

## ***In Situ* Flow Activates Endothelial Nitric Oxide Synthase in Luminal Caveolae of Endothelium with Rapid Caveolin Dissociation and Calmodulin Association\***

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**Acute changes in pressure or shear stress induce the rapid release of nitric oxide (NO) from the vascular endothelium resulting in vasodilation. Endothelial nitric oxide synthase (eNOS) regulates this flow-induced NO secretion. The subcellular location of flow-induced eNOS activity in the endothelium *in vivo* as well as the mechanisms by which hemodynamic forces regulate eNOS activity are unknown. The luminal cell surface of the endothelium, which is directly exposed to circulating blood stressors, has been examined for eNOS expression and functional activity. Immunoelectron microscopy of rat lung tissue shows eNOS labeling on the endothelial cell surface primarily within caveolae. Subcellular fractionation to purify luminal endothelial cell plasma membranes and their caveolae directly from rat lungs reveals that eNOS is not only concentrated but also enzymatically active in caveolae. Increasing vascular flow and pressure *in situ* rapidly activates caveolar eNOS with apparent eNOS dissociation from caveolin and association with calmodulin. Hemodynamic forces resulting from increased flow appear to transmit through caveolae to release eNOS from its inhibitory association with caveolin, apparently to allow more complete activation by calmodulin and other possible effectors. These data demonstrate a physiological relevant mechanotransduction event directly in caveolae at the luminal endothelial cell surface. Caveolae may serve as flow-sensing organelles with the necessary molecular machinery to transduce rapidly, mechanical stimuli and thereby regulate eNOS activity.**

The vascular endothelium has evolved a set of adaptive responses to the changing hemodynamic mechanical forces imposed by the circulating blood. Both vascular tone (1, 2) and vessel remodeling (3) are known to be regulated through endothelial cell responses to fluid mechanical forces. Inadequate or inappropriate adjustments to alterations in flow may result in pathophysiological consequences found in hypertension and atherosclerosis (4, 5). An understanding of the mechanisms by

which hemodynamic forces are detected and converted into a sequence of biological responses within the vascular endothelium presents a major challenge.

Flow-induced release of nitric oxide (NO)<sup>1</sup> from vascular endothelial cells appears to be an important and rapid mediator of cardiovascular function (6–9). Shear forces generated from fluid flow very rapidly stimulate NO production (10–13). NO is produced from the enzymatic conversion of L-arginine to L-citrulline through an isoform of nitric oxide synthase (eNOS) found in endothelial cells. How forces generated from fluid flow activate eNOS to rapidly release NO from vascular endothelial cells is unknown. It appears logical that the luminal surface of the endothelium, which is directly exposed to the blood flow and therefore expected to be sensitive to hemodynamic forces, would be a primary site for flow-responsive eNOS activity. However, cultured endothelial cells express eNOS primarily in the Golgi and cytoplasmic compartments (13, 14). More recent studies have shown that, when appropriately lipid acylated, eNOS can be targeted to membrane subfractions rich in caveolin and therefore assumed to be specialized plasmalemmal vesicles called caveolae (15, 16). Furthermore, eNOS may associate with the caveolar coat protein, caveolin, both in cultured endothelial cell lysates (15, 17) and in recombinant protein-protein assays (18–20). Although caveolin at the cell surface is quite specific for caveolae, it can also be quite abundant in the trans-Golgi network, at least in cultured cells (21). Probably because endothelial cells have remarkable plasticity with the ability to modulate their constitutive phenotype in response to their local tissue microenvironment (22), it is not surprising that they tend to adopt a “de-differentiated” phenotype when grown in culture. Isolating and growing endothelial cells in culture decreases the cell surface density of caveolae (23) and may redistribute caveolin and possibly other caveolar molecules such as eNOS to other compartments such as Golgi.<sup>2</sup> Last, one recent study has concluded from subfractionation analysis that caveolae lack signaling molecules such as eNOS and therefore cannot participate in cell surface signaling events (24). Thus, although critically important for understanding eNOS function, the precise subcellular location of eNOS and moreover, where physiologically active eNOS resides within the endothelium remain uncertain, especially under “native” conditions found *in vivo*.

Caveolae are small plasmalemmal invaginations that can

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<sup>1</sup> The abbreviations used are: NO, nitric oxide; eNOS, endothelial nitric oxide synthase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; mAb, monoclonal antibody; MES, 4-morpholineethanesulfonic acid; U, unbound; B, bound; H, rat lung homogenate; P, silica-coated luminal endothelial cell plasma membranes; V, caveolae; P-V, plasma membranes stripped of caveolae.

<sup>2</sup> V. Rizzo, P. Oh, and J. E. Schnitzer, unpublished observations.

function in endothelium as dynamic vesicular carriers (25, 34). They may also act as organized cell surface signal transduction centers (27–29). The localization of signaling molecules within a small microdomain is likely to provide the proximity necessary for rapid, efficient, and specific propagation of signals to downstream targets (29, 30). Caveolin is a structural coat protein of caveolae that may act as a scaffold protein for a variety of lipid-anchored signaling molecules such as eNOS, G-proteins, Ras, and Src-like kinases (18–20, 31). *In vitro* recombinant protein-protein interaction studies suggest that caveolin may inhibit the functional activity of eNOS (18–20). The physiological relevance of this ability to associate as recombinant proteins in the test tube, however, remains undefined, especially under physiological conditions experienced *in vivo*. Here, we attempt to define eNOS localization in more definitive terms as it exists in endothelium *in vivo* as well as to examine the regulation and mechanism of eNOS activation under changing hemodynamic conditions *in vivo*.

#### EXPERIMENTAL PROCEDURES

***In Situ Vascular Perfusion***—Male Sprague-Dawley rats were anesthetized with a 3:1 mixture of 10 mg/ml ketamine and 10 mg/ml xylazine per 100 g body weight. Following tracheostomy and thoracotomy, the lungs were inflated with approximately 3/4 tidal volume. The pericardium was removed and 200 units of heparin was injected into the right ventricle as an anticoagulant. After the thymus was removed to fully expose the pulmonary artery, a small cut was made in the right ventricle through which a catheter was fed and secured into the pulmonary artery. The left atrium was cut to allow for outflow and vascular fluid flow (mammalian Ringer's solution at 37 °C) was immediately established through the rat lungs at a pressure of 8–10 mm Hg (4–5 ml/min) for 5 min followed by perfusion for an addition minute at either 8–10 mm Hg (4 ml/min), 12–14 mm Hg (6.5 ml/min), or 18–20 mm Hg (10 ml/min). Flow rate was controlled by a syringe pump (Harvard Apparatus) and pulmonary pressure monitored during lung perfusions.

***Purification of Endothelial Cell Luminal Plasma Membranes and Caveolae***—Immediately following vascular perfusion as described above, the luminal endothelial cell plasma membranes and then their caveolae were isolated using an *in situ* silica coating procedure described previously (22, 30, 32). Briefly, the vasculature was immediately cooled to 10 °C by perfusion with cold MES-buffered saline (20 mM, pH 6.0) followed by a positively charged colloidal silica solution. Cross-linking of the silica particles by subsequent perfusion with polyacrylic acid created a stable adherent silica pellicle that marked this specific membrane of interest. This coating firmly attaches to the plasma membrane to increase its density and permit its purification by centrifugation to separate the silica-coated endothelial cell plasma membranes (P) from the starting whole lung homogenate (H). The silica-coated membrane pellets have many associated caveolae and display ample enrichment for various endothelial cell surface markers (22, 30, 32). Because eNOS is solubilized in detergents such as Triton X-100, even at low temperatures, the attached caveolae were sheared away from P in a detergent-free environment and purified by sucrose gradient floatation as described in our past work (22, 30, 32). A membrane band (V) detected at a density of 15–20% sucrose contained a homogeneous population of caveolae amply enriched in caveolar markers and depleted of noncaveolar markers (22, 30, 32). The remaining silica-coated membrane pellet stripped of caveolae was labeled P-V.

***Immunoaffinity Isolation of Caveolae***—Membrane vesicles were immunoaffinity isolated from V as described in our past work (29, 33, 34). Briefly, caveolin antibodies (10 µg; Transduction Laboratories, Lexington, KY) were preabsorbed onto goat anti-mouse IgG-coated magnetic beads (Dynal, Oslo, Norway) by a 2–4-h incubation at 4 °C. The antibody-bead conjugates were washed and incubated for 1 h at 4 °C with purified caveolar fraction V (10–15 µg of protein). Beads with any attached membranes (B) were separated magnetically from unbound material (U), washed, and then processed for SDS-PAGE and immunoblotting with eNOS antibodies as described below.

***Immunoprecipitation of Protein Complexes***—The rat lung vasculature was perfused at either 8–10 mm Hg (4 ml/min) or 18–20 mm Hg (10 ml/min) as described above. Following purification of luminal endothelial cell surface membranes (P), 500 µg of total protein from these preparations were solubilized for 1 h at 4 °C with a buffered solution (20 mM MES, 20 mM KCl, 135 mM NaCl, 2 mM dithiothreitol, 0.1 mM each EDTA and EGTA, 4 mM tetrahydrobiopterin, and 1 mM L-arginine)

containing either 20 mM CHAPS (17) or 60 mM *n*-octyl-β-D-glucopyranoside (35) (both detergents gave equivalent results). Supernatant material was divided equally (100 µl volumes) and incubated for 1 h separately with either eNOS mAb or caveolin mAb-coated magnetic beads prepared as described above. Beads with any attached proteins (B) were separated magnetically from unbound material (U) and subjected to Western analysis. Similar control experiments were carried out using a purified nonspecific mouse IgG (Sigma) to assess for possible nonspecific binding of the solubilized proteins.

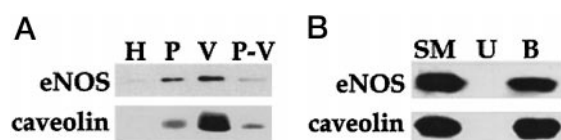
***Western Analysis***—Various membrane (H, P, V, and P-V) and immunoprecipitated protein samples were analyzed by SDS-PAGE (5–15% gradient gels) followed by electrotransfer to nitrocellulose filters for immunoblotting as in our past work (30, 33).

***eNOS Activity Assay***—Whole lung homogenate and purified luminal endothelial cell plasma membrane fractions were assayed for eNOS activity by measuring the conversion of L-[<sup>3</sup>H]arginine into L-[<sup>3</sup>H]citrulline as described previously (35, 36). Briefly, 5 µg of protein from H, P, V, or P-V subfractions were incubated (50 µl total volume) in assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 2 µM leupeptin, 1 µM pepstatin, 1 mM Pefabloc, 10 mM sodium fluoride, 1 mM vanadate) containing 1 mM NADPH, 4 µM tetrahydrobiopterin, 100 mM calmodulin, 2.5 mM CaCl<sub>2</sub>, 10 µM L-arginine and L-[<sup>3</sup>H]arginine (0.2 µCi, 55 Ci/mol) for 30 min at 37 °C. Addition of 1 ml of cold stop buffer (20 mM HEPES, pH 5.5, containing 2 mM of each EDTA and EGTA) served to quench the reaction. The reaction mixture was passed over a Dowex AG 50WX-8 resin packed (1 ml) column, washed with 1 ml of distilled water, and collected directly into a 20-ml liquid scintillation vial. In some experiments, caveolae isolated from either lowest or highest flow conditions were preincubated (15 min) with the NOS inhibitor, N<sup>G</sup>-mono-methyl-L-arginine (1 mM) and subsequently assayed for eNOS activity.

***Ultrathin Cyro- and Immunoelectron Microscopy***—Cryo-ultramicrotomy and immuno-gold labeling techniques were used to localize and quantify the distribution of eNOS in endothelium *in vivo* as described previously (26). For immunolabeling, thin frozen sections of the rat lung tissue were quenched with either 10% fetal calf serum or 0.5% fish skin gelatin (10 min) and incubated for 30 min with either the eNOS mAb or the control mAb TE-1, which does not recognize any protein in the lung by Western analysis. After washing (4 × 15 min in phosphate-buffered saline), sections were incubated with gold-labeled reporter secondary antibody (5 nm gold goat anti-mouse IgG) for 20–60 min. Labeled sections were extensively washed, contrasted with 2% methylcellulose, 3% uranyl acetate, and examined with a Philips 300 electron microscope operating at 60 kV.

#### RESULTS

***eNOS Concentrated on the Luminal Endothelial Cell Surface in Caveolae***—By using a purification scheme which permits isolation, directly from tissue, of the luminal endothelial cell plasma membranes normally exposed to the circulating blood which are then subfractionated to purify their caveolae (30), we found that eNOS was easily detected on the luminal endothelial cell plasma membranes (P) where it was enriched in the caveolae (5-fold more than P and 10–15-fold greater than plasma membranes stripped of their caveolae (P-V) (Fig. 1A). In these samples, as in our previous reports (30, 33), caveolin was enriched 15–25-fold in V relative to P. To be certain that eNOS was present within luminal plasma membrane caveolae, V was subjected to immunoaffinity isolation using a monoclonal antibody to caveolin. Consistent with a homogeneous population of caveolae in this fraction, silver stain following SDS-PAGE of immunisolated samples indicated that >95% of the protein contained in the starting material was found in the bound fraction (B) (37). As shown in Fig. 1B, Western analysis of the immunisolated caveolae revealed the presence of eNOS and caveolin in these same vesicles. Nearly all (>95%) the caveolin and eNOS in starting material was in B while little remained in the unbound fraction (U). Controls performed in the absence of primary antibody or with clathrin antibody showed little to no evidence of caveolae binding (37) and both eNOS and caveolin remained in U with no signal detected in B (data not shown). Thus, eNOS existed within the same low density caveolar vesicles that were coated with caveolin.



**FIG. 1. eNOS and caveolin are in the same caveolar vesicles.** *A*, proteins (2 mg) from indicated subcellular fractions of rat lungs (*H*, rat lung homogenate; *P*, silica-coated luminal endothelial cell plasma membranes; *V*, caveolae; *P-V*, plasma membranes stripped of caveolae) were subjected to Western analysis using eNOS and caveolin antibodies. Both eNOS and caveolin are concentrated in *V*. *B*, as the starting membrane material was subjected to affinity isolation using immunomagnetic beads with attached caveolin antibodies. The beads with any bound membranes (*B*) were separated magnetically from the unbound material (*U*). Both *U* and *B* were processed for Western analysis using eNOS and caveolin antibodies. The experiments shown are representative of at least two independent experiments. In each case, >90% of both the caveolin and eNOS signal was detected in the bound fraction when using the caveolin antibody.

**eNOS Immunolocalization to Caveolae**—To investigate caveolar localization of eNOS further, ultra-thin sections of whole rat lung tissue were probed with an eNOS monoclonal antibody and subsequently labeled with a gold-conjugated reporter antibody. Analysis by electron microscopy revealed significant eNOS labeling on the endothelial cell surface. As shown in Fig. 2, the eNOS distribution did not appear random over the endothelial cell surface but instead was found primarily within caveolae. eNOS was detected within single caveolae as well as caveolar clusters (Fig. 2, *A* and *B*). Caveolae associated with both the luminal and abluminal endothelial cell surface were labeled (Fig. 2*C*). In these same sections, much fewer gold particles was seen on other endothelial membranes including the Golgi network. Morphometric analysis (Table I) revealed that more than 60% (378 out of 615 particle counted) of the eNOS detected in the endothelium was associated with the plasma membrane with about one-half of the remaining 40% found to be “free” in the cytoplasm and the other 20% associated with other intracellular membranes. More importantly, the majority of eNOS (greater than 50% of all detectable eNOS) was found in caveolae. Thus, nearly 85% of the gold (eNOS) detected at the plasma membrane was associated with caveolae. Approximately 6.7 particles were found to be associated with each measured micron of caveolae membrane in comparison to only 0.92 particles per micron of plasmalemmal proper (Table I). Taken together with the biochemical analyses showing a similar level of enrichment in *V* versus *P*, it was apparent that eNOS resides primarily on the microvascular endothelial cell surface of the rat lung predominately concentrated in the caveolae.

**eNOS in Caveolae Is Functional**—The presence of eNOS in caveolin-coated caveolae does not necessarily confer functionality. In order to examine whether eNOS was enzymatically active at the endothelial cell surface and its caveolae, we incubated various membrane subfractions in an assay buffer system containing L-[<sup>3</sup>H]arginine and determined eNOS enzymatic activity by measuring its conversion to L-[<sup>3</sup>H]citrulline. eNOS in the caveolae purified from luminal membranes consistently demonstrated the greatest activity compared with the other membrane fractions tested (Fig. 3). The caveolar activity detected in *V* was more than 10-fold greater than that in the other fractions (*H*, *P*, and *P-V*), consistent with enrichment for eNOS in *V* as described above. Caveolar eNOS generated  $0.2 \pm 0.03$  pmol of L-[<sup>3</sup>H]citrulline/min/ $\mu$ g of protein. Past reports showed that immunoprecipitated eNOS obtained from whole cell lysates of untreated cultured bovine aortic endothelial cells ranged in activity from 0.1 to 2.0 pmol of L-[<sup>3</sup>H]citrulline/min/ $\mu$ g of protein (35). In addition, subcellular fractions containing caveolae from the cultured bovine aortic endothelial cells gen-

erated 0.01 pmol of L-[<sup>3</sup>H]citrulline/min/ $\mu$ g of protein (16). Our higher activity may represent differences in purity as well as differential levels, activity, and/or location (cytoplasm versus Golgi versus caveolae) of eNOS as it exists within the endothelium *in vivo* compared with cultured endothelial cells.

**Flow Activates Caveolar eNOS**—The luminal surface of the endothelium is directly exposed to the circulation and therefore is likely to be sensitive to hemodynamic forces. We recently showed that acute mechanotransduction from enhanced flow rates and pressures *in situ* occurs rapidly at the luminal cell surface primarily in caveolae (38). The localization of active eNOS within luminal surface caveolae may provide a physiological relevant site for flow-regulated activation of eNOS *in vivo*. We tested the rapid functional responsiveness of caveolar eNOS to hemodynamic forces *in situ* by increasing, for 1 min, the perfusion pressure and flow through the rat lung vasculature from 8–10 mm Hg (4 ml/min) to either 12–14 mm Hg (6.5 ml/min) or 18–20 mm Hg (10 ml/min). Caveolar eNOS activity in *V* was enhanced 1.5-fold when the vascular pressure and flow rate were increased to 12–14 mm Hg (6.5 ml/min). It increased further to 1.8-fold at 18–20 mm Hg (10 ml/min) (Fig. 3*B*). In this 1-min time period, the content of eNOS in *P* and *V* did not change with increasing flow (Fig. 3*C*). Thus, the observed enhancement of eNOS activity was not caused by flow-induced recruitment of eNOS to the caveolar compartment but rather an increase in eNOS enzymatic activity found in the caveolae.

To confirm that flow specifically activates caveolar eNOS, caveolae isolated from the microvascular endothelium of rat lungs subjected, *in situ*, to either lowest or highest flow conditions were preincubated with an L-arginine substrate analog, L-N<sup>G</sup>-mono-methyl-L-arginine. In these experiments, conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline was inhibited >85% (Fig. 4). Taken together, these observations indicated that eNOS, residing specifically within caveolae, was indeed responsive to a well known physiological vasoconstrictor, namely acute changes in vascular flow hemodynamics.

**Flow Induced Dissociation of eNOS-Caveolin Complex and Enhanced Association of eNOS-Calmodulin**—Our studies here showed that eNOS was compartmentalized on the endothelial luminal plasma membrane in the caveolar microdomain. More importantly, the caveolar eNOS could be activated by increasing pressure and flow through the rat lung vasculature *in situ*. Quite recently, *in vitro* studies using recombinant proteins to assess protein-protein interactions in a test tube have demonstrated that caveolin can bind to eNOS in a way that inhibits eNOS activity (18–20). Moreover, caveolin and eNOS derived from cultured bovine aortic endothelial cells have been shown to co-immunoprecipitate (17, 35). In this same system, the inhibitory eNOS-caveolin complex was disrupted by binding of Ca<sup>2+</sup>-calmodulin to eNOS and thereby serve as an allosteric modulator of enzyme activity (20, 39). Whether eNOS-caveolin and eNOS-calmodulin interactions even exist *in vivo* to provide a biologically relevant mechanism for regulating eNOS activity in the endothelium, especially in response to physiological effectors such as flow, remains unknown.

Here, we addressed this important question by utilizing the luminal endothelial cell plasma membranes (*P*) purified from rat lungs to examine the eNOS-caveolin and eNOS-calmodulin interaction as it exists *in vivo*. Under conditions where *P* was treated with a detergent (see “Experimental Procedures”) mild enough to liberate the eNOS-caveolin and eNOS-calmodulin complexes from the plasma membrane and yet apparently preserve native interactions, we found that caveolin, and to a much less extent calmodulin, could be co-immunoprecipitated with eNOS from samples subjected to pressure of 8–10 mm Hg

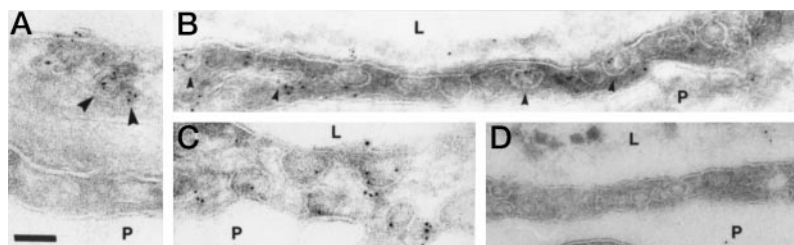


FIG. 2. **Immunolocalization of eNOS in caveolae of vascular endothelium by electron microscopy.** Rat lungs were perfused to remove blood, fixed and processed for cryo-ultramicrotomy and immunogold localization of eNOS (see "Experimental Procedures"). A, eNOS is detected predominately within luminal endothelial cell surface caveolae. A caveolar cluster (arrow) is amply labeled. B, a typical attenuated region of endothelium containing many eNOS-labeled caveolae (arrows). C, caveolae associated with both the luminal and abluminal cell surface contain eNOS. D, control primary antibody (TE-1) provides little to no gold-labeling in endothelium including caveolae. L, vessel lumen; P, perivascular space; bar in A and B = 91 nm; C = 86 nm; D = 146 nm.

TABLE I  
Analysis of eNOS localization in rat lung endothelium by immunogold electron microscopy

Ultrathin cryosections immunolabeled with the monoclonal antibody to eNOS were examined and photographed by electron microscopy. Electron micrographs taken at a magnification of  $\times 55,000$  were used for morphometric analysis as described previously (26). Au denotes the number of gold particles. Gold particles labeling the endothelium were counted (615 total) and their subcellular distribution was examined by assessing labeling associated with either plasma membranes (PM) or intracellular membranes (ICM) versus the gold labeling found apparently free in the cytoplasm. In this table, we provided the percentage distribution of the total number of gold particles counted in these compartments versus gold particles associated with all membranes or the plasma membrane. On close examination of the plasma membrane, gold particles in direct association with the non-caveolar plasma membrane (Proper) were scored separately from those directly labeling the caveolae. Also, the number of gold particles per  $\mu\text{m}$  of membrane was calculated for the caveolae and PM proper.

	Endothelial cell			Plasma membrane	
	PM	Cytoplasm	ICM	Proper	Caveolae
% of total Au	61.5	19.5	19	9	52.5
% of Au associated with membranes	76.4		23.6	11.2	65.2
% of Au associated with PM				14.6	85.4
Au/ $\mu\text{m}$ membrane				$0.92 \pm 0.62$	$6.7 \pm 1.6$

4–5 ml/min flow rates) (Fig. 5). Interestingly, after just 1 min of increased flow *in situ*, the caveolin signal detected in primary eNOS immunoprecipitates showed a loss of association and decreased an average of 2.4-fold after acute flow while the amount of immunoprecipitable eNOS remained the same (Fig. 5). On the other hand, calmodulin increased 1.8-fold with acute flow in these same eNOS precipitates. Similarly, the eNOS signal in the caveolin immunoprecipitates decreased by an average of 3-fold while the caveolin signal, as expected, remained constant. A very weak calmodulin signal was detected and remained constant in both caveolin precipitates. Thus, eNOS *in vivo* did indeed associate with caveolin at the luminal endothelial cell membrane surface. More importantly, under conditions where flow potentiated eNOS activity, eNOS did rapidly dissociate from caveolin with a reciprocal and concomitant rapid association of eNOS with calmodulin in response to a physiological stimulus (flow).

#### DISCUSSION

NO has been identified as an endothelial relaxing factor (1, 6, 40) which is released by changes in flow (10–13). The mechanisms of this basic cardiovascular response leading to activation of eNOS to generate NO has remained elusive, especially *in vivo*. Based on the short half-life of NO and the physiological effects that activate NO production such as shear stress, it is somewhat perplexing that in culture, eNOS appears to reside

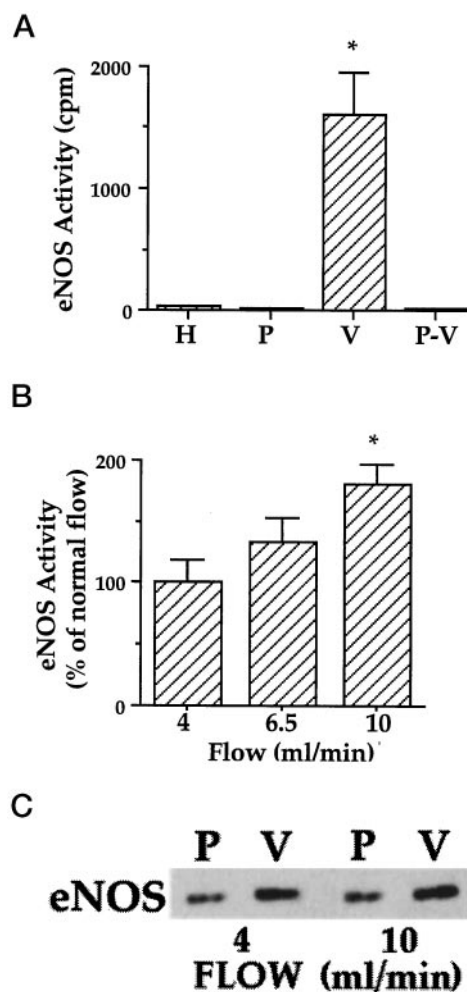
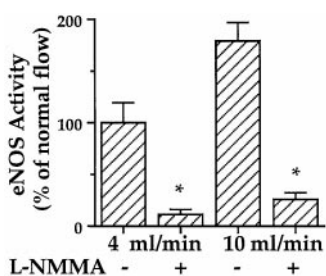
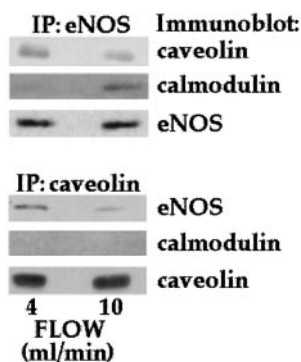


FIG. 3. **In situ flow activation of eNOS in caveolae at the luminal endothelial cell surface.** A, rat lungs were perfused at 4 ml/min (8–10 mm Hg) before their subfractionation (see "Experimental Procedures"). Proteins (5  $\mu\text{g}$ ) from indicated fractions were assayed for eNOS activity which was detected primarily within V. B, endothelial caveolae were purified from rat lungs subjected to the indicated perfusion rates and assayed for eNOS activity. Caveolar eNOS activity increased as the rate of flow increased. C, Western analysis of P and V following either 4 or 10 ml/min flow (8–10 and 18–20 mm Hg, respectively) *in situ*. Increases in eNOS activity is not the result of additional eNOS being recruited to the plasma membrane of enhanced flow *in situ* after 1 min. Results shown in A and B are mean  $\pm$  S.D. from three independent experiments (\*,  $p < 0.05$ ).

primarily in the Golgi and cytoplasmic compartments. It seems logical to expect that, at least for the flow responsive pool of eNOS, the enzyme would reside at the endothelial cell surface, especially the luminal surface which is directly exposed to the



**FIG. 4. Inhibition of flow-induced eNOS activation.** Caveolae were purified from rat lungs subjected to flow rates of either 4 or 10 ml/min (8–10 and 18–20 mm Hg, respectively). These caveolae were incubated with the eNOS inhibitor *N*<sup>G</sup>-mono-methyl-L-arginine (NMMA, 1 mM) before measuring eNOS activity. L-*N*<sup>G</sup>-mono-methyl-L-arginine blocked eNOS activity (>85%) in both cases (\**p* < 0.05). Results shown are mean ± S.D. from three independent experiments.



**FIG. 5. Flow induces eNOS to dissociate from caveolin and associate with calmodulin.** Western analysis for the indicated proteins were performed after immunoprecipitation (IP) of eNOS and caveolin from detergent-extracted luminal endothelial cell plasma membranes (see “Experimental Procedures”) purified from rat lungs that were subjected to either 4 ml/min (8–10 mm Hg) or 10 ml/min (18–20 mm Hg) *in situ* flow. This experiment was repeated 3–5 times with equivalent results.

forces of the circulation *in vivo*. Using immunogold electron microscopy as well as a unique methodology for purifying the luminal endothelial cell plasma membranes directly from tissue and subfractionating them to isolate plasmalemmal microdomains such as the caveolae (30), we show here that the luminal cell surface of the rat lung microvascular endothelium *in vivo* contains ample eNOS. Moreover, this eNOS appears to be concentrated in the caveolae where it is catalytically active and responds with enhanced activity to increasing flow through the rat lung vasculature *in situ*. Both immunoaffinity isolated caveolar vesicles and morphometric analysis of ultra-thin cryosections of rat lung endothelium probed for eNOS confirmed its subcellular location to caveolae at the luminal cell surface. Flow-induced eNOS activation is associated with a rapid decrease in eNOS and caveolin association whereas eNOS-calmodulin association is enhanced (after only 1 min of increased flow *in situ*). Thus, the release of eNOS from its inhibitory clamp, caveolin, may rapidly activate the enzyme which may be further potentiated by the binding of Ca<sup>2+</sup>-calmodulin, thereby providing a physiologically important mechanism for flow regulated NO production. Taken together, these data show that the endothelial cell surface and its caveolae from the rat lung vasculature are the primary subcellular site for eNOS and its flow-responsive activity *in situ*.

It has recently been reported that eNOS and other lipid-anchored signaling molecules are not present within caveolae (24). In this past study, immunoisolation of caveolae was performed overnight using a polyclonal antiserum made to a caveolin peptide. The caveolin antibody (monoclonal) used in

our studies recognizes caveolin oligomerized in caveolae and requires only a 1-h (not overnight) incubation to effectively immunoisolate >95% of the caveolae (Fig. 1B). This is significant because eNOS dissociates from the plasmalemma and caveolar membranes in a time-dependent manner (37). The caveolae in V are enriched in eNOS and after 1 h of incubation, eNOS is detected nearly completely in the caveolar immunoisolates (Fig. 1B). However, after overnight incubations, eNOS moves out of the caveolae into the soluble phase and is minimally detected in the immunisolated caveolae (37). Thus, performing the immunoisolations for brief periods preserved the original distribution of eNOS in caveolae and prevented artifactual dissociation of eNOS into solution. Our finding of enrichment of eNOS in caveolae is ultimately confirmed by our immunogold electron microscopy showing eNOS primarily in the caveolae of rat lung microvascular endothelium (Fig. 2).

The model for a role of caveolae in mechanotransduction were proposed in 1995 (41). Fluid mechanical forces may be transduced through caveolae as “mechanosensors” to initiate a signaling cascade that ultimately elicits appropriate endothelial cell responses to flow. Here, we show one such response, namely the specific flow-activation of eNOS in caveolae. It is known that the initial release of NO is dependent upon G-protein activation and increases intracellular Ca<sup>2+</sup> levels (42). Various G-proteins as well as Ca<sup>2+</sup>-ATPase and inositol 1,4,5-trisphosphate-activated channels have been found to reside in caveolae of endothelium (29, 30, 43). Other studies from this laboratory indicate that increased flow through the rat lung vasculature induces a rapid regionalized protein phosphorylation cascade that requires intact caveolae and involves local translocation of key signaling molecules (22, 38).<sup>2</sup> Therefore, caveolar compartmentalization of not only eNOS but several signaling molecules implicated in modulating eNOS activity, such as G-proteins, Ca<sup>2+</sup> entry regulators, and protein kinases and phosphatases, may serve to bring eNOS and its molecular modulators into close proximity for rapid, specific, and efficient mechanotransduction.

Caveolin may play a direct role in mechanotransduction, and especially in eNOS regulation, at the cell surface in caveolae. Because caveolin oligomerizes to form the substructural coat of the caveolae and its invaginated form (27, 44, 45), this geometric configuration may act as a loaded, tension-bearing, coiled spring acutely responsive to changes in hemodynamic forces experienced at the luminal cell surface. Hemodynamic forces are known to impose a strain on caveolae which can distort them (46) and may, for instance, modulate caveolin conformation sufficiently to permit local activation and translocation events. Because the immediate flow-activation of caveolar eNOS is not associated with an increase in eNOS content (*i.e.* translocation) (Fig. 2C), it is possible that caveolae permit activation of eNOS by a stress or pressure-induced alteration in caveolin conformation that releases eNOS from its functionally inhibitory clamp, caveolin. In addition, other possible effectors may also contribute to regulating eNOS activity such as protein phosphorylation (35, 47) as well as positive allosteric modulators of eNOS, such as calmodulin (20) and Hsp 90 (48). Indeed, here we show that Ca<sup>2+</sup>/calmodulin, which appears to be required for eNOS activity (20), can rapidly associate with eNOS after initiation of acute flow events *in situ*. (Fig. 4). One must also consider mechanical effectors located at the neck of caveolae that may participate in the transmission of certain mechanical stressors such as shear into the caveolae or even directly to caveolin. Recently, our laboratory has shown that dynamin forms an oligomeric structural collar around the neck of caveolae functioning in the fission of caveolae and their internalization (26). Obviously, further studies are needed to determine

whether these caveolar proteins play a role in caveolae-mediated mechanotransduction. Future studies focusing on caveolae, especially mechanostuctural relationships at the molecular level, and investigating flow activation of caveolin-associated signaling molecules and its downstream effectors, particularly in regard to eNOS activation, would greatly add to our understanding of mechanotransduction within caveolae of endothelium.

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