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Caveolin regulation of endothelial function

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Minshall, Richard D., William C. Sessa, Radu V. Stan, Richard G. W. Anderson, and Asrar B. Malik. Caveolin regulation of endothelial function. *Am J Physiol Lung Cell Mol Physiol* 285: L1179–L1183, 2003; 10.1152/ajplung.00242.2003.—Caveolae are the sites in the cell membrane responsible for concentrating an array of signaling molecules critical for cell function. Recent studies have begun to identify the functions of caveolin-1, the 22-kDa caveolar protein that oligomerizes and inserts into the cytoplasmic face of the plasma membrane. Caveolin-1 appears to regulate caveolar internalization by stabilizing caveolae at the plasma membrane rather than controlling the shape of the membrane invagination. Because caveolin-1 is a scaffolding protein, it has also been hypothesized to function as a "master regulator" of signaling molecules in caveolae. Deletion of the caveolin-1 gene in mice resulted in cardiac hypertrophy and lung fibrosis, indicating its importance in cardiac and lung development. In the endothelium, caveolin-1 regulates nitric oxide signaling by binding to and inhibiting endothelial nitric oxide synthase (eNOS). Increased cytosolic Ca^{2+} or activation of the kinase Akt leads to eNOS activation and its dissociation from caveolin-1. Caveolae have also been proposed as the vesicle carriers responsible for transcellular transport (transcytosis) in endothelial cells. Transcytosis, the primary means of albumin transport across continuous endothelia, occurs by fission of caveolae from the membrane. This event is regulated by tyrosine phosphorylation of caveolin-1 and dynamin. As Ca^{2+} influx channels and pumps are localized in caveolae, caveolin-1 is also an important determinant of Ca^{2+} signaling in endothelial cells. Many of these findings were presented in San Diego, CA, at the 2003 Experimental Biology symposium "Caveolin Regulation of Endothelial Function" and are reviewed in this summary.

caveolae; *Src*; dynamin; endothelial nitric oxide synthase; *CAV1* knockout; calcium; lipid raft domains

CAVEOLAE are cholesterol- and glycosphingolipid-rich membrane microdomains that function as mobile signaling platforms in the plasma membrane. They are ubiquitous features of endothelial cells comprising 95% of cell surface vesicles and ~15% of endothelial cell volume (41). Caveolin-1, the 22-kDa protein that coats the cytoplasmic surface of this specialized microdomain, is the defining protein constituent of caveolae (23, 45). Flask-shaped caveolar structures were generally absent in endothelial cells from caveolin-1 knockout mice (6, 44, 57). Caveolin-1 regulates the cholesterol content of caveolae because it binds to cholesterol and is involved in shuttling of cholesterol from the endoplasmic reticulum to the plasma membrane (33, 52). Numerous signaling molecules (G proteins, kinases, and others) are associated with caveolin-1 (3, 34, 38, 43), and this association may hold them in a quiescent or inhibited state (24). Caveolin-1 may concentrate signaling molecules in caveolae, allowing for their rapid activation by posttranslational protein modification, such as by phosphorylation (25). In this manner, caveolin-1 can function, in part, by organizing proteins in caveolae through protein-protein interactions, enabling finely tuned regulation of physiological responses [for example, Ca^{2+} entry and endothelial nitric oxide synthase (eNOS) activation (18)].

Caveolar release from the plasma membrane is an important mode of endocytosis in endothelial cells, which have few clathrin-coated pits (41), and is the first step in migration of vesicles to the basal membrane (31, 36, 37, 48). The vesicles detached from the plasmalemma shuttle to the basal membrane where they fuse and release their contents, a process termed transcytosis (13, 29, 31, 40, 56). Caveola-mediated transcytosis is an important mechanism of transendothelial transport of albumin and delivery of albumin-conjugated nutrients, fatty acids, and hormones across the endothelial barrier (32).

NITRIC OXIDE SIGNALING MECHANISMS IN CAVEOLAE

Nitric oxide (NO) generated by eNOS is important for maintenance of systemic blood pressure, vascular remodeling, angiogenesis, and wound healing (16, 47). eNOS is found enriched in caveolae where it associates with caveolin-1 by interacting with the scaffolding domain located between amino acids 82 and 101 (24). Binding of eNOS to caveolin-1 scaffold domain in vitro holds it in the inactive state (4). A direct relationship has been observed between the expression of caveolin-1 in endothelial cells and the inhibition of NO release

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(11). After increased $[Ca^{2+}]_i$, eNOS complexes with Ca^{2+}/calmodulin and dissociates from caveolin-1 and heat shock protein 90, thereby increasing eNOS activity and NO production (15). eNOS is also regulated by phosphorylation mediated by the Ser/Thr kinase Akt (10). Phosphorylation was found to both increase and decrease eNOS activity; that is, phosphorylation of Ser^{1179} increases eNOS activity and sensitivity to Ca^{2+}/calmodulin, whereas phosphorylation at Thr^{497} negatively regulates eNOS activity (11).

Bucci and coworkers (4) have addressed the caveolin-1 regulation of eNOS activity. They observed eNOS colocalization and coimmunoprecipitation with caveolin-1 and its enrichment in caveolae. Overexpression of caveolin-1 or delivery of the antennapedia-conjugated caveolin-1 scaffolding domain peptide decreased eNOS-dependent NO release (12, 21), indicating that binding of eNOS to the caveolin-1 scaffolding domain serves to inhibit the enzyme. To further characterize this phenomenon, the scaffolding domain peptide was tested in a mouse inflammation model induced by carrageenan (4). Delivery of the caveolin-1 scaffolding domain reduced the vascular permeability response and tissue edema formation by sequestering eNOS (4), indicating that eNOS activation and NO production are important determinants of the inflammatory response. The blockage of the vascular permeability response was similar to that observed in eNOS knockout mice (9). Thus the caveolin scaffolding domain peptide may provide a new therapeutic approach for regulation of NO production, perhaps mitigating the proinflammatory effects of excessive NO. The scaffolding domain peptide, by attenuating endothelial permeability, also reduced tumor progression in a mouse model (Sessa, unpublished observations), suggesting that the vascular leakage seen in angiogenesis is important in the mechanism of metastasis.

REGULATION OF CAVEOLA-MEDIATED TRANSCYTOSIS

Signaling pathways mediating release of caveolae from the plasma membrane are poorly understood. Phosphorylation events are likely important since caveolar fission is increased by phosphatase inhibition and decreased by kinase inhibition (30, 39). Caveolin-1 is phosphorylated by *Src* family kinases (14) on tyrosine *residue 14* (25), suggesting a relationship between tyrosine kinase activity and release of caveolae from the membrane (5, 31, 39, 55).

Immunoprecipitation studies showed that 60-kDa albumin-binding glycoprotein (gp60) associates with caveolin-1 after gp60 activation (31). In fact, both proteins are tyrosine phosphorylated during caveola-mediated endocytosis of albumin (31, 51, 55). In addition, inhibition of G protein signaling with either pertussis toxin or $G\alpha_i$ antagonist peptide, as well as dominant-negative *Src*, blocked albumin-activated endocytosis in endothelial cells (31). Because both gp60 and *Src* activation are required for albumin endocytosis, Minshall and coworkers (31) addressed the role of *Src* phosphorylation of caveolin-1 and dynamin-2 in endothelial cells in signaling the fission of caveolae and their release into the cytosol. Endocytosis of fluorescently tagged albumin or cholera toxin subunit B in endothelial cells was blocked by filipin and methyl-β-cyclodextrin (19, 31, 51), sterol binding agents that disassemble cholesterol-rich caveolae (46, 49). Coincident with the endocytosis of albumin (within 1 min after gp60 activation), caveolin-1 and dynamin-2 were tyrosine phosphorylated at *residues 14* and *597*, respectively (51). In both cases, pretreatment of cells with *Src* kinase inhibitor PP1 or PP2 abolished the phosphorylation. The functional importance of these events with respect to caveola-mediated endocytosis was investigated in pulmonary microvessel endothelial cells stably expressing non-*Src* phosphorylatable caveolin-1 or dynamin-2 mutants. Expression of either Y14F caveolin-1 or Y597F dynamin-2 abolished albumin and cholera toxin subunit B endocytosis, indicating *Src* phosphorylation of these residues is required for signaling caveola-mediated endocytosis (51). Furthermore, association between caveolin and dynamin was increased when dynamin was phosphorylated at Y597 and reduced by the nonphosphorylatable dynamin mutant (51).

Figure 1 presents a model of caveola-mediated endocytosis in endothelial cells. Caveolin-1 plays a central role because it serves a scaffolding function for components of the "caveolar release complex," G_i and *Src*, the signaling machinery responsible for endocytosis. *Src* family tyrosine kinases, which are activated by $G_{\beta\gamma}$-subunits on stimulation of G protein-coupled receptors (17, 28) or tyrosine kinase receptors (22), phosphorylate tyrosine residues on gp60 (55) as well as caveolin-1

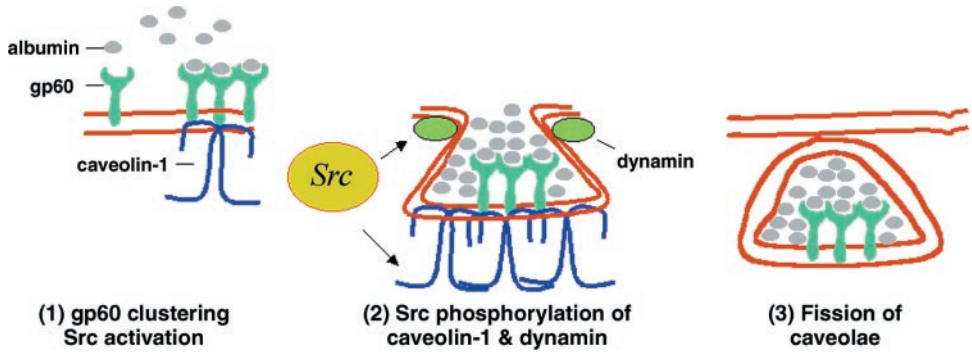

Fig. 1. Signaling mechanisms regulating endocytosis of albumin are shown. Endothelial caveolae are carriers of albumin. Albumin binding proteins (such as the 60-kDa albumin-binding glycoprotein, gp60) initiate the endocytosis of albumin by associating with caveolin-1 and activating *Src*. *Src*, in turn, phosphorylates caveolin-1 and dynamin-2, both of which are required for fission of caveolae and the internalization of bound and fluid-phase macromolecules within caveolae (32, 51).

(25, 31, 32, 55) and dynamin (1, 2, 22, 51). The caveolar release complex thus engaged (i.e., phosphorylated caveolin-1, dynamin-2, and *Src*) activates fission (32). Further interrogation of this model will define the relationships between gp60, caveolin-1, G_i, *Src*, and dynamin and the signals mediating transcytosis.

CAVEOLIN-1 KNOCKOUT MICE AND ENDOTHELIAL BARRIER FUNCTION

Caveolae transport plasma proteins across the vascular endothelial barrier and functionally organize signaling molecules. It stands to reason, therefore, that deletion of the caveolin-1 gene (*CAV1*) would result in the *1*) absence of plasmalemmal vesicles, *2*) inability to shuttle plasma constituents across the endothelium, and *3*) activation of signaling molecules that are normally held inactive by caveolin-1. Studies carried out in caveolin-1 knockout animals showed uncontrolled endothelial cell proliferation and lung fibrosis, increased NO production, impaired Ca^{2+} signaling, and defective endocytosis of albumin that could be reversed by expression of caveolin-1 cDNA (6, 44, 57). Deletion of the *CAV1* gene was not lethal, suggesting that compensatory mechanisms, such as increased junctional permeability of the endothelial barrier (50), are responsible for survival of the mice.

Ultrastructural analysis of microvessels in the caveolin-1 knockout mouse model showed the absence of caveolae (Fig. 2) but the presence of fenestrae and larger vesicular structures resembling vesicular-vacuolar organelles (Stan, unpublished observations). Thus assembly of these cellular structures does not require the presence of caveolin-1. Interestingly, somewhat larger (100- to 120-nm-diameter) uncoated vesicles resembling caveolae were present in endothelia of certain vascular beds, albeit fewer in number than caveolae in wild-type mice (57). This finding, reported by the three independent groups that have generated the *CAV1*$^{-/-}$ mice (6, 44, 57), indicates there may be an additional pool of vesicles in endothelial cells that is neither clathrin- nor caveolin-1-coated. Its role in endocytosis and transendothelial transport remains to be elucidated.

Perhaps the most striking observation regarding the phenotype of *CAV1*$^{-/-}$ mice was the fivefold increase in plasma NO levels (57). This finding supports the hypothesis that caveolin-1 has a function in regulating eNOS, thus removal of caveolin-1 from the cell increases basal eNOS activity and leads to higher constitutive plasma NO levels. The mechanism by which caveolin-1 regulates the activity of caveola-associated proteins could be by maintaining the correct lipid composition and interactions with protein kinase C and tyrosine kinases (for review, see Ref. 26).

These observations are consistent with data showing an important role of caveolin-1 in albumin transport and regulation of eNOS activity and cell proliferation (4, 31, 57). However, additional studies are needed to determine how caveolin-1 regulates endothelial barrier function, how elevated NO levels perturb endothelial barrier function, and how caveolin-1 keeps endothelial cells in a contact-inhibited state to maintain proper tissue organization in organs.

CAVEOLIN REGULATION OF CALCIUM SIGNALING

Caveolae are cholesterol-rich domains such that removal of cholesterol with filipin disrupts caveolae (45, 46, 49). Lipid rafts lack the caveolin-1 coat but may

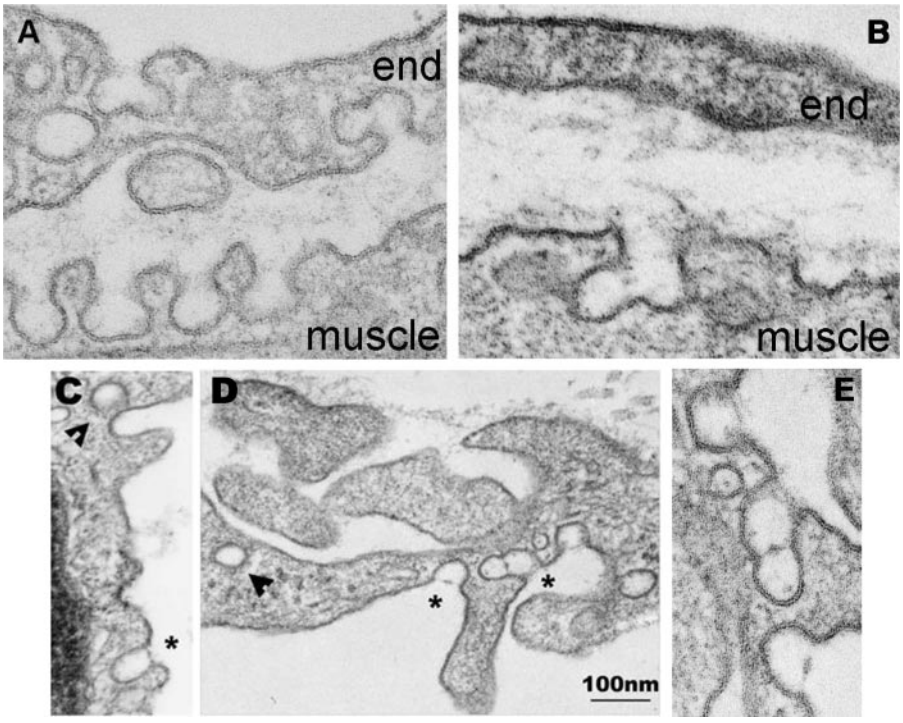

Fig. 2. Absence of caveolae in *CAV1*$^{-/-}$ mice. Transmission electron microscopy analysis of heart ventricular endothelial cells from *CAV1* wild-type mice (*A*) shows caveolae in both endothelial (end) and muscle cells, whereas caveolae were found in the muscle cells but not the endothelial cells in *CAV1* knockout mice (*B*). *C–E*: micrographs of endothelial cells from larger blood vessels in the *CAV1* knockout mice. Clathrin-coated vesicles are indicated (arrowheads) for size comparison. In both the heart and diaphragm muscle of *CAV1* knockout mice, larger caveola-like structures (*) could be occasionally observed in vessels with diameters >10 μm. These structures appear to be provided with stomatal diaphragms. [Modified from Zhao et al. (57).]

function in a similar manner to compartmentalize certain lipids and proteins (53). Environmental factors shift caveolin-1 localization (for example, to the trailing edge of a cell exposed to flow/shear stress). Thus caveolin-1 functions in lipid transport, membrane traffic, and cell signaling on the basis of its interacting partners (26).

Several molecules involved in Ca^{2+} translocation have been localized to caveolae, including a D-*myo*-inositol 1,4,5-trisphosphate receptor-like protein (8), dihydropyridine-sensitive Ca^{2+} channels (20), a Ca^{2+}-ATPase (7), and Trp1 channels involved in capacitive Ca^{2+} entry (27). Electron microscopy studies also showed that caveolae are in close proximity with the endoplasmic reticulum (ER) (54). The functional importance of caveolae with regard to Ca^{2+} release and re-uptake was assessed using live cell Ca^{2+} sensor yellow cameleon (18). With the use of fusion proteins of yellow cameleon and caveolin-1 or neuromodulin, which target the Ca^{2+} indicator to the caveolae or bulk plasma membrane, respectively, it was demonstrated that caveolae are the preferred sites of Ca^{2+} entry when ER Ca^{2+} stores are depleted. Caveolae are thus the compartment involved in regulating store-operated Ca^{2+} entry. Ca^{2+} that enters the cell via caveolae is coupled to activation of eNOS (18), which is a resident protein of caveolae.

The capacitive Ca^{2+} entry and eNOS model described by Isshiki and coworkers (18) suggest that caveolae function as organizers of signaling complexes, providing a mechanism for regulating the on and off state of an entire signaling circuit without the need to assemble any of its components. Caveolae are thus well suited to function as storage containers for preassembled signaling pathways that can be deployed on demand. They can also be used to carry signaling machinery to various locations in the cell, functioning as "mobile signaling platforms" that serve as the origin of specific signaling events in the cell. Although the role of caveolae in endothelial cells has been described primarily as transcellular carriers, the organization of various signaling modules appears to be very important as well.

SUMMARY

Caveolin-1, the defining protein of caveolae, regulates multiple functions of the vascular endothelium. Caveolin-1 is required for transcellular transport machinery of endothelial cells. It also forms mobile signaling platforms in that it concentrates and organizes signal transduction cascades that ultimately regulate tissue structure, vascular tone, and basal permeability. Current efforts directed at identifying the role of caveolin-1 in plasmalemmal vesicle formation and trafficking as well as regulation of signaling molecules such as capacitive Ca^{2+} entry channels, eNOS, *Src*, and G proteins should further our understanding of caveolin regulation of endothelial function.

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