Molecular determinants of endothelial transcytosis and their role in endothelial permeability

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Predescu SA, Predescu DN, Malik AB. Molecular determinants of endothelial transcytosis and their role in endothelial permeability. Am J Physiol Lung Cell Mol Physiol 293: L823–L842, 2007. First published July 20, 2007; doi:10.1152/ajplung.00436.2006.—Caveolae transcytosis with its diverse mechanisms—fluid phase, adsorptive, and receptor-mediated—plays an important role in the continuous exchange of molecules across the endothelium. We will discuss key features of endothelial transcytosis and caveolae that have been studied recently and have increased our understanding of caveolae function in transcytosis at the molecular level. During transcytosis, caveolae “pinch off” from the plasma membrane to form discrete vesicular carriers that shuttle to the opposite front of endothelial cells, fuse with the plasma membrane, and discharge their cargo into the perivascular space. Endothelial transcytosis exhibits distinct properties, the most important being rapid and efficient coupling of endocytosis to exocytosis on opposite plasma membrane. Endothelial transcytosis is a constitutive process of vesicular transport. Recent studies show that transcytosis can be upregulated in response to pathological stimuli. Transcytosis via caveolae is an important route for the regulation of endothelial barrier function and may participate in different vascular diseases.

caveolae; caveolin-1; endothelium; membrane fusion-fission

1 Caveolae comprise the caveolin-1 positive vesicles, whereas plasmalemmal vesicles refer to the entire nonclathrin vesicle population of endothelial cells, i.e., membrane-bound and apparently free in the cytoplasm. Because the true caveolae (plasmalemmal vesicles caveolin-1 positive) represent >95% of the whole vesicular population of the ECs, we will use the term caveolae as being synonymous with plasmalemmal vesicles.

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deficiency caused endothelial nitric oxide synthase (eNOS) upregulation and abnormal NO levels, leading to opening of the paracellular pathway (154, 167). Whereas the increase in the paracellular permeability is not a direct consequence of caveolae deficiency, it functionally compensates for deficient caveolae-mediated transcytosis (167). Despite severe EC abnormalities, life is possible without caveolin-1 and caveolae (21, 37, 167, 253). Although our understanding of transcytosis is as yet incomplete, recent studies have provided a molecular foundation for a more thorough future description. We will highlight the recent understanding of caveolae and transcytosis in ECs and their role in endothelial permeability with the hope of raising important questions for future studies.

ENDOTHELIAL CAVEOLAE: SPECIALIZED VESICULAR CARRIERS WITH DISTINCT STRUCTURAL, BIOCHEMICAL, AND FUNCTIONAL PROPERTIES

In the last few years, caveolae have been the center of numerous excellent reviews (3, 51, 127, 223). Here, we specifically focus and evaluate the morphological, biochemical, and functional evidence that make endothelial PVs a specialized class of vesicular carriers that actively mediate transcytosis.

Morphological features. PVs are the most characteristic organelle of the ECs defined by a unique morphology. They exist as either omega-like structures open to the extracellular environment, with their membrane in direct continuity, layer by layer, with the plasma membrane, or as detached spherical vesicles free in the cytosol, EM studies have also revealed caveolae in the endothelium of vascular segments (202). Therefore, the greater permeability of capillary and venular endothelia is consistent with markedly greater number of vesicles present in these vessels (213). Of note, among other structural features with a significant role in the control of blood-tissue exchanges are the intercellular junctions of the endothelium. Each microvascular segment and each capillary bed has its own characteristic junctional organization that reflects the strength of cell-cell contacts in a vascular endothelium (35, 188, 204, 205). A less elaborate organization of the intercellular junctions in the venous part was correlated to the low blood pressure and high permeability of the venular microvessels (205). By contrast, the most elaborate network of complex tight junctions from brain capillaries and the lowest frequency of transcytotic vesicles in the endothelium of cerebral vessels are in agreement with the high restrictiveness of the blood-brain barrier (14, 15, 85, 171, 226). Moreover, the strong tight junction-cytoskeleton interaction, the cross talk between components of the tight junction and the cadherin-catenin system, and the regulation of tight junction protein expression and distribution, may contribute to the specific characteristics of the blood-brain barrier (85, 178). Excellent reviews of work in this field have been published (85, 204, 205).

Morphometric analysis of vesicles distribution within the EC revealed that the majority of the total vesicle population (≈65%) is open to the extracellular environment, with more caveolar profiles open toward the abluminal front whereas the remaining 35% were found as discrete vesicular carriers within the cytoplasm (17, 118, 147, 202). Electron microscopy (EM) has shown that endothelial vesicles display a distinct surface structure consisting of alternating ridges and furrows giving a characteristic “striped” appearance to the cytosolic aspect of their membrane (76, 144, 181). These structural characteristics are present not only on the cytosolic aspect of these vesicles, but also on the plasma membrane microdomains with different degrees of curvature (181). These morphological features distinguish these caveolin-1 positive plasma membrane microdomains from the plasmalemma proper, coated pits, and coated vesicles and from noncaveolin PVs (144). The main constituent of caveolae is the 21- to 22-kDa protein caveolin-1 (181).

The loss of morphologically identifiable caveolae in caveolin-1 null mice demonstrates that the majority of the PVs in the endothelium are caveolae (37, 167, 253). The caveolin-1 coat renders caveolae unique and rules out random invaginations as being responsible for caveolae formation. Recent studies to establish in more detail the mechanisms of caveolae coat assembly have led to conflicting results. Using a combination of EM and circular dichroism complemented by analytical ultracentrifugation, Fernandez et al. (43) found that the caveolar coat comprises 50-nm long × 11-nm diameter subunits with each subunit consisting of a caveolin-1 oligomer that contains seven caveolin-1 molecules. Furthermore, the number of caveolin-1 molecules required to form the caveolar coat was estimated by Pelkmans and Zerial (143) for de novo generated caveolae in caveolin-1−/− fibroblasts transfected with caveolin-1-green fluorescent protein (GFP). Their computation analysis based on total internal reflection fluorescence microscopy (TIR-FM) indicated that the 50-nm diameter-assembled caveolae consisted of 144 molecules of caveolin-1 incorporated into 1 caveolar coat. On this basis, it can be presumed that 20 caveolin-1 heptameric subunits are sufficient to generate the caveolar coat.

Besides the numerous omega-shaped PVs open to the cell surface, either directly or through a neck, and vesicles apparently free in the cytosol, EM studies have also revealed caveolae in the different stages of their interaction with the plasma membrane during transcytosis (Fig. 1). The occurrence of these caveolar morphological intermediates in the fusion and formation of caveolae with the plasma membrane during the transendothelial transport is low (129, 160). This observation suggests that caveolae establish short-lived connections with the plasma membrane on both fronts of the ECs while maintaining their structure and shape. Recently, Pelkmans and Zerial (143) have shown that in HeLa cells in which caveolae do not function in a transcytotic process, caveolae shuttled between endocytic and exocytic compartments in a Rab5-dependent manner. Caveolae loaded and unloaded their cargo through membrane fission and fusion processes. Fluorescence recovery after photobleaching (FRAP) analysis indicated that in HeLa cells, caveolin-1-GFP domains are immobile in the membrane, suggesting that during their intracellular traffic, caveolae preserve, similar to endothelial caveolae (207), their morphology due to the stabilizing scaffold of caveolin-1. This feature distinguishes caveolae from other major vesicular carriers such as clathrin- or coat protein complex-coated vesicles that transiently lose their coat during membrane fusion-fission events (140). The caveolar coat comprises in addition to
caveolin-1, membrane proteins that cannot be removed by high salt or high pH (181), and thus it can be permanently attached to the caveolae and remains there during caveolae internalization steps (4). The presence of caveolae with elongated necks or caveolae with very narrow and constricted necks (Fig. 1A) suggests steps in the formation of membrane invaginations followed by release and formation of discrete vesicular carriers. Caveolae in proximity of the plasma membrane still attached by short staining-dense strands suggest caveolae in the late stage of pinching off from the membrane (129). Recently, it was shown that overexpression of intersectin-1s, a multimodular protein that recruits and scaffolds the endocytic protein machinery (73, 161), caused the appearance of caveolae with pleiomorphic necks frequently surrounded by dynamin collars (Ref. 161; Fig. 1A). Dynamin is the membrane fission GTPase thought to mediate scission of caveolae from the plasma membrane by a GTP-dependent process (120). Furthermore, caveolae were found close to the plasma membrane tethered and docked waiting for fusion of their membrane with plasmalemma (Refs. 129, 160; Fig. 1B). NEM sensitivity of transendothelial transport (149, 159) and the isolation, morphological, and biochemical characterization of the endothelial multimolecular transcytotic complexes (EMTCs) comprising a complete set of fusion and fission proteins are strong evidence supporting the concept of soluble NSF attachment protein receptor (SNARE)-mediated fusion of caveolae with the target membrane (160). The region of close apposition between the two membranes extends over 50 nm, i.e., half of the diameter of a typical caveola (160). The two membranes are <5 nm apart, about half of the length of the coiled bundle of vesicle- and target-SNARE proteins (v- and t-SNAREs) that form during exocytosis (78), suggesting that SNARE pairing has occurred. The existence of these intermediate stages of membrane fission and fusion on both sides of the ECs proves that caveolae are dynamic structures able to bud off, internalize, and discharge their content into the subendothelial space after fusing with plasma membrane.

Caveolae also have the ability to fuse into transendothelial channels either open on both fronts of the cell or isolated in the cytosol. Factors such as the high density of vesicle population, extreme attenuation of ECs, and existence of a large number of vesicles open at any time on the cell surface favor the formation of transendothelial channels (212). Frequently, caveolae fuse with each other to form clusters of interconnected caveolae (129, 161, 212). Caveolae can also fuse with large vacuoles and form vesiculo-vacuolar organelles (39, 40, 87). Interestingly, within transendothelial channels, clusters, or vesiculo-vacuolar organelles, the morphology of individual caveolae is still evident. Studies have also shown that caveolae have a relatively stable morphological structure, although they are dynamic in their ability to traffic across the endothelium. Caveolae structure, dimensions, and overall volume are not affected by anoxia, metabolic inhibitors, hypertension, change in temperature, or labeling with macromolecules (213). The membrane of caveolae open on the luminal side of the endothelium is in continuity, layer by layer, with the cell membrane, and the caveolar

Fig. 1. A and B: caveolae in intermediate stages of their interactions with the plasma membrane. A: highly magnified caveolar profiles in intermediate stages of the fission reaction. Overexpression of intersectin-1s in cultured human lung microvascular endothelial cells (ECs; Ref. 161) resulted in caveolae with narrow necks surrounded by staining-dense dynamin collars (a–d), caveolae attached to the plasma membrane by elongated necks (h and i), staining-dense strands (j and k), or extremely long necks (l–p). These morphological intermediates suggest steps in the release and formation of discrete vesicular carriers. Dynamin collars were also detected surrounding the constricted region between 2 adjacent caveolae (c and f). The micrograph in g shows in a favorable section a near complete dynamin ring encircling a caveolar neck. Bars: a–k and n, 20 nm; l, m, o, and p, 50 nm. B: caveolae in intermediate stages of the fusion reaction and cultured human lung microvascular ECs showing caveolae in close proximity to the plasma membrane but not yet opened to the subendothelial space. Caveolae are tethered and docked waiting for the fusion of their membrane with the basolateral plasmalemma. Note the wide region of close apposition between the caveolar membrane and plasma membrane (a–d) and the proximity and staining-dense strands between the 2 membranes (a1–d1). Bars: d, 50 nm; d1, 30 nm.
cavity is in direct communication and accessible to blood plasma, and thus they are exposed to the high blood pressure (30 mmHg at the level of capillaries to 140 mmHg at the level of large arteries). When caveolae are open on the tissue side, their cavity faces the interstitial pressure, which is much lower (0 to −5 mmHg; Fig. 2). This gradient in the hydrostatic pressure may influence caveolae trafficking. Based on this model, caveolae originating from the luminal side will have the pressure and content of plasma, whereas caveolae originating from the tissue front will have a much lower inside pressure and interstitial fluid content (Fig. 2). Caveolae, due to the permanent and stabilizing nature of their coat (4, 143, 213), resist these differences in hydrostatic pressures, when open luminally or abluminally, without expanding or collapsing during the transendothelial traffic. Caveolae starting their intracellular movement from the vessel lumen will fuse with the opposite plasmalemma and discharge their contents into the subendothelial space, presumably because of the higher pressure inside their cavity. Caveolae moving in the opposite direction when reaching the lumina will have a lower inside pressure favoring the uptake of macromolecules present in plasma. The role of the pressure gradient in caveolae trafficking across the endothelium is supported by studies in frog mesentery capillaries showing that the labeling of caveolae by ferritin is increased when microvascular pressure is raised (108), consistent with the role of transendothelial hydrostatic pressure gradient in regulating caveolar transport of macromolecules (108). Moreover, the ability of hemodynamic forces to modulate caveolae number at the cell surface (13, 175, 176) and the recent demonstration that caveolae and caveolin-1 are required for long- and short-term mechanotransduction in blood vessels (251) suggest that caveolae are sensitive to changes in the hemodynamic environment that directly affects the biochemical status of caveolin-1, the luminally open caveolae, and the process of their filling. The finding is significant, taking into consideration that the lack of coupling between transcytosis via caveolae and hydrostatic pressure was used as argument to discount the importance of transcytosis in the transendothelial transport of macromolecules (180).

Caveolae openings can display a stomatal diaphragm with a central density termed “knob” (201); in select microvascular beds (such as the lung), these diaphragms are found to be associated with ~95% of open caveolae. The stomatal diaphragm is a thin protein barrier anchored in the neck of the caveolae. Similar diaphragms occur at the stomata of caveolae in clusters or vesiculo-vacuolar organelles (213, 222, 225). Using a monoclonal antibody generated against a purified luminal plasma membrane preparation (60), it was shown that the protein, known initially as gp68 and renamed PV-1 (224, 225), is associated with caveolar stomatal diaphragms and is necessary for diaphragm formation (224, 225). The caveolar diaphragm may function as a structural feature to control the access of plasma proteins into the caveolar cavity (130, 225), but their role in regulation endothelial barrier function via transcytosis has not been examined.

Taken together, these morphological data establish that the endothelial vesicular system, comprising discrete caveolae and vesicles, fused clusters, channels of fused caveolae and vesiculo-vacuolar organelles, or the short-lived morphological intermediates described during fission-fusion events, is a system superbly designed for transendothelial transport function.

**Biochemical features.** Studies have documented that caveolar membrane displays a unique chemical profile: paucity of anionic sites (206, 210), high concentration of lectin receptors (203), presence of specific antigens such as gp68 (187), binding sites for albumin (59, 192, 201), and sterol-rich microdomains, defined as rings of cholesterol, formed at the line of fusion and fission between plasma membrane and membrane of caveolae (208). Identification of caveolin-1, major protein constituent of the caveolar membrane (90, 181), development of methods for purification and immunoisolation of endothelial caveolae (121, 193, 224), and proteomic analysis of purified caveolae fractions (103, 221) have provided new insights into the complex makeup of the endothelial caveolar membranes.

Biochemically, caveolae are best known by their association with caveolin-1, the integral membrane protein of the caveolar coat. Of the four caveolin gene products present in mammals, ECs have caveolin-1 with its two isoforms caveolin-1α and caveolin-1β (52, 86, 186) and caveolin-2 (56, 89, 137). Although deeply invaginated caveolae contain both isoforms, caveolin-1β was found to be associated only with caveolae in earlier stages of membrane invagination (52). Caveolin-2 has...
three isoforms (α, β, γ), and it was claimed to regulate caveolin-1-dependent caveola formation through its serine phosphorylation (on Ser23 and Ser36; Refs. 186, 220). However, ECs of caveolin-2 −/− mice display a caveolar population that is not modified morphologically or functionally (169). Caveolin-1 homo-oligomerizes or hetero-oligomerizes with caveolin-2; caveolin-1/caveolin-2 hetero-oligomers are thought to form the ridges of the caveolar coat (184). However, by immunogold labeling EM, only caveolin-1 was shown to localize on the caveolar ridges (181). Studies on the function and subcellular distribution of caveolin-1 revealed that it is not exclusively localized in caveolae. Caveolin-1 is also present, depending on the cell type, in the plasmalemma proper, mitochondria, and elements of the secretory pathway (96). Caveolin-1 can either be an integral membrane or a cytosolic soluble protein (96, 156, 158). As a soluble protein, caveolin-1 forms protein-lipid complexes containing cholesterol (158, 217, 238). Morphological and biochemical studies demonstrated that in ECs, 25% of the entire amount of caveolin-1 is present in the cytosol. Cytosolic caveolin was found in a protein-lipid complex comprising general components of the membrane fusion-fission machinery (158). The finding is also consistent with a role of caveolin in cholesterol trafficking (46). Although the cytosolic caveolin-1 has been detected in many cell types, its role in regulating endothelial permeability and the exact mechanism of shuttling between the two compartments remain to be established. The presence of soluble caveolin-1 suggests that the protein also functions outside of caveolae. However, these functions of caveolin-1 are less explored, and the caveolin-1 null mouse does not allow distinguishing between the functions of caveolin-1 within or outside of caveolae.

Caveolin-1 expression in cells deficient in both caveolae and caveolin-1 protein induces caveola formation (47). By contrast, caveolin-1 downregulation by either anti-sense (54) or small interfering RNA (siRNA; Refs. 62, 107) induced a dramatic decrease in the number of caveolae. Deletion of caveolin-1 protein expression in caveolin-1 null mice induced the loss in caveolae in ECs, adipocytes, and fibroblasts (37, 167, 168, 253), establishing the key role of caveolin-1 in caveola formation. Apparently, caveolin-1 number is dependent on caveolin-1 expression. In cultured cells, overexpression of caveolin-1 correlated with significant increase in caveola number (185). However, a direct correlation between the amount of caveolin-1 and caveola number cannot be established (241). This result is consistent with the existence in different cell lines of cytosolic pools of caveolin-1, which are not engaged in caveola formation (158, 217, 238). Likewise, in several pathological conditions, such as diabetes, the up-regulation of caveolin-1 was accompanied by hypertrophy of the caveolar compartment (139). The complexity of in vivo situation on one hand and the complexity of the pathological condition on the other hand make the correlation between the caveolin-1 expression and caveola number a controversial issue. Interestingly, in a transgenic mouse model, overexpression of caveolin-1 did not result in increased number of caveolae (7). The apparent discrepancy could be also explained by the fact that Bauer et al. (7) did quantify only the caveolae in direct relationship with plasmalemma, whereas the free caveolae, possibly the vesicular population accounting for the increase, was disregarded. On the other hand, the lack of correlation between the amount of caveolin-1 and caveola number in cultured non-ECs with the ECs from transgenic mice may suggest that in vivo there is a maximal number of caveolae or plasma membrane that cannot be increased under physiological conditions, despite increased caveolin-1 protein amount (7). Interestingly, caveolin-1 −/− mice displayed significant downregulation of caveolin-2 protein expression without any detectable change at the transcriptional level (169). However, caveolin-2 null mice showed proper localization of caveolin-1 and no caveola disruption (169), suggesting that caveolin-2 expression is not connected to the loss of caveolae in caveolin-1 −/− mice. Extensive morphological examination showed that the caveolin-1 null mice had in their endothelia very few caveolin-1-free membrane invaginations and vesicles (Fig. 3, A and B), especially in large vessels and in vascular beds from heart, lung, and diaphragm (37, 253).

Extensive studies to identify the interacting partners of caveolin-1 in different cells demonstrated that caveolin-1 interacts directly with numerous proteins (for a detailed description, see Refs. 3, 51, 170). Caveola fractions obtained by in situ silica coating of rat luminal endothelial plasma membranes, followed by physical detachment (using sonication) or GTP-dependent release, and relative purification by sucrose density centrifugation, or most recently by magnetic immunosolization, has led to a more detailed understanding of the molecular composition of this organelle (121, 193, 194, 224). Biochemical analysis of purified caveola fractions indicated enrichment in the signaling molecules Cr2−-ATPase and inositol 1,4,5-triphosphate receptor (193), flotillin-2, ATP synthase β-subunit, and annexin V (23), and some endothelial-specific markers such as gp62 (224). Caveolae purified from rat lung contain key proteins known to mediate different aspects of vesicle formation, docking, and fusion, including dynamin, the v-SNARE, cellubrevin, annexin II and VI, monomeric and trimeric GTPases, soluble NSF attachment protein-23 (SNAP-23), and NSF (190, 191). Importantly, in ECs, caveolin-1 is part of a supramolecular protein-lipid complex comprising general components of the fusion-fission machinery (NSF, α-, and γ-SNAP), targeting proteins, the t-SNAREs syntaxin-4 and SNAP-23, and the v-SNARE, cellubrevin, as well as regulatory components Rab5, the membrane fission GTPase, dynamin, and lipids such as cholesterol. EMTCs were found in both cytosol and membrane fractions (158).

Caveolae have a unique lipid composition that confers them Triton X-100 insolubility at low temperatures and low buoyant density (3, 194). Caveolae are enriched in free cholesterol, sphingomyelin, and, to lesser degree, ganglioside GMI, ceramide, diacylglycerol, and phosphatidylinositol diphosphate (16, 98, 136, 146, 216). Caveolae are also enriched in palmitoleic and stearic acids (53). The specific behavior of the lipids defines the uniqueness of the caveolar membrane. Only free cholesterol present in caveolae was found to be accessible to filipin (208) and reactive to cholesterol oxidase (46, 219). Caveolin-1 itself is a cholesterol-binding protein, with at least 1 mol of cholesterol associated per mole of caveolin-1 in the presence of 0.2% SDS (113). Binding of caveolin-1 to cholesterol is critical for the maintenance of the caveolar structure (181). The lipid composition of caveolae is similar to free cholesterol- and sphingolipid-rich microdomains (termed “rafts”) expressed in the plasma membrane of all mammalian cells (50, 148, 239). On this basis, caveolae can be considered
the mechanisms responsible for preferential protein localization in caveolae (125). Thus caveolae serve as structural platforms having specific protein-protein interactions that enable them to perform specific functions in different cell types.

Transcytosis in endothelium is usually a constitutive process of vesicular transport, implying that plasma solutes and macromolecules are taken up by a fluid phase or adsorptive process (201). Some plasma molecules, like low-density lipoprotein (LDL), β-very low-density lipoproteins (β-VLDL), and high-density lipoproteins (HDL), insulin, ceruloplasmin, albumin, and transferrin could be transported across the endothelium from specific vascular beds through a specific transcytotic process, namely receptor-mediated transcytosis (82, 201, 227, 240). The uptake of these molecules is mediated by specific receptors located mainly in caveolae and could follow not only the transcytotic pathway, but also endocytic pathways, serving in this way the needs of ECs (240). In cultured retinal vascular ECs, it has been shown that intracellular traffic of colloidal gold conjugates of insulin and LDL follow more than one intracellular pathway. About 40% of the gold particles were found at 1 h postincubation in the lysosomal compartment, whereas 20% were found in the subcellular space, suggesting that ECs are endowed with the molecular machinery needed for a selective sorting process along with the mechanisms necessary for transcytosis (201, 227). Insulin receptors are present in ECs (12, 177, 201), and they localize in caveolae (9, 177, 243). Transcytosis of insulin by cultured ECs is thought to be mainly receptor-mediated (9, 82, 227). ECs transport most of the insulin from the blood to the tissue in a way that prevents hormone degradation so that the insulin can interact with its receptor on subendothelial target cells. Presence of insulin receptor in caveolae and its direct interaction with caveolin-1 (119, 250) raise the possibility of caveolae involvement in insulin signaling events and thus in the pathogenesis of diabetes. Moreover, studies showed a close correlation among the high levels of cholesterol carrying lipoproteins and the incidence of atherosclerosis (48, 49). Since caveolin-1 and caveolae function in transcytosis of LDL cholesterol particles from the blood to the subendothelial space, they may play an important role in the development of atherosclerosis (49, 199, 215). Consistent with the cell type-dependent caveolae function, recent studies conducted in caveolin-1 null mice indicated that in ECs, caveolin-1 and caveolae may play a proatherogenic role, whereas in smooth muscle cells they may have an antiatherogenic effect (49).

Functional characteristics. Support for the role of caveolae in endothelial transcytosis was obtained as early as the 1960s using colloidal gold particles injected into the blood circulation of rats (128). Subsequent experiments using tracers with appropriate dimensions for the small and large “pore” systems (Table 1) were performed to delineate the location of the postulated “pores” (132, 173). The long series of tracer experiments established that caveolae account for the postulated efflux through large pores and, at least in part, for the function attributed to the postulated small pores in the pore theory of capillary permeability (80, 150, 159, 209, 212). A significant development in studying caveolae as transcytotic carriers was brought about by the introduction of dinitrophenylated proteins as tracers (Refs. 149, 152, 159; Table 1). Dinitrophenylated proteins with molecular diameters between 2–40 nm were found exclusively in caveolae while in transit across the EC (Fig. 4, A–D). No tracer particles were found leaving the

Fig. 3. A–C: caveolin-1-deficient vesicular structures and open intercellular junctions in caveolin-1 knockout mice. Segments of a heart capillary from caveolin-1 −/− mouse (B) and of a muscular venule (C) from caveolin-1 null mouse illustrate the presence of caveolin-1-independent vesicular profiles (v1–v4) and open intercellular junctions (j2 and j3). A shows a capillary from caveolin-1 wild-type (cav-1 wt) mouse with numerous caveolae and a normal intercellular junction (j1). Note in B a vesiculo-vacuolar-like organelle (VVO) and the presence in C of caveolin-3-dependent caveolae (*). pcs, perivascular space. Bars: A and B, 100 nm; C, 200 nm.

as the first specialized and stable lipid raft, described long before the term raft began to be used. The distinctive lipid composition of caveolae and presence of caveolin-1 on the inner leaflet of the caveolar membrane favors the localization of specific proteins in this specialized microdomain. Protein-protein interactions via the scaffolding domain of caveolin-1, N-linked myristoylation of proteins, palmitoylation of cysteine residues, and structural components of the transmembrane spanning region of proteins (hydrophobic regions) are some of the mechanisms responsible for preferential protein localization in caveolae (125). Thus caveolae serve as structural platforms having specific protein-protein interactions that enable them to perform specific functions in different cell types.

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lumina via interendothelial junctions. All probes with a diameter >2 nm generated "filtration residues" in the luminal introit of the intercellular junctions of arterioles and arteriolar end of the capillaries but did not penetrate the junctions (Fig. 4, E and F; Refs. 150, 159). Furthermore, thin serial sectioning performed in specimens perfused with electron-opaque tracers demonstrated that the tracers do not move via interendothelial junctions but via caveolae (242). These findings are consistent with the concept of endothelial junctions being impermeable to tracers >2 nm.

Perfusion experiments using tracers with molecular dimensions in the range of the diameter of the caveolar necks followed by morphological analysis of perfused specimens indicated that the access of tracer particles into the caveolar cavity is controlled by the size of the caveolar opening (152). Gold-conjugated orosomucoid particles (22 nm) plugged the necks of luminally or abluminally open caveolae (Fig. 5A), suggesting that the process of opening and closing of caveolae is a dynamic event. The time ratio between fully open caveolae and caveolae with very narrow or closed openings may represent a kinetic factor that controls the movement of molecules in and out of the caveolar cavity (127, 152, 159). The existence of plugged caveolae in ECs (Fig. 5) and the opening-closing cycle are graphically described in Fig. 2. A caveolar opening between 10–50 nm suggests that these caveolae can function as the large pore equivalents postulated in the literature, whereas caveolae openings below 10 nm represent the structural equivalents of the postulated small pore system (152). As evident from these data, the majority of open caveolae accept more easily small molecules than the larger ones. Thus the time ratio between fully open to restricted opening of caveolae (Fig. 2) is a distinctive caveolar feature that controls the access of different-sized molecules from the vascular lumen or interstitial space into the caveolar cavity as well as from the caveolar cavity into perivascular space (127).

In the last few years, the involvement of EC caveolae in transcytosis of macromolecules from the blood to the tissue has been addressed using the caveolin-1-deficient mice (195, 196). Studies of caveolin-1-deficient mice indicated a profound dysfunction of the vascular system (aberrations in the endothelium-dependent relaxation, contractility, maintenance of the myogenic tone, and, as a consequence, reduced physical ability), significant cardiopulmonary pathology including cardiomyopathy, pulmonary hypertension, thickening of the alveolar septa by uncontrolled EC proliferation, and lung interstitial fibrosis (37). Despite lack of caveolae, the albumin concentration in the cerebrospinal fluid and the extravascular oncotic pressure were normal. This finding suggested that an alternative pathway might compensate for albumin transport (37). Studies examining in detail albumin transport across the vasculature of mice

Table 1. Tracers used in studies of capillary permeability

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Molecular Mass, Da</th>
<th>Molecular Radius, nm</th>
<th>Ref. No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>15,000–300,000</td>
<td>7–10</td>
<td>211</td>
</tr>
<tr>
<td>Colloidal gold</td>
<td>440,000</td>
<td>5.5</td>
<td>18, 27, 42</td>
</tr>
<tr>
<td>Hemeoctapeptide</td>
<td>1,550</td>
<td>1.0</td>
<td>209</td>
</tr>
<tr>
<td>Hemenonapeptide</td>
<td>1,630</td>
<td>1.0</td>
<td>209</td>
</tr>
<tr>
<td>Hemeundecapeptide</td>
<td>1,880</td>
<td>1.0</td>
<td>209</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>12,800</td>
<td>1.5</td>
<td>80</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>1,700</td>
<td>1.7</td>
<td>212</td>
</tr>
<tr>
<td>Albumin-gold</td>
<td>8,000</td>
<td>7.5</td>
<td>55, 59, 105</td>
</tr>
<tr>
<td>Dinitrophenylated α-lactalbumin</td>
<td>14,000</td>
<td>1.5</td>
<td>159</td>
</tr>
<tr>
<td>Dinitrophenylated myoglobin</td>
<td>17,800</td>
<td>1.7</td>
<td>159</td>
</tr>
<tr>
<td>Myoglobin fragments</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>My6kDa-DNP</td>
<td>6,000</td>
<td>1.0</td>
<td>159</td>
</tr>
<tr>
<td>My2kDa-DNP</td>
<td>2,000</td>
<td>&lt;1.0</td>
<td>121, 159</td>
</tr>
<tr>
<td>Dinitrophenylated albumin</td>
<td>−68,000</td>
<td>6–7</td>
<td>58, 149</td>
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<tr>
<td>Dinitrophenylated orosomucoid</td>
<td>−46,000</td>
<td>&lt;2.5</td>
<td>152</td>
</tr>
<tr>
<td>Orosomucoid gold</td>
<td>6–7</td>
<td>152</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 4. A–D: transcytosis of α-lactalbumin-dinitrophenol (DNP) (A), myoglobin6kDa-DNP (B), myoglobin2kDa-DNP (C), and albumin-DNP (D) in the continuous endothelium of murine heart microvasculature. Myoglobin6kDa and myoglobin2kDa are 2 myoglobin fragments generated by cyanogen bromide cleavage of myoglobin as in Ref. 159. At 5 min postperfusion, the tracer molecules were detected in capillary lumen (1), luminal plasmalemma and plasmalemmal vesicles (PVs) open to the lumen (2), PVs within the endothelium (3), PVs discharging in the pericapillary space (4), and the pericapillary space (5). Endothelial junctions (EJs) unlabeled by tracer molecules are seen at j1 and j2 in A and B. B, inset, shows some PVs labeled by myoglobin6kDa-DNP molecules. Gold label is distributed over a radius of ~300 Å (the combined size of primary antibody and reporter antibody; Ref. 159). Filtration residues were generated by albumin-DNP (E) and myoglobin6kDa-DNP (F) in the luminal introit of the EJs after 5 min of perfusion. Bars: A and B, 200 nm; B, inset, 60 nm; C, 80 nm; E and F, 100 nm.
Fig. 5. Dynamic changes in dimensions of caveolae introits control the access of macromolecules into the caveolar cavity. A: mouse heart specimens perfused for 5 min with 22 nm of gold-orosomucoid show tracer particles associated with the plasmalemma proper (a and b), labeling caveolae open to the luminal (b, c, and d) and abluminal (d, e, and f) sides. Bars: 50 nm. B: in this model, caveolae with openings between 10–50 nm will accept molecules with dimensions within this range as well as molecules <10 nm in diameter. During the phase of the caveolar cycle when caveolae are fully open, they act as structural and functional equivalent for the postulated large pores (132, 173). When the caveolar opening is narrowed (2–10 nm), only molecules within these dimensions gain access into the caveolar cavity. The entry of larger molecules is restricted, suggesting that caveolae function as structural and functional equivalents of the postulated small pores. C: when caveolae are open on the luminal side and thus accessible to blood plasma, the high hydrostatic pressure of the lumen (30 mmHg) may equilibrate with the caveolar content, facilitating the access of tracer particles into the caveolar cavity. As the closing of the caveolar neck progresses, its diameter shrinks, the tracer particles are excluded, and, as a result, they plug the caveolar necks on this front. After release of caveolae from the plasma membrane, the free vesicles move inside the EC to fuse with the opposite plasmalemma and discharge their cargo in the pericapillary space. As the closing of the caveolar neck progresses, its diameter shrinks, the tracer particles are excluded, and, as a result, they plug the caveolar necks on this front. After release of caveolae from the plasma membrane, the free vesicles move inside the EC to fuse with the opposite plasmalemma and discharge their cargo in the pericapillary space. As the closing of the caveolar neck progresses, its diameter shrinks, the tracer particles are excluded, and, as a result, they plug the caveolar necks on this front. After release of caveolae from the plasma membrane, the free vesicles move inside the EC to fuse with the opposite plasmalemma and discharge their cargo in the pericapillary space. As the closing of the caveolar neck progresses, its diameter shrinks, the tracer particles are excluded, and, as a result, they plug the caveolar necks on this front. After release of caveolae from the plasma membrane, the free vesicles move inside the EC to fuse with the opposite plasmalemma and discharge their cargo in the pericapillary space.
1-deficient mice by reference to wild-type animals (196), it is possible that the altered intercellular junctions and the subsequent increase in the paracellular transport may not suffice for an efficient control over vascular permeability. Together, all these abnormalities cause a shorter lifetime for this mouse without caveolin-1 and caveolae (134). Opening of the interendothelial junctions and the resulting hyperpermeability are mediated by the increase in plasma NO content. Lack of caveolin-1 upregulates eNOS activity, causing a dramatic increase in NO and, as a consequence, paracellular hyperpermeability (196). The role of eNOS activation and abnormal NO production in regulating the integrity of the intercellular junction and paracellular permeability is well-documented (19, 20, 151, 153). Consistent with these findings, endothelial-specific overexpression of caveolin-1 reduced eNOS catalytic activity, preventing a large NO production, and thus reduced vascular permeability (7). In addition, delivery of caveolin-1 scaffolding domain in ECs of individual vessels or in a mouse model, facilitated by Antennapedia homeodomain, suffices to reverse the increase in microvessel permeability caused by pharmacological agents as platelet-activating factor or carrageenan (20, 254).

In other experiments in which RNA interference was used to knock down caveolin-1 expression in mouse lung microvascular endothelia, it was shown that 80–90% decrease in caveolin-1 expression reduced the number of caveolae in ECs and increased albumin transport via the paracellular pathway (107). The opening of the interendothelial junctions and the increased paracellular permeability to albumin were dependent on NO production. Whereas caveolin-1-dependent regulation of NO activity and NO production might be an important mechanism for regulation of microvessel permeability, evidence suggests that other factors are involved in this NO-mediated process. Studies on the effects of basal release of NO on microvessel permeability in individually perfused frog microvessels suggested that NO release may also act as an oxidant scavenger to maintain the normal permeability (68).

A recent study questioned the role of abnormal NO production in increased endothelial permeability and totally denied the existence of open interendothelial junctions as alternative pathway for deficient transcytosis in caveolin-1 null mouse (180). The authors reported no signs of morphological abnormality at the level of the interendothelial junctions and marked increase in the macromolecular transport. The authors suggest that an unperturbed two-pore system and an elevated capillary hydraulic pressure may explain the marked increase in the macromolecular transport (180). The grounds of these conflicting findings are not clear.

Caveolae trafficking also includes endocytic pathways. Caveolae endocytosis of membrane components, bacterial toxins, viruses, bacteria, and extracellular ligands was described in different cell types, where caveolin-1 is associated with endocytic compartments such as endosomes and caveosomes (38, 95, 140, 141, 172). In caveolin-1-deficient mouse embryonic fibroblasts, caveolae endocytic pathways are replaced by a caveolin-1- and clathrin-independent uptake, although some evidence suggests that the two pathways may coexist in the wild-type fibroblasts (34, 83).

Based on caveolin-1 interactions with numerous signaling proteins, caveolae may be well-equipped to function as centers for cellular signaling. Besides eNOS regulation (11, 20), studies implicated caveolin-1 and caveolae in channels and calcium signaling (24, 91, 244), G protein-coupled-receptors and G protein signaling (125), receptor internalization (36, 114, 200), cellular proliferation (167), polarized EC migration (8, 75, 133), and, more recently, in mechanosensation (251). Caveolin-1-deficient mice show defects in chronic flow-dependent remodeling and in acute flow-mediated dilation (251). These effects were rescued by reconstitution of caveolin-1 expression in the endothelium, suggesting that caveolae may play an important role as blood flow sensors (251). Mice deficient in caveolin-1 have alterations in signaling that ERK hyperactivation (30), insulin receptor defective expression and instability, as well as insulin signaling defects (29, 31, 123), altered GLUT4 glucose transporter localization (29), abnormal function of the plasma membrane calcium ATPase (21), hyperactivation of the Jak-2/STAT5a signaling cascade (79, 135), and disoriented migration (63, 67).

Caveolin-1 and caveolae have been linked to cholesterol homeostasis and lipid regulation, involvement in cellular sterol transport (45, 46, 74), and association with the surface of the lipid droplets (124) based on the ability of the caveolar membrane to bind cholesterol (113). Adipocyte abnormalities (reduced diameter, smaller lipid droplet size, hyperplasia of the brown adipose tissue, impaired lipolytic activity), the slight decrease in cholesterol synthesis in fibroblasts and macrophages isolated from caveolin-1-deficient mouse (48), and the dramatic reduction in lipid droplet formation during the liver regeneration of the caveolin-1 null mouse are observations consistent with a role of caveolin in regulation of cellular lipid homeostasis (44).

Although the phenotype of the caveolin-1-deficient mouse shows significant abnormalities and metabolic disorders, the molecular mechanisms involved are not yet fully understood. Still alive, but with a shorter lifespan (134), caveolin-1-deficient mouse shows significant cell- and tissue-specific abnormalities (70, 94) consistent with the idea that caveolae are not subcellular organelles with well-defined function; they provide a structural framework to accommodate different proteins performing different functions in different cell types. These findings suggest that in ECs, transcytosis via caveolae is the major transport pathway responsible for the exchange of macromolecules. Moreover, caveolin-1, due to its dual role—structural protein of caveolae and negative regulator of eNOS activity—may be a critical determinant of endothelial permeability at both transcellular and paracellular levels.

Lungs, which are extremely rich in caveolae (66), are significantly affected by caveolin-1 depletion. Lack of caveolae and caveolin-1 from ECs and type I pneumocytes caused hyperproliferation and hypercellularity with subsequent thickening of alveolar septa and decrease in the alveolar space (37, 167, 253). This histopathology was frequently associated with a wide range of lung diseases, suggesting that these mice may have dysfunction of pulmonary gas exchanges and perhaps general impairment of lung function. The thick alveolar septa showed immunoreactivity to fetal liver kinase-1 (Flk-1), a marker for nondifferentiated endothelial and hematopoietic progenitor cells but not to von Willebrand factor that labels progenitor cells but not to von Willebrand factor that labels endothelial markers (151, 167, 253). This histopathology was frequently associated with a wide range of lung diseases, suggesting that these mice may have dysfunction of pulmonary gas exchanges and perhaps general impairment of lung function. The thick alveolar septa showed immunoreactivity to fetal liver kinase-1 (Flk-1), a marker for nondifferentiated endothelial and hematopoietic progenitor cells but not to von Willebrand factor that labels markers (151, 167, 253). Loss of caveolin-1 and caveolae led to fibrosis and marked hypertrophy of type II pneumocytes (37, 167). Interestingly, loss of caveolin-1 caused significant decrease in caveolin-2 levels. Caveolin-2 requires...
coexpression with caveolin-1 for proper trafficking to the cell surface and plasmalemmal caveolae (110). Caveolin-2 deficiency did not affect caveolae formation, but the mice with no caveolin-2 show similar to caveolin-1-deficient animals alveolar septal thickening, EC hyperproliferation, and exercise intolerance. Their vasculature and vasoconstrictive-vasodilatory responses as well as normal lipid regulation were normal, suggesting that the caveolin-1 null mouse lung phenotype is secondary to caveolin-2 deficit (169). Caveolin-2 null mice show intercellular junctions that are not modified morphologically or functionally (169), indicating that as long as caveolin-1 is present and regulates eNOS function and thus the junctional integrity, the paracellular pathway is not affected.

**MOLECULAR DETERMINANTS OF ENDOTHELIAL TRANSCYTOSIS**

Decisive evidence in support of transcytosis was obtained in experiments in which the same tracers were perfused in situ or in vivo in the presence of NEM, a general alkylating reagent (99) that inhibits vesicular transport along exocytic (183), endocytic (246), and transcytotic pathways (228). NEM inactivates the membrane fusion ATPase, NSF, a critical component required for fusion of vesicular carriers with the plasma membrane (182). In ECs, assembly of the transcytotic fusion-fission complex is NSF-dependent and NEM-sensitive, suggesting that the ATPase activity of NSF is required for the assembly of these macromolecular complexes assumed to be involved in membrane fusion events during endothelial transcytosis (158).

Combined morphological and biochemical approaches established that transcytosis by PVs is NSF-dependent and NEM sensitive (149, 152, 159, 190). NEM inhibited by 75–80% the transport of tracers used (dinitrophenylated albumin, α-lactalbumin, myoglobin, small myoglobin fragments, orosomucoid, 125I-albumin, and 125I-gold-albumin) in mouse coronary microvessels and rat lung (149, 152, 159, 190). Caveolae were found heavily labeled with gold particles next to the abluminal plasmalemma but not yet opened to the subendothelial space (149, 159). Immunogold labeling of pericapillary spaces was drastically reduced compared with controls, and there was no evidence of tracer exit along interendothelial junctions. The NSF sensitivity of the transendothelial transport suggests that membrane fusion-fission events are involved in caveolae function during transcytosis. NSF had minimal effect on the transendothelial transport of [14C]sucrose and [14C]inulin tracers consistent with their exit via the paracellular pathway (159, 190).

Other cellular ATPases, such as kinesin and dynein, known to generate the movement of various cellular components along microtubules were also affected by NEM treatment (100, 145). Caveole dynamics are dependent on these two molecular motors (32), and thereby their inhibition by NEM may contribute to inhibition of caveolae transcytosis. The observation is consistent with the concept that caveolae are dynamic vesicular carriers involved in the transport across the endothelium. Several studies have indicated that NEM can significantly depress the contractile response of vascular smooth muscles to agonist challenge, and it may differentially affect the relaxation properties of the vascular smooth muscles after agonist-induced contraction depending on the type of blood vessel investigated (1, 234, 235). It has also been reported that high concentrations of NEM may induce a conformational change in albumin (71). In a culture cell system, NEM had inhibitory effects on cell adherence to substrates of different chemical composition, such as Falcon polystyrene, rubber, glass, polypropylene, Teflon, agar, etc. (64). However, it has been shown that sulfhydryl blocking reagents did not cause detachment of already adhered cells (65).

Physiological measurements of 125I-labeled radiolabeled albumin clearance from blood to muscle in isolated and maximally vasodilated perfused rat hindquarters in the presence of NEM indicated increased microvascular permeability, the result of marked vasoconstriction preferentially in precapillary vessels and toxic cellular damage caused by NEM treatment under the experimental conditions applied (22, 174). Based on these findings, the authors regard NEM, a reagent largely used for studying the molecular mechanisms of membrane fusion in different systems of vesicular transport, as unsuitable for studying endothelial transcytosis (22, 179). The findings must be tempered considering previous reports documenting significant decrease in the contractile response of vascular smooth muscle exposed to NEM before agonist challenge (234, 235). In addition, extensive EM morphological analyses of perfused specimens subjected to NEM treatment argue against a toxic effect of NEM (149, 152, 159, 190). No damage of the endothelium was detected. Endothelial junctions were intact; no tracer particles were found crossing the endothelial monolayer via open endothelial junctions or associated with the abluminal exit of the junction. The extent of transport inhibition by NEM was similar for all tracers used, ruling out an effect of 1 mM NEM on albumin conformation. The only detectable morphological effect of NEM treatment on perfused specimens was the accumulation of vesicles labeled by the tracer particles, unable to discharge their load, and as a consequence a reduced number of gold particles in the pericapillary space. These findings corroborated with the in vitro studies showing the NEM sensitivity and NSF dependence of endothelial transcytotic complexes (158) demonstrate that NSF alkylation by NEM interferes with membrane fusion events during caveolae transcytosis.

Significantly, progress has been made in understanding the molecular machinery regulating caveolae transcytosis in ECs. All biochemical, morphological, and functional approaches used indicated a similarity between the caveolar pathway and other major types of vesicular transport. The components of the general transport machinery (NSF, α-SNAP, γ-SNAP) and targeting components (syntaxin, SNAP-23, cellubrevin) are present in ECs prepared from cultured ECs (117, 149, 158, 191). They are also organized in large supramolecular protein-lipid complexes, described above as EMTCs, present in both cytosol and membrane fractions (Fig. 6A; Ref. 158). Small GTPases like Rab5 known to play regulatory roles in membrane transport are present in EMTCs. More recently, Pelkmans and Zerial (143) reported that in live HELa cells, Rab5 regulates caveolae endocytic traffic involving early endosomes. Immunogold labeling and negative-staining EM complemented by morphometric analysis indicated that endothelial fusion-fission complexes have high content of proteins and lipids and contain, singly or in pairs, the protein components of the fusion-fission machinery (Fig. 6B; Refs. 156, 158). Dynamic is present in these supramolecular complexes (156,
Dynamin has the ability to assemble into helical collars around the necks of endocytic vesicles (6, 33, 230). In ECs, dynamin mediates caveolae internalization through a GTP-dependent fission process requiring its presence at the neck of caveolae as well as its ability to hydrolyze GTP (120). Caveolae are efficiently released from rat endothelial plasma membrane patches in a GTP-dependent manner, and, significantly, the majority of the released vesicles remain associated with EMTCs as shown in Fig. 6C. Immunogold labeling EM showed that the complexes contain caveolin-1 and dynamin (158). The protein-lipid makeup of these complexes and their association with the GTP-dependent released caveolae suggest that they participate in fission, targeting, and fusion of caveolae with the endothelial plasmalemma or with the membrane of other caveolae.

It was shown that intersectin-1s plays an important role in dynamin recruitment to the caveolar necks (161). Intersectin-1s is a multifunctional adaptor protein component of the general endocytic machinery (41, 73, 161, 163). Both dynamin and intersectin were found to associate preferentially with caveolar necks (161). Intersectin-1s binds via three of its five SH3 domains to the proline-rich COOH terminus domain of dynamin (122, 249). The ability of intersectin to bind simultaneously more dynamin molecules likely creates a high local concentration of dynamin required for collar formation around caveolar necks in close proximity of the plasma membrane. A wide range of morphological changes (i.e., caveolae clustering, margination of caveolae at cell periphery unable to move through the cell cortex, caveolae with pleomorphic necks) and impaired membrane fission were observed in response to intersectin-1s overexpression in cultured microvascular ECs (161). These findings showing intermediate stages of caveolae budding and release from the plasma membrane during endocytosis, the first step in transcytosis, are consistent with the concept of caveolae as dynamic structures involved in transcytotic process. Once released from the plasma membrane, caveolae form free transport vesicles that reach the opposite front of the cell where they discharge their contents by fusion with the plasma membrane. Moreover, siRNA-mediated down-regulation of intersectin-1s protein expression in cultured ECs caused a significant decrease in caveolae number, consistent with intersectin-1s requirement for recruiting and organizing caveolae endocytic and transcytotic machinery (157).

Although the involvement of caveolae in the transendothelial transport of macromolecules is now accepted in the physiological literature (104), a clear distinction between caveolae functioning as shuttles between the two fronts of the ECs or as relays between one front of the cell and a chain of neighboring vesicles leading to the formation of transient transendothelial channels as Clough and Michel (28) suggested is still under debate. Recent work favors the targeted intracellular movement of caveolae as discrete transcytotic vesicular carriers (161, 242). Serial sectioning of eel rete mirabile specimens perfused with terbium indicates the presence of the tracer in the pericapillary spaces associated with labeled vesicles and vesicle clusters. No evidence for tracer crossing the capillary wall via the paracellular route was obtained (242). In addition, a biotin assay for caveolae internalization that allows detection of biotin internalized only in sealed vesicles (161) indicated that caveolae do not communicate with the extracellular environment after their release from the plasma membrane and during their intracellular movement. Caveolae were internalized together with biotinylated cell surface proteins. Since, in ECs, the population of clathrin-coated pits and vesicles is relatively small (comprising <3% of the total endothelial vesicles; Ref. 131) and with a minor contribution to the internalization process (201), it is likely that caveolae play the major role in the total uptake of cell surface proteins. The finding argues...
against the idea that the progressive labeling of caveolae by macromolecular tracers can be accounted for by the dispersion of tracer through the intricate invaginations that open onto the luminal surface of the ECs combined with “back filling” of abluminal caveolae by tracer (104). Moreover, the association of caveolae with protein components of actin cytoskeleton (97) and our recent observation that intersectin-2L (ITSN-2L), a protein able to regulate actin polymerization via Wiscott-Aldrich Syndrome protein (WASp)-Cdc42 pathway (72), interferes with caveolae dynamics (84) support the concept of caveolae as free vesicular carriers once released from the membrane.

The last step in transcytosis is exocytosis that involves SNARE-mediated targeting, docking, and fusion of the caveolar membrane with plasmalemma (160). Prefusion morphological intermediates, apparently docked caveolae, have been identified (Fig. 1B). This finding suggests that, as in other cells, SNARE pairing occurs in ECs during exocytosis. Endothelial t-SNARE proteins, syntxin-4, and SNAP-23 form cholesterol-dependent clusters that define the sites of caveolae fusion with the target plasma membrane during endothelial transcytosis (160). The v-SNARE cellubrevin, a component of the endothelial transcytotic complex (158), is present in the caveolar membrane and is required for targeted transcytosis in the vascular endothelium (102). Direct evidence for caveolae fusion with the target plasma membrane has been obtained in a cell-free system involving endothelial plasma membrane sheets and isolated biotin-labeled caveolae (160). Specific detection of biotin-streptavidin complex on the cytoplasmic aspect of the plasma membrane was studied by correlative gold-labeling EM of biotin-streptavidin complex on the cytoplasmic aspect of the plasma membrane (160). The results showed caveolae docking and fusing with the target plasma membrane at the level of SNAP-23 clusters. Thus specialized cholesterol-rich t-SNARE clusters on the basolateral endothelial plasma membrane are the preferential sites for caveolae docking and fusion. Docking and fusion of caveolae occurring at these clusters represent essential events in the control of exocytosis, the last step of endothelial transcytosis. Together, these findings further demonstrate that ECs and their vesicular carriers possess the molecules and the mechanisms required for efficient transendothelial transport.

In cultured cell systems, devoid of constitutive endocytosis and transcytosis processes via caveolae, analysis of caveolae dynamics using TIR-FM and FRAP indicated that caveolae functioned as endocytic vesicular carriers for which internalization is a cargo- (ligand-) activated event (143). In resting cells, 20–30% of caveolae were involved in localized cycles of membrane fusion-fission under the plasma membrane, whereas upon simian virus 40 (SV40) activation or pharmacological inhibition of phosphatases, the fraction of mobile caveolae doubles, and caveolae become engaged in long-range cytoplasmic transport (143). Therefore, in cell systems devoid of constitutive endocytosis/transcytosis, caveolae are able to pinch off and shuttle between membranes while maintaining their domain identity (34, 83, 229, 232, 236).

CLATHRIN- AND CAVEOLIN-1-INDEPENDENT VESICULAR CARRIERS IN ENDOTHELIAL CELLS

Recent studies have provided new insights into the existence of caveolin-1- and clathrin-independent pathways of endocytic trafficking in different cell types (summarized in Table 2). The recent availability of the caveolin-1-deficient mice has revealed the existence in ECs of vesicular membrane structures without clathrin or caveolin-1 coats (Fig. 3; Refs. 37, 167, 253) and their capability to function in transcytosis of gold-albumin transport pathway in the endothelium of caveolin-1 null mice (154, 167–170). Alternative nonclathrin and noncaveolin endocytic pathways have been better described in non-ECs of caveolin-1 knockout mice (34, 83). Using mouse embryonic fibroblasts from these mice, Kirkham et al. (83) demonstrated that trafficking of cholera toxin B involved cholesterol-dependent, dynamin-independent, endocytic vehicles identified as uncoated tubular and ring-shaped structures. However, the same pathway was present in embryonic fibroblasts from wild-type mice in which a certain part of cholera toxin B also entered the cell via caveolin-1 positive vesicles (83). In a similar study, Damm et al. (34) investigated the entry of SV40 in embryonic fibroblasts from caveolin-1–/– mice. They described a virus-activated pathway mediated by small vesicles that delivered the viruses to caveolin-1- and caveolin-2-devoid caveosome-like membrane structures (34). This pathway was tyrosine kinase- and cholesterol-dependent, dynamin II independent, and faster than caveolae-mediated pathway. Again, in

<table>
<thead>
<tr>
<th>Table 2. Caveolae and related types of vesicular carriers</th>
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<tr>
<td>Type of Vesicular Carrier (Ref. no.)</td>
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<tr>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Lipid raft-derived endocytic vesicles (10, 92, 93, 116)</td>
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<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
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<tr>
<td>Virus-activated endocytic vesicles (34)</td>
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<tr>
<td>Ring, tubules, endocytic vesicles (83)</td>
</tr>
</tbody>
</table>

Cav-1, caveolin-1; CT-B, cholera toxin subunit B; AMF, autocrine motility factor; SV40, simian virus 40; MEF, mouse embryonic fibroblast.
wild-type embryonic fibroblasts, this pathway functioned in parallel with caveolin-1-dependent entry, described earlier by the same group (142). Both pathways merged in the intermediate organelles (caveosomes) from where the viruses were transferred by a microtubule-dependent vesicular pathway to the tubular network of the smooth endoplasmic reticulum (34). Similar or related pathways involving the formation of small, noncoated vesicles at the plasma membrane have been described in NIH/3T3 cells (10, 93) and in cell lines devoid of caveolae, Caco-2, lymphocytes, and Jurkat lymphoma cells as well as in Cos-7 cells in which caveolin-1 protein expression was downregulated by siRNA (92, 116). The extent to which these findings from cells devoid of caveolae and efficient transcytosis could be applied to ECs remains to be determined.

OTHER MOLECULES REGULATING CAVEOLAE DYNAMICS

Endocytosis and transcytosis mediated by caveolae require a set of proteins and lipids as well as mechanisms common to clathrin-dependent and clathrin- and caveolin-1-independent endocytic pathways. During caveolar endocytosis, the caveolar ligands are sorted after internalization either to endosomes, lysosomes, and the Golgi complex (138), or to the caveosomes (116, 141). The caveosome is defined as a unique intermediate compartment with neutral pH and distinct from endosomes, lysosomes, and Golgi complex (141). Transcytotic caveolae bypass the lysosomes and discharge their cargo in the subendothelial space (128). The lack of knowledge regarding the molecular specificities of different caveolae pathways and related endocytic traffic makes it difficult at this time to define the specific mechanisms and requirements involved in sorting along different pathways. Frequently the findings obtained for caveolin-1 positive vesicles in a particular cell type are mistakenly generalized to all caveolin-1 positive vesicles in all cell types. Caveolae should be viewed as a subcellular component that can accommodate different proteins and perform specialized functions in different cell types or perhaps just in a particular compartment of the cell. Cumulated evidence from different cultured cell systems and experimental conditions applied indicates that caveolae internalization is dependent on the activation of protein kinase C (PKC; Ref. 197), actin cytoskeletal rearrangements (84, 147), dynamin recruitment (120), intersection-1s (161), syntaxin-6 (25), and glycosphingolipids and cholesterol (198). However, the role of these signaling pathways and the specific proteins involved are far from clear. In some cultured cell lines, the presence of certain ligands (SV40, Echovirus 1, and cholera toxin B) may activate signaling events associated with caveolae internalization (34, 83, 141, 142).

Src kinase. First evidence for the involvement of kinase activity in caveolae internalization came from studies in A431 cells indicating that okadaic acid, a specific inhibitor of phosphatases 1 and 2a, caused a dramatic removal of caveolae from the cell surface (137). Caveolae were found as clusters accumulated in the center of the cell (137). Caveolin-1, a major determinant of caveolar structure and a substrate for the src kinase family, is tyrosine-phosphorylated in response to Rous sarcoma virus transformation (61). Tyrosine phosphorylation of caveolin-1 occurs at tyrosine-14 in normal endothelium and induces decrease in caveolae open to the cell surface as well as accumulation of caveolin-1 positive structures larger than caveolae (5).

Src-mediated tyrosine phosphorylation of dynamin was also shown to be critical for dynamin self-assembly (2). Consistent
with these observations, src kinase activity was found to be required for receptor-mediated uptake and transcytosis of albumin in ECs (106), for internalization of SV40 (142) in fibroblasts from green African monkeys, and for lipid-stimulated caveolar endocytosis in human fibroblasts (198).

In fibroblasts from green African monkeys, a cell line in which caveolae display limited mobility and dynamics (34), it was reported that SV40, a nonenveloped DNA virus with a 50-nm diameter (142), activated tyrosine kinases and triggered a signaling cascade resulting in slow but efficient virus internalization. The pathway involves small, tight-fitting, noncoated vesicles that are caveolin-1-, dynamin II-, tyrosine kinase-, and cholesterol-dependent. SV40 is delivered to caveosomes and endoplasmic reticulum (141).

In human fibroblasts, elevation in cellular cholesterol, presence of lactosylceramide (214), or acute treatment with exogenous or synthetic glycosphingolipids selectively stimulated caveolar endocytosis in a Src- and PKCα-dependent manner (198).

PKC. The function and activity of signal transducers including members of the PKC family are closely regulated by their subcellular localization. PKCα resides in the caveolar membrane and phosphorylates a 90-kDa protein linked to caveolae internalization (218). Caveolin-1 itself also has sites for phosphorylation by PKC (231). PKCα traffics via caveolae to an endosomal compartment where it is inactivated by dephosphorylation and targeted for degradation (162). This is an activation-dependent process that requires PKC catalytic activity. Consistent with this observation, PKCα-deficient epithelial cells display inhibition of caveolae invagination and internalization (218). Recently, PKCα was implicated in albumin uptake via caveolae in human skin fibroblasts (198). These observations are especially important taking into account that PKC family is activated in response to several inflammatory mediators such as bradykinin, platelet-activating factor, and thrombin (252), resulting in significant caveolin-1-mediated endothelial barrier breakdown (245).

Actin cytoskeleton. Little is known about how the actin cytoskeleton controls the dynamics of caveolae. A number of studies provide evidence at light microscopy level suggesting that actin may be involved in caveolae internalization (84, 112, 137, 141, 142). Internalization of SV40 by monkey fibroblasts induced actin dynamics (142). Live cell imaging of SV40 uptake revealed a burst of actin polymerization associated with the formation of small actin patches and actin tails, followed by the movement of caveolae inside the cell (142). Latrunculin A (actin monomer-sequestering drug) and jasplakinolide (an actin polymer-stabilizing drug) reduced >60% virus internalization (142). Although changes in actin cytoskeleton were obviously induced by SV40 entry, the association of actin tails with single virus-containing vesicles needs to be further addressed with a higher level of resolution to establish with certainty the cause-effect relationship. In CHO cells, live cell fluorescence imaging of caveolae dynamics via GFP-tagged caveolin-1 indicated that both actin and microtubules act coordinately to regulate trafficking of the caveolar membrane system (112). In the absence of microtubules, cell surface caveolae increased more than twofold, and they became organized into linear arrays whereas complete depolymerization of the actin cytoskeleton with latrunculin A triggered rapid movements of caveolin-1-GFP positive structures toward the centrosomal region of the cell (112). Recent studies revealed that ITSN-2L could establish a connection between caveolae and actin polymerization pathway (84). Long ITSN isoforms interact with the WASp, a stimulator of actin filament nucleation (72, 84). In cultured ECs, binding of long ITSN-2 to the proline-rich domain of N-WASp stimulated the intrinsic GDP-GTP exchange activity of Cdc42 via the DH-PRH region of ITSN-2L (84). Stimulation of Cdc42 by ITSN-2L accelerated actin assembly via N-WASp and Arp2/3 complex (84). Augmented actin polymerization caused by overexpression of the DH-PRH region of ITSN-2L in cultured ECs interfered significantly with caveolae dynamics (84), suggesting a role of actin rearrangements in different steps of caveolae internalization.

Glycosphingolipids and cholesterol. The effects of cholesterol-depleting agents (181) and cholesterol oxidation (219) on caveolar architecture have suggested the involvement of lipids in caveolae dynamics. Moreover, in ECs, cholesterol, GTPγS, and some other lipids are components of the EMTCs (158). Identification of syntaxin-4 and SNAP-23 cholesterol-dependent clusters as preferential sites for caveolae fusion with the target membrane (160) further supported a role of cholesterol in caveolae function. Studies of caveolae endocytic pathway in human fibroblasts indicated that altering the balance of cholesterol, sphingolipids, and caveolin-1 at the plasma membrane (198) could modulate that caveolae endocytosis. Brief treatment with glycosphingolipids or increasing cellular cholesterol by growing fibroblasts in the presence of an excess of LDL (101, 164, 165) or by incubating the cells with a complex of methyl-β-cyclodextrin and cholesterol (26) stimulated the internalization of BODIPY lactosylceramide and Alexa Fluor 549 albumin via caveolae (198). It was suggested that sphingolipids stimulated the caveolar uptake either by a specific interaction with a membrane protein able to initiate a signaling cascade leading to caveolae internalization or by modifying the lipid organization and protein localization in both leaflets of the plasma membrane, resulting in kinase activation and stimulation of caveolae endocytosis. These observations raised the possibility that the lipid composition rather than specific proteins of caveolae may play a crucial role in maintaining and determining the distinctiveness of this membrane domain.

Caveolin-1. The same line of investigation indicated that in HeLa cells, caveolin-1 overexpression by adenoviral infection caused an increase in the density of caveolae at the cell surface but attenuated caveolar uptake of albumin (198). Addition of glycosphingolipids reversed this effect and further stimulated caveolae endocytosis as monitored by reduction in the number of surface-connected caveolae (198). This is consistent with the findings that caveolin-1 acts as a negative regulator of caveolae-mediated endocytosis of autocrine motility factor receptor in NIH/3T3-transformed cells that express little caveolin-1 and caveolae (93, 114).

Specific albumin receptors localized on the surface of endothelium: gp18, gp31, and gp60. Albumin is a protein that is both endocytosed and transcytosed by ECs through either fluid phase or receptor-mediated processes (105, 201); however, albumin transcytosis was found to be considerably more significant than endocytosis (201). No quantitative data are available on the fraction of albumin that is endocytosed vs. transcytosed. It can be assumed that the population of endothelial vesicles comprises at least two functionally distinct entities.
This assumption is substantiated by the existence of uncoated, caveolin-1-free vesicular carriers in the endothelium of caveolin-1 null mice (37, 167, 168) and by evidence obtained from double-labeling experiments of the rete mirabile capillary endothelium showing that transport of two physiological tracers, albumin and insulin, takes place through different populations of PVs and tubules (9). Only 1.4% of the vesicle population was labeled by both tracers (9).

Studies performed in different laboratories provided evidence that ECs express specific binding sites for albumin represented by albumin binding proteins (57, 189, 201, 233). Various experimental approaches indicated the existence of at least three albumin-binding proteins called gp18, gp31, and gp60. Based on the interaction between albumin-gold complexes with the EC surface (201), on limited immunocytochemical localization of albumin binding proteins on EC structures (201), and on colocalization studies by fluorescence microscopy (106), it was concluded that albumin binding proteins are concentrated in caveolae. Kinetic studies suggested that gp18 and gp30 have a significantly greater affinity for modified albumin than for native albumin, and, on this account, it was suggested that they behave as scavenger receptors (189), mediating binding, endocytosis, and lysosomal degradation of modified albumins (192). However, at least for gp18, the immunogold-labeling EM indicates its localization in caveolae open on the luminal front of the EC, caveolae apparently free in the cytosol and in caveolae open on the abluminal front, suggestive for a role of gp18 as a transcytotic receptor (201), perhaps for modified albumin.

Syntxin-6. Syntaxin-6 is a t-SNARE protein involved in membrane fusion events along the secretory pathway (88). Syntaxin-6 is primarily localized to the trans-Golgi network and endosomal structures, suggesting a role in trans-Golgi and post-Golgi fusion events (247). Inhibition of syntaxin-6 function in human fibroblasts resulted in dramatically reduced levels of glycosylphosphatidylinositol and G\textsubscript{M1} at the cell surface, reduced caveolin-1 and caveolae at the plasma membrane, and inhibition of albumin and BODIPY lactosylceramide caveolae-mediated uptake (25). These findings suggest that syntaxin-6 modulates caveolae endocytosis by controlling the delivery of microdomain-associated lipids and proteins from the Golgi complex to the cell surface, which are required for caveolar endocytosis.

CONCLUDING REMARKS

In this review, we have highlighted the morphological, biochemical, and functional characteristics of endothelial caveolae that make them a specialized class of vesicular carriers involved in transcytosis of protein molecules >2 nm across the endothelium. We argued that caveolae have the expected structure and chemistry of vesicular carriers needed to account for the extremely high microvascular permeability. The evidence presented indicates that a number of caveolin-1-dependent and -independent vesicular carriers exist in endothelium, but the molecular details that differentiate them are not fully clear. The challenges for the future of endothelial caveolae research are to provide a better description of the molecular machinery that is engaged by different caveolar pathways and the signaling mechanisms responsible for the parallel regulation of these pathways to establish the factors involved in cargo selection along a specific pathway and to try to understand the cross talk between transcellular and paracellular pathways. Caveolin-1 deficient cultured cells and caveolin-1 null mice may be useful in providing evidence concerning the role of caveolae and caveolins in the development of the atherosclerotic lesions, in the pathogenesis of diabetes, cancer, fibrosis, acute lung injury, and other diseases associated with increased vascular permeability, and in all major medical problems. The priority of future caveolae and ECs research is the use of appropriate mouse models to understand and define the role of endothelial caveolae and transcytosis in the molecular mechanisms of caveolae-based diseases. Such insights are likely to provide important information about the role of caveolae and increased vascular permeability in the disease pathogenesis as well as development of new drugs and therapeutic strategies important for preventing and treating human diseases.

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