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A Role for the Endothelial Glycocalyx in Regulating Microvascular Permeability in Diabetes Mellitus

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Abstract

Diabetic angiopathy is a major cause of morbidity and mortality in diabetes mellitus. Endothelial dysfunction and associated alterations in blood flow, pressure and permeability are widely accepted phenomena in the diabetic milieu and are understood to lead to microangiopathy. Despite the clinical importance of diabetic microangiopathy, the mechanisms of pathogenesis remain elusive. In particular, much is yet to be understood about the nature of the putative increased permeability with respect to diabetes. Microvessel permeability is intrinsically difficult to measure and a surrogate (solute or solvent flux) is usually reported, the measurement of which is hampered by haemodynamic factors, such as flow rate, hydrostatic pressure gradient, solute concentration and surface area available for exchange. Very few studies describing the measurement of permeability with respect to diabetes have controlled for all these factors. As a result, the nature of the increased microvessel permeability in diabetes mellitus and indeed its causes are poorly understood. Recent studies have shown that hyperglycaemia can alter the glycocalyx structure, and parallel findings have shown that the apparent increase in permeability demonstrated in hyperglycaemia may be due to an increase in the permeability of the vessels to water, and not an increase in protein permeability, an effect attributable to altered glycocalyx. This review focuses on the current understanding of microvascular permeability in terms of the endothelial glycocalyx-fibre-matrix theory, those methods used to determine permeability in the context of diabetes, and the more recent developments in our understanding of elevated microvascular permeability in the diabetic circulation.

Introduction

Diabetes mellitus is a heterogeneous disease, conventionally classified into two major types: insulin-dependent (IDDM, or type I) and non-insulin-dependent (NIDDM, or type II). Long considered a disease of minor significance to world health, diabetes is now emerging as one of the main threats to human health in the 21st century [1], not only in developed nations, but also in developing countries. The total number of diabetics is projected to reach 221

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million worldwide by 2010 [2]. Diabetes is characterised by hyperglycaemia, an increased vascular permeability and a propensity to develop vascular complications that contribute to the morbidity and mortality of the diabetic patient. In the UK, 35% of deaths are due to cardiovascular causes [3], however, this is increased to 60% in non-insulin dependent diabetics and 67% in insulin dependent diabetic patients over the age of 40 years despite management of conventional risk factors, e.g. blood pressure, obesity, dyslipidemia, salt intake and smoking [3]. Two types of vascular disease have been described [4]. Microangiopathy, which affects capillaries and arterioles, and is relatively unique to diabetes (for example retinopathy), and a macroangiopathy, which is morphologically very similar to atherosclerosis in non-diabetics, but more extensive, occur at an earlier age and has a characteristic distribution [5]. Alterations in microvessel permeability, and the possible role of the endothelial glycocalyx as a mediator of this diabetic endothelial dysfunction are the focus of this review.

Diabetes is Characterised by Endothelial Dysfunction

The endothelium is a key component of the cardiovascular system, which orchestrates many aspects of microvascular function, including pressure, flow, permeability, angiogenesis and rheology. It has long been recognized that the functional disturbance of the vascular endothelium is altered in diabetes mellitus, which gives rise to the clinical expression of microangiopathies. Diabetic endothelial dysfunction is thought to be manifest by an early increase in vascular permeability. Very few studies that determine permeability, however, have controlled for compounding haemodynamic factors (such as flow rate, hydrostatic and colloid osmotic pressure gradients). As will be discussed further, this is particularly problematic with respect to diabetes, since other haemodynamic changes occur in the microcirculation soon after the onset of disease [6]. Early increases in blood flow and pressure have been widely reported in those organs that develop diabetic microangiopathy [7-10]. In order to gain further insight into the nature of the increased permeability with respect to diabetes, it is first necessary to review our current understanding of microvessel permeability and how it is measured.

Microvascular Permeability and the Fibre-matrix Pore Theory

A determining factor affecting the rate of water and solute flux across the endothelial wall is the microvessel permeability. Permeability can be calculated from the amount of solute (g/s) or solvent (cm^3/s) moved through a unit area (cm^2) of capillary surface by a unit driving force (concentration gradient [for solutes] or hydrostatic pressure [for water]). Three parameters are commonly used to describe the permeability of a vessel wall: the diffusive permeability to a particular solute (P_s), the reflection coefficient (σ) and the hydraulic conductivity (L_p).

Diffusive Permeability (P_s)

The permeability of a membrane to a given solute (P_s)is defined as the rate of diffusion of solute across the vessel wall (J_s), per unit concentration difference (ΔC), per unit area (A), and is described by Fick's law ($J_s = PA(\Delta C)$).

Hydraulic Conductivity (L_p)

A characteristic of microvessel pores is that solute flux is coupled to water flux (convection). Indeed, for large solutes (such as albumin) convective flux dominates under normal physiological conditions. A measure of the ease by which a microvessel wall allows water to flow across it is the hydraulic conductivity (L_p) of a membrane. Hydraulic conductivity is a proportionality constant, which relates the bulk flow of water to its driving

force. It is defined as the volume of fluid flow, per unit area of vessel wall, per unit pressure gradient. Typical units are volume per second, per unit of driving pressure per unit area. It is related to the fluid filtration across the vessel wall by the differences of hydrostatic pressure (ΔP) and osmotic pressure ($\Delta\pi$) across the capillary wall. The principal mechanism for the regulation of fluid exchange is the adjustment of intracapillary pressure. Arteriolar constriction tends to decrease capillary pressure, thus, lowering the hydrostatic pressure that moves fluid from the capillary to the interstitial space. Venular constriction increases capillary pressure and thus promotes fluid loss to the interstitium. The balance of these regulates the flow of water across the vessel wall and is an important factor to consider in the context of diabetic angiopathy. The volume flux per unit area (J_V/A) across the vessel wall is described by Starling's equation for fluid filtration:

$$J_v/A = L_p\left[(P_c - P_i) - \sigma(\pi_c - \pi_i)\right],$$

where π_c and π_i are the capillary luminal and interstitial fluid osmotic pressures, respectively, P_c and P_i the capillary and interstitial hydrostatic pressures, respectively. J_V is the net volumetric rate of flow across the membrane per unit area (A), and σ is the mean oncotic (colloid osmotic) reflection coefficient of the plasma solutes. It can be seen from this that changes in L_p will drastically increase filtration rate, and hence convective flux of proteins.

Reflection Coefficient (σ)

The reflection coefficient (σ) expresses the fact that the microvessel wall is selective in the sense that water passes through it faster than larger solute molecules do. It is an index of the membrane's molecular selectivity. The reflection coefficient is defined as the osmotic pressure that a given difference in solute concentration exerts across a test membrane ($\pi_{\text{effective}}$), as a proportion of the full osmotic pressure that the same concentration difference exerts across a perfect semi-permeable membrane (π_{ideal}):

$$\sigma = \frac{\Delta\pi_{\text{effective}}}{\Delta\pi_{\text{ideal}}}.$$

If a solute crosses freely through the membrane it exerts no osmotic pressure (i.e. $\sigma = 0$). If a solute is completely reflected it exerts its full osmotic potential ($\sigma = 1$). The reflection coefficient is dependent on the ratio of a solute radius compared to the radius of the microvessel pore through which it passes, and thus gives useful information as to the nature of the pore size. Thus solute flux by convection will be dependent on the concentration of solute, the filtration rate and the non-reflected fraction ($1 - \sigma$),

$$J_s(\text{conv}) = C_p J_v(1 - \sigma).$$

Total solute flux per unit area (J_s/A) can be calculated from the non-linear flux equation

$$J_s/A = L_p \Delta P(1 - \sigma) C_p + P_d \Delta C \ \text{Pe}/\left(e^{\text{Pe}} - 1\right),$$

where Pe, the Peclet number is the ratio of convective to diffusive flux. So when the permeability is very low (e.g. to large solutes such as albumin) solute flux is dominated by

convection, and hence the primary factors driving solutes across the wall are the hydraulic conductivity (L_p) and effective pressure difference ($\Delta P = (P_c - P_i) - \sigma(\pi_c - \pi_i)$).

In order to yield a true assessment of microvessel permeability in diabetes, all three of the above permeability parameters should be considered.

The fibre-matrix theory of microvascular permeability, developed by Michel and Curry, holds that the capacity of the capillary wall to act as a molecular sieve is a characteristic bestowed upon it by the endothelial glycocalyx [11]. Consisting of a highly hydrated mesh of membraneassociated proteoglycans, glycosaminoglycans, glycoproteins and glycolipids [12, 13], the glycocalyx is present on the endothelial surface layer and within the intercellular cleft; it is not merely an inert intercellular cement as once thought [14], but understood to be a very dynamic structure. The possible existence of an ordered structure was first proposed by Curry and Michel [15] to explain why there is a sharp break in the solute permeability versus Stokes-Einstein relationship for molecules the size of albumin [16]. The ability to selectively allow passage of water and solutes across the vessel wall is described in terms of the spacing between the fibres of the glycocalyx (i.e. through small pores). Capillary permeability is thought to be determined both by a change in the size of small pores, and by an alteration in the number of pores—a function of the length and frequency of breaks in the tight region of the intercellular cleft between neighbouring endothelial cells. In an elegant study, Adamson gave experimental evidence for the glycocalyx as the site for exchange [17]. He reported that an increase in hydraulic conductivity (L_p), as measured by the Landis-Michel technique, is associated with partial digestion of the endothelial glycocalyx. The increased L_p was not accompanied by changes in dimensions of the intercellular cleft, and he postulated that the endothelial glycocalyx contributes as much as 60% of the hydraulic resistance of the capillary wall [17]. The ultrastructural properties of the glycocalyx were investigated by Squire et al. who carried out detailed computed autocorrelation functions of electron microscope images to identify its structure, including the anchoring foci that protrude from the underlying cytoskeleton [18]. Their findings confirmed the existence of a three dimensional fibrous meshwork with characteristic spacing of 20 nm in all directions, with an effective diameter of 10-12 nm. Using freeze-fracture replica of rare sections where the fracture plane passed parallel to the endothelial surface, they showed that anchoring foci are arranged in a hexagonal array with intercluster spacing of typically 100 nm in the frog capillaries [18]. More recent methods of fixation [19], which preserve the aqueous nature of the glycocalyx, combined with exclusion of fluorescently labelled macromolecules suggest that the glycocalyx and associated adsorbed proteins may extend as much as 0.5 μm into the vessel lumen [12, 20].

Measurement of Microvascular Permeability in Diabetes Mellitus

The investigation of microvessel permeability has been attempted with respect to diabetes using a variety of techniques. These usually investigate tracer clearance [21], where intravenously injected dye (solute) is seen to leak through the walls of hyperpermeable vessels. Dye leakage is correlated with permeability, and quantified, either spectrophotometrically, by extracting the solute from the tissue of interest [22, 23] or often by measurement of fluorescence intensity [24, 25]. These methods however, are complicated by haemodynamic factors. The amount of (solute) leakage from the vasculature can be increased by increases in surface area, plasma to tissue solute concentration gradient, capillary pressure or interstitial colloid osmotic pressure, or permeability to water, or solute. Likewise, decreasing interstitial pressure, oncotic reflection coefficient or plasma colloid osmotic pressure could increase solute flux. This is of particular importance in the diabetic milieu, where it has been demonstrated that an early increase in capillary pressure occurs in type I diabetes [26] compared to age- and sex-matched non-diabetic controls, and a

decreased interstitial pressure is reported to occur [27]. These confounding factors can be crucial in the interpretation of the experimental results. For instance an increase in tracer flux of 2-3-fold can easily be achieved by an increase in hydraulic pressure of just a few mmHg, purely by increasing convective solute flux, particularly to large molecular weight tracers. Equally a small increase in blood flow can drastically increase the delivery of solutes all along the microvessel wall and hence increase diffusive flux to small molecules such as glucose or fluorescein. It is therefore key that experiments are confirmed by methodologies that can distinguish haemodynamic changes from those of the endothelial barrier.

Microvascular barrier function may be estimated in single vessels, either in vitro or in vivo. The Landis-Michel micro-occlusion technique [28] allows the precise measurement of permeability characteristics, since the physical forces that determine transvascular permeability (hydrostatic and colloid osmotic pressures) are controlled. A vessel of known surface area is cannulated with a micropipette. The vessel is then perfused with solute at a known concentration and flow markers, usually red blood cells [29] or microspheres [30] at a defined pressure. Following gentle occlusion of the vessel, the filtration rate is determined from the velocity of the flow markers in the lumen. This method assumes that both the interstitial colloid osmotic and hydrostatic pressures are negligible. The permeability of vessels that are less accessible for this type of in vivo experiment may be used in an in vitro single vessel perfusion method [31]. Vessels are removed by micro-dissection and cannulated with four micropipettes (pipette in pipette), with each pipette connected to a reservoir to allow independent control of intraluminal pressure and flow [31]. Fluorescently labelled albumin is perfused through the vessel. As solute diffuses out of the lumen into the extravascular space it is tracked by measuring the rate of increase in fluorescence intensity [31]. The apparent solute permeability coefficient of albumin (P_a) is measured by the ratio of the flux of fluorescently labelled albumin to its transmural concentration gradient. The apparent permeability, P_a overestimates the true diffusive permeability for large molecular weight solutes, such as albumin, since it is dominated by convection (solvent drag) rather than by diffusion. Estimates of albumin clearance (P_a) as a permeability coefficient will vary with the net fluid filtration rate from plasma to tissue. Huxley et al. demonstrated that when the solute permeability coefficient (P_s) is plotted against pressure, it is apparent that the transcapillary solute flux increases as capillary hydrostatic pressure is increased, even though the solute permeability was unchanged. Extrapolation of this regression line shows that a significant flux occurs when the net filtration pressure is zero, indicating that both diffusive and solvent drag (convective) forces determine the flux of solute across vessel wall [32]. This convective flux may be the key to understanding changes in permeability in diabetes.

Diabetes Mellitus: Increased Permeability or Compounding Haemodynamic Factors?

There is a wealth of information regarding the putative increased endothelial macromolecular permeability in diabetes which is reviewed by Tooke [4]. Early studies by Parving et al. reported an increased transcapillary escape rate of albumin in poorly controlled short-duration diabetics, as measured by the disappearance of intravenously injected ^{125}I-labelled human serum albumin [8] and this has been more recently confirmed [33-35]. This increased transcapillary escape rate of albumin may reflect increases in vascular permeability or may be a result of an alteration in haemodynamic factors. Single capillary studies demonstrated an increase in transcapillary leakage of sodium fluorescein from nailfold capillaries in IDDM compared to age- and sex-matched non-diabetic controls [36, 37] and retina [38]. Oomen et al. documented that sodium fluorescein leakage is further elevated in patients with microalbumuria compared with patients in a long-duration

complication free group [39]. Diabetes compounded by peripheral neuropathy also results in an increased sodium fluorescein skin capillary leakage [25]. The determination of capillary filtration coefficient (CFC) by venous occlusion plethysmography (VOP) has been commonly used to describe the permeability characteristics of human diabetic subjects. The increased CFC in diabetics is more evident in patients of 20 years disease duration than newly-diagnosed diabetics [40]. However, it is likely that the increase in CFC is due to an increased hydrostatic pressure or surface area in these patients. Additionally, a lowered interstitial colloid osmotic pressure, as reported in diabetics without microvascular disease compared to non-diabetic control plasma [41] and in those patients with microvascular complications [27] may influence these results. The plasma colloid osmotic pressure does not appear to be altered in diabetes [27]. This decrease in interstitial osmotic pressure has been attributed to an increase net water flux out of the circulation with resultant increased lymph formation [41]. These results all point to an increased transcapillary convection of fluid in diabetic patients.

Many studies demonstrate an increased vascular permeability in diabetic animal models. Yuan et al. [42] measured venular permeability in isolated coronary venules of streptozotocin (STZ)-diabetic pigs, a model closely resembling the human disease (type 1 diabetes). The apparent solute permeability coefficient for albumin (P_a) was measured using fluorescently labelled albumin. The coronary venular P_a of STZ-diabetic animals was demonstrated to be significantly elevated compared to non-diabetic controls four weeks after the onset of diabetes [42]. Biobreeding rats (a model of type II diabetes) and STZ-induced diabetic rats had elevated albumin permeation as measured by ^{125}I-albumin in the vasculature of eyes, sciatic nerve, aorta, and kidney, those tissues predisposed to diabetic vascular disease in humans [43]. No evidence of increased albumin permeation was noted in heart, brain, testes or skeletal muscle [43]. Again, albumin permeation is particularly sensitive to changes in water flux, as it is diffusion limited, and therefore has a very high convective component to its transvascular transport. It is well documented that an increased permeability of retinal vessels occurs, which is thought to contribute to diabetic retinopathy in humans, but the permeability coefficients (P_d, L_p and σ) have not been clearly measured.

Hyperglycaemia and Increased Vascular Permeability

Although the precise mechanism(s) that lead to diabetic vascular disease have yet to be uncovered, hyperglycaemia has been identified as a culpable risk factor for micro- and macrovascular complications. This association first became evident from clinical studies. In a landmark study where over 4,000 patients with IDDM or NIDDM were followed [44], a relationship between the magnitude of hyperglycaemia (measured by blood glucose, glycosuria and episodes of ketoacidosis) and the incidence of nephropathy was identified. Large scale, randomised trials, such as the diabetes control and complications trial [45] and the UK Prospective Diabetes Study [46] also provide evidence of a causal relationship between chronic hyperglycaemia and diabetic (IDDM) microvascular disease by demonstrating that the relative risks for developing retinopathy, nephropathy, and neuropathy increase with increasing levels of mean glycosylated haemoglobin. It was also noted that strict glycaemic control both delayed the onset and slowed the progression of diabetic vascular complications [46]. Many studies, both in animal models [42, 47] and human clinical studies [26] strongly suggest hyperglycaemia is a cardinal feature of diabetic microvascular complications. Animal models of diabetes show an increased transcapillary solute flux (interpreted as increased albumin permeability) that may be observed just a few hours after hyperglycaemia. Experiments carried out by Algenstaedt et al. showed that vascular permeability, as assessed by TRITC-labelled albumin clearance within mice dorsal skin-fold chambers, significantly increased with elevated blood glucose [24]. This study did not control for changes in vascular haemodynamics. Yuan et al. [42] measured the effect of

elevated glucose concentrations on venular permeability in isolated porcine coronary venules from nondiabetic animals. A rapid increase in P_a was demonstrated following perfusion and superfusion with 12.5 mmol L^{-1} D-glucose compared to baseline 5 mmol L^{-1} D-glucose. This elevation in apparent solute permeability was dose-dependent, and a maximal response was demonstrated with 100 mmol L^{-1} D-glucose [42], but again this could have been due to an increased convective component. Using brown fat deficient transgenic mice, which have a metabolic disorder similar to type II diabetes in humans, Algenstaedt et al. described elevated subcutaneous TRITC-albumin permeation within a dorsal skin-fold chamber [24]. This increased flux was correlated with the intensity of the metabolic disorder, as represented by blood glucose levels. Interestingly, these mice also had an increased number of endothelial-leucocyte interactions, suggesting the involvement of an inflammatory response in the disorder. Rogers et al. noted that the elevated ^{125}I-albumin permeation reported in STZ-elicited diabetic rats was largely attenuated following a 14-day insulin treatment to reduce glucose levels [47].

We have recently obtained evidence for the direct effect of elevated glucose on the microvascular permeability in isolated capillaries and post-capillary venules of the mesentery [48]. Perfusion and superfusion of 20 mM glucose (a level likely to be found in the circulation of a poorly-controlled diabetic patient) caused a small but immediate 1.6-fold increase in L_p, but had no effect on the macromolecular permeability, as measured by the oncotic reflection coefficient, σ. No effect was seen following treatment with 20 mM mannitol, excluding the possibility that the permeability increase is the result of an osmotically active compound. It is tempting to speculate that that the chemical nature of glucose (an aldehyde sugar with oxidative potential) may deleteriously influence endothelial function. Indeed, supporting evidence is given by Zuurbier et al. who demonstrated an increased dye permeation through the glycocalyx layer of mouse capillaries without alterations in the permeability of other parts of the vessel wall following short-term treatment of glucose [49]. It remains to be seen whether this increase in water permeability correlates with morphological changes at the ultrastructural level.

The current fibre-matrix model determining capillary permeability describes the selectivity of the capillary wall (the pore size) in terms of the space between the fibres of the glycocalyx matrix on the endothelial cell surface, and the area for exchange (the pore number) in terms of the length and frequency of breaks in the tight junction strands [11]. It is possible, then, that the increased L_p following exposure to elevated glucose may be due to either an increase in pore frequency, or an increase in pore radius. A change in pore radius could be brought about by glycosylation of endothelial proteins thought to be involved in barrier function such as those at the tight juntion such as occludins [50], or the adherens junctions such as JAM-1 [51] or VE-cadherin [52]. However, if an increase in pore radius were the case, we would expect to measure a substantially reduced reflection coefficient to albumin, which was not the case [48]. Furthermore, since L_p is dependent on the fourth power of the radius of a pore, a 1.6-fold increase in L_p following treatment with elevated glucose would be the result of only a 1.13-fold increase in pore radius, which would barely increase solute permeability to albumin. Yuan et al., however, found that the P_a was dose-dependently increased following perfusion and superfusion of elevated glucose in coronary venules [42]. The P_a increased from 3.17 ± 0.28 to 6.45 ± 0.67 × 10^{-6} cm s^{-1} with 25 mM D-glucose; a mean fold increase of 2.03. This increase in P_a can be entirely explained by an increase in convective flux due to increased L_p, rather than increased solute permeability. In fact mathematical modelling based on the results of Yuan et al. show that the entire increase in P_a is accounted for by the measured increase in L_p [48]. Thus glucose is able to increase permeability to water without changing the permeability to proteins. Recently, direct evidence has been given for an effect of hyperglycaemia on the glycocalyx. Large molecular weight fluorescent moelcules are excluded from the glyococalyx layer to differing extents,

and less than that of red cells. The group of Vink and others in Amsterdam have recently demonstrated that the thickness of the exclusion layer to fluorescent moelcules and erythrocytes is reduced by hyperglycaemia, indicating that the glycocalyx structure has changed [53]. The details of these changes remain to be explored, but this direct evidence for altered glycocalyx by hyperglycaemia emphasises the role that the surface coat can play in microangiopathy induced by hyperglycaemic episodes. In particular, the effect of a sustained increase in hydraulic conductivity on the tissue is not known. Increased hydration of tissue can lead to fibrosis and laying down of collagen in chronically oedematous states [54], and basement membrane thickening is known to be a key component of diabetic angiopathy [55]. Whether recurrent chronic hyperglycaemic episodes result in recurrent episodes of sustainedly increased fluid flux due to a combination of increased capillary pressure and increased L_p, and this contributes to disease progression is yet to be determined.

Combined, these findings show that vessels exposed to elevated glucose are highly permeable to water without a change in the diffusive permeability to protein, yet still retain selectivity to large molecular weight solutes under convection. Thus, in early diabetic microangiopathy (such as microalbuminuria) the increase in solute flux may be entirely secondary to a glucose-mediated change in macrovascular convective flux. The mechanisms underlying this structural alteration, or its cell biological correlates are not known. However, the most likely explanation from a biophysical perspective is that there is an increase in density of small pores, or a reduction in their pathlength. If the ultrastructural correlates of this are the fibre-matrix components of the glycocalyx, then this could be brought about by a rearrangement of the fibres to reduce hydraulic friction (increase hydraulic permeability) without changing pore size. This could occur by a reduction in the fibre surface area without a change in its fractional solid volume of the matrix, which determines the reflection coefficient [56]. There is currently no ultrastructural evidence for changes in the glycocalyx matrix in direct response to glucose, but these experiments will need to be carried out.

The pathologic processes involved in the vascular dysfunction in diabetes are complex and far from being understood. This, in part, attests to the fact that many studies do not measure permeability per se, since confounding haemodynamic factors are poorly controlled. Whilst it appears to be evident that glucose at least in the early stages plays a contributing role to permeability changes in the disease, the mechanisms underlying these changes are unknown. Much more is to be gleaned from experiments that correctly and accurately measure permeability characteristics, and much of that information is likely to come from in vivo studies. This will deepen our understanding into the nature of microvascular permeability itself, and in the process(es) by which angiopathy occurs in the diabetic milieu. Elucidating the mechanisms involved in altered microvessel characteristics in diabetes may aid treatment of the microvessel disease, which affects the ever-increasing number of diabetic patients worldwide.

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