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SPOTLIGHT REVIEW

Vascular permeability modulation at the cell, microvessel, or whole organ level: towards closing gaps in our knowledge

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Multiple processes modulate net blood-to-tissue exchange in a microvascular unit in normal and pathophysiological conditions. These include mechanisms that control the number and type of microvessels perfused, the balance of adhesion and contractile forces that determine the conductance of the spaces between endothelial cells to water and solutes, the pressure and chemical potential gradients determining the driving forces through these conductive pathways, and the organization of barriers to macromolecules in the endothelial glycocalyx. Powerful methods are available to investigate these mechanisms at the levels of cultured endothelial monolayers, isolated microvessels, and the microvascular units within intact organs. Here we focus on current problems that limit the integration of our knowledge of mechanisms investigated in detail at the cellular level into a more complete understanding of modulation of blood-to-tissue exchange in whole organs when the endothelial barrier is exposed to acute and more long-term inflammatory conditions. First, we review updated methods, applicable in mouse models of vascular permeability regulation, to investigate both acute and long-term changes in permeability. Methods to distinguish tracer accumulation due to change in perfusion from real increases in extravascular accumulation are emphasized. The second part of the review compares normal and increased permeability in individually perfused venular microvessels and endothelial cell monolayers. The heterogeneity of endothelial cell phenotypes in the baseline state and after exposure to injury and inflammatory conditions is emphasized. Lastly, we review new approaches to investigation of the glycocalyx barrier properties in cultured endothelial monolayers and in whole-body investigations.

Keywords Perfusion and permeability • Endothelial cell heterogeneity • VE-cadherin and inflammation • Endothelial glycocalyx • Glycocalyx volume • Thrombin • Rac1 and endothelial permeability • Wound healing

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1. Introduction: multilevel studies of vascular permeability

A multilevel approach to understand the regulation of vascular permeability requires integration of our knowledge of the cellular and molecular mechanisms that modify the endothelial barrier as determined from studies in cultured endothelial cells into an understanding of both the normal endothelial barrier in intact organs and the effect of increased barrier permeability on organ function. At the cellular level, the goal is to identify the common endothelial cell mechanisms that increase permeability, then restore and maintain permeability after exposure to inflammation or injury. There has been significant progress towards this goal, particularly with respect to the regulation of the paracellular pathway, and the molecular components that regulate the passage of water, solutes, and inflammatory cells through junctions between the endothelial cells[1–3] (see review by Spindler *et al.* in this issue).[4] In spite of this progress, the contribution of these multiple modulators of the endothelial barrier has not been evaluated *in vivo*. Further, the translation of these studies to the improvement of clinical outcomes in inflammatory diseases remains limited at a time when there is increased clinical interest in disturbances of the microcirculation in many chronic diseases.[5] In this review, we focus on three problems that need to be understood in more detail to bridge the gap between studies at the molecular and cellular levels using cell culture experiments and studies of the function of the microvasculature in intact organs during physiological and pathophysiological modification of vascular permeability. The aim is to identify key areas for further investigation and the appropriate experimental methods to investigate regulation of the vascular permeability at different levels.

One problem is that the same agents that modulate vascular permeability may also act directly or indirectly on other cells in a microvascular unit. In addition to the well-recognized action of

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inflammatory agents to modulate microvascular perfusion by endothelial-dependent and -independent mechanisms,[6] inflammatory agents also act on tissue fibroblasts that change tissue pressure[7] (see review by Reed),[8] and on the lymphatics.[9] The net fluid and solute exchange due to an increase in permeability depends on some or all these additional mechanisms. It follows that such changes must be taken into consideration in the design and interpretation of experiments to evaluate vascular permeability when investigating measurements of blood-to-tissue exchange in intact tissues. As a step towards this goal, in the first part of this review, we describe recent refinements in methods to measure vascular permeability to macromolecular tracers such as serum albumin labelled with a fluorophore or isotope when there may be changes in vascular perfusion as well as increased permeability. In particular, we highlight examples of methods used to investigate vascular permeability regulation in transgenic mouse models.

A second problem in multilevel investigations is that there is no typical endothelium; endothelium is a highly heterogeneous organ and, in particular, the properties of endothelial cells available for culture from larger vessels can not be expected to be representative of microvascular cells.[10] Furthermore, endothelial cells undergo significant changes in phenotype under some culture conditions and *in situ* after exposure to inflammatory conditions. Overall they tend to change from a stable anti-inflammatory phenotype to a more pro-inflammatory phenotype under these conditions.[10–12] There is a need to understand this phenotype plasticity in the endothelial cells of intact microvessels and how experiments using cultured endothelial cells may be useful models to understand these more pro-inflammatory phenotypes. Third, barriers to exchange between blood and the tissues, in addition to those in the paracellular pathway between endothelial cells, play an important role in the intact circulation. In normal microvessels, an intact glycocalyx is part of the primary barrier which retains plasma proteins in the vascular space[13–16] (see reviews by Levick and Michel, Becker, and VanTeeffelen *et al*. in this issue).[17–19] However, the glycocalyx is often ignored in investigations of the endothelial barrier in cultured cell systems where it may be absent, or only partially expressed.[20,21] Each of these problem areas is discussed further below.

2. Blood flow and inflammatory responses: new methods

Depending on the microvascular bed and the inflammatory agent, there may be vasodilation or vasoconstriction at the same time as an inflammatory agent increases vascular permeability. Vasodilation increases the number of microvessels perfused during exposure to an inflammatory agent and increases the pressure within these vessels; both actions further increase the effect of increased permeability on blood-to-tissue exchange of fluid and solutes, while vasoconstriction will attenuate the effect. An example is the use of an inhibitor of nitric oxide synthase (NOS) to attenuate nitric oxide-dependent hyper-permeability in a microvascular bed. In tissues where nitric oxide is also an important endothelial-dependent vasodilator, the difference between the vascular leak of water and solutes with and without the NOS inhibitor likely reflects both the action of the inhibitor to reduce hyper-permeability in venular microvessels and the action of the inhibitor to reduce NO-dependent blood flow within the vascular bed[22] (see review by Durán *et al*. in this issue).[23] Because such interactions between the regulation of perfusion and permeability are common, some of the most widely used assays for increased permeability do not accurately measure the contribution of increased permeability to increase dye accumulation in a tissue unless there is no change in vascular perfusion or microvessel pressure (see review by Bates in this issue).[24] The problem is becoming more widely recognized and the recent review by Dvorak and co-authors[25] highlights the strengths and limitations of measurement based on single tracer studies such as various forms of the Miles Assay which do not take into account changes in vascular perfusion directly, and the more rigorous methods using two tracers, where one tracer measures total accumulation and the other measures vascular accumulation. While it is reasonable to argue that the error due to neglecting the vascular compartment may be small when there is a large vascular leak, important regulatory processes involve more subtle changes in vascular permeability where changes in extravascular tracer accumulation over typical measuring periods (30 min) are comparable to the amount in the local plasma volume.[26,27]

In collaboration with colleagues in Bergen, Norway, and Würzburg, Germany, our laboratory has modified a two-tracer method that was previously applied in rat tissue[26–29] for studies in wild-type and transgenic mice. The first of these experiments was recently published[30] using mice with an endothelial-specific deletion of the GC-A receptor for atrial natriuretic peptide (ANP). The detailed methods described therein can be used for practically any tissue in the mouse, and provide new ways to evaluate changes in basal, acute, and chronic changes in permeability in engineered mice including some of the unanswered questions discussed by Nagy *et al*. in models that include modulation of other ANP receptors, caveolin, eNOS, and Src/Yes knockouts.[25] Although technically demanding and time-consuming, they involve similar protocols and procedures to the various forms of the Miles Assay, but provide far more definitive information about true changes in permeability. Furthermore, refinements currently in development such as the use of long wavelength fluorescent dyes to label albumin overcomes limitations imposed by the use of radioactive tracers, and fluorescent labels such as FITC, which are significantly quenched in tissue samples.[31] In addition, in tissues such as hind-limb skeletal muscle, the coupling of solute flow to filtration (solvent drag) can be evaluated by adapting the approaches previously used in rats using a tourniquet to increase venous pressure.[27] The major limitation of the methods is that they can only be applied for one intervention at a time as the animal must be killed at the end of an experiment to collect tissue samples. Thus some alternate approaches which also measure the amount of a macromolecular tracer that accumulates in the tissue relative to the amount in the local vascular space are evaluated below.

Several new methods currently being tested include scanning and confocal imaging using albumin labelled with long wavelength fluorophores, MRI imaging with high molecular weight contrast agents, and microPET techniques using positron emitting probes. Their application is restricted to a more limited number of organs than the two-tracer technique, but some have the important advantage that they can be used repeatedly, enabling control and test experiments on the same animal, and longitudinal studies. They all extend the approaches initially developed in individually perfused microvessels, where rapid filling of the vascular space in a selected tissue region or volume of interest is measured as a step increase in the intensity signal of a tracer detected externally and measured

continuously.[32–35] Under conditions where the signal intensity is proportional to the amount of tracer in the tissue, and a constant amount of tracer is maintained in the vasculature, the rate of change of signal intensity after the filling of the vasculature measures the flux of tracer across the microvessel walls and into the tissue relative to the initial amount of tracer in the local vascular space. An apparent permeability coefficient (P_{app}) is estimated from this ratio if an appropriate average vascular volume to surface area ratio of the vessels in the sample can be estimated, i.e. P_{app} = [(extravascular amount/time)/(intravascular amount)] × volume/surface of exchange vessels.

The MRI method in *Figure 1* enabled repeated measurement of vascular permeability at intervals of about 2 h in the skin and muscle tissues of the mouse because the test probe used was small enough to be excreted by the kidney (35 kDa dendrimer labelled with gadolinium) over this period. Measurements of plasma level of the tracer monitored from a nearby artery are used to test when the tracer is excreted; they can also be used to correct for change in the amount of tracer in the vascular space during a single measurement. The measuring volume can be chosen to exclude all large vessels, enabling an estimate of

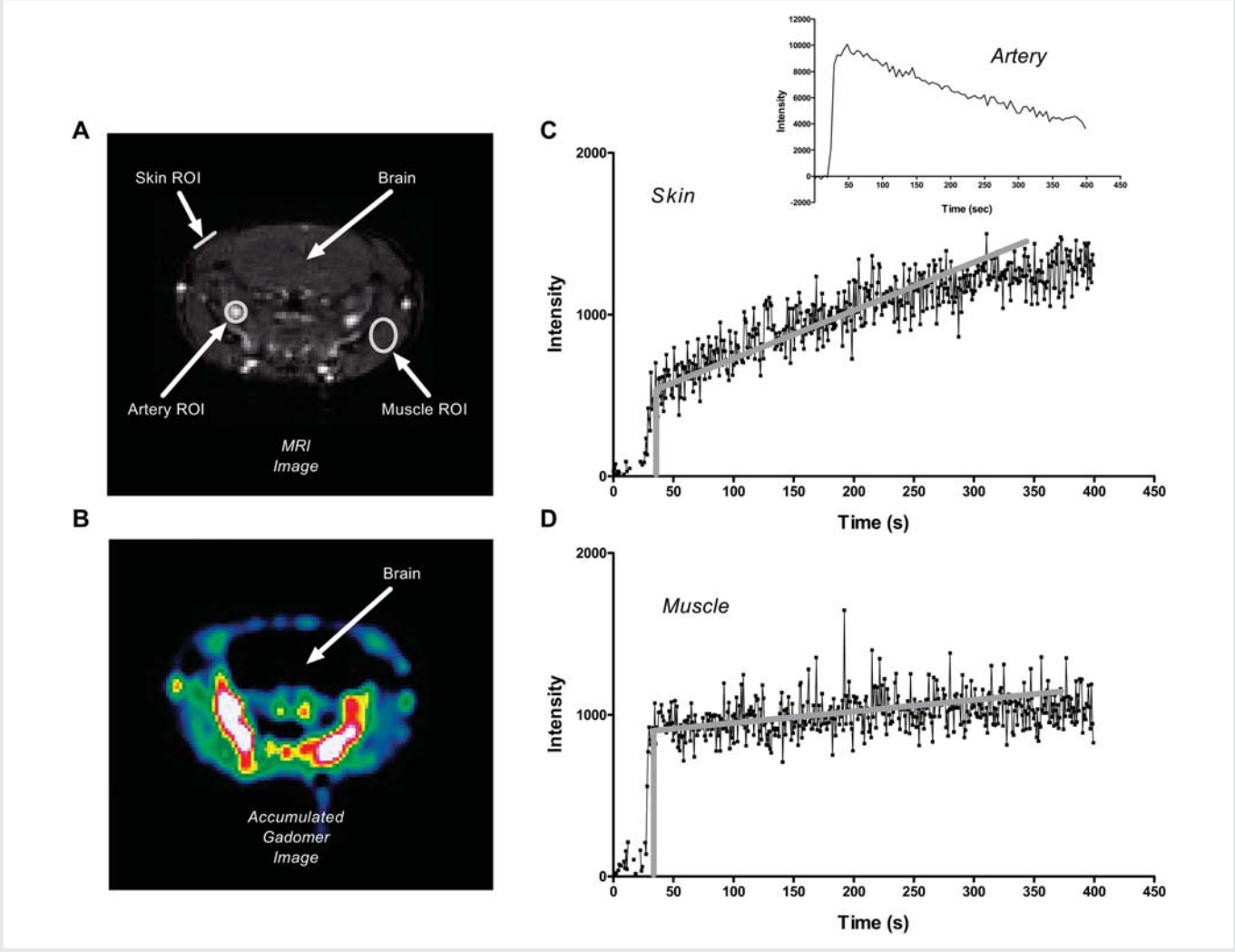

Figure 1 Measurement of 35 kDa Gadomer contrast agent apparent permeability coefficient in skin and muscle tissue of C57BL6 control mouse muscle and cheek during vehicle (saline) infusion. (*A*) MR Image (axial slice) of mouse head acquired 200 s after contrast agent injection via the tail vein. The regions of interest (ROIs) were carefully selected using anatomical references for muscle, skin, and vessels. (*B*) Shown is the subtracted image (image in A minus baseline image) recording the signal increase in tissue after injection of Gadomer, where cold colours indicate low signal enhancement and warm colours indicate high signal enhancement. Note the high signal intensity in large arteries showing that most of the high molecular weight Gadomer contrast agent is mainly in the vascular space. (*C*) Curve showing the signal intensity changes over time in an ROI used to estimate Gadomer permeability coefficient in the skin. As the contrast agent is injected, there is a step increase in tracer signal intensity above background as the vascular volume in the ROI is filled with the contrast agent. The tracer continues to accumulate in the ROI as it enters the extravascular space. The initial rate of tracer accumulation is estimated from the slope of the signal intensity over the first 100–150 s. An initial estimate of the vascular permeability is obtained from the magnitude of the initial slope and step. This initial estimate can be corrected for the fall in vascular tracer concentration (as measured from the signal intensity over an adjacent artery; see inset). (*D*) Signal intensity over time in an ROI over masseter muscle. Muscle permeability is less than in skin. The analysis to estimate vascular permeability is over an ROI containing no vessels larger than 100 μm diameter. Thus, assuming a mean plasma volume to exchange surface area of 4.4×10^{-4} cm, the vascular permeability coefficients in skin and muscle tissue were $4.6 \pm 0.6 \times 10^{-7}$ and $26 \pm 3 \times 10^{-7}$ cm/s, respectively. From Reference;[105] used with permission from Wiley–Blackwell.

the average volume to surface from the known microvessel size distribution in this tissue.[30] Even if there is increased perfusion in the presence of the test agent, it is possible to attribute most of the increased tracer accumulation to real increases in permeability if the volume to surface ratio for any newly perfused microvessels remains similar to that in the control state.[30] Examples of similar approaches include the use of long wavelength fluorophores attached to albumin to measure changes in permeability of mouse back skin wound using small animal whole fluorescent scanning methods,[36] and similar fluorescent labels in confocal microscopy.[37] In addition, a positron emitting isotope (^{64}Cu) attached to albumin for positron emitted tomography in microPET offers promise for measurements of tumour permeability.[38]

In summary, these methods not only enable reliable estimates of changes in permeability by comparing tissue accumulation to local vascular accumulation, but also enable longitudinal studies on a single animal. This greatly reduces the number of animals needed for a study, associated cost of animals, and the variability between animals. Methods based on fluorescent imaging in skin and superficial muscle using laser scanning confocal microscopy are more widely available but are limited by the penetration of the excitation light into the tissue. At the moment, the use of MRI and microPET techniques is restricted by the cost but collaborative efforts between groups with useful mouse models and groups with expertise in such imaging methods is strongly encouraged. We note that a suitable step input function is expected only for macromolecular tracers. When smaller tracers are used, or when the kinetics of tracer distribution is studied over longer time, a separate approach using an arterial input function and various forms of compartmental analysis have been used.[39,40]

3. Intact microvessels compared to cultured endothelial monolayers

3.1 Normal permeability

Before we discuss modulation of permeability, it is useful to summarize some of the structure–function relations in intact venular microvessels and compare them with data from cultured cells. The methods available for such studies use video densitometry and photometry methods to monitor the accumulation of a fluorescent tracer within a microvascular field. The approaches range from those in which a single microvessel is cannulated and perfused so that permeability to solute and water is measured under conditions where the surface area for exchange, microvascular pressure, and the composition of the superfusate are all controlled (as is possible in thin transparent tissues such as mesentery, thin muscle, surface vessels in the kidney, and completely isolated and perfused vessels[32,33,35,37,41–48]) to those in which localized leakage within a selected region of a microvascular bed is carefully quantified but microperfusion is not possible (see reviews by Durán *et al.* and Shen *et al.* in this issue).[23,49] We have used the microperfusion methods extensively in our laboratory to enable investigation of cellular mechanisms in microvessels with precisely known permeability properties (using immunofluorescence, electron microscopy, and fluorescence indicators of intracellular signalling). In the following sections, we review these methods as a bridge between investigations of cellular mechanisms to regulate the permeability barrier using cultured cells and those using intact microvessels.

The paracellular pathway is the focus because it is the primary pathway for water and solute exchange across the microvessel wall, and it is also where gaps form between cells. *Figure 2* shows ultrastructural data of (non-stimulated) rat mesenteric microvessels

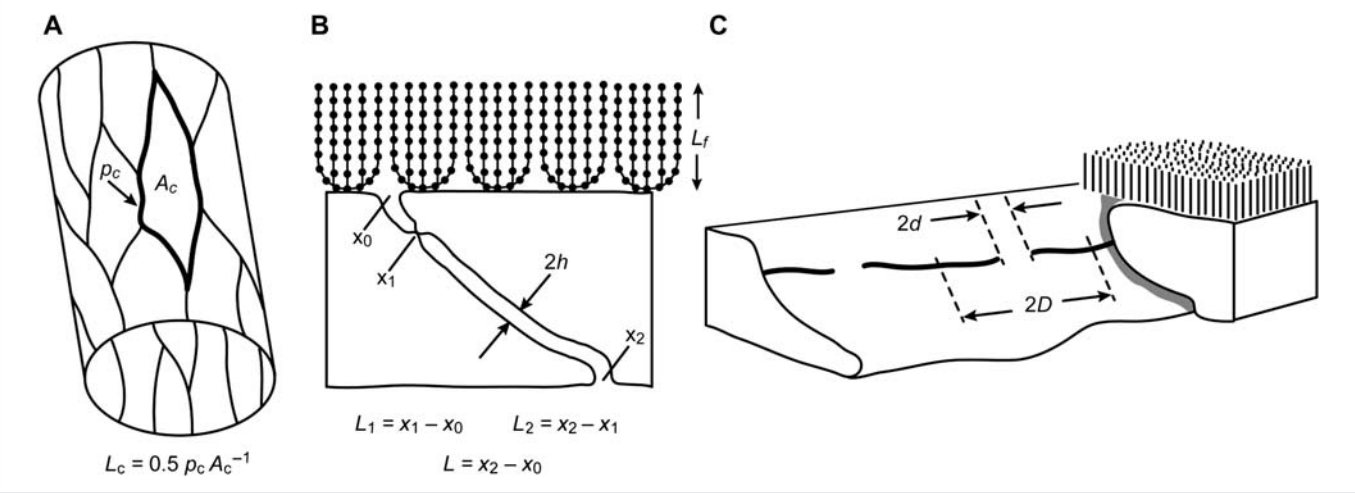

Figure 2 Measured parameters and diagram of the endothelial cleft. Diagrams illustrating measurement parameters used to construct mathematical model. (*A*) Area, A_c, and perimeter, p_c, measurements of individual cells seen in silver-stained whole mounts of venular microvessels were used to calculate cleft length per unit area, L_c. (*B*) From each electron microscopic image, the cleft depth, L, was measured along the contour between facing cells from the luminal cleft opening (x_0) to the abluminal cleft exit (x_2). The total distance from lumen to interstitium through the cleft is the sum of L_1, distance to the tight junction strand (x_1) from luminal cleft entrance, and L_2, distance from tight junction strand to the abluminal cleft exit. The cleft width is the distance between the outer leaflets of the cell membranes of the two facing cells. L_f, depth of the glycocalyx. (*C*) Oblique view of cleft segment reconstructed from serial sections illustrates the length of tight junction strand gaps, *2d*, and the mean distance between strand gap centres, equal to the functional unit length, *2D*. From Reference;[16] used with permission from Wiley–Blackwell.

(endothelial cell area, junction geometry, and glycocalyx organization). The endothelial cells (600–800 μm^2) overlap along their perimeter by 0.1 μm to more than 1 μm. The inter-endothelial cell spacing within this region of overlap (the cleft between adjacent cells) is remarkably constant, 18–22 nm, maintained by adhesion and spacer proteins within the junctional cleft. The spacing is determined by transmembrane adhesion proteins, such as VE-cadherin that provides a homophilic link between adjacent cells and is anchored to the cortical actin framework around the perimeter of each cell[1] (see review by Spindler *et al.* in this issue).[4]

When examined using confocal microscopy, the cell junctions contain a nearly continuous band of junctional proteins including occludin (a comparison with cultured endothelium is given below). First we note that Adamson *et al.*[16,50] have made detailed three-dimensional reconstructions of the arrangement of the tight junction strands in these vessels based on electron microscopy of ribbons of serial sections. As shown in the cartoon of *Figure 2*, the junction strands are not continuous but contain infrequent breaks, 100–300 nm long, separated 2–3 μm apart, forming very large pores which would allow relatively unrestricted passage of solutes including the plasma proteins, as well as water. Detailed 3D hydrodynamic models of water and solute flow through these junction breaks[16,51] confirm that their measured size and frequency account for most of the water flows and the exchange of small solutes (e.g. solutes close to 500 MW). A more limited reconstruction in heart microvessels by Bundgaard[52] also described tight junction strand breaks in venular vessels as well as much smaller gaps.[53] The permeability properties of these venular microvessels fall within the upper range of values reported in mammalian microvessels.[53] On the other hand, the permeability of these vessels is close to an order of magnitude smaller than typical water and large solute permeabilities of cultured endothelial monolayers.[13,53]

The breaks in the junctional strand are far too large to be a primary selective barrier to plasma proteins in these vessels, where the reflection coefficient to albumin is close to 0.9.[43] The selective barrier to plasma proteins is the endothelial glycocalyx which forms an additional barrier to the plasma proteins at the endothelial cell surface. By restricting diffusion of albumin within the fibres, and by both size and electrostatic exclusion, the glycocalyx barrier reduces the albumin permeability from values in the range of 10^{-6} cm/s, expected in the absence of the glycocalyx, to values 10^{-7} cm/s or lower.

An important observation is that significant changes in permeability can be accounted for without the formation of gaps. Michel and co-authors[54,55] report flow-dependent increases in small solute permeability with no change in hydraulic conductivity. The nature of this small solute regulation is still not well understood, but such changes could be accounted for if a flow-modulated (via NO) 'small pore' pathway in the tight junctions are stimulated up to a maximum capacity for the intercellular junctions when the whole line of contact between adjacent cells is effectively permeable to small solutes and the effective area of the entrance to the inter-endothelial cleft that is available for exchange approaches 0.2–0.3% of the endothelial surface area. On the other hand, two- to three-fold increases in albumin permeability (e.g. as measured in muscle and skin during a whole-body regulation mechanism to maintain plasma volume by ANP) could be accounted for by increased frequency of breaks or subtle changes in the glycocalyx. Permeability change of this order of magnitude may be important in the management of clinical situations involving trauma, surgery, and sepsis, where appropriate fluid balance and nutrient supply after surgery are essential and are likely to be more amenable to clinical interventions than fully developed high-permeability states characterized by gaps in venular microvessels[56] (see review by Becker *et al.* in this issue).[18] Thus inflammatory gaps in venular microvessels and cultured monolayers represent only one extreme expression of increased permeability.

3.2 Cellular mechanisms that regulate normal permeability and acute changes in permeability (comparison of culture and intact vessels)

The function of the endothelium as a permeability barrier can be broadly understood in terms of a balance between cell–cell and cell–matrix adhesion forces to maintain the integrity of the barrier and centripetal tension tending to pull the adjacent endothelial cells apart (see reviews by Shen *et al.* and Spindler *et al.* in this issue).[4,49] While this scheme usually is applied to understand increased tension, comparisons of intact and cultured cells suggest that balance of adhesion and tension is also important in determining the baseline permeability. The following section first compares the distribution of key components of the adhesion mechanisms in junctions such as VE-cadherin and occludin in these intact microvessels with their distribution in the junctions of endothelial cells cultured under control (non-stimulated) conditions and after treatment with agents to stabilize the barrier in the baseline. This is followed by a similar comparison after exposure to inflammatory conditions in the presence or absence of the stabilizers.

The complex interactions between signalling pathways that modify cell–cell adhesion are reviewed by Spindler *et al.* (this issue).[4] The picture that is emerging is that one common target of agents that stabilize the endothelial barrier is the small GTPase Rac1, which plays multiple roles in the organization of the cortical actin cytoskeleton and the distribution of endothelial junction proteins such as VE-cadherin and occludin. Rac1 is activated by sphingosine-1-phosphate (S1P), while cAMP acting via the Epac pathway activates Rap1 and indirectly activates Rac1 and downstream targets. Both S1P and agents that increase intracellular cAMP are therefore the focus of anti-inflammatory agents that stabilize adhesion between endothelial cells. Exposure of endothelial cells in culture to agents that activate Rac1 results in significant reorganization of key components of the adherens junction and focal adhesions, and a significant lowering of the baseline permeability.[57] S1P has been shown to enhance peripheral VE-cadherin and α-, β-, and γ-catenins.[58,59] S1P via activation of receptor S1P1 also reduces stress fibres and promotes cortical actin assembly[60] and improves monolayer barrier function, often measured as an increase in *trans*-monolayer electrical resistance.[59,60] However, a recent study clearly shows that when VE-cadherin is knocked down by use of siRNA, S1P still enhances barrier function in a Rho/Rho kinase-dependent manner[61], demonstrating that a strong component of the barrier-enhancing effect on cultured monolayers is due to cell spreading and closure of endothelial gaps that are often prominent in basal monolayers, but never seen in basal endothelium *in situ*. At the same time, recent evidence indicates that site-specific phosphorylation of VE-cadherin is not sufficient to promote barrier loss[62] and a separate study indicates that cAMP-Epac-Rap1-dependent stabilization of the peripheral actin band proceeds in the absence of VE-cadherin.[63] Thus modulation of

VE-cadherin is likely to be only one component of barrier regulation. The relative importance of cell spreading, pre-existing gap closure, and improved adhesion is an important area for further research to link investigations in cultured cells to anti-inflammatory processes in intact microvessels as illustrated by the following example.

Significant rearrangement of adhesion structures and lowering of baseline permeability in endothelial cultures is in sharp contrast to the response of *in situ* microvessels for which there is far less structural reorganization due to exposure to the same stabilizing agents. For example, increasing intracellular cAMP by treating perfused microvessels with a combination of rolipram (cAMP-specific phosphodiesterase 4 inhibitor) and forskolin (adenylate cyclase activator) reduces hydraulic permeability by only about 30% over 30 min, but no enhancement of VE-cadherin could be seen in vessels treated with rolipram/forskolin[64] (*Figure 3*); in the basal state, the adhesion protein VE-cadherin and the tight junction protein occludin are both continuous around the periphery of all endothelial cells. Similarly, in recent follow-up studies, S1P at 1 μM induced a 30% decrease in venular microvessel L_p within 30 min without any change in basal distribution of VE-cadherin, and in a separate study S1P was not seen to alter baseline L_p at all, yet had a strong effect on blocking permeability increase induced by platelet activating factor (PAF).[65] These significant differences between the state of the endothelial barrier in terms of VE-cadherin-dependent adhesion between cells, the presence of gaps, and cell spreading (often associated with increased RhoA activity)[66,67] in the baseline state of intact microvessels and cultured endothelial cell monolayers cannot be over emphasized. In particular, the results suggest that some of the properties associated with significantly increased permeability states in intact microvessels are already present in the baseline state of cultured monolayers. A specific example using endothelial cells of the blood-brain-barrier demonstrated loss of adhesion protein and alterations of tight junction morphology correlated with higher permeability in culture when compared with *in vivo*.[68] While little information is

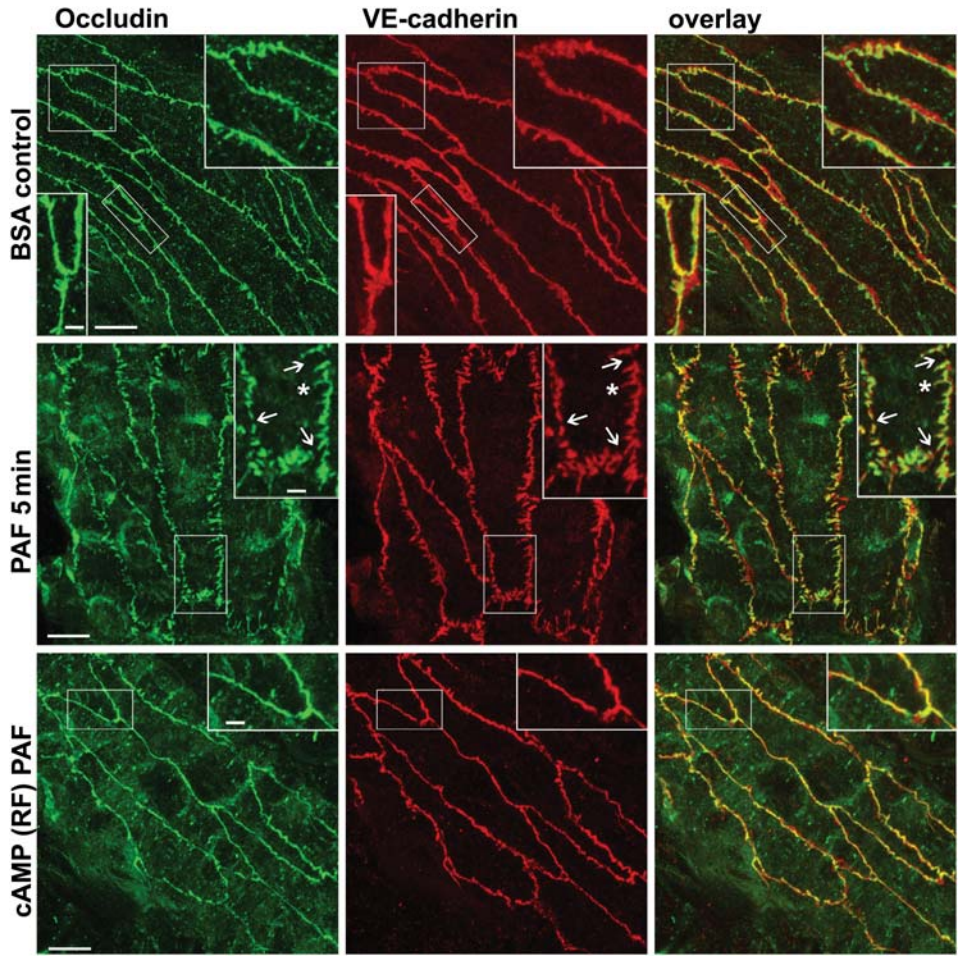

Figure 3 Increased cAMP inhibits PAF-induced changes in VE-cadherin and occludin *in situ*. Immunofluorescent localization of VE-cadherin (middle column) and occludin (left column) after perfusion with vehicle control (top row), PAF (middle row; 10 nM), and pre-treated with rolipram and forskolin to increase intracellular cAMP prior to PAF (bottom row).[64] After control perfusion VE-cadherin appeared in a continuous peripheral ribbon with frequent broad areas, likely corresponding to regions of extensive (order of 1–2 μm) endothelial overlap, while occludin was largely restricted to a narrow line. After PAF there were numerous spikes (asterisks) of VE-cadherin label oriented transverse to the endothelial perimeter and there were frequent discontinuities (arrows) in the label. Breaks in the continuity of occludin corresponded to similar discontinuities in the VE-cadherin, confirming loss of the tight junction barrier at sites of adherens junction loss. Increased cAMP prevented rearrangement of both VE-cadherin and occludin and very strongly inhibited any increase in Lp (not shown). Modified from Reference.[64]

available to directly compare expression profiles of cultured vs. *in situ* microvasculature, large vessel studies indicate modulation of protein expression resulting from shear stress both *in situ* and under culture conditions.[69,70] Thus it is not surprising that endothelium exhibits a different phenotype in culture when compared with *in situ* endothelium, which is embedded in native tissue.

It is also useful to compare the inflammatory gaps in venular microvessels (with no prior exposure to inflammatory conditions) to gaps reported in intact beds and in culture. In all inflammatory models (intact bed, isolated vessel, and cultured cells), gaps form at sites along the line of contact between the cells spaced between sites, where the cells maintain contact. In intact microvessels of rat trachea, stimulated by either Substance P or neurogenically, McDonald and co-authors described extensive finger-like projections that remain attached to the adjacent cells during an inflammatory response spanning the gaps.[71,72] Such gaps, rare or absent in basal conditions, were typically less than 1 μm in diameter and nearly all were formed between endothelial cells rather than by pathways through the endothelium.[71,73] These extensive results corresponded well to those of earlier work on rat mesentery.[74] Using electron microscopy of serial sections and reconstruction, we have demonstrated in rat mesentery that inflammatory gaps are typically 0.5 μm or less in diameter when venular microvessels are exposed to PAF or Bk.[75] The small size of these inflammatory gaps found *in situ* is in strong contrast to the large gaps that are seen in cultured endothelium. Endothelial cells in culture respond to inflammatory mediators by pulling apart (on the order of 5–10 μm) to such an extent that gaps are readily seen in fluorescence microscopy.[59,60,76,77] Thus adhesion between cultured endothelial cells often appears to be less well developed than that of endothelium in tissue. For *in situ* microvessels at the light microscopy level, the immunofluorescent localization of VE-cadherin and occludin illustrates the distribution of adherens and tight junction structures, respectively (*Figure 3*). In the basal state, both proteins are continuous around the endothelial periphery (*Figure 3*, top row). As a model of the acute inflammatory response for a vessel responding to PAF (*Figure 3*, middle row), both VE-cadherin and occludin undergo significant widespread rearrangement along the junction, but there are no inflammatory gaps between cells large enough to see with light microscopy. The numerous short breaks in the label suggest local loss of the adhesion proteins, but these breaks in the fluorescent label do not necessarily correspond to the classical inflammatory gaps seen at the level of electron microscopy. The size and density of gaps in relation to the reorganization of other key components of the junction is not well understood.

A further difference between inflammatory gap formation in culture and that *in situ* is that formation of such gaps *in situ* does not depend on contractile mechanisms. Rho kinase, myosin light-chain kinase (MLCK), and myosin ATPase are all implicated in the contractile mechanism proposed for formation of endothelial gaps based largely on cultured cell work[67] (evidence for contractile activity *in vivo* in review by Shen *et al.* in this issue).[49] However, neither direct inhibition of RhoA by bacterial toxin, nor pharmacological inhibition of Rho kinase fully inhibited the acute L_p response to PAF or to Bk in mesenteric venules.[78] Similarly, inhibition of MLCK with ML-7 failed to block either PAF or Bk and inhibition of myosin ATPase showed no effect on PAF.[64] In contrast to the preceding results using *in situ* microvascular endothelium, formation of paracellular gaps in cultured endothelial monolayers, which are most often studied by stimulation with thrombin, can be strongly blocked by inhibition of Rho kinase or MLCK, indicating that gap formation in cultured endothelium is not necessarily representative of gap formation in intact microvessels. The change in endothelial phenotype *in situ* with respect to responsiveness to thrombin is discussed further below.

3.3 Responses to thrombin: an example of the changes in endothelial cell phenotype and response to mediators under inflammatory conditions

Investigations of permeability regulation in cultured endothelial monolayers often use thrombin as an acute inflammatory stimulus and describe active contractile mechanisms as contributing to acute increases in barrier permeability characterized by large gap formation.[76] The problem with linking these observations with the control of vascular permeability in intact microvascular beds is that evidence for a direct acute action of thrombin to increase permeability in intact microvascular beds is limited. Some of the effects of thrombin on vascular permeability appear to be secondary to the release of other inflammatory mediators from mast cells or of neurogenic inflammation.[79] Even in the lung, where thrombin has been shown to cause an increase in lung weight indicating oedema, its action is attributed mainly to haemodynamic changes to increase filtration rather than to increase permeability.[80] Although the RhoA-dependent pathways activated by thrombin are well understood, the reason why they are so robust in some cultured endothelial cell monolayers is not understood. One possibility suggested previously[81] is that exposure of endothelial cells to injury (including some steps in cell culture) or inflammatory conditions may up-regulate key elements of the thrombin-induced contractile mechanisms. Below we review experiments that begin to test this idea by identifying conditions where endothelial barriers are modified from being unresponsive to thrombin to clearly responsive.

The first is from our laboratory using venular microvessels such as those in *Figures 2* and *3* in rat mesentery exposed for cannulation and perfusion with a micropipette under aseptic conditions. Hydraulic conductivity (L_p) was used as an index of normal and increased permeability. The experiments were designed to measure permeability in a selected microvessel first under normal conditions with no prior exposure to inflammatory conditions, and then in the same microvessels 24 h after blood perfusion had been restored. The key observation is that vessels that responded to the PAF by transiently increasing permeability failed to respond to thrombin when this was applied at any time during the first 1–2 h after the experiment began. However, after 24 h the vessels responded to thrombin.[79] The vessels had increased baseline permeability and a moderate, but not statistically significant increase in permeability relative to Day 1 in response to PAF. Additional experiments demonstrated that the minimum time to observe a thrombin-induced response was 6 h.[82] Further, whereas pre-treatment with inhibitors of contractile mechanisms (ML-7 to inhibit myosin light chain kinase, Y27632 to inhibit Rho kinase, and BDM to inhibit myosin ATPase) failed to block the PAF response, pre-treatment with the Rho kinase inhibitor (Y27632) attenuated the response to thrombin.[75,79,83] The experiment shows that a vessel that previously failed to respond to thrombin, and which significantly increased permeability in response to PAF independent of contractile mechanisms, later demonstrated both contraction-dependent (thrombin) and contraction-independent (PAF) increases in permeability. The microvessels on Day 2 showed

many signs of being exposed to sustained inflammatory conditions including both attached and migrated inflammatory cells and enlargement of the endothelial cells (*Figure 4A and B*).

Kim and co-authors[36] have reported changes in the response to thrombin over a 24 h period using a 6 mm skin wound in mouse back skin. The permeability of the vessels in the wound (mainly in the wound periphery) to albumin was measured using one of the new fluorescence scanning methods discussed above. Within the first hour after the wound, VEGF induced a 3- to 4-fold increase in albumin permeability, but there was no response to thrombin. After 24 h the vessels in the peripheral region of the wound responded to thrombin, with an increase in permeability similar to that elicited by VEGF. The Rho Kinase inhibitor Y27623 reduced the thrombin-dependent increase in permeability to the Day 2 baseline permeability. The inhibitor also attenuated a larger fraction of the VEGF response after 24 h (average response was reduced to 50% of control vs. 75% of control) (*Figure 4C*).

The results in both venular microvessels and skin in which thrombin-increased permeability are consistent with the hypothesis that there was up-regulation of thrombin-dependent contractile pathways in the endothelial cells after exposure to inflammatory conditions. Such mechanisms modify the balance of adhesion and contractile forces and contribute to the more inflammatory phenotype. Changes in the endothelial cells may not be the only mechanisms to account for the results in the intact microcirculation. Systemic depletion of circulating PMNs with antibodies blocked their extravasation into the wound (as measured by accumulation of EGFP-expressing neutrophils) but did not alter the thrombin-induced increase in permeability.[36] However, depletion of circulating platelets significantly attenuated the response, suggesting that thrombin

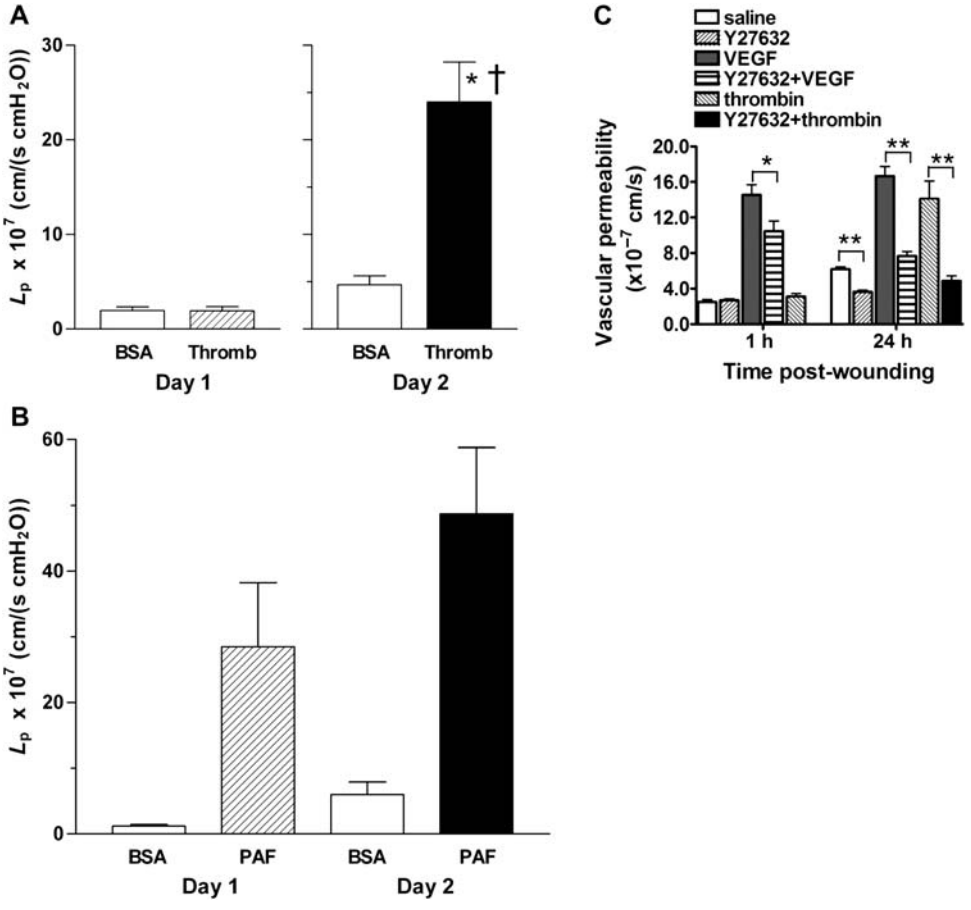

Figure 4 (*A*) Comparison of thrombin response in inflamed and non-inflamed rat mesentery venules. In vessels not previously manipulated (Day 1) L_p measured during thrombin perfusion is not different from that measured during perfusion with vehicle control solution ($n = 8$). When L_p is measured 24 h after initial perfusion, thrombin stimulates a large L_p response (Day 2), approximately five times greater than Day 2 vehicle control L_p ($n = 11$; $P < 0.05$ *different from paired Day 2 vehicle control, †different from Day 1 thrombin, non-paired). (*B*) Response to PAF 24 h after initial perfusion in rat mesentery venules. Paired L_p measurements in response to PAF (10 nM) on the second day of a 2-day experiment (solid bar, Day 2) were not significantly different from the response measured initially in 6 venules (striped bar, Day 1). Corresponding vehicle control measurements are shown (open bars). (*C*) Rho-kinase inhibition reduces thrombin- and VEGF-stimulated vascular hyperpermeability in a mouse skin wound model. (C) Vascular permeability at 1 h and 24 h post-wounding and effect of Rho-kinase inhibition with Y-27632 on basal, thrombin-, and VEGF-induced vascular permeability. *$P < 0.05$ and **$P < 0.01$ between groups; $n = 5–7$ mice in each group. From Curry *et al.*[79] for (*A*) and (*B*) and Kim *et al.*[36] for (*C*), used with permission from the American Physiological Society.

activation of platelets in the vicinity of the wound contributed indirectly to its action to increase permeability. The main conclusion is that the mechanism of increased permeability depends not only on the specific inflammatory mediators but on the state of the endothelial cells with respect to prior exposure to inflammatory conditions. It is not clear what aspect of endothelial cell culture (source of the cells, injury during harvesting, or other mechanisms) may lead to acute thrombin-induced increases in permeability but the answers to such questions are essential if the results from cultured monolayers are to be appropriately used to understand responses in intact microvessels.

3.4 Expression of phosphodiesterases in cells: possible role of differential expression of phosphodiesterases in modulation of permeability

There is now clear evidence that many of the agents that act to increase permeability do so by reducing the level of cAMP, while increased cAMP strongly attenuates increased permeability (see reviews by Spindler *et al.* and Shen *et al.*).[4,49] Because the level of cAMP is regulated by phosphodiesterases, an important area for further research is modulation of phosphodiesterase activity. Two phosphodiesterases are of particular interest in this regard. PDE2A and PDE3A can both reduce the level of cAMP. However, whereas cGMP is a competitive inhibitor of PDE3A, cGMP activates PDE2A. Thus the action of an agent that stimulates the production of endothelial cGMP will modulate the permeability barrier differently depending upon the concentration of cGMP and the corresponding relative activity of PDE3A and PDE2A. Beavo and co-authors[84] have shown in HUVECs that low concentrations of cGMP (less than 100 nM) inhibit PDE3A without activating PDE2A, thereby raising cAMP levels and tending to lower permeability. On the other hand higher concentrations of cGMP activate PDE2A, overcoming the effect of inhibiting PDE3A and tend to lower local cAMP to increase permeability. An example of the bimodal regulation determined by these mechanisms is the action of a low concentration of ANP to induce low intracellular cGMP and attenuate increased permeability while a higher concentration of ANP increases permeability.[84]

Finally, it is likely that the balance of cGMP- and cAMP-dependent mechanisms are further modulated by experimental conditions. For example shear stress applied to endothelial cells in culture stimulates the production of both NO (acting to increase cGMP) and prostacyclin (acting to increase cAMP). Furthermore, different levels of phosphodiesterase expression are likely under different conditions. Beavo and co-authors[84] also demonstrated that exposure of the cells in culture to TNFalpha for 24 h upregulated PDE2A. It is reasonable to suggest that exposure of PDE2A may contribute to the development of a more pro-inflammatory phenotype under some inflammatory conditions. An emerging concept is that a balance of pro-inflammatory and anti-inflammatory mechanisms may determine baseline permeability and responses to inflammatory agents. As well as the examples cited above, recent observations demonstrate contributions of different S1P receptors,[85] a role for Rac1 to generate reactive oxygen species as well stabilize adhesion,[86,87] and differences in the expression of phosphodiesterases in males and females as determinants of responses to vascular injury.[88]

4. Modification of the glycocalyx

The picture of barriers on the endothelial cell surface that is emerging is of a multifunctional layer that not only forms the primary permeability barrier to the plasma proteins on the luminal surface of the endothelium,[53,89] but also plays a key role to reduce resistance to blood flow through narrow microvessels,[90,91] and reduces the access of circulating peptides and ligands on inflammatory cells to receptors on the endothelial cell surface.[92,93] Some of the known structures of the glycocalyx at a cellular level are reviewed by Tarbell in this issue.[94] Some of the key components are linked into raft-like structures in the endothelial cell membrane and extend a maximum of 40–60 nm from the surface. Side chains of these membrane-attached molecules may extend to a maximum of 100–200 nm from the cell surface. However, it is becoming clear that the glycocalyx as expressed in cultured endothelial cells is not necessarily representative of the structure on the cell surface of intact endothelial barriers[21,95] (and see review by Becker *et al.* in this issue).[18]

Imaging studies using individually perfused vessels in which the structure and composition of the glycocalyx is modulated suggest that a structure formed by core proteins and side chains with bound plasma proteins can form a reasonably periodic matrix close to the endothelial cell membrane.[96] Furthermore, hydrodynamic models of such quasi-periodic structures demonstrate that the size and spacing of core proteins and their side chains in the presence of bound plasma proteins are sufficient to account for the glycocalyx as a primary diffusion and osmotic barrier to plasma proteins.[97,98] A less ordered and more dynamic structure, possibly better described as an endothelial surface layer[91] extends further into the lumen of some microvessels for distances of at least 0.5 micron. This structure plays a critical role in the flow of red cells, leucocytes, and plasma through microvessels.[21,90,99,100] A distinction between an inner quasi-ordered structure and a dynamic outer structure for the glycocalyx appears to be critical to account for some of the discrepancies between estimates of glycocalyx thickness made from haemodynamic and permeability measurements. Another way to highlight differences in functions of the glycocalyx is to distinguish between a relatively stable inner structure and a dynamic outer structure whose thickness and composition is determined by a balance between shedding and enzymatic breakdown and replacement by cellular synthesis and adsorption from circulation.[92]

A major contribution to our understanding of glycocalyx function would be provided by new *in vitro* models to study the composition and modulation of the endothelial glycocalyx. At present most investigations of permeability regulation using cultured endothelial monolayers ignore the glycocalyx, and there is disagreement about the extent to which a functional glycocalyx barrier is even present in monolayers that are not conditioned by flow.[20,21] An extension of the approaches reviewed by Tarbell in this issue[94] to include investigations of the glycocalyx in endothelial cell monolayers conditioned by chronic shear and as yet poorly understood conditions that protect or stabilize the glycocalyx is required.

In intact organs the reviews by Becker and VanTeeffelen describe the growing evidence that changes in the glycocalyx alter blood-to-tissue exchange in a variety of clinical conditions including ischaemia, reperfusion, diabetes, and chronic infectious disease. Because loss of components of the glycocalyx may be some of the earliest signs of endothelial dysfunction (increased fragments in

circulating plasma, increased renal excretion of glycocalyx fragments) measurement the glycocalyx function using methods applicable in the whole body are of significant interest as they provide new diagnostic tools[101–103] (see review by VanTeeffelen *et al*. in this issue).[19] One approach is to define a 'glycocalyx volume' as a distribution volume estimated as the difference between the exclusion volumes measured by two separate probes; one volume measured by a preparation of clinical dextrans assumed to penetrate the glycocalyx, and the other the more limited distribution volume available to circulating red cells.

In spite of the promise of this approach, there are major concerns with attempts to associate changes in the glycocalyx volume index as presently measured solely to changes in the glycocalyx because several technical problems are still to be overcome.[104] One problem is the heterogeneity of molecular weight components in clinical dextran solutions used to probe the glycocalyx. Some small components of the probe are lost from the circulation early and are likely to cross through the glycocalyx and enter the interstitial fluid, resulting in an overestimate of the glycocalyx volume. Another problem is that the concentrations of the larger components of the dextran probe that do enter the glycocalyx cannot be equal to their concentrations in the circulating plasma as assumed in the estimate of a distribution volume because of the well-known effects of steric and charge exclusion. These problems also likely lead to significant overestimates of the true glycocalyx volume. Refinements to the approach to overcome these deficiencies are essential if it is to provide a reliable tool for both clinical and experimental investigations of the glycocalyx.

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