Functional and morphological studies of protein transcytosis in continuous endothelia

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Invited Review

Functional and morphological studies of protein transcytosis in continuous endothelia. Am J Physiol Lung Cell Mol Physiol 287: L895–L901, 2004; doi:10.1152/ajplung.00075.2004.—Continuous microvascular endothelium constitutively transfers protein from vessel lumen to interstitial space. Compelling recent biochemical, ultrastructural, and physiological evidence reviewed herein demonstrates that protein transport is not the result of barrier “leakiness” but, rather, is an active process occurring primarily in a transendothelial vesicular pathway. Protein accesses the vesicular pathway by means of caveolae open to the vessel lumen. Vascular tracer proteins appear in free cytoplasmic vesicles within minutes; contents of transport vesicles are rapidly deposited into the subendothelial matrix by exocytosis. Caveolin-1 deficiency eliminates caveolae and abolishes vesicular protein transport; interestingly, exchange vessels develop a compensatory transport mode through the opening of a paracellular permeability pathway. The evidence supports the transcytosis hypothesis and the concept that transcytosis is a fundamental component of transendothelial permeability of macromolecules.

albunin; caveolae; filipin; microvascular endothelium

CONTINUOUS MICROVASCULAR ENDOTHELIUM is often compared with a molecular sieve containing pores of two discrete sizes, but compelling recent morphological and biochemical data have established that the endothelium is much more than a passive filter. Approximately 20% of its cellular volume consists of vesicles (28), and the endothelial cell is extremely rich in caveolae, the uncoated pits originally described in 1953 by Palade (23) in endothelial cells, which are the vesicular carriers for transcytosis. The two-pore theory, a product of the work of Pappenheimer et al. (26) and Grotte (11), attempts to explain the paracellular movement of fluid and solutes through the microvessel wall in terms of 1) the density of “large” and “small” pore populations, 2) the pore dimensions, and 3) the net transcapillary filtration pressure given by the algebraic sum of the four components of the Starling equation, $P_{f}$, $P_{f,oc}$, $P_{f,ht}$, and $P_{f,oc}$ (where $P$ and $\pi$ are hydrostatic and oncotic pressure, and the subscripts refer to the capillary or interstitial fluid compartment). The small pores are generally presumed to account for transvascular movement of water and dissolved solutes up to a limiting size of $\sim$4 nm; the large pores are supposed to account for the leakage of any plasma protein through microvascular walls, as these pores are nondiscriminatory by size and accommodate molecules $>12$ nm in diameter (29). The macromolecular transport pathway is described as “nonhydraulic” because large pores make a minor contribution to overall transvascular water movement, given the theoretical estimate of their numbers (ranging from 0.003 to 0.1% of small-pore population) (17, 18). Although the physiology of the microvessel wall is often viewed from the perspective of the pore theory or its variants, the postulated pore systems, whose calculated dimensions are well within the resolution of the electron microscope, have never been proven to exist by the extensive ultrastructural studies of the lining endothelial cells of intact microvessels carried out over the years. The apparent inconsistency between the physiological and ultrastructural approaches has fueled a vigorous debate about the true nature of the pore systems, and whether large pores, in particular, are in fact pores at all. Initial inquiries into macromolecular permeability of microvessels were often unsuccessful attempts to reconcile the differences between the pore theory and the actual ultrastructural observations. However, a truly complete definition of macromolecular permeability must include the structure of the “permeability pathway.” In this article, we evaluate the recent biochemical, ultrastructural, and physiological evidence that supports the transcytosis hypothesis and the concept that transcytosis is a fundamental component of transendothelial permeability of macromolecules. We will review data derived from studies in the endothelial cell monolayer as well as the intact lung in support of the transcytosis hypothesis. Regardless of the investigational approach used, the results described below indicate that macromolecular transport through continuous endothelia (including pulmonary microvessels) involves caveolae.

TRANSPORT OF ALBUMIN: THE TRANSCYTOSIS HYPOTHESIS

Albumin is implicated in the facilitated transfer of numerous ligands (fatty acids, amino acids, peptides, steroids, metals, and xenobiotics) from the vascular to interstitial compartment (27) and has additional functions as the primary circulating carrier and probable extravascular carrier for intermediate and long-chain fatty acids that are otherwise insoluble (3, 47). About 60% of albumin in the body is outside of blood vessels (12). In a “dry” organ such as the lung, serum albumin is presumed to

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utilize a minute number of large pores to reach the interstitium without provoking edema (18). However, the underlying issue is the true nature of macromolecular transport (i.e., the large pore system). Studies of the endothelial barrier (8, 9, 19, 25) to define the transport pathway at an ultrastructural level conclusively showed the involvement of vesicles or vesicle-derived structures in the transport of macromolecules such as albumin through continuous endothelia under physiological conditions. Extensive biochemical and functional studies have proven the existence of albumin-binding proteins on the endothelial cell surface (7, 9, 21, 38, 39, 44). On the basis of the accumulated ultrastructural, biochemical, and functional evidence, the transcytosis hypothesis asserts that the nonhydraulic pathway in the intact lung is through the endothelial cell itself, i.e., transcytosis via noncoated plasmalemmal vesicles, or caveolae (45).

**ULTRASTRUCTURAL AND FUNCTIONAL EVIDENCE IN SUPPORT OF TRANSCYTOSIS**

Palade and colleagues (25) have made a powerful case for the involvement of vesicular carriers in macromolecular transport through continuous microvascular endothelia. A series of early studies described noncoated vesicles (first termed plasmalemml vesicles and later caveolae) in endothelial cells that can be labeled by electron-dense tracers (enzymatic reaction products, gold-labeled macromolecules, or more recently, haptened plasma proteins) circulating in the bloodstream or in the perfusate of isolated organs (23, 24). Macromolecular tracers accessed interstitial space only via noncoated vesicular carriers that internalized the tracer at the luminal membrane and deposited it in the interstitium by exocytosis of the contents of vesicles. The intercellular junctions of continuous endothelium were never detectably permeable to tracers of diameter ≥18–20 Å (24, 33). Thus an alternative hypothesis to the traditional pores was proposed suggesting that shuttling of vesicles between luminal and abluminal surfaces of endothelial cells accounts for macromolecular transport.

However, arguments have been made questioning the transport function of plasmalemml vesicles, because of evidence that a direct path of diffusion may exist between apparently free cytoplasmic vesicles and the cell exterior facing the capillary lumen (2). Electron-microscopy studies exploring this idea made use of serial, thin sections through capillary endothelial cells of the frog. It was proposed that cytoplasmic vesicles in endothelial cells are actually immobile elements of branching, permanent, or semipermanent invaginations of the plasmalemma.

Wagner and Chen (50) combined electron microscopy with use of electron-opaque tracers to rigorously test the hypothesis that plasmalemml vesicles have a transport function. These investigators prepared serial, ultrathin sections through the rete mirabile of the eel, enabling them to reconstruct the movement of the vascular tracer terbium (Tb3+) through the wall of nonfenestrated capillaries. Tb3+ left an electron-opaque trail on being released into the interstitial space between the endothelial cell layer and the overlying pericytes. Reconstruction of the samples in three dimensions revealed that the path of tracer deposits between the vessel wall interstitium and abluminal endothelial membrane did not lead to interendothelial clefts but rather to abluminal endothelial caveolae and clusters of fused vesicles. These observations showed that the structures involved in the transfer of the tracer were the endothelial vesicular carriers. Moreover, the finding that interendothelial clefts were impermeable to the tracer ruled out the possibility that tracer retrogradely filled abluminal caveolae after accessing the interstitium by the paracellular pathway. The results provide a strong demonstration that transcellular transport in endothelial vesicles is the only route by which electron-dense tracers cross the endothelial cells of continuous capillaries.

Milici et al. (19) addressed an important technical concern about the analysis of macromolecular transport through continuous endothelia, namely, whether electron-dense macromolecule tracers use a different transport process from their native counterparts. They employed immunocytochemical procedures to localize native albumin in transit through the continuous endothelium of perfused microvessels from murine myocardium. Native albumin was supplied in the perfusing liquid after an initial washout of the vascular bed without albumin. The results showed that albumin 1 binds (with low affinity) to the luminal surface of the capillary endothelium, 2 is restricted in transit through the endothelium to plasmalemml vesicles, and 3 appears in the pericapillary space <15 s after the beginning of its perfusion. Albumin was never found within interendothelial junctions and was not associated with the abluminal exits of interendothelial spaces. Therefore, native albumin utilizes the vesicular transport pathway to cross continuous mammalian endothelia.

Figures 1 and 2 show electron micrographs demonstrating, respectively, the distribution of gold-labeled (A-Au) and haptened albumin [albumin derivatized with 2,4-dinitrobenzenesulfonic acid (A-DNP)] in pulmonary capillaries or venules 5–10 min after being injected into vascular space. Regardless
of the tracer type used, albumin is seen to associate with 1) endothelial caveolae open to the vessel lumen, 2) caveolae apparently free inside of endothelial cells, and 3) caveolae open to the abluminal side of the endothelial barrier during various stages of albumin transcytosis. Endocytic and exocytic omega-figures that contain the tracer are evident at luminal and abluminal endothelial cell surfaces, respectively, and transitional free caveolae capable of moving in the luminal-to-abluminal direction, are observed. Albumin did not use a paracellular route in exiting from the nonfenestrated capillary, and the transcellular route should account for most of tracer appearing in the interstitial space.

The contribution of transcytosis to protein transport is difficult to evaluate in a quantitative fashion solely on the basis of electron micrographs that sample a minute percentage of exchange microvessels in a vascular bed. Dinitrophenylated albumin (A-DNP) is an acceptable substitute for native albumin (8) yet is advantageous as a tracer, because A-DNP 1) can be visualized by electron microscopy using an electron-dense reporter antibody and 2) is biochemically detectable using the combination of a primary antibody plus a secondary antibody coupled to horseradish peroxidase, thereby allowing correlation of ultrastructural and transport studies under identical conditions. Predescu et al. (28) showed that A-DNP in transit through the endothelium of perfused coronary microvessels (murine) was present on the luminal cell surface, in plasmalemma vesicles, or in interstitial spaces following exocytosis of the tracer. A-DNP did not accumulate in or pass through interendothelial junctions. N-ethylmaleimide (NEM) rapidly (<5 min) blocked the transendothelial trafficking of albumin (28). To quantitate the effect of NEM on vesicular transport, an ELISA was used to detect A-DNP in supernatants of perfused whole heart homogenate. NEM blocked the transport of A-DNP through murine coronary microvessels by 80%. The decrease of vesicular transport in the presence of NEM is likely the result of the known action of NEM to inhibit the fusion of vesicular carriers to their target membranes by alkylation of NEM-sensitive factor (36, 53). Similar results were obtained with other dinitrophenylated plasma proteins such as α1-acidic glycoprotein (orosomucoid), α-lactalbumin, and myoglobin (31, 33). NEM did not significantly affect the transport of tracers assumed to follow the paracellular pathway (i.e., [3H]sucrose or [14C]inulin). These findings show that transcytosis can account for transcapillary protein transport in nonfenestrated endothelia.

It is important to note that the transcytotic pathway mediates the extravasation of intact serum proteins. An illustrative study by Baldus et al. (1) demonstrated that the enzyme myeloperoxidase (MPO, ~150 kDa) uses the transcytotic pathway to reach the subendothelial matrix of isolated thoracic aorta. MPO in subendothelial space was shown to be an intact molecule with fully functional catalytic activity. This results in formation of the potent nitrating species NO3 from nitrate, which in turn causes tyrosine nitration of target proteins (chiefly fibronectin) in the subendothelial matrix.

The availability of transgenic mice affords new opportunities to directly test the transcytosis hypothesis. Drab et al. (4) provided an excellent first description of the phenotype of caveolin-1-deficient mice. These mutants lacking caveolae have a distribution of albumin in vascular space and cerebrospinal fluid that is identical to the wild type, but so far no explanation has been provided. This observation has been cited as evidence against the transcytosis hypothesis. However, steady-state distribution of a solute does not assess permeability, which fundamentally depends on the rate of transport and indicates nothing about its mechanism(s). Moreover, Lisanti and colleagues (34, 42), in recent comparative studies of wild-type and caveolin-1-null mice, have presented evidence that endothelial caveolae are required for vesicular uptake and transport of albumin tracers in pulmonary microvessels in vivo and in isolated aortic ring segments. In caveolin-1 deficiency, electron microscopy demonstrates that perfused A-Au tracer remained bound to the luminal endothelial cell surface of coronary capillaries (see Figs. 3 and 4) did not reach the perivascular space. Even though the interendothelial space (IES) appears patent in this image, the tracer did not penetrate the interendothelial junctional region because of its large (20 nm) molecular size. In contrast to the characteristic wild-type phenotype (see Figs. 1 and 2), internalization of the tracer in the knockout was extremely rare and caveolae were absent (Fig. 3). Thus both ultrastructural and functional evidence indicates critical involvement of endothelial caveolae in macromolecular transport.

Our analysis of lung and heart vascular beds of caveolin-1-deficient mice perfused with A-Au also showed, in capillaries and venules (but not arterioles), a marked increase in the incidence of interendothelial gaps (Fig. 3, inset). Continuous capillary endothelia are normally devoid of such gaps. In contrast, venule IESs are present in wild-type mice but normally exclude 20-nm tracer molecules. It is thus likely that
caveolin-1-null mice were compensating for their deficiency in caveolae by inducing a paracellular permeability pathway in exchange vessels. Such compensation provides a potential explanation for the minimal impact of caveolin-1 deficiency on transvascular protein distribution (4). Evidence indicates that constitutive activation of endothelial nitric oxide synthase activity in the caveolin-1-deficient mouse contributes to increased paracellular permeability of vessel walls (43).

MONOLAYER STUDIES IN SUPPORT OF THE TRANSCYTOSIS HYPOTHESIS

A series of studies in cultured microvascular endothelial cells has implicated the transcellular pathway in the transport of albumin. These in vitro studies identified four groups of proteins (molecular mass: 16–18, 30–32, 60, and 72 kDa) that specifically bind albumin and are expressed on the endothelial cell surface (7, 20, 21, 39). For gp60, in particular, it was demonstrated that native albumin has a high affinity (38, 40, 48).

John et al. (13) demonstrated that methyl-β-cyclodextrin, a caveola-disrupting agent, inhibits up to 80% of albumin transport through rat pulmonary microvascular endothelial monolayers. This study addressed the agonist property of albumin and whether the albumin transport process is saturated at physiological albumin concentrations. They demonstrated, in monolayers of rat endothelial cells, 1) that vesicular carriers (endothelial caveolae) are involved in the transport process (i.e., the cellular uptake rate of albumin was comparable to overall probe transport), 2) that endothelial albumin uptake depends on albumin concentration, and 3) that transport is not maximally activated at a physiological level of albumin. These findings suggest that the albumin receptor is not saturated under physiological conditions and that the transport process is capable of modulation.

STUDIES IN THE INTACT LUNG PREPARATION IN SUPPORT OF THE TRANSCYTOSIS HYPOTHESIS

Lung preparation for vessel wall permeability measurements and its caveats. The perfused lung is frequently used to study barrier function in an intact vascular bed because ex vivo preparations afford experimental control of perfusate parameters (i.e., temperature, composition, and flow), vascular pressures, and timing of tracer-molecule application and washout. Moreover, gravimetric methods offer the advantage of a quantitative assessment of the presence of edema fluid while avoiding many of the pitfalls of traditional in vivo approaches. Several laboratories have used the isogravimetric lung preparation in studies of microvessel fluid and protein transport via a cellular pathway. Attempts to observe a transcellular transport process in this model using tracer molecules can be complicated by 1) the paracellular convective pathway, which may shunt the cell pathway, and 2) the low tolerance for edema formation as a reflection of modified interendothelial junctions that cause increased fluid and protein leak compared with the in vivo case (10). To control the indicated problems, the isolated lung preparations are usually weighed to verify the stability of lung fluid balance and hypoperfused (<10 ml·min⁻¹·g wet lung⁻¹) to prevent fulminant edema (indicated by continuous lung weight gain). Given such problems, it is important to mention that, with the necessary precautions, the endothelial barrier properties may be quantified as albumin permeability-surface area product (a measure of vessel wall albumin permeability) and the albumin reflection coefficient (σ_ab). The importance of the paracellular pathway in vessel-wall permeability of isolated lung preparations is indicated by measurements of σ_ab that vary between baseline values of ~0.65 (35) and those >0.93 (14, 52) that are within a physiological range (10).

Fig. 3. Absence of vesicular transcytosis of albumin in perfused heart microvessels from caveolin-1-null mouse. The heart in a caveolin-1-null mouse was perfused for 10 min with a solution containing A-Au followed by a washout period (10 min) and the introduction of a fixative (15 min); tissue was processed for electron microscopy. Main electron micrograph: coronary capillary in full cross section (bar, 150 nm); inset: postcapillary venule (bar, 150 nm). Plasmalemmal vesicles are absent in both cases. Note open interendothelial space (IES) in capillary and actual penetration of the tracer (molecular diameter 20 nm) via IES in the venule (inset). Bottom left and top right corners of main micrograph show abnormal expansion of the PVS that appears to contain deposits of a fibrous material.
Evidence for protein transcytosis in intact pulmonary microvessels. The polypeptide antibiotic filipin, a natural product of *Streptomyces filipinensis*, is a useful pharmacological tool for addressing the mode of transvascular albumin transport, because this membrane cholesterol-sequestering agent selectively blocks transport via noncoated vesicles in the rat lung preparation (41). In the study of Schnitzer et al. (41), filipin significantly reduced the transeellular transport of albumin but not insulin, and microvascular permeability to albumin (but not insulin) was significantly decreased (up to 80%) in the ex vivo rat lung preparation after filipin perfusion. Filipin and other drugs (most frequently, methyl-β-cyclodextrin) that are used to reduce the level of plasma membrane cholesterol dismantle endothelial caveolae and hence should selectively modify the cell pathway rather than the paracellular pathway. Filipin treatment did not affect the endocytosis and degradation of activated α2-macroglobulin, a ligand of the clathrin-dependent pathway, implying that this pathway continues to function normally after removal of caveolae; by contrast filipin blocked the transcytosis of insulin, which utilizes noncoated vesicular carriers to reach the pulmonary interstitium (41). These findings are significant as they suggest that filipin has specificity toward noncoated vesicles. In this regard, Simionescu et al. (46) had previously worked out the ultrastructural correlates of filipin binding in mouse capillary beds (including the lung), which were perfused with the cholesterol probe in situ and rapidly fixed for electron microscopy. Short exposure (10 min) to filipin-glutaraldehyde solution was shown to cause caveolae to lose their characteristic flask shape and to flatten out. Prolonged (e.g., 30–60 min) filipin exposure randomly labeled the cell membrane in a far less specific manner. These studies clearly validate the use of filipin as a reagent to perturb noncoated vesicular carriers in isolated lung preparations, provided that exposure time is kept brief.

The study of Vogel et al. (49) showed that filipin inhibited uptake of tracer albumin >50%. Albumin uptake includes all tracer compartmentalized at membrane binding sites, in cytoplasm, and in extravascular space following a 3-min labeling period and a washout period sufficient to reduce tracer counts in venous effluent to an undetectable level. Albumin binding to specific membrane sites in this study was minimized by providing in the perfusate an excess of unlabeled albumin (5 g/100 ml). Filipin, in accord with the work cited above, is expected to block only caveolae-mediated transport of labeled albumin into endothelial cells (endocytosis) or across the cell layer (transcytosis).

However, caution must be exercised in filipin studies, particularly when control experiments show that filipin has a direct effect to increase vascular permeability. Filipin has often been used as a permeabilizing agent (16) and has an ionophorous action on lipid bilayers (15, 37). Thus in intact lung preparations, increased membrane permeability of any contaminant [125]I that is not associated with albumin could conceivably reduce or mask an inhibitory effect of filipin on [125]I-albumin uptake. The Vogel et al. study (49), which attempted to minimize the direct effect of filipin on endothelial [125]I permeability, confirms and extends at a functional level the existence of a filipin-sensitive transport process in the intact lung. According to this study, filipin inhibited both the total albumin uptake and the osmotically sensitive fraction, thus indicating that albumin tracer reaches the interstitial compartment by a transcellular process that is sensitive to filipin. An entirely different experimental approach used by Feng and colleagues (5) is of interest in this context and leads to a similar conclusion in support of transcytosis. Feng et al. (5) studied transport of various solutes between the plasma and the epithelial lining fluid (ELF) of air-filled rat lungs. Solutes that were injected into the bloodstream were recovered in bronchoalveolar lavage fluid following a labeling period. Diffusion of proteins ([131]In-labeled transferrin and human albumin, and Evans blue-labeled rat albumin) plus two labeled chelates ([55]Tc-diethylenetriaminepentaacetate and [51]Cr-(ethylene dinitroil)tetraacetate] into the ELF before lavage, occurred at similar rates, suggesting vesicular transport. These findings imply that the fate of albumin in transit through the endothelial barrier is not to remain trapped in endothelial cells following endocytosis but to exit into pulmonary interstitial space, where it subsequently accesses the distal epithelial cell layer. The conclusions drawn from the described functional studies dovetail with the body of compelling evidence for the transcytosis hypothesis provided by combined analyses of endothelial cell ultrastructure and transport of labeled macromolecules (see above).

**Agonist property of albumin.** Native and liganded albums sometimes have strikingly different transport rates across microvascular endothelia; behavior of this kind is difficult to reconcile with pore theories. Galis et al. (6) compared the rate of transport of albumin-oleic acid complexes and albumin-palmitic acid complexes with that of defatted albumin. The uptake into lung tissue of the ligand-loaded albumin was consistently two to three times higher than that of defatted albumin, and morphometric analysis demonstrated that the binding of the albumin complexes to plasmalemma vesicles was two to three times greater than that of defatted albumin. Analysis of the crystal structure of fatty acid-albumin complexes has demonstrated that the size and shape of native albumin are virtually unchanged even after all five of albumin’s fatty acid-binding pockets are occupied (3). Hence, the functional data (6) would be difficult to explain under pore theory but are compatible with the idea of a vesicular transport process regulated by ligand binding. Along the same lines, conjugation of labeled riboflavin with albumin resulted in a tracer flux twice that of native albumin across the alveolar-capillary barrier of both the in vivo and ex vivo rat lung (51).

Another aspect of transendothelial transport of albumin that is not easily reconciled with traditional pore theories concerns the presence of modified albumins (glycated, nitratated, and nitrosylated species) that account for an appreciable fraction of circulating albumin (22). The modified albumins do not differ from native albumin in terms of molecular dimensions and net charge, yet modified albumins studied thus far have a transport rate two to three times higher than that of native albumin (30, 32). These studies provide circumstantial evidence that certain albumin-ligand complexes may have higher affinity or efficacy of binding than native albumin at critical endothelial cell-surface sites.

**CONCLUSIONS**

A growing body of evidence indicates a high contribution of noncoated vesicles (caveolae) to protein transport across continuous endothelia. Methodologies combining complementary biochemical, morphological, immunocytochemical, and func-
tional analyses hold promise for characterizing the transcellular pathway in microvascular endothelia. An important unanswered question is the identity of the caveola-associated molecules and signaling pathways by which cell-surface albumin-binding proteins such as gp60 regulate the movement of vesicular carriers involved in transcytosis.

REFERENCES


