion was reversible when those vessels were reperfused with frog Ringer solution containing albumin (10 mg ml^{-1}).

Additional control experiments

The L_p measurements in all the above experiments were determined at one capillary pressure to enable the time course of changes to be followed. To verify that the increased filtration rates were due to a real change in L_p, rather than to a change in driving force (for example an osmotic gradient established across the capillary wall), we measured J_v/S at different hydrostatic pressures in the microvessels. The results of a representative experiment in which the microvessel was exposed to hypertonic solutions after BSA was removed from the perfusate is shown in Fig. 6 as the relationship between J_v/S and the capillary pressure. The lower and upper lines are regression lines obtained when the vessel was perfused with isotonic and hypertonic frog Ringer solution, respectively. The fact that the slope of the regression line increased with no change in the intercept on the pressure axis indicates that L_p was increased by the hypertonic solution. The same results were obtained in five additional independent experiments (Table 2).

DISCUSSION

Our results demonstrate that cell shrinkage caused by exposure to hypertonic solutions has no effect on the endothelial barrier permeability when the barriers are in a low permeability state, established by the presence of albumin in the perfusate. However, cell shrinkage does increase microvessel barrier permeability after albumin is removed from the perfusate. These experiments, done both on cultured endothelial cell monolayers and on intact microvessels, demonstrate that cell shrinkage exerts similar effects on permeability, i.e. that hypertonic solutions increase barrier permeability in the absence but not presence of albumin. Thus, our results demonstrate that a change in endothelial cell volume is not always transduced into a change in the permeability of the endothelial barriers. We also found in the present study that hypertonic solutions increased the permeability of microvessels in the presence of albumin when the microvessels were first perfused with GRGDTP, a peptide which loosens EC–ECM attachment by competing for the integrin-binding site between endothelial cells and ECM proteins. This finding supports the hypothesis that the state of EC–ECM attachment sites determines the permeability response to cell shrinkage. We further hypothesize that the enhanced permeability response to cell shrinkage which occurs upon albumin removal may involve modification of EC–ECM attachment. This is further discussed below.

Cell volume after exposure to hypertonic solutions

The bovine aortic endothelial cell monolayer system provides a useful model to investigate the role of intracellular volume on the properties of endothelial barriers

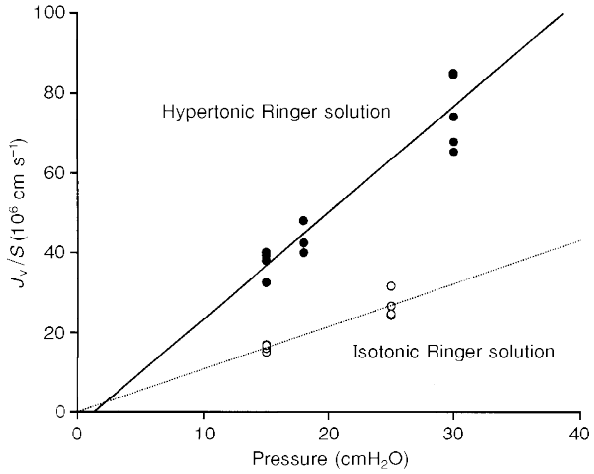

Figure 6. Measurements of filtration rate at different pressures: absence of osmotic gradients effects

Determinations of the relationship between filtration rate per unit area of capillary wall (J_v/S) and capillary pressure for a single vessel. Control measurements were made in the absence of BSA (○). The capillary was then exposed to hypertonic solution. The measurements between 2 and 5 min after exposure to hypertonic solution are shown (●). L_p, equal to the regression line, increased from $10{\cdot}7 \times 10^{-7}$ (control) to $26{\cdot}8 \times 10^{-7}$ cm s^{-1} $(cmH_2O)^{-1}$ (hypertonic solution). The fact that the intercept did not change suggests that osmotic pressure across the capillary wall ($\Delta\pi$) was not altered.

Table 2. Hypertonic solutions increase L_p but do not change the pressure intercept (in the absence of BSA)

	Hydraulic permeability ($L_p \times 10^{-7}$ cm s^{-1} (cmH$_2$O)$^{-1}$)		Intercept ($\sigma\Delta\Pi$ cmH$_2$O)	
Vessel number	Isotonic	Hypertonic	Isotonic	Hypertonic
1	3·2	10·8	4·8	1·9
2	6·2	30·1	−0·1	0·2
3	9·3	25·5	2·7	3·1
4	5·4	14·3	1·4	4·5
5	10·7	26·8	−0·1	1·3
6	10·5	27·8	0·6	4·6

$\sigma\Delta\Pi$ is the effective osmotic pressure exerted by all the solutes across the endothelial barrier. The value is expected to be close to zero if there is no effective osmotic pressure difference.

because these cells have been well characterized previously in our laboratory. In this regard, we have examined the effects of hypertonic solutions on intracellular volume of BAECs using two different methods: (1) radioisotopic evaluation of attached endothelial cell monolayer intracellular water space using ^{14}C-urea and ^{14}C-sucrose as markers of total and extracellular space, respectively; and (2) electronic cell sizing of suspended endothelial cells using a Coulter Counter (O'Donnell, 1993). These studies demonstrated that the volume of BAECs decreased to 68% of control within 1 min after exposure to hypertonic solutions (400 mosmol l^{-1}, hypertonic; 300 mosmol l^{-1}, control), followed by a recovery of volume to 90% of control by 30 min in the sustained presence of hypertonic media. Thus, BAECs undergo an initial shrinkage of the magnitude predicted by the increase in osmolarity and in so doing behave as perfect osmometers initially, i.e. before a subsequent slower restoration of cell volume occurs. We have evaluated the volume of cultured BAECs both in the presence of albumin (present as 10% FBS) and in the absence of albumin in numerous previous studies. In experiments performed in both albumin-free media and albumin-containing media, shrinkage occurred within 1 min to the volume predicted for a perfect osmometer, when the cells were switched from isotonic to hypertonic media. This rapid shrinkage was followed by a recovery of cell volume. That is, at 1 min after switching cells from 300 to 350 and 400 mosmol l^{-1} media, their intracellular volumes were 85 and 75% of control (isotonic) volume, respectively. Thus, the cells behave initially as perfect osmometers whether they are maintained in the presence or absence of albumin.

One of the central assumptions in our experiments *in vivo* using intact microvessels is that the frog endothelial cells forming the microvessel wall also undergo cell shrinkage upon their exposure to hypertonic solutions in the same manner that BAECs shrink. Although the cell volume of the frog endothelium was not determined here, studies of many different types of vertebrate cells have demonstrated that in general, vertebrate cells shrink rapidly in response to hypertonic solutions (Eveloff & Warnock, 1987). The exception to this occurs in a limited number of cell types that exhibit very low water permeability. What is less clear about the frog endothelial cells is the speed of recovery from the initial cell shrinkage and the mechanism employed to recover volume. It is possible that the frog endothelial cells might fail to recover volume after exposure to hypertonic solutions, as has been described for lymphocytes (Grinstein, Rothstein, Sarkadi & Gelfand, 1984), frog skin epithelial cells (Ussing, 1982) and frog urinary bladder epithelial cells (Davis & Finn, 1985). However, even in these cells that do not recover volume quickly, exposure to hypertonic solutions causes a rapid shrinkage and thus, the assumption remains that frog endothelial cells shrink in the presence of hypertonic solutions.

Removal of albumin enhances the extent of the cell shrinkage-induced increase in the endothelial barrier permeability

We have shown that exposure to hypertonic solutions does not increase endothelial barrier permeability when albumin is present in the perfusate. In contrast, the same perturbation does increase permeability after albumin has been removed from the perfusate. This observation was consistent for both BAEC monolayers and intact frog microvessel studies. As we have previously shown that endothelial cell volume is rapidly reduced to the same extent in both the presence and absence of albumin, a reduction in cell volume alone is not sufficient to increase permeability. Thus, these observations suggest that there are differences in the way that a volume change in the endothelial cell modifies the principal water pathway, which lies between the endothelial cells.

One simple mechanism to link a change in cell volume to a change in the resistance of the pathway between the cells involves a rounding up of the endothelial cell as cell volume is reduced. Thus, if the tendency to round up is reduced, the

magnitude of the permeability change associated with a volume change would be reduced. This argument suggested to us that one difference between endothelial cells in contact with albumin-free *versus* albumin-containing solutions might be the degree to which the endothelial cells resist rounding up. This difference may, in turn, reflect the degree to which the endothelial cells are attached to their basement membranes. We do not have direct proof that this mechanism underlies our observations, however, our results are consistent with this hypothesis. That is, we found that hypertonic solutions do increase the permeability of albumin-perfused microvessels if the microvessels have been perfused first with an RGD-containing hexapeptide which competes for a binding site between endothelial cells and their basement membranes.

Although a direct effect of albumin on EC–ECM attachment sites described above is one of the simplest explanations of our data, it is likely that one or more indirect actions also play a role. One possibility is that albumin alters calcium fluxes into the endothelial cell and indirectly changes integrin–ECM attachments by intracellular calcium-dependent processes. This idea is supported by the experiments of He & Curry (1993) in which removal of albumin caused a transient increase in cytoplasmic calcium concentration. We also know that albumin removal modifies the structure and arrangement of the endothelial cell glycocalyx by a process which depends on binding of albumin to the cell surface (Adamson & Clough, 1992). Thus, albumin removal may directly or indirectly, via a calcium-dependent signalling pathway, also modify other cell surface molecules, including integrins, which form part of the bridge between the cell cytoskeleton and attachment sites in the basement membrane.

An interesting variation on the mechanism described above is that exposure to albumin-free Ringer solution (and RGD peptides) does not modify existing EC–ECM attachments, but may reduce the ability to form additional attachment sites after cell volume or shape begins to be disturbed. We do not have any direct evidence for a tendency to increased adhesion, but increased adhesion of endothelial cells to the ECM may account for the trend for L_p to fall below control values when vessels were exposed to hypertonic solutions in the control state (Fig. 2*A*, with albumin at the concentration of 10 mg ml^{-1}; and Fig. 5*B*, with the control peptide, GRGETP). These possibilities require further investigation. We examine EC–ECM attachment in more detail below.

Cell shrinkage increases microvessel permeability when the EC–ECM attachment has been disrupted by GRGDTP

The literature suggests that the integrin family of adhesion receptors is directly involved in EC–ECM attachments (Cheresh, 1987; Albelda, Daise, Levine & Buck, 1989). Studies on cultured endothelial cells have indicated that maintenance of the endothelial barrier involves attachments to adhesive glycoproteins and the basement membrane. Dejana and her colleagues demonstrated that antibodies directed against the fibronectin receptor, $\alpha_5\beta_1$ causes dose-dependent inhibition of the adhesion of endothelial cells to substrate matrix protein (Dejana, Colella, Conforti, Abbadini, Gaboli & Marchisio, 1988). Also Cheng & Kramer (1989) have shown that adhesion of human microvascular endothelial cells to fibronectin is blocked by peptides containing the RGD sequence.

Our own observation that conditions expected to reduce endothelial cell volume by 30% did not substantially change L_p in normal vessels, but did increase permeability when the vessels were pretreated with GRGDTP, is consistent with the hypothesis that reduced endothelial cell volume changes the structure of the cleft between adjacent cells in a manner that is dependent on the EC–ECM attachment. It is possible that a change in the endothelial cell volume may have little effect on the geometry of the junction between endothelial cells if a significant cell shrinkage can be accommodated simply by reducing the thickness of the cell over most of the area where the cells are attached tightly to the ECM. On the other hand, when the cells are more loosely attached to the ECM, a similar cell shrinkage could disrupt the geometry of the junction between the cells if they tend to round up as observed in cultured endothelial cells with disrupted attachment sites. Thus, the assumption is made that after GRGDTP perfusion, the number of attachment sites is reduced, and therefore the strength of EC–ECM adhesion is reduced. During perfusion of microvessels with isotonic solutions, this reduction in attachment appears to be sufficient to induce only a small change in permeability. For example, an isotonic perfusate containing albumin and GRGDTP (GRGDTP/albumin ratio = 20) increased L_p by only 27% during the first 4 min of perfusion (Fig. 5*A*; isotonic). In contrast, when endothelial cell volume was reduced in the presence of GRGDTP, the disturbance was sufficient to increase permeability by 4·6-fold (Fig. 5*A*; hypertonic), presumably by an alteration in inter-endothelial cleft structure. In another study of RGD peptide effects of microvessel permeability, which used an RGD peptide/albumin ratio of 5 for perfusion of rat pulmonary venules, no effect on L_p was observed (Tsukada *et al.* 1995). Whether the discrepancy between that study and our present study is due to a lower RGD peptide/albumin ratio in the perfusate or to a difference in species remains to be clarified.

The structure and function of integrins have been extensively studied in a variety of species and cell types but little is known at present about integrins of frog endothelia. Many studies, however, have investigated the role of integrins on morphogenetic cell movements during embryogenesis of *Xenopus* or *Rana pipiens* (see reviews by DeSimone, 1994). In addition, Chen & Grinnell (1995) found that stretch enhancement of neurotransmitter release at the frog neuromuscular junction is modulated by RGD-dependent integrin bonds. Thus, integrins are known to play an important role in cell–ECM adhesion in frog as well as higher vertebrates. An important finding of our study is

that, whereas GRGDTP altered the permeability of frog microvessels, GRGETP, a structurally similar but biologically inactive peptide, had no effect on permeability. This suggests that the permeability increasing effect of GRGDTP is due to the specific inhibition of integrin function in the frog endothelium.

We assume that the action of the RGD peptide is primarily at the EC–ECM site. This does not, however, rule out other sites of action of GRGDTP. For example, Conforti *et al.* (1992) reported that β_3 and β_1 integrins were detected on abluminal as well as luminal surfaces of cultured human umbilical vein endothelial cells. Tsukada *et al.* (1995) found that ligation of a luminally located $\alpha_v\beta_3$ integrin increased transcapillary fluid flux in lung *in vivo*. Moreover, Lampugnani and her colleagues have shown that the integrin heterodimers $\alpha_2\beta_1$ and $\alpha_5\beta_1$ are located at cell-to-cell borders in human umbilical vein endothelial cell monolayers and antibodies to $\alpha_2\beta_1$, $\alpha_5\beta_1$ and the RGD peptide cause a dose-dependent increase in the leakage of the high molecular weight probe, horseradish peroxidase (Lampugnani, Resnati, Dejana & Marchisio, 1991). This raises the possibility that the RGD peptide may bind to the endothelial cell at sites other than the abluminal surface. Further experiments are required to investigate the specificity of RGD binding to the endothelium at each of the possible binding sites.

We have focussed on the paracellular pathway because this is the principle water pathway across the endothelial barrier. However, water may cross the endothelial barriers via an exclusive water pathway (possibly across the cells (Curry *et al.* 1976) and by gaps which may form close to the junctions in the high permeability state (Neal & Michel, 1995)). We note that the original measurement of the exclusive water pathway by Curry *et al.* (1976) were made with the same levels of osmotic pressure difference across the endothelial cells as in the present experiments. Thus, we do not expect even larger effects in these experiments. We cannot exclude the possibility that there may be changes in pathways close to the junctions as described by Neal & Michel (1995). The idea that we may create other pathways by bringing adjacent membranes nearer together is a closely related idea and needs to be investigated further. However, it should be noted that a change in cell geometry alone does not explain the results as hypertonic solutions in the presence of albumin alone do not increase L_p.

Comparison of results with other experiments with increased osmolarity

Our results may help to explain some inconsistencies in the published data regarding the response of the endothelial barrier to increased osmolarity. The present finding that cell shrinkage in the normal BSA perfused microvessels did not increase L_p (Fig. 2) is consistent with previous observations by Curry *et al.* (1976) in experiments using perfused frog microvessels. This finding does not appear to be restricted to perfused frog vessels; because hypertonic solutions were also not found to increase permeability in mammalian hindlimb (Pappenheimer *et al.* 1951; Wolf & Watson, 1989). Our observations are consistent with the hypothesis that the increased permeability induced by albumin-containing hypertonic solutions, as has been observed by some investigators (Granger *et al.* 1979; Rapoport *et al.* 1980; Rasio *et al.* 1981; Shepard *et al.* 1987), could have resulted from experimental conditions in which cell shrinkage was transduced into a permeability-increasing cell shape change (i.e. rounding *versus* flattening).

Our study is one of the first to investigate mechanisms that change permeability in both individually perfused microvessels and in cultured endothelial cell monolayers using a similar approach to modify permeability in both endothelial barriers. The observation that the response of monolayers to albumin-containing *versus* albumin-free solutions was the same as microvessel responses to these solutions suggests that the monolayers may provide a valuable model for further evaluating mechanisms underlying regulation of permeability. Although a similar qualitative effect of cell shrinkage on endothelial permeability was observed in both intact microvessels and cultured endothelial monolayer studies, we note that permeability characteristics of cultured endothelial cell monolayers differ from those of intact microvessels. For example, the P_s ($8{\cdot}7 \times 10^{-6}$ cm s^{-1}) observed for monolayers in these studies is significantly higher than the P_s ($2{\cdot}1 \times 10^{-6}$ cm s^{-1}) previously observed in perfused microvessels (Adamson, Huxley & Curry, 1988). Furthermore, the magnitude of the permeability change due to hypertonic solutions in the absence of albumin is much smaller in the cultured monolayer study (the mean value for the ratio of P_s in hyperRinger to P_s in Ringer was 1·3) compared with intact microvessels (the mean value for the ratio of L_p in hyperRinger to L_p in Ringer was 3·5). This may well be the result of cultured monolayers exhibiting a baseline permeability that is sufficiently high to preclude a large additional increase after the perturbation.

Physiological relevance of this work

In summary, our experiments help to distinguish the contributions of two mechanisms which increase the permeability of intact microvessels. One is a change in the shape of the endothelial cells (in this case caused by a reduction in cell volume) and the other is a change in endothelial cell attachment to the ECM. In many high permeability states, both mechanisms may act in a co-ordinated manner to increase permeability. Our findings may help us to establish a model *in vivo* to investigate conditions in which the EC–ECM attachment has been impaired, but which may not significantly increase permeability until an additional perturbation is added. One example of such a condition may be the untreated hyperglycaemic state. The interaction of glycated albumin with the ECM in microvessels may compromise EC–ECM attachments by mechanisms similar to the Ringer solution effect described above, predisposing the endothelial cells to

increased permeability during acute changes in plasma glucose levels. Studies of the hyperglycaemic state have documented a correlation between morphological changes in basement membrane induced by hyperglycaemia and microvessel permeability (Bendayan & Rasio, 1981).

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