Novel Mechanism of Endothelial Nitric Oxide Synthase Activation Mediated by Caveolae Internalization in Endothelial Cells

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Abstract—Caveolin-1, the caveolae scaffolding protein, binds to and negatively regulates eNOS activity. As caveolin-1 also regulates caveolae-mediated endocytosis after activation of the 60-kDa albumin-binding glycoprotein gp60 in endothelial cells, we addressed the possibility that endothelial NO synthase (eNOS)-dependent NO production was functionally coupled to caveolae internalization. We observed that gp60-induced activation of endocytosis increased NO production within 2 minutes and up to 20 minutes. NOS inhibitor N\textsuperscript{G}-nitro-L-arginine (L-NNA) prevented the NO production. To determine the role of caveolae internalization in the mechanism of NO production, we expressed dominant-negative dynamin-2 mutant (K44A) or treated cells with methyl-\(\beta\)-cyclodextrin. Both interventions inhibited caveolae-mediated endocytosis and NO generation induced by gp60. We determined the role of signaling via Src kinase in the observed coupling of endocytosis to eNOS activation. Src activation induced the phosphorylation of caveolin-1, Akt and eNOS, and promoted dissociation of eNOS from caveolin-1. Inhibitors of Src kinase and Akt also prevented NO production. In isolated perfused mouse lungs, gp60 activation induced NO-dependent vasodilation, whereas the response was attenuated in eNOS\(^{-/-}\) or caveolin-1\(^{-/-}\) lungs. Together, these results demonstrate a critical role of caveolae-mediated endocytosis in regulating eNOS activation in endothelial cells and thereby the NO-dependent vasomotor tone. (\textit{Circ Res.} 2006;99:870-877.)

Key Words: caveolin-1 ■ endocytosis ■ vasomotor tone ■ albumin ■ transcytosis

Endothelial NO synthase (eNOS) is modified by N-myristoylation and palmitoylation, which targets the enzyme to caveolae,\(^1\) the plasma membrane cholesterol-rich microdomains.\(^2,3\) Multiple mechanisms are involved in regulating NO production following eNOS activation. eNOS activity is regulated by Ca\(^{2+}\)-calmodulin, phosphorylation activated by kinases such as Src and interactions with caveolin-1, dynamin, and heat shock protein 90 (HSP90).\(^4\) The effects of phosphorylation are complex. Phosphorylation of Ser116 and Thr497 negatively regulates eNOS activity, whereas phosphorylation at Ser635 and Ser1179 has the opposite effect.\(^5-7\) Src kinase activated eNOS by inducing phosphorylation at Tyr83.\(^8\) Phosphorylation at Ser617 functions by affecting the phosphorylation of the above residues.\(^9\) Insulin, estrogen, and shear stress were shown to induce phosphorylation-dependent activation of eNOS at Ser1179 independent of increased intracellular [Ca\(^{2+}\)].\(^10-12\)

eNOS in caveolae is held inactive by its association with caveolin-1,\(^13\) but eNOS activity can be increased by Ca\(^{2+}\)/calmodulin\(^3\) and binding to HSP90 and dynamin-2.\(^14,15\) HSP90 facilitates the phosphorylation of eNOS by forming a ternary complex with eNOS and Akt.\(^16\) Dynamin-2 regulates eNOS activity through the binding of its proline-rich domain to the FAD domain of eNOS, promoting electron transfer between the bound flavins of the reductase domain and increasing NO production.\(^15\)

We have shown that activation of the albumin-binding protein gp60 in microvascular endothelial cells (ECs) induces Src activation,\(^17,18\) resulting in phosphorylation of both dynamin-2 and caveolin-1.\(^18,19\) This response required activation of the heterotrimeric GTP-binding protein, G\(_i\), and release of the heterodimer G\(_{\beta\gamma}\).\(^19\) The G\(_{\beta\gamma}\)-induced Src kinase activation signaled the scission of caveolae from the endothelial cell plasma membrane.\(^17-21\) Because caveolin-1 regulates eNOS activity and caveolae-mediated endocytosis, we addressed the possibility that these are coupled processes requiring activation of similar signaling pathways. In the present study, we determined the role of caveolae-mediated endocytosis induced by caveolin-1 phosphorylation in signaling the uncoupling of eNOS from caveolin-1 and regulating NO production.
Materials and Methods

Antibodies

Rabbit anti-eNOS antibody and goat anti-phospho-Ser1179-eNOS antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif); gg60 antibody was generated as described.13 Rabbit anti-phospho-Ser73-Akt antibody was purchased from Cell Signaling Technology (Danvers, Mass); mouse pTyr14-caveolin-1 antibody and rabbit anti-caveolin-1 antibody were purchased from Transduction Laboratories (Franklin Lakes, NJ).

Reagents

Fraction V BSA was purchased from Fisher Scientific (Pittsburgh, Pa), 2× recrystallized BSA from ICN Biomedicals (Solon, Ohio), acetylated BSA from Electron Microscopy Sciences (Hatfield, Pa), and 25% BSA solution in Tyrode’s buffer (used in isolated lung studies) from Sigma Chemical Co (St Louis, Mo). P22 (4-amino-5-(4-chlorophenyl)-7-(1-buty1)pyrazolo[3,4-d]pyrimidine), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), A23187 (calcimycin), SH-6 (0-2,3-dideoxy-myo-inositol 1-(R)-2-methoxy-3-(octadecyloxy)propyl hydrogen phosphate), and mSRRK (myristoylated-SIRKALNILGYPYD) were purchased from Calbiochem (La Jolla, Calif). All other reagents were purchased from Sigma unless otherwise indicated.

Cell Culture and Transfection

Rat lung microvascular endothelial cells (Vac Technologies, Rensselaer, NY) were cultured using DMEM (Invitrogen, Carlsbad, Calif) supplemented with 10% FBS, 100 U/mL penicillin, and 50 µg/mL streptomycin as described.23 Transfection withAdv-c-ARK1 and Dyn-2-K44A were performed as described.18,19 The cultures were maintained in 5% CO2/95% air at 37°C.

Mice

Animal studies were approved by the University of Illinois Animal Care and Use Committee. Caveolin-1 knockout (Cav1−/−; Cav1+/#/#) breeding pairs were purchased from The Jackson Laboratory (Bar Harbor, Me). Adult male Black Swiss mice were purchased from Taconic (Hudson, NY) for use as strain-matched controls. Adult female C57Black6 and age matched eNOS knockout mice (eNOS−/−) were purchased from The Jackson Laboratory.

NO Measurement

NO produced by cells cultured on 6-well plates was measured using a porphyrinic NO electrode as described.24 Briefly, the electrode is created by coating carbon fibers with a metalloporphyrinic conductive polymer and subsequently sealed with Nafion. Each electrode is created by coating carbon fibers with a metalloporphyrinic conductive polymer and subsequently sealed with Nafion. The electrode is then inserted into the Nafion membrane where it is oxidized to the nitrosyl ion. The electron is transferred to the porphyrin of the conductive polymer and proceeds along the copper wire to a detector. The NO electrode is placed onto the surface of an endothelial cell monolayer and 2 additional electrodes are added to the solution to generate a 650-mV potential. The system was coupled with a FastS femtostat and a personal computer with electrochemical software (Gamry Instruments, Warminster, Pa). Electrode current, which is proportional to NO concentration, is measured as a function of time. The cell culture medium temperature is kept at 37°C.

Intracellular Ca2+ Measurements

Intracellular Ca2+ was measured using a ratiometric fluorescence detection method using fura-2 AM (5-oxazoledioneoxycarbonyl acid, 2-(6-(bis-(2-acetyloxy) methoxy)-2-oxoethyl)amino)-5-(2-(2-(bis-(2-acetyloxy)methoxy)-2-oxoethyl)amino)-5-(methylphenoxy)ethoxy)-2-(benzofuranyl)), (acetyloxy)methyl ester). NO production was measured in cells treated with BAPTA-AM (1.2-bis(2-aminoethoxy)ethane-N,N,N′,N′-tetra-acetic acid) with albumin stimulation. Cells grown on 25-mm diameter glass coverslips was washed twice with Hank’s balanced salt solution (HBSS). Cells were loaded with either 3 µmol/L fura-2 AM alone or with 10 µmol/L BAPTA-AM for 30 minutes at 37°C. Cells were imaged using an Attofluor RatioVision digital fluorescence microscopy system (Atto Instruments, Rockville, Md) equipped with a Zeiss Axiovert S100 inverted microscope and F-Fluar ×40, 1.3 NA oil immersion objective. Regions of interest in individual cells were marked and excited at 334 and 380 nm with emissions collected at 520 nm at 5-second intervals. At the end of each experiment, 10 µmol/L ionomycin was used to obtain fluorescence of Ca2+ -saturated fura-2 (high [Ca2+]) and 10 mM/L EGTA to obtain fluorescence of free fura-2 (low [Ca2+]). [Ca2+]0 was calculated based on a dissociation constant (Kd) of 225 mM/L with a 2-point curve fit.

Immunostaining and Confocal Microscopy

Confluent RLMVECs were washed with PBS, fixed, permeabilized, and stained with anti-eNOS polyclonal antibody (1 µg/mL) and the nuclear marker 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/mL), as described.19 A Zeiss LSM 510 META microscope was used for confocal microscopy. Nonconfocal DAPI images were acquired using Hg lamp excitation and UV filter set with the LSM 510. Fluorescence emission was detected in optical sections <1 µm in thickness (pinhole set to achieve 1 Airy unit).

Immunoprecipitation and Western Blotting

For Western blot analysis, cells were lysed with lysis buffer (30 minutes at 4°C in 50 mM/L Tris-HCl, pH 7.5, containing 150 mM/L NaCl, 1 mM/L EDTA, 0.25% sodium deoxycholate, 1.0% Nonidet P-40, 0.1% sodium dodecyl sulfate, 1 mM/L Na3VO4, 1 mM/L NaF, 44 µg/mL phenylmethylsulfonyl fluoride, and protease inhibitor mixture) and insoluble materials were removed by centrifugation (14 000g for 15 minutes). For immunoprecipitations, the lysates were incubated with 10 µg/mL primary antibodies overnight at 4°C followed by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) for 4 hours. Proteins were then run on a 5% to 20% gradient SDS-PAGE gel, transferred to a nitrocellulose membrane, and blotted with the appropriate primary antibody overnight at 4°C and with horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature. Proteins were detected using an ECL kit (Amersham, Piscataway, NJ).

Endothelial Nos and Akt Phosphorylation

Rat lung microvascular endothelial cells (RLMVECs) were grown on 60-mm dishes to 80% to 90% confluence, subsequently serum deprived for several hours in serum-free DMEM, then treated with BSA (30 mg/mL) for various time periods ranging from 30 seconds to 30 minutes. Cell lysates were used for Western blot analysis as described above. In experiments designed to assess inhibition of phosphorylation of eNOS and Akt, cells were pretreated for 20 minutes with inhibitors, BSA was added to activate gg60, and subsequently the cells were lysed and processed as above.

Mouse Lung Preparation

Mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (0.3 mL/30 g of a 10 mg/mL solution) and xylazine (0.1 mL/30 g of a 2 mg/mL solution) to facilitate surgical anesthesia. Heparin was administered intravenously via retroorbital injection and following tracheostomy; the lungs were ventilated with room air at a positive pressure of 15 cm H2O. A thoracotomy was performed, PE10 tube was inserted into the pulmonary artery through the right ventricle, and the left atrium was incised. Pulmonary arterial pressure was monitored continuously through the pulmonary cannula, which was connected to a fluid-filled pressure transducer. The lungs were subsequently perfused with HEPES-buffered RPMI medium 1640 (pH 7.4) at the baseline flow of 1.8 mL/min, and pulmonary venous pressure was held constant. After a period of 15 minutes, isogravimetric conditions were achieved, defined as stable pressure and lung weight (which was also continuously monitored as described18). A continuous infusion of the stable thromboxane A2 mimetic U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α) (100 mM/L) was then initiated to induce vasoconstriction and was maintained until the end of the experiment. Endothelin-1 (400 mM/L) was infused as a bolus for 5 minutes and then washed out for...
5 minutes. Administration of these vasoactive substances resulted in an increase in pulmonary arterial pressure of 2 to 3 times the baseline. At the point of maximum vasoconstriction, the perfusate was switched to HEPES-buffered RPMI medium 1640 with 2% BSA to activate gp60 and the pressure response of the lung preparation was recorded. Pulmonary vascular resistance (PVR) (cm H₂O/mL per minute) was derived as the ratio of the pulmonary arterial pressure (Ppa) and left atrial pressure divided by the perfusate flow.

**Statistical Analysis**

Data are presented as mean±SEM. Means were compared using 1-way ANOVA. Dunnett’s test was used for post hoc comparison of experimental groups to controls.

**Results**

**Ca²⁺-Independent eNOS Activation**

We previously showed that albumin binding to gp60 induces Gp60-activated Src kinase signaling and activation of caveolae-mediated endocytosis.17–23 To address the role of endocytosis in regulating activation of eNOS, RLMVECs were serum deprived for 2 hours and BSA (5 mg/mL) was added while measuring NO production using the porphyrin-coated electrode. This increased NO production lasting up to 20 minutes compared with the transient response observed in cells stimulated with Ca²⁺ ionophore, A23187 (2 µmol/L) (Figure 1a). Albumin did not increase intracellular [Ca²⁺] (Figure 1b), and preincubation of RLMVEC with 1 µmol/L BAPTA-AM (intracellular Ca²⁺ chelator) had no effect on NO production compared with control (Figure 1c).

**NO Production Induced by Gp60 Activation**

We observed that albumin-activated NO production was saturable between 20 to 30 mg/mL albumin with an EC₅₀ of 5 to 7 mg/mL albumin (Figure 2A). EC₅₀ value of 100 µmol/L albumin (6.5 mg/mL) (n=6/point). This concentration/effect relationship is indicative of a receptor-mediated event and similar to the dose-response relationship of gp60-induced endocytosis (see inset, replotted from John et al,23 with permission). B. Similarity between gp60 cross-linking and albumin-induced NO generation. Cold-alcohol precipitated and endotoxin-free Cohn fraction V BSA, double-recrystallized BSA, highly pure albumin, at 30 mg/mL, increased NO production (NO concentration present at 20 minutes is tabulated). The amount of NO produced in response to anti-gp60 antibody (Ab) (10 µg/mL), fraction V BSA (fV-BSA), or doubly recrystallized BSA (2xC-BSA) were not statistically different. A partly linearized and acetylated form of BSA (Acet-BSA) (30 mg/mL) induced significantly less NO production compared with gp60 cross-linking or native BSA, indicating the importance of charge and/or tertiary structure for the albumin-mediated effect (n=6 wells/group).
Dissociation of eNOS From Caveolin-1
We observed punctate localization of eNOS in vesicle-like structures in endothelial cells (Figure 3A). Caveolin-1 and eNOS were coimmunoprecipitated using anti-eNOS antibody (Figure 3B). The association was markedly reduced following gp60 activation used to induce caveola internalization.20 Gp60 induced the phosphorylation of caveolin-1 (p-Cav1-Tyr14), peaking after 1 minute. Akt phosphorylation (p-Akt-Ser473) steadily increased for up to 15 minutes, at which time it declined. Phosphorylation of eNOS (p-eNOS-Ser1179) showed a biphasic response, increasing at 1 minute and again at 30 minutes. Results shown are representative of 3 experiments.

Gβγ Dependence of NO Production
We measured NO production in endothelial cells transfected with adenoviral vector containing the C terminus of β-adrenergic receptor kinase (ct-βARK), a selective inhibitor of Gβγ signaling.23 Ct-βARK expression reduced gp60-activated NO production by 72% indicating the important involvement of Gβγ in signaling both NO production and caveolae-mediated endocytosis (Figure 4). This role of Gβγ was directly addressed using mSIRK, an independent activator of Gβγ-signaling.19,28 We observed that mSIRK stimulated NO production independent of gp60 activation and the effect was prevented in endothelial cells expressing ct-βARK (Figure 4).

Impaired NO-Dependent Pulmonary Vasodilation Induced by Gp60 Activation in Caveolin-1–Null Mice
We used nonblood perfused mouse lungs, which were perfused at constant flow, to address the effects of gp60 activation on eNOS activation and NO production in the intact microcirculation. In lungs prevasoconstricted with the thromboxane A2 mimetic U46619, infusion of albumin to activate gp60 resulted in phosphorylation of caveolin-1 following gp60 activation (Figure 5B). Preconstriction with endothelin-1 also decreased Akt and eNOS phosphorylation induced by gp60 (Figure 5A). Intact caveolae were also required for NO production (Figure 5A) and the gp60-mediated increase in NO production was markedly reduced in eNOS+/− and caveolin-1−/− mouse lungs. Similar results were obtained in lung preparations...
Gp60 activation by either means activated the Src kinase signaling pathway.\textsuperscript{17–21} Cohn fraction V, fatty acid-free, and double-recrystallized albumin used to activate gp60 induced NO production, whereas denaturation of albumin did not result in NO production. The effect of albumin on NO production was also saturable, similar to the effect of albumin in activating gp60.\textsuperscript{23} The sigmoid concentration-response curve showed maximal NO production between 20 to 30 mg/mL albumin, the range of the normal plasma albumin concentration to which endothelial cells are exposed. The concentration-response curve correlated precisely with the EC\textsubscript{50} of albumin-activated endocytosis,\textsuperscript{23} suggesting that the plasma concentration bathing endothelial cells is itself an important determinant of the constitutive NO production.

We have demonstrated previously that gp60-induced phosphorylation of caveolin-1 and Src is required for the engagement of the signaling machinery responsible for caveolae-mediated endocytosis.\textsuperscript{17} As Src also phosphorylates eNOS via the PI3K/Akt pathway,\textsuperscript{30} we investigated the possible relationship between caveolae-mediated endocytosis and eNOS-derived NO production. Activation of gp60 rapidly induced eNOS and Akt phosphorylation with concomitant caveolin-1 phosphorylation at Tyr14. Importantly, eNOS became uncoupled from caveolin-1 following gp60 activation, suggesting that Akt mediates the response downstream of PI3K.

Using PP2, a Src kinase inhibitor, and SH-6, an Akt inhibitor, we observed that inhibition of G\textsubscript{βγ}-subunits with the synthetic peptide mSIRK induced NO production independent of gp60 and this response was abolished in ct-βARK–transfected cells. These findings demonstrate an important role for G\textsubscript{βγ} in signaling NO production, consistent with the functional coupling of eNOS-derived NO production with caveolae-mediated endocytosis.

In a series of studies, we addressed the signaling roles of Src kinase and Akt in activating eNOS induced by gp60. Using PP2, a Src kinase inhibitor, and SH-6, an Akt inhibitor, we observed that both inhibitors significantly reduced NO production induced by gp60 activation. This was associated with reduced Akt and eNOS phosphorylation. However, the decreased NO production ranged from 40% to 80%, suggesting that other pathways are also involved in signaling eNOS activation by gp60. Additionally, the inhibitor of PI3K, LY294002, abrogated the Akt and eNOS phosphorylation induced by gp60, suggesting that Akt mediates the response downstream of PI3K.

To address the relationship of eNOS activation to endocytosis, methyl-β-cyclodextrin, a cholesterol-depleting agent, and Dyn-2 K44A, a GTPase-defective form of dynamin-2, were used to block caveolae-mediated endocytosis. Cyclo-dextrin functions by disrupting the caveolae structure,\textsuperscript{23,31} and dynamin mutant prevents scission of caveolae from the membrane.\textsuperscript{18} We have shown that both agents effectively

**Discussion**

This study shows that activation of the albumin-binding protein gp60, which signals caveolae-mediated endocytosis in pulmonary microvascular endothelial cells,\textsuperscript{17} induces eNOS activation and NO production. The gp60-mediated eNOS activation occurred by a Ca\textsuperscript{2+}-independent mechanism. Quenching the Ca\textsuperscript{2+} signal with the intracellular Ca\textsuperscript{2+} chelator BAPTA-AM had no effect on NO production. We used 2 independent approaches to activate gp60: (1) gp60 cross-linking involving a primary rabbit anti-gp60 antibody\textsuperscript{22} and (2) ligation of gp60 by albumin addition to serum-deprived endothelial cells.\textsuperscript{20} In previous studies, gp60 cross-linking was shown to promote plasmalemmal gp60 clustering.\textsuperscript{17}
promoting vasodilation. In this preparation, on the basis of
addressing the role of gp60-activated NO production in
maintaining solution at constant flow and venous pressure to
lungs perfused with nonblood RPMI medium 1640–con-
motor tone. We assessed the vasomotor tone of mouse
sis, specifically its involvement in the regulation of vasculo-
production associated with caveolae-mediated endocyto-
plasma membrane is a crucial mechanism of NO produc-
(93/350) supports an important role of caveolae
endothelium to regulate vascular tone. Other vasodilators, such
as carbon monoxide32 and prostacyclin,33 release of which
have been localized to caveolae, may also be generated
secondary to caveolae-mediated endocytosis. In addition,
albumin likely interacts in multiple ways with the endo-
from eNOS- or caveolin-1–null mice, suggesting that
disruption of caveolae internalization partially reduced NO
significantly reduced, but it was not prevented in lungs obtained
from eNOS knockout (n=5) and caveolin-1 knockout mice (n=4). L-NAME–pre-
treated lungs (100 μmol/L) showed similar responses to those observed in eNOS
knockout lungs. Data are expressed as percentage of pulmonary vascular resis-
tance (PVR) recorded during U46619 infusion (^P<0.05 vs wild type). B, Exam-
ple tracing of an experiment shown in a
contrasts the vasodilation seen in eNOS
knockout and wild-type mouse lungs in
which pulmonary vessels were precon-
stricted. In both cases, activation of
gp60 led to initial, transient pressure
increase followed by rapid vasodilation in
wild-type mouse lungs; a much slower
and overall attenuated response in eNOS
knockout lungs. C, Gp60-induced vaso-
dilation of endothelin-1 (40 nmol/L) pre-
constricted lungs (n=4). Endothelin-1 was infused for 5 minutes followed by 5
inutes washout period before introduc-
Figure 6. Albumin induced vasorelaxation
of preconstricted perfused lung
preparations. A, RPMI medium 1640–
perfused mouse lungs (perfused at con-
stant flow) were treated with a continu-
ous infusion of 100 nmol/L U46619,
resulting in a 2- to 3-fold increase in pul-
monary arterial pressure. Significant
vasodilation was observed following
addition of 2% albumin-containing RPMI
medium 1640 in wild-type (WT) mouse
lungs to activate gp60 (n=6). This effect
was significantly reduced in lungs from
eNOS knockout (n=5) and caveolin-1
knockout mice (n=4). L-NAME–pre-
treated lungs (100 μmol/L) showed simil-
ar responses to those observed in eNOS
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dilation of endothelin-1 (40 nmol/L) pre-
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inutes washout period before introduc-
ction of 2% albumin containing perfusate to activate gp60 (red tracing). After withdrawal of BSA from the preparation and perfusion with
RPMI medium 1640 alone, there was rebound of vasoconstriction. Comparison is made to endothelin-treated preparation without the
addition of BSA (black tracing, n=3). D, intact mouse lungs were perfused with RPMI medium 1640 containing 2% albumin at baseline
followed by vasocostriction with U46619 (100 nmol/L). After steady-state pulmonary arterial pressure was achieved, perfusing solution
was changed to RPMI medium 1640 containing 2% IgG. This change was associated with a greater increase in U46619-induced vaso-
constriction (n=3).

blocked caveolae-mediated endocytosis induced by
gp60.18,23 In the present study, they resulted in ∼50% reduction in NO production. This finding coupled with
similarities between EC50 of albumin-mediated endocytosis
(93 μmol/L) and EC50 of gp60-mediated NO production
(100 μmol/L) supports an important role of caveolae
endothelium in the mechanism of NO production. As
disruption of caveolae internalization partially reduced NO
production, it is probable that other mechanisms are also
involved. One possibility is the recent finding that dy-
namin directly regulates eNOS activity independent of the
scission function of dynamin.29 Thus, although activation
of eNOS resulting from scission of caveolae from the
plasma membrane is a crucial mechanism of NO produc-
tion as identified in the present study, it is clear that eNOS
is also subject to regulation by other signaling pathways.

We next determined the functional significance of NO
production associated with caveolae-mediated endocyto-
sis, specifically its involvement in the regulation of vaso-
motor tone. We assessed the vasomotor tone of mouse
lungs perfused with nonblood RPMI medium 1640–con-
taining solution at constant flow and venous pressure to
address the role of gp60-activated NO production in
promoting vasodilation. In this preparation, on the basis of
the Poiseuille’s equation, any change in pulmonary artery
pressure directly reflects a change in pulmonary vasomotor
tone. We observed that gp60 activation induced rapid and
profound dilation of vessels preconstricted with either
endothelin-1 or U46619. This response was independent of
sex and strain, at least in the 3 mouse strains tested (CD1,
Swiss Black, C57/Black6). The vasodilation was signifi-
cantly reduced, but it was not prevented in lungs obtained
from eNOS- or caveolin-1–null mice, suggesting that
albumin likely interacts in multiple ways with the endo-
thelium to regulate vascular tone. Other vasodilators, such
as carbon monoxide32 and prostacyclin,33 release of which
have been localized to caveolae, may also be generated
secondary to caveolae-mediated endocytosis. In addition,
albumin may bind NO and form
S-nitroso-albumin, which

Drab et al,36 showed in caveolin-1–/– mice that the
vasodilator response to acetylcholine (as assessed by
relaxation of preconstricted aortic rings) was enhanced.
The greater relaxation induced by acetylcholine in caveo-
lin-1–/– aortic rings was ascribed to a higher production of
NO.36 Our finding that the vasodilator response to gp60-
induced caveolae endocytosis was reduced in caveolin-1 null lungs seems to contradict the data found by Drab et al. We observed that impairment of caveolae endocytosis in methyl-β-cyclodextrin–treated endothelial cells was coupled to reduced eNOS activity and NO production. Therefore, a likely explanation of our data is that caveolae-mediated endocytosis as regulated by Src-induced phosphorylation of caveolin-1 is, itself, an important signal for eNOS activation. Thus, in the absence of caveolae-mediated endocytosis as in caveolin-1−/− lungs, there is diminished NO production and hence an impairment of gp60-activated pulmonary vasodilatation. This concept is supported by evidence of a marked decrease in eNOS activation and NO production on stimulation of caveolin-1 null endothelial cells with VEGF.37

In conclusion, we have uncovered a novel mechanism of eNOS activation and NO production in endothelial cells involving caveolae-mediated endocytosis induced by gp60. Inhibition of endocytosis resulted in the marked impairment of NO production. eNOS activity induced by gp60 was mediated by Gpα1 activation of downstream Src, Akt, and PI3K pathways. As caveolae internalization is a constitutive process in endothelial cells,38,39 this mechanism of NO production may be important in regulating basal pulmonary vaso-motor tone. Thus, strategies interfering with caveolae endocytosis may have a deleterious effect on eNOS activation and NO production and lead to pulmonary arterial hypertension.

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Disclosures

None.

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Internalization in Endothelial Cells

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