Regulation of endothelial nitric oxide synthase by the actin cytoskeleton

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Su, Yunchao, Sophia Edwards-Bennett, Michael R. Bubb, and Edward R. Block. Regulation of endothelial nitric oxide synthase (eNOS) with the actin cytoskeleton. Am J Physiol Cell Physiol 284: C1542–C1549, 2003. First published January 29, 2003; 10.1152/ajpcell.00248.2002.—In the present study, the association of endothelial nitric oxide synthase (eNOS) with the actin cytoskeleton in pulmonary artery endothelial cells (PAEC) was examined. We found that the protein contents of eNOS, actin, and caveolin-1 were significantly higher in the caveolar fraction of plasma membranes than in the noncaveolar fraction of plasma membranes in PAEC. Immunoprecipitation of eNOS from lysates of caveolar fractions of plasma membranes in PAEC resulted in the coprecipitation of eNOS, actin, and caveolin-1. The binding of Ca2+/calmodulin to eNOS disrupts the inhibitory eNOS-caveolin or eNOS-bradykinin B2 complex, leading to enzyme activation. Heat shock protein 90 (HSP90) is known to serve as a molecular chaperone in protein folding and maturation events. Recent studies indicate that it also serves as an allosteric activator of eNOS. Histamine, estrogen, vascular endothelial growth factor, and shear stress promote the association between eNOS and HSP90 and increase the activity of eNOS. Hypoxia has been shown to inhibit the eNOS-HSP90 association and eNOS activity through a calpain-mediated mechanism. eNOS is localized to specific cellular domains in endothelial cells, including Golgi and plasmalemmal caveolae, and NO synthesis. Our laboratory has recently shown that shear stress induces endothelial cell rearrangement of actin filaments and microtubules with the major axes of the cell, and Hutcherson and Griffith (15) reported that shear stress-induced endothelial cell rearrangement of actin filaments and microtubules provides a transduction pathway between shear stress and NO synthesis. Our laboratory has recently shown that the state of actin microfilaments regulates L-arginine transport and NO production by pulmonary artery endothelial cells (PAEC). We also found that the polymerization state of the microtubule cytoskeleton regulates NO production and eNOS activity in endothelium; nitric oxide; caveolae; endothelial nitric oxide synthase

THE ENDOTHELIAL ISOFORM of nitric oxide synthase (eNOS) catalyzes the reaction to produce nitric oxide (NO) from l-arginine (27, 28, 31). It is known that eNOS is tightly regulated by a variety of transcriptional, posttranscriptional, and posttranslational mechanisms (13, 18, 27, 29). Protein-protein interactions represent an important posttranslational mechanism for regulation of eNOS activity (13, 18). It has been shown that calmodulin serves as an allosteric activator for eNOS and that caveolin directly interacts with and inhibits eNOS (13, 17, 18, 26). Bradykinin B2 receptor has been shown to reside in endothelial caveolae and to interact with eNOS in a ligand- and calcium-dependent manner via its COOH-terminal intracellular domain (16). The binding of Ca2+-calmodulin to eNOS disrupts the inhibitory eNOS-caveolin or eNOS-bradykinin B2 complex, leading to enzyme activation. Heat shock protein 90 (HSP90) is known to serve as a molecular chaperone in protein folding and maturation events. Recent studies indicate that it also serves as an allosteric activator of eNOS (14). Histamine, estrogen, vascular endothelial growth factor, and shear stress promote the association between eNOS and HSP90 and increase the activity of eNOS (14, 18), whereas hypoxia has been shown to inhibit the eNOS-HSP90 association and eNOS activity through a calpain-mediated mechanism (33).
PAEC (35). An earlier report by Venema et al. (36) suggested that there is a significant amount of eNOS in the insoluble portion of the Triton extraction of aortic endothelial cells. The Triton-insoluble fraction represents mainly the actin microfilaments. Therefore, reorganization of the actin cytoskeleton may affect eNOS activity, leading to the alteration of NO production. In the present study, we found that eNOS is associated with actin and that actin polymerization state plays an important role in the regulation of eNOS activity in PAEC.

**MATERIALS AND METHODS**

**Materials.** Anti-actin antibody and phallolidin were obtained from Sigma-Aldrich (St. Louis, MO). L-[3H]arginine was obtained from Amersham (Arlington Heights, IL), and mouse anti-eNOS monoclonal antibody and anti-caveolin-1 polyclonal antibody were obtained from Transduction Laboratories (Lexington, KY). Texas red-phallolidin and deoxyribonuclease I conjugated with Texas red was obtained from Molecular Probes (Eugene, OR), FITC-goat anti-mouse IgG was from Jackson Immunoresearch Laboratories (West Grove, PA), and swinholide A was from Alexis (San Diego, CA).

**Cell culture.** Endothelial cells were obtained from the main pulmonary artery of 6- to 7-mo-old pigs and were cultured as previously reported (34). Third to sixth passage cells in monolayer culture were maintained in RPMI-1640 medium containing 4% fetal bovine serum and antibiotics (10 μg/ml penicillin, 100 μg/ml streptomycin, 20 μg/ml gentamycin, and 2 μg/ml Fungizone) and were used 2 or 3 days after confluence.

All monolayers were initially identified as endothelial cells by phase-contrast microscopy. Selected dishes of cells were further characterized by electron microscopy or by indirect immunofluorescent staining for factor VIII antigen or both. By use of these techniques, monolayer cultures were estimated to be pure endothelial cells.

**Preparation of caveolar fractions of plasma membrane.** Caveolar fractions of plasma membranes were prepared according to the method reported by Lisanti et al. (21). Briefly, PAEC were washed and homogenized in 0.25 M sucrose using nitrogen cavitation. After unbroken cells were removed by centrifugation, the homogenates were centrifuged again at 10,000 g for 20 min. The pellets were then suspended in 0.25 M sucrose buffer. The suspensions were layered on a discontinuous sucrose gradient of 45, 30, and 15%. The gradients were centrifuged at 25,000 rpm for 1 h in a Beckman SW 28 rotor. The hazy band in the 30% layer is the caveolar fraction and was collected. The remaining bands were collected and represented the noncaveolar fraction.

**Western blot analysis of eNOS, actin, and caveolin-1.** Control PAEC or PAEC exposed to swinholide A, phallolidin, or hypoxia (95% N\(_2\) and 5% CO\(_2\), 24 h) were washed with PBS and then resuspended into 1% Triton X-100 buffer containing 20 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 100 mM NaCl, 10 mM NaF, 1 mM Na\(_2\)VO\(_4\), 1 mM Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μM calpain inhibitor-1, and 1 μg/ml pepstatin A. The lysates were incubated at 4°C for 20 min and centrifuged at 10,000 g for 20 min. The supernatants constitute the Triton-insoluble fractions. Half of the Triton-insoluble fraction was mixed 1:1 (volume) with Western blot sample buffer (0.06 M Tris·HCl, 2% SDS, and 5% glycerol, pH 6.8) to measure the protein contents of eNOS and actin, as described above, and another half was mixed 1:1 (volume) with immunoprecipitation buffer. Immunoprecipitations were performed using eNOS antibody as described in the following section. The protein contents of eNOS and actin were measured as described above. The pellets constitute Triton-insoluble fractions, which were lysed in Western blot sample buffer to measure the protein contents of eNOS and actin.

**Coimmunoprecipitation of eNOS and actin.** Caveolar fractions of PAEC plasma membranes were lysed in ice-cold immunoprecipitation buffer containing 20 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na\(_2\)VO\(_4\), 1 mM Pefabloc, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 μM calpain inhibitor-1, and 1 μg/ml pepstatin A. The lysates were centrifuged at 10,000 g for 20 min to remove insoluble material. Lysates (500 μg, 500 μl) were incubated with anti-eNOS antibody, nonimmune IgG, or anti-actin antibody at 4°C overnight. Protein A Sepharose (30 μl) was added, and the samples were further incubated for 2 h at 4°C. The immunoprecipitates were recovered by centrifugation and washed three times in buffer containing 50 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 0.1% Triton X-100. Immunoprecipitated proteins were eluted from the Sepharose beads by boiling the samples for 5 min in 50 μl of SDS immunoblotting sample buffer. The Sepharose beads were then pelleted by centrifugation at 10,000 g, and eNOS or actin protein contents in the supernatants were analyzed by Western blot as described above.

**Immunofluorescence confocal microscopy.** Confluent control PAEC or PAEC exposed to swinholide A, phallolidin, or hypoxia (95% N\(_2\) and 5% CO\(_2\), 24 h) were fixed in 4% paraformaldehyde and then incubated with 0.1% Triton X-100 for 10 min and with 5% goat serum for 30 min. eNOS, F-actin, and G-actin were stained with mouse anti-eNOS antibody labeled with FITC-goat anti-mouse IgG, Texas red-phallolidin, and deoxyribonuclease I conjugated with Texas red, respectively. After the unbound molecules were washed off, eNOS and actin immunofluorescence were assessed using a Zeiss LSM 510 laser scanning confocal microscope.

**Actin purification and polymerization.** Rabbit skeletal muscle Ca\(^{2+}\)-actin was prepared from frozen muscle (Pel-Freez, Rogers, AR) in Tris·HCl (5.0 mM, pH 7.8) containing 0.2 mM ATP, 0.2 mM diethiothreitol, 0.1 mM CaCl\(_2\), and 0.01% sodium azide, according to the method of Spudich and Watt (30). The actin was further purified by gel filtration on a 100-ml Sephacryl HR300 column (Amersham-Pharmacia, Piscataway, NJ). G-actin was polymerized into F-actin by incubation with 2% SDS, 0.25 M sucrose buffer containing 20 mM Tris·HCl at room temperature for 2 h, as reported by Cerven (7).

**Expression and purification of eNOS protein in Escherichia coli.** E. coli containing the plasmid Bov-eNOSPCw (kindly provided by Dr. Bettie Sue Masters, University of Texas, San Antonio, TX) were inoculated in 0.5 liter of TB (terrific broth)
containing ampicillin (50 μg/ml) and chloramphenicol (35 μg/ml) (24). The cultures were grown in a shaking incubator to an OD600 of ~0.8 at 22°C (200 rpm) and induced with 0.5 mM isopropyl β-d-thiogalactopyranoside (IPTG). One hour before IPTG induction, δ-aminolevulinic acid (0.5 mM final) was added and, at the time of IPTG induction, riboflavin (3 μM final) and ATP (1 mM final) were also added. After induction, the flasks were kept in the dark at 22°C (200 rpm). The cells were harvested 48 h after induction. Bovine eNOS protein was purified using a 2′,5′-ADP Sepharose 4B column, as described previously (32).

**eNOS activity assay in total membrane fractions.** Control PAEC or PAEC exposed to swinholide A, phalloidin, or hypoxia (95% N2–5% CO2, 24 h) were scraped and homogenized in buffer A (50 mM Tris·HCl, pH 7.4, containing 0.1 mM each of EDTA and EGTA, 1 mM PMSF, 1.0 μg/ml leupeptin, and 10 μM calpain inhibitor-1). The homogenates were centrifuged at 100,000 g for 60 min at 4°C, and the total membrane pellets were resuspended in buffer B (buffer A + 2.5 mM CaCl2). The resulting suspensions were used for determination of eNOS activity by monitoring the formation of L-[3H]citrulline from L-[3H]arginine (34). The total membrane fractions (100–200 μg of protein) were incubated (total volume 0.4 ml) in buffer B containing 1 mM NADPH, 100 mM calmodulin, 10 μM BH4, and 5 μM combined L-arginine and purified L-[3H]arginine (0.6 μCi; specific activity 69 Ci/mmol; NEN, Boston, MA) for 30 min at 37°C. Purification of L-[3H]arginine and measurement of L-[3H]citrulline formation were carried out as described previously (34). The specific activity of NOS is expressed as pmol L-citrulline·min⁻¹·mg protein⁻¹ and reflects eNOS activity because our cells do not exhibit basal or swinholide-induced iNOS activity. Protein contents in the total membrane fractions were determined by the method of Lowry et al. (22).

**Statistical analysis.** In each experiment, control and experimental endothelial cells were matched for cell line, age, seeding density, number of passages, and number of days postconfluence to avoid variation in tissue culture factors that can influence the measurement of eNOS activity and eNOS protein analysis. Results are shown as means ± SE for n experiments. Student’s paired t-test was used to determine the significance of differences between the means of treated and control groups, and a P value of <0.05 was taken as significant.

**RESULTS**

**eNOS is colocalized with the actin cytoskeleton in PAEC.** To determine whether eNOS is colocalized with actin, PAEC were double labeled with anti-eNOS antibody coupled to FITC-labeled goat anti-mouse IgG and with either Texas red-phalloidin or deoxyribonuclease I conjugated with Texas red and then examined by laser scanning confocal microscopy. As shown in Fig. 1, A1–A3, eNOS is primarily distributed in two areas in PAEC, the plasma membrane and the perinuclear region. eNOS localized to the plasma membrane is colocalized with cortical F-actin. eNOS that is located in the perinuclear area is colocalized with G-actin (Fig. 2, A1–A3).

**eNOS and actin preferentially distribute within caveolae.** To investigate whether eNOS and actin are colocalized within caveolae of PAEC, the protein contents of eNOS, actin, and caveolin-1 in the caveolar fractions and the noncaveolar fractions of plasma membranes from PAEC were measured by Western blot analysis. As shown in Fig. 3, the protein contents of eNOS, actin, and caveolin-1 are significantly higher in the caveolar fractions of the plasma membranes than in the noncaveolar fractions of the plasma membranes in PAEC, suggesting that eNOS and actin are colocalized within caveolae.

**eNOS and actin are coimmunoprecipitated from caveolar membrane fractions.** The association of eNOS and actin in PAEC was studied further using coimmunoprecipitation methods. Lysates from PAEC caveolar membrane fractions were immunoprecipitated with antibody directed against eNOS or against actin. Immunoprecipitation of eNOS by anti-eNOS antibody coupled with FITC-labeled goat anti-mouse immunoglobulin G (IgG) (A1, B1, C1, D1) and Texas red-phalloidin (A2, B2, C2, D2) and examined with a Zeiss LSM 510 laser scanning confocal microscope. A3, B3, C3, and D3 are overlays of A1, B1, C1, D1 and A2, B2, C2, D2, respectively. Areas of yellow staining represent colocalization of eNOS and cortical F-actin. The images shown are representative of the results observed in 3 experiments.
These results suggest that eNOS is associated with actin in PAEC caveolae.

Effect of actin on eNOS activity. To investigate the effects of actin on eNOS activity, purified F-actin and G-actin were isolated from PAEC plasma membranes and measured by immunoblot analysis using anti-eNOS antibody coupled with FITC-labeled goat anti-mouse IgG. Incubation of purified eNOS with 100 mM KCl did not affect eNOS activity significantly. The magnitude of increase in eNOS activity induced by G-actin was much greater than that induced by F-actin. The effect of G-actin appeared to be dose-dependent, and denaturing actin protein by boiling for 10 min abolished the effect of actin. These results indicate that actin and its polymerization state play an important role in the regulation of eNOS activity.
be used to modulate actin function in endothelial cells (6). As shown in Table 1, incubation of PAEC with 50 nM swinholide A, a concentration shown to disrupt actin filaments without causing cytotoxicity (38), resulted in increases in eNOS activity and protein content. In contrast, exposure of PAEC to 50 μM phalloidin for 4 h or to hypoxia for 24 h resulted in a decrease in eNOS activity, although the protein contents of eNOS were not altered by phalloidin or hypoxia. The magnitude of the increase in eNOS activity in swinholide A-exposed cells is much greater than that in eNOS protein content. Swinholide A and phalloidin did not alter actin protein contents in intact cells, but hypoxia significantly reduced actin protein contents (Table 1). These results suggest that eNOS activity is regulated by actin polymerization state and/or actin protein contents in a posttranslational manner.

**The effect of swinholide A, phalloidin, or hypoxia on eNOS-actin interactions in the Triton-insoluble and -soluble fractions.** To investigate whether the effects of actin polymerization on eNOS activity are due to effects on eNOS-actin interaction, we measured eNOS activity and the protein contents of eNOS and actin in cells, and the protein contents of eNOS and actin and the actin/eNOS ratio in Triton-insoluble fractions, which contain only F-actin, and in Triton-soluble fractions, which contain mainly G-actin. We also measured the actin/eNOS ratio in the immunoprecipitated pellets induced by eNOS antibody in the Triton-soluble fractions. Incubation of PAEC with swinholide A resulted in a decrease in actin protein content and the actin/eNOS ratio in Triton-insoluble fractions. However, the protein contents of eNOS and actin in the Triton-soluble fractions and the actin/eNOS ratio in the precipitation pellets induced by eNOS antibody were decreased in phalloidin-treated cells (Table 1). Hypoxia did not influence eNOS contents in the Triton-insoluble or -soluble fractions, but it did decrease actin protein contents in Triton-insoluble and -soluble fractions, and consequently, the actin/eNOS ratios in the Triton-insoluble fractions and in the precipitation pellets induced by eNOS antibody in the Triton-soluble fractions were decreased (Table 1).

The effect of swinholide A, phalloidin, and hypoxia on the colocalization of eNOS and actin. To further confirm the interaction of eNOS with the actin cytoskeleton, the effects of swinholide A, phalloidin, and hypoxia on the colocalization of eNOS with F-actin and G-actin were studied by confocal fluorescence microscopy. Incubation of PAEC monolayers with swinholide A (50 nM, 4 h) resulted in an accumulation of eNOS and G-actin in the perinuclear area of PAEC, a disappearance of eNOS located at the plasma membrane, a disruption of actin stress fibers and cortical F-actin, and, consequently, diminished colocalization of eNOS with cortical F-actin and increased colocalization of eNOS with G-actin (Figs. 1 and 2, B1–B3). Incubation of PAEC with phalloidin (50 μM, 4 h) caused an increased formation in cortical F-actin and more colocalization of eNOS with cortical F-actin (Figs. 1 and 2, C1–C3). Exposure of cells to hypoxia for 24 h resulted in a disruption of actin stress fibers and cortical F-actin and diminished colocalization of eNOS with cortical F-actin (Figs. 1 and 2, D1–D3).

**DISCUSSION**

The present study indicates that eNOS is associated with actin within caveolae in PAEC. This is supported by the fact that eNOS and actin colocalize with caveolin-1 in caveolar fractions of PAEC plasma membranes. Moreover, in coimmunoprecipitation studies, we found that immunoprecipitation of eNOS from the caveolar fraction of PAEC plasma membranes resulted in the
results show that the magnitude of increase in eNOS activity caused by swinholide A-induced disruption of F-actin is much greater than that in eNOS protein content. Stabilization of F-actin by phalloidin decreases eNOS activity without affecting eNOS protein content. These results suggest that disruption of the actin cytoskeleton increases eNOS activity not only through a pretranslational mechanism, as reported by Laufs et al. (19), but also through a posttranslational one and that stabilization of the actin cytoskeleton decreases eNOS activity primarily, if not exclusively, via a posttranslational mechanism.

To investigate the possible posttranslational mechanism responsible for the regulation eNOS activity by actin and its polymerization state, we studied the effects of swinholide A, phalloidin, and hypoxia on the interactions of eNOS with actin in the Triton-insoluble and Triton-soluble fractions of PAEC. Our results demonstrate that disruption of the actin cytoskeleton by swinholide A decreases the association of eNOS with F-actin and increases the association of eNOS with G-actin. In contrast, stabilization of the actin cytoskeleton by phalloidin increases the association of eNOS with F-actin and decreases the association of eNOS with G-actin. We also found that hypoxia decreases the associations of eNOS with F-actin and G-actin. The altered associations of eNOS with F-actin or G-actin might be caused by changes in availabilities of F-actin or G-actin due to the effects of swinholide A, phalloidin, or hypoxia on the concentrations of F-actin or G-actin in PAEC. Together, our data indicate that the effects of actin and its polymerization state on eNOS activity are due to their effects on F-actin-eNOS and G-actin-eNOS interactions. Associations of eNOS with F-actin or G-actin increase eNOS activity. Association of eNOS with G-actin causes more significant increase in eNOS activity than that caused by association with F-actin.

eNOS is localized to specific cellular domains, including Golgi and plasmalemmal caveolae, in endothelial cells (10, 12). The relative distribution between the Golgi and caveolae is related to the pattern of endothe-
lial cell heterogeneity and function (3, 12). Our results indicate that eNOS is primarily distributed in two areas in PAEC, the plasma membrane and the perinuclear region. eNOS localized to the plasma membrane is colocalized with cortical F-actin. eNOS that is located in the perinuclear area (probably Golgi) is colocalized with G-actin. Thus eNOS localized to either the plasma membrane or the perinuclear area appears to be subject to regulation by actin. Because association of eNOS with G-actin causes more significant increase in eNOS activity than that caused by association with F-actin, eNOS that is located in the perinuclear area (probably Golgi) might be more active. This is consistent with the observation that swinholide A increases accumulation of eNOS and G-actin in the perinuclear area and also increases eNOS activity to a greater degree than eNOS protein content in PAEC.

We have previously reported that an actin-binding protein, fodrin, is linked to CAT-1 (37), the major arginine transporter in endothelial cells, and that CAT-1 forms a caveolar complex with eNOS in lung endothelial cells (25). Therefore, association of eNOS with actin within caveolae may not only directly enhance eNOS activity and NO release but may also serve to stabilize the eNOS-CAT-1-fodrin-actin complex, thereby facilitating directed delivery of substrate (L-arginine) and optimal NO production.

Regulation of eNOS function by the actin cytoskeleton is a novel concept that may help to advance our understanding of the regulation of NO production in the pathophysiology of hypoxia, pulmonary hypertension, and angiogenesis. For example, hypoxia causes disruption of actin filaments (4) and alterations in eNOS expression and activity (33) and NO production (20). Increases in shear stress in pulmonary hypertension also change actin cytoskeletal organization (9, 23), and several studies have reported altered eNOS activity and/or NO production in the presence of pulmonary hypertension (1, 2). Recent reports indicate that NO is an important mediator of angiogenesis (5). Because angiogenesis is regulated by the cytoskeleton, this suggests that the eNOS-actin association described here plays a role in angiogenesis. Finally, demonstration of eNOS association with actin filaments opens the door to the possibility that manipulation of the cytoskeleton may provide a new avenue for preventing or reversing impaired eNOS activity and vascular NO production in the presence of endothelial dysfunction.

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