Microvascular Hyperpermeability in Caveolin-1 (−/−) Knock-out Mice

TREATMENT WITH A SPECIFIC NITRIC-OXIDE SYNTHASE INHIBITOR, l-NAME, RESTORES NORMAL MICROVASCULAR PERMEABILITY IN Cav-1 NULL MICE*

William Schubert‡§, Philippe G. Frank‡§†, Scott E. Woodman‡§, Hideyuki Hyogo**, David E. Cohen**, Chi-Wing Chow‡, and Michael P. Lisanti‡§‡‡

From the ‡Department of Molecular Pharmacology, the §Division of Hormone-Dependent Tumor Biology at the Albert Einstein Comprehensive Cancer Center, and the **Departments of Medicine and Biochemistry, Marion Bessin Liver Research Center, Albert Einstein College of Medicine, Bronx, New York 10461

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Microvascular permeability is mediated by (i) the caveolar transcytosis of molecules across endothelial cells and (ii) the paracellular movement of ions and nutrients. Recently, we derived Cav-1 (−/−) knock-out mice using standard homologous recombination techniques. These mice are viable but show a loss of endothelial cell caveolae and striking defects in caveolae-mediated endocytosis. Thus, a compensatory mechanism must be operating in these mice. One possible compensatory response would be an increase in the paracellular pathway, resulting in increased microvascular permeability. To test this hypothesis directly, we studied the microvascular permeability of Cav-1 null mice using a variety of complementary in vivo approaches. Radio-iodinated bovine serum albumin was injected into Cav-1-deficient mice, and its rate of clearance from the circulatory system was compared with that of wild type control mice. Our results indicate that iodinated bovine serum albumin is removed from the circulatory system of Cav-1-deficient mice at a substantially faster rate. To determine whether this defect is restricted to the paracellular movement of albumin, lungs from Cav-1-deficient mice were next perfused with the electron dense dye Ruthenium Red. Micrographs of lung endothelial cells from Cav-1-deficient mice demonstrate that the paracellular movement of Ruthenium Red is dramatically increased. In addition, electron micrographs of Cav-1-deficient lung capillaries reveal defects in tight junction morphology and abnormalities in capillary endothelial cell adhesion to the basement membrane. This defect in cell-substrate attachment is consistent with the postulated role of caveolin-1 in positively regulating integrin signaling. Because loss of caveolin-1 expression results in constitutive activation of eNOS activity, we also examined whether these increases in microvascular permeability are NO-dependent. Interestingly, treatment with l-NAME (a well established nitric-oxide synthase inhibitor) successfully reversed the microvascular hyperpermeability phenotype of Cav-1 knock-out mice. Thus, caveolin-1 plays a dual regulatory role in controlling microvascular permeability: (i) as a structural protein that is required for caveolae formation and caveolar transcytosis and (ii) as a tonic inhibitor of eNOS activity to negatively regulate the paracellular pathway.

Vascular endothelial cells form a continuous and semipermeable barrier that is responsible for maintaining the homeostatic regulation of fluid balance between the circulatory system and surrounding tissues. These cells also regulate vascular permeability to plasma proteins and various cells within the blood. Vascular endothelial cells have two different mechanisms that appear to act in concert to control vascular permeability. The first mechanism is the paracellular (between cells) pathway, first reported by Majno and Palade in 1961 (1), in which transported material passes across endothelial tight junctions. The second mechanism is via the transcytotic (through cells) pathway (2), in which transported material is taken up by the endothelial cell from the apical surface, transported across the cell, and released at the basolateral surface. This latter pathway is mediated by caveolae organelles, which are also known as plasmalemmal vesicles.

Caveolae, 50–100-nm vesicular invaginations of the plasma membrane, were first described morphologically in the 1950s by Yamada (3) and Farquhar and Palade (4) and are most abundant in endothelial cells, adipocytes, smooth muscle cells, and fibroblasts. Caveolae have been implicated in vesicular permeability since the 1950s because of their role in the fluid phase transcytosis of both large and small molecules across endothelial cells (3, 4). Caveolin-1, a 21–24-kDa integral membrane protein, is a principal component of caveolar membranes (5, 6). It has been proposed that caveolin-1 acts as a scaffolding protein to concentrate and organize specific lipids, such as sphingolipids and cholesterol (7–9), and lipid-modified signaling molecules, such as G-proteins, Ha-Ras, and Src-like kinases (10–12), within caveolae microdomains. Interestingly, endothelial nitric-oxide synthase (eNOS)1 is also lipid-modified, targets to caveolea mem-

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‡‡ To whom correspondence should be addressed: Dept. of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Tel.: 718-430-8828; Fax: 718-430-8830; E-mail: lisanti@aeon.yu.edu.

1 The abbreviations used are: NOS, nitric-oxide synthase; eNOS, endothelial NOS; BSA, bovine serum albumin; CSD, caveolin-1 scaffolding domain.
branes, and interacts directly with caveolin-1. In addition, interaction of caveolin-1 with eNOS inhibits its catalytic activity, thereby preventing NO production (13).

NO and its endothelial-specific synthase (eNOS) are important signaling molecules in determining the action of various vasomediators in the control of vascular permeability (14–17). Although the role of eNOS and NO in vascular permeability has been widely studied through the use of eNOS inhibitors to block permeability and NO donors to accentuate these effects (18, 19), the exact mechanism by which eNOS and NO regulate vascular permeability remains unclear. However, one possible mechanism through which NO can influence vascular permeability is via the regulation of endothelial cell shape and intercellular junction formation (20, 21). In support of this model, pulmonary microvascular endothelial cell adhesion is regulated by NO (22).

Thus, the role of caveolin-1 in the regulation of endothelial cell permeability may be 2-fold. First, caveolin-1 expression drives caveolae formation, thereby directly regulating the capacity of the endothelial cell for transporting molecules such as albumin via caveolar transcytosis. Second, caveolin-1 may indirectly regulate the paracellular pathway via its ability to tonically inhibit eNOS, preventing NO production.

Recently, we have generated Cav-1-deficient mice (23). Interestingly, these mice are viable and fertile despite a loss of caveolae organelles in the cell types where the caveolin-1 protein is normally expressed. We have shown that fibroblasts and endothelial cells derived from Cav-1 null mice are incapable of endocytosing albumin, an abundant serum protein. Albumin is normally transported across endothelial cells via caveolar transcytosis (25).

Here, we show that despite a loss of endothelial cell caveolae, Cav-1 null mice show dramatic increases in microvascular permeability. Because loss of caveolin-1 expression results in constitutive activation of eNOS, we also examined whether these increases in microvascular permeability were NO-dependent. Interestingly, treatment with l-NAME (a well established NOS inhibitor) successfully rescued the microvascular hyperpermeability phenotype of Cav-1 knock-out mice.

**Materials and Methods**

**Serum Albumin Concentration**—Blood was collected from both wild type and Cav-1-deficient mice. Serum was then isolated by centrifuging the blood at 6000 rpm for 6 min at 4 °C. The amount of serum albumin
was then determined by using a color-metric Sigma diagnostics kit (Reactiv Albumine).

**In Vivo BSA Clearance**—Female wild type and Cav-1-deficient mice at 2 months of age were anesthetized using pentobarbital (Abbott labs) at 136 mg/kg of body weight. Once anesthetized, 15 μl of 125I-BSA at a concentration of 10 mCi/ml was mixed with 150 μl of 1× phosphate-buffered saline and introduced into mice via tail vein injection. The blood samples were then collected from the tail at 2, 5, 15, 30, 45, and 60 min post-injection. The samples were stored at 4°C until all time points were obtained. Serum was then isolated by centrifuging the blood at 6000 rpm for 6 min at 4°C. The amount of radioactivity in a 10 μl of serum was determined using a γ-counter. The rate of clearance for each mouse was determined by using the number of cpm obtained from the 2-min time point as the starting point, i.e. 100%, and shown graphically.

**In Vivo Tissue Uptake of 125I-BSA**—After the last blood sample was taken at 60 min post-injection, the mice were euthanized, and various organs were isolated. The tissues were washed thoroughly in 1× phosphate-buffered saline to remove any residual blood. The amount of 125I-BSA in each tissue sample was then determined. Each tissue sample was weighed, and the amount of radioactivity in a given tissue sample was expressed as cpm/g.

**Tight Junction Morphology**—Untreated lung samples from both wild type and Cav-1-deficient mice were prepared for electron microscopy to examine the microvascular system of the lung (24). Tight junctions from capillary endothelial cells of both wild type and Cav-1 null mice were examined, and micrographs containing 40 tight junctions/condition were generated. These tight junctions were then measured, and the averages were determined and plotted (n = 40 for each genotype).

**Ruthenium Red**—Wild type and Cav-1-deficient mice were anesthetized, as described above. The heart was then exposed, and the descending aorta was cut. A saturated solution of Ruthenium Red in 1× phosphate-buffered saline warmed to 37°C was injected into the right ventricle at a rate of 0.5 ml/min for 10 min. Lung tissue that had been perfused with the Ruthenium Red was removed and placed in a standard fixative for electron microscopy (2.5% gluteraldehyde in 0.1M cacodylate buffer) for 2 h. The lung samples were then processed and examined by electron microscopy, as described previously (24). The intensity of staining from apical and basolatral plasma membranes was determined for both wild type and Cav-1 null endothelial cells using ImageQuant software by Molecular Dynamics.

**L-NAME Treatment**—Wild type and Cav-1-deficient mice were treated with or without the eNOS inhibitor, L-NAME. Mice were given an intraperitoneal injection of L-NAME (30 mg/kg; Fisher) 1 h prior to injection of the 125I-BSA solution. Clearance of 125I-BSA from the blood was then determined, as described above.

**FIG. 4. Cav-1-deficient mice show alterations in lung endothelial cell tight junctions.** A. Electron micrographs of Cav-1-deficient lung endothelial cells (KO) show alterations in their tight junctions as compared with wild type controls (WT). Note that the tight junctions in endothelial cells from Cav-1-deficient mice are smaller in size and shape. Arrows point at the tight junctions. RBC, red blood cells. Scale bar, 500 nm. B, higher magnification views of wild type and knock-out tight junctions are shown. Arrows point at the two ends of the tight junction. Scale bar, 100 nm. C, quantitation of tight junction length. Forty tight junctions from wild type and Cav-1 endothelial cells were examined and measured (n = 40 for each genotype). Note that Cav-1-deficient tight junctions were found to be ~3.5 times smaller than wild type tight junctions.
RESULTS

Cav-1-deficient Mice Show Large Increases in Microvascular Permeability—To examine the in vivo role of Cav-1 in albumin uptake, female age-matched Cav-1-deficient and wild type mice were given tail vein injections of $^{125}$I-BSA. The blood samples were taken at various time points, and the rate of clearance for radio-iodinated BSA was determined (Fig. 1).

At 5 min post-injection, the majority ($\approx 70\%$) of iodinated BSA was cleared from the circulatory system of the Cav-1-deficient mice. In contrast, only a small fraction ($\approx 25\%$) of the iodinated BSA was immediately cleared from wild type mice. Interestingly, the level of iodinated BSA in the circulatory system of the wild type mice at 60 min post-injection is greater than the levels seen in Cav-1-deficient mice at 5 min post-injection.

At 60 min post-injection, the mice were euthanized, and various tissue types were isolated to determine that amount of iodinated BSA that accumulated in a given tissue. These results are shown graphically in Fig. 2; the fold differences between the levels of iodinated BSA in the Cav-1-deficient and wild type mice show defects in cell-substrate attachment. A, electron micrographs of Cav-1-deficient (KO, panels c and d) lung endothelial cells show weakened attachment to the basement membrane as compared with wild type cells (WT, panels a and b). Note that multiple sites where endothelial cells have become detached from the basement membrane ($) can be observed in Cav-1-deficient mice. L, lumen of capillaries; RBC, red blood cell. The scale bar indicates 500 nm and applies to panels a–d. B, a higher magnification view is shown that convincingly demonstrates that these blebs seen in Cav-1-deficient endothelial cells are delineated by the endothelial cell plasma membrane and the basement membrane. ENDO, endothelial cell; BM, basement membrane. Scale bar, 500 nm. C and D, quantitation of the number of detachment sites/capillary. C, of the 100 capillaries examined, the majority of capillaries (93) were found to have no sites of detachment from the basement membrane. D, of the 100 capillaries examined, the majority of capillaries were found to have multiple sites of detachment. Frequency of occurrence is plotted versus the number of detachment sites/capillary.
Cav-1-deficient mice were examined by electron microscopy to identify any possible ultrastructural changes. Interestingly, endothelial cell tight junctions in Cav-1-deficient mice (Fig. 4, A and B) appeared to be significantly smaller than tight junctions seen in wild type animals (Fig. 4A and B). Forty tight junctions from wild type and Cav-1 endothelial cells were examined and measured (Fig. 4C). Such quantitation revealed that Cav-1-deficient tight junctions are on average ~3.5 times smaller than wild type tight junctions.

In addition, many of the Cav-1-deficient endothelial cells forming lung capillaries (Fig. 5A, panels c and d) have regions that have clearly become detached from the basement membrane. Importantly, this cell-substrate adhesion defect was not routinely observed in wild type animals (Fig. 5A, panels a and b). A higher magnification view is shown in Fig. 5B that convincingly demonstrates that these “blebs” seen in Cav-1-deficient endothelial cells are delineated by the endothelial cell plasma membrane and the basement membrane.

To quantitate the number of detachment sites and their frequency, 100 capillaries from wild type and Cav-1-deficient lung samples were examined, and the number of detachment sites were counted. On average, 2.19 endothelial detachment sites/capillary were observed in Cav-1-deficient lung samples. However, only 0.12 endothelial detachment sites/capillary were observed in wild type mice. In wild type lung samples, 93 of the 100 capillaries examined were found to have no sites of detachment from the basement membrane (Fig. 5C), whereas the Cav-1-deficient lung samples had as many as nine points of detachment for an individual capillary (Fig. 5D). These results are consistent with the proposed role of caveolin-1 in cell-substrate attachment, i.e. as a positive regulator of integrin signaling (reviewed in Ref. 26). Interestingly, neither of these abnormalities was detected in endothelial cells lining the aorta (data not shown).

To further examine the possibility that capillaries in the Cav-1-deficient mice are hyperpermeable, a solution of Ruthenium Red (an electron-dense dye) was perfused into the lungs of both wild type and Cav-1-deficient mice. Normal tight junctions should block the Ruthenium Red staining from reaching the basal membrane of the endothelial cells, whereas tight junctions that are defective or hyperpermeable should allow the Ruthenium Red to penetrate to the basal membrane, resulting in both membranes being darkly stained.

As expected, electron micrographs of wild type lung capillary endothelial cells (Fig. 6, A and B) do show a difference in staining between the luminal and basal membranes. The luminal membrane (arrows) is clearly densely stained, and the basal membrane (arrowheads) shows little or no staining. In contrast, the luminal (arrows) and basal (arrowheads) membranes in the Cav-1-deficient endothelial cells (Fig. 6, C and D) are both densely stained. The staining intensity of the apical and basolateral membranes was quantitated for wild type and Cav-1-deficient endothelial cells. The apical/basolateral ratio for wild type endothelial cells was ~5.6:1, whereas the apical/basolateral ratio for Cav-1-deficient cells was 1.7:1. Therefore, there appears to be an approximately 3-fold increase in the permeability of Cav-1-deficient endothelial cells.

**Microvascular Hyperpermeability Is Rescued by Treatment with l-NAME, a Well Established NOS Inhibitor**—To examine the role of NO production in the hyperpermeability phenotype of Cav-1-deficient mice, we employed a well characterized NOS inhibitor, i.e. l-NAME. Cav-1-deficient and wild type mice were injected with l-NAME 1 h prior to the injection of iodinated BSA. Clearance studies were then performed on these animals.

Fig. 7 shows that the rate of radio-iodinated BSA clearance in l-NAME-treated Cav-1-deficient mice is dramatically re-
Reduced. Note that the rate of BSA clearance for L-NAME-treated Cav-1-deficient mice approaches the clearance rates observed for wild type mice. However, treatment with L-NAME did not affect BSA clearance rates in wild type mice. The accumulation of radio-iodinated BSA in the tissues of Cav-1-deficient mice was also determined in the presence of L-NAME (Fig. 8). As predicted, treatment with L-NAME restored iodinated BSA levels in Cav-1-deficient mice to near wild type levels in all of the tissues examined. Thus, the microvascular hyperpermeability phenotype of Cav-1-deficient mice appears to be depend-

**FIG. 7.** Treatment with the NOS inhibitor, L-NAME, rescues the hyperpermeability phenotype of Cav-1-deficient mice. Cav-1-deficient mice treated with L-NAME show a dramatic decrease in the clearance of radio-iodinated BSA, as compared with untreated Cav-1-deficient mice. Note that the rate of BSA clearance for L-NAME treated Cav-1-deficient mice approaches the clearance rates observed for wild type mice. However, treatment with L-NAME did not affect BSA clearance rates in wild type mice.

**FIG. 8.** Cav-1-deficient mice treated with L-NAME have reduced 125I-BSA accumulation in their tissues. Treatment of Cav-1-deficient mice with L-NAME reduces the amount of 125I-BSA found in the various tissues. The levels of 125I-BSA accumulation observed for Cav-1-deficient mice treated with L-NAME are similar to levels seen in wild type animals (percentage of control).

**FIG. 9.** eNOS protein expression is not up-regulated in Cav-1-deficient mice. eNOS expression levels in lung tissue samples derived from Cav-1 wild type (WT, +/+), Cav-1 heterozygous (HET, +/−), and Cav-1-deficient (KO, −/−) mice were determined by Western blot analysis. Note that eNOS protein expression is not altered in Cav-1-deficient mice. Immunoblotting with anti-β-actin IgG is shown as a control for equal loading.

**FIG. 10.** Caveolin-1 expression differentially regulates microvascular permeability. A schematic diagram summarizing the functional roles of caveolin-1 is shown. Briefly, caveolin-1 drives caveolae formation and is therefore a positive (+) regulator of caveolar transcytosis. Conversely, caveolin-1 acts as a tonic inhibitor of eNOS activity, thereby preventing NO production and negatively (−) regulating the paracellular pathway. Our current experiments with Cav-1-deficient mice directly support these assertions.
ent on NO production, because treatment with a NOS inhibitor clearly rescues this phenotype.

**eNOS Protein Levels Are Not Up-regulated in Cav-1-deficient Mice**—We next examined eNOS expression levels in lung tissue samples derived from wild type (+/+), heterozygous (+/−), and Cav-1-deficient (−/−) mice. However, Fig. 9 shows that eNOS protein expression levels are not altered in Cav-1-deficient mice. These results demonstrate that the defects we observe in Cav-1-deficient mice are not due to overexpression of eNOS. Because caveolin-1 is thought to function as a tonic inhibitor of eNOS activity, these defects may be due to an increase in eNOS activity, as we and others have reported previously (23, 26, 27).

**DISCUSSION**

Vascular endothelial cells form a continuous barrier separating the circulatory system from the surrounding tissues, and disruption of the endothelial barrier and subsequent increases in vascular permeability can lead to a wide variety of pathological conditions (for reviews see Refs. 28 and 29). Vascular permeability is mediated by two separate pathways. Material taken up by the cell and actively transported through the cell to the opposite side is said to move via the transcellular pathway, whereas material that exits the circulatory system by passing between endothelial cell tight junctions occurs via the paracellular pathway. Previously, we have shown that loss of caveolae in fibroblasts and endothelial cells abolishes the ability of these Cav-1-deficient cells to take up albumin (24). These results demonstrate that vascular endothelial cell caveolae are directly involved in the transcellular movement of albumin. Caveolae also appear to play an indirect role in regulating vascular permeability through the ability of the caveolin-1 protein to control NO production within endothelial cells. Caveolin-1 has been shown to bind eNOS, and this binding event maintains eNOS in an inactive state. Upon receiving the proper stimulus, eNOS is released from Cav-1 binding and becomes catalytically active, and NO production ensues (13). Higher levels of nitric oxide are in turn responsible for increasing vascular permeability in endothelial cells (18, 30).

In this report, we demonstrate that Cav-1-deficient mice have a hyperpermeable or "leaky" microvascular system, such that the clearance rate of BSA injected into the circulatory system is significantly increased. In addition, endogenous levels of serum albumin are significantly reduced in Cav-1-deficient mice, supporting a loss of proper control over vascular permeability in these mice. Initially, these findings may seem to contradict our earlier study in which aortic rings and perfused lung tissue were shown to have defects in the uptake of BSA (24). However, the aortic samples taken from Cav-1-deficient mice in the current study also show a decrease in the amount of BSA that accumulated, as compared with aortic samples derived from wild type mice (Fig. 2), in accordance with our previous findings. In addition, coupling of BSA to 5-nm colloidal gold particles is expected to dramatically alter the conformation and/or size of the BSA molecule, such that it can no longer pass through the tight junctions and remains in the blood vessel lumen (24). This is in contrast to our current findings demonstrating that free BSA undergoes increased paracellular uptake. Thus, gold-conjugated BSA may be a good probe to follow caveolae-mediated endocytosis, but it cannot be used to mimic the transport of free BSA via the paracellular pathway.

In support of our current findings that Cav-1-deficient mice have a hyperpermeable microvascular system, electron micrographs of Cav-1-deficient lung capillaries reveal alterations in tight junction morphology and defects in capillary endothelial cell adhesion to the basement membrane. In addition, injection of an electron-dense dye (Ruthenium Red) that is incapable of passing through wild type tight junctions further demonstrated that the circulatory system of Cav-1-deficient mice is hyperpermeable. Interestingly, the increased microvascular permeability observed in Cav-1-deficient mice was corrected by treatment with L-NAME, a well known NOS inhibitor. These results directly demonstrate that the observed hyperpermeability phenotype is dependent on NO production. Thus, we conclude that caveolin-1 plays two distinct roles in the regulation of vascular permeability: (i) as structural protein that is required for caveolae formation and caveolar transcytosis and (ii) as tonic inhibitor of eNOS activity to negatively regulate the paracellular pathway (summarized schematically in Fig. 10).

Over the past several years, it has become apparent that vascular permeability is regulated by many complex signaling pathways within endothelial cells. One of these pathways involves regulation by eNOS. Caveolin-1 acts as a natural endogenous inhibitor of eNOS by maintaining the enzyme in a catalytically inactive state. This binding and inhibitory activity are mediated by the scaffolding domain (residues 82–101) of caveolin-1 (23, 26, 31–33). Upon its stimulus-mediated release from caveolin-1 binding, eNOS becomes catalytically active, and NO production ensues. Furthermore, our current studies directly support the findings of Sessa and co-workers (13), who used a chimeric peptide containing a cellular internalization signal and the caveolin-1 scaffolding domain (CSD, residues 82–101). Using wild type mouse ears treated with an irritant (mustard oil) to induce the extravasation of Evan’s blue dye, they showed that the CSD could potentely act as an inhibitor of microvascular permeability/vascular leakage. The actions of the CSD could be mimicked by using the NOS inhibitor L-NAME. In further support of the specificity of the CSD, a scrambled peptide version of the CSD had no activity in this assay system.

NO has been shown to increase cGMP levels by regulating the action of guanylate cyclase (17). The exact mechanism as to how cGMP regulates vascular permeability is poorly understood, but one method may be by affecting the cAMP levels in endothelial cells. Levels of cAMP may be lowered by a cGMP-activated phosphodiesterase such as phosphodiesterase 2, which has been identified in endothelial cells (34, 35); He et al. (36) have shown that increased cGMP stimulated phosphodiesterase 2 and lowered cAMP, resulting in increased permeability in rat and frog mesenteric vessels. Another possible action of cGMP is through its ability to stimulate the phosphorylation of VASP, a protein associated with focal adhesion sites and adherens junctions (37). However, the exact role that VASP plays in regulating vascular permeability has yet to be studied, but it could explain the observation that endothelial cell adhesion is altered upon treatment with NO (22), as well as our current findings of altered endothelial tight junction morphology and detachment from the basement membrane.

Although the majority of Cav-1-deficient tissues examined in this study contained higher levels of iodinated BSA, as compared with wild type mice, segments of aorta taken from Cav-1-deficient mice showed significantly lower levels of BSA accumulation. These current findings are consistent with our previous data showing that caveolar endocytosis of radio-iodinated albumin is blocked in isolated aortic ring segments derived from Cav-1-deficient mice (24).

In summary, we have shown that Cav-1-deficient mice demonstrate significant increases in microvascular permeability. In addition, we demonstrate that this phenotype is an NO-dependent phenomenon. As such, caveolin-1 plays a crucial role in regulating microvascular permeability via (i) caveolar transcytosis, as a structural protein that is required for caveolae
formation, and (ii) the paracellular pathway, as a tonic inhibitor of eNOS activity.

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